·Basic Research·

Effect of the dipeptide Arg-Gln on retinopathy of prematurity in mice

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Received:2009-02-26 Accepted:2009-07-04

Abstract

• AIM: To investigate the effect of the dipeptide Arg-Gln on retinal neovascularization of retinopathy of prematurity (ROP) in the oxygen-induced retinopathy (OIR) animal model.

• METHODS: Forty-eight 7-day-old C57BL/6J mice were exposed to 750mL/L oxygen for 5 days and then to normal situation to produce the murine model of oxygen-induced retinopathy (OIR). All mice received twice daily intraperitoneal injections of PBS or the dipeptide Arg-Gln (1.0, 3.0, 5.0g/kg per day), starting on postnatal day 12 and continuing till postnatal day 17. Experimental groups (36 mice, 12 in each group) received Arg-Gln, while the control group (12 mice) received PBS. All mice were executed at postnatal day 17. The changes of retinal vessels of mice were observed by ADPase histochemical technique and HE staining was used to count preretinal neovascular nuclei. RNA was isolated from retinas of 28 mice (7 in each group) selected at random and VEGF mRNA level of each group was measured by real-time RT-PCR.

• RESULTS: Neovascularization reduced in retinas of the dipeptide Arg-Gln treated group in a dose-dependent manner. Compared with control group, experimental group had diminished non-perfusion area and neovascular tufts in retinal flatmount. The number of the endotheliocyte nuclei of new vessels extending from retina to vitreous was significantly less in the eyes of the experimental group than in control group. Arg-Gln at 5g/kg per day reduced preretinal neovascularization by about 75% (P <0.01). There was a significant reduction in VEGF mRNA at the 17th day in Arg-Gln treated group compared with control group(P < 0.01).

• CONCLUSION: Arg-Gln dramatically inhibits retinal angiogenesis in OIR and this effect is associated with a reduction in retinal VEGF mRNA level. It appears to be a safe way to prevent and treat some neovascular retinal diseases including retinopathy of prematurity.

• KEYWORDS: retinal neovascularization; arginine-glutamine; retinopathy of prematurity; oxygen-induced retinopathy

Wang AY, Chai J, Chen XL. Effect of the dipeptide Arg-Gln on retinopathy of prematurity in mice. *Int J Ophthalmol* 2009;2(3):250-252

INTRODUCTION

With improved survival of very low-birth-weight infants in China, retinopathy of prematurity (ROP) is emerging as a significant problem which is the major cause of blindness in children at present [1]. The common pathologic features of ROP are ischemia-induced retinal neovascularization. These changes lead to hemorrhage, tissue damage, and retinal scarring, which ultimately lead to vision loss and blindness. At present, retinal laser photocoagulation and cryotherapy appear to be the most effective treatment for retinal neovascularization [2,3]. However, these procedures can destroy postmitotic retinal neurons and permanently affect visual function. It is important to find safe preventive measures or early intervention modalities of treatment for ROP. In this study, we examined the effect of Arg-Gln on animal models of ROP.

MATERIALS AND METHODS

Mouse Model of Oxygen-induced Retinopathy Mouse model of oxygen-induced retinopathy (OIR) was produced in C57BL/6J mice by a method described by Smith *et al*[4]. Forty-eight seven-day-old (postnatal day 7) mice and their mothers were placed in an airtight incubator and exposed to an atmosphere of 750±50mL/L oxygen for 5 days. Incubator temperature was maintained at 23±2℃. Then they were returned to room air at postnatal day 12. At the 12th day, 36 mice in experimental group received twice daily intraperitoneal injections of the dipeptide Arg-Gln (1.0, 3.0, 5.0g/kg per day, each group consists of 12 mice), starting on postnatal day 12 and continuing till postnatal day 17, and 12 mice in control group received twice daily intraperitoneal injections of PBS at the same time.

Observation of Retinal Neovascularization by ADPase Histochemical Technique On the 17th day, 8 mice (2 mice in each group) were anesthetized with either an intramuscular injection of ketamine(80mg/kg) and xylazine (15mg/kg). To evaluate vessel morphology, all eyes were removed and fixed with 40g/L paraformaldehyde in phosphate-buffered saline overnight. The cornea, lens, and vitreous were surgically removed and retinas were dissected. Retinas were processed for magnesium-activated adenosine diphosphatase (ADPase) staining as previously described by Lutty and McLeod [5]. ADPase-stained retinas were flatmounted on microscope slides with gelatin-coated cover slips. The vasculature was then examined under microscopy.

Quantification of Retinal Neovascularization On the 17th day, 12 mice (3 mice in each group) were sacrificed and the eyes were enucleated, immersed in 40g/L paraformaldehyde in PBS for at least 24 hours, and embedded in paraffin. Serial 6μm sections from whole eyes were cut sagittally parallel to the optic nerve and stained with hematoxylin and eosin according to a standard protocol. The extent of neovascularization was determined by counting neovascular cell nuclei extending through the internal limiting membrane into the vitreous. All counting was done based on a masked protocol. For each eye, 10 intact sections of equal length, each 30μm apart, were evaluated. The mean number of neovascular nuclei per section per eye was then determined.

VEGF mRNA Detected by RT-PCR On the 17th day, 28 mice (7 mice in each group) were sacrificed and retinas were then dissected from the eye. Total RNA was isolated from mouse retina (TRIzol reagent; Invitrogen) according to the manufacturer's protocol. The cDNA was synthesized by using 2μg of total RNA and reverse transcription reagents (DRR037, TakaRa) in a 50μL RT reaction. Real-time PCR analysis was applied with 1μL cDNA per reaction (SYBR PrimeScript RT-PCR kit DRR041, TakaRa). 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 (Ct) for each gene was determined. Relative mRNA levels were calculated based on the Ct and normalized to β-actin. The level of VEGF mRNA determined for the injection of PBS was set to 100%. These experiments were performed using the mouse VEGF primer pair and the primer pair (D3751β-actin internal standards, TakaRa).

