Chapter 22

Models of Oxygen Induced Retinopathy in Rodents

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Abstract

Much of the knowledge we have gained into the development of pathological ocular angiogenesis has come from the development of in vivo models that enable functional assessment of key components of signaling pathways in disease progression. Indeed, rodent models have facilitated identification of several therapeutics that target pathological angiogenesis. Two of the most widely used rodent models of oxygen induced retinopathy (OIR), Smith's mouse model and Penn's rat model reproducibly induce neovascularization reminiscent of the disease retinopathy of prematurity (ROP). In this chapter we discuss development of ROP in humans and compare features with that of the rat and mouse models, focusing both on the benefits and caveats of using such models. Furthermore, we discuss in detail the methodology of both procedures and discuss the importance of various features of the model.

Key words Retinopathy of prematurity, Oxygen-induced retinopathy, Neovascularization, Angiogenic cascade

1 Introduction

Retinopathy of Prematurity (ROP) is a potentially blinding disease affecting premature infants. Like other retinopathies including age-related macular degeneration (AMD) and proliferative diabetic retinopathy (PDR), ROP is characterized by pathological ocular angiogenesis and retinal neovascularization (NV) [1, 2]. Premature birth alters the development of the retinal vasculature resulting in the delayed maturation of vessels, retinal ischemia at the peripheral retina causes a decrease in available oxygen that is detected in the retina by Müller cells which, in turn secrete large amounts of growth factors, namely vascular endothelial growth factor (VEGF). VEGF is a potent endothelial cell-selective mitogen detected by receptors on retinal microvascular endothelial cells which, together with other signals leads to the induction of an angiogenic cascade [3]. The angiogenic signaling cascade makes for an attractive therapeutic target in ocular diseases that exhibit pathological neovascularization.

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Knowledge gained from in vivo models of retinal diseases have yielded much of what we know about physiological and pathological blood vessel growth in the retina [4], knowledge that has also impacted our understanding of non-ocular angiogenic conditions. In this chapter we discuss two oxygen-induced retinopathy (OIR) models of ROP that have been developed over the years and have both been used to identify and therapeutically target molecular mechanisms that control the angiogenic cascade [5, 6]. We outline in detail both the advantages and disadvantages of each model, and the methodological procedures necessary to complete each model.

1.1 Human ROP Characteristics

ROP alters the normal development of retinal blood vessels in premature infants. Premature infants are susceptible to ROP due to the incomplete development of the retinal vasculature on birth. During normal retinal development vascularization begins at approximately 16-weeks gestational age initially provided by the hyaloid vessels traversing from the optic nerve to the anterior segment. Hyaloid vessels must regress for clear vision which usually occurs at 34-weeks gestational age, and by this time the intraretinal vasculature is well advanced; however, vessels do not reach the ora serrata (serrated junction between the retina and the ciliary body) until week-40. At birth the hyperoxic postnatal environment (partial pressure of dissolved arterial oxygen (PaO_2) is 55–80 mmHg compared with the uterine environment PaO_2 of 30 mmHg) is believed to stimulate reduction of growth factor production [7]. In premature infants this occurs prior to the completion of the vascular development and retards developmental angiogenesis.

ROP is a biphasic disease; initially characterized by the hyperoxic postnatal environment inducing vasoattenuation or the cessation of retinal vascular development [8, 9], as the vasculature is incomplete the retina becomes hypoxic inducing vasoproliferation and preretinal NV [10]. This preretinal NV predisposes the infant to plasma leakage, intravitreal hemorrhages, retinal detachment, and in some cases subsequent visual loss [11].

Improvements to the survival rates of premature babies have resulted in an increase in the prevalence of ROP in recent years. The Supplemental Therapeutic Oxygen for Premature Retinopathy of Prematurity (STOP-ROP) multicenter trail in 2004 showed 65 % of premature babies routinely placed in oxygen therapy on birth went on to develop ROP (Dale Phelps, personal communication, information derived from the STOP trial [12]). However, the development and severity of ROP is a multifactorial process where there is not one sole determinant, factors including temporal development and gene expression combine with clinical care to impact ROP pathogenesis.

1.2 Experimental Models of ROP

In 1954, Patz first demonstrated preretinal NV in rats that had been exposed to a constant level of extreme hyperoxia [13]. Similar

results were seen in the same year in mouse models of retinal NV [14, 15]. However, such results were difficult to replicate in the mouse for many years. Ashton failed to produce a proliferative retinopathy using oxygen concentration of 80–90 % [16].

