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TITLE:

A Model of Glaucoma Induced by Circumlimbal Suture in Rats and Mice

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KEYWORDS:

Animal model, glaucoma, circumlimbal suture, intraocular pressure, chronic ocular hypertension, retinal ganglion cells

SUMMARY:

Chronic ocular hypertension is induced by applying a circumlimbal suture in rats and mice, leading to functional and structural deterioration of the retinal ganglion cells consistent with glaucoma.

ABSTRACT:

The circumlimbal suture is a technique for inducing experimental glaucoma in rodents by chronically elevating intraocular pressure (IOP), a well-known risk factor for glaucoma. This protocol demonstrates a step-by-step guide on this technique in Long Evans rats and C57BL/6 mice. Under general anesthesia, a “purse-string” suture is applied on the conjunctiva, around the equator and behind the limbus of the eye. The fellow eye serves as an untreated control. Over the duration of our study, which was a period of 8 weeks for rats and 12 weeks for mice, IOP remained elevated, as measured regularly by rebound tonometry in conscious animals without topical anesthesia. In both species, the sutured eyes showed electroretinogram features consistent with preferential inner retinal dysfunction. Optical coherence tomography

showed selective thinning of the retinal nerve fiber layer. Histology of the rat retina in cross-section found reduced cell density in the ganglion cell layer, but no change in other cellular layers. Staining of flat-mounted mouse retinae with a ganglion cell specific marker (RBPMS) confirmed ganglion cell loss. The circumlimbal suture is a simple, minimally invasive and cost-effective way to induce ocular hypertension that leads to ganglion cell injury in both rats and mice.

INTRODUCTION:

Animal models provide an important platform for laboratory investigation of cellular processes underlying glaucoma pathogenesis, as well as to evaluate potential therapeutic interventions. Several inducible models have been developed to produce sustained intraocular pressure (IOP) elevation, the most important risk factor for glaucoma. Methods that have been applied to elevate IOP include: hypertonic saline injection in episcleral veins[1], laser photocoagulation of the trabecular meshwork[2] or of the limbal veins[3], and intracameral injection of substances such as ghost red blood cells[4], microbeads[5,6] and viscoelastic agents[7]. Each approach has its advantages and limitations.

A good model for glaucoma should mimic the disease process, with minimal complication such as trauma, inflammation and media opacities. These complications are frequently associated with the procedures used to induce IOP elevation, and can confound interpretation of outcomes. For example, paracentesis of the anterior chamber, even when foreign substances are not introduced, has been shown to cause trauma and inflammation that is not representative of typical glaucomatous change[8,9]. In addition to the importance of avoiding inflammation, maintaining optical clarity facilitates *in vivo* imaging and electrophysiology to monitor disease progression. Although it is unclear to what extent these complications may affect disease investigations, it may be better to avoid penetrating the eye during model induction. The circumlimbal suture approach avoids penetration of the globe and facilitates *in vivo* longitudinal assessment of retinal structure and function. More importantly, this model differs from previous ones in its capacity to return IOP to baseline values by removal of the suture when required. IOP normalization may be useful for studying the cellular and molecular correlates of reversible and irreversible ganglion cell injury[10-14].

This article focuses on the technique for model induction. Characterization of retinal injury caused induced by this model in rats and mice can be found in greater detail elsewhere[15-19].

PROTOCOL:

All experimental procedures were conducted according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, set by the National Health and Medical Research Council in Australia. Ethics approval was obtained from the Howard Florey Institute Animal Ethics Committee (approval number 13-044-UM and 13-068-UM for rats and mice, respectively).

1. Intraocular Pressure Measurement in Conscious Rats

1.1. Set the laboratory rebound tonometer to the rat setting. Swaddle the awake rat in a piece of soft cloth to calm the animal. Expose the head and neck. Gently hold the torso in one hand, with the animal's back resting against the investigator's chest.

Note: Topical anesthesia is not required.

1.2. Use the other hand to bring the rebound tonometer near the rat's eye, so that the tip of the IOP probe is approximately 2 – 3 mm away from and perpendicular to the corneal apex. Use the right hand to measure IOP in the animal's right eye, and left hand for the left eye.

1.3. Wait a few seconds for the rat to calm and press the measurement button once. Observe the tip of the IOP probe gently hit the corneal apex once; and hear the rebound tonometer beep once.

Note: A single beep of the tonometer confirms successful measurement, which can be read from the LCD screen. A double beep indicates a measurement error. Measurement errors can arise from factors such as inappropriate working distance between the probe and the cornea, an excessive tilt in the orientation of the tonometer, or the probe striking the eyelid or a non-central part of the cornea. Refer to the rebound tonometer manual from the manufacturer for further detail regarding measurement errors.

1.4. Repeat step 1.3 ten times at an interval of 1 – 2 second, from these measurements derive an average IOP value for that time point. Reset the tonometer after the 5th reading.

1.5. For serial monitoring, measure IOP at the same time of the day and under consistent lighting conditions to minimize variation due to the diurnal IOP cycle[20,21].

2. Intraocular Pressure Measurement in Conscious Mice

2.1. Set the rebound tonometer to the mouse setting according to manufacturer's instruction.

2.2. To restrain the mouse by hand, place the mouse on a grill cage top and gently pull the tail backwards.

Note: This will prompt the animal to grip onto the metal grill with its front legs and attempt to pull itself forward, which will slightly stretch its body.

2.2.1. Use the other hand to grasp the loose skin immediately behind the ears. Secure the lower body of the animal by holding the tail between the ring finger and middle finger (or between the little finger and your palm).

Note: Try not to grasp the skin too tight, to avoid suffocation and applying pressure on the eyes.

2.3. With the now free hand (initially holding the tail), bring the rebound tonometer near the mouse's eye, so that the tip of the IOP probe is approximately 2 – 3 mm from and perpendicular to the corneal apex. To measure the other eye, rotate the mouse so that the other eye is now in front of the tonometer.

2.4. Wait for the mouse to calm and press the measurement button once. Observe the tip of the IOP probe gently hit the corneal apex; with a single beep confirming successful measurement.

Note: A double beep indicates a measurement error. It may help to have a second experimenter read and document the IOP readings whilst the first experimenter takes the measurements.

2.5. Repeat step 2.4 ten times with an interval of 1 – 2 second to derive an average IOP. Reset the tonometer after the 5^{th} reading.

2.6. As per serial measurement in rats, measure mouse IOP at the same time of the day and under consistent lighting conditions.

