Cedilanid inhibits retinal neovascularization in a mouse model of oxygen-induced retinopathy

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Purpose: This study investigated the effect of cedilanid on retinal neovascularization in a mouse model of oxygen-induced retinopathy.

Methods: Seven-day-old C57BL/6 mice were exposed to 75% ± 1% oxygen for 5 days and were then returned to room air to induce retinal neovascularization. Cedilanid (0.025–0.2 μg) was intravitreally injected into the left eye of each mouse on postnatal day 12 (P12) and P15. PBS was intravitreally injected into the right eye as a control. Retinal neovascularization was evaluated with isolectin GS-IB4 staining of the retinal blood vessels. The function of reestablishment blood vessels was evaluated with angiography with the injection of fluorescein isothiocyanate (FITC)-dextran followed by isolectin GS-IB4 staining. Real time (RT)–PCR and western blot were used to examine the mRNA and protein expression of hypoxia inducible factor 1 alpha (HIF-1α) and vascular endothelial growth factor (VEGF), respectively.

Results: Retinal neovascular areas and obliterative areas were statistically significantly smaller in the eyes injected with cedilanid (0.05 μg, 0.1 μg, and 0.2 μg) compared with the control eyes. The inhibitory effect of cedilanid was observed in a dose-dependent manner. In addition, the retinal neovascular areas and the obliterative areas in the eyes injected with 0.2 μg cedilanid on P12 were statistically significantly smaller than those in the eyes injected with the same dose of cedilanid on P15. Cedilanid promoted the circulative function of reestablished blood vessels in the obliterative areas. Cedilanid inhibited the expression of HIF-1α and VEGF in mice treated with hyperoxia.

Conclusions: Cedilanid inhibits retinal neovascularization in a mouse model of oxygen-induced retinopathy. Early treatment with cedilanid produces better inhibition of retinal neovascularization. Cedilanid may be a potential treatment of neovascular diseases.

Retinal neovascularization is a pathological feature of many retinal diseases, such as retinopathy of prematurity and diabetic retinopathy, and can lead to severe vision loss and even blindness [1]. Inhibition of retinal neovascularization is a therapeutic strategy for treating these retinal diseases. Several therapeutic strategies have been developed to inhibit retinal neovascularization, including inhibition of vascular endothelial growth factor (VEGF), hypoxia inducible factors (HIFs), and erythropoietin (EPO), and transplantation of myeloid progenitors [2-5]. Therefore, identifying agents that inhibit retinal neovascularization is important for treating neovascular diseases.

It is known that ischemia or hypoxia contributes to the initiation and development of retinal neovascularization [6].

One of the key oxygen homeostasis regulators, HIF-1α, is a transcription factor that regulates a variety of proangiogenic factors, such as VEGF, stromal-derived factor-1 (SDF), stem cell factor (SCF), platelet-derived growth factor B (PDGFB), and placental growth factor (PlGF), and EPO [6-10]. It has been reported that increased expression of HIF-1α is sufficient to induce neovascularization [11]. Therefore, inhibition of HIF-1α may block several proangiogenic signaling pathways, thus possessing advantages over monotherapy that targets a single proangiogenic factor. Several studies have demonstrated that inhibition of HIF-1α reduces retinal neovascularization in the murine model of oxygen-induced retinopathy [2,3,12]. Therefore, identifying agents that inhibit HIF-1α represents a therapeutic strategy for the treatment of neovascular diseases.

Digoxin and other cardiac glycosides have been found to inhibit HIF-1-dependent gene transcription of target genes in many hepatic, lymphoid, and prostate cell lines and inhibit tumor growth in tumor xenografts in vivo [13]. In addition, digoxin has been reported to inhibit retinal ischemia-induced

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HIF-1α expression in a mouse model of choroidal neovascularization and oxygen-induced ischemic retinopathy [12]. However, digoxin used for the treatment of congestive heart failure has a narrow therapeutic window, and digoxin overdose is associated with lethal toxicity, such as ventricular arrhythmia. Therefore, the oral use of digoxin for the treatment of neovascular diseases may be limited by its toxicity. Similar to digoxin, cedilanid is a cardiac glycoside used for the treatment of congestive heart failure. Compared with digoxin, cedilanid has some advantages, such as faster onset of action, less accumulation in the body, wider therapeutic window, and less toxicity. Therefore, it is necessary to test whether cedilanid can inhibit retinal neovascularization. In this study, we investigated the effect of cedilanid on retinal neovascularization in a mouse model of oxygen-induced retinopathy. The purpose of this study was to explore whether cedilanid inhibited retinal neovascularization in a dose- and time-dependent manner and to investigate whether cedilanid promoted reestablishment of blood vessels in obliterative areas.

METHODS

Animals: The Animal Ethics Committee of Beijing Tongren Hospital of Capital Medical University approved all experimental protocols. This study was performed in accordance with the ARVO Statement for Use of Animals in Research. Adult C57BL/6 mice were obtained from Vital River Laboratories (Beijing, China). Animals were housed at room temperature (25 °C) with 60% humidity and a 12 h:12 h light-dark cycle. Mice were fed standard rat chow and water ad libitum. Experiments were performed on litters (male and female) of these mice at postnatal day (P) 7.

To study the dose-dependent effect of cedilanid on oxygen-induced retinal vascularization, 40 mice were randomly assigned to four groups: the 0.025 μg cedilanid group (n = 10), the 0.05 μg cedilanid group (n = 10), the 0.1 μg cedilanid group (n = 10), and the 0.2 μg cedilanid group (n = 10). One and two mice died after intravitreal injection in the 0.025 μg and 0.2 μg cedilanid groups, respectively. To study the time-dependent effect of cedilanid on oxygen-induced retinal vascularization, ten mice received cedilanid (0.2 μg) and PBS (1X; 137 mM NaCl, 2.7 mM KCl, 20 mM NaPO₄, 1.76 mM KPO₄, pH 7.4; in the control eyes) on P15. To study the restoration of circulation in retinal blood vessels in the obliterative area, cedilanid (0.2 μg) was intravitreally injected into the left eyes of the mice.

