In Vivo Retinal Vascular Oxygen Tension Imaging and Fluorescein Angiography in the Mouse Model of Oxygen-Induced Retinopathy

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Submitted: March 28, 2013
Accepted: September 5, 2013

Citation: Mezu-Ndubuisi OJ, Teng P, Wanek J, et al. In vivo retinal vascular oxygen tension imaging and fluorescein angiography in the mouse model of oxygen-induced retinopathy. *Invest Ophthalmol Vis Sci*. 2013;54:6968–6972. DOI:10.1167/iovs.13-12126

Purpose. Oxygenation abnormalities are implicated in the development of retinopathy of prematurity (ROP). The purpose of this study is to report in vivo retinal vascular oxygen tension (PO_2) measurements and fluorescein angiography (FA) findings in the mouse model of oxygen-induced retinopathy (OIR).

Methods. We exposed 19 neonatal mice to 77% oxygen from postnatal day 7 (P7) to P12 (OIR), while 11 neonatal mice were kept under room air (control). Using phosphorescence lifetime imaging, retinal vascular PO_2 was measured followed by FA. Repeated measures ANOVA was performed to determine the effects of blood vessel type (artery and vein) and group (OIR and control) on PO_2. Avascular retinal areas were measured from FA images in OIR mice.

Results. There was a significant effect of vessel type on PO_2 ($P < 0.001$). The effect of group on PO_2 was not significant ($P = 0.3$), indicating similar PO_2 between OIR and control mice. The interaction between group and vessel type was significant ($P = 0.03$), indicating a larger arteriovenous PO_2 difference in OIR mice than control mice. In control mice, FA displayed normal vascularization, while FA of OIR mice showed abnormalities, including dilation and tortuosity of major retinal blood vessels, and avascular regions. In OIR mice, the mean percent avascular retinal area was 33% ± 18%.

Conclusions. In vivo assessment of retinal vascular oxygen tension and vascularization patterns demonstrated abnormalities in the mouse model of OIR. This approach has the potential to improve understanding of retinal vascular development and oxygenation alterations due to ROP and other ischemic retinal diseases.

Keywords: retina, vascular oxygen tension, oxygen-induced retinopathy, retinopathy of prematurity, fluorescein angiography

Retinopathy of prematurity (ROP) occurs when in utero normal retinal blood vessel development in physiologic hypoxia is disrupted by preterm birth into a relatively hyperoxic extrauterine environment. These oxygenation abnormalities, further exacerbated by the use of supplemental oxygen, inhibit normal retinal vasculature development. This can impair the retinal supply of oxygen and nutrition, leading to pathologic neovascularization, cicatrization, and detachment. Even with current therapies, many preterm infants still have long-term visual deficits[1] and ROP remains a major cause of childhood blindness worldwide.[2]

Postnatal vascular development has been studied in retinal tissue removed from murine models of oxygen-induced retinopathy (OIR) using methods such as paraffin-embedded retinal histology sections,[3] cryosections of whole mount retinas,[4] and fluorescence immunohistochemistry of retinal flat mounts.[5–7] Although in vivo retinal fundus imaging in OIR mice has been reported recently,[8] to our knowledge, fluorescein angiography (FA), which allows better visualization of retinal vascular abnormalities in live OIR mice, has not been performed previously.

Inspired level of oxygen is the most controllable risk factor for ROP; hence, knowledge of retinal oxygenation is important for advancing therapeutic interventions. Currently, no clinical methods are available to assess retinal oxygenation in newborn humans. In OIR mice, the response of retinal oxygenation to a carbogen challenge has been studied by magnetic resonance imaging.[9,10] In healthy mice, direct measurements of retinal tissue oxygen tension (PO_2) by oxygen-sensitive microelectrodes[11] and retinal vascular PO_2 by phosphorescence lifetime imaging[12] have been performed only in adults. In our study, we report measurements of retinal vascular PO_2 combined with FA in neonatal healthy and OIR mice using in vivo imaging techniques.

