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The Effects of Anesthesia, Mouse Strain, and Age on Intraocular Pressure and an Improved Murine Model of Experimental Glaucoma

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Abstract

The purpose of this study was to improve a mouse model of chronic intraocular pressure (IOP) elevation utilizing microbead injection in two strains of mice and to assess the effect of age and anesthesia on measured IOP. We compared our previous model with two modified protocols for injecting polystyrene microbeads and viscoelastic material in CD1 or C57BL/6 mice. The measured outcomes were degree of IOP elevation and production of axonal loss. The first new protocol was injection of 3μL of equal volumes of 6μm and 1μm diameter beads, followed by 2μL of viscoelastic (3+2). The second new protocol injected 4μL of the two bead mixture, then 1μL of viscoelastic (4+1). Both were compared to injection of 2μL of 6μm beads with 3μL of viscoelastic (2+3). We also compared the effects of age and of two anesthetic regimens (intraperitoneal ketamine/xylazine/acepromazine versus isoflurane gas) on measured IOP in untreated eyes of both strains. IOP was 2 mm Hg lower with intraperitoneal than with gas anesthesia in both strains ($p=0.003$, $p<0.0001$, t-test). IOP measurements were lower in untreated young (2 months) compared to older (10 months) C57BL/6 mice ($p=0.001$, t-test). In the experimental glaucoma mouse model, mean IOP and number of elevated IOP measurements were higher in newer protocols. Mean axon loss with the 4+1 protocol (all strains) was twice that of the 2+3 and 3+2 protocols (36% vs. 15% loss, $p = 0.0026$, ANOVA), and mean axon loss in CD1 mice (21%) was greater than in C57BL/6 mice (13%) ($p = 0.047$, ANOVA). Median axon loss in 4+1 protocol treated C57BL/6 mice expressing yellow fluorescent protein in 2% of retinal ganglion cells (RGCs) had greater median axon loss than C57BL/6 4+1 protocol treated mice (26% vs. 10%, $p=0.03$). The 4+1 protocol provided higher, more consistent IOP elevation and greater axonal loss. The effects of age, strain, and anesthesia on induced IOP elevation and axon damage must be considered in mouse experimental glaucoma research.

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1. Introduction

Glaucoma is a leading cause of vision loss worldwide (Quigley and Broman 2006). The disease results in axonal degeneration in the optic nerve, as well as death of retinal ganglion cells (RGC). Animal models have contributed substantially to our knowledge of the effects of intraocular pressure (IOP) on RGC survival in glaucoma. A number of mouse strains that spontaneously develop elevated IOP and damage similar to that in human glaucoma have been identified (John et al, 1998; Zhou et al, 2009; Aihara et al, 2003b; Fijukawa et al, 2009), including the DBA/2J mouse. In another recently developed strain, the GLAST-deficient mouse, RGC death occurs spontaneously at normal IOP (Harada et al, 2010). To test hypotheses regarding features that contribute to RGC loss in such models, one approach is to breed a spontaneous glaucoma mouse strain with a strain carrying another genetic alteration of interest, and then to assess the effect of genetic alteration on the glaucoma phenotype. While such experiments are elegant, the time needed to produce these doubly affected strains is considerable (Libby et al, 2005).

To allow for more rapid experiments and to produce experimental glaucoma in a wide variety of strains, methods to induce elevated IOP in mice have been pursued. IOP increase has been accomplished by laser treatment of the outflow area, cautery or osmotic damage to the episcleral and vortex veins, or bead injection into the anterior chamber (Grozdanic et al, 2003; Aihara et al, 2003a; Gross et al, 2003; Nakazawa et al, 2006; Ruiz-Ederra and Verkman, 2006; McKinnon et al, 2009; Sappington et al, 2010). Previously, we produced a model of experimental glaucoma with the intracameral injection of polystyrene microbeads and viscoelastic material (Cone et al, 2010) modifying the approach suggested by Sappington et al. (2010) in mice and that of Urcola et al. (2006) in rats. Although these original approaches successfully created RGC soma and axonal damage, we sought to generate longer and more consistent IOP elevation by varying the bead injection procedure. Chen et al. (2011) have since reported variations in the original bead injection technique of Sappington et al. The goal of continued improvements in these bead injection models is to achieve 4 main features: 1) prompt and consistent IOP increase in most eyes, 2) lack of detrimental effects on the cornea and sclera, 3) substantial and consistent death to RGC and their axons without injury to other retinal neurons, and 4) easy, low cost methods.

IOP measurement in mouse glaucoma models is indispensible. The IOP of mice of several common strains can be accurately measured with the TonoLab instrument (Morris et al, 2006; Pease et al, 2006; Saeki et al, 2008; Wang et al, 2005.), even after the cornea and sclera have been altered by chronic IOP elevation (Pease et al, 2011). The age effect on untreated IOP in mice has been infrequently studied in C57BL/6 and other strains of mice. Further assessments seem appropriate in larger sample sizes, in particular in the CD1 strain (Savinova et al, 2001).

While methods have been devised to obtain IOP measurements in awake mice (Nissirios et al, 2007), it has been found that anesthetics are necessary in some strains. It is well known that IOP in anesthetized mice (Wang et al, 2005) and rats (Jia et al, 2000) is lower than IOP in awake or restrained rodents (Ding et al, 2011); however this effect has not been studied in the CD1 strain. Mice anesthetized with a mixture of ketamine, acepromazine and xylazine have lower IOP measurements than with ketamine alone (Danias and Kontiola, 2003).

Overall, it is important to understand the effect of anesthesia on IOP in mice of commonly used strains and in both younger and older animals after experimental glaucoma has been induced.

In this report, we provide detailed methods for an improvement in our bead injection technique to induce IOP and RGC loss in mice. We also compared IOP measurements taken with two forms of anesthesia (parenteral sedation compared to isoflurane inhalation) and measured age-related and strain-related differences in IOP and axon loss that may be relevant in murine glaucoma research.

2. Methods

2.1. Animals

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, using protocols approved and monitored by the Johns Hopkins University School of Medicine Animal Care and Use Committee. CD1 albino mice were obtained from Charles River Laboratories, Wilmington, MA, USA. We obtained C57BL/6 pigmented mice as well as transgenic C57BL/6 pigmented mice which selectively express yellow fluorescent protein (YFP) in RGC under the control of the Thy-1 promoter, B6.Cg-Tg(Thy1-YFPH)2Jrs/J, (denoted: C57BL/6 YFP), both from Jackson Laboratories, Bar Harbor, ME, USA. We used data from 669 mice in the study, with ages ranging from 2 to 22 months.