Oxygen-induced retinal neovascularization: The mouse model of oxygen-induced retinal neovascularization was induced as previously reported by Xiong et al. [4]. Briefly,

7-day-old C57BL/6 mice (P7) with their nursing mothers were exposed to 75% ± 1% oxygen in an oxygen chamber with an oxygen-regulating instrument (Biosphenx Ltd, New York, NY). The mice were exposed to the high concentration of oxygen between P7 and P12. The oxygen levels were checked twice a day. On P12, the mice were removed from the chamber and were exposed to room air for 5 days. Intravitreal injection was performed on P12, and the mice were euthanized by cervical dislocation on P17 for fluorescein isothiocyanate (FITC)-dextran and isolectin GS-IB4 staining of the retinal blood vessels.

Intravitreal injection: Mice were anesthetized with an intraperitoneal injection of 10% chloral hydrate (4 ml/kg, Sinopharm Chemical Reagent Co., Ltd. Shanghai, China). The lid fissure was opened, and the eye was proptosed. Intravitreal injection was performed as previously described [4]. Briefly, after tropical anesthesia on the cornea, intravitreal injection was performed with a 33-gauge needle (Hamilton, Bonaduz, Switzerland) at 1 mm behind the limbus. Cedilanid (1 μl) was intravitreally injected into the left eye of each mouse. An equal amount of PBS was intravitreally injected into the right eye of each mouse as a control. After the injections, treatment with ofloxacin (Shenyang Xingqi Pharmaceutical Co., Ltd., Shenyang, China) was used to prevent infection.

To study the dose-dependent effect of cedilanid on oxygen-induced retinal vascularization, cedilanid (0.025 μg, 0.05 μg, 0.1, μg and 0.2 μg) or PBS was intravitreally injected into the left or right eye of each mouse on P12, respectively. To study the time-dependent effect of cedilanid on oxygen-induced retinal vascularization, cedilanid (0.2 μg) or PBS was intravitreally injected into the left or right eye of each mouse on P15. To study the restoration of circulation in retinal blood vessels in the obliterative area, cedilanid (0.2 μg) was intravitreally injected into the left eye of each mouse on P12. The right eye without intravitreal injection was used as a control.

Isolectin GS-IB4 staining of retinal blood vessels: Isolectin GS-IB4 staining was performed as previously described by Banin et al. [14]. Briefly, on P17, the mice were euthanized by cervical dislocation, and the eyes were enucleated and fixed in 4% paraformaldehyde for 20–30 min at room temperature. The retinas were isolated under an operating microscope and then were fixed with methanol on ice. After three washes with PBS, the retinas were incubated with 20% goat serum (GS) and 20% fetal bovine serum (FBS) for 1 h to block the nonspecific staining. Then, the retinas were stained overnight at 4 °C with isolectin GS-IB4 (1:200 dilution, Invitrogen) in PBS containing 10% GS and 10% FBS. The retinas were then whole mounted, and images were taken under a fluorescence microscope (DP72; Olympus, Tokyo, Japan).

Angiography with FITC-dextran: The mice were deeply anesthetized with an intraperitoneal injection of 10% chloral hydrate. Then the mice were transcardially perfused via the left ventricle with PBS containing 0.25 ml of 2.5% FITC-dextran (molecular weight, 1.5×10^5; Sigma-Aldrich, Saint Louis, MO). The mice were then euthanized by cervical dislocation, and the eyes were enucleated and fixed in 4% paraformaldehyde for 1 h at room temperature. The retinas were isolated under an operating microscope and flatmounted on the slides. The retinas were then examined and photographed under a fluorescence microscope (Olympus). Then the retinas fixed with methanol on ice and stained with isolectin GS-IB4 as previously described.

Assessment of retinal angiogenesis and obliteration: Images were taken at 40X magnification with a resolution of 1360 × 1024 dpi. Each pixel represents an area of 6.529 μm². Four to six images were taken and merged together to produce an image of the entire retina, using Photoshop CS4 software (Adobe, San Jose, CA). The hyperfluorescent area was measured using Photoshop software. The border of the hyperfluorescent area was determined using the lasso tool (Figure 1A,C,E). The area of the neovascular regions was calculated in pixels and converted to the actual area using the following formula: area (mm²) = pixel × 6.529×10^{-6}. The area of the obliterative region was calculated using the same method (Figure 1B,D,F). The neovascular (obliterative) area ratio was calculated according to the following formula: neovascular (obliterative) area ratio = the neovascular (obliterative) areas of eyes treated with cedilanid / the neovascular (obliterative) areas of eyes treated with PBS.

Real-time PCR: Total RNA was isolated from retinal tissues using TRIzol reagent (Invitrogen, Carlsbad, MA), according to the manufacturer's protocol. RNA was reverse transcribed into cDNA using a reverse transcription system (Invitrogen). Real time (RT)–PCR was performed in a final 20 μl mixture containing 2 μl cDNA, 0.5 μl of each primer, and 10 μl SYBR Green (Invitrogen). Primers used in this study were as follows: 5′-TCT CCA AGC CCT CCA AGT AT-3′ (forward) and 5′-GAT TCA TCA GTG GTG GCA GT-3′ (reverse) for HIF-1α, 5′-CAC CCA CGA CAG AAG GAG A-3′ (forward) and 5′-ATG TCC ACC AGG GTC TCA AT-3′ (reverse) for VEGF, and 5′-AGG CCG GTG CTG AGT ATG TC-3′ (forward) and 5′-TGC CTG CTT CAC CAC CTT CT-3′ (reverse) for GAPDH as an internal control. The reaction conditions were as follows: 94 °C for 3 min; 94 °C for 20 s, 60 °C for 20 s, 72 °C for 20 s with 40 cycles; and a final extension of 72 °C for 7 min. The mRNA expression of HIF-1α and VEGF was calculated by normalizing with the

internal control, GAPDH. The relative expression of HIF-1α and VEGF was calculated using the 2^{-ddCT} method.