The inconsistencies and lack of quantification associated with this model led Smith and colleagues [6] to redefine exposure parameters to ensure consistent reproducible neovascularization in the mouse retina [6]. Smith's oxygen induced retinopathy (OIR) model was used on C57BL6 mice after hyaloid regression; in brief 1-week-old (P7) mice are exposed to 75 % oxygen for 5 days (P7–P12), during the first 48 h immature capillaries in the central retina rapidly regress leading to central vaso-obliteration, in the latter part of the hyperoxic exposure revascularization of this central retina begins. In addition, during this exposure vessels constrict to regulate PO_2. At P12 mice are returned to room air, leading to hypoxia in the vaso-obliterated central retina and an induction of Hif-1α-dependent angiogenic signaling. Hyperoxia exposure produced a far more profound and reproducible retinal NV in P7–P12 pups compared to previous experiments on P0 pups [6]. Strict regulation of this protocol successfully models the defining disease characteristics observed during the development of human ROP in an efficient experimental setting. This model his since been adopted as the benchmark model of mouse OIR, contributing to over 30,000 publications since its description in 1994 (Google Scholar search on keywords "oxygen induced retinopathy mice," June 19, 2014).

The development of the rat model of ROP experienced similar issues, Ashton and Blach failed to reproduce Patz's initial data [17], concluding previous investigators claims were "inadequately substantiated on the evidence provided," due to the inconsistent nature of the vasoproliferative response. Again like the mouse model the rat model has experienced numerous changes to make it more reliable, including alterations to staining employing histochemical methods as described by Flower et al., using ADPase staining [18]. The constant level of extreme hyperoxia in rat models, like in the mouse, led Ricci and colleagues to observe "*marked peripheral retinal neovascularization*" after 5 days of continuous exposure to 80 % oxygen in newborn rat pups [19]. Both rat and mouse models demonstrate retinal NV and boast different advantages and disadvantages making both therapeutically applicable. However, both also lack pathophysiological features observed clinically in newborn babies, a factor that then sparked Penn's enthusiasm into making the rat model more clinically robust.

Premature infants who develop the ROP experience rapid fluctuations in PaO_2 resulting in alternating periods of hyperoxemia and hypoxemia. This exposure paradigm is not explicitly defined in previous models where rats are maintained in severe hyperoxia (80 %) for 5 days, although they may experience relative hypoxia

during the experiment if oxygen levels are not meticulously monitored. During periods where gas cylinders were changed or bedding and water replaced the oxygen levels within the chamber will rapidly fluctuate, and this may explain why various investigators saw inconsistencies in neovascular growth.

This led Penn and colleagues to pose the theory that it was in fact the transient fluctuations in oxygen levels during the experiment that stimulated neovascular growth and so he set up an experiment where newborn rats where exposed to a cycle of 80 % oxygen for 12 h followed by 40 % oxygen for 12 h for a number of days followed by a short period of time in normoxia. Indeed 66 % of rats developed preretinal NV during this insult compared to none exposed to a continuous 80 % followed by room air [5]. Penn subsequently went on to show that the range of PaO_2 variation determines the severity of OIR in newborn rats, more specifically showing fluctuations between 80 % and 40 % in healthy animals with normal lung functioning do not reflect the PaO_2 experienced by neonates in the neonatal intensive-care unit (NICU). Consequently he set the oxygen levels to fluctuate between 50 % and 10 % on a 24 h basis; this not only better reflected the arterial blood gases experienced by sick premature infants in the NICU but also resulted in a greater retardation of retinal blood vessel development and increase NV severity comparatively. After 4 days postexposure in room air, the incidence of preretinal neovascularization was 97 % in the 50/10 % rats and 72 % in the 80/40 % group [4, 5, 20].

Exposure to the 50/10 regime retards both the superficial and deep retinal vessels, thus causing the avascular periphery. Removal to normal air causes neovascular growth at the vascular boundary, the joining of neovascular tufts results in a ridge of preretinal vessel growth that is highly reminiscent of the mesenchymal ridge seen in human ROP [21].

1.3 Benefits and Caveats of the Rat and Mouse Models

Both the mouse OIR developed by Smith and the rat 50/10 OIR model developed by Penn have proved useful scientific models which have aided in furthering knowledge of pathological oxygen-induced ocular angiogenesis, and in testing novel treatments for therapeutic application. However, like most scientific models, each boasts both advantages and disadvantages, and requires careful control of a number of variables.

Unlike the development of the human disease the pathology induced in both models are induced by oxygen fluctuations postpartum. Eyes open once initial development of retinal vasculature is complete, in humans this coincides with birth whereas in rats and mice this starts to occur at around P12 in normoxic conditions. The high ex-uterine oxygen environment premature babies experience prior to complete retinal development can thus be easily modeled in pups after birth by manipulation of oxygen levels, making rodents excellent for studying pathological as well as

developmental angiogenesis. Both models, when correctly monitored, provide a robust induction of pathological neovascularization in a time-efficient manner.

