Enteral Arg-Gln Dipeptide Administration Increases Retinal Docosahexaenoic Acid and Neuroprotectin D1 in a Murine Model of Retinopathy of Prematurity

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PURPOSE. Low levels of the long chain polyunsaturated fatty acid (LCPUFA) docosahexaenoic acid (DHA) have been implicated in retinopathy of prematurity (ROP). However, oral DHA suffers from poor palatability and is associated with increased bleeding in premature infants. We asked whether oral administration of the neutraceutical arginine-glutamine (Arg-Glu) could increase retinal DHA and improve outcomes in a mouse model of oxygen-induced retinopathy (OIR).

METHODS. Postnatal day 7 (P7) pups were maintained at 75% oxygen for 5 days and then returned to room air on P12. Pups were gavaged twice daily with Arg-Gln or vehicle from P12 to P17 and eyes were harvested for analysis on P17. Vaso-obliteration and vascular density were assessed on retinal flat mounts and preretinal neovascularization was assessed on retinal cross sections. Retinas were used for measurement of DHA and 10,17S-docosatriene (neuroprotectin D1, NPD1), a key DHA-derived lipid, and for analysis by reverse-phase protein array (RPPA).

RESULTS. With Arg-Gln treatment, retinal DHA and NPD1 levels were increased in OIR pups. Arg-Gln reduced preretinal neovascularization by 39 ± 6% (P < 0.05) relative to vehicle control. This was accompanied by a restoration of vascular density of the retina in the pups treated with Arg-Gln (75.0 ± 3.0%) compared to vehicle (53.1 ± 3.4%; P < 0.05). Arg-Gln dipeptide restored OIR-induced signaling changes toward normoxia and was associated with normalization of insulin-like growth factor receptor 1 signaling and reduction of apoptosis and an increase in anti-apoptosis proteins.

CONCLUSIONS. Arg-Gln may serve as a safer and easily tolerated nutraceutical agent for prevention or treatment of ROP.

Keywords: neutraceuticals, oxygen-induced retinopathy, retinal proteomic analysis, arg-gln dipeptide, docosahexaenoic acid
Dipeptidases cleave Arg-Gln once the peptide enters the circulation.\textsuperscript{5} Stress, common in premature infants in neonatal intensive care units, can induce amino acid deprivation, specifically arginine and glutamine deficiency.\textsuperscript{6,7} Deprivation of retinal glutamine increases the expression of VEGF\textsuperscript{8-10} a potent retinal angiogenic factor. Supplementation of glutamine in low-birth-weight infants is safe as shown by multicenter trials using either intravenous or enteral glutamine supplementation.\textsuperscript{6,11} Arginine supplementation in low-birth-weight infants results in a reduction in necrotizing enterocolitis likely due to improved intestinal blood flow mediated by increased local nitric oxide production.\textsuperscript{12} In this study, we sought to determine whether Arg-Gln has beneficial effects when given by the enteral route as would occur for use in infants and, if so, the potential mechanism for this effect. We found that enteral treatment of dipeptide prevented high oxygen–induced vaso-obliteration and pathologic neovascularization while promoting vascular regrowth in the oxygen-induced retinopathy (OIR) model. These changes were accompanied by an increase in retinal levels of docosahexaenoic acid (DHA), the major omega-3 polyunsaturated fatty acid (PUFA) in the retina that plays a significant role in maintaining the health of the retina.

**Materials and Methods**

**Mouse Model of Oxygen-Induced Retinopathy**

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The Institutional Animal Care and Use Committees of the University of Florida and of Indiana University approved animal procedures. C57BL6/J timed-pregnant mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). The mice were housed in the University of Florida Health Science Center Animal Care facilities or in the Indiana University Laboratory Animal Resource Center at Indiana University–Purdue University (Indianapolis campus). In the neonatal mouse model of OIR, 7-day-old mice were placed with their nursing dams in a 75% oxygen atmosphere for 5 days. On postnatal day 12 (P12) and continuing through postnatal day 17 (P17), mouse pups received twice-daily gavage feedings (20 \(\mu\)L). The types of gavage feedings included vehicle (0.9% sodium chloride), Arg-Gln dipeptide (1, 2.5, or 5 g/kg body weight per day as a hydrochloride salt; Bachem, Babendorf, Switzerland), or the ethyl ester form of DHA (2.5 g/kg body weight per day). For each type of gavage feeding, we used three litters of pups. On average, a litter consists of six pups. Therefore, each data point represents a minimum of one eye each from six pups and was repeated three times. On the fifth day after return to normoxia (P17), the animals were euthanized. Additional controls included nongavaged pups that underwent the OIR model and pups maintained at normoxia.