Western blot: Retinal tissues from eyes in each group were homogenized on ice in lysis buffer. Proteins were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto polyvinylidene fluoride membranes with electroblotting. Membranes were then incubated with 5% nonfat dry milk, followed by incubation with primary antibodies against HIF-1α (rabbit anti-HIF-1α polyclonal antibodies, dilution 1:400, Santa Cruz Biotechnology, Dallas, TX), VEGF (rabbit anti-VEGF polyclonal antibodies, dilution 1:300, Santa Cruz Biotechnology), or β-actin (rabbit anti-β-actin monoclonal antibody, 1:400, Santa Cruz Biotechnology) at 4 °C overnight. Membranes were then incubated with horseradish peroxidase–linked goat anti-rabbit secondary antibodies (dilution 1:1,000, Santa Cruz Biotechnology) at room temperature for 2 h. Bands were visualized using a chemiluminescence detection system.

Statistical analysis: Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL). Numerical data are presented as the mean and standard deviation. Paired *t* tests were used to compare the difference between the eyes injected with cedilanid and the contralateral control eyes within the same group. A p value of less than 0.05 was considered statistically significant.

RESULTS

Cedilanid inhibits oxygen-induced retinal neovascularization in a dose-dependent manner: We first investigated the dose-dependent effect of cedilanid on oxygen-induced retinal neovascularization. On P12 after the mice were removed from the oxygen chamber, cedilanid was intravitreally injected into the left eye of each mouse, and PBS was injected into the right eye as a control. The retinal neovascular areas and the obliterative areas were not statistically significantly different between the eyes injected with 0.025 μg cedilanid and the control eyes injected with PBS (Figure 2; p>0.05, n = 9). The retinal neovascular areas and the obliterative areas were statistically significantly smaller in the left eyes injected with high concentrations of cedilanid (0.05 μg, 0.1 μg, and 0.2 μg), compared with the corresponding control right eyes (Figure 2; p<0.05, n = 10, n = 10, n = 8, respectively). The retinal neovascular areas in the eyes injected with 0.05 μg, 0.1 μg, and 0.2 μg cedilanid were reduced to 58%, 42%, and 19% of the neovascular areas in the corresponding control eyes injected with PBS, respectively. These results suggest that cedilanid inhibits oxygen-induced neovascularization in a dose-dependent manner. The obliterative areas in the eyes injected with 0.05 μg, 0.1 μg, and 0.2 μg cedilanid were

reduced to 74%, 54%, and 61% of the obliterative areas in the corresponding control eyes injected with PBS, respectively. These results suggest that cedilanid reduced the obliterative areas in a dose-dependent manner, and the maximum effect occurred at the dose of 0.1 μg. In addition, the neovascular area ratio was reduced with an increasing dose of cedilanid (Figure 2M), suggesting cedilanid inhibited neovascularization in a dose-dependent manner.

Early cedilanid treatment on P12 produced better inhibition of retinal neovascularization than on P15: We then investigated the time-dependent effect of cedilanid on retinal

neovascularization in the mouse model of oxygen-induced retinopathy. On P12 and P15, cedilanid (0.2 μg) and PBS were intravitreally injected into the left and right eyes of each mouse, respectively. On P15, the retinal neovascular areas were statistically significantly reduced in the left eyes injected with 0.2 μg cedilanid compared with the control eyes injected with PBS (Figure 3; p<0.05, n = 10 per group). However, there was no statistically significant difference in the obliterative areas between the eyes treated with cedilanid and PBS (Figure 3; p>0.05, n = 10 per group). Furthermore, the retinal neovascular areas and the obliterative areas in the
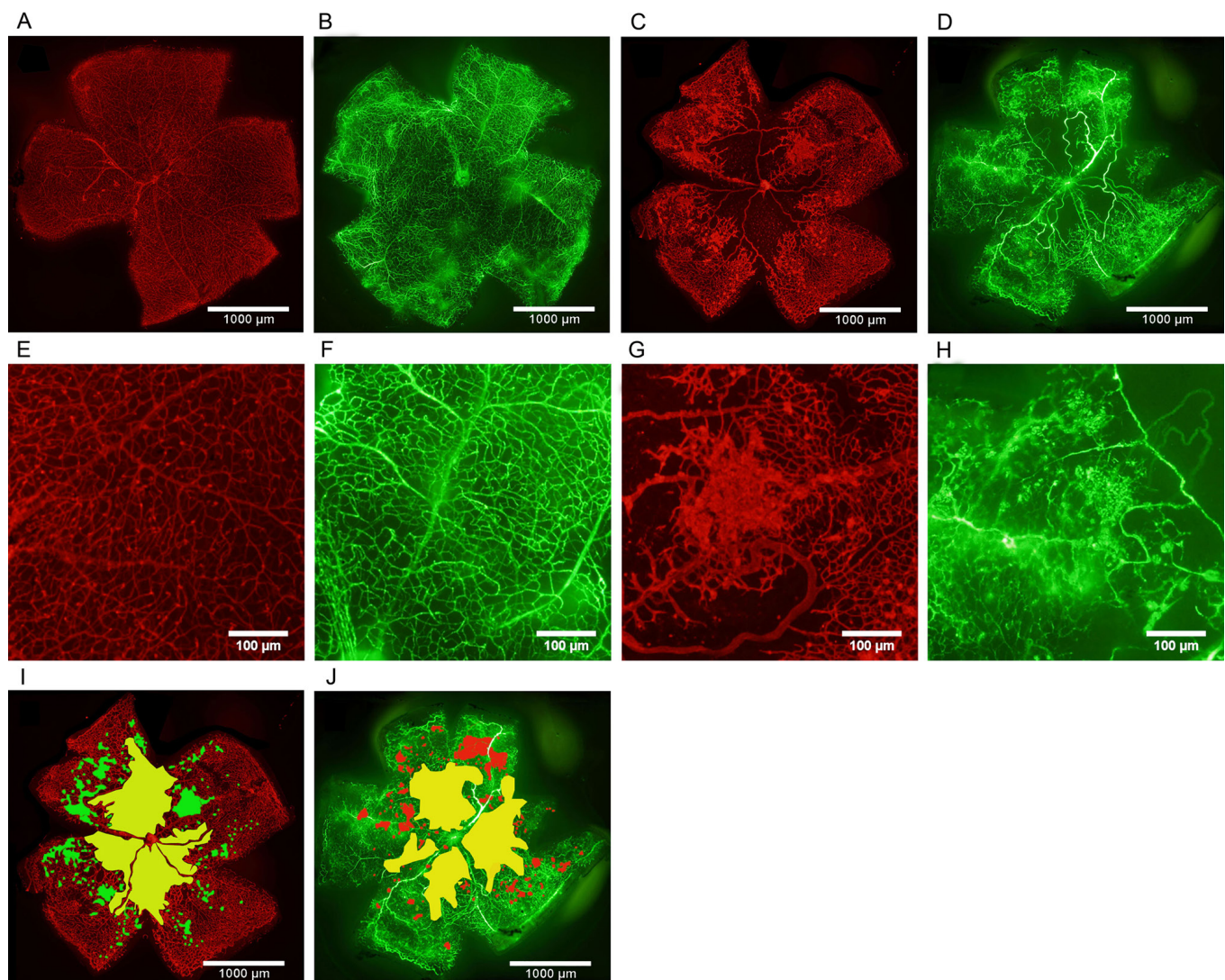

