Neuroprotective Effects of Omega-3 Polyunsaturated Fatty Acids in a Rat Model of Anterior Ischemic Optic Neuropathy

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Purpose. The purpose of this study was to investigate the therapeutic effect of omega-3 polyunsaturated fatty acid (ω-3 PUFA) administration in a rat model of anterior ischemic optic neuropathy (rAION).

Methods. The level of blood arachidonic acid/eicosapentaenoic acid (AA/EPA) was measured to determine the suggested dosage. The rAION-induced rats were administered fish oil (1 g/day EPA) or phosphate-buffered saline (PBS) by daily gavage for 10 consecutive days to evaluate the neuroprotective effects.

Results. Blood fatty acid analysis showed that the AA/EPA ratio was reduced from 17.6 to ≤1.5 after 10 days of fish oil treatment. The retinal ganglion cell (RGC) densities and the P1-N2 amplitude of flash visual-evoked potentials (FVEP) were significantly higher in the ω-3 PUFA–treated group, compared with the PBS-treated group ($P < 0.05$). The number of apoptotic cells in the RGC layer of the ω-3 PUFA–treated rats was significantly decreased ($P < 0.05$) compared with that of the PBS-treated rats. Treatment with ω-3 PUFAs reduced the macrophage recruitment at the optic nerve (ON) by 3.17-fold in the rAION model. The M2 macrophage markers, which decrease inflammation, were induced in the ω-3 PUFA–treated group in contrast to the PBS-treated group. In addition, the mRNA levels of tumor necrosis factor-alpha, interleukin-1 beta, and inducible nitric oxide synthase were significantly reduced in the ω-3 PUFA–treated group.

Conclusions. The administration of ω-3 PUFAs has neuroprotective effects in rAION, possibly through dual actions of the antiapoptosis of RGCs and anti-inflammation via decreasing inflammatory cell infiltration, as well as the regulation of macrophage polarization to decrease the cytokine-induced injury of the ON.

Keywords: rat anterior ischemic optic neuropathy (rAION), inflammation, neuroprotection, omega-3 polyunsaturated fatty acids, optic nerve, retinal ganglion cells

Nonarteritic anterior ischemic optic neuropathy (NAION) is a visually disabling disease caused by the primary damage of retinal ganglion cells (RGCs) and is the most common type of ischemic optic neuropathy in people over the age of 50, affecting somewhere between 2.3 and 10.3 individuals per 100,000 in the United States and Taiwan.[1–2] The visual loss has a sudden onset and may be unremitting. Risk factors, especially nocturnal hypotension, impaired autoregulation of the microvascular supply, systemic vasculopathy occlusion, crowded disc appearance, and venous insufficiency, play important roles in NAION development.[1–5]

To date, there is no effective treatment for NAION. Systemic corticosteroid therapy has been shown to improve both visual acuity and visual fields in a prospective study of NAION.[6] However, the results have been controversial.[7] Conclusions regarding the efficacy of systemic corticosteroid therapy vary between different clinical studies. While there have been several therapies proposed, most have not been sufficiently studied. Translational preclinical research may lead to the improved medical management of NAION.[8]

Inflammation is involved in the pathogenesis, development, and progression of NAION, with the recruitment of both polymorphonuclear leucocytes and macrophages, following an optic nerve (ON) infarct.[9–12] Our recent study demonstrated that the early administration of granulocyte-colony stimulating factor (G-CSF) can stabilize the blood–ON barrier (BOB) to reduce the macrophage infiltration and can induce M2 microglia/macrophage polarization to decrease the expression of proinflammatory cytokines in the rat anterior ischemic optic neuropathy (rAION) model. Moreover, we found that the early administration of G-CSF can provide neuroprotection of RGCs and ONs, as well as preserve visual function.[13] Thus, we considered that the stabilization of the BOB, activation of M2 macrophages, and reduction of proinflammatory cytokine

expression should constitute three important approaches for ON protection in the rAION model.

Numerous studies have demonstrated that omega-3 polyunsaturated fatty acids (ω-3 PUFAs) have a protective role against inflammatory-, ischemia-, light-, oxygen-, and age-associated pathologies of the vascular and neural retina.[14] High doses of PUFAs were previously found to have a hopeful effect in several animal models of ocular pathologies, including age-related macular degeneration (AMD), retinitis pigmentosa, and Stargardt's disease.[15–19] The resolution of inflammation contributed by ω-3 PUFAs is an active progression mainly driven by a series of mediators, termed resolvins, docosahexaenoic acid (DHA)-derived protectin D1, 13-EFOX derived from the ω-3 PUFAs, eicosapentaenoic acid (EPA), and DHA.[20,21] Among the main mediators of the inflammatory response is the production of proinflammatory eicosanoids derived from the ω-6 PUFA, arachidonic acid (AA). The balance between the pro- and anti-inflammatory compounds plays a crucial role in the disease development and the resolution of an inflammatory response.[21] Literature reported that the anti-inflammatory effects of ω-3 PUFAs lie in their capability to guide the polarization of macrophages from the proinflammatory to the proresolving M2 phenotype.[22] Moreover, ω-3 PUFAs can protect from blood–brain barrier (BBB) damage after hypoxic–ischemic brain injuries.[23,24] Thus, we considered that the administration of ω-3 PUFAs may provide anti-inflammation activity, M2 macrophage activation, and BOB stabilization, which is a potential treatment for rAION.

