Video Article

Puncture-Induced Iris Neovascularization as a Mouse Model of Rubeosis Iridis

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Abstract

We describe a model of puncture-induced iris neovascularization as a general model for noninvasive evaluation of angiogenesis. The model is also relevant for targeting neovascular glaucoma, a sight-threatening complication of diabetic retinopathy. This method is based on the induction of iris vascular response by a series of self-sealing uveal punctures on BALB/c mice and takes advantage of the postpartum maturation of mouse ocular vasculature. Mouse pups undergo uveal punctures from postnatal day 12.5, when the pups naturally open their eyes, until postnatal day 24.5. Due to the transparency of the cornea, iris vasculature can be analyzed easily through time by noninvasive *in vivo* methods. Furthermore, the semitransparent iris of BALB/c mice can be flatmounted for detailed immunohistologic analysis with minimal non-specific background staining. In this model, angiogenesis is mainly driven by the inflammatory and plasminogen activating systems. The puncture-induced model is the first to induce iris neovascularization in small rodents, and has the advantage of allowing direct noninvasive *in vivo* analysis of the angiogenic process. Moreover, the model can be combined with angiogenic modulating substances, which highlights its potential in the study of angiogenesis with an *in vivo* perspective.

Video Link

The video component of this article can be found at https://www.jove.com/video/57398/

Introduction

The iris, together with the ciliary body and the choroid, comprises the uvea, which is the most vascularized tissue of the eye. Iris vasculature is essential in maintaining homeostasis in the anterior chamber of the eye. As a result of abundant anastomotic connections between arteries and veins, iris blood vessels provide nutrients and supply of oxygen not only to the iris itself, but to the entire anterior segment of the eye[1].

The formation of new blood vessels, or angiogenesis from pre-existing ones, is fundamental in physiological processes, such as development and wound healing[2]. Angiogenesis is finely regulated by a multitude of canonical factors, such as vascular endothelial growth factor (VEGF) and plasminogen activator inhibitor (PAI), as well as multiple inflammatory factors, and an imbalance of these factors can lead to pathologic angiogenesis[3].

In the eye, neovascularization is the cause of sight-threatening diseases, such as proliferative diabetic retinopathy (PDR) and neovascular glaucoma (NVG). In these ocular diseases, the focal neovascularization is commonly located in retinal tissues, yet the imbalance in inflammatory and angiogenic factors in both the posterior and anterior ocular chambers of the eye has been associated with rubeosis iridis, the clinical term for iris pathological neoangiogenesis[4]. These pathologies indicate the capability of the adult iris to undergo angiogenesis. In mice, ocular vasculature is immature after birth and continues maturation postpartum. This peculiarity of development is exploited in the mouse model of oxygen-induced retinopathy, a model that closely mimics the clinical condition of retinopathy of prematurity[5]. In addition, angiogenesis and inflammation play a pivotal role in wound healing mechanisms[6], and wound healing itself has been associated with angiogenesis models[7].

In this study, we describe a model of puncture-induced iris neovascularization. Uveal punctures are performed near the outer limit of the limbus, which induce iris neovascularization by triggering the wound healing system. Due to the transparency of the cornea, iris vasculature can be analyzed easily *in vivo* by noninvasive methods. Punctured eyes present an increase of vascular bed in the iris, which has been associated with an increase of plasminogen activating and inflammatory markers[8]. The presented model has great potential as a new tool to study angiogenesis and screening angiogenic compounds, and allows direct *in vivo* visualization of the angiogenic processes.

Protocol

BALB/c mouse pups of either sex were used in accordance with the statement for the Use of Animals in Ophthalmologic and Vision Research, and the protocols were approved by Stockholm's Committee for Ethical Animal Research. Mice were housed in litters, together with the nursing mother, with a 12 h day/night cycle, free access to food and water, and monitored daily.

NOTE: For the surgical procedure, mice were kept under anesthesia with volatile isoflurane. Ocular ointments are discouraged during ocular procedures, as they might interfere with treatments and substances used. If necessary, to prevent dry-eye, a drop of sterile normal saline solution

can be applied.Though uveal punctures are self-healing , care was taken during uveal punctures to ensure sterility with surgical instruments. Post-surgical treatment included hydration with normal saline solution subcutaneously, and analgesia with ocular topical administration of tetracaine hydrochloride. The pups were allowed to recover to sternal recumbency on a heating pad before being returned to the nursing mother, in a clean cage. Litters were kept with the same nursing mother to avoid stress.