Figure 1. Isolectin GS-IB4 and FITC-dextran staining shows the distribution of healthy and neovascular blood vessels in the retina. Retinal whole mounts stained with isolectin GS-IB4 and FITC-dextran in eyes from healthy mice and mice treated with hyperoxia. Representative images with isolectin GS-IB4 (**A**, **C**, **E**, **G**, **I**) and FITC (**B**, **D**, **F**, **H**, **J**). **A**, **B**: Staining in eyes from healthy mice. **C**, **D**: Staining in eyes from mice treated with hyperoxia. **E**, **F**: Magnified images showing the distribution of the retinal blood vessels. **G**, **H**: Magnified images showing neovascular tufts. **I**: Image produced in Photoshop. The neovascular areas are green, and the obliterative area is yellow. **J**: Image produced in Photoshop. The neovascular areas are red, and the obliterative area is yellow.
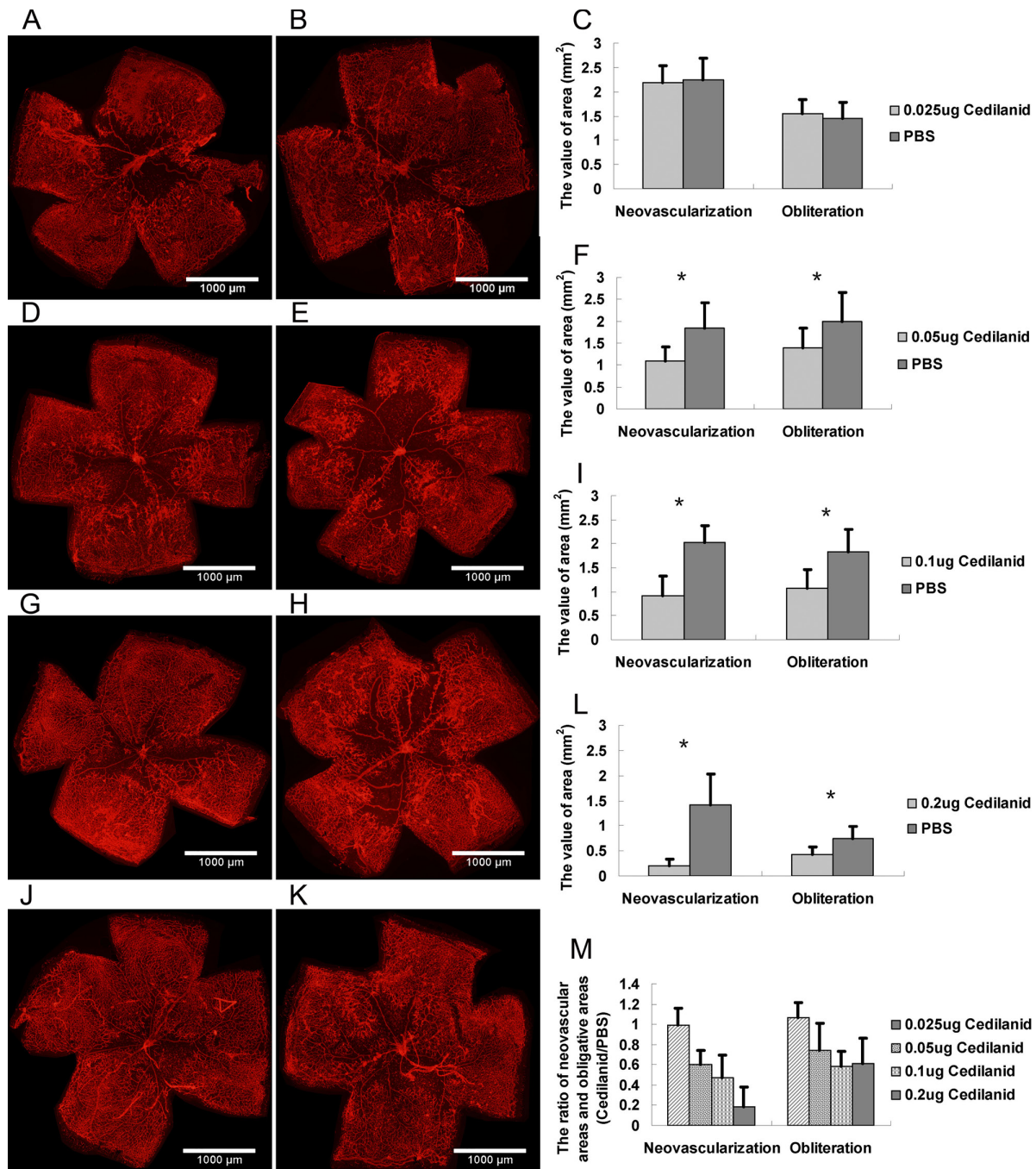
Figure 2. The dose-dependent effect of cedilanid on oxygen-induced retinal neovascularization. Immunofluorescence images of retinal whole mounts showed the neovascular area and the obliterative area in the left eyes injected with 0.025 μg (**A**; n = 9), 0.05 μg (**D**; n = 10), 0.1 μg (**G**; n = 10), and 0.2 μg (**J**; n = 8) and the corresponding control eyes injected with PBS (**B**, **E**, **H**, **K**). Cedilanid and PBS were intravitreally injected on P12. The quantification of the neovascular area and the obliterative areas in eyes injected with 0.025 μg (**C**), 0.05 μg (**F**), 0.1 μg (**I**), and 0.2 μg (**L**) and control eyes injected with PBS. **M**: The ratios of the neovascular areas and the obliterative areas in eyes injected with different doses of cedilanid to the corresponding control eyes injected with PBS. *p<0.05 versus PBS control.