Selected eyes were removed and fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned as previously described.\textsuperscript{13} Other eyes were prepared for quantitative retinal flat-mount analysis. These mice at the time of euthanasia were perfused with FITC-labeled dextran (MWt = 2 \times 10^{6}) (Sigma-Aldrich Corp., St. Louis, MO, USA) to visualize the vasculature. Eyes were then enucleated, incubated in 4% formaldehyde, and then in PBS. Neural retina was dissected from the RPE-choroid-sclera complex. Four to seven radial cuts were made in the retina to allow the retina to lie flat for imaging using confocal microscopy (MRC-1024 Confocal Laser Scanning System; Bio-Rad, Hercules, CA, USA).

**Determination of Periretinal Neovascularization in the OIR Model**

Serial sections (6 \(\mu\)m) of whole eyes were cut sagittally, and approximately 150 serial sections were cut from each eye. Between two and four sections on each side of the optic nerve, 30 to 90 \(\mu\)m apart, were counted for neovascularization as described by Smith et al.\textsuperscript{13} We excluded cross sections that included the optic nerve because of the normal vessels emanating from the optic nerve. Vascular cell nuclei, identified under light microscopy with hematoxylin staining, were considered to be associated with new vessels if they were found on the vitreal side of the internal limiting membrane. Masked observers counted preretinal nuclei. Efficacy of treatment was calculated as the percentage of average of nuclei per section in the eyes of treated animals versus control animals.\textsuperscript{5}

**Determination of Retinal Vaso-Obliteration in the OIR Model**

The FITC-labeled dextran retinal flat mounts were used to determine the relative levels of vaso-obliteration. TIF images of the retinal flat mounts were used to determine the areas of the retina and of the vaso-obiterated regions using ImageJ.\textsuperscript{14}

**Determination of Retinal Vascular Density in the OIR Model**

The FITC-labeled dextran retinal flat mounts were used to determine the relative levels of vascular density. The retina was divided arbitrarily into 12 sectors, akin to the 12 hours of a clock. Images were taken in 10 of the 12 sectors. TIF images of the retinal flat mounts were used to segment each retina into central retina, midperipheral retina, and peripheral retina regions. Each image was overlaid with a grid of equally spaced squares using Adobe Photoshop V5.0 (Adobe Systems, San Jose, CA, USA) with the number of grids for each region ranging from two to six.\textsuperscript{15} Fields of view selected for analysis in peripheral retinas included regions of capillary-sized vessels directly adjacent to radial arterioles, whereas areas selected for analysis in central and midperipheral retinas included the radial arterioles. The actual grid was superimposed onto each image. The intersections of the grid with the dextran-labeled vasculature were shown as yellow dots. The mean vascular density incidence was determined for each area and compared to its control. The data are presented as means \(\pm\) SD.\textsuperscript{16}

**Tissue Fatty Acid Analysis by Reverse-Phase–High-Performance Liquid Chromatography (RP-HPLC)**

An aliquot of total lipids from neural retina was saponified (0.4 N KOH in 80% methanol, 50°C for 1 hour). Saponified fatty acids were acidified and extracted with diethyl ether according to Wang et al.\textsuperscript{17} and stored in methanol containing 1 mM butylated hydroxytoluene. Saponified free fatty acids were fractionated and quantitated by RP-HPLC using a YMC J-Sphere (ODS-H80) column (Allentown, PA, USA) and a sigmoidal gradient starting at 86.5% acetoniitile + acetic acid (0.1%) (Sigma-Aldrich Corp.) and ending at 100% acetoniitile + acetic acid (0.1%) over 50 minutes with a flow rate of 1.0 mL/min using a Waters Corporation 600 controller (Milford, MA, USA). Fatty acids were introduced to the HPLC by injection in methanol and detected using ultraviolet absorbance and evaporative light scatter as previously described.\textsuperscript{17} Authentic fatty acid standards (Nu-Chek Prep, NIC, Elysian, MN, USA) were used to generate calibration curves for verification and quantification of fatty acids.
Neuroprotectin D1 (NPD1) Analysis