Methods

Animals

Neonatal C57BL/6J mice were obtained from breeding colonies maintained at the University of Illinois at Chicago (Chicago, IL). All experimental procedures received approval from the Animal

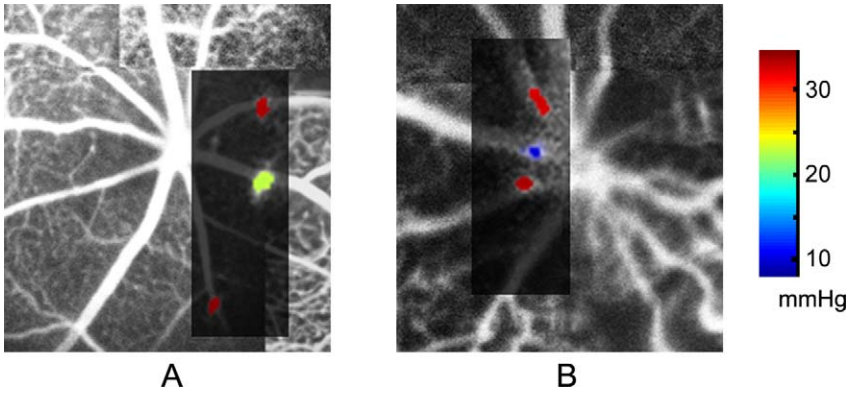

FIGURE 1. Examples of cross-sectional vascular PO_2 maps (*rectangles*) superimposed on fluorescein angiograms in control (**A**) and OIR (**B**) mice depicting PO_2 measurements in major retinal vessels. *Color bar* displays PO_2 in mm Hg.

Care Committee of the University of Illinois at Chicago, and were in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. To induce OIR, mice were exposed to 77% ± 2% (mean ± SD) oxygen in a regulated hyperoxia chamber (Biospherix Ltd., Redfield, NY) from postnatal day 7 (P7) to P12, after which they were returned to room air.[13] The control mice were kept under room air continuously throughout the length of the experiment. The control and OIR litters were placed with their mothers in separate cages. The mothers of the OIR mice were alternated with extra nursing adult mice every 24 hours to prevent the nursing females from incurring pulmonary toxicity from prolonged exposure to hyperoxia.

Before imaging, mice were anesthetized by intraperitoneal injections of ketamine (100 mg/kg) and xylazine (5 mg/kg), and anesthesia was maintained by supplemental injections of ketamine (20 mg/kg) and xylazine (1 mg/kg) as needed. The pupils were dilated with 1% tropicamide. Retinal vascular PO_2 imaging and FA were performed sequentially in one eye of each mouse. For retinal vascular PO_2 imaging, Pd-porphine (Frontier Scientific, Logan, UT), an oxygen-sensitive albumin-bound phosphorescence molecular probe, was injected intravenously (20 mg/kg) using a 30-gauge needle (Becton, Dickinson, and Co., Franklin Lakes, NJ), while viewing the tongue vein with an operating microscope. Before imaging, 1% hydroxypropyl methylcellulose and a glass cover slip were applied to the cornea to prevent corneal dehydration, and to eliminate the cornea's refractive power. For FA, 10% fluorescein sodium (AK-FLUOR; Akorn, Decatur, IL) was injected intraperitoneally (100 mg/kg) and imaging was performed in the same eye within 5 minutes. Before FA, the glass cover slip was removed and the cornea irrigated with saline.