eyes injected with 0.2 μg cedilanid on P12 were statistically significantly smaller than those in the eyes injected with 0.2 μg cedilanid on P15 (Figure 3D). These results suggest that only cedilanid treatment on P12 produced better inhibition of hypoxia-induced neovascularization.

Cedilanid promoted the circulative function of reestablished blood vessels in obliterative areas: We further investigated the restoration of circulation in obliterative areas by examining whole mount retinas after the FITC-dextran perfusion and isolectin GS-IB4 staining. Cedilanid (0.2 μg) was intravitreally injected into the left eyes on P12, and the retinas were examined with FITC-dextran perfusion and isolectin GS-IB4 staining on P17. FITC-dextran staining showed that most of the neovascular blood vessels were FITC-positive, suggesting that most neovascular blood vessels support

circulatory function (Figure 4A,B). Compared with the control eyes, the neovascular areas and the obliterative areas were statistically significantly reduced in the eyes injected with cedilanid (0.2 μg), and many reestablished blood vessels were stained with FITC-dextran in the eyes treated with cedilanid, suggesting functional blood vessels (Figure 4C,D). However, some neovascular blood vessels adjacent to the obliterative area showed only isolectin GS-IB4 staining without FITC-dextran staining, suggesting that these reestablished blood vessels are nonfunctional (Figure 4E).

Cedilanid inhibited the expression of HIF-1α and VEGF: We examined the mRNA and protein expression of HIF-1α and VEGF in retinal tissues from mice treated with normal air, mice treated with hyperoxia, and mice treated with cedilanid after hyperoxic exposure, using RT–PCR and western
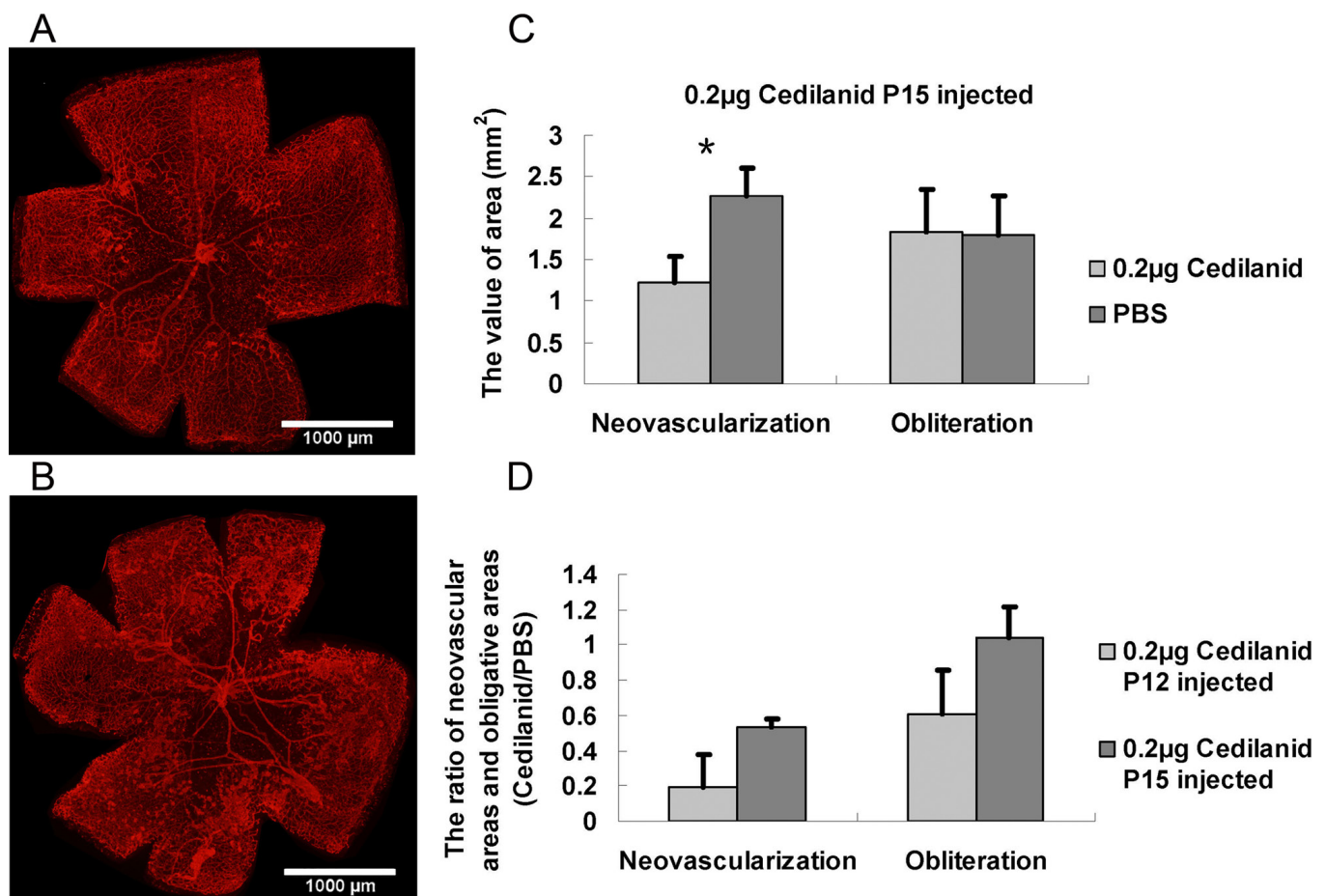

