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# Intraocular pressure measurements in a rat glaucoma model

Michael Doyle
*Western Michigan University*, michael.p.doyle@wmich.edu

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Michael Doyle

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## Abstract

The main objective of this study was to determine if consistent intraocular pressure (IOP) measurements could be obtained from an *in vivo* Long Evans rat glaucoma model. An increase in IOP is the primary risk factor associated with glaucoma and reliable IOP measurements would validate our *in vivo* glaucoma model that we are inducing in rats. The study used nine Long Evans rats. Three rats severed as controls, three received surgery to induce glaucoma and three were administered PNU-282987 neuroprotective eye drops and then surgery to induce glaucoma. IOP measurements were obtained by tapping the retina of the rat eye through use of a Tono-Lab. Theoretically, IOP should have increased in six of the nine rats, since the surgery created scar tissue and decreased the efficiency of the aqueous drainage system of the eye. The results of this study were somewhat inconclusive in obtaining reliable IOP measurements but are promising. Significant differences were found between the experimental groups and internal controls as well as the control group. In order to claim significantly reliable results, there should not be statistical significance in the control group. This study recommends that future techniques used to obtain consistent IOP measurements should include, longer training periods, increase the number of IOP measurements taken at one time and disregard the first few readings until consistency is observed. This overall significance of this study reveals how future experiments may obtain reliable IOP measurements to validate the surgical procedure of inducing glaucoma.

# Introduction

Glaucoma is known as a neurodegenerative disorder that has prolonged destruction on both the retinal ganglion cells (RGCs) and axons in optic nerve fibers. Glaucoma is the number one cause of permanent blindness throughout the world and is the second overall leading cause of blindness in general, affecting on average 60.5 million people on the planet (Nucci et al. 2013). The actual cause of glaucoma is currently unknown but clinical studies have shown that the primary risk factor for glaucoma is an increase in intraocular pressure (Vickers et al., 1995; Brooks et al., 1997; Dkhissi et al., 1999; Dong et al., 2008; Seki et al., 2010; Guerin et al., 2011). The main objective of this study is to determine if reliable IOP measurements can be obtained through an *in vivo* Long Evans rat glaucoma model.

The approaches used to currently treat glaucoma involve a reduction of IOP via medical, laser or surgical technique (Nucci et al. 2013). However, reducing the overall pressure is frequently insufficient to inhibit the progression of the disease and visual reduction (Vickers et al., 1995; Brooks et al., 1997; Dkhissi et al., 1999; Dong et al., 2008; Seki et al., 2010; Guerin et al., 2011). Many patients still exhibit signs and symptoms of the disease after their IOP is lowered to within normal therapeutic ranges. Additionally, in normal tension glaucoma, the onset of the disease is seen to progress in a significant number of patients who exhibit regular untreated IOP levels (Nucci et al. 2013).

IOP is due to a decrease in outflow through the trabecular meshwork and the uveoscleral blood vessels of the aqueous drainage system. As IOP builds up in the anterior part of the eye, it puts pressure on the lens. This consequently puts pressure on the vitreous humour in the poster chamber of the eye, which puts pressure on the retina. This destroys some neurons containing glutamate to produce an over-abundance of glutamate in the retina. Over stimulation of the

amino acid glutamate receptor leads to neuronal cell death, also known as excitotoxicity (Olney and de Gubareff, 1978; Ankarcrona et al., 1995). Large amounts of glutamate within the eye have been shown to induce apoptosis (cellular death) of the RGCs. This is due to an over extended influx of calcium ions through appropriate receptor channels that triggers apoptotic pathways to induce cell death. As the axons from the RGC sends visual information to the brain, if this information is halted due to cell death, blindness occurs (Vickers et al., 1995; Brooks et al., 1997; Dkhissi et al., 1999; Dong et al., 2008; Seki et al., 2010; Guerin et al., 2011).

RGCs contain axons that form the optic nerve. These axon fibers transmit visual stimuli to the brain from the retina (Wehrwein et al., 2004). The information that is transmitted through the optic nerve to the brain contains information of simple visual contrast, hue (main property of color), direction, orientation and motion. RGCs respond to light stimulation through complex pathways using neurotransmitters of glutamate, gamma-aminobutyric acid and glycine, including ion channels of sodium, potassium, calcium and chloride. RGCs contain more types of ion channels than have been found in any other class of retinal neuron (Ishida, 1995).

