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Intraocular injection of KH902 alleviates retinal hypoxia in a mouse model of oxygen-induced retinopathy

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Abstract: Inhibition of vascular endothelial growth factor (VEGF) has been widely applied in anti-neovascularization therapies. As a novel anti-VEGF agent, KH902 (conbercept) is designed to restrain pathological angiogenesis. However, the effects of KH902 on retinal hypoxia have not been well studied. In a mouse model of oxygen-induced retinopathy (OIR), we assessed retinal hypoxia at postnatal days 14 (P14) and P17, as well as retinal neovascularization (RNV) at P17. In addition, we evaluated the protein level of VEGF and galectin-1 (Gal-1). Changes of the neuroretinal structure were also examined. Our results indicated that KH902 could remit retinal hypoxia in OIR at P14 and P17, which was an exciting novel finding for KH902 function. Additionally, we confirmed that KH902 markedly reduces RNV. Our results indicated that administration of KH902 downregulated VEGF expression, as well as Gal-1. Damage of neuroretinal structure after KH902 injection was not observed, which was also an encouraging result. Our study suggests that KH902 plays a role in alleviating retinal hypoxia and that it could be used for the treatment of other neovascular ocular diseases.

Keywords: KH902; retinal neovascularization; retinal hypoxia; retinopathy of prematurity; oxygen-induced retinopathy

INTRODUCTION

Pathological angiogenesis is the hallmark of numerous retinal diseases, such as proliferative diabetic retinopathy (PDR) and retinopathy of prematurity (ROP) [1-5]. Increasing evidence indicates that vascular endothelial growth factor (VEGF) plays a central role in pathological angiogenesis [6]. Thus, some anti-VEGF agents have been developed for the treatment of angiogenic conditions, including cancer and age-related macular degeneration [7,8]. However, the major flaw of all these agents is the requirement for repeated administration [9-11].

KH902 (conbercept) is a novel recombinant fusion protein containing extracellular domain 2 of VEGF receptor 1 and extracellular domains 3 and 4 of VEGF receptor 2, which are fused to the constant region (Fc) of human IgG1 [12,13]. Recent studies have shown that KH902 could be used as an antiangiogenic agent [14-16]. It has been used for the treatment of retinal diseases such as age-related macular degeneration (AMD) [17]. Recently, KH902 has been reported to have good clinical efficacy and safety for treating wet AMD [18]. In addition, KH902 has therapeutic efficacy for the treatment of diabetic macular edema [19]. Nevertheless, the effect of intravitreal injection of KH902 on retinal hypoxia as well as retinal structure has not been investigated in depth. Oxygen-induced retinopathy (OIR) is a widely used animal model to study the mechanism and treatment of retinal neovascularization (RNV) [20,21]. Furthermore, retinal hypoxia and RNV can be quantified using the retinal immunostaining technique [21]. Thus, we chose the OIR mouse model in our study.

Galectin-1 (Gal-1) has been indicated to be a key player in the process of angiogenesis [21-23]. After hypoxia, Gal-1 gene expression was upregulated in different human cell lines [23], and inhibition of Gal-1 has been reported as an effective anti-neovascularization intervention [22]. Furthermore, Gal-1 has been indicated to be involved in the pathology of hypoxia in RNV [21].

Taking all of this into consideration, in this study we investigated the effect of KH902 on retinal

neovascularization, especially on retinal hypoxia. Moreover, we evaluated the impact of KH902 on the retinal structure after intraocular injection in the mouse model of oxygen-induced retinopathy (OIR). In addition, we studied the expression of VEGF and Gal-1 after intravitreal injection of KH902.

MATERIALS AND METHODS

Ethics statement

Every effort was made to lower the suffering of animals. All protocols were approved by the Committee on the Ethics of Animal Experiments of Wuhan University, and all animal experiments were carried out strictly according to the Wuhan University Guide for the Care and Use of Laboratory Animals' recommendations. Also, this study conformed to the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research.

OIR model

The Laboratory Animal Center of Wuhan University provided the C57BL/6J mice. An OIR mouse model was generated as previously described [20,21,24,25]. Briefly, at postnatal day 7 (P7), newborn pups were exposed to an environment of oxygen at 75±2% for 5 days and then returned to room air (RA).