The AA/EPA ratio is considered to be a clinically relevant measurement. For evaluating the effects from proinflammatory eicosanoids and cytokines, the AA/EPA ratio was recognized as a diagnostic index.[25] The value of AA/EPA reaches to 11.1∼2.1, which is associated with a reduced production of proinflammatory eicosanoids and cytokines.[25] The higher level of EPA in plasma was inversely related to the risk of major coronary diseases in a Japanese population.[26] In addition, the AA/EPA ratio proved to have a linear relationship with the ratio of prostaglandin (PG)I3 and PGI2 to thromboxane (TXA2).[27] A higher serum AA/EPA ratio is correlated to a higher risk of cardiovascular disease.[28] In addition, among patients with acute ischemic stroke, AA/EPA was reported to be an important factor related to early neurologic deterioration.[29] Thus, the manipulation of the AA/EPA ratio to a low level may also provide potential benefits in ischemic optic neuropathy.

To date, ω-3 PUFAs have never been evaluated in the rAION model. Therefore, in this preclinical trial, we examined the therapeutic effects of ω-3 PUFA treatment in a rat model of anterior ischemic optic neuropathy.

Methods

Animals

Adult male Wistar rats weighing 150 to 180 g (7–8 weeks old) were used in this study. The rats were obtained from the breeding colony of BioLASCO Co., Taipei, Taiwan. Animal care and experimental procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. In addition, the Institutional Animal Care and Use Committee (IACUC) at Tzu Chi Medical Center approved all the animal experiments. All manipulations were performed with the animals under general anesthesia, achieved by intramuscular injections of a mixture of ketamine (40 mg/kg body weight) and xylazine (4 mg/kg body weight; Sigma-Aldrich Corp., St. Louis, MO, USA), and by the use of topical 0.5% Alcaine eye drops (Alcon, Puurs, Belgium). The rats had free access to food and water and were maintained in cages in an environmentally controlled room with a temperature of 23 ± 1°C, a humidity of 55 ± 5%, and a 12-hour light/dark cycle.[13]

Study Design

To monitor the blood AA/EPA ratio, six wild-type rats were administered ω-3 PUFAs (1 g/day EPA and 0.5 g/day DHA, Zone OmegaRx; Zone Labs, Marblehead, MA, USA) once daily for 14 days. The Zone OmegaRx consisted of purified ethyl esters rich in EPA (400 mg) and DHA (200 mg) per gram for the liquid formulation. During the daily gavage, the blood samples were collected once daily to determine the AA/EPA ratio. We found that the AA/EPA ratio was stable (at less than 1.5) after 9 or 10 days of gavage. In the following rAION experiments, 24 rats received rAION experiments and received a once-daily gavage administration of either fish oil (1 g/day EPA and 0.5 g/day DHA, $n = 6$) or phosphate-buffered saline (PBS, serving as the control; 0.2 mL; $n = 6$) from day 3 before rAION to day 6 post rAION induction. The other six rats received a sham operation (Fig. 1). The rats were euthanized at the fourth week post infarct by CO_2 inhalation. Retinal ganglion cell density was measured by retrograde labeling with Fluoro-Gold (Fluorochrome, LLC, Denver, CO, USA), and visual function was assessed by flash visual-evoked potentials (FVEPs) at the fourth week post infarct. A terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay of the RGC layer was performed. The newly synthesized (extrinsic) macrophage (ED1) and M2 macrophage (CD206) markers were examined by immunohistochemistry (IHC) in the ON sections. The mRNA levels of the inflammatory cytokines tumor necrosis factor-alpha (TNF-α), inducible nitric oxide synthase (iNOS), interleukin-1 beta (IL-1β), and M2 macrophage polarization were measured in the ON samples by quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis.

Fatty Acid Analysis

Sample Preparation for Analysis. To assess the AA/EPA ratios following treatment, blood samples were collected, processed for fatty acid separation, and analyzed using gas chromatography.[30] Blood samples were collected on a Whatman filter paper and stored at −20°C until ready for analysis. In each sample, sodium hydroxide (NaOH)/methanol (MeOH) was added and heated for 1 minute at 85°C, and 14% boron trifluoride (BF_3)/MeOH was then added and heated for 7 minutes at 85°C. Once the samples reached room temperature, 0.3 g NaCl was added to ensure complete migration of the total fatty acid methyl ester (FAME) fraction to the organic phase, followed by 1 mL n-hexane. When the samples were separated into the organic and aqueous phases, the organic phase was collected, and this was repeated three times. To remove any impurities, 0.9 g Na_2SO_4 was added to the samples and then centrifuged at 1350*g* for 5 minutes. Supernatants were collected and dried using an analytical evaporator at approximately 45°C under a nitrogen stream. Once dried, the samples were redissolved in n-hexane and analyzed using a gas chromatography–flame ionization detector (GC-FID).

Gas Chromatography–Flame Ionization Detector. An Agilent GC-6890 system (Santa Clara, CA, USA) was equipped with an FID.[30] An H_2 flow rate of 35 mL/min and an air flow rate of 350 mL/min were used. The flow rate of carrier gas (He) was set at 2.5 mL/min. The temperature of the injection port and detector was set at 280°C and 300°C, respectively. The oven temperature was programmed to initiate at 160°C for 3 minutes; then, the temperature was raised to 200°C at a rate of 20°C/min, held there for 4 minutes, and finally increased to 250°C at a rate of 5°C/min and held there for 23 minutes. The injection volume was 1 μL in the split-less injection mode. A

