·Basic Research·

Bevacizumab treatment reduces retinal neovascularization in a mouse model of retinopathy of prematurity

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Abstract

• **AIM: To evaluate the effect of different bevacizumab concentrations on retinal neovascularization in a retinopathy of prematurity (ROP) mouse model.**

• **METHODS: A total of 60 of C57BL/6 J mice were exposed to 75% ±2% oxygen from postnatal d7 to postnatal d12. Fifteen nonexposed mice served as negative controls (group A). On d12, 30 mice (group C) were injected with 2.5 μg intravitreal bevacizumab (IVB), 30 mice (group D) were injected with 1.25 μg IVB in one eye. The contralateral eyes were injected with balanced salt solution (BSS) (control group =group B). The adenosine diphosphatase (ADPase) histochemical technique was used for retinal flat mount to assess the oxygen –induced changes of retinal vessels. Neovascularization was quantified by counting the endothelial cell proliferation on the vitreal side of the inner limiting membrane of the retina. Histological changes were examined by light microscopy. The mRNA levels of vascular endothelial growth factor (VEGF) were quantified by Real–time PCR. Western –blotting analysis was performed to examine the expression of P–VEGFR.**

• **RESULTS: Comparing with the control group B, regular distributions and reduced tortuosity of vessels were observed in our retinal flat mounts in groups C and D. The endothelial cell count per histological section was lower in groups C(P<0.0001) and D(P<0.0001) compared with the control group B. Histological evaluation showed no retinal toxicity in any group. In all oxygen treated groups VEGF mRNA expression was significantly increased as compared to age –matched controls. No significant change in VEGF mRNA expression could be achieved in either of the treatments or the oxygen controls. The results of the Western blot were consistent with that of the Real–time PCR analysis.**

• **CONCLUSION: An intravitreal injection of bevacizumab is able to reduce angioproliferative retinopathy in a mouse model for oxygen–induced retinopathy.**

• **KEYWORDS:** mouse; retinopathy of prematurity; retinal neovascularization; bevacizumab (Avastin); intravitreal injection
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INTRODUCTION

Retinopathy of prematurity (ROP) is a pathologic condition of the retina in which abnormal angiogenesis (called retinal neovascularization) can lead to devastating consequences, as a result of vitreal bleeding and tractional retinal detachment. The increased incidence of ROP is linked to the implementation of new neonatal care technologies that enhance the survival of very low birth weight infants, and the continued need for supplemental oxygen to maintain arterial oxygen tension in an appropriate range.

A mouse model of oxygen-induced retinopathy was established by Smith *et al*[1] that closely recapitulates many key aspects of ROP in human beings. The model entails exposure to hyperoxia during early retinal development leading to the arrest or retardation of normal retinal vascular development. When the animals are returned to the normoxic environment, they are under a relative hypoxic situation where the retina now lacks its normal vasculature that is required to adequately support the neural tissue in normoxic conditions. This ischemic situation results in unregulated, abnormal neovascularization[1].

Bevacizumab is a complete humanized murine monoclonal antibody against all isoforms of human vascular endothelial growth factor (VEGF). It has been approved by the Food and Drug Administration (FDA) for treatment of metastatic colorectal cancer. Intravitreal bevacizumab, in off-label use, is also of benefit in the treatment of many retinal diseases with intraocular vascular proliferation in patients with corneal neovascularization, ischaemic retinopathy, such as diabetic retinopathy, retinopathy of prematurity(ROP) and retinal vein occlusion [2-4]. VEGF has been implicated as the major

angiogenic stimulus responsible for the formation of intraocular neovascularization in these diseases, and drugs that inhibit the biological activity of VEGF represent a new paradigm in the treatment of intraocular neovascular diseases [5]. There are limited data indicating structural and ultrastructural effect of intravitreally administered bevacizumab on retinal cells and organelles [6-8]. The aim of our study was to investigate the effect of intravitreal-injected bevacizumab in different concentrations on retinal neovascularization in an oxygen-induced retinopathy (OIR) of prematurity mouse model. The OIR in the mouse, with vascular development similar to the human, with reproducible and quantifiable proliferative retinal neovascularization, is useful for study of pathogenesis of retinal neovascularization as well as for the study of medical intervention for ROP and other retinal vasculopathies[1].