Intraperitoneal injection of pimonidazole

Sixty mg/kg of pimonidazole HCl (Hypoxyprobe, Inc. Burlington, MA, USA) was intraperitoneally injected 60 min before the mice were killed [21,24,26].

Intravitreal injection of KH902

A microinjection device was used for precise intraocular injection [21,24,27]. KH902 was purchased from Chengdu Kanghong Biotech Co. Ltd. (Conbercept, Chengdu, China). At P12, the OIR-KH902 group received 2.5 μg per eye with 1 μL volume, which was diluted in sterile phosphate-buffered saline (PBS). The concentration of KH902 injected was less than clinically recommended in humans (10 mg/mL) [19], which was safe and effective in our preliminary experiment. The vehicle control group (OIR-PBS) received an equal amount (1 μL) of PBS. No obvious complications related to the injection procedure and KH902 itself were observed. Mice were randomly divided into four groups: a room-air (RA) group, an OIR group (without any intervention), an OIR-KH902 group (treated with intravitreal injection of KH902), and an OIR-PBS group (treated with PBS as vehicle control).

Hematoxylin-eosin staining

As previously reported [21,24,25], 4% paraformaldehyde (PFA) was used to fix the removed eyes for 24 h, which were embedded in paraffin. Eyeballs were cut serially (6 μm). Only sections that passed through the optic nerve were chosen and stained with hematoxylin and eosin (H&E). Using light microscopy (BX63, Olympus, Tokyo, Japan), neovessel cells were identified and calculated. The density of retinal ganglion cells (RGCs) and retinal thickness were measured as described [21,28-30]. Images of the central retina (450-750 μm from the optic disk) were taken. The thickness of the whole retina, inner nuclear layer (INL) and inner plexiform layer (IPL) were quantified, as well as the RGCs density [21].

Flat-mount retinal immunostaining

Eyeballs were fixed in 4% PFA for 1 h. The retina was flattened with four cuts. After rinsing in PBS and blocked in a buffer containing 5% bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS for 90 min, the retina was incubated with hypoxyprobe (HP) antibody (anti-pimonidazole rabbit antiserum, 1:100, Hypoxyprobe, Inc. Burlington, MA, USA) and *Griffonia simplicifolia* isolectin B4 (IB4) conjugated to Alexa Fluor 594 (1:200, Invitrogen/Thermo Fisher Scientific, MA, USA) for 2 nights at 4°C. After rinsing in PBS with tween-20 (PBST), the retinas were incubated with FITC-AffiniPure goat anti-rabbit IgG (1:200, Jackson ImmunoResearch Laboratories, PA, USA) for one night at 4°C and then mounted. Images of the retinas were taken using fluorescence microscopy (BX63; Olympus, Tokyo, Japan). The data for the hypoxic area, vaso-obliteration (VO) and retinal neovascularization (RNV) were collected as described [21,24,31,32].

Retinal cryosection and confocal laser scanning microscopy

Specimens were processed as described [21,24]. The posterior eyecups were fixed in 4% paraformaldehyde (PFA). After graded dehydration in 10%, 20% and 30% sucrose, respectively, the eyecups were embedded in the optimum cutting temperature compound (Sakura Finetek, CA, USA). Sections were cut at 12-μm thickness by a Leica CM1950 cryostat (Leica Microsystems, Wetzlar, Germany) and then mounted. Sections were incubated with HP antibodies (anti-pimonidazole rabbit antiserum, 1:100, Hypoxyprobe Inc. Burlington, MA, USA). After being rinsed with PBST, cryosections were incubated with FITC-AffiniPure goat anti-rabbit IgG (1:200, Jackson ImmunoResearch Laboratories, PA, USA) for 2 h. The sections were stained with DAPI for 20 min and then rinsed. Images of the section were captured using confocal laser scanning microscopy (FV1200; Olympus, Tokyo, Japan) [21,24].

Western blot (WB) analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the total protein of the retinas, and the proteins were blotted onto polyvinylidene fluoride (PVDF) membranes. After blocking, the PVDF membranes were incubated overnight at 4°C with primary antibodies as follows: a polyclonal goat antibody against galectin-1(Gal-1) (mouse Gal-1 antibody, 0.1 μg/mL, R&D System, Minneapolis, MN, USA), a polyclonal rabbit antibody against VEGF (1:500, Santa Cruz, Dallas, TX, USA), a monoclonal rabbit antibody against β-actin (β-Actin Rabbit mAb, 1:1000, Cell Signaling Technology, Danvers, MA, USA). After washing with TBST, the PVDF membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or donkey anti-goat IgG (1:5000, Jackson ImmunoResearch Laboratories, PA, USA) for 1.5 h [21,24,33]. Chemiluminescence was used to develop the protein band, which was then analyzed using Image J.