In both models the severity induced can vary in different wild-type strains [22, 23] and because of vendor-related sub-strain differences [24, 25]. Smith et al. observed higher NV induction using C57Bl/6 wild-type mice from Jackson Laboratories (Bar Harbor, ME) compared to Taconic Farms (Germantown, NY) [25], and Penn et al. noted greater induction of NV in Sprague-Dawley rats from Charles River (Charles River Laboratories, Wilmington, MA) compared with several other vendors, observations that were confirmed in 2002 by a study comparing Charles River to Harlan [24]. For both the rat and mouse model the susceptibility to, and severity of, NV is very much dependent on genetic background and environment.

The mouse model is both highly reproducible and less complex than the rat model in terms of protocol. A strong advantage in using the mouse model is the ability to study gene knockouts relatively easily. Knockout mice allow investigation into the importance of a gene throughout various stages of pathological development (providing the knockout is not embryonic lethal), furthermore rescue experiments can be achieved in inducible knockouts where genes can be turned on and off at different times during development.

With current advances in technology the rat genome is becoming increasingly easier to manipulate and transgenic rats are available; however, at present for gene-knockout studies the mouse model is easily the preferred choice.

In the mouse model, pups do not enter the protocol until P7, so the hyaloid vessels have already significantly regressed and do not need to be removed from the retina following enucleation. In the rat model, however, pups are exposed to alternating oxygen levels immediately after birth, which substantially delays the regression of the hyaloid vessels thus requiring their careful removal during dissection. Despite the development of the improved 50/10 OIR model, getting a reproducible system requires detailed precise following of the methodology. For example as the procedure begins at birth it is important litters are monitored carefully, litter size is important for pathological induction thus it is often necessary to coordinate dams to give birth at a similar time so that litters can be pooled if necessary (observations from experimental data).

An adequate model should replicate key characteristics observed in the human pathology. ROP is a biphasic disease consisting of vaso-obliteration followed by neovascularization; two phases reflected in both the rat [5] and mouse [6] models of OIR. The rat model is widely accepted as the most clinically relevant model of ROP, OIR rat pups present of a ridge of preretinal vessel growth at P20 that is highly reminiscent of the mesenchymal ridge seen in human ROP [21].

However, a clear disadvantage of both models from a clinical perspective is that the pathology observed recovers after time. In the rat model NV regresses after P25 [26], and in the mouse model regression begins at P17 with little or no vaso-obliteration or NV present by P25 [27]. If one requires a model that tests drug dependence and/or the effect of drug withdrawal over a prolonged period of time, then this model is not going to be appropriate.

1.4 Assessment of Induced Pathology

Assessment of the pathology must be carried out blinded to treatment for both rat and mouse models. The retinae need to be coded so that the measurer is unaware of the treatment of the retinae.

1.4.1 Rat Model

One of the factors that make this model more robust compared to other OIR models is the ability to quantify retinal NV with clinical correlation. One method, although only semiquantitative and poses the possibility of becoming subjective, gives a quick read out of NV severity that correlates well with more quantitative and reliable methods. In this method a clock face is superimposed onto a flat mounted retina with each retinal quadrant containing 3 clock hours, investigators sum the number of clock hours containing the pathology. The standard method for quantifying NV in rodent OIR is counting cell nuclei above the internal limiting membrane in histologic sections; however, the much quicker clock-hour analysis demonstrated high correlation ($r_s = 0.95$, $P = 0.0001$) with nuclei counts [28]. Initial PRNV is very difficult to quantify by looking at images, as you are looking for growth through the retina into the vitreous it is essential that you are able to focus the microscope up and down to properly visualize the abnormal growth. Although severe NV can be observed at low magnification you really need to analyze each retina at least at 40× magnification to be sure. More recent publications have more quantitatively outlined NV tufts and expressed as a percentage of total retinal area [29].

1.4.2 Mouse Model

In the mouse model two main features are assessed; during the first phase the area of vaso-obliteration can be determined (P8–P12) and during the second phase the area of NV can be assessed (P14 onwards). These features can both be assessed in retinae at P17 during maximal proliferation [6]. Vaso-obliteration and NV can be measured in whole retinal flat mounts stained for endothelial cell marker, isolectin-B4, where the vaso-obliterated and NV areas can be separately outlined and expressed as a percentage of the total retinal area using image-processing software (for example ImageJ or Photoshop) [25].

This chapter describes in detail the methodological procedures necessary to complete both the mouse and the rat model, both which have significantly advanced knowledge surrounding the factors involved in the progression of retinopathy.