MATERIALS AND METHODS

Materials All experimental mice were neonatal C57BL/6J mice, purchased from Animal Experimental Center of Science Academy (Shanghai, China). All animals were cared for in accordance with the Nanjing Medical University for Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Oxygen Exposure and Drug Treatment Protocol Newborn mice were randomly assigned to experimental and control groups. At P7 (postnatal d7), 60 pups in the experimental group were exposed to hyperoxia (75%±2%O_2) for 5d (P7 to P11) and then returned to normoxia (room air) for 5d. Oxygen concentration was measured with an Oxygen Monitor (Ceramatec Inc., China). A commercially available formulation of bevacizumab (Avastin; Genentech, Inc., South San Francisco, California, USA) was used.

For the ROP mouse model, we used doses of 2.5 μg and 1.25 μg bevacizumab, whereas 1.25 μg bevacizumab as being almost representative of the equivalent dosage of 1.25 mg bevacizumab given intravitreally in humans. For a 2.5 μg injection, 1 μL of the original suspension was injected. For 1.25 μg injection, 1 mL of the original suspension was diluted with 1 mL sterile saline and resuspended, then 1 μL of the new suspension was immediately injected into the vitreous. One microliter of bevacizumab was intravitreally injected with a 32-gauge needle under direct observation with a stereoscopic microscope, at the corneascleral junction, at the 6 o'clock position. The mice were deeply anaesthetized by means of an inhalation of aether. Intravitreal injections were performed on P12 by delivering of 1 μL bevacizumab into the vitreous cavity of the right eye. The contralateral eye received an intravitreal injection of 1 μL balanced salt solution (BSS) in the same position. Fifteen mice of the same age that had been kept in room air without exposure to high levels of oxygen were used as negative controls. On P17, the mice were killed and the eyes were enucleated.

On d12, 30 mice (group C, n=30 eyes) were injected with 2.5 μg intravitreal bevacizumab (IVB), 30 mice (group D, n=30 eyes) were injected with 1.25 μg IVB in one eye. The contralateral eyes were injected with BSS (control group, group B, n=60 eyes). Fifteen age-matched mice, kept in room air, were used as negative controls (negative control, group A, n=30 eyes). Enucleated eyes were examined on light microscopy (LM), (10 eyes for each group).

Quantitative Assessment of Retinal Neovascularization by Counting Vascular Lumens As our previous description, at P17, the eyes were removed, fixed in 4% paraformaldehyde for 24h, and embedded in paraffin [9]. Sagittal sections of 5 μm, each 30 μm apart, were cut through the cornea parallel to the optic nerve. The sections were stained with hematoxylin and eosin to assess retinal vasculature *via* light microscopy (Carl Zeiss, Chester, VA, USA). Neovascularizations were quantified by counting the endothelial cell proliferation on the vitreal side of the inner limiting membrane (ILM) per histological section of the retina in at least 10 sections from each eye by two independent observers blind to treatment. The average intravitreal vessels/section was calculated for each group. There were at least 10 eyes in each group. The difference of each group was compared. For quantitative assessment of retinal neovascularization, results are shown as the mean ± standard deviation (SD) count of the endothelial cell nuclei.