Statistical analysis

The data are presented as the mean±standard deviation (SD). Student's *t*-test was used to compare the difference between the two groups. One-way ANOVA was used to analyze group differences; multiple comparisons were carried out with the Bonferroni post hoc test. P<0.05 was considered statistically significant.

RESULTS

Increase of vaso-obliteration and hypoxia in OIR at P12

The retinal vasculature and hypoxic area were stained with IB4 and HP, which served for the immunostaining of retinal vasculature and hypoxia, respectively. The results indicated that the RA group had no VO and hypoxic zone at P12, while severely hypoxia and a large area of VO emerged in the central retina in the OIR group (P<0.001; Fig. 1).

KH902 ameliorates retinal hypoxia in OIR at P14

Two days after injection of KH902, we examined the retinal vasculature and hypoxia and found aberrant vessels growing at the junction between perfused and non-perfused areas in the OIR group. Quantification

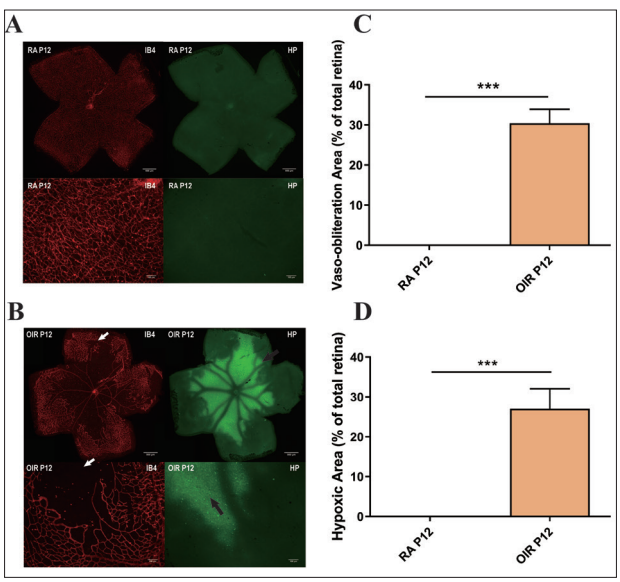

Fig. 1. Vaso-obliteration (VO) and hypoxia in oxygen-induced retinopathy (OIR) at postnatal day 12 (P12). **A** – Room air (RA) group. No VO and the hypoxic area were observed. **B** – OIR group. VO (white arrow) and hypoxia (black arrow). *Griffonia simplicifolia* isolectin B4 (IB4) (red) and hypoxyprobe (HP) (green). Upper panel scale bar, 500 μm; lower panel scale bar, 100 μm. **C**, **D** – Quantification of VO area and hypoxic area. RA group vs. the OIR group, ***P<0.001; n=12 in both groups.

analysis revealed that intravitreal injection of KH902 significantly decreased hypoxic area at P14 compared to those in the OIR (P<0.001) and the OIR-PBS groups (P<0.001; Fig. 2), indicating amelioration of retinal hypoxia by KH902.

KH902 ameliorates retinal hypoxia and inhibits retinal neovascularization at P17

In OIR at P17, the H&E results showed that the number of preretinal neovascular cell nuclei in the OIR-KH902 group was reduced significantly compared to those in the OIR group (P<0.001) and in the OIR-PBS group (P<0.001; Fig. 3).

