

The Dipeptide Arg-Gln Inhibits Retinal Neovascularization in the Mouse Model of Oxygen-Induced Retinopathy

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PURPOSE. Premature infants undergoing intensive care are highly vulnerable to amino acid deprivation. Supplementation of glutamine or arginine has resulted in beneficial effects in human neonates. This study was conducted to examine the effect of the dipeptide arginyl-glutamine (Arg-Gln) on vascular endothelial cell growth factor (VEGF) levels in primary human retinal pigment epithelial (hRPE) cell cultures and on inhibition of neovascularization in the oxygen-induced retinopathy (OIR) model.

METHODS. The effects of Arg-Gln on VEGF levels were measured in supernates from hRPE cells by using ELISAs. For in vivo studies, mouse pups received twice-daily intraperitoneal injections of Arg-Gln, a control dipeptide (Ala-Gly) or were not injected. Retinal flatmounts from one cohort were prepared and retinal vessel morphology examined. The contralateral eyes were embedded, sectioned, and stained to count preretinal neovascular nuclei. RNA was isolated from retinas of selected animals and was used to quantify VEGF mRNA by real-time RT-PCR.

RESULTS. Treatment of hRPE cells with Arg-Gln decreased VEGF levels in a dose-dependent manner. In the OIR model, Arg-Gln at 5 g/kg per day reduced preretinal neovascularization by 82% \pm 7% ($P < 0.005$), when compared with the control dipeptide Ala-Gly, and reduced VEGF mRNA by 64% \pm 9% ($P < 0.001$).

CONCLUSIONS. Arg-Gln dramatically inhibited retinal neovascularization in the OIR model. This effect was associated with a reduction in retinal VEGF mRNA levels. Similarly the dipeptide reduced VEGF expression in hRPE cells, a cell type likely to respond to retinal hypoxia by expressing VEGF. Arg-Gln appears to be safe and, with future studies in human infants, may prove beneficial in the prevention of ROP. (*Invest Ophthalmol Vis Sci.* 2006;47:3151-3155) DOI:10.1167/iov.05-1473

Vascular retinopathies are the leading causes of blindness in the Western world, with retinopathy of prematurity (ROP) being the major cause of blindness in children under the age of seven.^{1,2} Salient pathologic features are neovascularization of

the retinal vascular endothelium with edema and breakdown in the blood-retinal barrier (BRB). These changes lead to hemorrhage, tissue damage, and retinal scarring, which ultimately lead to vision loss and, in the most severe cases, blindness. Panretinal laser coagulation surgery, which has major drawbacks, is currently the only option for delaying blindness in infants with vascular retinopathies.³⁻⁵

There are no preventive or early intervention modalities of treatment available for retinopathy of prematurity or for diabetic retinopathy, the leading cause of blindness in adults of working age.⁶ The outcome of these diseases may be greatly impacted if a safe therapy was made available early in the course of retinal ischemia and neovascularization.

One of the proposed mechanisms for the pathogenesis of ROP includes overproduction of the angiogenic growth factors including VEGF,⁷ resulting in vasoconstriction; poor blood flow; and, ultimately, retinal ischemia. Nutritional factors may play a major role in regulation of these putative mechanisms. Recent studies of retinal pigment epithelial cells in culture have demonstrated that glutamine deprivation results in a dramatic elevation of VEGF.⁸ In studies of mammary epithelium, glutamine deprivation increased both VEGF and IL-8, a potent neutrophil chemoattractant, and these same changes were evident with arginine deprivation.⁹ In addition, several studies in animals suggest that glutamine supplementation reduces inflammation.^{10,11}

Premature infants undergoing intensive care are also frequently deprived of both arginine and glutamine^{12,13} because of stress. They are unable to maintain endogenous synthesis of these conditionally essential amino acids, making these infants highly vulnerable to glutamine and arginine deprivation.

Glutamine and arginine supplementation have both been shown to be safe in low-birth-weight infants. Recent multicenter trials of intravenous and enteral glutamine supplementation have demonstrated safety.^{14,15} A recent randomized study of arginine supplementation in low-birth-weight infants demonstrated a decreased incidence of necrotizing enterocolitis (NEC).¹⁶ It has been speculated that this effect is secondary to improved blood flow to the microvasculature of the intestine via increased local nitric oxide production through the L-arginine-nitric oxide synthase pathway.