ADPase Histochemistry [10] Mice were deeply anesthetized with an inhalation of aether. Eyes were removed and fixed in 4% paraformaldehyde overnight at 4℃. Under a dissecting microscope, the retina was removed, and flat-mounted by making radial cuts. Retinas for flat mounting were processed by a modification of the lead sulfide technique. The retina was fixed overnight at 4℃ in 10% neutral buffered formal in (3.7% formaldehyde), washed in cold tap water, and then incubated for ADPase activity at 37℃ for 15min. The reaction solution contained 0.2 mol/L of TRIS maleate buffer (pH 7.2), 3 mmol/L of lead nitrate, 6 mmol/L of magnesium chloride, and 1 mg of ADP per milliliter. The retina was then rinsed five times in distilled water, and the reaction product was developed with 2% ammonium sulfide for 1min. After washing in distilled water, the retina was flat mounted in glycerogel. The deposition of ADPase reaction product was sometimes developed by exposing sections before staining to ammonium sulfide (Light Solution diluted 1:10 and incubated for 1min). Five retinas from both group A and group B and ten retinas from both group C and group D were processed by this method on P17. The flat-mounted retinas were visualized under a microscope (LEICA DMLS) and photographed by a digital camera(Olympus Camedia C-3030 Zoom).

Ribonucleic acid Extraction and Real-Time Polymerase Chain Reaction Eyes were enucleated from the mice at P17. Retinas were separated from eyeballs (n=10) on an iced plate and immediately frozen in liquid nitrogen until further use. Total RNA (Ribonucleic acid) was extracted from the frozen retina tissues using Trizol reagent according to the manufacturer's instructions. The total RNA concentrations from each group were determined by measuring the optical density at 260 and 280 nm using an ultraviolet light spectrophotometer. Aliquots of 20 μL RNA from each group were applied for production of cDNA. One microliter of cDNA from each group was amplified in 25 μL of reactive mixture with 0.25xSYBR Green Supermix (Molecular Probes). The following primers were used: VEGF, 5'AGACACGGTGGTGGAAGAAG 3' and 5'GGAAGATGA GGAAGGGTAAGC 3'; β-actin, 5'CCTCTATGCCAACAC AGTGC3' and 5'GTACTCCTGCTTGCTGATCC3'. With these primers the mRNA expression of VEGF, together with β-actin as calibrator were analyzed simultaneously in quadruple reactions. The PCR reaction was performed under the following conditions: an initial denaturation at 94℃ for 4min followed by amplification for 40 cycles (94℃ for 10s, 64℃ for 15s, and 72℃ for 20s). The final elongation step was performed for 5min at 72℃. Control β-actin PCR reactions were run simultaneously. Quantitative real-time PCR analysis was done using the LightCycler Detection System (Roche Diagnostics). Results are expressed as the ratio of the target gene to control gene for each sample.

Western Blot Analysis Western Blot was performed using standard Western blot methods. After homogenization and centrifugation, the supernatant was collected for total protein values. The amount of protein was measured with the BCA protein assay kit (KangChen KC-430). Fifty micrograms of protein from each sample was loaded on sodium dodecyl sulfoxide polyacrylamide gel electrophoresis (SDS-PAGE) and then proteins were transferred to polyvinylidence difluoride membranes at 60 mA for 1h. Antibody reaction was performed after blocking of nonspecific binding sites with 5% bovine serum albumin (BSA). The membranes after blocking were incubated overnight with the primary antibodies (anti-phospho-VEGFR, Cell Signaling Technology, Beverly, MA, USA) at 4℃. Then, membranes were washed with TBS and incubated with horseradish Peroxidase(HRP)-conjugated secondary antibody (anti-mouse antibody; Kang Chen, Shanghai, China) in blocking buffer for 1h at room temperature. After 3 washes, the proteins were visualized with enhanced chemiluminescence (Amersham) detection. To ensure the equal loading of protein in each lane, the blots were stripped and reprobed with an antibody against β-Actin. Experiments were repeated 3 times. Intensity values were normalized relative to control values. The blots were scanned using a flatbed scanner and the band intensity analyzed using the Image Jsoftware (NIH, USA).

Statistical Analysis Statistical analysis was performed using SAS 8.2 statistical software. The data were analyzed with descriptive statistics. The data were analyzed with t test, paired t test, ANOVA test or q test for paired figures. $P<0.05$ was considered statistically significant.