3. Induction of Intraocular Pressure Elevation in Anesthetized Rats and Mice

3.1. Clean the surgical bench with 0.5% chlorhexidine in 70% ethanol. Cover the bench with sterile drapes. Autoclave all surgical equipment beforehand. Ensure all experimenters wear appropriate personal protective equipment (surgical masks, gowns and sterilized gloves).

3.2. To induce general anesthesia, place the animal in an induction chamber. Deliver 3 – 3.5% isoflurane with O_2 at a flow rate of 3 L/min.

3.2.1. Maintain anesthesia with 1.5% isoflurane at 2 L/min delivered *via* a rodent face mask throughout the surgery. Ensure sufficient depth of anesthesia by the absence of a paw pinch reflex.

3.2.2. Avoid respiratory depression by adjusting the flow rate when necessary to maintain the respiratory rate at approximately 60 breaths/min.

3.3. Randomly select one eye to induce ocular hypertension, with the contralateral eye to serve as an untreated control. Instill one drop of 0.5% proxymetacaine ophthalmic solution for topical anesthesia. To clean the ocular surface, rinse the eye with 3 mL of sterile normal saline.

3.4. Cover the animal with a sterile, fenestrated surgical drape, exposing the eye to be sutured.

3.5. Perform a purse-string suture on the bulbar conjunctiva around the globe. In rats, weave the 7/0 nylon suture parallel and 2 mm posterior to the limbus (**Figure 1**). In mice, place the 10/0 nylon suture at 1 mm posterior to the limbus.

3.5.1. Take care not to penetrate the sclera. A sudden pupillary dilation during the surgical procedure indicates the sclera has likely been penetrated.

3.5.2. Anchor the suture on the conjunctiva using 5–6 anchor points in rats, and 4–5 anchor points in mice.

3.5.3. Avoid direct compression on the major episcleral veins by threading the suture underneath the conjunctiva at the crossing of these veins.

Note: While we recommend avoiding compression of the major episcleral vein in rats, this is not routinely done in mice due to low visibility of these veins in mouse eyes. Even though the major veins are not directly compressed, it is likely that the smaller vessels in the episcleral vein plexus are under pressure, which may be a contributing factor to the sustained IOP elevation (please see Discussion for mechanism of IOP elevation).

3.6. Fasten the purse-string suture by tying a slipknot then followed by a second simple knot (**Figure 1**). To avoid an excessively high post-surgical IOP spike, have an assistant measure the IOP immediately before fastening the second knot.

3.6.1. If the IOP is found to be too high, adjust the slip knot by partially releasing the tension on one end of the suture (arrow in **Figure 1A**).

3.6.2. After the desired IOP is achieved (ideally 30 – 60 mmHg in rats or 30 – 40 mmHg in mice), tie off the second knot while maintaining a continuous pulling force on that end of the suture (arrow in **Figure 1A**).

3.6.3. After the second knot has been tightened, trim the ends of the suture to minimize any foreign body sensation. Monitor the animal during recover from general anesthesia.

Note: It is important to use the slipknot when tying the first knot to ensure adequate inward compression on the eye. After several weeks it is usually noted that the ends become embedded in the conjunctiva.

4. Monitoring IOP

4.1. Take the first IOP measurement at 2 minutes post-operatively under isoflurane anesthesia. Subsequently, monitor IOP when the rodent has regained consciousness as per the aforementioned steps 1 and 2.

Note: Monitor the IOP twice during the first day (2 minutes and 1 hour), daily in the first week and once or twice per week thereafter.

5. Assaying Retinal Structure and Function

5.1. At the desired experimental end point (in this case after 8 weeks in rats and 12 weeks in mice), under general anesthesia using intraperitoneal injection with ketamine/xylazine, measure retinal function with the dark-adapted electroretinogram (ERG) as described in greater detail elsewhere.[15-17]

Note: We have found robust ganglion cell dysfunction, retinal nerve fibre layer thinning and ganglion cell loss for durations between 8-12 weeks. Others have successfully employed longer periods of IOP elevation[14,15].

5.2. Immediately after ERG measurement, measure the thickness of retinal nerve fiber layer (RNFL) and total retinal thickness using spectral domain optic coherence tomography (SD-OCT) [16,18].

5.3. At the end of the longitudinal study, euthanize the animals under deep anesthesia.

5.3.1. Dissect the retina for histology[18], for example immunostaining of whole-mount retina using a retinal ganglion cell (RGC) specific antibody such as RNA-binding protein with multiple splicing antibody (RBPMS) or brain-specific homeobox/POU domain protein 3A (Brn3a).[16,19,22]

REPRESENTATIVE RESULTS:

The following results in rats[18] and mice[16] have been previously reported and are summarized here. The circumlimbal suture produced a similar pattern of IOP elevation in rats and mice (**Figure 2**). A brief IOP spike, up to 58.1 ± 2.7 mmHg in rats and 38.7 ± 2.2 mmHg in mice, was found immediately after the suture procedure. In rats, IOP magnitude gradually reduced over time to be 44 ± 6 mmHg and 32 ± 2 mm Hg, at 3 and 24 hours, respectively[15]. After this initial IOP spike IOP remained relatively stable for several weeks. Over the experimental period, IOP in the ocular hypertensive (OHT) eyes remained elevated by ~ 9 mmHg for 8 weeks in rats, and by ~ 5 mmHg for 12 weeks in mice.

To assess RGC function, scotopic ERG at very dim stimulus energies elicits the positive Scotopic Threshold Response (pSTR), which was found to be reduced in the OHT eyes, relative to control eyes in both rats and mice (**Figure 3**). There was also a small reduction of the ERG a- and b-wave, which is likely to reflect a mild dysfunction of the photoreceptors and bipolar cells, respectively. The largest deficit however was found in the pSTR, confirming preferential inner retinal dysfunction subsequent to the mild chronic IOP elevation.

Consistent with inner retinal dysfunction, a selective loss of cell density in the RGC layer was also evident in the cross-sections of OHT retina (**Figures 4A – 4C**). In contrast, cell numbers in the outer and inner nuclear layers remain unaltered[18], suggesting that off-target ischemic

effects are minimal. Such findings in rats are corroborated by cell counts on whole-mount mouse retinae stained using an RGC specific antibody and confocal microscopy (**Figures 4E – 4G**). Similarly, OCT scans around the optic nerve head shows that chronic IOP elevation results in reduced RNFL thickness, whilst total retinal thickness remained unaltered in both species (**Figures 4D** and **4H**).