Neural retinas were homogenized with glass homogenizer in cold methanol (total of 3 mL). After the homogenization, 6 mL cold CHCl₃ was added. Internal standard mixture (AA-d₈, EPA-d₁₅, ILTH-d₁₅, 15HETE-d₈, and PGD₂-d₄) (Cayman Chemical, Ann Arbor, MI, USA) was added, then sonicated. Samples were centrifuged and pellets were collected for protein measurement. Supernatant was mixed with pH 3.5 water, and the pH of aqueous phase was adjusted. After discarding upper phase, the bottom phase was dried and resuspended in 40 µL methanol : H₂O = 1:1 for mass spectrometry. Xevo TQS equipped with Acquity 1 Class UPLC (Waters Corporation) was used for lipidomics. Acquity UPLC HSS T3 1.8-µm 2.1 × 50-mm column was used for separation of fatty acids and their derivatives. Forty-five percent of solvent A (H₂O + 0.01% acetic acid) and 55% of solvent B (methanol + 0.01% acetic acid) with 0.4 mL/min flow were used initially, followed by gradient to 15% of solvent A for the first 10 minutes, then gradient to 2% of solvent A at 18 minutes. Two percent of solvent A ran for 25 minutes, then gradient back to 45% of solvent A for re-equilibration for 30 minutes. The capillary voltage was −2.5 kV, desolvation temperature at 600°C, desolvation gas flow at 1100 L/h, cone gas at 150 L/h, and nebulizer pressure at 7.0 bar with the source temperature at 150°C. Mass Lynx 4.1 software (Waters Corporation) was used for operation and recording of the data. Mass spectrometry data were analyzed and calculated using Excel (Microsoft Corp., Redmond, WA, USA).

Proteomic Analysis

For protein expression assays, retinas from each pup were disrupted in the TissueLyser LT (Qiagen, Germantown, MD, USA) in 200 µL lysis buffer provided by the Functional Proteomics Reverse Phase Protein Array (RPPA) Core Facility (M.D. Anderson Cancer Center, Houston, TX, USA): 1% Triton X-100, 50 mM HEPES pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 100 mM NaF, 10 mM Na pyrophosphate, 1 mM Na₃VO₄, 10% glycerol, and freshly added protease and phosphatase inhibitors (Roche Diagnostics Corporation, Indianapolis, IN, USA). After pelleting the debris, the protein concentration was determined with the Pierce BCA microplate procedure (Thermo Fisher Scientific, Waltham, MA, USA) on a Synergy H1 plate reader (BioTek, Winooski, VT, USA), adjusted to ~1 µg/µL and sent to RPPA Core for proteomic analysis using their standard procedures (https://www.mdanderson.org/research/research-resources/core-facilities/functiona-proteomics-rppa-core.html; in the public domain).

Statistical Analysis

All results are expressed as the mean ± SEM. The values were processed for statistical analyses by ANOVA followed by t-test assuming unequal variances using statistical analysis software (SPSS, Chicago, IL, USA). Differences were considered statistically significant at P < 0.05. The number of animals is indicated in the figure legends, with a minimum of five mice per group.

Results

The Arg-Gln Dipeptide Significantly Reduced Pre-retinal Neovascularization in the OIR Mouse Model

Gavage with the Arg-Gln dipeptide showed a dose-dependent reduction in pre-retinal neovascularization similar to the intraperitoneal injection of Arg-Gln with the maximum dose of 5 g/kg body weight/day, resulting in the largest reduction in preretal neovascularization (39 ± 6%; P < 0.05 relative to vehicle control) (Fig. 1). The maximal dose of 5 g/kg per day was used in all subsequent studies.

In this model, body weight can affect the formation and regression of retinal neovascularization. However, no difference was noted as the average weight of the OIR controls (pups treated with vehicle) was 7.16 g ± 0.4, while the average weight of the pups in the experimental cohorts was 6.62 g ± 0.8 (P = 0.198).