RESULTS

Retinal Flat-mount On P17, the normal untreated mouse (group A) retina is completely vascularized. No avascular area and no blood vessel tufts are present. The main vessels are straight and show no tortuosity. After oxygen treatment (group B) from P7 to P12 there are many avascular areas in the middle of the retina around the vessel origin, many blood vessel tufts, and most of the main vessels show different degrees of tortuosity. Treatment with bevacizumab(groups C, D) decreased the number of blood vessel tufts and changed the main vessel tortuosity significantly (Figure 1).

Quantification of Proliferative Retinopathy with and Without Intravitreal Bevacizumab Injection The degree of hyperoxia-induced neovascularization was quantified in serial paraffin sections by counting the endothelial cell nuclei on the vitreal side of the ILM. In group A (negative control group), there were no endothelial cell nuclei detected on the vitreal side of the ILM of the retina (Figure 2A). In group B (control group), the retina contained multiple neovascular tufts extending into the vitreous (Figure 2B). These tufts originated from the retinal vessels, forming clusters of immature endothelial cells. In this group, the mean ±SD number of endothelial nuclei anterior to the ILM was 23.15± 1.31 per histological section. While in the IVB-injected groups (groups C, D), the administration reduced these lesions greatly, the new blood vessels appeared sparse (Figure 2C, 2D). Endothelial cell nuclei counts were 1.70± 0.8 in group C (2.5 μg IVB), 1.83±0.75 in group D (1.25 μg IVB). Regarding endothelial cell nuclei counts groups C and D displayed significant differences compared with group B, the control group ($P<0.01$). Howere, there was no significant difference between groups C and D ($P>0.05$). Significant differences were obtained, when group A was compared with groups B ($P<0.01$). Compared with the control group (group B), endothelial cell nuclei counts were reduced by 93% in group C (2.5 μg IVB), 92% in group D (1.25 μg IVB) (Table 1).

Morphological Analysis Using Light Microscopy Morphological analysis of retinal layers using LM, neither IVB-injected groups (groups C, D) nor the control group (group B) and the negative control group (group A) reveal signs of cystic degeneration, hypocellularity or loss of the nuclear layer (Figure 2).

mRNA Expression of Vascular Endothelial Growth Factor by Real-Time Polymerase Chain Reaction Real-Time PCR analysis was performed to assess the mRNA levels of VEGF in the mice retinas (Table 2). In all oxygen

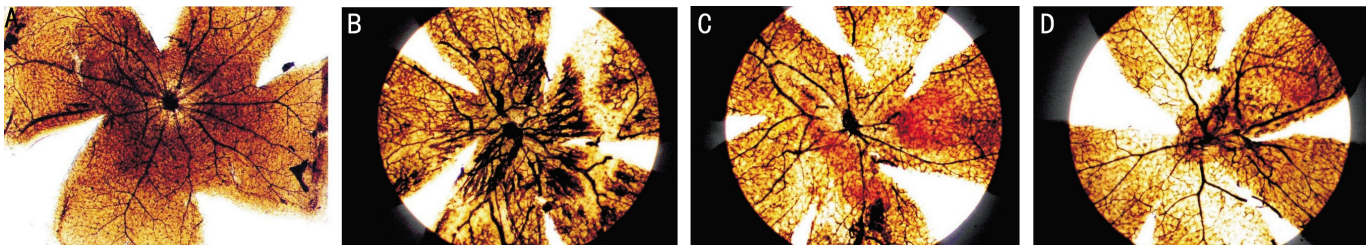

Figure 1 The flat-mounted retinas were visualized under a microscope (LEICA DMLS) and photographed by a digital camera (Olympus Camedia C-3030 Zoom), ×25, ADPase staining A: Retinal flatmount of group A on P17. Retinal vessels are regularly distributed, without obstruction; B: Retinal flatmount of group B. At the posterior of retinas, tortuous vessels and nonperfusion areas are showed; C: Retinal flatmount of group C. Nonperfusion areas are significantly reduced, with disappearance of new vessels; D: Retinal flatmount of group D. Retinal vessels, distribution are regular, without obstruction.