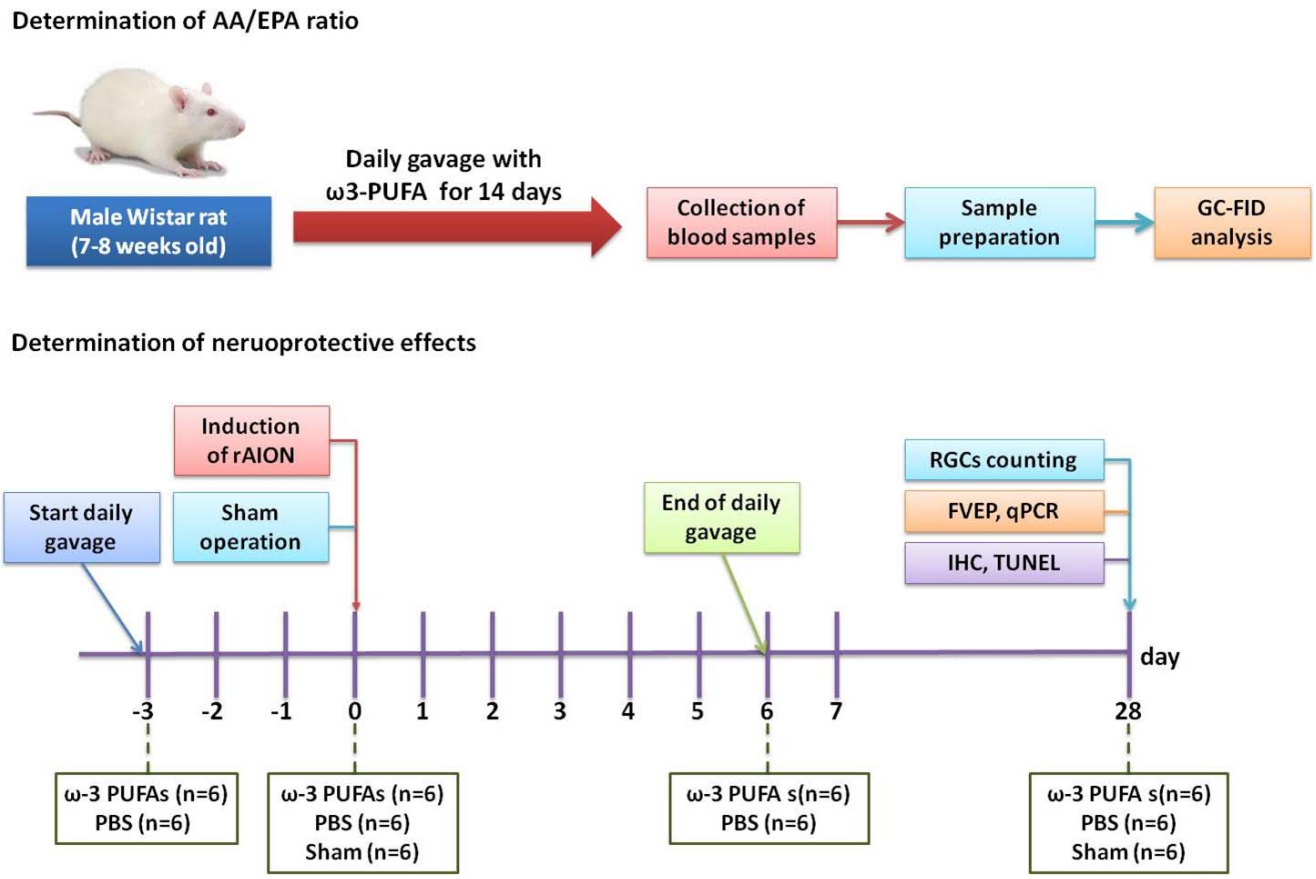

Figure 1. Illustration of the study design to investigate the therapeutic effects of ω-3 PUFA treatment and the role of ω-3 PUFAs in regulating optic nerve (ON) inflammation in a rAION model.

capillary column (DP-23 fused-silica capillary, 30 m × 0.25-mm I.D. × 0.25-μm film thickness; Supelco, Bellefonte, PA, USA) was employed.

Induction of rAION

As previously described,[13,31] the optic nerve head (ONH) of rats was treated with an argon laser immediately after intravenous injection of the photosensitizing agent Rose Bengal (RB; Sigma-Aldrich Corp.). Sham laser treatment consisted of illuminating the ON region with an argon laser without RB administration. Briefly, after general anesthesia, RB was administered intravenously through the tail vein using a 28-gauge needle (2.5 mM RB in PBS/1 mL/kg animal weight). After the administration of RB, the right optic discs were directly treated with an argon green laser 532 nm in wavelength, 500 mm in size, and 80 mW in power (MC-500 Multi-color laser; Nidek Co., LTD, Tokyo, Japan). There were 12 pulses of 1-second duration for each laser procedure. When RB is activated by green laser light, it glows a bright golden color, which can be evidenced as successful photodynamic therapy (PDT).[32] Following this procedure, Tobradex eye ointment (Alcon) was administered. Subsequently, the rats were kept on electric heating pads at 37°C for recovery.

Retrograde Labeling of RGCs With Fluoro-Gold

The detailed procedure has been previously described in our reports.[13,31,33–38] The retrograde labeling of the RGCs was performed 1 week before the rats were euthanized. Briefly, the rats were anesthetized with a mixture of ketamine and xylazine and placed in a stereotactic apparatus (Stoelting, Wood Dale, IL, USA). The skin covering the skull of the rats was opened, and 1.5 μL 5% Fluoro-Gold was injected into the superior colliculus on each side using a Hamilton syringe. One week later, the eyeballs were harvested after euthanization. The eyeballs were placed in 10% formalin for 1 hour, and the retina was then carefully dissected and flat mounted on a slide. The retinas were examined with a ×400 epifluorescence microscope (Axioskop; Carl Zeiss Meditech, Inc., Thornwood, NY, USA) equipped with a filter set (excitation filter = 350–400 nm; barrier filter = 515 nm). The retinas were examined for RGCs at a distance of 1 or 3 mm from the center of the ONH to provide the central and midperipheral RGC densities, respectively. We counted at least five randomly chosen areas of 62,500 μm^2 in the central and midperipheral regions of each retina, and their averages were estimated as the mean density of RGCs per retina ($n = 6$ in each group). The uneven RGC death of the topographic image (such as difference between upper and lower; temporal and nasal retinas) in flat-mounted retinas, the sample will be discarded without use to reduce variation.

