Exogenous erythropoietin aggravates retinal neovascularization in a murine model of proliferative retinopathy

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1. Introduction
Retinopathy of prematurity (ROP) is a blinding eye disorder that affects premature babies (1,2). In ROP, retinal neovascularization (RNV) plays a central role and may lead to blindness (1,3). The first phase of ROP is mainly characterized by delayed vascular growth, and the second phase is mainly present as RNV (4,5). The survival of low-birth-weight infants is increasing due to the development of neonatal care. However, the continuous demand for supplementary oxygen probably increases the incidence of ROP (6,7).

Erythropoietin (EPO), a hematopoietic glycoprotein generated from the fetal liver and adult kidneys, is also produced in low levels by central nervous system tissue. EPO stimulates production of red blood cells. Recombinant human EPO (rEPO) has been widely used in the treatment of neonatal anemia (8,9). However, the relationship between EPO and ROP still needs deeper investigation. EPO has been reported as a retinal angiogenic factor in proliferative diabetic retinopathy (PDR), as well as a risk factor for developing ROP (10,11). In the vaso-obliterative phase, supplementary EPO may protect the retina from vessel loss, preventing oxygen-induced retinal angiogenesis. On the other hand, suppression of EPO would be beneficial for the inhibition of retinal pathological vessels in the second phase (12). Intravitreal injection of small interference RNA is effective in hindering the development of RNV (12). Moreover, a recent study indicated that early EPO administration did not raise the risk of ROP (13). A clinical analysis found that rEPO was an independent risk factor in the development of ROP (14). However, the underlying mechanism by which exogenous EPO induces angiogenesis is still uncertain.

In the present study, we investigate the effect of rEPO treatment on the severity of retinal angiogenesis and its possible mechanisms, which provides a better understanding of RNV and a reasonable application of rEPO.

2. Materials and methods
2.1. Animals
C57BL/6J mice were purchased from the Animal Center of Wuhan University. About 10–16 mouse pups were used per group, and both eyes were removed for experimental
analysis. All experimental protocols related to animals were approved by the Committee on the Ethics of Animal Experiments of Wuhan University. This study was implemented in accordance with the recommendations in the Guide for the Care and Use Committee of Wuhan University. All surgeries were performed under sodium pentobarbital anesthesia, and every effort was made to minimize suffering.

2.2. Oxygen-induced retinopathy
The mouse model of oxygen-induced retinopathy was performed according to previous protocol (15). In brief, postnatal mice at day 7 (P7) were exposed to 75% oxygen conditions for 5 days. At P12, the mice were returned to room air until P17 (Figure 1).

2.3. Intraperitoneal injection of rEPO
At P12, the mice were given daily intraperitoneal (IP) injections of vehicle (saline) or 10 IU, 50 IU, or 100 IU recombinant erythropoietin (rEPO, Roche Pharma Ltd., Basel, Switzerland). Mice were randomly divided into the following groups: a room air (RA) group, an oxygen-induced retinopathy (OIR) group, an OIR treated with saline (vehicle control) group, an OIR treated with 10 IU rEPO group, an OIR treated with 50 IU rEPO group, and an OIR treated with 100 IU rEPO group.

2.4. Retinal flat-mount fluorescent staining.
Retinal whole-mount staining was processed as previously reported (16). At P17, eyes were enucleated and then fixed in 4% paraformaldehyde (PFA) for 1h at room temperature. Retinas were isolated and incubated with *Griffonia simplicifolia* isolectin B4 conjugated to Alexa Fluor 594 (1:200; Invitrogen, Carlsbad, CA, USA) for 48h at 4 °C. The retinas were then mounted onto slides with antifading medium. Images were taken by fluorescent microscope (BX63; Olympus, Tokyo, Japan). Adobe Photoshop CS5 was used to quantify the area of RNV, which was processed as previously described (17).

2.5. Hematoxylin and eosin (H&E) staining
After removal, eyeballs were fixed and embedded in paraffin. Serial sections (6 µm) of eyes were cut sagittally at 32-µm intervals. Only cross-sections through the optic nerve were selected. Images were taken by light microscope (BX63; Olympus, Tokyo, Japan). Preretinal neovascular nuclei were distinguished from other structures and counted via light microscopy.

2.6. Western blot analysis
Western blot was performed according to a standard protocol (18). First, retinas were prepared for protein extraction. Proteins were isolated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were incubated overnight at 4 °C with primary antibodies as follows: rabbit monoclonal antibody against VEGF (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal rabbit antimouse iNOS (1:500; Beijing Bios Biotechnology Co. Ltd., Beijing, China), GAPDH antibody (GAPDH rabbit mAb, 1:1000; Cell Signaling Technology, Danvers, MA, USA). GAPDH was set as the loading control. After being washed thoroughly with TBS-T, the membranes were incubated with HRP-conjugated goat antirabbit IgG (1:5000; Wuhan Boster Biological Technology Co. Ltd., Wuhan, China) at room temperature for 90 min. The bands were visualized with chemiluminescence and detected by photographic film. Prestained marker (Fermentas, Baltimore, MD, USA) was used to estimate the molecular weight of proteins. The experiments were repeated 3 times.