FIGURE AND TABLE LEGENDS:

Figure 1. Circumlimbal suture application around the equator of the eye. A: Firstly, use a slipknot to tighten the purse-string suture by pulling only one string (arrow), which will ensure adequate inward compression. An assistant can measure the IOP immediately before fastening the second knot. **B**: Subsequently tie a second simple knot to lock the first knot. **C**: Photograph of circumlimbal suture on a mouse eye. (Data in **A** and **B** are reused with permission from previous work, see references [18] and [16], respectively)

Figure 2. The circumlimbal suture raised intraocular pressure in this case for 8 weeks in rats (A, n = 8) and 12 weeks in mice (B, n = 23). IOP remained unchanged in contralateral control eyes. (individual OHT eyes represent by red symbols and control eyes by grey symbols). Average and standard deviations are overlaid in black. Data are replotted with permission from previous work [16,18]).

Figure 3. Chronic IOP elevation induced functional deficits particularly in the inner retina in both rats (A & B) and mice (C & D). A: Average ERG waveforms (n = 8 rats) in response to a bright and dim stimulus (2.07 and -5.31 log cd.s.m^{-2} for top and bottom trace respectively) after 8 weeks of IOP elevation. **B**: The relative amplitude of the pSTR, indicative of RGC function, was more affected than the photoreceptoral a-wave and the bipolar cell driven b-wave. **C** and **D** are as per **A** and **B** but derived from the average of 23 mice after 12 weeks of IOP elevation. Again, RGC dysfunction was more severe than photoreceptoral and bipolar cell dysfunction. ERG: electroretinogram; OHT: ocular hypertension; IOP: intraocular pressure; pSTR: positive Scotopic Threshold Response; RGC: retinal ganglion cells; * P< 0.05. Error bars: standard error of mean. Data are reused with permission from previous work.[16,18]

DISCUSSION:

The circumlimbal suture is a new model of chronic ocular hypertension. In addition to the studies from which the representative results are sourced[16,18], this animal model has been utilized in a number of recent studies[15,23-26]. Comparison across these previous reports shows that the method produces repeatable outcomes, including the magnitude of IOP elevation, as well as the brief IOP spike during model induction (see later discussion). Although the duration of IOP elevation needed to induce robust RGC changes is between 8 and 12 weeks, the model can be maintained for longer, with studies reporting outcomes for 15-16 weeks of IOP elevation[14,15]. In addition to repeatability, this method is relatively simple, cost effective, and can be used in both rats and mice. When compared with other approaches that involve penetrating the eye at model induction, this model is amenable to investigations that require clear optical media, such as electrophysiology or *in vivo* retinal imaging. One reason for this is

that by avoiding paracentesis, the circumlimbal suture method aims to preserve the immune privilege of the eye and therefore minimize trauma-related inflammation and cataract. A previous study employing this technique, found that Iba-1 expression, a marker for inflammation, was not upregulated in the retina[15], however the presence of other inflammatory markers or anterior chamber inflammation have not yet been quantified in this model. Another advantage is that the IOP elevation can be reversed by suture removal, which is a simple procedure that can be done under light sedation and topical anesthesia[14,15]. This renders the circumlimbal suture a unique model for investigating the potential reversibility of ganglion cell injury in glaucoma[24].

Although the mechanism by which the suture procedure raises IOP is not completely understood, obstruction of aqueous outflow is the likely cause after ruling out several other factors. From previous studies, we have shown that the circumlimbal suture does not significantly alter anterior chamber depth or iridocorneal angle in both rats[15] and mice[16] and is therefore not a model of angle closure glaucoma. Additionally, as pupillary dilation and pupil size were not altered, the clarity of the optical media was preserved, and no frank inflammatory changes was observed with anterior chamber OCT or with retinal cross sections, we do not believe that intraocular pressure elevation arises through an inflammatory mechanism. Finally, our finding that IOP could be rapidly normalized after removal of the circumlimbal suture suggests that remodeling of the trabecular meshwork as a result of inflammation would be an unlikely cause of the IOP elevation[16,24]. Thus, it is likely that IOP elevation arises from aqueous outflow obstruction, either *via* compression of Schlemm's canal or the episcleral veins. Further investigation is underway to determine the precise cause of aqueous outflow obstruction induced by this model.

The circumlimbal suture has several limitations. One obvious concern is the initial IOP spike that occurs during the application of the suture, which gradually reduces over several hours. Indeed, an excessive IOP spike has the potential to induce ischemic-reperfusion injury, which is not typical of chronic open angle glaucoma. In this regard it is prudent to post surgically confirm normal retinal perfusion using ophthalmoscopy or OCT angiography.

The potential contribution of the IOP spike was recently addressed by comparing untreated control eyes with a sham control group where the suture was applied as per methods described above, and then removed after 2 days. In other words, these sham control eyes were subjected to the same acute IOP spike but not the chronic IOP elevation beyond 48 hours. We found that the long term outcomes, measured by ERG, OCT and RGC counts, remain unaltered in the sham controls when compared with untreated controls[16], showing that the initial IOP spike did not have an important role in the RGC deficit seen in this model. This is also supported by the fact that in the ocular hypertension (OHT) eyes, there was no correlation between the magnitude of the IOP spike and the RGC dysfunction in the long term, whereas there was a significant correlation with chronic IOP elevation[15]. Additionally, one study where the suture was removed after 8 weeks shows that ganglion cell fully recovered, as measured by pSTR[24], which supports the idea that the brief IOP spike resulting from the model induction makes little contribution to the retinal dysfunction found after chronic IOP elevation. Had the transient IOP spike been a

contributing factor to the ganglion cell injury, one would not expect such recovery after suture removal at week 8. Therefore, despite having the limitation of a transient IOP spike, the circumlimbal suture model of ocular hypertension is a useful addition to currently available small animal glaucoma models.