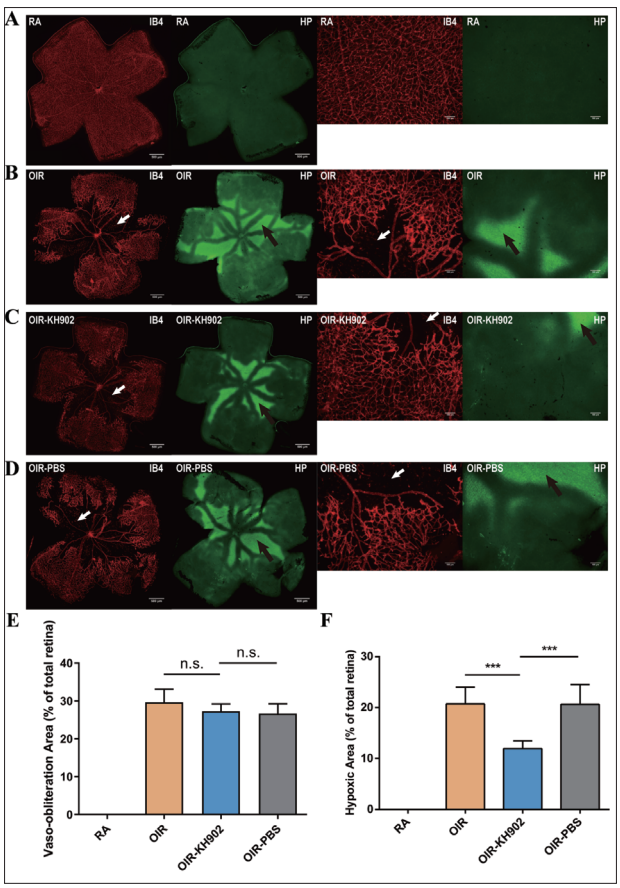

Fig. 2. KH902 ameliorates retinal hypoxia at P14. **A** – RA group. **B** – OIR group. **C** – OIR-KH902 group. **D** – OIR-PBS group. Left panel scale bar, 500 μm; right panel scale bar, 100 μm. IB4 (red) and HP (green). VO (white arrow) and hypoxia (black arrow). *Griffonia simplicifolia* isolectin B4 (IB4) (red) and hypoxyprobe (HP) (green). **E, F** – Quantification of VO and hypoxic area, respectively. The hypoxic area was slightly but significantly decreased in the OIR-KH902 group compared to the OIR group (***P<0.001) and the OIR-PBS group (***P<0.001); n=12 in each group.

We observed that KH902 ameliorated the retinal hypoxia in OIR at P14. Staining of the hypoxic area of the retina to investigate the role of KH902 at P17 showed that KH902 intravitreal injection significantly reduced the retinal hypoxia (P<0.001; Fig. 4). Thus, we propose that intravitreal injection of KH902 can ameliorate hypoxia in OIR, as well as inhibit the RNV. We also stained the retinal vessels to analyze the RNV at P17. Although no RNV was observed in the RA group, the outcome showed obvious neovascular tufts