2 Materials

For oxygen exposure protocol

- Oxycycler (Biosperix) or ProOx110 (Biospherix)—if using a ProOx110 a Perspex chamber to house the cages will need to be made; we recommend this be sized to comfortably house two cages.
- Carbon dioxide sensor—calibrated (this may be included with your oxygen controller).
- Rat/mouse housing consumables—bedding, water, feed, replacement cages, etc.
- Animals: Rat strain—Sprague-Dawley (Recommended vendor Charles River), Mouse strain—C57/B6 (Recommended vendor: Jackson Laboratories).
- Nitrogen and oxygen cylinders (BOC).

For intravitreal injection (if necessary)

- 10 μl Hamilton syringe (WPI).
- 35 gauge needle (for murine eyes).
- 33 gauge needle (for rat eyes).
- Injectable drug made up in vehicle.
- Vehicle control (whatever the treatment is made up in minus the active ingredient).
- Isoflourane rig connected to mask that will comfortably fit the pup.
- Heat mat.

For dissection and staining

- Phosphate buffered saline (PBS).
- Angled Vannas scissors (WPI).
- 15° ophthalmic knife (for initial incision into eye, if not available one can use a needle).
- Forceps x2—fine pointed, select to suit individual preferences.
- Petri dishes 35 mm.
- 96-well round bottom well plate (or similar—for storage of retinae during staining protocol).
- 4 % paraformaldehyde in PBS.
- PBS–Triton X-100: 1× PBS pH 7.4, 1 % bovine serum albumin, 1 % Triton X-100.
- PBlec: 1× PBS pH 6.8, 10 mM calcium chloride, 10 mM magnesium chloride, 0.5 % Triton-X100.

- Isolectin IB4.
- Streptavidin conjugated Alexa Fluor 488 or 546/555/594.
- Microscope slides
- Coverslips
- Vectashield mounting medium.

3 Methodology

3.1 Oxygen Exposure Protocol for Rat 50/10 OIR

1. Order several Sprague-Dawley (*see* **Note 1**) prepartum, multiparous female rats, and receive them at approximately gestational Day 13 or 14 upon arrival. Stress of shipping can severely affected pregnant dams so changes in environment any closer to birth should be avoided. Alternatively rats can be bred in-house following standard animal husbandry procedures.
2. Check cages three times daily for newborn pups, dams usually give birth in the early hours of the morning; however, this is not always the case.
3. After parturition (ideally within 4 h), place mother and pups into an oxygen chamber (*see* **Note 2**), and begin specific oxygen-exposure profile (50–10 % O_2 alternate every 24 h). The mother and pups will remain in the oxygen chamber for maximum 14 days. If multiple mothers whelp within 24 h of each other, all pups will be pooled and each mother will be given 17 randomized pups. The minimum number of pups per litter at the beginning of each study is targeted at 14 pups per mother. This will help to ensure optimal litter sizes; litter size is a dependent variable on preretinal NV in this model [30]. Normal lighting cycles will be maintained, as well as standard room temperature (22 °C). On Day 7/0, animals are weighted (pups are expected to weigh less than aged matched conventionally reared controls), and given fresh bedding and water (*see* **Note 3**) (Fig. 1a).
4. Fourteen days postpartum (Day 14/0) or occasionally less than 14 days for some specific studies, the mother and pups will be removed from the oxygen chamber and placed in room air conditions for up to 6 days (Days 14/0–14/6). Again, animals are weighted and cages are changed. If the pup numbers drop below 13, this litter is usually not used for a formal study, since the NV score will be low for many pups.
5. At Day 14/0 or occasionally at another time point that is designed for the individual study, begin the delivery of test compounds via most appropriate route of administration and at predetermined frequency (e.g., topical drops, intravitreal injection(s)—*see* **Note 4**, subcutaneous injection(s) and pump