Arg-Gln Dipeptide Significantly Reduced Vaso-oblitration in the OIR Mouse Model

Treatment with the Arg-Gln dipeptide showed a significant reduction in vaso-oblitration compared to vehicle-treated mice (Fig. 2). The average level of vaso-oblitration in vehicle-treated mice was 28 ± 4% (Fig. 2B) when compared to normoxic pups (Fig. 2A), whereas treatment with the Arg-Gln dipeptide significantly reduced vaso-oblitration to 6.7 ± 5% (P = 0.03; Fig. 2C). Areas outlined in yellow represent areas of vaso-oblitration. Figure 2D shows the graphical summary of these results.

Retinal Vascular Density in the OIR Model Is Significantly Higher in Mice Treated With the Arg-Gln Dipeptide

Retinal flat mounts from FITC-dextran-perfused mice are shown in Figures 3A through 3C. The OIR vehicle-treated pups demonstrated a large area with loss of vasculature (Fig. 3A) compared to the normoxia control (Fig. 3C), pups not undergoing OIR. Treatment with the dipeptide markedly increases vascular regrowth (Fig. 3B). A graphical summary of all the vascular density area represented as a percent of the analysis of preretinal neovascularization levels in the OIR mouse model following gavage treatments. The numbers in brackets refer to gavage doses in g/kg body weight/day. *Indicates P values < 0.05 compared to vehicle (n = 11 for vehicle, n = 18 for dipeptide concentration, n = 6 for normoxia).
Retinal DHA and NPD1 Levels Are Restored to Normal in the OIR Model by Treatment With Arg-Gln Dipeptide

In the neural retina of the mouse pups, we examined whether the dipeptide could change levels of DHA, the omega-3 PUFA that is concentrated in phospholipids of synaptic membranes, retinal pigment epithelium cell (RPE), and photoreceptors. In normoxic mice the molar percentage (mol%) of DHA in the neural retina was 25.4 ± 3.2% (Fig. 4A). In the OIR model, mice treated with vehicle demonstrated a significantly lower mol% of DHA of 16.7 ± 2.6% (P < 0.05). DHA levels were restored to normoxic levels by treatment with Arg-Gln dipeptide (23.4 ± 1.6%; P > 0.2, when compared to normoxic). DHA mediates its protective effects in part by increasing generation of neuroprotectants. A specific mediator generated from DHA that contributes largely to its
biological significance in the retina is 10,17S-docosatriene (neuroprotectin D1, NPD1). In normoxic (non-OIR) mice, the level of NPD1 in the neural retina was set at 100\% (Fig. 4B). The level of NPD1 in OIR mice was 356\% (P < 0.05). NPD1 level in Arg-Gln–treated mice was 3150\% (P < 0.05).

DHA Supplementation of ROP Pups Results in Reduction of Preretinal Neovascularization, Reduction in Vaso-Obliteration, and Increase in Retinal NPD1 levels

We next asked whether directly supplementing mouse pups with DHA by gavage administration would result in a similar beneficial effect as we observed with the dipeptide. Treatment with DHA shows a reduction in preretinal neovascularization of 49\% (P < 0.05) (Fig. 5A).

DHA treatment resulted in a reduction in vaso-oblation (representative retinal flat mount in Fig. 5B; graphical summary in Fig. 5C) compared to vehicle-treated control (Fig. 2A) and comparable to dipeptide-treated mice (Fig. 2C). The average level of vaso-oblation in vehicle-treated mice was 28\% whereas treatment with DHA resulted in a significant reduction in vaso-oblation to 6.3\% (P = 0.02). There was no significant difference in the level of vaso-oblation between the mice treated with Arg-Gln (6.7\%) or DHA (6.3\%). However, unlike the dipeptide, DHA did not have a beneficial effect in restoring retinal vascular density when compared to vehicle controls (Figs. 5D, 5E; central: vehicle = 30.8\% ± 22.7\%, DHA = 46.4\% ± 13.7\%, P value = 0.06; midperiphery: vehicle = 68.4\% ± 3.8\%, DHA = 78.6\% ± 5.7\%, P value = 0.1; periphery: vehicle = 81.7\% ± 5.3\%, DHA = 84.9\% ± 4.6\%, P value = 0.4).