Previous studies conducted in this laboratory have shown that acetylcholine acts as a neuroprotective agent against excitotoxicity in RGCs (Wehrwein et al., 2004; Thompson et al., 2006; Asomugha et al., 2010; Brandt et al., 2011; Iwamoto et al., 2013). The effect of acetylcholine is utilized through nicotinic acetylcholine receptors and initiates several signal transduction pathways to increase cell survival (Asomugha et al., 2010). Recent results from this laboratory have indicated that eye drop application of 10mM PNU-282987, an α7 nicotinic acetylcholine receptor agonist, on Long Evans rats can prevent the loss of RGCs associated with the progression of glaucoma. As previously stated, decreasing the IOP and maintaining it at a therapeutic level does not always prevent the destruction of RGCs as the disease progresses.

Therefore, this is significant to potential treatment approaches to glaucoma in the future, which would not solely concentrate on reducing IOP.

The chemical compound of PNU-282987 is known as a potent agonist, which binds to the α7 nicotinic acetylcholine receptor (Bodnar et al. 2005). Pfizer developed the chemical using robotics and automated systems and the term PNU was created as an acronym by Pharmacia and Upjohn. PNU-282987 reduces the loss of RGCs that are normally seen in an *in vivo* rat glaucoma model. PNU-282987 directly affects the RGCs in the posterior part of the eye and has not interfered with IOP. Furthermore, the chemical is physiologically a specific agonist toward α7 nicotinic acetylcholine receptors and would theoretically exhibit less potential side effects on the body. Broad general agonists can interfere with many subtypes of receptors (Iwamoto et al., 2013).

IOP measurements will be taken for the duration of this study, before and after inducing glaucoma, to determine if consistent IOP measurements can be obtained and to determine if the IOP measurements change significantly after surgery to induce glaucoma. Before inducing glaucoma, rats received the α7 specific agonist to determine if IOP varied with or without a progression of RGCs. In order to induce glaucoma in the eyes of adult Long Evans rats, hypertonic saline was injected into the episcleral veins of the retina. This creates scar tissue, decreases aqueous humor drainage, increases the IOP in the anterior chamber of the eye and mimics the effects of actual glaucoma. Previous results from this laboratory show that a significant amount of RGCs are lost in the retinal periphery four weeks after the injection of hypertonic saline (Iwamoto et al., 2013). However, this loss of RGCs is eliminated if retinas are treated with PNU-282987 before surgery designed to induce glaucoma. Because of these previous results, the hypothesis to be tested in this study is that surgery to induce glaucoma

should lead to an increase in IOP compared to control untreated conditions. These studies were conducted to determine if IOP measurements could be obtained to verify or refute this hypothesis.

## Materials and Methods

### *Recordings*

Before the surgery, all nine rats were handled for four weeks before surgery and then for 4 weeks after surgery by taking measurements of IOP in both the right and left eye 4 times each week. The readings were recorded with use of a Tono-Lab XL (Mentor, Norwell MA). A Tono-Lab contains a transducer that measures the tension and pressure produced by the cornea. The rats were slowly desensitized over the weeks to get use to the procedure by being brought out of their cage and allowed to explore on a lab bench at their will, before a measurement was attempted. To obtain an IOP measurement, the Tono-Lab is held parallel to the lab bench and positioned close to tap the cornea. The researcher calmly keeps the rat stationary on the lab bench while taking a pressure measure with the Tono-Lab. Once a successful reading resulted, it was recorded, the rat was given a Cheerio and the standard deviation was calculated.

### *Animal and experiment preparation for inducing glaucoma*

Nine adult Long Evans rats (Charles River Labs, Portage, MI) were used for the duration of this study. The rats were all female and between the ages of three to six months old. The animals were kept in Western Michigan University's animal research facility. Three rats were used as controls, three received PNU neuroprotective eye drops and glaucoma surgery and the other three received the surgery with no neuroprotective eye drops. The three rats receiving the

neuroprotective eye drops were administered 10 mM PNU-282987 eye drops two times each day (30 µl) for three days before the surgery. The eye drops were given at least four hours between each application and the rats received a Cheerio after each treatment of eyedrops. The six rats that received the surgical procedure were weighed using a standard scale.