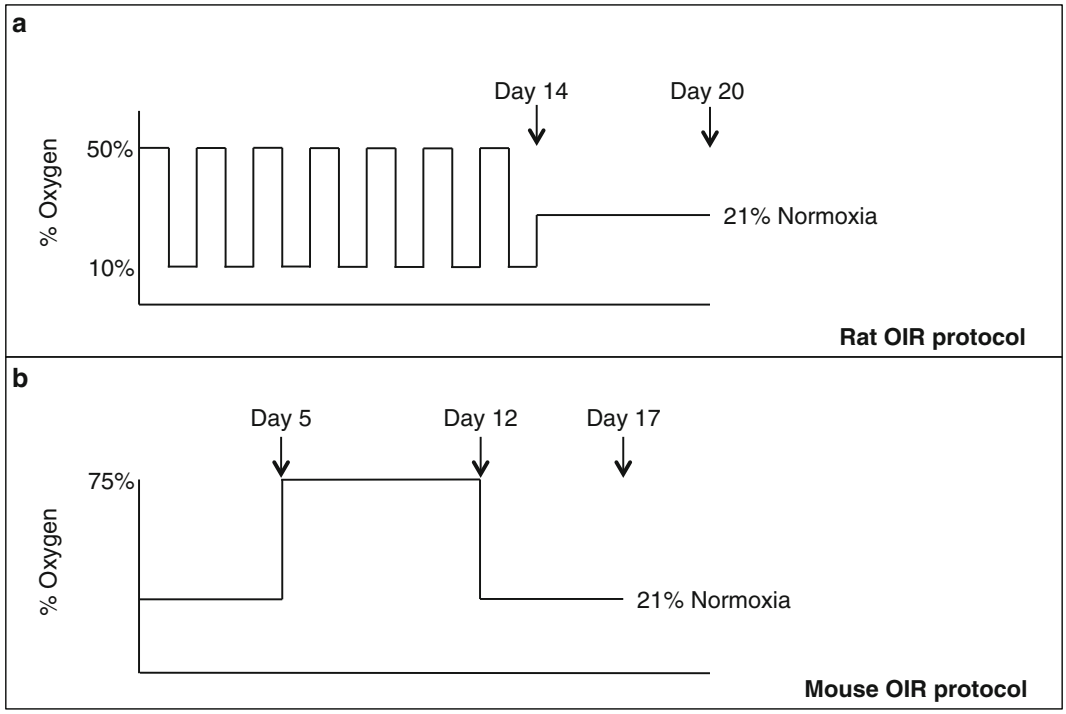

Fig. 1 Diagram representing oxygen insult protocol for rat model (**a**) and mouse model (**b**)

implantation, intraperitoneal injection(s), or oral gavage). If the procedure requires anesthesia, isoflourane inhalation is used.

6. After predetermined period of room air exposure, most frequently 6 days (*see* **Note 5**) (Day 14/6), euthanize mother and pups and enucleate eyes (pups only) for histologic and biochemical evaluations.

3.2 Oxygen Exposure Protocol for Mouse OIR

1. Order male and female mice of desired breed from desired vendor (*see* **Note 1**) for in house breeding or order prepartum, multiparous female mice, and receive them at approximately gestational Day 13 or 14 upon arrival.
2. Mice should be checked daily for pups and the day of parturition recorded, including litter size. Seven day old pups are placed into an oxygen chamber with their nursing dams and subjected to 5 days of 75 % oxygen—oxygen sensors should be calibrated prior to each experiment according to manufacturer's specification. During these 5 days the chamber should not be opened unless to replace food, water or bedding (*see* **Note 5**) (Fig. 1b).
3. At P12 mice are removed from the chamber to room air and retinal regrowth will occur. Pups should be weighed and

documented. As with the rat model treatments can be administered on or after exposure to normoxia—the same guidelines listed above should be adhered to (*see* Subheading 3.1, **step 5**).

4. By P17 neovascularization begins to spontaneously regress and is resolved by P25 so experiments should be concluded at P17 for maximal NV. Pups should, as with the rat model, be culled by lethal injection or isoflourane inhalation (*see* **Note 6**).

3.3 Intraocular Injections

1. It is important to collect and sterilize all equipment prior to placing the pups under anesthesia. Sterilizing injection equipment is essential to minimize risk of endophthalmitis.
2. Ensure pups are under anesthesia (*see* **Note 6**).
3. Using a 33-gauge needle for rat pups or a 35-gauge needle for mouse pups (NanoFil; World Precision Instruments, Sarasota, FL), connected to the Hamilton 10 μl syringe (WPI) insert at 90° posterior to the limbus to avoid lens damage, angle the needle at 45° so the tip sits beneath the lens in the vitreous (*see* **Note 7**).
4. Between 2–5 μl injections for rats and 1 μl injections for mice should be performed on both eyes using a Hamilton syringe (WPI) (*see* **Note 8**), one eye acting as a control (*see* **Note 9**).
5. The injection should be done slowly and is often easier if another investigator is present to push the syringe plunger. After the injection the needle should be held steady in the eye for approximately 30 s.
6. Animals should be monitored until recovery (~2 h) and then returned to normoxia. Any issues with the injection for example large backflow upon removal of the needle, external or internal vessel hemorrhaging of vessels should be noted and if necessary eyes excluded from the study.