1. Anesthesia

1. Prepare the anesthesia induction chamber, to administer isoflurane anesthetic and ensure that a small mouse mask is also coupled.
   NOTE: Perform all animal experiments in agreement with all applicable ethical permits.
2. Prefill the induction chamber with 3-4% volatile isoflurane in atmospheric air.
   NOTE: Different anesthetic substances other than isoflurane may be used, according to ethical permits. Volatile anesthetic procedures should be carried on a ventilated bench or fume hood.
3. Place a postnatal (P)12.5-day-old BALB/c mouse pup in the induction chamber. Confirm that the anesthesia apparatus is ready to deliver isoflurane to the induction chamber.
   NOTE: Mouse pups are fragile and best handled by the scruff. Keep the pups together as a litter or with the nursing mother to avoid stress.
4. Allow the pup to be fully anesthetized.
5. Gently, pick up the mouse pup by the scruff.
   NOTE: Keep the induction chamber closed at all times for safe and stable induction.
6. Transfer the mouse to the rodent mask. Make sure to switch the anesthetic flow from the induction chamber to the mask when moving the mouse.
7. Perform a toe pinch to confirm anesthesia.

2. Puncture Procedure under Surgical Stereoscope

1. Position the mouse in lateral recumbency position. Confirm the mask is well positioned so the mouse eye is in the field of view of the stereoscope.
2. Gently rotate the mouse head so the eye is facing up toward the stereoscope.
   NOTE: Clear focus of the eye should be achieved under the stereoscope. For positioning, a magnification of 16X is recommended, while 40X should be used to perform the surgical procedure. If ethical permits allow it, trimming the tip of the whiskers can facilitate the visualization of the eye.
3. Using small tying forceps, carefully protrude the pup's eye by applying downward pressure to the eyelids dorsal and ventral to the eye.
4. Keep a gentle but firm hold of the pup's eye lid, with small forceps.
   NOTE: It is recommended to perform the eye protrusion and lid holding with the non-dominant hand, as the dominant hand should perform the puncture procedure.
5. Use the stereoscope to locate the corneal limbus. Albino BALB/c limbus can be easily identified by the circular vascular plexus posterior to the cornea.
6. With a 0.25 mm diameter (30 G) beveled needle, perform a small uveal puncture, near the posterior limbal limit of the uvea. Use only the tip of the needle, and no more than half the bevel (equivalent to 0.5 mm), puncture the uvea. If executed correctly, the uveal punctures are self-sealing, yet intraocular injection of study substances can be administered through the same puncture wound.
   NOTE: If experienced with mouse ocular anatomy, the uveal puncture is executed between the ciliary body and ora serrata. Care must be taken while performing the uveal puncture to avoid rupturing the lens.
7. Perform the second uveal puncture in the opposite site of the eye from the first puncture site. Optimize the distance and position of the punctures; consider 12 and 6 o'clock punctures, with 12 o'clock being as dorsal as possible.
8. Repeat the uveal punctures procedure every four days until P24.5. Before every successive uveal puncture, monitor animal status paying particular attention to presence of traumatic cataract due to lens damage during the uveal puncture, or obvious diminished ocular pressure. Aim for executing repeat punctures at the same positions as previous punctures for optimal effects.

3. Noninvasive *in Vivo* Monitoring

1. Prior to the puncture or injections on each experimental day, anesthetize the mouse as performed above.
2. With the mouse in lateral recumbency position, gently protrude the eye.
3. Focus on iris vasculature with the surgical stereoscope.
4. Take a picture of the iris vasculature with a camera fitted to the surgical stereoscope.
   NOTE: Though not necessary, inducing meiosis can be helpful to normalize iris area per animal. Consider ethical permits when selecting a meiosis-inducing agent. Careful focus of the iris vasculature will facilitate further quantitative analysis. A magnitude of 40X is recommended.