Pulled glass electrodes were used as a glass needle for insertion of 50 µl of 2M NaCl into the rat's episcleral veins. Glass electrodes were pulled using a Warner Instrument 2-phase patch pipette puller and bevelled. Once the glass electrodes were pulled, they were attached to a 1 cc syringe needle through use of tapered polyethylene tubing (PE-50, Clay Adams, Parsippan, NJ) that was backfilled with the 2M NaCl solution before injection into the episcleral veins.

*Surgical procedure of inducing glaucoma*

The aseptic setup of the procedure was first laid with lab bench paper consisting of the plastic side down and the absorbance side up. The animals were then anesthetized with KAX (ketamine, xylazine HCl, acepromazine and sterile water) (0.1ml/100g) through an intraperitoneal injection into the rat's belly. Before the start of the surgery, the rats were anesthetized until no reflexes were observed. One drop of Alcaine (proparacaine hydrochloride 0.5%) was added to the right eye after testing reflexes and the rat's whiskers were cut. A hemostat was used to clamp the tissue around the eye, which slightly bulged the right eye up, revealing more of the rat's episcleral vein. 50 µl of 2M sterile hypertonic saline was then injected into the vein in both the superior and inferior quadrant of the eye after piercing through the conjunctiva and injected into the radial aqueous vein of the rat's superior and inferior right eye under a light microscope. After the completion of the surgery, triple antibiotic was applied

around the right eye. The rats were fully anesthetized for the entire duration of the surgery and then closely watched for full recovery before being returned to WMU's animal facility.

*Drugs and solutions for inducing glaucoma*

Alcaine (proparacaine HCl 0.5%) and 2 M sodium chloride solution was purchased from Sigma. KAX is a combination cocktail consisting of 5 ml ketamine (100 mg/ml), 2.5 ml xylazine (20 mg/ml), 1 ml acepromazine (10 mg /ml), and 0.5 ml sterile water. A 1 cc (1ml) heparin syringe was used to extract 1ml of acepromazine and a 10 ml syringe was used to extract 5 ml of ketamine. A scale was tared and 50 mg of xylazine was measured out in a weigh boat. The ingredients were mixed and sterile filtered into an autoclaved vial. This was done by using a 22 micron paper filter in a Sterilgard Hood, while wearing gloves. The α7 nAChR specific agonist, PNU-282987, was purchased from Sigma.

*Animal and experiment preparation for harvesting retinas*

One month following surgery to induce glaucoma, the animals were sacrificed and the retinas were removed for quantification of RGCs. The euthanize chamber was lined with a paper towel. Each rat was individually placed in the chamber and was euthanized with carbon dioxide for three minutes until dead. The euthanized rats were tested for any respiratory activity or reflexes by pulling on the animal's feet. The euthanized rat was then covered with paper towel and transported from the animal research center to Dr. Linn's laboratory.

The surgical setup in the lab was laid out with lab bench paper arranged under a dissecting microscope with fiber optic illumination. The station was prepared with a pair of

small scissors, large scissors, a scalpel with scalpel blades, appropriate labeled sylgard dishes for placement of both right and left retinas and phosphate buffered saline (PBS).

*Surgical procedure for harvesting retinas*

To remove the retinas from euthanized rats, the connective tissue around both the control (left eye) and experimental eye (right eye) was carefully cut without puncturing the eyeball. The optic nerve was severed and the eyeball was then removed from the eye socket and placed into a dish with PBS solution. The cornea, lens, and vitreous humour were then removed from each eye to expose the retina lying in the back of eye. The retina was carefully peeled away from the remaining sclera surrounding the eye and pinned down flat with the RGC layer facing up, in a labeled sylgard dish using cactus needles. The retinas were fixed using a 4% paraformaldehyde solution for 12 hours at 4 degrees Celsius, washed with PBS three times, incubated in goat serum for one half hour and then again incubated with monoclonal antibody against a glycoprotein that is only found in the plasma membrane of RGCs in the retina (Swadzba et al., 2012). The Thy 1.1 antibody (mouse anti-rat) was applied at a dilution of 1:300 in 0.02% saponin in PBS for one week at 4 degrees Celsius. One week later, the retinas were rinsed again 3 times using PBS and incubated in a fluorescent secondary antibody (Alexa Fluor 595, goat anti-mouse, dilution 1:300) 4 degrees Celsius. After the third day in the secondary antibody, the retinas were rinsed and mounted on glass slides in 50% PBS and 50% glycerol and viewed under a Zeiss confocal microscope.

