

Erythropoietin receptor antibody inhibits oxidative stress induced retinal neovascularization in mice

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Abstract

• **AIM:** To observe the effect of erythropoietin receptor antibody (EpoRA) on oxygen-induced retinal neovascularization.

• **METHODS:** C57BL / 6J mice, newly born 7 days, were exposed in high oxygen for 5 days and then placed in normal air for another 5 days, thus the animal models of retinal neovascularization were made. Experimental animals were allocated into 3 groups: normal, experimental and therapeutic. The normal group was fed in the normal environment. Into the vitreous cavity of mice in the therapeutic group were injected 2 μ L of EpoRA for 5 successive days. And the experimental group was injected the same amount of normal saline. Mice were sacrificed 17 days after birth and their eyeballs were removed for detection of malonaldehyde (MDA) content in the retina and by HE staining endothelial cells were counted the breaking through internal limiting membrane.

• **RESULTS:** In the experimental group, MDA content in the retina was 25.11 \pm 3.46 μ mol/g, which was obviously less than those in the normal group (5.34 \pm 1.79 μ mol/g, P <0.01) and those in the therapeutic group (12.04 \pm 1.91 μ mol/g). Pathological sections showed the nuclear number of the endothelial cells breaking through internal limiting membrane was 0.7 \pm 0.2 in normal group, and 46.2 \pm 6.5 in high oxygen induced experimental group. In the therapeutic group injected with EpoRA, it was lowered to 24.0 \pm 5.0 (P < 0.01).

• **CONCLUSION:** EpoRA can effectively inhibit oxygen-induced neovascularization in retina of mouse by reducing oxidative damage.

• **KEYWORDS:** oxygen-induced; retinal neovascularization; erythropoietin receptor antibody; malonaldehyde
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INTRODUCTION

Retinal neovascularization is a common pathophysiological basis for retinopathy of prematurity (ROP), diabetic retinopathy (DR), and many other retinopathy, and also a main cause of morbidity for secondary vitreous hemorrhage, vitreous fibrosis and traction blinding eye diseases such as retinal detachment^[1,2]. Recent studies have shown that erythropoietin (Epo) has the activity similar to angiogenic factors, which is an independent and important factor of promoting the retinal neovascularizations^[3]. The cause of retinal neovascularization is very complex, and its mechanisms are not yet very clear. Retina has a large number of polyunsaturated fatty acids, and its oxygen uptake is higher than that of other organs, which makes the retina more sensitive to oxidative stress. Latest research shows that oxidative stress is one of the most important reasons for promoting the formation of retinal neovascularization^[4-6]. Many studies asserted that Epo played an important role in the formation of retinal neovascularization by inducing hypoxia and ischemia in target organ. Erythropoietin receptor antibody (EpoRA) has a significant protective effect on the response of retinal ischemia and hypoxia. In our research we established an hypoxia-inducible animal model of retinal neovascularization in order to observe whether EpoRA could inhibit the oxidative stress by reducing retinal neovascularization.

MATERIALS AND METHODS

Materials C57BL/6J mice and pregnant mice were provided by Shanghai Animal Center of Chinese Academy of Sciences; animal experimental chamber(Second Military

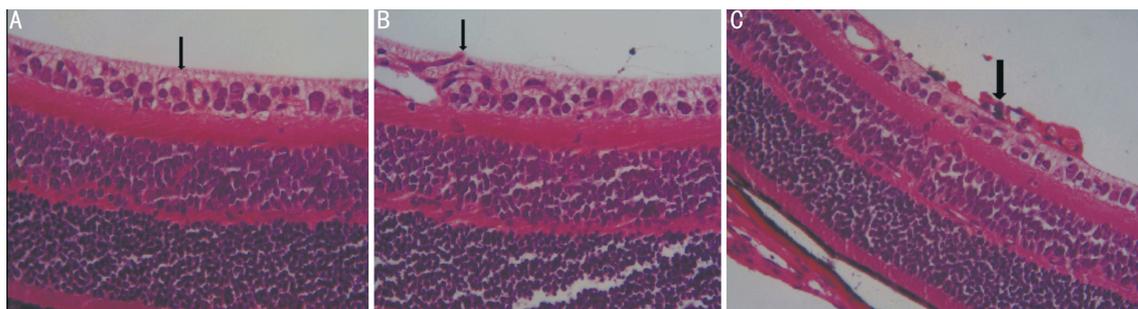


Figure 1 Nuclear number of endothelial cells breaking through internal limiting membrane (400 ×) A: Normal group; B: Experimental group; C: Treatment group