Retinal Vascular Oxygen Tension Imaging

Retinal vascular PO_2 was measured using an established optical section phosphorescence lifetime imaging system.[14,15] A laser beam was focused to a line and projected at an oblique angle on the retina. Using an intensified charge-coupled device camera attached to a slit-lamp biomicroscope (Carl Zeiss, Oberkochen, Germany), optical section phosphorescence images were acquired nasal and/or temporal to the optic disc. Since the incident laser and imaging axis were not coaxial, retinal vessels were displaced laterally from the choroidal vessels in the section image according to depth. Additionally, due to the binding of the oxygen-sensitive molecular probe to albumin, measurements were obtained only within the retinal blood vessels and not tissue. Images were analyzed to measure phosphorescence lifetimes, which were converted to PO_2 using the Stern-Volmer relationship.[16] In each mouse, two or three repeated PO_2 measurements in the same retinal blood vessel were averaged. Overall, PO_2 measurements were obtained in 6 ± 2 blood vessels in one eye of each mouse. The number of vessels in which PO_2 was measured varied due to anatomic differences among mice. In addition, small pupil size also limited phosphorescence imaging of some vessels. In OIR mice, abnormalities in the retinal vasculature often precluded a clear distinction between arteries and veins based on FA. Since the blood vessel with the highest PO_2 had to be an artery and the blood vessel with the lowest PO_2 had to be a vein, in each mouse, the maximum and minimum of all vascular PO_2 measurements were designated as retinal arterial and venous PO_2, respectively. The arteriovenous PO_2 difference was calculated as the difference between maximum and minimum PO_2 measurements. Although retinal arterial and venous PO_2 are related to retinal oxygenation, the arteriovenous PO_2 difference is of particular interest, because it is the vascular parameter related directly to the amount of oxygen consumed by the retinal tissue.[17,18] Therefore, comparison of arteriovenous PO_2 difference between control and OIR mice will determine the presence of abnormal retinal oxygen extraction. Due to the small size of the mice, it was not feasible to measure systemic arterial PO_2 directly. Therefore, to reduce variability due to systemic physiologic condition and anesthesia, and ensure that control and OIR mice had similar retinal arterial oxygen levels, data were included from 11 control and 19 OIR mice that had retinal arterial PO_2 within one SD of the mean in control mice (35 ± 11 mm Hg, $N = 13$). Data from 2 outliers from each of the control and OIR groups were excluded.

Retinal Fluorescein Angiography

A scanning laser ophthalmoscope (Spectralis; Heidelberg Engineering, Heidelberg, Germany) was used to perform FA immediately after retinal vascular PO_2 imaging. Multiple FA images, each encompassing a field of view approximately 5 optic disc diameters, were acquired superior, inferior, temporal, and nasal to the optic disc. These images were aligned based on the continuity of blood vessels to generate a montage FA, displaying a larger field. Customized software was developed with the use of Matlab software (Mathworks, Natick, MA) to determine the percent avascular retinal area from the montage FA image. All avascular retinal regions were identified manually, and their areas were summed automatically and divided by the total retinal area imaged. Percent avascular retinal areas were determined by three independent

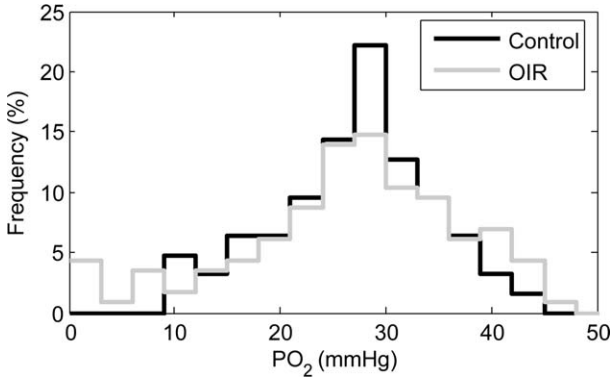

FIGURE 2. Normalized distribution profiles of retinal vascular PO_2 measurements obtained in 63 vessels of control and 115 vessels of OIR mice.

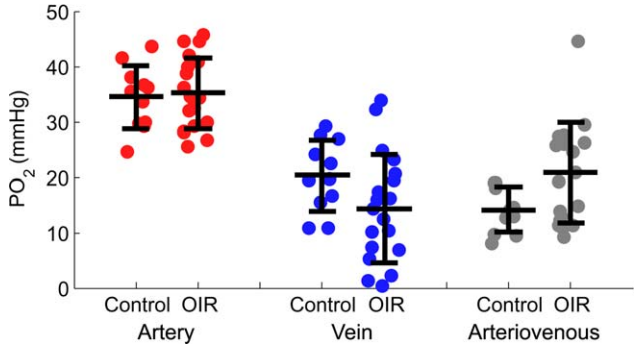

FIGURE 3. Retinal arterial PO_2, venous PO_2, and arteriovenous PO_2 difference in 11 control and 19 OIR mice. *Symbols* represent measurements in individual mice, while *horizontal bars* represent the mean ± SD in each group.

observers (OJM-N, NPB, FYC) from gradable FA images in 18 of 19 OIR mice.