In normoxic mice the mol% of DHA was 25.4\% (Fig. 5F). In the OIR model, mice treated with vehicle demonstrated...
levels of caveolin-1, Axl, Bcl-3d, Mnk1, and WIP12 toward normal (shown in green in Table).

DHA treatment resulted in a reduction in the expression of the autophagy protein LC3A toward normoxia and reductions of phosphor-iboylaminimidazole succinocarboxamide synthase (PAICS) and RPA32 and increases of E-cadherin and p70S6K1 toward normoxia levels (shown in blue in Table).

Some retinal proteins upregulated by the OIR model (XRCC1, collagen VI, JNK2, ATM, IRb, Pdc6b, and PI3K) were downregulated by either the dipeptide or DHA. Some proteins downregulated by the OIR model were increased by both the dipeptide and DHA, including Akt, eEF2K, HSP27, HSP70, MSH6, c-Jun, and merlin (shown in beige in Table). The complete series of proteins examined in the retina of the OIR pups are shown in Figure 6 (higher expression in Fig. 6A or lower expression Fig. 6B).

**DISCUSSION**

Currently, there are limited safe pharmacologic options for the treatment of ROP. Discovery of a new, easily affordable treatment and preventative strategy, such as the Arg-Gln dipeptide described in this study, may be a highly relevant approach of global interest. Treatments such as systemic administration of propranolol or ocular administration of antivegf are associated with side effects in premature infants. Intravenous propranolol has been associated with arrhythmias. Intraocular anti-VEGF has been associated with systemic inhibition of VEGF for up to 8 weeks after a single injection. The effects of systemic VEGF inhibition on other developing vascular beds such as the lungs are unknown.

Treatment failure and adverse events such as persistent avascular retina, retinal detachment, and blindness have also been reported. While studies have aimed to decrease intraretinal concentrations of growth factors such as VEGF levels, other treatment strategies have supported the need to safely increase levels of the growth factor, insulin-like growth factor-1 (IGF-1), to improve VEGF signaling and promote physiological revascularization in premature infants in need of oxygen therapy.

Our studies support that the Arg-Gln dipeptide serves to increase endogenous DHA production in a safe and beneficial manner for premature infants. Massive maternal-fetal transfer of long chain polyunsaturated fatty acids (LCPUFAs) occurs during the third trimester of a healthy pregnancy; thus preterm infants prevented DHA levels from declining in plasma, allowing generation of cerebral phospholipids needed for maturation of visual acuity and cognitive function.

Administration of fish oil to preterm infants from the first day of life showed a significantly lower incidence and severity of ROP and risk of laser therapy in the treated group. A randomized controlled trial demonstrated that either breast milk or formula supplemented with LCPUFAs when given to premature infants prevented DHA levels from declining in blood, allowing generation of fetal phospholipids needed for maturation of visual acuity and cognitive function.

In agreement with Connor et al. we show that DHA levels are reduced in the retina in the OIR pups from 25 mol% in normal pups to 17 mol% at P17, and this lower level of retinal DHA levels is associated with an increase in central retinal obliteration and an increase in neovascularization. The retina contains the highest concentration of DHA of all tissues, where it is cytoprotective and in particular neuroprotective.
FIGURE 5. DHA given by gavage reduces preretinal neovascularization and vaso-obliteration but not vascular density in the OIR mouse model. (A) Graphical summary of the analysis of preretinal neovascularization levels in the OIR mouse model following gavage treatments of DHA (n = 15) and vehicle. *Indicates P values < 0.05 compared to vehicle. (B) Representative retinal flat mount from FITC-dextran–perfused mouse undergoing OIR and treated with vehicle. Scale bar: 1 mm. (C) Graphical representation of reduction in vaso-obliteration with treatment of pups with DHA via gavage in the OIR model. *Indicates a P value < 0.05 (n = 8). #Indicates a P value > 0.05 (n = 8). (D) Representative retinal flat mount from FITC-dextran–perfused DHA-treated OIR mouse showing reduced vascular density suggesting that, unlike the dipeptide-treated OIR mice, DHA does not promote vascular regrowth. Scale bar: 0.2 mm. (E) Graphical summary of the analysis of retinal vascular density in the mice undergoing OIR and gavage treatments with vehicle or DHA (n = 5) compared to mice maintained under normoxia. *Indicates P values < 0.05 compared to normoxia. (F) Retinal DHA levels in the pups receiving DHA or vehicle compared to normal room air controls. (G) Retinal NPD1 levels in the pups receiving DHA (n = 6) or vehicle (n = 6) compared to normal room air controls (n = 6). The yellow line in (B) delineates the areas of retinal vaso-obliteration. *Indicates P values < 0.05 compared to normoxia.
A concentration of DHA in membranes of retinal photoreceptors and neurons is indicative of the importance of DHA in membrane-associated functions. DHA is required for the regeneration of rhodopsin and signal transduction in photoreceptor cells. LCPUFAs derived from DHA are required to maintain the highly curved edges of the photoreceptor discs of the outer segment. Not surprisingly, levels of DHA are reduced in not only ROP but also in other ocular diseases such as diabetic retinopathy and age-related macular degeneration. When considering DHA therapeutically, several limitations exist. DHA is unstable and has a short shelf life. Furthermore, it is not tolerated by some infants, resulting in reduced feeding because of difficulty in camouflaging the flavor of DHA. Use of DHA has been associated with increased bleeding at the time of teething.