Figure 3. The time-dependent effect of cedilanid on oxygen-induced retinal neovascularization. **A**: Immunofluorescence images of retinal whole mounts show the neovascular area and the obliterative area in the left eyes injected with 0.2 μg cedilanid. **B**: Immunofluorescence images of retinal whole mounts show the neovascular area and the obliterative area in the corresponding control eyes injected with PBS. Cedilanid and PBS were intravitreally injected on P15. **C**: Quantification of the neovascular area and the obliterative areas in eyes injected with 0.2 μg and control eyes injected with PBS; n = 10. *p<0.05 versus PBS. **D**: The ratios of the neovascular areas and the obliterative areas in eyes injected with cedilanid to the corresponding control eyes injected with PBS on P12 and P15.

blotting. The RT–PCR results showed that the mRNA expression of HIF-1α and VEGF was statistically significantly increased in the mice treated with hyperoxia, and intravitreal injection of cedilanid statistically significantly inhibited the expression of HIF-1α and VEGF in the mice treated with hyperoxia (Figure 5A). Consistent with the RT–PCR results, western blot analysis showed that cedilanid statistically significantly inhibited the protein expression of HIF-1α and VEGF in mice treated with hyperoxia (Figure 5B,C).

DISCUSSION

Several studies have shown that many antiangiogenic treatments, such as the use of VEGF inhibitors [15], anti-SDF-1 signaling [16], and EPO inhibition [4], suppress retinal neovascularization. However, because a wide range of proangiogenic factors contribute to retinal neovascularization, inhibition of a single proangiogenic pathway may be not

sufficient to reduce retinal neovascularization. HIF-1α has been found to regulate the expression of many proangiogenic factors, including VEGF, DSF-1, PDGFB, and PlGF, and EPO [6-9]. Theoretically, HIF-1α inhibition can suppress the role of multiple proangiogenic factors in retinal neovascularization, leading to potent inhibition of retinal neovascularization. This idea was supported by several previous studies showing that inhibition of HIF-1α by YC-1 reduced the expression of several proangiogenic factors (such as VEGF, SDF-1, SCF, PDGFB, and PIGF) and inhibited retinal neovascularization in a mouse model of oxygen-induced retinopathy [10,17]. Furthermore, Zhang et al. identified digoxin and other cardiac glycosides as potent HIF-1α inhibitors, and HIF-1α inhibition by digoxin reduced tumor growth in tumor xenografts [13]. Recently, Yoshida et al. reported that digoxin inhibited the expression of HIF-1α, reduced the expression of several proangiogenic factors (such as VEGF, SDF-1,
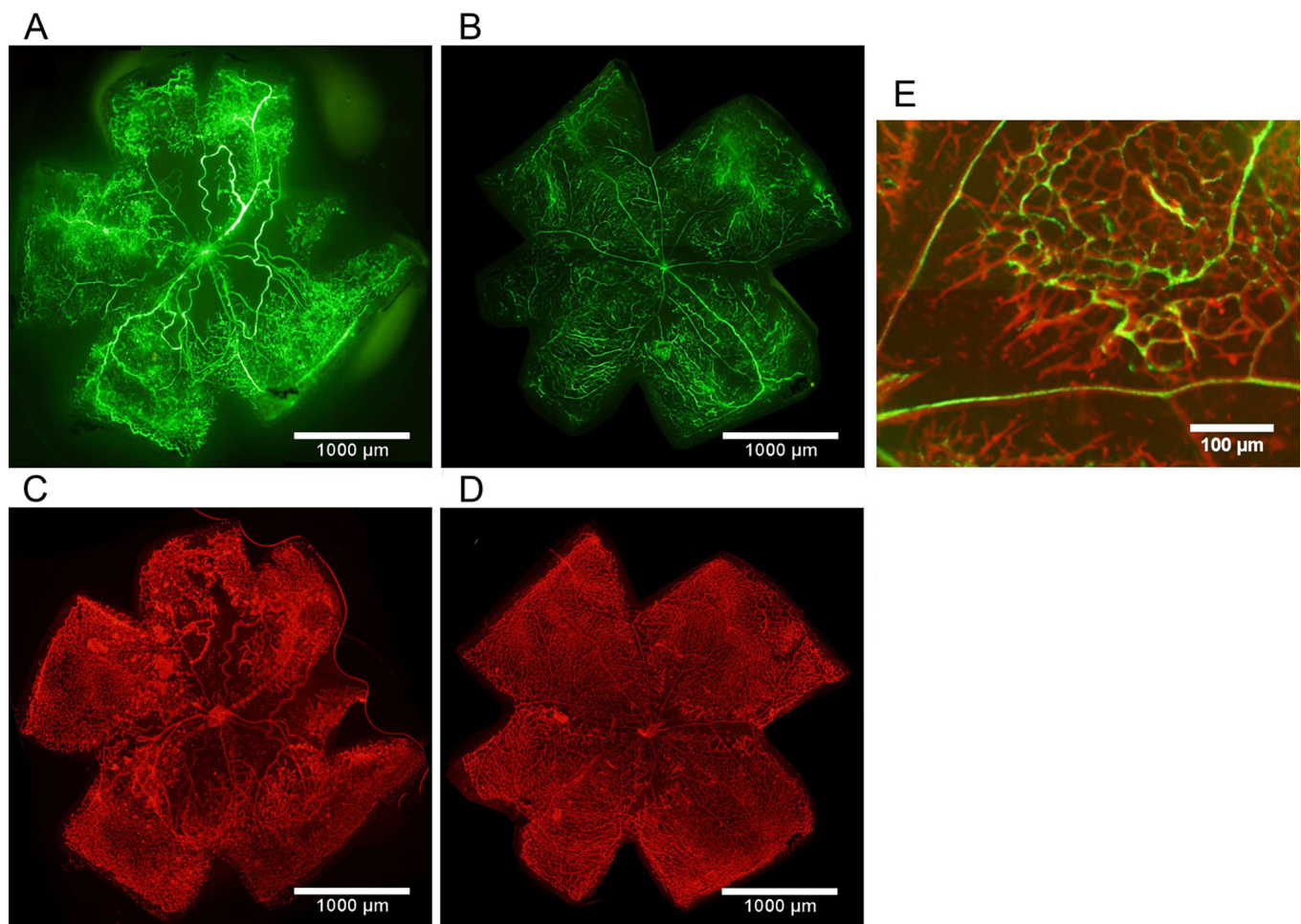