Statistical Analysis

Repeated measures ANOVA was performed to determine the effects of blood vessel type (artery and vein) and group (OIR and control) on vascular PO_2. The agreement among the three observers' gradings of percent avascular retinal area was determined by calculating the intraclass correlation coefficient (ICC). Percent avascular retinal areas were calculated in each OIR mouse by averaging the three measurements. Statistical significance was accepted at $P < 0.05$.

Results

Examples of cross-sectional vascular PO_2 maps overlaid on montage FAs of control (P19) and OIR (P17) mice are shown in Figures 1A and 1B, respectively. In these two mice, retinal arterial PO_2 measurements were similar, while retinal venous PO_2 was lower in the OIR mouse compared to that of the control mouse.

Retinal vascular PO_2 measurements were obtained in 63 and 115 retinal blood vessels of 11 control and 19 OIR mice, respectively. The normalized distribution profiles of PO_2 measurements in control and OIR mice were similar, both displaying a single peak (Fig. 2). The PO_2 measurements averaged over all blood vessels in control (27 ± 6 mm Hg, $N = 11$) and OIR (25 ± 7 mm Hg, $N = 19$) mice were similar ($P = 0.48$). The variation (SD) of PO_2 among blood vessels of the same animal were on average 7 and 9 mm Hg in control and OIR mice, respectively. Values of vascular PO_2 less than 10 mm Hg were present only in OIR mice.

Measurements of retinal arterial PO_2, venous PO_2, and arteriovenous PO_2 difference in individual control and OIR mice are plotted in Figure 3. Mean retinal arterial and venous PO_2 in control mice were 34 ± 6 and 20 ± 6 mm Hg ($N = 11$), respectively. Mean retinal arterial and venous PO_2 in OIR mice were 35 ± 6 and 14 ± 10 mm Hg ($N = 19$), respectively. Mean arteriovenous PO_2 difference in control and OIR mice was 14 ± 4 and 21 ± 9 mm Hg, respectively. As expected, there was a significant effect of vessel type (artery and vein) on PO_2 ($P < 0.001$). Furthermore, there was no significant effect of group (OIR and control) on PO_2 ($P = 0.3$), indicating similar vascular PO_2 between OIR and control mice. However, there was a significant interaction between group and vessel type ($P = 0.03$), demonstrating a significantly larger arteriovenous PO_2 difference in OIR mice compared to control mice.

An example of an FA image in a control mouse shows normal vascularization and a uniform capillary network between major retinal blood vessels (Fig. 1A). In contrast, FA images of OIR mice displayed dilation and tortuosity of major retinal vessels, venous beading, dilation of capillaries, microaneurysms (Fig. 4A), arteriovenous shunting (Fig. 4B), and regions of avascularity (Figs. 1B, 4A, 4B). Manually selected avascular retinal regions in one OIR mouse are shown in Figure 4A. Based on compiled data in all OIR mice, the ICC of the percent avascular retinal areas graded by the three observers was 0.95. The mean percent avascular retinal area in OIR mice was 33% ± 18% ($N = 18$).

Discussion

Oxygen has been implicated directly in the pathogenesis of ROP.[13] Therefore, it is important to measure variations in retinal oxygenation that can trigger biochemical and metabolic changes leading to abnormal vascularization. However, the lack of available methods for measuring retinal oxygenation in ROP has led to studies of OIR using ex vivo techniques in mice and rats,[4,6,7,13] which can provide only limited information about retinal physiology. In our study, in vivo quantitative assessment of abnormalities in retinal vascular PO_2 and vascular patterns in OIR mice were demonstrated.