Intraperitoneal injection of the Arg-Gln dipeptide at 5 g/kg body weight reduced neovascularization by 52 ± 6% whereas oral Arg-Gln dipeptide reduced neovascularization by 61 ± 6% as would be the preferred route in preterm infants. Feeding the OIR pups with Arg-Gln dipeptide restores DHA to normoxic levels by P17. The magnitude of the dipeptide effect was similar to that of direct DHA supplementation, and both the dipeptide and the DHA supplement restored retinal DHA levels.

**Figure 6.** Cluster of proteins that have altered expression in the OIR model. Retinas of cohorts of DHA-treated (n = 6) and Arg-Gln-treated OIR mice are compared to saline-injected OIR pups (n = 6). (A) Proteins with higher expression in OIR control (saline-injected pups) compared to both Arg-Gln and DHA treatments, which reduce their expression often more in the Arg-Gln group. Red: upregulation; green: downregulation. (B) Proteins that have lower expression in OIR control (saline-injected pups) while both Arg-Gln and DHA treatments increase their expression, often more in the DHA group. Red: upregulation; green: downregulation.
to that seen in healthy normoxic pups that did not undergo the OIR model. However, some important caveats need to be considered. Vasoobliteration and neovascularization were similarly prevented by Arg-Gln and DHA, but vascular density was restored only by Arg-Gln. These differences in the effects of Arg-Gln and of DHA are likely not due to differences in the levels of retinal DHA or NPD1, as levels were higher in the DHA-treated pups compared to the dipeptide-treated mice. This also suggests that the beneficial effects of the dipeptide cannot be exclusively due to the changes in DHA and NPD1. While we do not have a mechanism accounting for the increase in capillary regrowth in the dipeptide-treated mice, our results would suggest that these events are independent of changes in LCPUFA and NPD1.

Interestingly, NPD1 is increased in the retina of OIR pups treated with the vehicle, compared to normoxia. This highlights the role of NPD1 biosynthesis as an adaptive response of the retina to stress. NPD1 homology 6 (MSH6), Jun proto-oncogene, AP-1 transcription factor subunit (c-Jun).

Table 1: Proteomic Analysis of Retina From Normoxia Pups, OIR Pups, and Pups Undergoing Gavage With the Arg-Gln Dipeptide or DHA

Table

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<th>Protein</th>
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<th>P Value</th>
<th>DP</th>
<th>P Value</th>
<th>DHA</th>
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<td>Conexin-43</td>
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In summary, oral administration of Arg-Gln is associated with increases in endogenous retinal DHA and NPD1 production. However, the beneficial effect observed with use of the dipeptide cannot be explained solely on the increase in DHA or NPD1, as exogenous administration of DHA did not result in increased capillary regrowth. This important difference suggests that dipeptide treatment likely modulates unique signaling pathways that are not influenced by exogenous DHA supplementation. Thus, our findings suggest that oral treatment with the dipeptide Arg-Gln to premature infants may represent a safe and highly beneficial therapy for prevention and treatment of ROP.

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