3.4 Retinal Dissection and Flat-Mounting

1. Rats or mice should be culled with an overdose of inhalation isoflourane anesthetic (*see* **Note 10**), weighed and eyes removed.
2. Eyes should be fixed in 4 % PFA dissolved in 1× PBS (pH 7.4) for 60 min and enucleation performed using fine curved forceps and curved tip scissors (World Precision Instruments, WPI). Care needs to be taken to avoid excessive pressure on the globe. Retinal flat mounts are prepared with a modification of the methods of Chan-Ling [31].
3. Dissection is performed in a petri dish adapted to hold an eye, in 1–2 ml of chilled 1× PBS (4 °C) under a dissection microscope. Peribulbar fat and connective tissue are dissected away to expose the sclera. A short stump of optic nerve is left protruding from the globe. An incision is made immediately anterior to the corneoscleral limbus with a 15° ophthalmic scalpel.

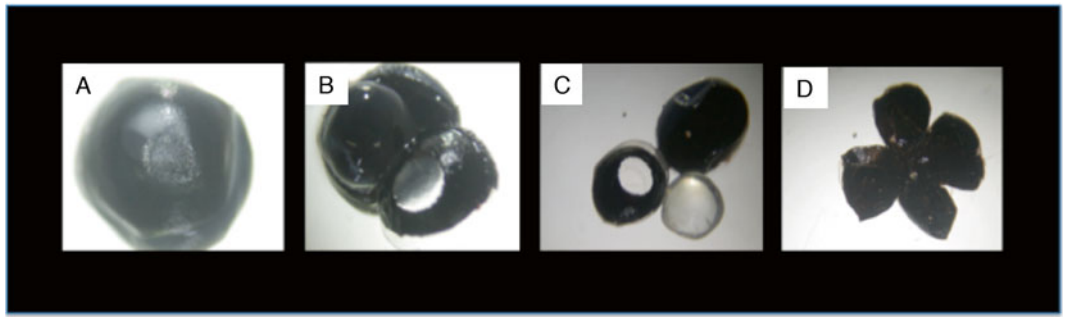

Fig. 2 Dissection of a mouse eye. (**a**) Whole eye removed. (**b**) Incision made at the level of the ora serrata, and cut to expose lens. (**c**) Lens and anterior chamber removed. (**d**) Four incisions made in the posterior cup prior to separating retina from sclero-choroidal complex

The cornea is grasped with fine-toothed forceps and an incision is continued circumferentially with curved-tip microdissection scissors. The cornea, iris, and crystalline lens are discarded (Fig. 2).

4. For rat preps only: forceps are used to avulse the hyaloid vessels and vitreous gel from their retinal attachments (*see* **Note 11**): particular attention should be paid to the regions posterior to the ora serrata and around the optic nerve head, where the attachments are most marked.
5. Four equally spaced radial relaxing incisions, extending two thirds of the way from the retinal periphery to the optic nerve head, are made with scissors to flatten the eyecup. The sclerochoroid is removed leaving just the retina, and two pairs of fine forceps used simultaneously to tease away residual vitreous and hyaloid vessels.
6. The fully dissected retina is returned to 1× PBS for staining.

3.5 Isolectin-B4 Histochemistry

1. Fixed retinas in 1× PBS are permeabilized for 2 h in PBS–Triton X-100 and retinae then washed twice in PBlec.
2. Fluorophore-conjugated isolectin-B4 with specificity for alpha-galactosylated glycoprotein residues on vascular endothelial cells and macrophages (*Griffonia simplicifolia* type I isolectin-IB4), should be diluted (4 μg/ml) in PBlec, 50 μl added to each retina and retinae left overnight in the dark at 4 °C (*see* **Note 12**).
3. The following day retinas are washed five times in 1× PBS and secondary antibody Alexa Fluor 488 conjugate (Molecular Probes, OR, USA) added 2 μg/ml in 1× PBS for at least 2 h.
4. Stained retinas should be washed and carefully flat-mounted on microscope slides, drop Vectashield (Vector Labs) onto the retinas and coverslip. Coverslips require sealing with nail varnish to prevent retinas from drying out.

3.6 Analysis and Quantification of Retinal Flat Mounts

3.6.1 Rat 50/10 OIR

1. Early stages of PRNV will be observed as small round clumps of cells protruding through the retinal surface, these have adopted the term "popcorn" swellings based on their phenotype.
2. PRNV always occurs close to the edge of vascular development, just prior to the avascular area in a brush border type manner. It is important to note that the budding of the most peripheral vessels is not PRNV, PRNV occurs slightly more centrally.
3. PRNV characteristically occurs at the ends of veins, not at the end of arteries. Arteries are identifiable as following oxygen insult the periarteriolar space is increased and you see an absence of vessels surrounding the arteries.
4. Initial "popcorn" swelling merge to form PRNV brush borders, characterized by dark staining.
5. In a pup who has undergone the 50/10 OIR insult one should expect to see PRNV in at least 6 clock hours (Fig. 3a).
6. For more quantitative analysis retinal flat mounts are imaged and if necessary separate images merged to form a full retinal image, the area of retina possessing PRNV is calculated and expressed over total retinal area (Fig. 3b and c).
7. Other features of pathology can be further detailed, for example vascular area, vessel tortuosity and vessel diameter [29].