Retinal vascular PO_2 measurements were reported in neonatal control mice for the first time to our knowledge. These measurements were lower than previously reported values in adult mice, using a similar phosphorescence imaging technique, but with lower depth discrimination.[12,19] As noted in these studies, measurements may have been artificially high due to contribution from the choroidal phosphorescence signal. Retinal venous PO_2 measurements in our study were in agreement with inner retinal tissue PO_2 measured with oxygen-sensitive microelectrodes in adult mice.[11]

The arteriovenous PO_2 difference in OIR mice was significantly larger than in control mice. This finding is in line with previously reported differences in the retinal oxygenation response between OIR and control mice by MR spectroscopy.[10] The higher arteriovenous PO_2 difference in OIR may be a consequence of the sparseness of the vascular network, such that more oxygen extraction must have occurred from these blood vessels to supply a larger volume of retinal tissue.

Retinal vascular patterns observed in FA images of OIR mice were grossly abnormal. The OIR mice showed avascular regions as well as dilation and tortuosity of major retinal vessels. The observed avascular retinal regions in the vicinity of the optic nerve head were similar to those reported previously

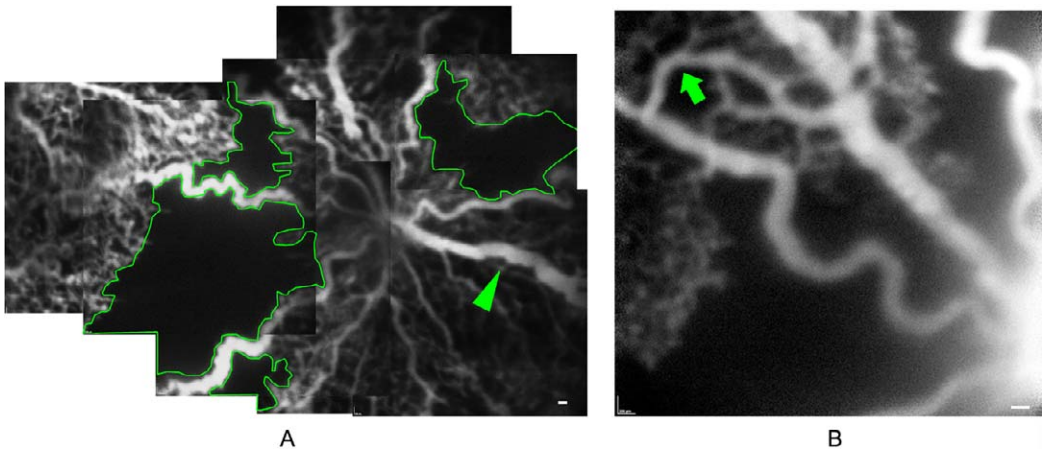

FIGURE 4. Examples of fluorescein angiograms in 2 OIR mice displaying retinal vascular abnormalities that include venous beading (*arrowhead* in [**A**]), arteriovenous shunting (*arrow* in [**B**]), dilation and tortuosity of major retinal vessels, dilation of capillaries, microaneurysms, and regions of avascularity. Manually selected avascular retinal regions are outlined with *green lines* (**A**).

in ex vivo studies in OIR mice.[6,13,20,21] Our measurement of avascular retinal area (33%) is consistent with several reported values (22% to 35%) in similarly-aged OIR mice using the ex vivo retinal flat mount technique.[8,10,22] Comparable vascular abnormalities, but in peripheral retina, are observed in human ROP,[23–25] as well as in a rat OIR model.[7]

One might have expected the measured degree of retinal avascularity in our study to result in an even larger arteriovenous PO_2 difference in OIR mice. However, it appears that substantial regions of avascular retina were not able to extract oxygen by diffusion due to absence of nearby retinal vessels. As a result, these avascular retinal regions did not likely impact the arteriovenous PO_2 difference. Accordingly, arteriovenous PO_2 difference measurements in our study almost certainly underestimated the severity of retinal hypoxia in OIR mice.