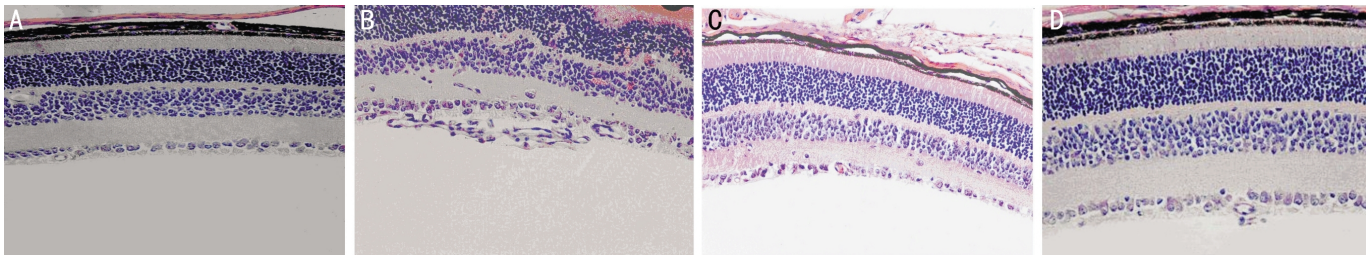

Figure 2 The average intravitreal vessels/section was calculated for each group (×200, H&E staining) A: Histological sections of the inner retina of group A. No vessel buds protruded into the vitreous; B: Histological sections of the inner retina of group B. New vessel buds are found protruding into the vitreous; C: Histological sections of the inner retina of group C. No vessel buds are found protruding into the vitreous; D: Histological sections of the inner retina of group D. Few vessel buds are found protruding into the vitreous.

Table 1 Average number of blood vessels lumen that passed the ILM on every sections of each group

Groups	Sections (pieces)	Blood vessel tufts ($\bar{x} \pm s$)
A	50	0.80±0.61
B	50	23.15±1.31
C	50	1.70±0.80
D	50	1.83±0.75

Table showing the count of neovascular cells [mean (SD)] on the vitreal surface of the inner limiting membrane of the retina with an experimental model of retinopathy of prematurity with C57BL/J6 mouse in each group.

Table 2 The expression of retinal VEGF mRNA in each group

Groups	β-actin	VEGF	VEGF/β-actin
A	0.00385±0.00013	0.00097±0.00002	0.25264±0.00377
B	0.00033±0.00002	0.00016±0.00003	0.47191±0.06146
C	0.00025±0.00002	0.00012±0.00001	0.48578±0.02282
D	0.00037±0.00001	0.00019±0.00002	0.49724±0.04866

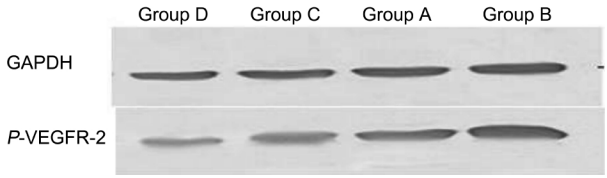

Figure 3 Western blot analysis for *P*-VEGFR in the retinas of experimental mice.

Table 3 Western blot analysis for *P*-VEGFR in the retinas of experimental mice

Groups	*P*-VEGFR/GAPDH
C	0.349054
D	0.530978
A	0.634097
B	1.266709

treated groups mRNA expression of VEGF was significantly increased as compared to age-matched controls (group A). The VEGF/β-actin expression in the group C was 0.48±0.02, while it was 0.47±0.06 in the group D (P>0.05). The highest VEGF/β-actin expression was in the group B at 0.49±0.04 compared with 0.25±0.01 in the group A (P<0.01). No significant differences in VEGF/β-actin expression levels were found in groups B, C and D (P>0.05) (Table 2).

Protein Expression of Phosphorylated Vascular Endothelial Growth Factor Receptor by Western Blot Analysis The protein expression of phosphorylated VEGFR in mice retinas from each group was studied by Western blot analysis (Figure 3, Table 3). In the normal group (group A), the protein expression of phosphorylated VEGFR was relatively strong. As for the untreated oxygen group (group B), the expression of this protein was significantly increased. However, the expression of this protein was markedly suppressed by the administration of intravitreal bevacizumab (P<0.05, group B compared with groups C and group D). No significant differences in protein expression of phosphorylated VEGFR were found in group C and D (P>0.05).