Optic Nerve and Retinal Sample Preparation

Optic Nerve Preparation. A segment of the ONs approximately 5 to 7 mm in length was collected upon euthanasia of the rats at 4 weeks. The ONs were immediately frozen in liquid nitrogen for histologic and IHC study.[13]

Retinal Section Preparation. To collect the eye cups, the lens and vitreous body were removed from the eyeball. The remaining eye cups, containing the sclera and the retina, were fixed in 4% paraformaldehyde for 2 hours. Each retinal cup was

cut adjacent to the disc into two pieces. The tissues were then dehydrated in 30% sucrose overnight and kept at −20°C until further processing.[13]

In Situ Nick End-Labeling (TUNEL) Assay for Apoptotic Cell Measurements

All frozen sections of the retinas were prepared with samples cut at 1 to 2 mm in distance from the ON head to ensure the use of equivalent fields for comparisons.[31] A TUNEL assay (DeadEnd Fluorometric TUNEL System; Promega Corporation, Madison, WI, USA) was performed to detect apoptotic cells. The TUNEL-positive cells in the RGC layer of each sample were counted in 10 high-powered fields (HPF, ×400 magnification).

Flash Visual-Evoked Potentials (FVEPs)

Flash visual-evoked potentials were recorded 4 weeks post infarction. To avoid the VEP responses of albino Wistar rats being contaminated from the contralateral side, we performed the same surgery in both eyes for FVEP examination. This procedure was approved by the IACUC and is detailed in our previous reports.[13,31,33–38] A visual electrodiagnostic system (UTAS-E3000; LKC Technologies, Gaithersburg, MD, USA) was used to measure photopic FVEPs.[31,39] The first positive-going wavelet was defined as the P1 wave[40] and was compared to the latency of the P1 wave and the amplitude of the P1-N2 wave in each group ($n = 6$ rats in each group) to evaluate visual function.

Immunohistochemistry Staining

The ED1 antibody reacts against the newly synthesized/extrinsic macrophages, respectively, and we used monoclonal antibodies of ED1 (1:50; AbD Serotec, Oxford, UK) in this procedure.[13] Briefly, the frozen ON sections were fixed with acetone at −20°C for 30 minutes and then blocked with 5% fetal bovine serum containing 1% bovine serum albumin for 15 minutes. The primary antibody was applied and incubated overnight at 4°C. The secondary antibody conjugated with fluorescein isothiocyanate (FITC, 1:100, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was incubated at room temperature for 1 hour. Counterstaining was performed using 4′,6-diamidino-2-phenylindole (DAPI, 1:1000; Sigma-Aldrich Corp.). For comparisons, the ED1-positive cells were counted in six HPFs (×400 magnification) at the ON lesion site.

Quantitative Reverse Transcription–Polymerase Chain Reaction (qRT-PCR)

Arg 1, CD206, and Fizz1 are markers of M2 macrophages.[13,41] Tissue RNA was extracted using the Qiagen RNeasy Mini Kit (Hilden, Nordrhein-Westfalen, Germany) from ON lysates obtained by the sonication method. All RNA samples were reverse transcribed for 30 minutes at 42°C with a High Capacity cDNA Reverse Transcription Kit according to the standard protocol of the supplier (Applied Biosystems, Foster City, CA, USA). Quantitative RT-PCR was performed on an AB PRISM 7300 Sequence Detection System (Applied Biosystems) using the QuantiTect SYBR green qRT-PCR kit (Qiagen). Expression levels of each target gene were normalized to those of GypA mRNA. The data are reported as the mean relative expression levels ± standard deviation (SD). Forward and reverse primers used in this study for target gene amplification were as follows:

Arg1-F-TCGGAGCGCCTTTCTCTAAG;
Arg1-R-ATCCCCGTGGTCTCTCACAT;

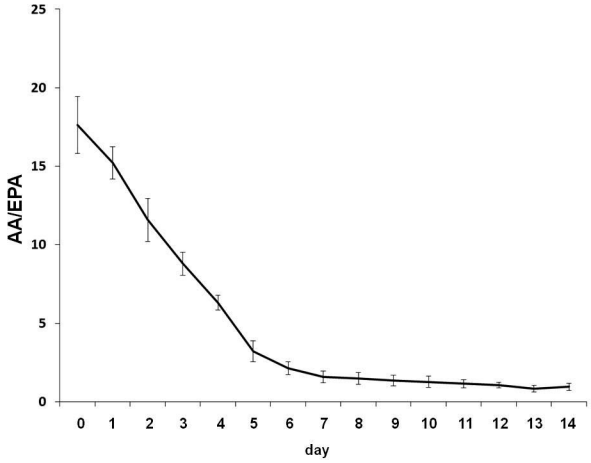

Figure 2. The level of blood AA/EPA ratio changes from day 0 to 14 after feeding with ω-3 PUFAs. Six adult male Wistar rats were gavaged with ω-3 PUFAs once daily for 14 days and were monitored for their daily blood AA/EPA ratio.

CD206-F-AACGTTCGCTGATGCAAACC;
CD206-R-TGTAAACTGCACCTGCTCGT;
Fizz1-F-CAACAGGATGAAGACTGCAACCT;
Fizz1-R-GGGACCATCAGCTAAAGAAG;
GypA-F-CACCGTGTTCTTCGACATCAC;
GypA-R-CCA GTGCTCAGAGCACGA AAG.

Statistical Analysis

All measurements in this study were performed in a masked fashion: The mean ± SD were obtained. Statistical analysis was performed with commercial software (SPSS, version 19.0, Chicago, IL, USA). The Mann-Whitney *U* test was used to evaluate the differences among treated groups. Statistical significance was declared if $P < 0.05$.