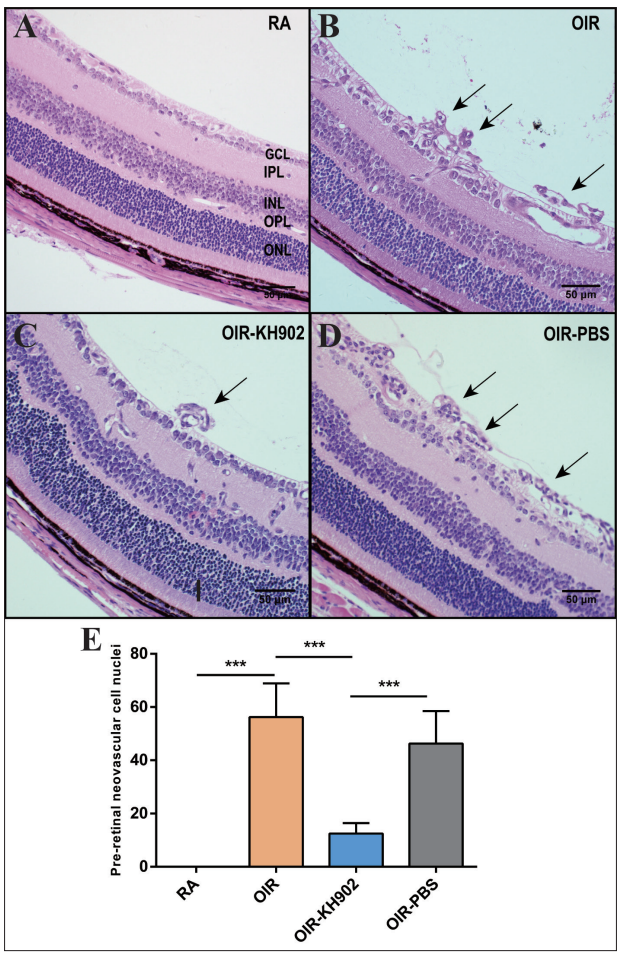

Fig. 3. KH902 reduces the number of preretinal neovascular cells. Quantification of preretinal neovascular cells in OIR at P17. Sections from four groups were stained with hematoxylin and eosin (H&E). **A** – RA group. **B** – OIR group. **C** – OIR-KH902 group. **D** – OIR-PBS group. Black arrows indicate preretinal neovascular cells. Scale bar, 50 μm. **E** – Quantification of preretinal neovascular cell nuclei from four groups. The number of preretinal neovascular cells from the OIR-KH902 group was reduced compared to those from the OIR and the OIR-PBS group. RA group vs. the OIR group, ***P<0.001; OIR-KH902 group vs. the OIR group, ***P<0.001, OIR-KH902 group vs. the OIR-PBS group, ***P<0.001; n=10 in each group.

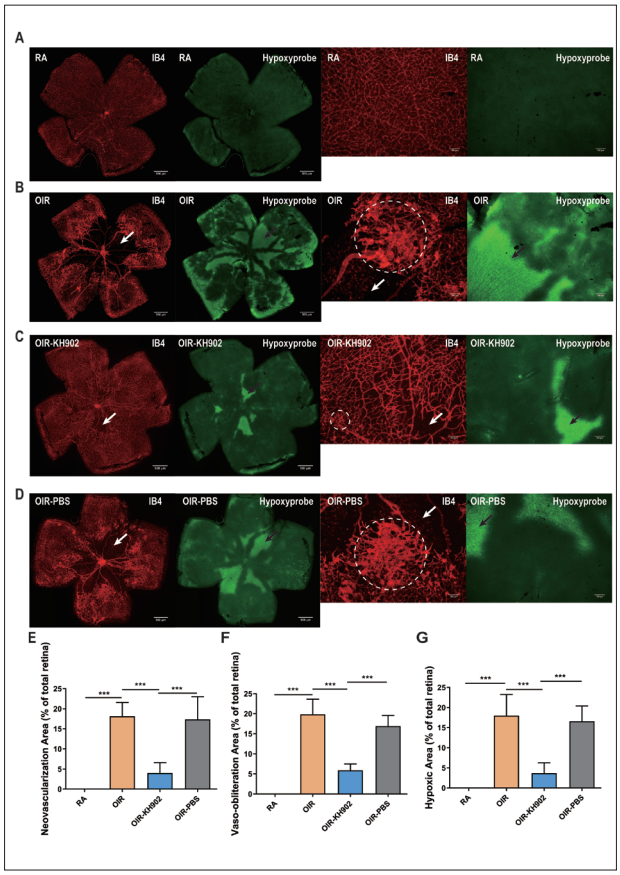

Fig. 4. KH902 ameliorates retinal hypoxia and inhibits retinal neovascularization at P17. **A** – RA group. **B** – OIR group. **C** – OIR-KH902 group. **D** – OIR-PBS group. VO (white arrow) and hypoxia (black arrow). Neovascular tufts (white cycle). *Griffonia simplicifolia* isolectin B4 (IB4) (red) and hypoxyprobe (HP) (green). Left panel scale bar, 500 μm; right panel scale bar, 100 μm. **E** – Quantification of retinal neovascularization (RNV). **F** – Quantification of VO. **G** – Quantification of hypoxia. RNV, VO, and hypoxia area were decreased from the OIR-KH902 group compared to the OIR group and the OIR-PBS group; RA group vs. the OIR group, ***P<0.001; OIR-KH902 group vs. the OIR group, ***P<0.001, OIR-KH902 group vs. the OIR-PBS group, ***P<0.001; n=10 in each group.

emerging in both the OIR group and the OIR-PBS group. RNV was considerably decreased in the OIR-KH902 group compared to the OIR group (P<0.001) and OIR-PBS group (P<0.001; Fig. 4). These results confirmed that KH902 can reduce oxygen-induced neovascularization.