The concept of combining arginine and glutamine as a dipeptide (Arg-Gln) stems from two lines of reasoning. First, this dipeptide combination obviates the decomposition of aqueous glutamine into the cyclic product associated with ammonia liberation and improves its limited solubility in water (35 g/L H₂O at 20°C).^{17,18} Second, dipeptides are better absorbed than single amino acids.¹⁹ The use of stable dipeptides shows great promise as a method for provision of glutamine, which is otherwise difficult to deliver.^{17,18} Because of the low intake of these amino acids in premature infants and the in vitro studies demonstrating high VEGF and IL-8 output from cells deprived of glutamine and/or arginine,^{8,9} we hypothesized that the endoplasmic stress system is particularly responsive to these amino acids. Furthermore, we postulated that

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VEGF homeostasis in the neonatal retina is dependent on maintenance of these amino acids within a suitable range. To evaluate the efficacy of Arg-Gln, we administered it in dose-dependent fashion in a mouse model of retinopathy of prematurity, the OIR model developed by Smith et al.,^{7,20} and retinal neovascularization and VEGF production were examined. There is ample evidence showing that retinal pigment epithelial cells are a source of VEGF in ischemic retina.²¹⁻²⁹ Thus, we examined glutamine-deprived human cell cultures to evaluate whether Arg-Gln affects VEGF levels. Treatment of hRPE cells with Arg-Gln decreased VEGF levels. In the OIR model, Arg-Gln dramatically reduced preretinal neovascularization, and this reduction corresponded with reduced retinal VEGF mRNA levels.

METHODS

Cell Culture

Human eyes were obtained from the National Disease Resource Interchange (Philadelphia, PA) within 36 hours of death ($n = 4$ donors), in accordance with the provisions of the Declaration of Helsinki for research involving human tissue. hRPE cells were prepared and maintained as previously described.^{30,31} For cell culture experiments, hRPE from passages 3 to 5 were used. All tissue culture media were purchased from Mediatech, Inc. (Herndon, VA). hRPE cultures were placed in glutamine-free medium for 24 hours and then were exposed to 0, 0.5, 1, 2.5, or 5 mg/mL of dipeptide for 48 hours.

VEGF ELISA

VEGF protein concentration was determined on cultured hRPE cells by ELISA (Quantikine Human VEGF Immunoassay ELISA kit; R&D Systems, Minneapolis, MN). Cells were grown to confluence to form monolayers and were kept in either the full medium containing FBS, for an untreated control, or with glutamine-free DMEM (Cellgro, Herndon, VA) with serum extender (MITO+; R&D Systems). The cells were serum starved for 72 hours, during which time the cells had the serum extender in the media. After 72 hours of serum starvation, dipeptide was added to the cells at a concentration of 0.5 mM for 48 hours, and the supernatants were collected. Total protein from all the treated wells was harvested and used to normalize the VEGF ELISA results. Total protein concentration was measured with a protein assay (Pierce Biotechnologies, Rockford, IL). VEGF protein levels in the supernatants were determined with the VEGF ELISA (R&D Systems). Each experiment was performed in duplicate ($n = 2$), and each data point was performed in triplicate, according to the manufacturer's protocol.

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. Animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Florida. C57BL6/J timed-pregnant mice were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were housed in the University of Florida Health Science Center Animal Care facilities. In the neonatal mouse model of oxygen-induced retinopathy, 7-day-old mice were placed with their nursing dams in a 75% oxygen atmosphere for 5 days.²⁰ Mouse pups received twice daily intraperitoneal injections (50 μ L) starting on postnatal day 12 (P_{12}) and continuing through postnatal day 17 (P_{17}). In one experiment, injections included vehicle (0.9% sodium chloride) and the test compounds Ala-Gly (5 g/kg per day), Arg-Gln dipeptide (5 g/kg per day as a hydrochloride salt; Bachem, Babendorf, Switzerland); and, in a second experiment, different doses of Arg-Gln (1.0, 2.5, or 5 g/kg per day) were tested. For each type of injection, we used one or two 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. After the fifth day after return to normoxia (P_{17}), the animals were euthanized

by injection of a lethal dose of a combination of ketamine (70 mg/kg body weight) and xylazine (15 mg/kg body weight) followed by cervical dislocation. The eyes were removed and fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned as previously described.²⁰ Preretinal nuclei were counted by masked observers. Efficacy of treatment was calculated as the percentage of average of nuclei per section in the eyes of Arg-Gln-treated animals versus control animals. For total RNA isolation from retina the animals were killed and the eyes removed. The retina were then dissected from the eye and stored in preservative (RNAlater buffer; Ambion, Austin, TX) at 4°C for subsequent isolation of RNA.