Results

Determination of the Blood AA/EPA Ratio

During the daily gavage with ω-3 PUFAs, the blood AA/EPA ratio gradually decreased from 17.63 ± 1.82 to 0.95 ± 0.22 (Fig. 2). The AA/EPA ratio stably dropped to 1.5 following daily gavage with ω-3 PUFA for 10 days. Thus, we considered daily gavage with ω-3 PUFA for 10 days for the following rAION experiments.

Treatment With ω-3 PUFA Preserved the Postinfarct Survival of RGCs

Treatment with ω-3 PUFAs preserved a higher density of RGCs in both central and midperipheral retinas in contrast to treatment with PBS (Fig. 3A). The quantification data showed that the RGC densities in the central and midperipheral retinas in the sham group were 1737 ± 185/mm^2 and 1315 ± 80/mm^2, respectively. Four weeks after infarction, the RGC densities of the central and midperipheral retinas in the PBS-treated group were 524 ± 201/mm^2 and 374 ± 107/mm^2, respectively. In the ω-3 PUFA–treated group, the RGC densities of the central and midperipheral retinas were 1190 ± 450/mm^2 and 771 ± 201/mm^2, respectively (Fig. 3B). The number of RGCs in the central and midperipheral retinas was 2.27- ($P =$

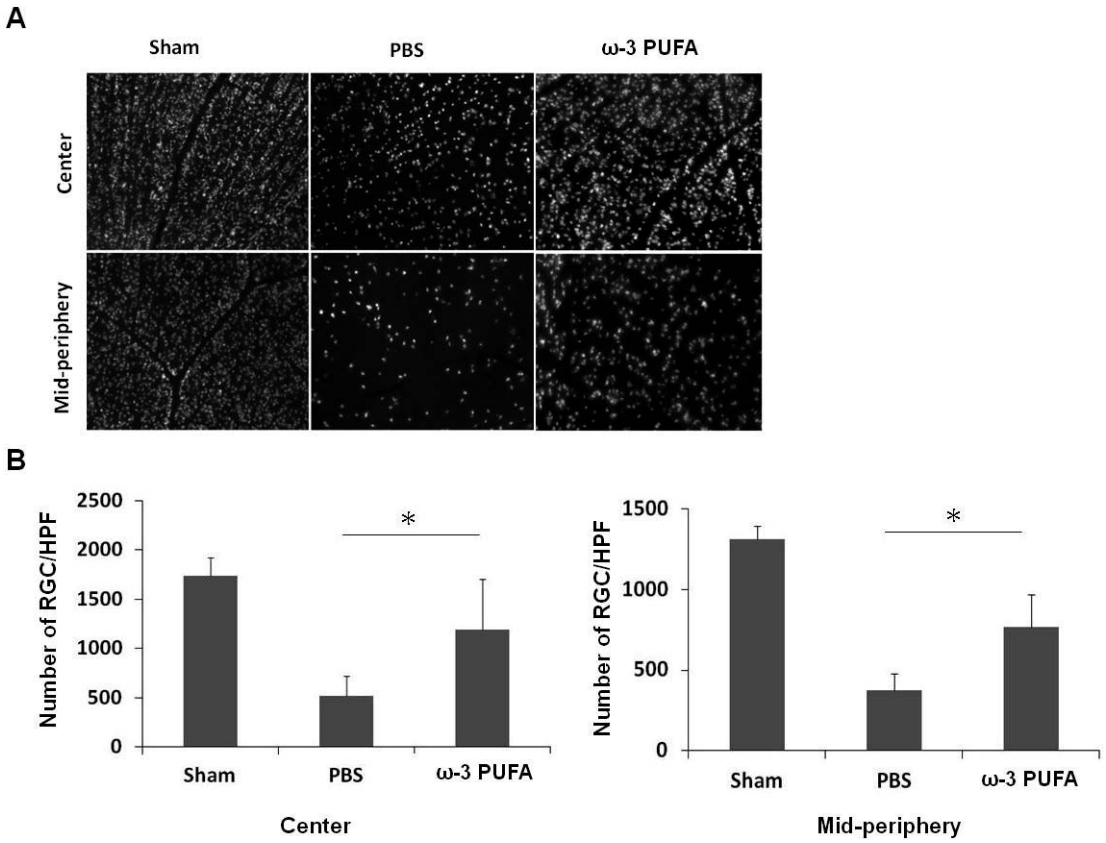

Figure 3. Survival of RGCs in rAION-induced rats with PBS treatment and ω-3 PUFA treatment at 28 days post rAION induction. (**A**) Representative of flat-mounted central and midperipheral retinas and the morphometry of RGCs in each group ($n = 6$ per group) by Fluoro-Gold retrograde labeling at 4 weeks after rAION induction. (**B**) The RGC density of the central retina and midperipheral retina in each group. The data are expressed as the mean ± SD for each group ($n = 6$). The number of RGCs in the central and midperipheral retinas was 2.27- ($P = 0.03$) and 2.06-fold ($P = 0.03$) higher in the ω-3 PUFA–treated group than in the PBS-treated group. The *asterisk* indicates $P < 0.05$ using the Mann-Whitney U test.