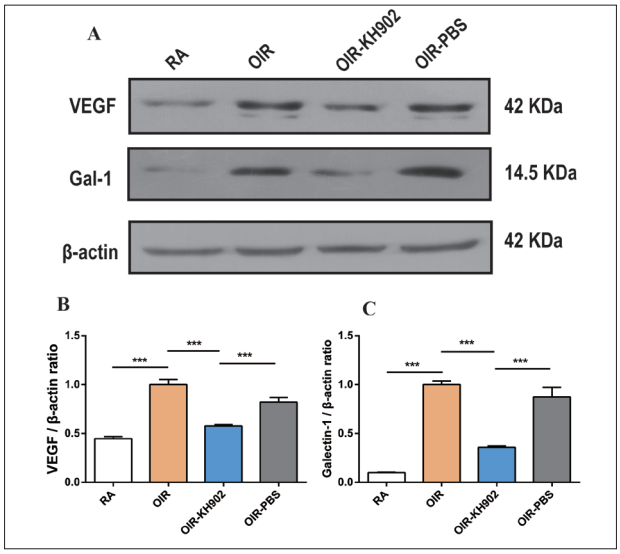

Fig. 5. KH902 downregulates the expression of VEGF and Gal-1 at P17. **A-C** – Protein bands and quantification analysis of VEGF and Gal-1 from four groups. RA group vs. the OIR group, ***P<0.001; OIR-KH902 group vs. the OIR group, ***P<0.001, OIR-KH902 group vs. the OIR-PBS group, ***P<0.001. Data are presented as the mean±SD of three independent experiments.

KH902 downregulates the expression of VEGF and Gal-1 at P17

Western blot analysis performed to assess the relative protein level of VEGF and Gal-1 (Fig. 5) demonstrated that KH902 suppressed the expression of VEGF as well as Gal-1 compared to those in the OIR group (P<0.001) and in the OIR-PBS group (P<0.001; Fig. 5).

KH902 does not significantly damage neuroretinal structure at P17

The alteration of the neuronal structure after injection of KH902 was examined. Normally, there will be a significant retinal neuronal degeneration in OIR after oxygen exposure [22]. In our study, at P17, cryosection staining of HP indicated that the hypoxic area emerged mainly from INL to ganglion cells layer (GCL), but to a less extent in outer nuclear layer (ONL) and photoreceptor layer. Therefore, H&E staining was performed to assess retinal thickness, INL thickness, IPL thickness and RGCs density, which did not change significantly in the OIR-KH902 group compared to those in the OIR group (P>0.05) and in the OIR-PBS group (P>0.05; Fig. 6).

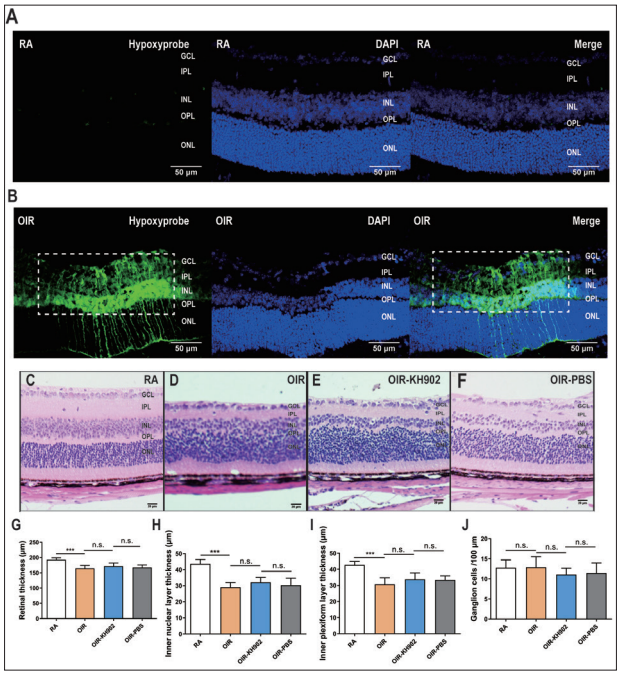

Fig. 6. KH902 does not significantly impact retinal neuronal structure at P17. **A** – RA group. **B** – OIR group. Scale bar, 50 μm; Hypoxic condition of the retina in OIR at P17 staining by HP (green), enrichment of staining (white rectangle) was observed mainly from inner nuclear layer (INL), inner plexiform layer (IPL) to ganglion cells layer (GCL). **C-F** – H&E staining of cross-sections. Scale bar, 20 μm. **G-J** – Quantification analysis of retinal thickness, INL thickness, IPL thickness and retinal ganglion cell density. RA group vs. the OIR group, ***P<0.001; OIR-KH902 group vs. the OIR group, P>0.05, OIR-KH902 group vs. the OIR-PBS group, P>0.05; n.s.: no significant; n=12 in RA group and n=15 in other groups.