4. Post-Operative Care

1. Remove the BALB/c pup from the rodent mask, and gently transfer it to a heating pad layered with a surgical mat.
2. Apply one drop of 1% tetracaine solution to punctured eyes.
   NOTE: Other analgesic solutions can be used, according to ethical permits.
3. Hydrate the animal with a subcutaneous injection of 200 µL of sterile normal saline solution.
4. Return the pup to the nursing mother in a clean mouse cage.
5. Monitor the recovery process. The pup should be able to deambulate and return to the litter.

5. Eye Enucleation

1. Euthanize the animal by cervical dislocation. Perform enucleation and dissection under a stereoscope for better visualization of the procedure.
   NOTE: Different euthanasia procedures can be used. It is recommended to strictly adhere to ethical permits.
2. Pull apart the eyelids to improve the exposure to the eye.
3. Make a small cut (approximately 0.5 cm) near the canthus of the eye lids with curved Bonn eye scissors.
4. Use the exposed periocular space to insert micro tying forceps behind (under) the globe in the orbit.
5. Grab the tissue surrounding the eye and gently pull upwards. Avoid holding onto the eyeball, use the surrounding tissues closer to the eye socket.
6. Insert the curved Bonn eye scissors behind the eye globe in the orbit.
7. Cut through the surrounding tissues until the eye is released from the socket.
   NOTE: This dissection technique maintains eye anatomy and benefits subsequent analysis.
8. Proceed to the removal of extraneous tissue from the eye. The removal of extraneous tissue can be performed after fixing, if preferred.
   NOTE: BALB/c mice are melanin deficient, therefore, to increase contrast, a dark background is recommended to facilitate dissection procedure.
9. Briefly rinse the whole-eye in a Petri dish filled with sterile phosphate-buffered saline (PBS).
10. Transfer the eye to a 2 mL tube containing 1.5 mL of 4% buffered-formalin solution.
11. Fix the eye for 6 h at room temperature.
    NOTE: Fixing confers the rigidity of the tissue and facilitates dissection of the iris. Fixing time should be tested and adjusted for different applications.

6. Iris Dissection Procedure under Stereoscope

1. Remove the fixative solution from the tube with a 1.5 mL plastic Pasteur pipette (or similar), and rinse the eye three times with fresh PBS.
2. Place the fixed eye in a smooth dry surface (dissection stand) with the anterior chamber facing upwards.
3. With a beveled 30 G needle, perform an entry point posterior to the limbus.
4. Holding the anterior segment with small tying forceps, insert one tip of Clayman-Vannas straight scissors into the opening previously created.
   NOTE: It is recommended to control the forceps with the non-dominant hand, and the scissor with the dominant.
5. While rotating the eye with the tying forceps, cut around the limbus to remove the posterior segment of the eye.
   NOTE: Performing partial cuts will allow the inner tip of the scissors to continue in the tissue and greatly facilitates the process.
6. Position the anterior segment facing downward, exposing the lens.
7. Remove the lens carefully by grabbing it with small forceps and pulling upwards.
   NOTE: A beveled needle can be used to puncture and pull the lens.
8. Position the anterior segment with the cornea facing down and the cut perpendicular to the field of view.
9. Gently grab the tissue posterior to the ciliary body with the small tying forceps.
10. With Clayman-Vannas straight scissors, proceed to trim just anterior to the ciliary body to remove the trabecular meshwork and isolate the iris. Confirm that the trabecular meshwork is removed; the iris should move within the cornea.
11. Carefully transfer the anterior eyecup with the small tying forceps, holding the cornea, to a 96-well plate containing 200 µL of PBS.
12. Continue holding the cornea in the well and dissect the iris by flushing with PBS with a pipette.
    NOTE: If the iris remains adherent to the cornea, some trabecular meshwork might be present, and the bevel of a 30 G needle can be used to help displacing the iris.
13. Remove the cornea from the 96-well with the small tying forceps, and keep the isolated iris in the well for further analysis.
14. Store free-floating iris samples at 4 °C.
    NOTE: Despite being fixed, long-term storage of the iris free-floating samples is not recommended.