Some of the eyes were taken for qualitative retinal flatmount analysis. At the time of euthanasia, these mice were perfused with FITC-labeled dextran to visualize the vasculature. The eyes were enucleated and incubated in 4% formaldehyde and then in PBS. The neural retina was dissected from the RPE-choroid-sclera complex and flatmounted with four to seven radial cuts and examined and photographed separately by confocal microscopy (MRC-1024 Confocal Laser Scanning System; Bio-Rad, Hercules, CA).

Real-Time RT-PCR

For VEGF mRNA measurements, total RNA was isolated from mouse retina (TRIzol reagent; Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. For each experiment, RNA from at least six mouse retinas was pooled to generate one data point. The cDNA was synthesized using either 2 or 4 μ g of total RNA and reverse transcription reagents (*TaqMan*; Applied Biosystems, Inc. [ABI], Foster City, CA) in a 100- μ L RT reaction. Real-time PCR analysis (*TaqMan*; ABI) was applied with 1 μ L cDNA per reaction and sequence detection (SYBR Green PCR Core Reagents and the model 5700 Sequence Detection System; ABI). At the end of the PCR cycle, a dissociation curve was generated to ensure the amplification of a single product, and the threshold cycle time (C_t) for each gene was determined. Relative mRNA levels were calculated based on the C_t and normalized to β -actin. The level of VEGF mRNA determined for the injection of vehicle was set to 100%. These experiments were performed using the mouse VEGF primer pair (catalog RDP-73-025; R&D Systems) and the primer pair (QuantumRNA β -actin Internal Standards, catalog 1720; Ambion).

Data Analysis

All data represent the mean \pm SEM. ANOVA was used to evaluate differences among groups, and individual comparisons were made with Student's *t*-tests with the Bonferroni correction. $P < 0.05$ was considered significant.

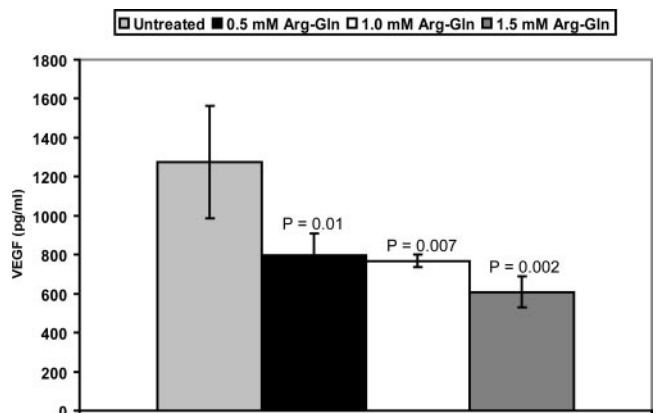


FIGURE 1. Effects of dipeptide on VEGF secretion in hRPE cells. In comparison to basal VEGF expression, exposure to the dipeptide resulted in a statistically significant dose-dependent decrease in VEGF expression.

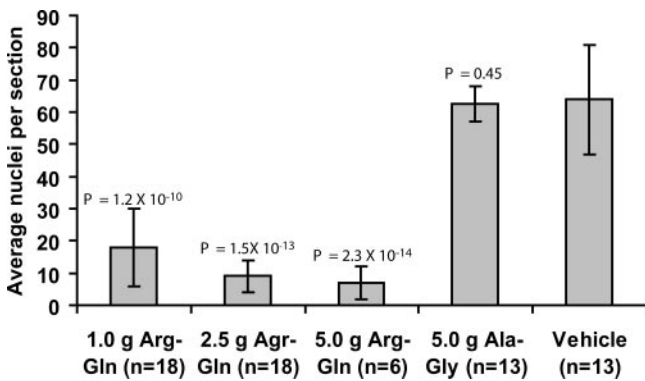


FIGURE 2. Dose effect of the Arg-Gln dipeptide on retinal proliferation. Even at the lowest concentration tested (1 g/kg per day), there was a significant reduction in preretinal nuclei. At the highest dose tested, there was an ~80% reduction. All dipeptide-treated samples showed significant decreases in preretinal nuclei. The Ala-Gly peptide showed no decrease in preretinal nuclei when compared to vehicle.