Figure 1. Cartoon schematic of the murine model of OIR. Newborn mice are kept in room air from birth to postnatal day 7 (P7). At P7, mice are exposed to 75% hyperoxegen, which suppresses normal retinal development and leads to significant vessel loss. Mice are then back to room air at P12; hypoxic avascular area of retina triggers pathological neovascular response. RNV reaches its peak at P17, which is visualized and quantified.
2.7. Statistical analysis
All values are presented as the mean ± standard deviation (SD). Group differences were compared by one-way analysis of variance followed with the Bonferroni post hoc test for multiple comparisons. P < 0.05 was considered as statistically significant.

3. Results
3.1. Establishment of OIR model
At P17, retinal flat-mount fluorescence and H&E staining were conducted to determine the successful establishment of OIR. Whole-mount staining of isolectin B4 revealed a clear central area of nonperfusion (Figure 2). Neovascular tufts were present at the junction between the vascularized area and the nonperfused region, mainly in the midperipheral area of the retina. However, no vasoobliteration area or pathological vessels were found in the RA group. Consistently, H&E staining showed that many preretinal neovascular cells emerged from the inner retinal layer extending to the vitreous chamber (Figure 3). The results proved that RNV was substantially induced after hyperoxygen exposure.

3.2. Administration of rEPO promotes RNV in OIR
After daily administration of rEPO from P12 in OIR, whole-mount staining of retinas and calculation of RNV indicated that no significant difference was found in the neovascular tuft area between the vehicle control (saline) and 10 IU groups (Figure 4; 16.48% ± 3.075% vs. 17.33% ± 2.97%; t = 0.641; P > 0.05), while 50 IU and 100 IU rEPO injections significantly aggregated the severity of RNV compared to the saline group (Figure 4; 20.37% ± 1.678% and 22.25% ± 3.705% vs. 16.48% ± 3.075%; t = 2.949 and 4.379, respectively; P < 0.05). The outcome indicated that rEPO increased the neovascular tufts dose-dependently. However, a low dose (10 IU) of rEPO did not significantly promote the growth of aberrant vessels.

3.3. Injection of rEPO increased the number of preretinal neovascular cells
H&E staining of sections and quantification of preretinal neovascular cells showed that, after injection of rEPO, the number of preretinal neovascular cells obviously increased in the 50 IU and 100 IU groups compared with that from the saline group (Figure 5; 51.30 ± 5.14 and 56.90 ± 7.85 vs. 38.90 ± 8.66; t = 3.759 and 5.457, respectively; P < 0.05). However, no significant difference was found between the saline group and the 10 IU rEPO group (Figure 5; 38.90 ± 8.66 vs. 47.40 ± 7.382; t = 2.577; P < 0.05). The outcome also demonstrated that a low dose (10 IU) of rEPO did not significantly increase the number of neovascular cells.

3.4. Effect of rEPO on the expression of VEGF and iNOS
Changes in the expression of proangiogenic proteins such as VEGF and iNOS were evaluated via western blot analysis (Figure 6). Compared with those in the vehicle control group, protein levels of VEGF and iNOS were significantly upregulated in the 100 IU rEPO group (P < 0.05). However, no statistical significance was found when comparing the expression of VEGF and iNOS between the saline group and the 10 IU rEPO group or 50 IU rEPO group.

Figure 2. Flat-mount retinal fluorescent staining: a) RA group; b) OIR group. Vasculatures stained by isolectin B4 (red) show the construction of retinal vessels. In the room air (RA) group, no vaso-obliteration (VO) area or neovascular tufts were observed while large nonperfused zones (white irregular circle) and RNV (white arrows) were discovered in the OIR group at P17 (magnification 40×; scale bar = 500 μm).
4. Discussion

EPO has been used in the treatment of neonatal anemia (8,13,19). Many studies have shown the close relationship between administration of rEPO and the development of ROP (8,14). A recent metaanalysis found that late EPO supplementation does not significantly reduce or increase any fatal adverse outcomes; there is only the increased risk of ROP (8). However, early injection of rEPO is not associated with the risk of retinopathy in premature infants (13,19). The development of ROP has been divided into 2 phases according to the alteration of vasculature. The vessel attenuation in the first phase leads to the hypoxia-induced vasoproliferation in the second phase. Chen et al. (20) indicated that EPO treatment in the first phase protected the retina from vessel regression, eventually preventing RNV.