Statistical Analysis All data were rpresented as the mean± SD. ANOVA was used to evaluate differences among groups. $P < 0.05$ was considered to be significant.

RESULTS

ADPase Histochemical Technique in Retinal Flatmounts The pattern of vascular development and neovascularization was seen readily in retinal flat-mounts by ADPase histochemical technique. The retinal vascular patterns in the mice of control group were characterized by the neovascular tufts and avascular area, a typical pattern of pathological retinal neovascularization. The retinal vessels from the Arg-Gln-treated mice had reduced avascular area and neovascular tufts in a dose-dependent manner compared with control group (Figure 1).

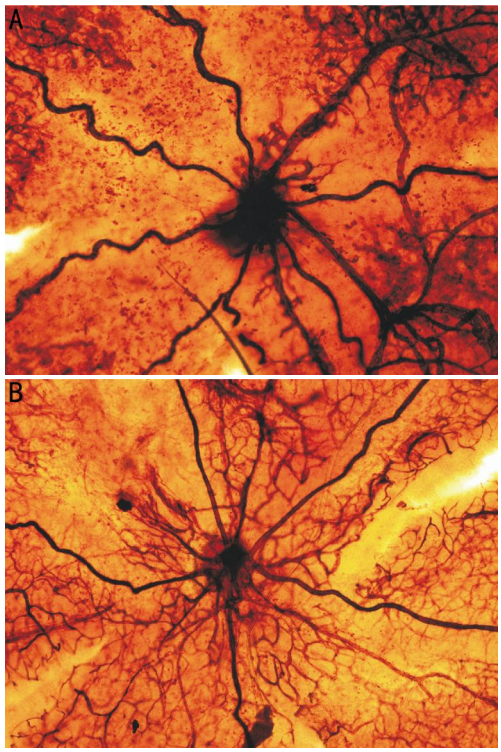

Figure 1 The pattern of vascular development in retinal flat-mounts by ADPase histochemical technique A:indicates retinal flat-mount of control group; B:indicates retinal flat-mount of experimental group. Compared with control group, the experimental group had reduced avascular area and neovascular tufts

Tissue Slice of HE Staining Five days of treatment of mice with various doses of Arg-Gln (1.0, 3.0, and 5.0g/kg per day) resulted in a significant reduction in preretinal nuclei. At the highest dose tested, there was an about 75% reduction. All dipeptide-treated samples showed significant decreases in preretinal nuclei compared with control group ($P < 0.01$, Figure 2).

Effect of Arg-Gln Administration on VEGF mRNA in OIR Mice There was a significant reduction in VEGF mRNA at the 17th day in Arg-Gln treated group in a dose-dependent manner compared with control group ($P < 0.01$). At the dose of 5.0g/kg per day, there was an about 68% reduction in the level of VEGF mRNA (Figure 3).

DISCUSSION

Many studies indicate that one of the proposed mechanisms for the pathogenesis of ROP includes overproduction of the

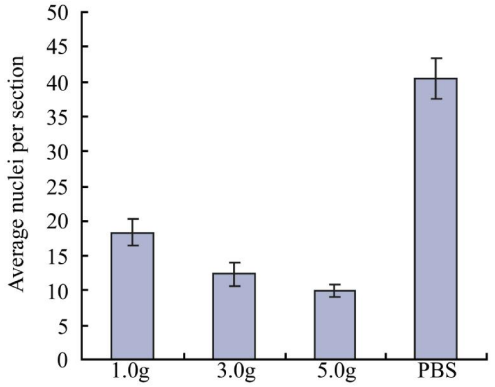

Figure 2 Dose-effect of the Arg-Gln dipeptide on retinal proliferation Even at the lowest concentration tested (1g/kg per day),there was a significant reduction in preretinal nuclei. At the highest dose tested, there was an about 80% reduction. All dipeptide-treated samples showed significant decrease in preretinal nuclei

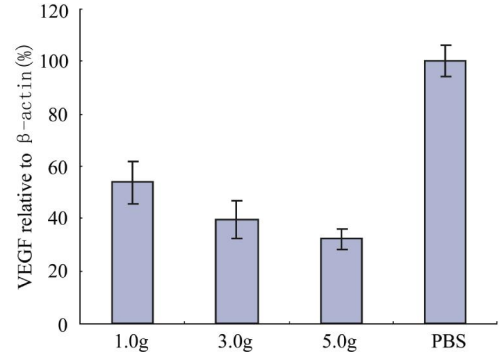

Figure 3 Effect of the Arg-Gln on VEGF mRNA There was a significant reduction in VEGF mRNA at the 17th day in Arg-Gln treated group in a dose-dependent manner compared with control group

angiogenic growth factors including VEGF [6,7], resulting in vasoconstriction, poor blood flow, and ultimately retinal ischemia. Nutritional factors may play a major role in regulation of these 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 shown to be safe in low-birth-weight infants [14,15]. Recent studies indicated that arginine and glutamine (Arg-Gln) can improve blood flow to the microvasculature via increased local nitric oxide production through the L-arginine-nitric oxide synthase pathway [16,17]. Supplementation of glutamine or arginine has resulted in beneficial effects in human neonates. This study is to demonstrate the beneficial effects of a nutraceutic agent, Arg-Gln dipeptide, in inhibiting retinal angiogenesis. The proposed mechanism is a reduction of VEGF expression *in vivo*. It may provide a safe way to prevent and treat some forms of proliferative retinopathies including ROP.

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