One limitation of our study was distinguishing retinal arteries and veins in the OIR mice, due to gross vascular abnormalities. Therefore, in each mouse, the maximum and minimum PO_2 measurements were designated as retinal arterial and venous PO_2, respectively. Since in individual mice, all retinal arteries are supplied by the same parent source of blood, arteries should have small variations and higher levels of PO_2 than retinal veins. Thus, in each mouse, the maximum PO_2 was likely a reasonable estimate of retinal arterial PO_2. In contrast, the lowest retinal venous PO_2 (minimum PO_2) may have been less representative of PO_2 values in all veins due to local retinal physiologic and, in the case of OIR mice, pathologic variations. Another limiting factor was the lack of monitoring of the systemic arterial PO_2 which may have been a source of measurement variability.

The normalized PO_2 distribution profiles in neonatal control and OIR mice both displayed a single peak. However, two distinct peaks, corresponding to retinal arterial and venous PO_2, were expected, similar to profiles obtained in healthy ventilated adult rats (our unpublished data). The expected existence of two peaks may have been obscured because the peak separation was smaller than the distribution widths. The magnitude of the peak separation is determined by the oxygen metabolic rate, while the distribution widths represent variability in retinal arterial and venous PO_2. Variability in retinal arterial PO_2 is dependent on variations in the systemic physiologic condition, while variability in the retinal venous PO_2 is determined by variations in oxygen metabolic rate and arterial PO_2. Since the PO_2 distribution profiles in the neonatal control and OIR mice displayed only a single peak (not two peaks, one for arteries and one for veins), one may speculate that the oxygen metabolic rate is likely lower (less separation between peaks, lower arteriovenous difference), and the physiologic condition is more variable (wider distributions) in neonatal mice than in adults. Although the shapes of the distribution profiles in control and OIR mice were similar, abnormally low values of vascular PO_2 ($<$10 mm Hg) were present only in OIR mice, which may be attributed to veins that collected blood from large hypoxic regions of avascularity.

In summary, to our knowledge this is the first report of quantitative measurements of retinal vascular PO_2 and avascularity from FA in live OIR mice. Further longitudinal studies using in vivo imaging techniques are necessary to elucidate the spatial relationship between retinal vascularization and oxygenation in OIR mice during vaso-obliteration and revascularization phases. This may facilitate better understanding of oxygen-induced disease processes and, thereby, advance development of therapies for ROP. Furthermore, since the OIR model causes retinal avascularity, the findings of our study also may be relevant to other ischemic retinopathies, such as diabetic retinopathy.[26]

Acknowledgments

Supported by National Institutes of Health Grants R01 EY017918, P30 EY001792, R01 HL66109, and R01 ES11863, and Research to Prevent Blindness

Disclosure: **O.J. Mezu-Ndubuisi**, None; **P.-Y. Teng**, None; **J. Wanek**, None; **N.P. Blair**, None; **F.Y. Chau**, None; **N.M. Reddy**, None; **J.U. Raj**, None; **S.P. Reddy**, None; **M. Shahidi**, P

References

1. Rivera JC, Sapieha P, Joyal JS, et al. Understanding retinopathy of prematurity: update on pathogenesis. *Neonatology.* 2011; 100:343–353.
2. Gilbert C. Retinopathy of prematurity: a global perspective of the epidemics, population of babies at risk and implications for control. *Early Hum Dev.* 2008;84:77–82.
3. Gu X, Samuel S, El-Shabrawey M, et al. Effects of sustained hyperoxia on revascularization in experimental retinopathy of prematurity. *Invest Ophthalmol Vis Sci.* 2002;43:496–502.
4. Saito Y, Geisen P, Uppal A, Hartnett ME. Inhibition of NAD(P)H oxidase reduces apoptosis and avascular retina in an animal

model of retinopathy of prematurity. *Mol Vis*. 2007;13:840–853.