RESULTS

Effect of Arg-Gln Concentration and Exposure Time on VEGF Production by hRPE Cells

Human RPE cells were exposed to various concentrations of Arg-Gln for 48 hours. In comparison to basal VEGF expression, exposure to the dipeptide resulted in a statistically significant decrease (reduced by $52.3\% \pm 6.2\%$ for 1.5 mM Arg-Gln, $P = 0.002$) in soluble VEGF expression into the culture medium (Fig. 1).

Effect of Arg-Gln Administration on OIR

Five days of treatment of mouse pups with various doses of Arg-Gln (1.0, 2.5, and 5.0 g/kg per day) resulted in a significant reduction in preretinal nuclei. At the highest dose tested, there was an ~80% reduction. All dipeptide-treated samples showed significant decreases in preretinal nuclei ($P < 0.00001$; Fig. 2). The control dipeptide, Ala-Gly showed no significant reduction in preretinal nuclei when compared with vehicle-treated pups ($P = 0.45$).

Retinal flatmounts were prepared from Arg-Gln-treated pups and compared with those of the control vehicle-treated pups. The fluorescently labeled (FITC) high-molecular-weight dextran delineated the vasculature and demonstrated that the

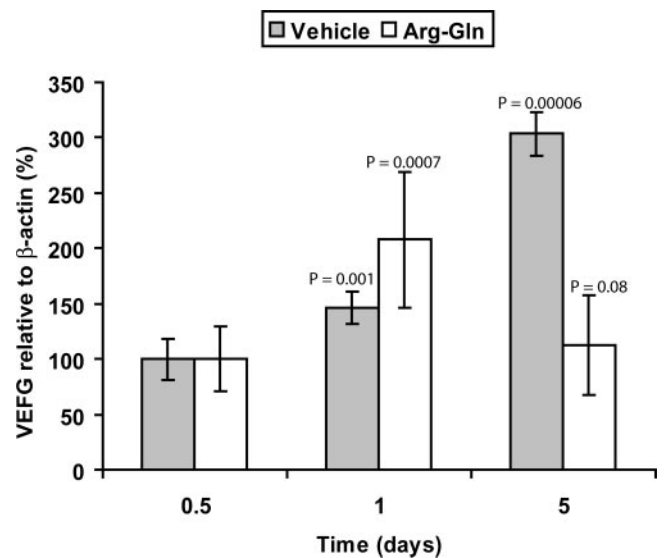


FIGURE 4. Effect of the dipeptide on VEGF mRNA. Eyes enucleated at 0.5, 1, and 5 days after removal from high oxygen ($n = 14$ eyes for each data point). Vehicle-treated animals were compared to dipeptide-treated animals. In the vehicle and dipeptide-treated animals, there was an increase in VEGF mRNA during the period examined. However, there was a significant reduction in VEGF mRNA at day 5 in the dipeptide-treated mice compared with the control.

retinal vessels from the Arg-Gln-treated pups had reduced neovascular tufts and vascular leakage compared with the control samples (Fig. 3).

Effect of Arg-Gln Administration on VEGF mRNA in OIR Mice

In the dipeptide-treated animals (Fig. 4), there was a clear increase in the VEGF mRNA at day 1 ($207\% \pm 61\%$; $P = 0.0007$) compared with vehicle at day 0.5 ($100\% \pm 29\%$; $P = 0.0007$). At day 5 in the dipeptide-treated pups, there was a significant reduction in the VEGF mRNA ($112\% \pm 44\%$; $P = 0.08$) returning VEGF levels to that of vehicle on day 0.5 ($P = 0.08$). VEGF production in vehicle-treated pups increased at day 1 ($P = 0.001$) and day 5 ($P = 0.00006$) compared with vehicle at day 0.5. $P < 0.05$ was considered significant.