2.2. Anesthesia

For anterior chamber injections, mice were anesthetized by either an intraperitoneal injection of 50 mg/kg of ketamine, 10 mg/kg of xylazine, and 2 mg/kg of acepromazine or by inhalation of isoflurane. For the latter, we used the RC^2 - Rodent Circuit Controller (VetEquip, Inc., Pleasanton, CA, USA). This instrument supplies oxygen from an attached tank at 50–55 pounds per square inch. Oxygen is mixed with isoflurane and sent to two outflows at 500 cc/minute, delivering 2.5% of isoflurane in oxygen to the animal. One outflow enters a box where mice were placed into for initial sedation. After about 2 minutes, the sedated animal was positioned for IOP measurement and clinical examination with a nose cone delivering the isoflurane gas/ oxygen mixture. The nose cone permitted access to the eyes. Animals that were anesthetized with isoflurane did not receive topical anesthesia. Animals under ketamine/ xylazine/acepromazine anesthesia received topical anesthesia of 0.5% proparacaine hydrochloride eye drops (Akorn Inc. Buffalo Grove, IL, USA).

2.3. New Bead Sterilization Protocol

Our previous protocol used beads with a diameter of 6 μm. In the new protocol, we injected an equal volume mixture of 1 μm and 6 μm beads (Polybead Microspheres®, Polysciences, Inc., Warrington, PA, USA). First, we sterilized the beads with 100% ethanol, which caused clumping of the 1μm beads. To separate beads, they were placed in 0.5% Triton in sterile phosphate-buffered saline and heated in a water bath at 65°C for one hour. Then, each bead size was twice centrifuged to a pellet and resuspended in sterile, phosphate buffered saline. After the final centrifugation, the pellet was aspirated directly into a glass cannula, which was pulled to a tip diameter of 50 μm for intracameral injection. We previously estimated the final concentration of beads as 3×10^6 beads per μL for 6 μm beads, and we estimate the concentration of 1 μm beads as 1.5×10^7 beads per μL.

2.4. Bead Injection Protocols

Animals received one of two new bead injection protocols, both of which were different from our previously reported protocol. Both newer approaches had higher volumes of beads

per injection and both used two different sizes of beads. In all protocols, a single glass cannula with a 50 μm diameter tip was connected by polyethylene tubing to a Hamilton syringe (Hamilton Company Reno, NV, USA). First, we drew into the cannula the viscoelastic solution (10 mg/ml sodium hyaluronate: Healon, Advanced Medical Optics Inc., Santa Ana, CA, USA). In the new protocols, we then drew up 1 μm beads, and finally the 6 μm beads. The old protocol used only 6 μm beads. In the new protocols, the larger beads entered the eye first, followed by the smaller beads, though some mixing may have occurred. In all protocols, the Healon entered last, pushing beads into the peripheral anterior chamber and the trabecular meshwork area, and preventing egress of beads after withdrawal of the needle.

Forceps were used to hold the conjunctiva to stabilize the eye before penetrating the inferior portion of the anterior chamber with the glass cannula. The injection was made over 45 to 60 seconds, and the cannula was held in place for 2 minutes before being withdrawn, to reduce egress of Healon. As the needle was being removed from the chamber, we applied gentle pressure at the exit site with forceps to retain the beads and Healon in the eye. Injections were made as close to the limbus as possible. In some eyes, the iris could be seen occluding the internal corneal opening after the cannula was removed.

In the first of the two new protocols (referred to here as 3+2), we injected 1.5 μL of 6 μm beads, 1.5 μL of 1 μm beads, and 2 μL of Healon. For the second protocol (referred to as 4+1), we injected 2 μL of 6 μm beads, 2 μL of 1 μm beads, and 1 μL of Healon. The previously reported protocol involved the injection of 2 μL of 6 μm beads and 3 μL of Healon (referred to as 2+3).

2.5. Mouse Groups

In the bead injection portion of this study, 8 groups of mice received a bead injection; either the previously published 2+3 protocol or one of the new protocols, 3+2 or 4+1. Four groups were CD1 mice, two were C57BL/6 mice, and two were C57BL/6 YFP expressing. Of the four groups of CD1 mice, one received the 2+3 protocol, another 3+2 protocol, and two groups received the 4+1 protocol. One of the 4+1 protocols treated a CD1 cohort that also received three subconjunctival buffer injections as part of another study (denoted CD1 Buffer group in Tables). Of the C57BL/6 mouse groups, two were C57BL/6, receiving the 2+3 protocol or the 4+1 protocol, and two were C57BL/6 YFP, receiving the 4+1 protocol, one group of the latter was sacrificed at 3 weeks after injection and the other at 6 weeks.

The anesthetic and age effects on IOP were studied in uninjected control eyes of animals of both CD1 and C57BL/6 mouse strains.

2.6. Intraocular Pressure Measurement

All IOP measurements were made using the TonoLab tonometer (TioLat, Inc., Helsinki, Finland), recording the mean of 6 readings with optimal variability grade. We measured baseline IOP prior to injection. Then, we measured the IOP 10 minutes after bead and Healon injection, and at various times up to 6 weeks after injection. Prior to IOP measurement, animals were anesthetized by one of two methods. One method was intraperitoneal injection of a mixture of ketamine, xylazine, and acepromazine (50, 10 and 2 mg/kg, respectively). The other was inhalation of isoflurane as described above.

2.7. Sacrifice and axial length measurements

Animals were sacrificed under general anesthesia by exsanguination, then perfused intracardially with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH = 7.2). Enucleated eyes were inflated to 15 mm Hg with a needle connected to a fluid-filled

reservoir for measurement of axial length and width with a digital caliper (Instant Read Out Digital Caliper, Electron Microscopy Sciences, Hatfield, PA, USA). The length was measured from the center of the cornea to a position just temporal to the optic nerve, while the width was measured at the largest dimension at the equator, midway between cornea and optic nerve.

2.8. Optic nerve axon counts

To assess RGC damage, we estimated axon loss in optic nerve cross-sections by a quantitative, sampling technique. After perfusion fixation, we enucleated the globes and optic nerves. We then placed the nerves in 1% osmium, dehydrated them in ascending alcohol concentration, and then placed them in 1% uranyl acetate in 100% ethanol for 1 hour. Tissues were embedded in epoxy resin mixture at 60°C for 48 hours. One micron thick cross-sections of the optic nerve were cut and digital images of the nerves were taken at low power to measure each optic nerve area. Then, high power images were taken (100x, oil immersion objective) using a Cool Snap camera and Metamorph Image Analysis software (Molecular Devices, Downington, PA, USA). For each nerve, five 40 × 40μm fields were acquired, with the total of sampled nerve equaling 9% of the overall nerve area. Masked observers edited non-axonal elements from each image, generating an axon density from the software. The average axon density/mm^2 was multiplied by the individual nerve area to estimate the axon number (for further protocol details, see Levkovitch-Verbin et al, 2002 or Marina et al, 2010). Experimental eyes were compared to the mean axon number in pooled, fellow eye nerves of the appropriate strain, age, and tissue fixation to yield percent axon loss.

2.9 Histology of Retina

To check for inner retinal ischemic atrophy, control and bead injected superior retinas were cryopreserved in 20% sucrose in 0.1MPO_4 and OCT (Sakura, Alphen aan de Rijn, The Netherlands). Eight μm thick sections were made using a Microm Cryostat (Richard-Allen, Kalamazoo, MI, USA). Tissue was then exposed to a 1:1,000 dilution of DAPI (Invitrogen, Carlsbad, CA, USA) for 2 minutes before mounting with Fluorescent Mounting Medium (DakoCytomation, Carpinteria, CA, USA). Images were taken using the Zeiss LSM 510 Meta Confocal Microscope (Zeiss MicroImaging, Thornwood, NY, USA).