Medical University); oxygen analyzer (XPO-318, Cosmos, Japan); surgical microscope (OPMJ- CS, Germany); fluorescence microscope and digital camera (Nikon, Japan); EpoRA (Sigma, USA); MDA kit (Jiancheng company, Nanjing, China); homogenizer (TP2100, Kinematica, Switzerland).

Methods Animal model of high-dense-oxygen-induced retinal neovascularization was used. Seven-day old 30 C57BL/6J mice were randomly divided into control group, experimental group and treatment group, $n=10$. Control group: The same age (7 days after birth) mice were placed in $23\pm 2^{\circ}\text{C}$ and sacrificed at P17. Seven-day old mice and the lactating mice were placed in the same cabin. Before that, the chamber had been vacuumized, and then 100% humid medical pure oxygen (flow rate 1.5L/min) had been aerated. The condition and the parameters of the container were as follows: partial pressure of oxygen: $75\%\pm 2\%$, temperature: $23\pm 2^{\circ}\text{C}$, with fluorescent lighting. And the mice were fostered under these conditions for 5 days. Treatment group: At P7 we injected EpoRA $2\mu\text{L}$ into the vitreous space of mice with a micro-like gauges before they were placed into the chamber. Experimental group: Mice were injected the equivalent amount of saline. At P12, the immature mice were replaced into the chamber and fed with the maternal. At P17, the mice were sacrificed and the retinal tissue and eyeballs were obtained. The retinal tissue was added into $100\mu\text{L}$ saline, homogenized in ice bath 3-5 minutes, 500 turn/min. After centrifuged 10 minutes, 12 000r/min, $50\mu\text{L}$ supernatant was obtained for determination of malonaldehyde (MDA) according to the kit instructions. The eyeballs were fixative fixed, embedded, and the specimens were serially sectioned paralleling to optic nerve sagittal axis. Each slice was $6\mu\text{m}$ thick, and 20 slices per specimen were selected, xylene dewaxed, ethanol rehydrated, hematoxylin after stained, gradient ethanol dehydrated, transparent and mounted. Finally optical microscopy was used to observe the nuclear number of the endothelial cells that had broken

into the internal limiting membrane. The experiment was repeated three times.

Statistical Analysis The three groups were compared by using single factor analysis of variance (ANOVA) and SNK pairwise comparison methods. Testing results were denoted by mean \pm standard deviation (mean \pm SD). SPSS 13.0 software was used for statistical analysis with $P<0.05$ as statistically significant.

RESULTS

MDA Content At P17, EpoRA was found to significantly inhibit the content of MDA in retina in immature mice in treatment group: As normal group was with content of $5.34\pm 1.79\mu\text{mol/g}$, experimental group was with $25.11\pm 3.46\mu\text{mol/g}$ and treatment group with $12.04\pm 1.91\mu\text{mol/g}$. The result of randomly designed analysis of variance was $F=7.59$, $P<0.01$. SNK method in every two group, $\alpha=0.05$, MDA content in the experimental group was significantly higher than that in the normal group ($P<0.01$), while in high-dense-oxygen-induced models in the treatment group, MDA content was significantly less than that in the experimental group, and the difference was statistically significant ($P<0.01$).

Neovascularization EpoRA significantly inhibited the nuclear number of the endothelial cells breaking into internal limiting membrane in immature mice exposed to high-dense oxygen (Figure 1). Normal group (Figure 1A) showed smooth internal limiting membrane without abnormal structure and clear retinal layers. There were only a small number of the endothelial cells breaking into internal limiting membrane: $0.7\pm 0.2/\text{piece/eye}$. The experimental group (Figure 1B) showed that neovessels can be seen invading the internal limiting membrane, and significant change in neovascularization can be seen: $46.2\pm 6.5/\text{piece/eye}$; it (Figure 1C) showed that neovascularization in treatment group was significantly less than those in experimental group: $24.0\pm 5.0/\text{films/eye}$. The result via randomly designed analysis of variance was $F=9.23$, $P<0.01$.