EPO is known as an oxygen-regulated hormone that promotes erythrogenesis (12). Endogenous EPO in the retina is crucial for retinal angiogenesis (10,20). A previous study showed that mRNA expression of EPO is increased greatly in the second phase of retinopathy (12). After intravitreal injection of small interference RNA (siRNA), expressions of EPO as well as RNV are significantly suppressed through RNA interference. This result indicates that the silencing of EPO in the OIR retina inhibits the RNV (12). Another work also found that antibodies against EPO hindered RNV by reducing EPO to the physiologic level (10). These findings are consistent with the present study, which has shown that exogenous EPO has a proangiogenic effect on promoting RNV. In the OIR model, we administered the rEPO daily from P12 to P17, which is equal to the second phase of ROP. The results suggested the proangiogenic effect of rEPO in the process of RNV, which is consistent with a recent study that demonstrated that the late use of rEPO increased the risk of ROP (8).

Quantification of RNV via retinal flat-mount staining and section H&E staining has been used extensively to evaluate the establishment of OIR and the severity of RNV (15–17). In the present study, injection of rEPO led to an increase of RNV, significantly so in the 50 IU and 100 IU groups. Our results showed that a high dose of rEPO significantly promotes the development of RNV in mice.

Despite the obvious demonstration of rEPO’s role as a proangiogenic protein, the underlying mechanism by which this works is a matter for speculation. We found that a high dose of rEPO (100 IU) significantly upregulated the expression of VEGF and iNOS. VEGF is a key and well-known factor in the process of angiogenesis, which stimulates endothelial cell activation and proliferation (21–23). A previous study demonstrated a higher vitreous level of EPO in stage 4 ROP, which was significantly correlated with the level of VEGF in the vitreous chamber of the eyes (24). Moreover, VEGF has been suggested to mediate angiogenesis through erythropoiesis-stimulating factors (25). The present investigation revealed that treatment with 100 IU of rEPO exacerbated RNV via increasing the level of VEGF expression, suggesting that rEPO induced upregulation of VEGF, which contributed to the pathogenesis of RNV.

Nitric oxide (NO) is synthesized by 3 kinds of NO synthase, including neuronal nitric oxide synthases (nNOS), endothelial nitric oxide synthases (eNOS), and inducible nitric oxide synthase (iNOS). Nitric oxide is a critical molecule that takes part in angiogenesis (26,27). iNOS is expressed in the ischemic retina and has been proven to be
Figure 4. Quantification of RNV after rEPO administration: a) saline group; b) 10 IU rEPO group; c) 50 IU rEPO group; d) 100 IU rEPO group; e) quantification analysis of RNV. Data are presented as mean ± SD. Whole-mount staining showed that central ischemic area and RNV tufts were present in all groups that were exposed to hyperoxxygen at P12 and received administration of saline or a gradient dose of rEPO (magnification 40×; scale bar = 500 µm). Significantly, 50 IU and 100 IU rEPO exacerbated development of RNV at P17 when compared to the saline group (n = 10; *P < 0.05, ***P < 0.001).
Figure 5. Measurement of preretinal neovascular cell nuclei: a) saline group; b) 10 IU rEPO group; c) 50 IU rEPO group; d) 100 IU rEPO group; e) quantification of preretinal neovascular cells. Data are presented as mean ± SD. H&E staining indicated that neovessels extending to the vitreous chamber were present in every group (magnification 200×; scale bar = 50 µm). High doses of rEPO (50 IU and 100 IU) significantly increased the number of preretinal neovascular cells at P17 compared to the saline group (n = 10; *P < 0.05, ***P < 0.001).
involved in tumor-induced angiogenesis and ischemic RNV (28–30). Previous studies showed that inhibition of iNOS significantly suppressed RNV in the OIR model (31,32). He et al. suggested that iNOS mediates hypoxia inducible factor 1 alpha (HIF-1α) activation and VEGF upregulation in RNV through the PI3K/Akt pathway (31). NO-dependent pathways refer to a potential mechanism by which EPO stimulates bone repair and angiogenesis (33). EPO was attributed to an upregulation of iNOS, as previously reported (34). We consistently found that 100 IU rEPO injection promoted RNV and increased expression of iNOS in the OIR mouse model, indicating that rEPO aggregated RNV by inducing the expression of iNOS and VEGF.

In conclusion, this investigation reveals that administration of rEPO exacerbates RNV in a mouse model of OIR, possibly via activation of VEGF and upregulation of iNOS. The study not only indicates that the dose and timing of rEPO treatment are important to preterm infants, but also provides theoretical evidence for restraint of rEPO-induced RNV clinically.

**Figure 6.** Effects of rEPO on retinal protein levels of VEGF and iNOS. The protein levels were normalized to GAPDH. Data are presented as mean ± SD. Western blot analysis indicated that 100 IU rEPO injection significantly upregulated the protein levels of VEGF and iNOS (*P < 0.05).
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References