Western blot analysis for P-VEGFR-2 was performed. GAPDH was served as the loading control. Quantitative analysis was performed by measuring protein expression relative to the control. Figures were selected as representative

data from three independent experiments. Each value represents the mean±SD of three independent experiments.

DISCUSSION

As the most well known, retinal ischemia followed by tissue hypoxia is the most important cause of blindness including ROP, DR, and AMD. Neovascularization generated from VEGF plays an important pathogenic role in hypoxia-induced retinal damage. Retinal hypoxia first induces compensatory changes of blood flow, excessive expression of cytokines and angiogenesis. VEGF is an essential cytokine responsive to oxygen sensing mechanisms in the retina and regulates retinal compensatory changes, which could be however disorganized and adversely result in vascular leakage and neovascularization [11,12]. It has been shown that hyperoxic treatment followed by normoxic conditions lead to the development of retinal neovascularization, for which VEGF has been shown to be the predominant inducer [13]. Bevacizumab (Avastin), a humanized monoclonal antibody, was approved by the FDA in February 2004 for the treatment of metastatic colorectal cancer. It has been widely used as an off-label treatment for age-related macular degeneration and retinal vascular disorders [14-16]. Recently, some authors have reported intravitreal injection of bevacizumab for severe and advanced forms of ROP [17-21]. Ameri *et al* [22] evaluated the effect of intravitreous bevacizumab (1.25 mg) after single injection in the pigmented rabbit retinal neovascularization model and demonstrated suppression of endothelial cell growth and reduction in vascular permeability.Zhang *et al* [23] described significantly reduced neovascularization,which was assessed by counting the endothelial cell nuclei in the nerve fibre layer and ganglion cell layer in the C57BL/J6 mouse retina in OIR model after intravitreal injection of bevacizumab. Based on electrophysiological findings, bevacizumab was found to be nontoxic to the retina of rabbits and on the mouse retina and had no harm on retinal function [24,25]. Kim *et al* [26] observed no definite histological abnormalities after IVB injection at concentration 2.5 mg/mL in C57BL/6 mouse. Inan *et al* [27] reported normal retinal function and structure in electrophysiological investigation and in LM in rabbits after intravitreally injected bevacizumab at doses of 1.25 and 3.0 mg.

In the present study, treatment with bevacizumab (groups C, D) decreased the number of blood vessel tufts and changed the main vessel tortuosity significantly, and increased mRNA and protein expression of VEGF was observed in the untreated ROP group. The administration of bevacizumab markedly decreased these expressions, and resulted in reduced angiogenesis. We have shown that an IVB is able to reduce angioproliferative retinopathy in a mouse model for oxygen-induced retinopathy. Compared with the control group (group B), endothelial cell nuclei counts were reduced by 93% in group C (2.5 μg IVB), 92% in group D (1.25 μg IVB). Our data strengthen the idea that the mechanism does not involve a direct or indirect reduction of the VEGF mRNA level, but acts by binding to VEGF and blocking its interaction at the receptor on the surface of the endothelial cell. These data underline the potential utility of bevacizumab in oxygen-induced retinopathy and other hypoxic conditions of the retina. Morphological analysis of retinal layers using LM, neither IVB-injected groups (groups C, D) nor the control group (group B) and the negative control group (group A) did not reveal signs of cystic degeneration, hypocellularity or loss of the nuclear layer.

Further immunohistochemical and functional studies are necessary to determine toxic effects of intravitreal-injected bevacizumab, especially in different doses and after repeated injections. Testing of bevacizumab in the context of randomized experimental and clinical trials are warranted before definitive statements can be made on the safety and efficacy of bevacizumab in intraocular neovascular diseases.

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Conflicts of Interest: Feng F, None; **Cheng Y,** None; **Liu QH,** None.

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