7. Possible Experimental Read-Outs

1. To visualize the blood vessels in the dissected irises, use free-floating whole-mount immunohistochemistry staining methods for markers such as platelet endothelial cell adhesion molecule (PECAM)-1 or isolectin B4. Alternatively, use whole-body perfusion with vascular staining, such as fluorescent-labeled high molecular weight dextrans.
2. Use *in vivo* noninvasive images to perform *in silico* flat-mounting and quantify full iris vasculature with appropriate imaging software.
   NOTE: Vasculature can be quantified, both *in vivo* and *in vitro*, using densitometry or with vasculature analysis software[9].
3. Process the whole eyeball for nucleic acids and protein extraction, followed by PCR or immunoblotting methods, to perform molecular analysis of the punctured eyes.
   NOTE: Molecular analysis of tissues is best performed with non-fixed tissue, but dissection of non-fixed iris is extremely challenging, and the use of the whole eye has rendered statistically significant results in previous studies[8].

Representative Results

Albino BALB/c mouse pups at P12.5 were subjected to uveal punctures, repeated every fourth day (experimental day 0, 4, 8, 12), until P24.5. At P27.5, mice were euthanized and irises carefully dissected (experimental day 15). Pictures of mouse eyes were taken with a camera attached to a surgical stereoscope before every puncture series in each experimental day to assess noninvasive evaluation of the iris vascular response. Uveal punctures induce a vascular response from the iris by triggering the wound healing system (**Figure 1**). Due to transparency of the cornea and melanin-deficient BALB/c mice, increased iris vasculature is readily visible from experimental day 4 (P16.5) and intensifies throughout the duration of the protocol. Immunohistochemistry images for PECAM-1 denote an increase in overall vasculature in irises of punctured eyes compared to controls (**Figure 2**).

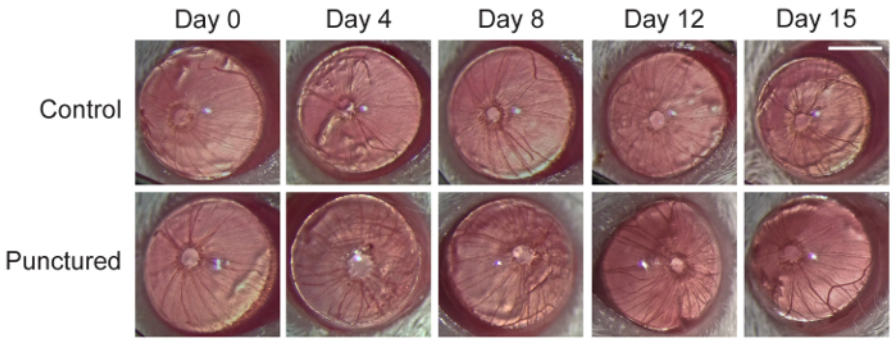

Figure 1: Noninvasive monitoring of puncture-induced iris angiogenesis. Representative images of day 0, 4, 8, 12, and 15 of control and punctured eyes. Iris vascular response is evident from day 4 onwards in the punctured eyes. Scale bar = 1 mm. Please click here to view a larger version of this figure.