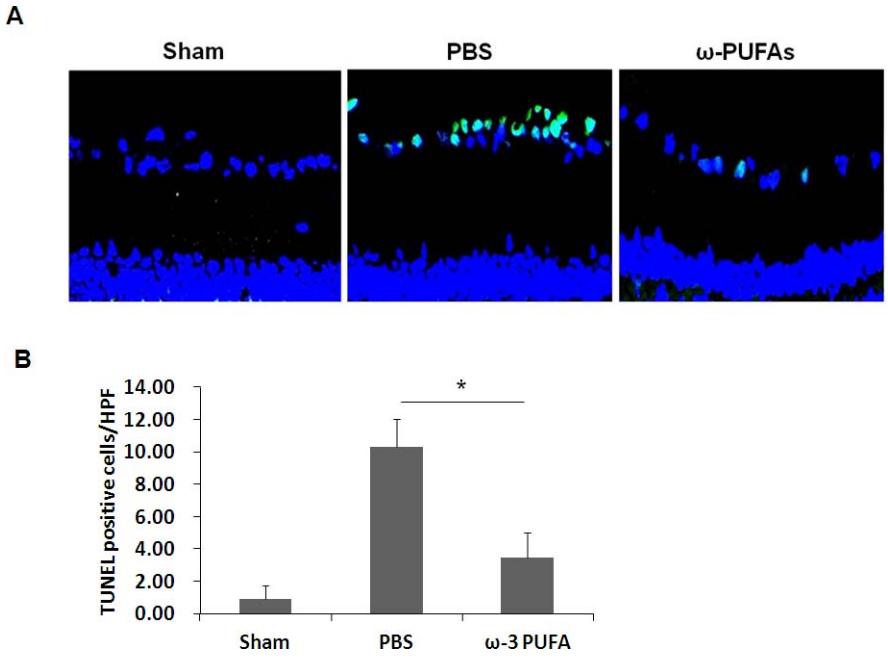

Figure 4. Analysis of RGC death in the RGC layer using a TUNEL assay at 4 weeks after rAION induction. (**A**) Representative pictures of apoptotic cells in the RGC layers among each group, double stained. The apoptotic cells (TUNEL-positive cells) in *green* were stained with TUNEL staining, and the nuclei of RGCs in *blue* were stained with DAPI staining. (**B**) Quantification of TUNEL-positive cells per high-power field. The data are expressed as the mean ± SD in each group ($n = 6$). The apoptotic cells in the ω-3 PUFA–treated group were significantly decreased by approximately 2.94-fold ($P = 0.007$) compared to the PBS-treated group. The *asterisk* indicates $P < 0.05$ using the Mann-Whitney U test.

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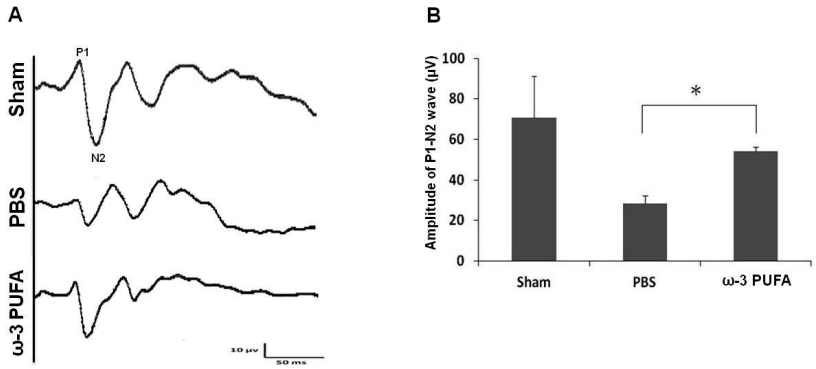

Figure 5. Evaluation of the recovery of injured optic nerves by FVEPs in the rAION model. (**A**) Representative FVEP tracings at 4 weeks following rAION induction in the sham group, the PBS-treated group, and the ω-3 PUFA–treated group. (**B**) *Bar charts* showing the P1-N2 amplitude. The data are expressed as the mean ± SD in each group ($n = 6$ in each group). The amplitudes of the P1-N2 waves in the ω-3 PUFA–treated group were significantly higher than those in the PBS-treated group (54.3 ± 2.1 vs. 26.5 ± 1.4 μv, $P = 0.005$). The *asterisk* indicates $P < 0.05$ using the Mann-Whitney *U* test.

0.03) and 2.06-fold ($P = 0.03$) higher, respectively, in the ω-3 PUFA–treated group than in the PBS-treated group.

Treatment With ω-3 PUFAs Reduced the Postinfarct Apoptosis of RGCs

The TUNEL-positive cells/HPF in the RGC layers of the retina were quantified in the sham group as 0.93 ± 0.81, in the PBS-treated group as 10.32 ± 1.74, and in the ω-3 PUFA–treated group as 3.50 ± 1.51 (Fig. 4). The apoptotic cells in the ω-3 PUFA–treated group were significantly decreased at approximately 2.94-fold ($P = 0.007$) compared with the PBS-treated group.

Treatment With ω-3 PUFAs Preserved Visual Function in the rAION Model

To evaluate the ON function after ω-3 PUFA treatment, the FVEPs of the sham group, the PBS-treated group, and the ω-3 PUFA–treated group were recorded (Fig. 5A). The latency of the P1 wave was not significantly different among groups in the FVEP tests. The amplitudes of the P1-N2 waves in the ω-3 PUFA–treated group were significantly higher than those of the PBS-treated group (54.3 ± 2.1 vs. 26.5 ± 1.4 μv, $P = 0.005$, respectively, Fig. 5B).

Evaluation of Extrinsic Vascular-Borne Macrophage Recruitment to the ON

The cells on the ON were counterstained with 4′,6-diamidino-2-phenylindole (DAPI), while the newly synthesized (extrinsic) macrophages were stained with ED1 (Fig. 6A). The quantification data showed 0.57 ± 0.33 ED1-positive cells/HPF in the sham group, 157.67 ± 28.15 ED1-positive cells/HPF in the PBS-treated group, and 49.80 ± 54.71 ED1-positive cells/HPF in the ω-3 PUFA–treated group (Fig. 6B). The macrophage recruitment was decreased 3.17-fold in the ω-3 PUFA–treated group compared with the PBS-treated group ($P = 0.008$).