*Statistical analysis of data*

In order to declare the data between the right and left eyes of the rats to be significant, t-tests were calculated. These numbers were calculated using Microsoft Excel 2011. The right eye data points were selected for array 1 and the left eye data for array 2. 2 tail and type 3 was specifically chosen for each t-test calculated. Significance was indicated if the t-test was $P<0.05$.

## Results

*Average IOP measurements before surgery in control subjects*

Three rats served as a control group for the duration of this study. IOP measurements were recorded using a Tono-Lab and a new probe was used during each new set of readings. These measurements were recorded for duration of four weeks before surgery. The bar graphs in figure 1 represent the average IOP measurement obtained from the right eye and the left eye under control untreated conditions. As no procedure was conducted on either eye, this experiment was designed to determine if consistent IOP measurements could be obtained in the right and left eye. As demonstrated by the bar graphs below there was a significant difference obtained between right and left IOP measurements from untreated control retinas before surgery was performed to induce glaucoma. The right eye had an average IOP of 22.11, while the left eye had an average measurement of 18.93.

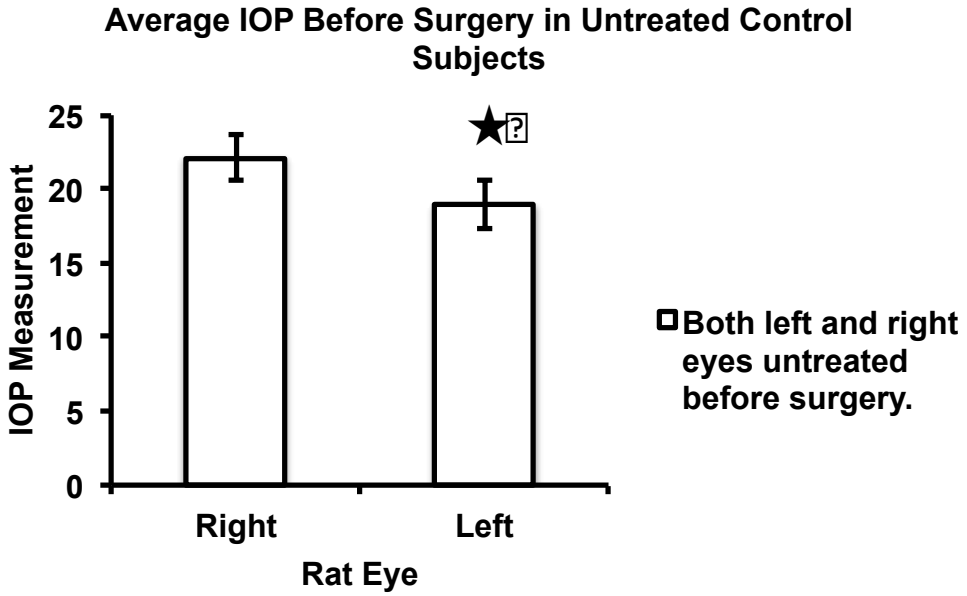


Figure 1: Average IOP measurements in the untreated control group. The star symbol indicates significant difference.

*Average IOP measurements after surgery*

In this part of the experiment, we expected there to be a significant increase in IOP measurement in the right eye after surgery. Three rats received the surgical procedure to induce glaucoma in the right eye. The left eye severed as an untreated *in vivo* control. The rats had their IOP measured for four weeks after the surgery that induced glaucoma in the right eye. The average IOP in the right eye was 18.59 and the left eye measurement was 17.49. The average IOP measurement in the right eye was significantly larger than the average IOP measurement in the left eye.