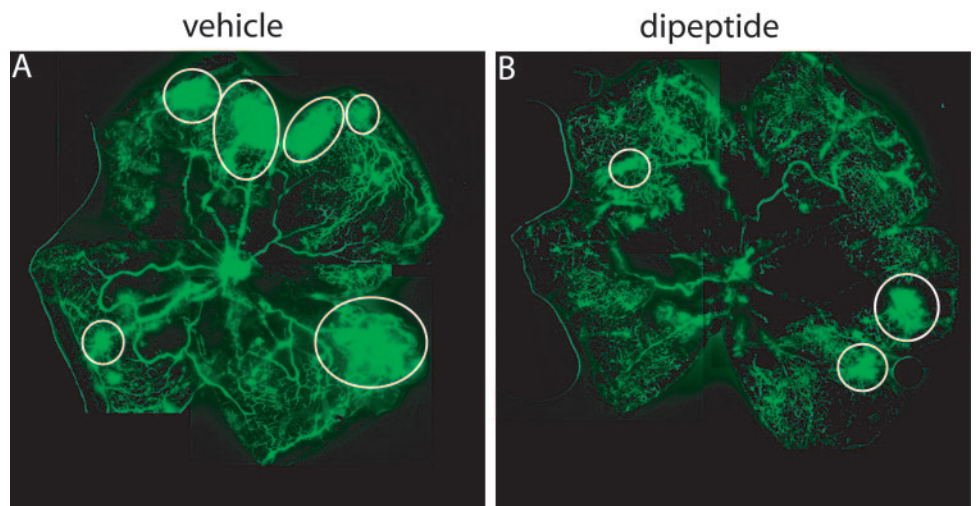


FIGURE 3. Arg-Gln dipeptide reduced leakage from neovascular vessels. Retinal flatmounts from a vehicle-treated animal (A) and an Arg-Gln-treated animal (B) showed a decrease in leaky neovascular tufts (circle) in the retina of Arg-Gln-treated mice.

DISCUSSION

The major findings of the study were the dramatic reduction of preretinal neovascularization in the OIR model. Five days of treatment with Arg-Gln reduced preretinal neovascularization by $82\% \pm 7\%$ ($P < 0.005$) when compared with vehicle injected and control noninjected eyes. We also examined the levels of VEGF mRNA expression in the retina in the OIR model. Pierce et al.,³² found that VEGF mRNA levels drop by 55% 24 hours after placement of mice into high oxygen. Simpson et al.,³³ showed in the OIR model that the level of the VEGF mRNA approximately doubles within 24 hours after removal from high oxygen. Our results extend these results and show a continued increase of VEGF mRNA levels up to 5 days after removal from high oxygen. Our results also demonstrate that treatment with the dipeptide, beginning immediately after removal from high oxygen, reduced VEGF mRNA levels by $64\% \pm 9\%$ ($P < 0.001$). We next examined the effect of the dipeptide on VEGF production by the hRPE cells. Treatment of hRPE with Arg-Gln decreased VEGF levels, supporting a direct effect on RPE function. These results suggest that by reducing the levels of the key growth factor VEGF, the dipeptide may have an effect on both angiogenesis and BRB permeability.

Although it is well appreciated that ischemia leads to VEGF expression, amino acid deprivation may also be a critical component in this pathologic process. Abcouwer et al.⁸ previously demonstrated that in retinal pigment epithelial cell cultures, VEGF mRNA levels increased in response to glutamine deprivation by greater than 10-fold compared with physiologically relevant glutamine concentrations.⁸ The half-life of VEGF mRNA was also increased 2.5-fold by glutamine starvation.⁸

Several nutritional agents have already been shown to be beneficial in neonates at risk for ROP. The relationship between sugar inositol intake, serum inositol levels, and ROP severity has been examined in low-birth-weight infants receiving various concentrations of inositol.³⁴ After 30 days, infants with higher serum inositol levels at birth and receiving high inositol formula showed a significantly lower incidence of severe ROP than infants with lower serum inositol levels receiving the lower inositol formula. More recently, the effects of inositol supplementation on respiratory distress syndrome were examined.³⁵ A significant reduction in ROP of any stage was observed after addition of inositol to the infant diet.