Although the aforementioned evidence supports the usefulness of this model, every effort should be made to minimize the transient IOP spike. The following may assist with model induction. First, the most common problem encountered is that IOP can return to normal a few days after suture application. The probable cause is that the suture knot gradually loosens over time. To troubleshoot, ensure the first (slip) knot is securely fastened before tying the second knot. This can be achieved by continuously maintaining tension on one end of the slip knot (arrow in **Figure 1A**) until the second knot is tied. The second most common issue is hyphema which can occur in the first few hours after suturing. In our experience, this was commonly associated with an excessively high IOP spike (usually ≥ 80 mmHg in rats and mice) or perforation of the eye when weaving the suture. Other complications of the procedure include cataract (usually reversible) in the short term, and loss of the suture in the long term due to suture slippage or tearing of the conjunctiva. We have not noted the development of any ocular surface infections in any cohort of rats or mice. For novices to microscopic surgery, some practice is required to master circumlimbal suture application. We have reported an initial success rate of 50% in our first cohort of mice (40 out of 81 mice)[16]. In our experience, this improves to 70 – 80% with practice. In a subsequent cohort of 60 mice, we found a total success rate of 70%, with hyphema (13%) and suture loss (17%) accounting for the 30% failure rate. In a cohort of 20 rats, we find a higher success rate (90%) than in mice, with only 2 rats being excluded due to hyphema (10%), and no animals were excluded due to suture loss. Perforation during surgery are rare occurrences in both rat and mouse models (~1%).

ACKNOWLEDGMENTS:
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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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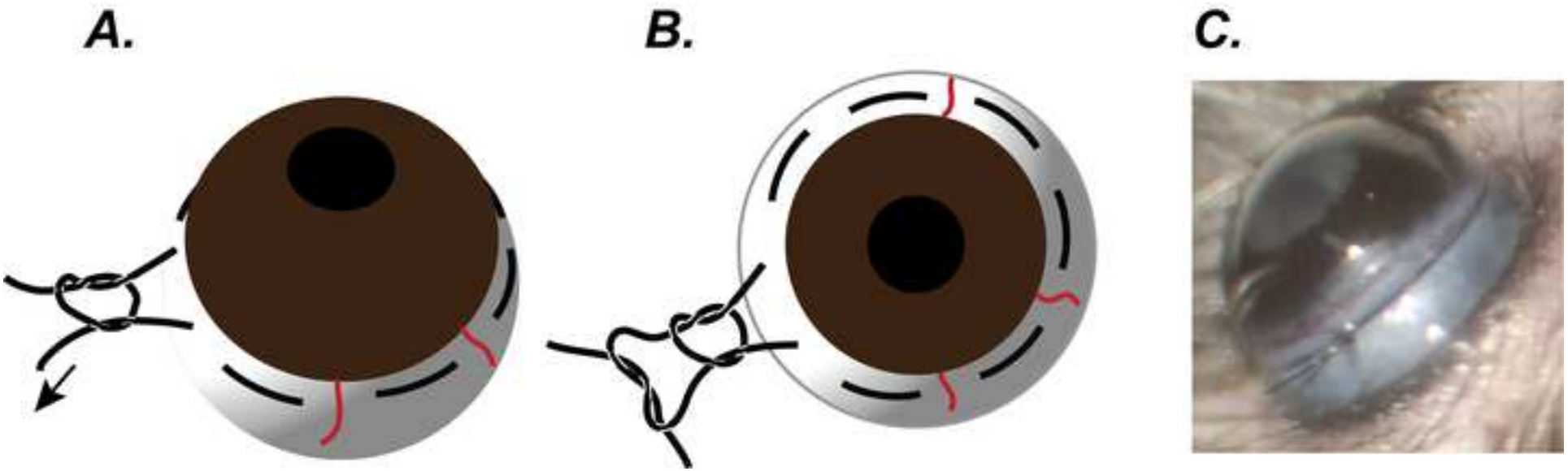

Figure 2

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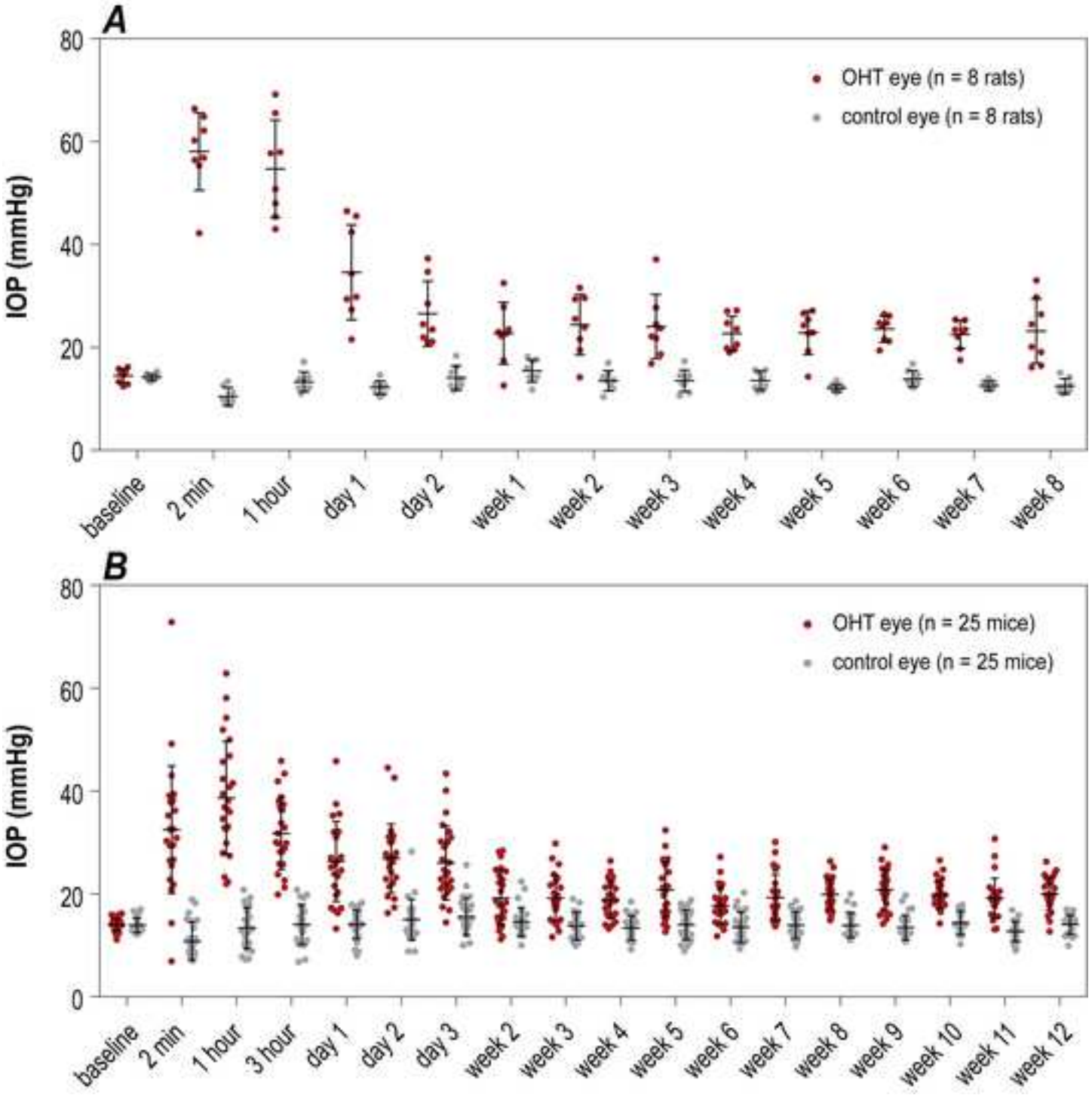