By SNK method ($\alpha=0.05$), the number of the endothelial cells' nuclear that invade the internal limiting membrane in treatment group was higher than that of control group ($P < 0.01$); but lower than the experimental group significantly ($P < 0.01$). The number of invading endothelial cells' nuclear in experimental group was larger than that of control group ($P < 0.01$).

DISCUSSION

Retinal neovascularization is one of the common pathological changes in Preterm children retina, *et al* The precise mechanisms have not yet been fully elucidated and there is no effective clinical treatment currently [7,8]. Retina contains large amounts of unsaturated fatty acids, and oxygen uptake was higher than those in other tissues, which makes retinal cell more sensitive to the changes of oxygen in the microenvironment. Studies have shown that hypoxia oxidative stress can cause the generation of neovascularization, which is a normal physiological response in all types of cells [9,10]. However, since retinal cell has special ability of visual response, once retinal neovascularization formed, this process will seriously affects the function of retinal cells that lead to the loss of visual function. Epo is a glycoprotein hormone that can specifically control the formation of red blood cells by promoting survival, proliferation, differentiation and maturation of the erythroid progenitor cell. Recent studies have shown that Epo is of angiopoietin activity, and can promote the proliferation and migration of endothelial cells both *in vivo* and *in vitro*. The production of Epo is mainly in the retinal Muller cells [11]. Hernández *et al* [12] have reported that in the retinal tissue, Epo and its receptor's mRNA can be detected. The expression of Epo's receptor can be observed in the retinal ganglion cell layer, external plexiform layer and photoreceptor cell layer. Chen *et al* [13] confirmed that ischemia and hypoxia could lead to increasing expression of Epo in retina, stimulating Epo-dependent neovascularization. Retinal ischemia and hypoxia can also lead to oxidative damage. HIF-1 α is nuclear transcription factor produced by ischemic and hypoxic cell, which can trigger the number of genes activation and process of angiogenesis related to ischemic response, thus HIF-1 α and retinal oxidative damage are closely related. Studies have shown that Epo and vascular endothelial growth factor are both downstream of HIF-1 α target genes [13]. Ischemia and hypoxia are main signals that Epo were produced in retina. It is proved that the increasing expression of Epo is paralleled with the level of HIF-2 α expression in the formation of retinal neovascularization in mice, which suggest that Epo may serve as HIF-2 α target

gene and play an important role in retinal neovascularization. Experiments also proved that Epo played an essential role in formation and maintenance of retinal neovascularization in the course of environment changes from high oxygen to normal, indicating that in the neovascularization induced by oxidative damage in retina, Epo and its receptor are fundamental.

Our study finds that, EpoRA can inhibit hyperoxia-induced retinal neovascularization in immature mice. Oxidative stress is a disturbance in the prooxidant-antioxidant balance and in favor of the former, thus leading to potential damage. Indicators of oxidative stress include damaged DNA bases, protein oxidation products, and lipid peroxidation products. MDA is an end product of unsaturated fatty acid components of biofilm lipid peroxidation, under the action of reactive oxygen species. The density of MDA can reflect the level of oxidative stress. In retinal neovascularization, oxidative stress is an important factor and in this process, Epo is one of the most important inducer. It was proved that in our established oxygen-induced retinal neovascularization model, compared with that in experimental group, the level of MDA in treatment group was significantly reduced; the nuclea number of the endothelial cells breaking into internal limiting membrane was markedly decreased. Our experiment suggested that EpoRA could effectively inhibit oxygen-induced neovascularization in retina of mouse by reducing oxidative damage.

In summary, the present study demonstrates that EpoRA can reduce oxidative stress induced by high oxygen in the process of retinal neovascularization, which can reduce retinal neovascularization. Epo is of a variety of biological activity, so Epo and its receptors are closely related to the formation of retinal neovascularization. Using EpoRA to treat retinal neovascularization has a bright future. Studies for Epo, its receptor and its receptor antibodies may provide a new way for the control of retinal neovascularization in the coming days. Therefore, we will next focus on the mechanism of Epo, its receptor and its receptor antibody. We hope to provide a basis for the clinical treatment of retinal neovascularization.

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