Vitamins E and A have also been evaluated in trials of premature infants in the prevention of ROP. In very-low-birth-weight infants, vitamin E supplementation reduced the risk of severe retinopathy and blindness among those examined, but increased the risk of sepsis.³⁶ A meta-analysis of three studies examining the effects of Vitamin A supplementation on ROP suggested a trend toward reduced incidence.³⁷

In a series of studies similar to the one reported herein, the antiangiogenic agent, squalamine was given during the evolution of OIR in the mouse to determine whether it could improve retinal neovascularization.³⁸ Squalamine doses were begun at various times after OIR induction. In this 17-day model, there was improvement in the degree of blood vessel tuft formation, blood vessel tortuosity, and central vasoconstriction after squalamine treatment on day 15 or 16. Single-dose squalamine at day 12 was effective at reducing subsequent development of retinal neovascularization at doses as low as 1 mg/kg. Thus, squalamine appeared to be an active inhibitor of OIR in mouse neonates at doses as low as 1 mg/kg given once. Furthermore, squalamine, given late in the course of OIR, improved retinopathy by inducing regression of retinal neovascularization and abrogating the invasion of new vessels beyond the inner-limiting membrane of the retina.

Despite these studies, none of these agents is currently being used routinely for the specific prevention of ROP. It is likely that the mechanisms of action of vitamins A and E would be through their antioxidant activity, whereas the mechanism of squalamine is poorly understood, but it may be acting via interconversion of arginine and/or glutamine, a phenomenon known to occur.³⁹

In one study, glutamine deprivation increased VEGF and IL-8 mRNA expression in mammary adenocarcinoma and other breast cancer cell lines cultured under both normoxic and hypoxic conditions.⁹ Of all amino acids tested, glutamine had the largest effect on VEGF and IL-8 mRNA expression. Arginine was the second most critical of the amino acids that resulted in VEGF and IL-8 responses. This leads to a hypothetical framework as to how the individual amino acids of Arg-Gln dipeptide may alter proliferative retinopathy and potentially prevent retinal damage.

This study is the first to demonstrate the beneficial effects of a nutraceutical agent, Arg-Gln dipeptide, in inhibiting retinal angiogenesis. The proposed mechanism is a reduction of VEGF expression *in vivo*. The data also demonstrate that Arg-Gln decreased VEGF expression in a cell culture from a cell type known to produce growth factors in the ischemic retina. This finding supports the beneficial effect of the dipeptide on maintaining the function of the anterior and posterior BRB by reducing VEGF, a factor known to be key in this dysfunction.

It is likely that the Arg-Gln dipeptide would be safe when provided to low-birth-weight human infants because both arginine and glutamine have been administered individually in previous studies with no ill effects. For example, arginine supplementation has been demonstrated to decrease necrotizing enterocolitis in low-birth-weight infants.¹⁶ Single-center studies of glutamine supplementation in low-birth-weight infants have demonstrated decreased hospital-acquired sepsis,⁴⁰ an amino acid profile that suggests less catabolism, and decreased hospital costs.⁴¹ Multicenter trials have not supported a beneficial effect on sepsis, but a trial of enteral glutamine supplementation showed a decrease in intraventricular hemorrhage and periventricular leukomalacia.^{14,15} Studies of glutamine supplementation in adult bone marrow transplant recipients,⁴² trauma patients,⁴³ and burn patients⁴⁴ have also demonstrated beneficial effects.

The benefits of administering the dipeptide (as opposed to individual amino acids) to neonates are many. A greater quantity of dipeptide can be administered in a smaller volume because of better solubility of the dipeptide compared with the individual amino acids. This volume consideration may be critical in infants who are highly volume sensitive—in particular, low-birth-weight infants with lung disease. Oligopeptides are better absorbed than individual amino acids.^{45,46} Finally, the osmolality of the dipeptide is lower than if each of the amino acids is administered individually.

In summary, the development of a safe and noninvasive therapy for treatment of ROP is needed because the current therapies of laser photocoagulation and cryotherapy are destructive. Arginyl-glutamine is a novel dipeptide with the potential to be used as a nutritional adjunct in critically ill infants at high risk of ROP. Additional studies to evaluate further the mechanisms of action in cell cultures and safety and efficacy in animals as well as studies pertaining to mode of administration (oral, versus parenteral, versus ocular application) are necessary before trials are initiated in human infants.

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