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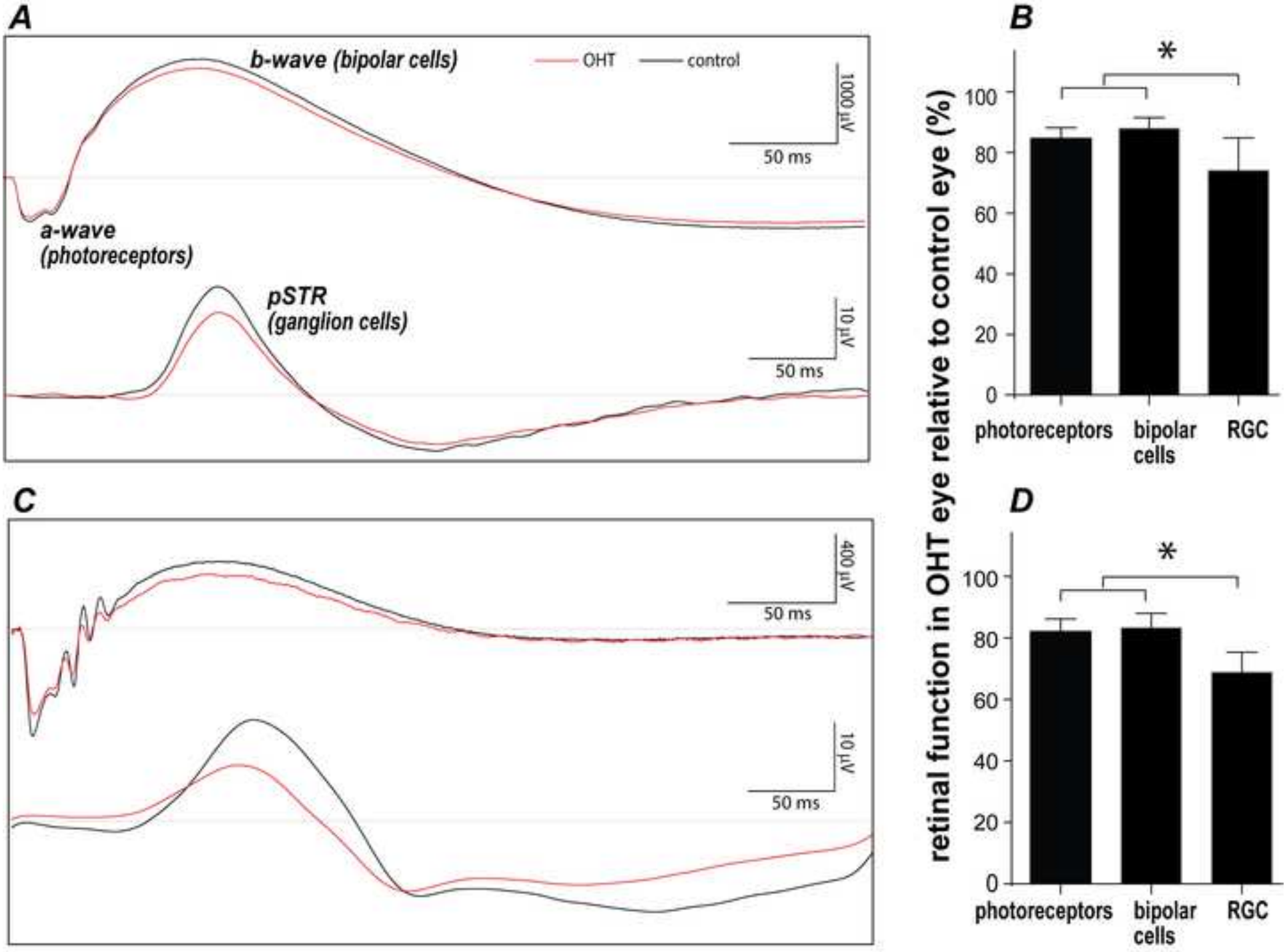

Figure 4

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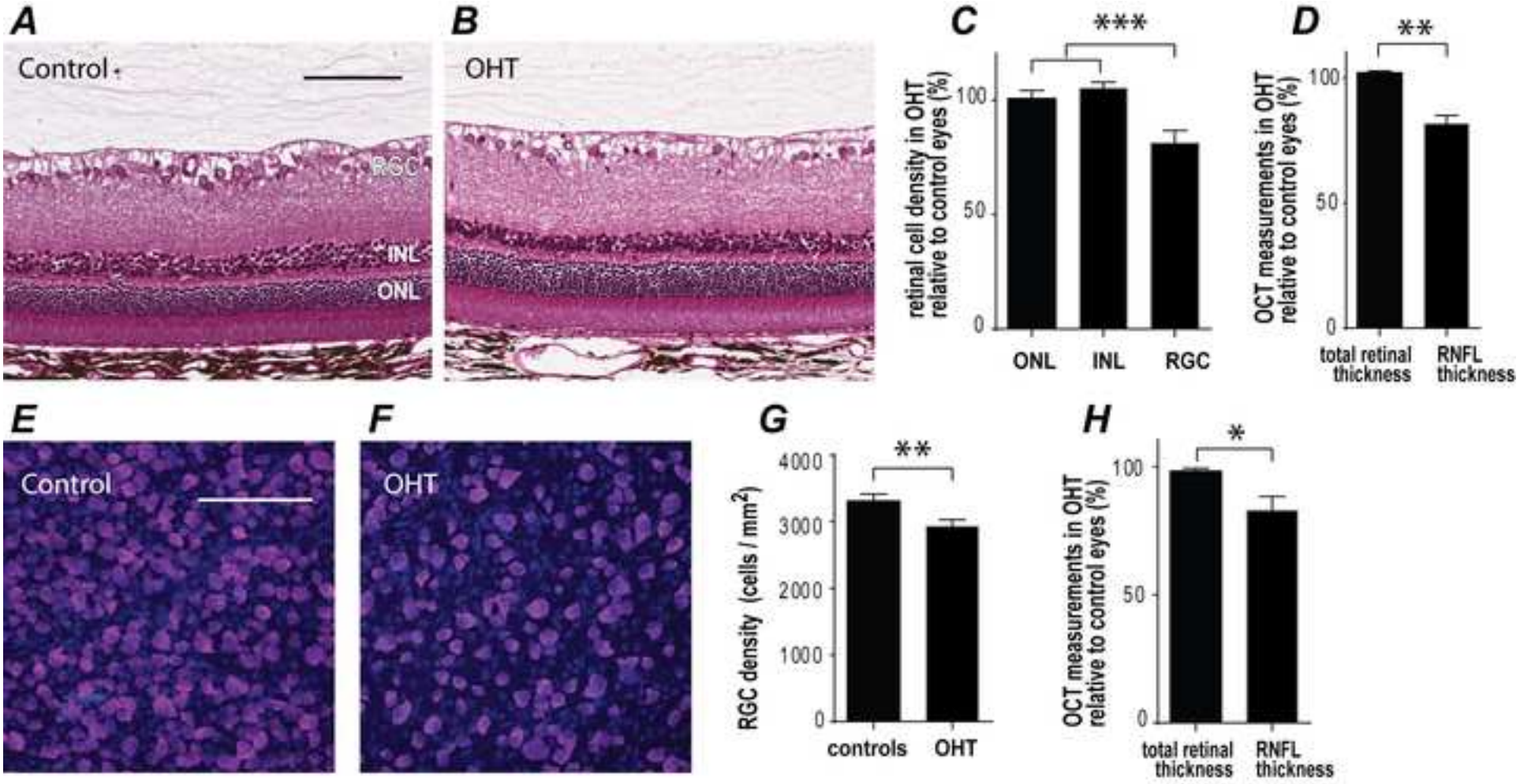