Figure 4. Evaluation of the circulative function of reestablished blood vessels. Representative image of whole mount retinas after fluorescein isothiocyanate (FITC)-dextran perfusion (green, **A**, **B**) and isolectin GS-IB4 staining (red, **C**, **D**) in the control eyes (**A**, **C**) and eyes (**B**, **D**) injected with 0.2 µg cedilanid. **E**: Merged images at a high magnification (x400).

SCF, PDGFB, and PIGF), and suppressed retinal neovascularization and choroidal neovascularization [12]. In this study, we investigated the effect of another cardiac glycoside cedilanid on retinal neovascularization in a mouse model of oxygen-induced retinopathy. Similar to digoxin, cedilanid is an inhibitor of Na$^+$/K$^+$ ATPase that is used for the treatment of congestive heart failure. In this study, we found that similar to digoxin, cedilanid inhibited oxygen-induced retinal neovascularization in a dose- and time-dependent manner. Early treatment of cedilanid at P12 produced a better inhibitory effect on oxygen-induced retinal neovascularization than treatment at P15. In addition, cedilanid promoted reestablishment of blood vessels in obliterative areas. Furthermore, we found that cedilanid inhibited the expression of HIF-1α and VEGF in mice treated with hyperoxia, suggesting the cedilanid inhibited oxygen-induced retinal neovascularization by inhibiting the expression of HIF-1α. Our findings suggest that cedilanid may be useful for the treatment of neovascular diseases.

In the present study, we tested the dose-dependent effect of cedilanid on retinal neovascularization in a mouse model of oxygen-induced retinopathy, using doses ranging from 0.025 μg to 0.2 μg. At the dose of 0.025 μg, cedilanid did not

statistically significantly inhibit retinal neovascularization, suggesting that this dose of cedilanid may not be sufficiently potent to inhibit retinal neovascularization. At higher doses (0.05 μg to 0.2 μg), cedilanid statistically significantly suppressed retinal neovascularization. The inhibition of cedilanid on retinal neovascularization is in a dose-dependent manner. This dose-dependent effect of cedilanid is similar to that of digoxin as previously reported by Yoshida et al. [12]. At the dose of 0.2 μg, cedilanid resulted in an approximate 80% reduction in the neovascular areas, suggesting that cedilanid is a potent inhibitor of retinal neovascularization.