Treatment With ω-3 PUFA Induced the Expression of M2 Microglia/Macrophage Markers

The results showed that the mRNA levels of *Arg 1*, *CD206*, and *Fizz1* (markers of M2 macrophages) were increased in the ON following treatment with ω-3 PUFAs and rAION compared with those in the PBS treatment (Fig. 7A). In addition, the IHC results showed that CD206 was highly expressed in the ON following treatment with ω-3 PUFAs compared to the PBS treatment and sham control (Fig. 7B).

Treatment With ω-3 PUFA Significantly Reduced the Proinflammatory Cytokine Production in the Damaged ON

The mRNA levels of the proinflammatory cytokines *TNF-α*, *iNOS*, and *IL-1β* in the ON samples were evaluated using qRT-PCR analysis (Fig. 8). The expression levels of *TNF-α*, *IL-1β*, and *iNOS* in the ON tissues were significantly decreased by 3.6-, 3.2- and 2.8-fold, respectively, in the ω-3 PUFA–treated group compared with those of the PBS-treated group ($P < 0.05$).

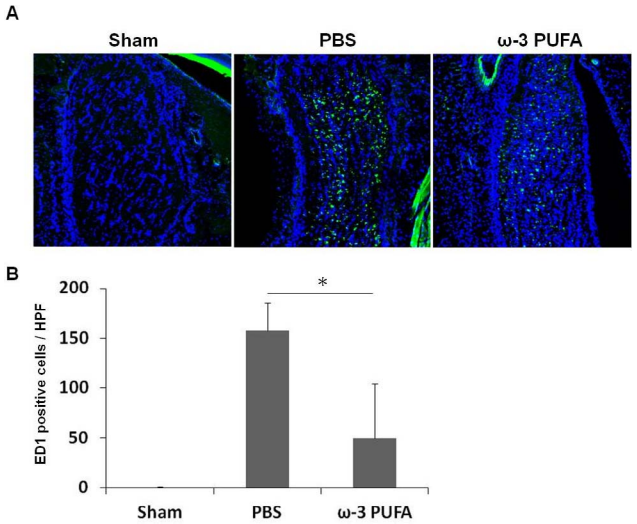

Figure 6. Immunohistochemistry of ED1 in ONs at 4 weeks after rAION induction for evaluating inflammatory infiltration. (**A**) The representative figures display ED1 staining in the longitudinal sections of the ON. The ED1-positive cells in *green* were labeled with FITC, and the nuclei in *blue* were stained with DAPI. (**B**) Quantification of ED1-positive cells per high-power field. The data are expressed as the mean ± SD in each group ($n = 6$). The macrophage recruitment was decreased 3.17-fold in the ω-3 PUFA–treated group compared to the PBS-treated group ($P = 0.008$). The *asterisk* indicates $P < 0.05$ using the Mann-Whitney *U* test.

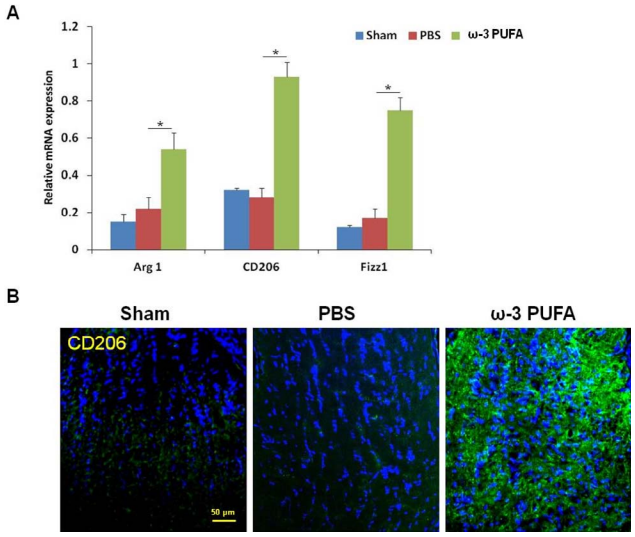

FIGURE 7. Evaluation of M2 macrophage polarization in the rAION model. (**A**) Relative mRNA expression levels of the markers of M2 macrophages in the ONs are shown as histograms. Each value was normalized to *GypA*. The statistical analysis indicated that the expression of *Arg 1*, *CD206*, and *Fizz1* (markers of M2 macrophages) increased after treatment with ω-3 PUFAs compared to treatment with PBS ($P < 0.05$). (**B**) Immunohistochemistry of CD206 in the ONs at 4 weeks after rAION induction for evaluating M2 macrophage polarization. CD206 was highly expressed in the ON following treatment with ω-3 PUFAs compared to the PBS treatment and sham control. The *asterisk* indicates $P < 0.05$ using the Mann-Whitney *U* test.

DISCUSSION

Our findings analyzed herein suggest that the administration of ω-3 PUFAs has a neuroprotective effect in rAION, as evidenced by both RGC morphometry and visual functional preservation. Treatment with ω-3 PUFAs preserved the survival of RGCs by reducing the apoptotic RGCs post infarct. In addition, ω-3 PUFA supplementation preserved the visual function, as evidenced by FVEP. Dietary ω-3 PUFA supplementation is reported to have a protective effect against cerebral and retinal ischemic injury.[42–45] In these cases, ω-3 PUFA administration prevents the decrease of electroretinogram a- and b-wave amplitudes following transient retinal ischemia.[42,43] Furthermore, increasing dietary omega-3 has beneficial effects across the retina, with the greatest improvement occurring in ganglion cell function.[45]