2.10. Statistical Analysis

The following data were tabulated and compared between treated groups and between treated and control eyes: mean IOP, IOP exposure over time (positive integral = area under the IOP versus time curve in the treated eye that exceeded the area under the IOP versus time curve in the control eye), peak IOP (mean of the highest IOP reading in each mouse by protocol and strain), number of weekly measures in which the IOP in the bead-injected eye exceeded that in its fellow eye by ≥5mmHg, which we have previously published is the 97.5% confidence limit for normal intraocular difference in mice (Cone et al, 2010), axial length and width, and axon count. Primary outcomes were compared, taking into account the following: height and length of IOP exposure, type of anesthesia, age, and mouse strain. Mean values were compared with parametric statistical tests for data that were normally distributed and median values with non-parametric testing for those whose distributions failed normality testing. Repeated measures ANOVA models were constructed to account for multiple variables. In some models, animals receiving the 4+1 protocol were the reference group, comparing them to the combined 2+3 and 3+2 protocol groups, with inclusion of the variables: strain and weeks of followup. Similar ANOVA analysis was performed in models in which C57BL/6 animals were used as the reference to compare to CD1 animal groups. The C57BL/6, 4+1 treated group followed for 3 weeks was not included in the multivariate analysis in either the strain or protocol comparisons.

3. Results

3.1. Anesthesia effects

To compare the IOP between intraperitoneal and gas anesthesia, we used IOP data taken on two different days in the same animal (these were control animals that did not receive any treatment), the first measurement was with intraperitoneal anesthesia and the second was with either intraperitoneal or gas anesthesia. The intraperitoneal then intraperitoneal IOP values were compared to the intraperitoneal then gas IOP values. IOP was measured at an average of 10 minutes after anesthesia was administered. Both C57BL/6 and CD1 strain animals were evaluated in this manner at 3.8 to 4.2 months of age (Table 1).

For intraperitoneal to isoflurane anesthesia, IOP was >2 mm Hg higher under gas anesthesia in both strains, equal to about a 20% higher IOP compared to the baseline mean IOP of 10 mmHg in both strains. The difference between two intraperitoneal anesthesia measurements in CD1 mice was not significant (Table 1), but in C57BL/6 animals, the second measure was slightly higher than the first (1.1 mm Hg, $p = 0.02$, t-test).

With ketamine/xylazine/acepromazine intraperitoneally, we found that approximately 2% of animals died due to anesthesia prior to completing follow-up (10 out of 512). Anesthesia deaths were more common in C57BL/6 mice (13%; 8/61 in one group). By contrast, all mice recovered successfully from isoflurane anesthesia. The most frequent exposure to either anesthetic was twice in one week.

3.2. IOP comparison of younger and older control mice

We compared the IOP (using intraperitoneal anesthesia only) between young (2 months old) and older, untreated mice of C57BL/6 (8 months old) and CD1 (10 months old) strains. In C57BL/6, IOP was significantly higher in 8 month old compared to 2 month old mice ($p = 0.001$, t-test, Table 2). The IOP did not significantly differ between 2 month old and 10 months old CD1 mice, but we tested only a small number of older CD1 animals.

3.3. IOP comparison among bead model protocols

As expected, the mean IOP difference (treated compared to control eyes) was significantly higher for all bead injected eyes than untreated eyes ($p < 0.0001$, ANOVA, adjusted by group, strain, and weeks followed, Table 3). The mean IOP difference between treated and control eyes was significantly higher for the 4+1 protocol than for the 2+3 protocol ($p \leq 0.0001$, t-test), and IOP difference was greater among C57BL/6 mice than CD1 mice ($p = 0.004$, ANOVA, Table 3). The IOP data of combined groups (2+3/3+2 protocols) can be seen in Table 4, while data for each specific protocol and strain are compared in Table 5.

We calculated exposure to IOP over time as the difference between the treated and control eye pressures in mmHg—days, a value we have named the positive integral IOP (Cone et al, 2010). Animals that received the 4+1 protocol had significantly higher positive IOP integral than the combined 2+3 & 3+2 protocols ($p = 0.003$, ANOVA, adjusting for group and strain). An ANOVA model found a borderline difference in positive IOP integral by strain ($p = 0.06$).

Mean peak IOP readings were determined by taking the highest peak IOP during the study and averaging by protocol and strain group. The 4+1 protocol was found to have a higher peak IOP for both CD1 and C57BL/6 mice than the 2+3 protocol ($p \leq 0.0001$ and $p \leq 0.001$, respectively, t-test) and higher peak IOP than the 3+2 protocol ($p \leq 0.0001$, t-test).

When we considered the course of IOP over time, the 4+1 protocol had larger mean differences between treated and control eyes than the 2+3 protocol at the 7 day and 14 day

time points in both strains of mice (Figure 1), but was generally similar to the original protocol thereafter. The C57BL/6 treated eyes had dramatically elevated mean IOP readings at 3 and 7 days with the 4+1 protocol ($p = 0.001$ and $p < 0.0001$, respectively, t-test, Figure 1).

Previously, we demonstrated that the IOP difference between untreated, fellow eyes in anesthetized mice exceeded 5 mmHg less than 5% of the time (as previously published in Cone et al., 2010). We used this standard to judge the number of occasions in which the treated eyes have a significant IOP elevation (outside the 95% confidence limit). The number of times the treated eye IOP exceeded the control eye by this standard was significantly greater in the 4+1 protocol than in the 2+3 protocol in ANOVA models for 5 of 7 time points and for the combined data from days 3, 7, and 14 days ($p = 0.001$, Table 6). The ANOVA model showed that C57BL/6 mice had significantly more 5 mm Hg elevations over control than did CD1 mice at some time points, but not overall ($p = 0.42$, Table 6).

In the CD1 4+1 & buffer group, the baseline IOP in both injected and uninjected eyes was 3 mmHg higher than some other controls, possibly relating to systemic effects of prior recent treatments including subconjunctival saline injections. The bead-injected eyes of this group had comparably higher IOP than controls and comparable positive integral IOP to other groups. Also, C57BL/6 YFP animals share the same general background with C57BL/6, but due to some selective breeding to develop their specific labeling of ganglion cells, differences that may influence baseline IOP could exist, potentially explaining higher control IOP values.

3.4. Axial Length

Axial length increased significantly after exposure to chronically elevated IOP with all groups combined, as well as in the 4+1 and in the 2+3/3+2 combined groups (ANOVA, Table 7). Both CD1 and C57BL/6 mice had significant axial length increases (Table 7). However, ANOVA models showed that the differences between protocol groups (4+1 compared to 2+3 or 3+2 protocols) or between mouse strains were not significant (ANOVA).