We also investigated the time-dependent effect of cedilanid on retinal neovascularization with intravitreal injection of cedilanid at different time points (P12 versus P15). At the same dose of cedilanid (0.2 μg), early treatment on P12 produced greater inhibition of retinal neovascularization than treatment on P15. Yoshida et al. found that a single intravitreal injection of digoxin starting on P12 inhibited the expression of HIF-1α and suppressed retinal neovascularization by approximately 75% in a mouse model of oxygen-induced retinopathy [12], suggesting that HIF-1α possibly started to be induced to promote neovascularization on P12 when the mice were removed from a hyperoxic environment to a hypoxic
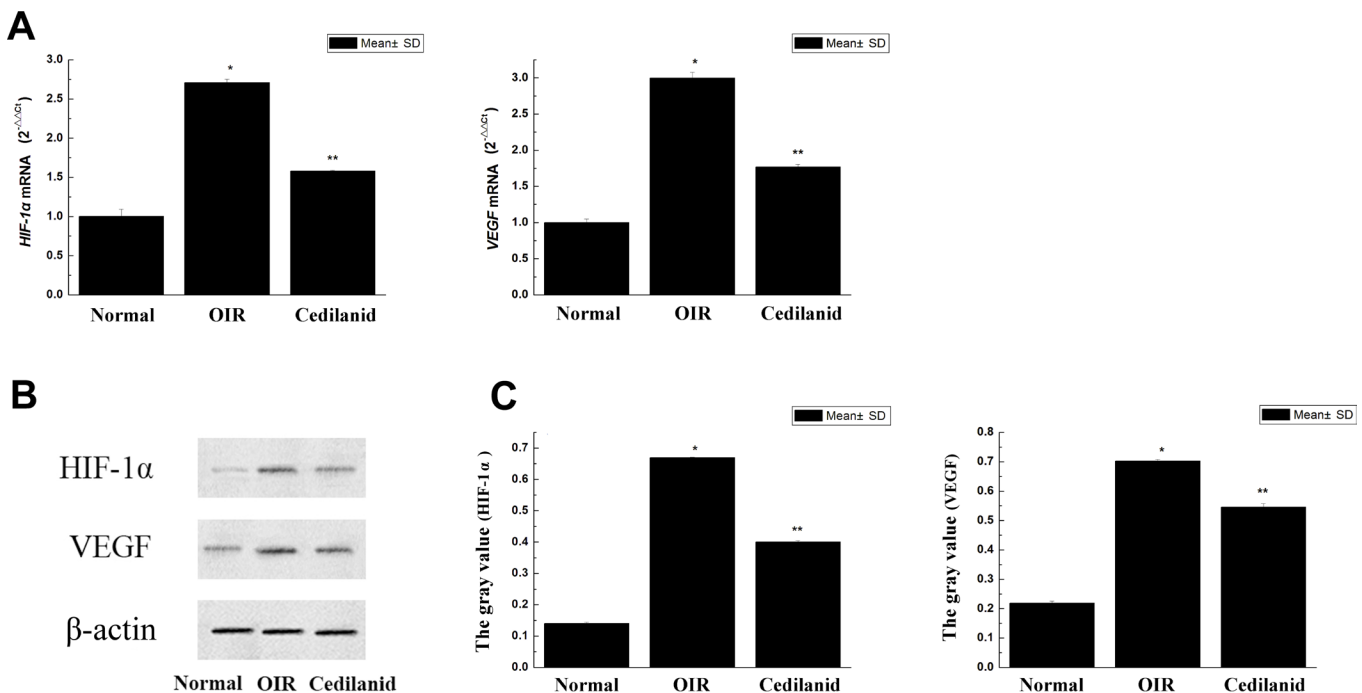

Figure 5. Cedilanid inhibited the expression of HIF-1α and VEGF in retinal tissues from mice treated with normal air, mice treated with hyperoxia, and mice treated with cedilanid after hyperoxic exposure. **A**: Real time (RT)–PCR results show the relative mRNA expression of hypoxia inducible factor 1 alpha (HIF-1α) and vascular endothelial growth factor (VEGF). **B**: Representative western blot results show the protein expression of HIF-1α and VEGF. **C**: Quantification of the protein expression of HIF-1α and VEGF; n = 3. *p<0.05 versus mice with normal air, **p<0.05 versus mice treated with hyperoxia.

environment. Consistent with this idea, it has been reported that an increase in HIF-1α expression peaked at 2 h after the mice were removed from hyperemic conditions to normal air and that the expression of VEGF temporally and spatially correlated with the expression of HIF-1α [18]. Therefore, at P15, retinal neovascularization is formed after the activation of HIF-1α and the expression of proangiogenic factors. Therefore, our findings that late treatment of cedilanid on P15 produced less inhibition of retinal neovascularization suggests that cedilanid may be less effective when given after retinal neovascularization has occurred. Therefore, early treatment is important for effective inhibition of retinal neovascularization by cedilanid.

We also found that intravitreal injection of cedilanid promoted the restoration of blood vessels in the obliterative regions. Most of the reestablished blood vessels in the eyes treated with cedilanid were stained with isolectin GS-IB4 and FITC-dextran, indicating that they were functional vessels. This may explain why cedilanid treatment resulted in a statistically significant reduction of the obliterative areas. Because FITC-dextran is a big molecule (molecular weight, 1.5×10^5) that cannot exudate from the blood vessel into the surrounding tissues, we could not evaluate the permeability of these reestablished blood vessels and the function of the blood–retina barrier. It has been reported that the blood–retina barrier is disrupted in animal models of retinal neovascularization [19,20]. Further studies are required to investigate whether cedilanid treatment improves the function of the blood–retina barrier in the mouse model of oxygen-induced retinopathy.

Compared with digoxin, cedilanid has lower bioaccumulation and a wider therapeutic window. Despite these advantages, use of cedilanid for the treatment of neovascular diseases may be associated with toxicity. In this study, we selected low doses (0.025–0.2 µg) of cedilanid for intravitreal injection. Because the blood–retina barrier can prevent the diffusion of cedilanid into the systemic circulation, theoretically, cedilanid should produce minimal systemic toxicity. However, it remains to be determined whether oral doses that produce inhibition of retinal neovascularization can cause severe toxicity.

The current study has several limitations. First, because intravitreal injection was performed in neonate mice on P12, the difficulty of intravitreal injection led to the deaths of several mice (n = 3), thus leading to the different numbers of animals in each group. The mortality rate was 7.5%, which is similar to that reported in the literature [12]. Second, we did not test the safety of cedilanid after intravitreal injection and oral administration. Future studies should investigate the safe dose and therapeutic window of cedilanid after intravitreal and oral administration.

In summary, we found that intravitreal injection of cedilanid inhibited retinal neovascularization in a mouse model of oxygen-induced retinopathy. Early treatment of cedilanid on P12 produced a better inhibitory effect on retinal neovascularization than on P15. Our findings suggest that cedilanid is a potent inhibitor of retinal neovascularization and may show promise as a treatment targeting neovascular diseases. Early treatment is important for the use of cedilanid to inhibit retinal neovascularization.

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