Table of Materials

Click here to download Table of Materials list of materials revised.XLSX

Name of the Material/Equipment	Company	Catalogue Number
normal saline	Baxter International Inc	AHB1323
Chlorhexadine 0.5%	Orion Laboratories	27411, 80085
Isoflurane 99.9%	Abbott Australasia Pty Ltd	CAS 26675-46-7
ocular lubricant	Alcon Laboratories	1618611
Needle holder (microsurgery)	World Precision Instruments	555419NT
Proxymetacaine 0.5%	Alcon Laboratories	CAS 5875-06-9
Scissors (microsurgery)	World Precision Instruments	501232
Surgical drape	Vital Medical Supplies	GM29-612EE
Suture needle for rats (microsurgery)	Ninbo medical needles	151109
Suture needle for mice (microsurgery)	Ninbo medical needles	160905
Tweezers (microsurgery)	World Precision Instruments	500342
rebound tonometer	TONOLAB, iCare, Helsinki, Finland	TV02

Comment/Description

Maintain corneal hydration during surgery

Disinfection of surgical instrument

Proprietory Name: Isoflo(TM) Inhalation anaaesthetic. Pharmaceutical-grade inhalation anesthetic mixed with oxygen gas for suture procedure

Proprietory Name: Genteal, ocular lubricant to keep the other eye moist

To hold needle during ocular surgery

Topical ocular analgesia

To cut excessive suture stump during ligation

Ensure sterile enviornment during surgery

8-0 nylon suture attached with round needle, cutting edge 3/8, dual-needle, suture length 30cm
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Manuscript #: JoVE58287

Title of Article: A model of glaucoma induced by circumlimbal suture in rats and mice

Author(s): Zheng He, Da Zhao, Anna van Koeverden, Christine Nguyen, Jeremiah Lim, Vickie Wong, Algis Vingrys, Bang Bui

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We have proofread the manuscript and made amendments as required.

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3. Figure 3 and 4: The bottom half of the error bar is obscured by the filled in chart.

Thank you, we have changed the bar charts in Figure 3 and 4 as suggested by the Editorial Reviewer.

4. Please do not abbreviate journal titles.

This has been amended.

Reviewers' comments:

We thank the reviewers for their useful comments. We have attempted to address all their queries and have amended this within the manuscript (track changes) and also detailed a response to each individual comment below. We hope that this has strengthened the paper.

Reviewer #1:

1) What is the mechanism of the IOP elevation in this model? External globe compression alone cannot account for persistent IOP elevation over weeks, as aqueous humor dynamics should eventually lead to re-equilibration of IOP. Most likely there is an effect of compressing the episcleral outflow system in the eye, leading to reduced trabecular outflow. It is interesting to note, however that the authors specifically discourage the investigator from placing suture over large episcleral veins, and instead suggest the suture be placed underneath the veins. Has the mechanism of IOP elevation been investigated? More information is needed here.

We thank the reviewer for this comment. To incorporate this suggestion, the following paragraph has been added to the Discussion (Page 9):

Although the mechanism by which the suture procedure raises IOP is not completely understood, obstruction of aqueous outflow is the likely cause after ruling out several other factors. From previous studies, we have shown that the circumlimbal suture does not significantly alter anterior chamber depth or iridocorneal angle in both rats (Liu et al 2015) and mice (Zhao et al 2017) and is therefore not a model of angle closure glaucoma. Additionally, as pupillary dilation and pupil size were not altered, the clarity of the optical media was preserved, and no frank inflammatory changes was observed with anterior chamber OCT or with retinal cross sections, we do not believe that intraocular pressure elevation arises through an inflammatory mechanism. Finally, our finding that IOP could be rapidly normalized after removal of the circumlimbal suture suggests that remodelling of the trabecular meshwork as a result of inflammation would be an unlikely cause of the IOP elevation (Liu et al 2017, Zhao et al 2017). Thus it is likely that IOP elevation arises from aqueous outflow obstruction, either via compression of Schlemm's canal or the episcleral

veins. Further investigation are underway to determine the precise cause of aqueous outflow obstruction induced by this model.

2) The authors need to include more information on success rates and the need to censor animals - beyond the note at the end of the discussion at success rate is only 50% when starting out and only 70-80% under best circumstances. At what rates do animals need to be excluded for different reasons such as scleral perforation, inadequate IOP elevation, and excessive IOP elevation? Given the presence of a foreign body for several weeks, do any animals acquire infections?