3.5. Axon Loss

Animals that received the 4+1 protocol had significantly higher axon loss than 2+3 protocol, ($p=0.003$, t-test). The 4+1 protocol animals also had a significantly higher axon loss than the 2+3/3+2 protocol groups combined ($p = 0.003$, ANOVA, Table 8). CD1 bead treated animals had greater axon loss than C57BL/6 ($p = 0.04$, ANOVA).

C57BL/6 mice had significant axon loss with the 2+3 protocol and comparable loss with the 4+1 protocol (Table 9). C57BL/6 YFP 4+1 eyes also had significant axon loss at both 3 and 6 weeks ($p = 0.003$ and $p \leq 0.001$, respectively, t-test). Their loss at 6 weeks was significantly greater than the C57BL/6 with the same 4+1 protocol ($p = 0.03$, t-test). The loss of axons in damaged nerves was clearly seen in epoxy embedded cross-sections (Figure 2). Damaged nerves had degenerating axons and macrophages filled with clear vacuoles.

3.6 Histology of the Retina

Cross-sections of retina showed no abnormality in any retinal layer other than the nerve fiber, ganglion cell and inner plexiform layers in bead-injected eyes. Even in eyes from the C57BL/6 4+1 protocol group that had the highest IOP elevations in the study, there was no evidence of inner retinal ischemic atrophy (Figure 3).

4. Discussion

We have modified our previously described model for experimental glaucoma in the mouse to improve its usefulness for studying the effects of IOP increase. An increase in the volume of injected beads combined with the mixture of smaller and larger beads in the new protocols produced several desirable features. First, the IOP elevation was greater, particularly evident in the 4+1 protocol during the first two weeks after bead injection. The 4+1 protocol also produced more RGC axon loss than the previous protocol. The median axon loss reached half of the optic nerve in the CD1 animals with the 4+1 protocol. Axon loss of this magnitude is advantageous for experiments that seek to measure neuroprotective treatments for experimental glaucoma. Detailed study of the axonal pathology in laser-induced glaucoma in CD1 mice was published by Fu and Sretavan (2010). The 4+1 protocol in C57BL/6 YFP mice led to significantly greater axon loss than we have observed previously in C57BL/6 animals. To determine whether this is a true strain difference between C57BL/6 groups will require further study. In fact, in 2 subsequent groups of C57BL/6 animals to be reported from our group mean axon loss using the 4 + 1 protocol was 31% in one group of 29 mice and 26% in the other group of 30 mice. It is important to have models that produce significant damage in C57BL/6 animals, since they represent the background strain for so many transgenic mice.

The new data confirm our prior finding (Cone et. al. 2010) that C57BL/6 mice are less susceptible to RGC death from experimental IOP elevation than the albino CD1 mice and the difference seen with the C57BL/6 YFP strain deserves further study. In this experiment, we used axon loss as the RGC damage criterion, but the same susceptibility difference was identified among C57BL/6 animals by RGC layer neuron counts in our prior report. Although pressure elevations were high for the C57BL/6 4+1 group, retina cross sections found no ischemia or damage to layers other than the RGC layer. The apparently lower susceptibility of C57BL/6 mice occurred despite the fact that in both our reports they had higher IOP and higher positive integral IOP in the treated eyes than CD1 animals. A recent presentation (McDowell C et.al., IOVS 2011; 52: ARVO E-Abstract, 2440) also found strain-specific susceptibility using another mouse glaucoma model. The basis for such strain-specific differences in damage could provide important clues to the risk factors in glaucoma pathogenesis. We are now investigating whether differences in the biomechanical behavior of the sclera in C57BL/6 and CD1 mice contribute to the susceptibility differences were observed in RGC axon loss.. We have found that C57BL/6 mice differ in properties of scleral stiffness and scleral response to elevated IOP from CD1 mice, potentially contributing to their difference in RGC loss susceptibility.

Intraperitoneal anesthesia significantly lowered IOP in C57BL/6 mice, but not CD1. We have no specific data that indicate whether this is a true strain difference or was a result of the smaller sample size of CD1 mice. Intraperitoneal anesthesia also led to more frequent premature death of mice (Schulz et al, 2002), particularly in C57BL/6 animals. While the use of isoflurane requires the purchase of an anesthesia machine, the rapid and safe recovery of animals is a true advantage. There is some evidence in humans that anesthetic agents have both neuroprotective and neurodegenerative effects (Schifilliti et al, 2010; Karmarkar et al, 2010) that could produce undesired confounding factors in neuroprotection research. Interestingly, in children, IOP measurements under ketamine are closer to the awake IOP than the IOP under gas anesthesia (Blumberg et al, 2007); this is the opposite of the rodent data. Both forms of anesthesia are associated with lower measured IOP than in awake, restrained mice (Johnson et al, 2008). Higher IOP level during an experiment would tend to increase the ability to detect subtle differences in IOP between eyes of an animal, and therefore would be desirable. Others have found that isoflurane is a suitable alternative to ketamine in mouse research (Woodward et al, 2007). Some investigators have found that

measurement of IOP in awake mice can be accomplished with patience and training, though our experience is that this is strain specific.

We found a significantly higher IOP in untreated C57BL/6 mice that were several months older (10 months) compared to younger (2 month old) C57BL/6 mice. With a smaller number of animals, we did not detect a similar age-related IOP difference in CD1 mice. In a survey of several mouse strains,Savinova et al. (2001) found lower IOP with age in some strains of mice, including C57BL/6; however, they did not test CD1 mice and their method of IOP measurement was different from ours. Since there may be a difference in measured IOP with age, it is likely that the age of animals should be taken into account in experimental glaucoma research. In our previous research, we found that older C57BL/6 mice could be paradoxically less susceptible to RGC loss than younger mice of the same strain. Insofar as mice can be considered appropriate models for human disease, it is probable that older animals should be included in work dealing with diseases that become more prevalent with age in humans.

Previous investigators have utilized microbead injections into the anterior chamber to cause experimental glaucoma in rodents, including Urcola et al (2006) and Chen et al (2011). While Chen et al report greater axonal loss in 12 mice with their method, the precise age of the animals was not given, and they gave 2 sequential injections, used larger beads, and had longer follow-up than the present report. Additionally, Chen et al report that 7% were euthanized early due to a corneal opacity or sign of inflammation, a rate higher than observed with our method. However, due to the substantial differences in technique, the two methods require further study to determine their relative merits.

In summary, we found that a newer 4+1 protocol for experimental glaucoma in mice was as simple to implement as our previous method and produced higher IOP and more RGC axon loss. There are many other possible variations of the protocol that could be attempted, so the present protocol may not be the ultimate or the ideal solution.

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References

Aihara M, Lindsey JD, Weinreb RN. Experimental mouse ocular hypertension: establishment of the model. Invest Ophthalmol Vis Sci. 2003a; 44:4314–4320. [PubMed: 14507875]

Aihara M, Lindsey JD, Weinreb RN. Ocular hypertension in mice with a targeted type 1 collagen mutation. Invest Opthlamol Vis Sci. 2003b; 44:1581–1585.