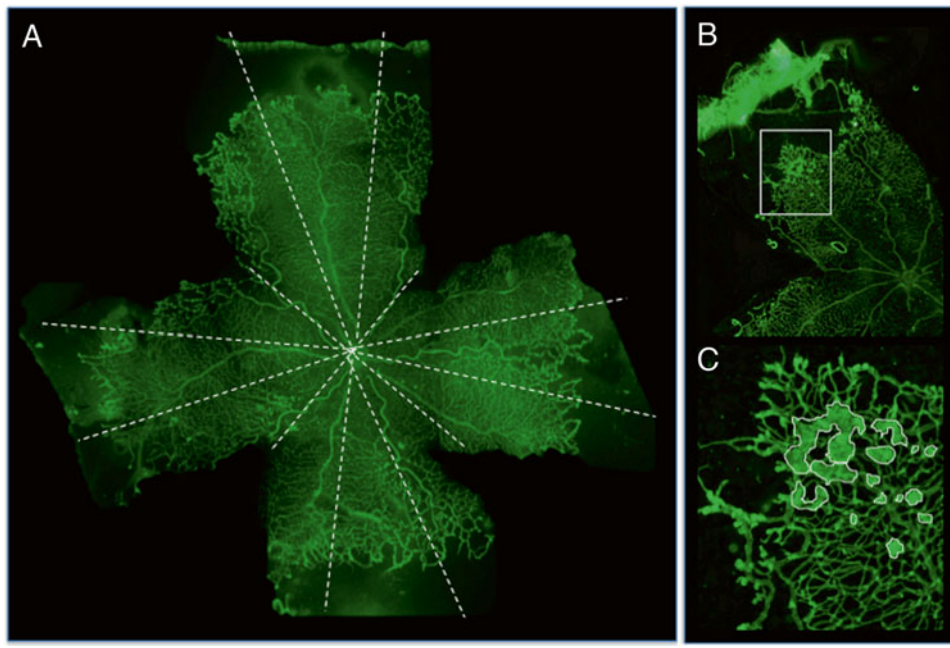

Fig. 3 Rat OIR analysis. (**a**) Stained flat-mounted retina split into clock hours for analysis. (**b**) Identification of pre-retinal neovascularization at the periphery. (**c**) Pre-retinal neovascularization outlined for area quantification

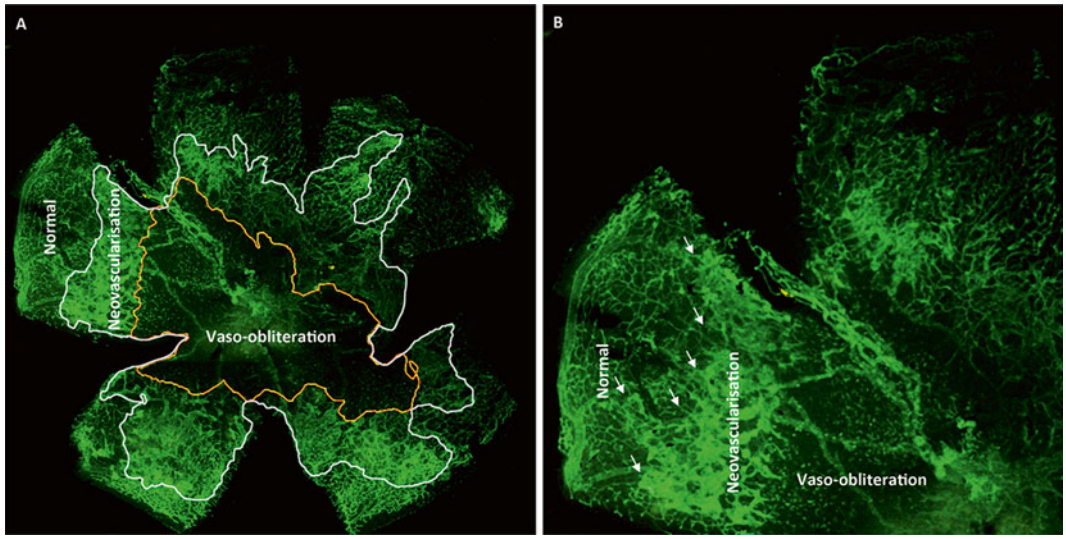

Fig. 4 Mouse OIR analysis. (**a**) Low power visualization (4× objective) of regions of the retina stained with IB4. The VOA is delineated from the NVA by an *orange line*. The NVA is separated from the Normal region by a *white line*. (**b**) Higher power (10× objective) with *arrows* showing where the neovascular front is