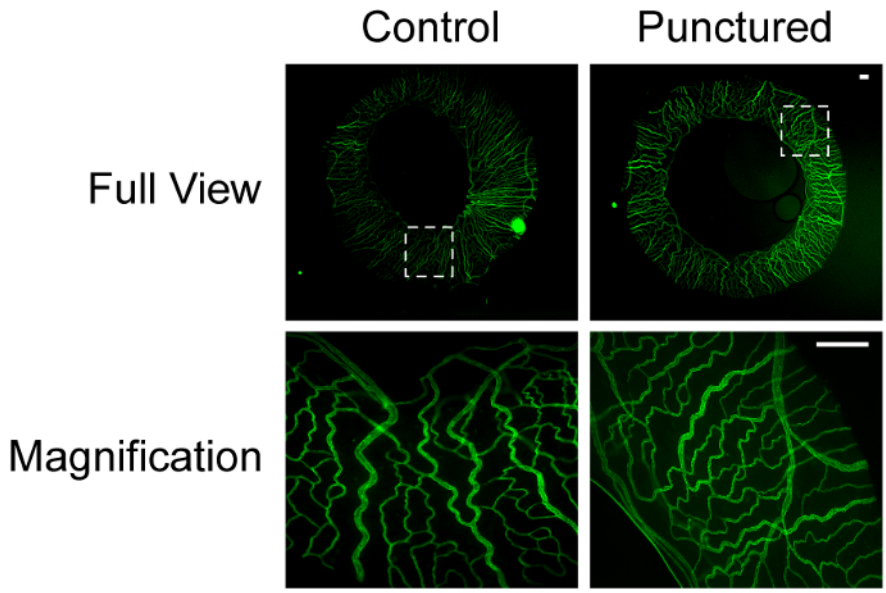

Figure 2: Iris vascular response in puncture-induced eyes. Illustrative images of PECAM-1 staining from day 15 irises, displayed as full view and magnified area (square), from control and punctured eyes. Scale bar = 400 µm (top); 200 µm (bottom). Note the increase of vascular branches in punctured irises. Please click here to view a larger version of this figure.

Discussion

In the present protocol, a novel method for induction of iris vascular response by uveal puncture is presented. The puncture triggers wound healing mechanisms and promotes vascular responses in the iris[10,11]. This is in agreement with ocular pathologies, such as PDR and NVG, where exacerbated angiogenic responses from the retina in the posterior segment of the eye culminate in the clinical condition of rubeosis iridis, an increased vascularization of the iris[12,13].

The cornea is avascular and possesses a peculiar collagen structure resulting in transparency. In line with this, angiography of the anterior segment of the eye for visualization of iris vasculature is achievable by direct visualization of iris blood vessels with *in vivo* noninvasive methods[13,14]. Previous animal models of iris neovascularization relied on large-eyed animals where complex surgical interventions were performed[15,16,17], or by intraocular injection of specific angiogenic substances[18,19]. In this method, the use of pigment deficient BALB/c mice

enables direct observation of the iris vasculature *in vivo*, and allows noninvasive quantification of the iris neovascularization in mice, thus providing a new tool to study *in vivo* angiogenesis.

Furthermore, the presented method for mouse iris dissection allows analysis of puncture-induced iris neovascularization with vascular specific markers. Here, iris vessels were illustratively stained with PECAM-1 antibodies and visualized by fluorescence microscopy. Free-floating, whole-mount immunostaining protocols are easily achieved and fairly routine. In addition, perfusion methods for blood vessels can be performed in this model. Vascular specific staining grants assessment of iris blood vessel structure and thus quantification, by user-based or software analysis, of the iris neovasculature[9]. As previously described[8,20], the illustration of iris neovasculature was assessed at day 15 post-puncture-induction. Nevertheless, effects on iris vascularization can be observed as early as day 4 post-puncture-induction, suggesting that evaluation of the puncture-induced iris neovascularization model could be carried out at different time points, based on specific experimental requirements.

It is noteworthy to state that this protocol presents some challenges, particularly related to the size of a mice eyes relative to other animal model. Extreme care should be taken while executing the uveal puncture procedure, to avoid lens touches or damage to the eye. Observation of ocular condition is paramount, and animals displaying experimental-related cataracts or drop in ocular pressure should be excluded and appropriately euthanized. Mouse irises are extremely fragile and somewhat tacky. Proper dissection and isolation of the tissue presents another critical step for the protocol. A correct separation from the trabecular meshwork is necessary for displacement of the iris from the anterior chamber. Moreover, due to fragility, iris isolation requires fixation of the tissue, which impairs downstream molecular analysis. Though, a molecular analysis of an unfixed whole-eye has been successfully applied to the presented method[8].

In summary, the described protocol presents a novel puncture-induced iris neovascularization mouse model. This model has the advantage of allowing *in vivo* noninvasive direct visualization of angiogenesis. In addition, the use of small rodents renders the presented model attractive for studies of rubeosis iridis, as it avoids the use of large-eyed animals and accompanied ethical restriction. Furthermore, the procedure can be combined with injection of pro- or anti-angiogenic substances through the self-sealing puncture, enhancing its usefulness as a new model of *in vivo* angiogenesis.

Disclosures

The authors have nothing to disclose.

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