Blumberg D, Congdon N, Jampel H, Gilbert D, Elliott R, Rivers R, Munoz B, Quigley HA. The effects of sevoflurane and ketamine on intraocular pressure in children during examination under anesthesia. Am J Ophthalmol. 2007; 143:494–499. [PubMed: 17317393]

Chen H, Wei X, Cho KS, Chen G, Sappington R, Calkins DJ, Chen DF. Optic neuropathy due to microbead-induced elevated intraocular pressure in the mouse. Invest Ophthalmol Vis Sci. 2011; 52:36–44. [PubMed: 20702815]

Cone FE, Gelman SE, Son JL, Pease ME, Quigley HA. Differential susceptibility to experimental glaucoma among 3 mouse strains using bead and viscoelastic injection. Exp Eye Res. 2010; 91:415–424. [PubMed: 20599961]

Danias J, Kontiola A, Filippopoulos T, Mittag T. Method for the noninvasive measurement of intraocular pressure in mice. Invest Ophthalmol Vis Sci. 2003; 44:1138–1141. [PubMed: 12601041]

Ding C, Wang P, Tian N. Effect of general anesthetics on IOP in elevated IOP mouse model. Exp Eye Res. 2011 E-pub ahead of print.

Fijukawa K, Iwata T, Inoue K, Akahori M, Kadotani H, Fukaya M, Watanabe M, Chang Q, Barnett EM, Swat W. VAV2 and VAV3 as candidate disease genes for spontaneous glaucoma in mice and humans. PLoS ONE. 2009; 5:1–11.

Fu CT, Sretavan D. Laser-induced ocular hypertension in albino CD-1 mice. Invest Ophthalmol Vis Sci. 2010; 51:980–990. [PubMed: 19815738]

Gross RL, Ji J, Chang P, Pennesi ME, Yang Z, Zhang J, Wu SM. A mouse model of elevated intraocular pressure: retina and optic nerve findings. Trans Am Ophthalmol Soc. 2003; 101:163–169. [PubMed: 14971574]

Grozdanic SD, Betts DM, Sakaguchi DS, Allbaugh RA, Kwon YH, Kardon RH. Laser-induced mouse model of chronic ocular hypertension. Invest Ophthalmol Vis Sci. 2003; 44:4337–4346. [PubMed: 14507878]

Harada C, Namekata K, Guo X, Yoshida H, Mitamura Y, Matsumoto Y, Tanaka K, Ichijo H, Harada T. ASK1 deficiency attenuates neural cell death in GLAST-deficient mice, a model of normal tension glaucoma. Cell Death Differ. 2010; 17:1751–1759. [PubMed: 20489729]

Jia L, Cepurna W, Johnson E, Morrison JC. Effect of General Anesthetics on IOP in Rats with Experimental Aqueous Outflow Obstruction. Invest Ophthalmol Vis Sci. 2000; 41:3415–3419. [PubMed: 11006233]

John SW, Smith RS, Savinova OV, Hawes NL, Change B, Turnball D, Davisson M, Roderick TH, Heckenlively JR. Essential iris atrophy pigment dispersion, and glaucoma in DBA/2J mice. Invest Opthalmol Vis Sci. 1998; 39:951–962.

Johnson TV, Fan S, Toris CB. Rebound tonometry in conscious, conditioned mice avoids the acute and profound effects of anesthesia on intraocular pressure. J Ocul Pharm Ther. 2008; 28:175–185.

Karmarkar SW, Bottum KM, Tischkau SA. Considerations for the use of anesthetics in neurotoxicity studies. Comp Med. 2010; 60:256–262. [PubMed: 20819374]

Libby RT, Li Y, Savinova OV, Barter J, Smith RS, Nickells RW, John SW. Susceptibility to neurodegeneration in a glaucoma is modified by Bax gene dosage. PLoS Genet. 2005; 1:17–26. [PubMed: 16103918]

Levkovitch_Verbin H, Quigley HA, Martin KR, Valenta D, Baumrind LA, Pease ME. Translimbal laser photocoagulation to the trabecular meshwork as a model of glaucoma in rats. Invest Ophthalmol Vis Sci. 2002; 43:402–410. [PubMed: 11818384]

Marina N, Bull ND, Martin KR. A semi automated targeted sampling method to asses optic nerve axonal loss in a rat model of glaucoma. Nat Protoco. 2010; 5:1642–1651.

McKinnon SJ, Schlamp CL, Nickells RW. Mouse models of retinal ganglion cell death and glaucoma. Exp Eye Res. 2009; 88:816–824. [PubMed: 19105954]

Morris CA, Crowston JG, Lindsey JD, Danias J, Weinreb RN. Comparison of invasive and non-invasive tonometry in the mouse. Exp Eye Res. 2006; 82:1094–1099. [PubMed: 16466714]

Nakazawa T, Nakazawa C, Matsubara A, Noda K, Hisatomi T, She H, Michaud N, Hafezi-Moghadam A, Miller JW, Benowitz LI. Tumor necrosis factor-alpha mediates oligodendrocyte death and delayed retinal ganglion cell loss in a mouse model of glaucoma. J Neurosci. 2006; 26:12633–12641. [PubMed: 17151265]

Nissirios N, Goldblum D, Rohrer K, Mittag T, Danias J. Noninvasive determination of intraocular pressure (IOP) in nonsedated mice of 5 different inbred strains. J Glaucoma. 2007; 16:57–61. [PubMed: 17224751]

Pease ME, Cone FE, Gelman SE, Son JL, Quigley HA. Calibration of the TonoLab tonometer in mice with spontaneous or experimental glaucoma. Invest Ophthalmol Vis Sci. 2011; 52:858–864. [PubMed: 20720229]

Pease ME, Hammon JC, Quigley HA. Monometric calibration and comparison of TonoLab and TonoPen tonometers in rats with experimental glaucoma and in normal mice. J Glaucoma. 2006; 15:512–519. [PubMed: 17106364]

Quigley HA, Broman A. The number of persons with glaucoma worldwide in 2010 and 2020. J Ophthalmol. 2006; 90:151–156.

Ruiz-Ederra J, Verkman AS. Mouse model of sustained elevation in intraocular pressure produced by episcleral vein occlusion. Exp Eye Res. 2006; 82:879–884. [PubMed: 16310189]

Saeki T, Aihara M, Ohashi M, Araie M. The efficacy of TonoLab in detecting physiological and pharmacological changes of mouse intraocular pressure- comparison with TonoPen and microneedle manometery. Curr Eye Res. 2008; 33:247–252. [PubMed: 18350435]

Sappington RM, Carlson BJ, Crish SD, Calkins D. The microbead occlusion model: A paradigm for induced ocular hypertension in rats and mice. Invest Ophthalmol Vis Sci. 2010; 51:207–216. [PubMed: 19850836]

Savinova OV, Sugiyama F, Martin JE, Tomarev SI, Paigen BJ, Smith RS, John SWM. Intraocular pressure in genetically distinct mice: an update and strain survey. BMC Genetics. 2001; 2:12. [PubMed: 11532192]

Schifilliti D, Grasso G, Conti A, Fodale V. Anaesthetic-related neuroprotection: intravenous or inhalational agents? CNS Drugs. 2010; 24:893–907. [PubMed: 20932063]

Schulz H, Johner C, Eder G, Ziesenis A, Reitmeier P, Heyder J, Balling R. Respiratory mechanics in mice: strain and sex specific differences. Acta Physiologica. 2002; 174:367–375.