3.6.2 Mouse OIR

1. Two main features can be clearly observed in retinal flat mounts (usually from P17 pups) stained for isolectin-B4, areas of vaso-obliteration and neovascularization (Fig. 4).
2. Retinal flat mounts are imaged and images captured as formats compatible with ImageJ [32], and if necessary separate images merged to form a full retinal image. The central vaso-obliterated area (VOA, observed by a lack of vessels present) is outlined using the freehand selection tool in NIH image, and the area determined using the measure command in ImageJ.
3. The same is done for the neovascular area (NVA) where each area of angiogenesis, determined by the overgrowth and lack of definition of individual microvessels (*see* Fig. 4b) is outlined using the freehand selection tool and this area calculated using ImageJ. The area of normal vasculature (normal), as defined by the clear two layers of vessel network stained without areas of vascular growth is also measured.
4. Each of the calculated areas can be expressed as a percentage of the total retinal area (i.e., $100 \times \text{VOA}/(\text{VOA} + \text{NVA} + \text{normal})$). Some more automated methods have been developed and are discussed in [25].

4 Notes

1. Studies have identified that the same strain of rat or mouse ordered from a different supplier can have profound differences in pathology reproducibility. Smith et al. observed higher

NV induction using C57Bl/6 wild-type mice from Jackson Laboratories compared to Taconic Farms [25], and Penn et al., noted greater induction of NV in Sprague-Dawley rats from Charles River. We were able to successfully induce NV using C57/B6 and Sprague-Dawley from Charles River, though we did not compare strains in house [29, 33].

2. Should a Biospherix oxycycler not be available a similar system can be created using one or two ProOx controllers connected to a gas input (nitrogen for 10 % oxygen for 50 %) and an in-house built perspex chamber. The caveat being oxygen levels require daily manual switching and close monitoring to ensure correct oxygen levels are maintained. Should a manual operation procedure be used take care to ensure chamber is ventilated to prevent humidity and carbon dioxide build up (Carbon dioxide can be scrubbed using soda lime).
3. Unnecessary opening of the oxygen chamber during the procedure should be avoided to prevent pups acclimatizing to normoxia. Bedding and water will require changing at least once during the procedure, this should be done quickly and whilst changing the oxygen levels from 10 % to 50 % for the rat model.
4. Conventionally reared rat pups open their eyes following complete development of the retinal vascularization at P12 (postnatal day 12); however, pups exposed to alternating oxygen levels during development experience retarded growth of the retinal vasculature and thus delayed opening of the eyes. It may be necessary to surgically open the eyes in order to administer intravitreal injections; it is of paramount importance to perform control injections in the contralateral eye of the same pup as intralitter variation is often high in this model.
5. The 50/10 OIR model develops vascular tortuosity at P12, peripheral avascular retina at p14 and intravitreous neovascularization at P18. Note that in this model pre-retinal neovascularization recovers by P25 [26], thus to observe differences in pathology we recommend ending the experiment at P20.
6. For isoflourane anesthesia we recommend 2–4 % isoflourane with an oxygen flow of 1–2 l/min.
7. If available use an operating/surgical microscope to visualize the correct position of the needle in the eye during the injection. You should be able to move the tip of the needle without altering the position of the lens. If you can't do this it is likely you have injected into the lens and thus the injection is void. It will be clear if the injection has penetrated the lens as it will cloud the eye. You must also take care to avoid hemorrhaging of large vessels as you penetrate the eye.

8. For rat pups we recommend a maximum injection volume of 5 μl but a preferred volume of 1–3 μl and for mice pups a recommended injection volume of 1 μl, larger volumes will significantly increase intraocular pressure (IOP) and disrupt retinal vessels. The larger the volume the greater the chance of losing drug via backflow during removal of the needle post-injection. Injection volume will be determined by the characteristics of the injected drugs.
9. Control will depend of the vehicle used to dissolve or suspend the drug, for example DMSO. If injecting antibodies we recommend using the equivalent concentration of IgG from the animal the antibody was raised in. If your treatment requires more than one injection take care not to inject into the same location as the previous injection.
10. Avoid methods such as cervical dislocation as the pressure in the eye will increase and lead to the rupture of small blood vessels.
11. Removal of the hyaloid vessels is important for staining of the retinal vessels. Usually the vessels regress during development; however, this is retarded under the variable oxygen conditions experienced during this procedure. Take care to carefully remove all the vessels for a clean prep.
12. If necessary, time can be reduced to 2 h at room temperature.

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