DISCUSSION

In the mouse model of OIR, we confirmed that KH902 has an antiangiogenesis property in RNV diseases. Moreover, our results indicate that intravitreal injection of KH902 ameliorated retinal hypoxia both at P14 and P17, which is a new finding that could extend the application of KH902 in clinical treatment. Additionally, the KH902 injection did not significantly damage the neuroretinal structure in the OIR model at P17, which is also a novel observation. We also showed that the KH902 injection downregulated Gal-1, which is also important in the process of angiogenesis [21-23].

In the OIR model, oxygen overexposure leads to a degeneration of the retinal capillaries from P7 to P12, whereas relative hypoxia after P12 induces aberrant neovascularization [20]. Hypoxia, a critical feature of pathological conditions, activates angiogenic signals by preventing hypoxia-inducible factors (HIFs) from degradation [34,35]. Recently, HP has been broadly applied as a marker to detect and quantify hypoxia [36-38]. Our results showed that retinal hypoxia existed at P12, and previous studies showed that retinal hypoxia triggers the pathological angiogenic process, leading to RNV [39,40]. Thus, we speculated that ameliorating retinal hypoxia in the early stage should be important in inhibiting the formation of RNV. Accordingly, we investigated the status of retinal hypoxia at both P14 and P17 and found that intravitreal injection of KH902 significantly weakened the retinal hypoxia in both stages. The outcome also implied that retinal hypoxia could be a more effective and sensitive indicator than the RNV, due to its earlier emergence.

The pharmacokinetic profile of KH902 is very similar to aflibercept, with the main difference being the presence of a portion dedicated to VEGFR2, which was designed to increase the efficacy and stability and to produce relative affinity for VEGF-C [41,42]. In clinical practice, KH902 has been used for the treatment of retinal diseases such as wet AMD [17,18]. One experimental study indicated that KH902 inhibited RNV *in vivo* [16]. It was previously shown that KH902 had marked inhibitory effects on angiogenesis both *in vitro* and *in vivo* [15], which was consistent with our confirmation in the OIR model at P17. However, the role of KH02 in remitting retinal hypoxia was not revealed. We discovered that KH902 alleviates the retinal hypoxia in the early stage at P14, as well as the late stage at P17, which is a promising observation. Our findings suggest that KH902 possesses the potential for treatment of retinopathy of prematurity (ROP) and other hypoxic angiogenic diseases.

Gal-1 plays a critical role in promoting angiogenesis in anti-VEGF refractory tumors [22]. In our study, VEGF and Gal-1 both decreased after KH902 injection, which was consistent with previous studies [21,23,24]. As Gal-1 is also a critical factor in the process of pathological angiogenesis, further studies should investigate the correlation between VEGF and Gal-1 and the relationship between KH902 and Gal-1.

Retinal neuronal degeneration occurs in OIR and not in control mice raised in room air [29,30]. In the

inner layer, the number of TUNEL-positive cells was shown to be increased, but not in the outer layer of the retina in OIR [43]. Our results also indicate that the retina suffered from serious hypoxia in OIR, especially from INL to GCL. Previous studies of KH902 did not investigate the influence of KH902 on neuroretinal structure [15,16]. In the present study we found no significant difference in INL thickness, IPL thickness or RGC density after the application of KH902. However, further studies should evaluate different dosages and timing of KH902 administration, as well as assess the long-term effects of KH902 on retinal development and functions, which may provide a more profound understanding. In addition, further studies should examine the molecular mechanism of KH902 in remitting retinal hypoxia.

Our study indicates that intravitreal injection of KH902 alleviates retinal hypoxia in OIR, which is a novel and promising finding for the treatment of RNV diseases. We showed that KH902 injection does not damage the neuroretinal structure, thereby enhancing the safety of the application. Additionally, we confirmed the anti-neovascularization property of KH902, suggesting its efficacy in treating other neovascular ocular diseases as well. In summary, our study supports the advantages of KH902 for the treatment of RNV and offers a promising strategy for other neovascular ocular diseases.

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Author contributions: Experimental design and supervision: NY and YX; experimental implementation: NY, XH, and NZ; manuscript writing and review: NY and YX.

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