5. Fruttiger M. Development of the mouse retinal vasculature: angiogenesis versus vasculogenesis. *Invest Ophthalmol Vis Sci*. 2002;43:522–527.
6. Connor KM, Krah NM, Dennison RJ, et al. Quantification of oxygen-induced retinopathy in the mouse: a model of vessel loss, vessel regrowth and pathological angiogenesis. *Nat Protoc*. 2009;4:1565–1573.
7. Penn JS, Tolman BL, Lowery LA. Variable oxygen exposure causes preretinal neovascularization in the newborn rat. *Invest Ophthalmol Vis Sci*. 1993;34:576–585.
8. Furtado J, Davies M, Choi D, et al. Imaging retinal vascular changes in the mouse model of oxygen-induced retinopathy. *Trans Vis Sci Technol*. 2012;1:5.
9. Ito Y, Berkowitz BA. MR studies of retinal oxygenation. *Vision Res*. 2001;41:1307–1311.
10. Roberts R, Zhang W, Ito Y, Berkowitz BA. Spatial pattern and temporal evolution of retinal oxygenation response in oxygen-induced retinopathy. *Invest Ophthalmol Vis Sci*. 2003;44:5315–5320.
11. Yu DY, Cringle SJ. Oxygen distribution in the mouse retina. *Invest Ophthalmol Vis Sci*. 2006;47:1109–1112.
12. Shonat RD, Kight AC. Oxygen tension imaging in the mouse retina. *Ann Biomed Eng*. 2003;31:1084–1096.
13. Smith LE, Wesolowski E, McLellan A, et al. Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci*. 1994;35:101–111.
14. Shahidi M, Shakoor A, Blair NP, Mori M, Shonat RD. A method for chorioretinal oxygen tension measurement. *Curr Eye Res*. 2006;31:357–366.
15. Shahidi M, Wanek J, Blair NP, Mori M. Three-dimensional mapping of chorioretinal vascular oxygen tension in the rat. *Invest Ophthalmol Vis Sci*. 2009;50:820–825.
16. Lakowicz JR, Szmacinski H, Nowaczyk K, Berndt KW, Johnson M. Fluorescence lifetime imaging. *Anal Biochem*. 1992;202:316–330.
17. Teng PY, Wanek J, Blair NP, Shahidi M. Inner retinal oxygen extraction fraction in rat. *Invest Ophthalmol Vis Sci*. 2013;54:647–651.
18. Wanek J, Teng PY, Albers J, Blair NP, Shahidi M. Inner retinal metabolic rate of oxygen by oxygen tension and blood flow imaging in rat. *Biomed Opt Express*. 2011;2:2562–2568.
19. Wilson DF, Vinogradov SA, Grosul P, Kuroki A, Bennett J. Imaging oxygen pressure in the retina of the mouse eye. *Adv Exp Med Biol*. 2005;566:159–165.
20. Stahl A, Connor KM, Sapieha P, et al. The mouse retina as an angiogenesis model. *Invest Ophthalmol Vis Sci*. 2010;51:2813–2826.
21. Dorfman A, Dembinska O, Chemtob S, Lachapelle P. Early manifestations of postnatal hyperoxia on the retinal structure and function of the neonatal rat. *Invest Ophthalmol Vis Sci*. 2008;49:458–466.
22. Li J, Wang JJ, Peng Q, et al. Macrophage metalloelastase (MMP-12) deficiency mitigates retinal inflammation and pathological angiogenesis in ischemic retinopathy. *PLoS One*. 2012;7:e52699.
23. Raghuveer TS, Bloom BTA. Paradigm shift in the prevention of retinopathy of prematurity. *Neonatology*. 2006;100:116–129.
24. Flynn JT, O'Grady GE, Herrera J, Kushner BJ, Cantolino S, Milam W. Retrolental fibroplasia: I. Clinical observations. *Arch Ophthalmol*. 1977;95:217–223.
25. Ober R, Palmer E, Drack A, Wright K. Retinopathy of prematurity. In: Wright K, Spiegel P, Thompson L, eds. *Handbook of Pediatric Retina Disease*. New York, NY: Springer; 2006:284–349.
26. McVicar CM, Hamilton R, Colhoun LM, et al. Intervention with an erythropoietin-derived peptide protects against neuroglial and vascular degeneration during diabetic retinopathy. *Diabetes*. 2011;60:2995–3005.

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