For have added more information regarding the success rate of the procedure, as follows :

Although the aforementioned evidence supports the usefulness of this model, every effort should be made to minimize the transient IOP spike. The following may assist with model induction. First, the most common problem encountered is that IOP can return to normal a few days after suture application. The probable cause is that the suture knot gradually loosens over time. To troubleshoot, ensure the first (slip) knot is securely fastened before tying the second knot. This can be achieved by continuously maintaining tension on one end of the slip knot (arrow in Figure 1A) until the second knot is tied. The second most common issue is hyphema which can occur in the first few hours after suturing. In our experience, this was commonly associated with an excessively high IOP spike (usually ≥ 80 mmHg in rats and mice) or perforation of the eye when weaving the suture. Other complications of the procedure include cataract (usually reversible) in the short term, and loss of the suture in the long term due to suture slippage or tearing of the conjunctiva. We have not noted the development of any ocular surface infections in any cohort of rats or mice. For novices to microscopic surgery, some practice is required to master circumlimbal suture application. We have reported an initial success rate of 50% in our first cohort of mice (40 out of 81 mice).[16] In our experience, this improves to 70 – 80% with practice. In a subsequent cohort of 60 mice, we found a total success rate of 70%, with hyphema (13%) and suture loss (17%) accounting for the 30% failure rate. In a cohort of 20 rats, we find a higher success rate (90%) than in mice, with only 2 rats being excluded due to hyphema (10%), and no animals were excluded due to suture loss. Perforation during surgery are rare occurrences in both rat and mouse models (~1%).

3) The authors should provide data on range of IOP to permit sample size calculations, which will also need to take into account success rate of inducing the model. Examples of sample sizes should be included. Figure 2 should include standard deviation of IOP on error bars rather than standard error of the mean, since the purpose of these graphs is descriptive of the technique and not in representing an experimental sample. A better way to depict the data for a reader interested in using this model would be a scatter plot with individual animal data points included.

We thank the reviewer for this suggestion. We have changed Figure 2 to a scatter plot of individual data points along with the group average ± SD.

4) The authors need more precise details regarding their materials. The sutures specifically need catalog numbers in the table at the end of the manuscript. The authors specify in the text that the suture should be nylon, but this also needs to be included in the table. There also is a question regarding the suture needle specified in the table - this needle is noted to be "used with 3-0 suture" and is 15mm long. Please check this - specific characteristics of the needle type (shape, size, cutting surface, length) with catalog number should be provided for both the 7-0 nylon and 10-0 nylon sutures (and they are likely not the same for both sizes of suture).

Thank you for your suggestion. Detail in the material list have been updated. The line with "3-0 suture" has been deleted.

5) Given that early spikes in IOP occur and IOP is the main outcome being tested, I was surprised that the main protocol did not specify that slip knot suture tension should be titrated to IOP prior to tying the second knot - though I see that this is recommended as a "technical tip" in line 279. Please consider making this part of the specified procedure.

We agreed with the reviewer. We have moved this "technical tip" into the main text of the protocol. Step 3.6 has been amended to the following:
Fasten the purse-string suture by tying a slipknot followed by a second simple knot (Figure 1). To avoid an excessively high post-surgical IOP spike, have an assistant measure the IOP immediately before fastening the second knot. If the IOP is found to be too high, adjust the slip knot by partially releasing the tension on one end of the suture (arrow in Figure 1A). After the desired IOP is achieved (ideally 30 – 60 mmHg in rats or 30 – 40 mmHg in mice), the suture will be continuously pulled to maintain the force, and then the second knot will be tied. After the second knot has been tightened, the ends of the suture should be trimmed to minimize any foreign body sensation. After several weeks it is usually noted that the ends of the suture become embedded in the conjunctiva. Allow the animal to recover from general anesthesia.

6) I would recommend including detailed instructions on tying a slip knot, as non-surgeons may be unfamiliar with this.

We thank the reviewer and agree that experimenters lacking surgical experience may require additional instruction in the tying of the slip knot. We believe that this technique can be better demonstrated by video rather than with instruction in the body of the text.

7) The IOP spike is a concerning issue, despite the electrophysiology and OCT data provided in the discussion (which are rather low-sensitivity methods to look for effects of a transient IOP spike). What is the range IOP in early spikes? How long do IOP spikes last? ("several hours" - line 247 - is nonspecific and an IOP of 80 for several hours would undoubtedly produce many off-target ischemic effects that would likely necessitate exclusion of the animal) How many animals have an IOP spike? Do histologic photoreceptor counts change in animals that have an IOP spike?

The individual IOPs are now given in the revised Figure 2, which shows that transient IOP spikes occurs in all rats and majority of the mice.

The following results in rats[18] and mice[16] have been previously reported and are summarized here. The circumlimbal suture produced a similar pattern of IOP elevation in rats and mice (Figure 2). A brief IOP spike, up to 58.1 ± 2.7 mmHg in rats and 38.7 ± 2.2 mmHg in mice, was found immediately after the suture procedure. In rats IOP magnitude gradually reduced over time to be 44 ± 6 mmHg and 32 ± 2 mm Hg, at 3 and 24 hours respectively[15]. After this initial IOP spike IOP remained relatively stable for several weeks. Over the experimental period, IOP in the ocular hypertensive (OHT) eyes remained elevated by ~ 9 mmHg for 8 weeks in rats, and by ~ 5 mmHg for 12 weeks in mice.

We have also make reference to our observation that outer retinal cell counts were unaltered.

Consistent with inner retinal dysfunction, a selective loss of cell density in the RGC layer was also evident in the cross-sections of OHT retina (Figure 4A – C). In contrast, cell numbers in the outer and inner nuclear layers remain unaltered, suggesting that off-target ischemic effects are minimal.

We therefore believe that by controlling the post-surgical IOP spike the model provides a means to study chronic IOP effects outcomes. However we agree that animals with very high IOP spike may need to be excluded.

8) Given the information provided about IOP spikes, it would seem prudent to visually monitor retinal perfusion after the suture is tied off.

Thank you for this suggestion. The following has been added to the discussion section:
The circumlimbal suture has several limitations. One obvious concern is the initial IOP spike that occurs during the application of the suture, which gradually reduces over several hours. Indeed, an excessive IOP spike has the potential to induce ischemic-reperfusion injury, which is not typical of chronic open angle glaucoma. In this regard it is prudent to post surgically confirm normal retinal perfusion using ophthalmoscopy or OCT angiography.

9) I recommend not listing ERG, OCT, or histology as required steps of the protocol (as in lines 153-163) as they are not required and this protocol does not provide any information on how to carry this out, but only refers to other protocols.