Urcola JH, Hernandez M, Vecino E. Three experimental glaucoma models in rats: Comparison of the effects of intraocular pressure elevation on retina ganglion cell size and death. Exp Eye Res. 2006; 83:429–437. [PubMed: 16682027]

Wang WH, Millar JC, Pang IH, Wax MB, Clark AF. Noninvasive measurement of rodent intraocular pressure with rebound tonometer. Invest Opthamlmol Vis Sci. 2005; 46:4617–4621.

Woodward WR, Choi D, Grose J, Malmin B, Hurst S, Pang J, Weleber RG, Pillers DA. Isoflurane is an effective alternative to ketamine/xylazine/acepromazine as an anesthetic agent for the mouse electroretinogram. Doc Opthalmol. 2007; 115:187–201.

Zhou Y, Grinchuk O, Tomarev SI. Transgenice mice expressing the Tyr437His mutant of human myocilin protein develop glaucoma. Invest Opthalmol Vis Sci. 2009; 49:1932–1939.

Highlights

- In this improved mouse model, a higher intraocular pressure (IOP) elevation was produced.
- Along with a greater retinal ganglion cell injury in two strains, CD1 and C57BL/6.
- Intraperitoneal anesthesia causes a lower IOP than isoflurane gas, in control eyes of both strains.
- Young C57BL/6 mice present a significantly lower IOP in control eyes than their older counterparts.

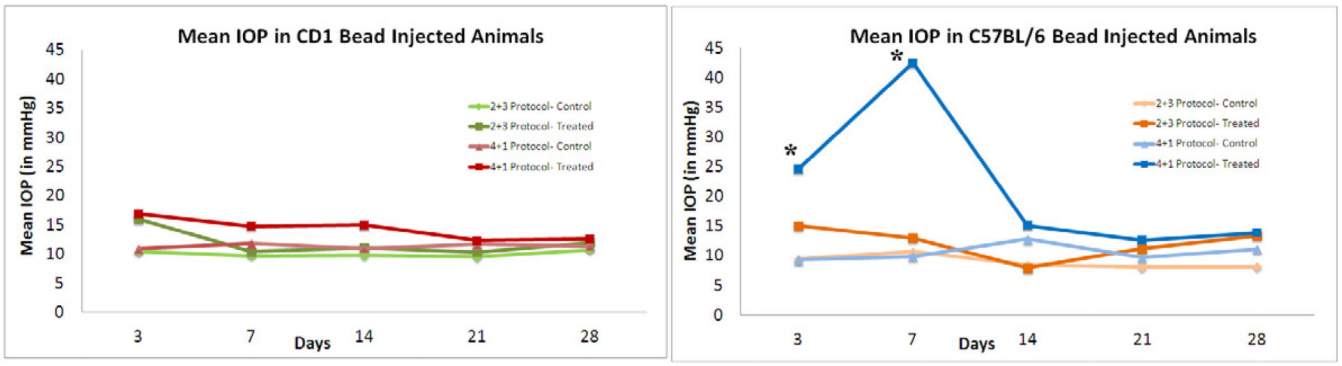

Figure 1.

Mean IOP readings of the right, untreated eye, and left, bead and Healon injected eye, of two protocols, 2+3 and 4+1 protocols, in two strains, CD1 and C57BL/6 mice. * IOP differences between 2+3 protocol and 4+1 protocol were found to be statistical significant by $p \leq 0.001$, t-test. Data presented as mean and standard error.

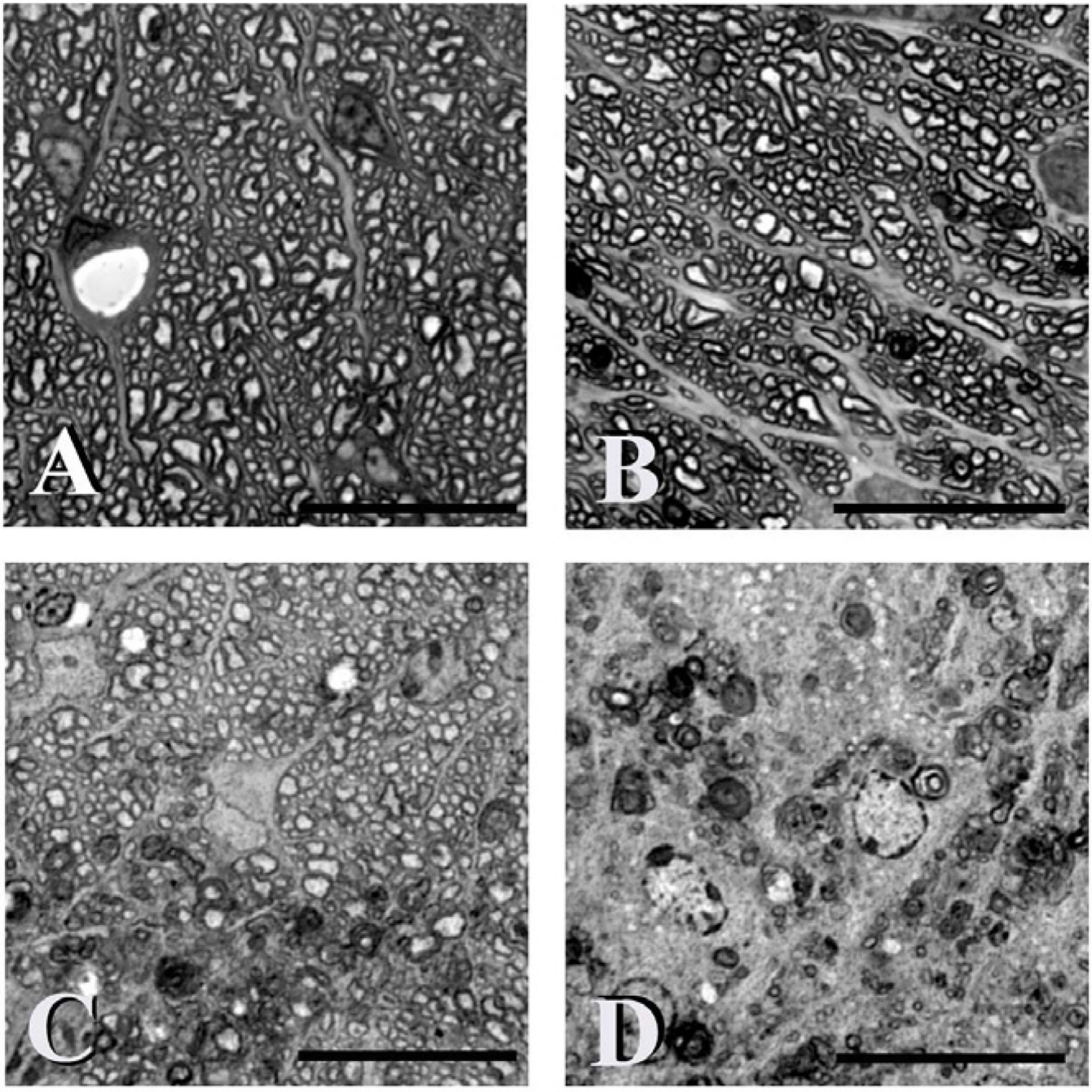

Figure 2. Optic nerve cross-sections from CD1 mice

A) normal untreated nerve, B) a nerve from 2+3 protocol with mild damage, C) a moderately damaged nerve from 3+2 protocol, and D) nerve from 4+1 protocol which shows severe axon loss (scale bar = 30μm).