Inflammation has been shown to play a major early role following ON infarct.[12,13,31,46–48] Dietary supplementation with ω-3 PUFAs has been of great interest in the prevention of inflammation.[49] Oral ω-3 PUFA supplementation (EPA and DHA) for 3 months can inhibit the cytokine expression of IL-1β, TNF-α, and interleukin-6 (IL-6) in the blood mononuclear cells of both young and older women.[50] A reduced production of IL-1β, TNF-α, and IL-6 was also observed in the plasma of C57BL/6 mice following 5-week dietary supplementation with EPA and DHA in a preclinical study.[51] Similar data were published by Wallace et al.,[52] who reported that C57BL/6 mice fed for 6 weeks on dietary fats (fish oil) demonstrated decreased production of TNF-α and IL-1β by macrophages. In our observations, treatment with ω-3 PUFAs reduced macrophage recruitment at the ON site after rAION induction. Previous studies also demonstrated that ω-3 PUFAs can protect from BBB disruption after hypoxic–ischemic brain injuries.[23,24] In addition, one study demonstrated that ω-3 PUFA treatment may protect the BBB by inhibiting matrix metalloproteinase (MMP) production and activity.[24] The ω-3 PUFAs have shown the ability to inhibit inflammation-induced MMP-9 production by reducing nuclear factor (NF)-κB– and AP-1–mediated MMP-9 gene transcription.[53,54] In our previous study, we also found that the ON vascular permeability was highly increased by rAION induction in the first 2 days post infarct.[13] Thus, treatment with ω-3 PUFAs in rats with rAION may reduce macrophage infiltration into the ON site via prevention of BOB disruption.

One mechanism recently invoked to explain the anti-inflammatory effects of ω-3 PUFAs is their ability to guide the polarization of macrophages from the proinflammatory to the proresolving M2 phenotype.[22] The activation of peroxisome proliferator–activated receptor gamma (PPARγ) is considered to be a critical step in monocyte polarization toward the M2 phenotype, and the M2-macrophage polarizing effect of DHA was recently related to its ability to function as a natural ligand for PPARγ.[22,55] Furthermore, the ability of two metabolic products of DHA, namely, maresin 1 and resolvin D1 (ReVD1), in promoting the switch of macrophage M1 to the proresolving M2 phenotype has been recently reported.[56,57] Thus, treatment with ω-3 PUFAs may induce these beneficial effects by altering M2 macrophage polarization during the inflammatory

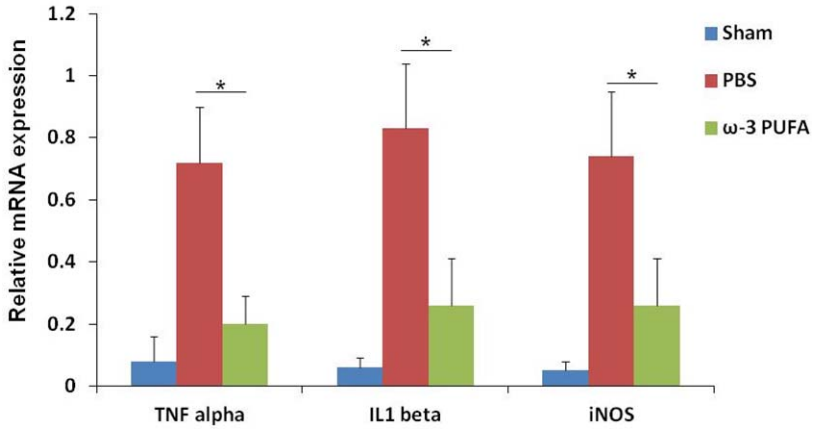

FIGURE 8. The relative mRNA expression levels of *TNF-α*, *IL-1β*, and *iNOS* in the ON tissues. Each value was normalized to GypA. The data are expressed as the mean ± SD in each group ($n = 6$ in each group). The statistical analysis indicated that the relative mRNA levels of TNF-α, IL-1β, and *iNOS* were reduced after treatment with ω-3 PUFA compared to PBS treatment ($P < 0.05$). The *asterisk* indicates $P < 0.05$ using the Mann-Whitney *U* test.

response following an ON infarct. Our observations demonstrated that ω-3 PUFA supplementation could alter the M1 macrophage to a M2 macrophage, with a subsequent reduction in proinflammatory *TNF-α*, *iNOS*, and *IL-1β* expression. Proinflammatory cytokines are highly increased in the ON and may cause further ON injuries in ischemic optic neuropathy.[12,13,46,47,58] Thus, a reduction in the level of proinflammatory cytokines can prevent cytokine-induced ON injuries.

The significance of monitoring the AA/EPA ratio is correlated to the fact that lower levels of PUFAs in either the circulating blood or the retina are associated with certain retinopathies. In particular, eyes from AMD donors exhibit significantly decreased levels of very long-chain PUFAs and high ω-6/ω-3 ratios.[59] A lower AA/EPA ratio is required for reducing proinflammatory eicosanoids and cytokines.[25] Therefore, examining systemic biomarkers may be a good indication of the disease progression and treatment success, such as AA/EPA to protect RGCs from death; the optimal AA/EPA for rAION requires further investigation.

It is possible that earlier administration of ω-3 PUFAs may have better rescue effects in rAION. The limitation of this study is that we did not investigate the best therapeutic dosage of ω-3 PUFAs in this rAION model. This important issue needs a well-designed study to compare different dosages and administration durations.

In conclusion, these novel findings provide insight into the role of inflammation and its involvement in the pathogenesis of rAION, indicating that ω-3 PUFA administration has neuroprotective effects via the dual actions of the antiapoptosis of RGCs and the anti-inflammation of the ON. Through decreasing inflammatory cell infiltration in the ON and regulating macrophage polarization, ω-3 PUFAs can decrease the cytokine-induced inflammation of the ON. Our results may foster strategies to treat patients with NAION.

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