Thank you for this suggestion. We agree that the outcome measures may differ depending on what aspect of glaucoma pathophysiology is to be investigated. Therefore, the ERG, OCT and histology will not be included in the video protocol. In addition we have amended the title of this section as follows.

5. The following methods can be used to assay retinal structure and function, and were used to obtain the representative results that follow

10) Line 276 - it is unclear to me how an IOP spike to > 80mmHg would cause a hyphema. Hypotony could cause reflux from the collector system back into the anterior chamber, but very high IOP should not cause reflux. If there is true bleeding in the anterior chamber, it seems most likely that it would be due to ocular penetration with the suture needle.

Thank you for this comment. We acknowledge that raised IOP is unlikely to cause a hyphema, and that the hyphema is likely to be associated with perforation of the sclera, however we did observe the IOP to be very high in animals with hyphema. The text has been amended to the following:

The second most common issue is hyphema which can occur in the first few hours after suturing. In our experience, this was commonly associated with an excessively high IOP spike (usually ≥ 80 mmHg in rats and mice) or perforation of the eye when weaving the suture.

11) Why is a different duration of experiment recommended for mice and rats (i.e. in the abstract)?

The duration mentioned reflects the examples that have been given in the document, referencing our previously published manuscripts (Zhao et al 2017; van Koeverden et al 2018). This is not restrictive however and different durations of IOP elevation could be used. The text in step 5 of the main protocol has been amended to reflect this:

5.1) At the desired experimental end point (in this case after 8 weeks in rats and 12 weeks in mice), under general anesthesia using intraperitoneal injection with ketamine/xylazine we measured retinal function with the dark-adapted electroretinogram (ERG) as described in greater detail elsewhere.[15-17]

Note: We have found robust ganglion cell dysfunction, retinal nerve fibre layer thinning and ganglion cell loss for durations between 8-12 weeks. Others have successfully employed longer periods of IOP elevation[14,15.]

12) The IOP measurement section states that a double beep from the rebound tonometer indicates "the probe was either too far or two close to the cornea surface". This is an oversimplication - a double beep indicates an error, and there are many reasons for errors in addition to the two suggested by the authors, including the probe being held too vertically (either tip up or tip down) with regard to the gravity horizon. The authors should instead refer to the error codes listed in the tonometer instructions.

Thank you for this suggestion. The text in section 1.3 has been amended to the following: 1.3)
1.3) Wait a few seconds for the rat to calm and press the measurement button once. Observe the tip of the IOP probe gently hit the corneal apex once; and hear the rebound tonometer beep once.
Note: A single beep of the tonometer confirms successful measurement, which can be read from the LCD screen. A double beep indicates a measurement error. Measurement errors can arise from factors such as inappropriate working distance between the probe and the cornea, an excessive tilt in the orientation of the tonometer, or the probe striking the eyelid or a non-central part of the cornea. Refer to the rebound tonometer manual from the manufacturer for further detail regarding measurement errors.

The text in section 2.4 has been amended to the following:
2.4) Wait for the mouse to calm and press the measurement button once. Observe the tip of the IOP probe gently hit the corneal apex; with a single beep confirming successful measurement.
Note: A double beep indicates a measurement error. It may help to have a second experimenter read and document the IOP readings whilst the first experimenter takes the measurements.

13) Line 133 - rinsing the ocular surface with normal saline will not "disinfect" it. Either disinfect with something like betadine, or specify that the ocular surface is being rinsed or cleaned, but not necessarily disinfected.

Thank you for this suggestion. The word "disinfect" has been amended to "clean".

Reviewer #2:
Manuscript Summary:
In this manuscript, He and colleagues describe step-by-step the technique of circumlimbal suture as a means for elevating intraocular pressure in rodents, inducing experimental glaucoma. The authors described the methodology in two rodents: rats and mice. This techniques requires some expertise, as with any micro-surgery. However, it is efficient and reproducible.

Major Concerns:
The authors mentioned there is not inflammation associated to the technique and that immune privilege is preserved. This was not addressed in the publication.

Thank you for raising this issue. In previous work using the circumlimbal suture, we found that Iba-1 expression in the retina was not upregulated after chronic IOP elevation. Additionally, the surgery does not breach the anatomical structures that contribute to the immune privilege of the eye. The presence of other inflammatory markers or cells in the anterior chamber with the use of this model has not yet been quantified and would be useful in the future. The text has been amended to the following (line 258):

One reason for this is that by avoiding paracentesis, the circumlimbal suture method aims to preserve the immune privilege of the eye and therefore minimize trauma-related inflammation and cataract. A previous study employing this technique, found that Iba-1 expression, a marker for inflammation, was not upregulated in the retina[15], however the presence of other inflammatory markers or anterior chamber inflammation have not yet been quantified in this model. Another advantage is that the IOP elevation can be reversed by suture removal, which is a simple procedure that can be done under light sedation and topical anesthesia[14,15]. This renders the circumlimbal suture a unique model for investigating the potential reversibility of ganglion cell injury in glaucoma[24].

Minor Concerns:
All looks good, well-written

Reviewer #3:
Manuscript Summary:
The authors present a well written protocol to perform a novel approach to inducing ocular hypertension in rats/mice. I have a few minor comments only below that ought to be addressed.

Major Concerns:
None

Minor Concerns:
1. Line 95-96. It would be useful to add a reference to highlight the nature of diurnal IOP fluctuations in rodents at this point eg. PMID: 10798653.

The suggested reference has been added.

2. Line 159. It would be helpful for the ready to have recommendations for antibodies appropriate for whole mount analysis of rat retina for RGC assays eg. Brn3a, Isl1 etc

Thank you for this recommendation. The text has been amended to the following:
5.3) At the end of the longitudinal study, euthanize the animals under deep anesthesia. Dissect the retina for histology[18], for example immunostaining of whole-mount retina using a retinal ganglion cell (RGC) specific antibody such as RNA-binding protein with multiple splicing antibody (RBPMS) or brain-specific homeobox/POU domain protein 3A (Brn3a)[16,19,22]

3. Line 246. The authors ought to comment on the possibility that circumlimbal sutures will likely compress episcleral venous outflow and lead to an increase in eVP with secondary congestion which is likely a contributory mechanism to the IOP elevation, which is not found in human open angle glaucoma.

Thank you for this comment. Please see our response to reviewer one, point 1.

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Title:	Systemic hypertension is not protective against chronic intraocular pressure elevation in a rodent model
Author:	Anna K. van Koeverden, Zheng He, Christine T. O. Nguyen, Algis J. Vingrys, Bang V. Bui
Publication:	Scientific Reports
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