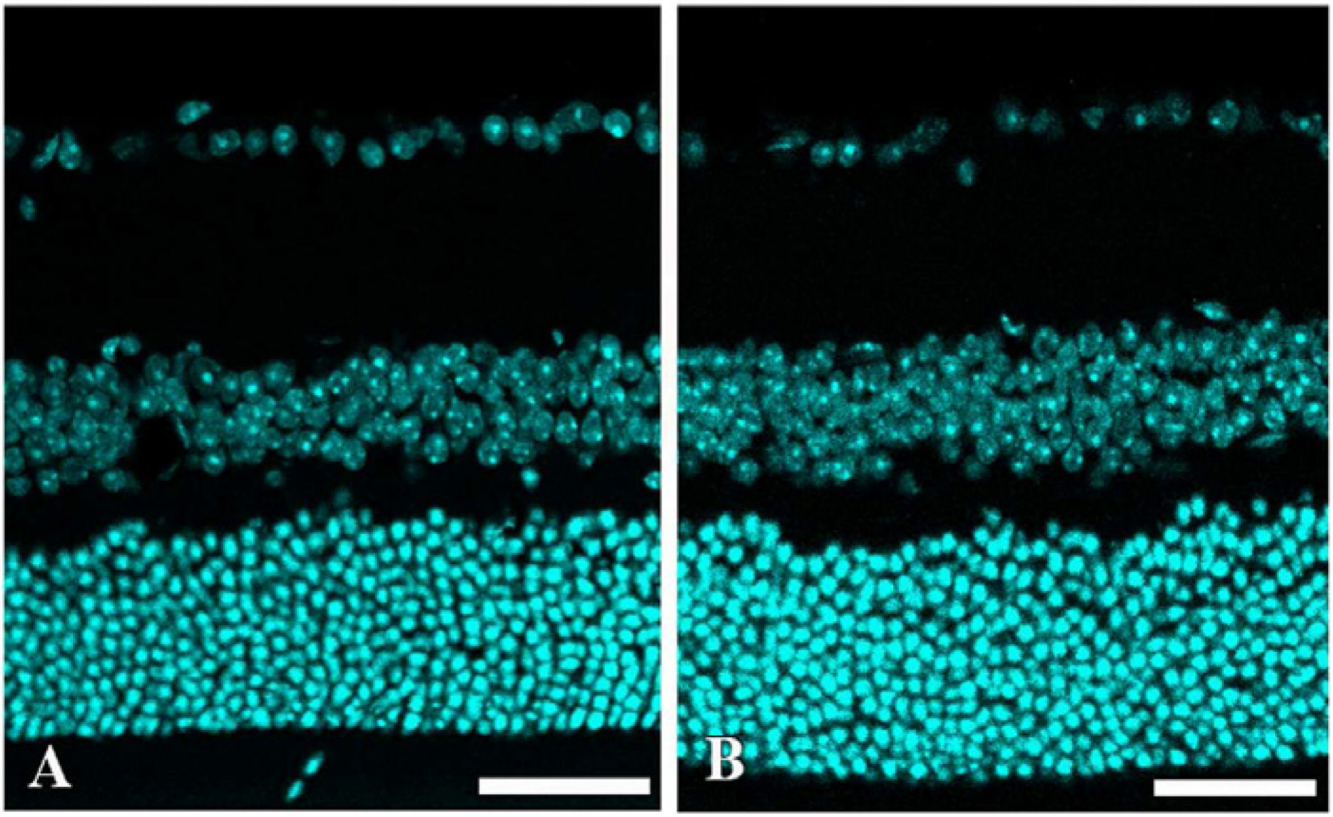

Figure 3. Retinal cross-sections from C57BL/6 4+1 mice

A) Retinal cross-section from an untreated eye. B) Retinal cross-section from a C57BL/6 4+1 protocol treated eye. No loss of mid-retinal or outer retinal layers is seen in the glaucoma retina (scale bar = 50μm).

Table 1

IOP Difference between Intraperitoneal and Gas Anesthesia.

CD1	**IOP Difference between 2 I.P. anesthesias**	**I.P.-- I.P. difference p value**	**IOP difference between I.P. and gas anesthesia**	**I.P.-- gas difference p value**	**I.P.-I.P vs I.P.- gas difference p value**
Mean ± SD	−0.3 ± 3.0 mm Hg	0.54	−2.2 ± 3.5 mm Hg	**<0.0001**	**<0.001**
Median	−0.5		−3		
Number	36		82		
C57BL/6	**IOP Difference between 2 I.P. anesthesias**	**I.P.- I.P. difference p value**	**IOP difference between I.P. and gas anesthesia**	**I.P.- gas difference p value**	**I.P.-I.P vs I.P.- gas difference p value**
Mean ± SD	−1.1 ± 4.5 mm Hg	**0.02**	−2.2 ± 4.3 mm Hg	**0.003**	0.11
Median	0		−2		
Number	122		41		

Table 2

Age and Strain Effect on IOP.

CD1	Baseline IOP, (mmHg) in young mice (at 2 months)	Baseline IOP (mmHg) in older mice (at 10 months)	P value
Mean ± SD	10.3 ± 3.3	10.1 ± 1.3	0.49
Median	9	10	
Number	129	9	
C57BL/6	**Baseline IOP (mmHg) in young mice (at 2 months)**	**Baseline IOP (mmHg) in older mice (at 8 months)**	**P value**
Mean ± SD	10.4 ± 2.9	12.4 ± 4.2	**0.001**
Median	10	12	
Number	168	51	

Table 3

Grouped IOP Data after Bead + Heal on Injection.

				IOP Difference (mmHg)			p-value			
Strain, Protocol	**N**	**RE IOP (mmHg) Mean ± SD**	**LE IOP (mmHg) Mean ± SD**	**Mean ± SD**	**Median**	**Positive Integral**	**2+3 vs. 3+2 Protocol**	**2+3 vs. 4+1 Protocol**	**3+2 vs. 4+1 Protocol**	**CD1 vs. C57BL/6**
2+3 protocol	35	9.8 ± 1.0	12.5 + 3.1	**2.6 + 3.3***	1.9	104.0 + 104.5	0.67	**<0.0001**	**<0.01**	
3+2 protocol	9	10.7 + 1.2	12.9 + 2.8	**2.2 + 3.1†**	1.2	74.1 + 93.0				
4+1 protocol	77	13.0 + 1.7	18.6 + 5.1	**6.2 + 4.3***	5.0	212.0 + 171.9				
CD1	85	12.2 ± 2.0	15.7 ± 4.9	**4.1 ± 3.8***	4.0	151.3 ± 159.0				**<0.01**
C57BL/6	36	11.2 ± 2.4	18.0 ± 6.1	**6.7 ± 5.0***	5.6	229.0 ± 159.0				

Table 4

Group IOP Data after Bead + Healon Injection, 2+3 and 3+2 protocols combined.

Strain, Protocol	N	RE IOP (mmHg) Mean ± SD	LE IOP (mmHg) Mean ± SD	IOP Difference (mmHg)		Positive Integral	p- value	
				Mean ± SD	Median		Control vs. Bead Injected	4+1 vs. 2+3/3+2 Protocol
2+3/3+2 protocol	44	10.0 ± 1.2	12.5 ± 3.1	2.6 ± 3.3	2	96.1 ± 101.1	**<0.0001**	**0.001**
4+1 protocol	73	13.0 ± 1.8	18.3 ± 5.2	6.1 ± 4.3	5	205.8 ± 177.1	**<0.0001**	

Table 5

All IOP Data after Bead + Healon Injection.

Strain, Protocol	N	Weeks Followed	Control IOP (mmHg) Mean ± SD	Treated IOP (mmHg) Mean ± SD	IOP Difference (mmHg)		Positive Integral	Peak IOP Mean ± SD (mmHg)
					Mean ± SD	Median		
CD1, 2+3	21	6	10.3 ± 1.8	11.8 ± 2.0	**1.5 ± 2.5***	1.5	67 ± 65	20 ± 6
CD1, 3+2	9	6	10.4 ± 0.9	12.9 ± 2.7	**2.4 ± 2.7***	2.0	74 ± 93	19 ± 7
CD1, 4+1	10	5	10.5 ± 0.8	13.9 ± 3.9	**3.4 ± 3.7***	3.5	93 ± 114	24 ± 9
CD1, 4+1 & Buffer	46	6	13.5 ± 1.4	21.6 ± 4.9	**8.1 ± 4.5‡**	7.7	197 ± 176	36 ± 10
C57BL/6, 2+3	15	6	9.8 ± 1.1	15.3 ± 4.6	**5.5 ± 4.2‡**	5.0	196 ± 162	22 ± 11
C57BL/6, 4+1	10	6	10.2 ± 0.8	18.5 ± 3.2	**8.3 ± 3.3‡**	7.5	315 ± 116	45 ± 16
C57BL/6 YFP, 4+1	8	3	13.7 ± 1.1	24.6 ± 3.0	**10.9 ± 3.3‡**	11.4	266 ± 111	40 ± 5
C57BL/6 YFP, 4+1	7	6	14.4 ± 0.7	22.7 ± 4.5	**8.3 ± 4.4‡**	6.5	329 ± 198	35 ± 7

Table 6

IOP Elevation in Bead + Healon Injected Animals.

		Number of IOP Elevations for respective days							
Strain, Protocol	**N**	**3**	**7**	**14**	**21**	**28**	**35**	**42**	**3+7+14***
Group 2+3	35	53%	22%	8%	17%	31%	19%	36%	28%
Mean ± SD (mmHg)		15.3 ± 5.6	11.8 ± 4.5	10.1 ± 3.7	10.9 ± 4.0	12.2 ± 8.9	12.7 ± 7.0	14.0 ± 5.9	12.4 ± 5.1
Group 3+2	9	56%	11%	22%	22%	22%	56%	-	30%
Mean ± SD (mmHg)		16.3 ± 7.8	12.2 ± 5.4	12.1 ± 3.3	10.8 ± 3.0	12.8 ± 2.9	13.4 ± 3.7		13.6 ± 5.9
Group 4+1	81	72%	73%	43%	13%	12%	11%	9%	63%
Mean ± SD (mmHg)		22.3 ± 8.3	23.9 ± 12.3	17.3 ± 7.9	14.3 ± 5.0	15.7 ± 6.8	14.5 ± 6.8	12.5 ± 5.3	21.1 ± 10.1
p-value 2+3 vs 3+2		0.88	0.47	0.25	0.7	0.63	**<0.05**	**<0.05**	0.85
p-value 2+3 vs 4+1		**<0.05**	**<0.0001**	**<0.001**	0.66	**<0.05**	0.23	**<0.001**	**<0.0001**
p-value 3+2 vs 4+1		0.32	**<0.001**	0.22	0.48	0.41	**<0.001**	0.36	**<0.01**
Group CD1	85	62%	45%	30%	8%	12%	9%	11%	46%
Group C57	40	73%	73%	35%	30%	33%	30%	25%	60%
p-value		0.23	**<0.05**	0.59	**<0.001**	**<0.001**	**<0.001**	**<0.01**	**<0.05**

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Table 7

Axial Length in Bead + Healon Injected Animals.

Strain, Protocol	N	Control	Treated	% change	p-value for treatment effect
2+3 and 3+2 protocol	44	3.43 ± .15	3.70 ± .19	7.12%	**<0.0001**
4+1 protocol	73	3.49 ± .15	3.81 ± .29	7.86%	**<0.0001**
CD1	85	3.50 ± .15	3.82 ± .22	8.03%	**<0.0001**
C57BL/6	32	3.38 ± .13	3.64 ± .33	6.50%	**0.0002**

Table 8

Axon Loss after Bead + Healon Injection, ANOVA models.

Strain, Protocol	N	Mean ± SD	Median	p-value beads vs pooled control	p-value 4+1 vs 3+2/2+3	p-value CD1 vs C57
2+3/3+2 protocol	44	15 ± 24%	8%	**0.0013**	**0.0026**	
4+1 protocol	73	36 ± 34%	27%	**<0.0001**		
CD1	85	33 ± 35%	21%	**<0.0001**		**0.047**
C57BL/6	32	19 ± 23%	13%	**0.0004**		

Table 9

Axon Loss after Bead + Healon Injection, by Group.

					p value
Strain, Protocol	**N**	**Weeks Followed**	**Mean ± SD**	**Median**	**Left - right**
CD1, 2+3	10	6	7 ± 13%	6%	**0.02**
CD1, 3+2	9	6	23 ± 30%	7%	< **0.001**
CD1, 4+1	9	5	32 ± 33%	50%	< **0.001**
CD1, 4+1 & Buffer	41	6	42 ± 36%	35%	< **0.001**
Strain, Protocol	**N**	**Weeks Followed**	**Mean ± SD**	**Median**	**Left - right**
C57BL/6, 2+3	14	6	16 ± 25%	9%	**0.04**
C57BL/6, 4+1	6	6	10 ± 12%	10%	0.056
C57BL/6 YFP, 4+1	8	3	19 ± 11%	19%	**0.003**
C57BL/6 YFP, 4+1	7	6	32 ± 23%	25%	< **0.001**