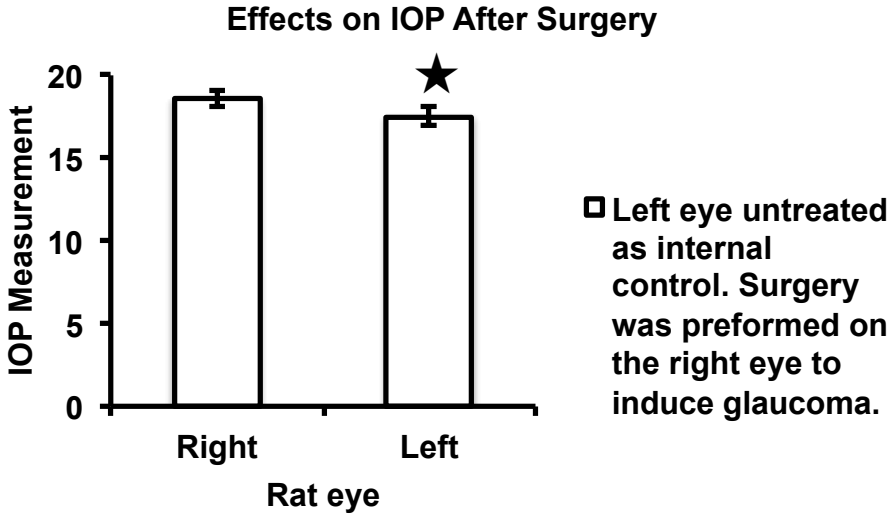


Figure 2: Average IOP measurements after surgery to induce glaucoma. The star symbol indicates significant difference.

*Average IOP after PNU treatment and surgery to induce glaucoma*

The right eye IOP measurements were expected to significantly increase compared to the left internal control eye. The PNU application was not expected to affect IOP since it acts on the retina and not the pressure in the anterior part of the eye. The other three remaining rats used in this study received both PNU-282987 neuroprotective eye drops and surgery to induce glaucoma in the right eye. The eye drops were administered twice a day, four hours apart for three days before the surgical procedure. The left eye served as an untreated internal control. These average IOP measurements were recorded for four weeks after the surgery to induce glaucoma. The average right eye IOP measurement was 18.63 and the left eye was 15.11. The average IOP measurement in the right eye was seen to be significantly larger than the average IOP measurement in the left eye.

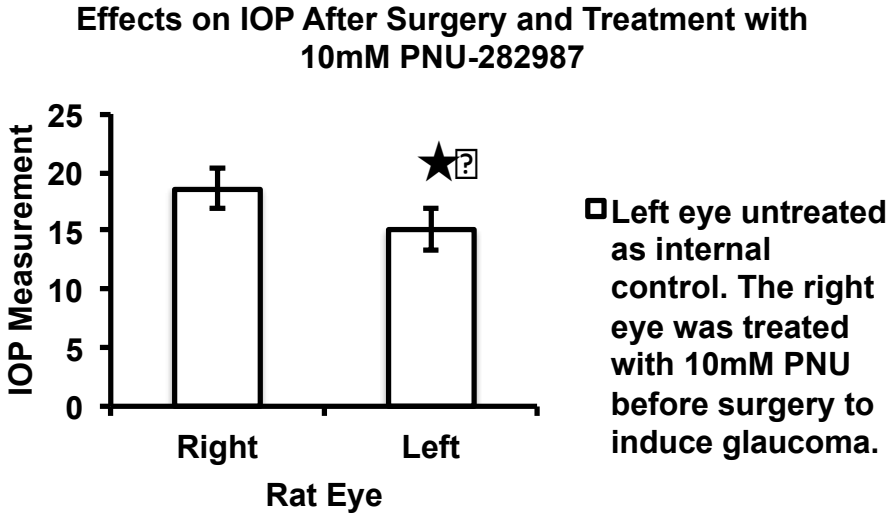


Figure 3: Average IOP after PNU application and surgery to induce glaucoma. The star symbol indicates significant difference.

*Differences between average IOP measurements taken amongst researcher*

The main objective of this next graph was to compare IOP measurements between two researchers. This was done to indicate if there is difference between results. The same animals were used, but the measurements were altered between researchers every other day from Monday through Thursday. Depending on who was taking the day's IOP measurements, technique and the amount of recordings could vary amongst researcher. The next figure summarizes the IOP data collected from the same animals from different researchers. Although there was some variation in average IOP measurements, the effects were not significant.

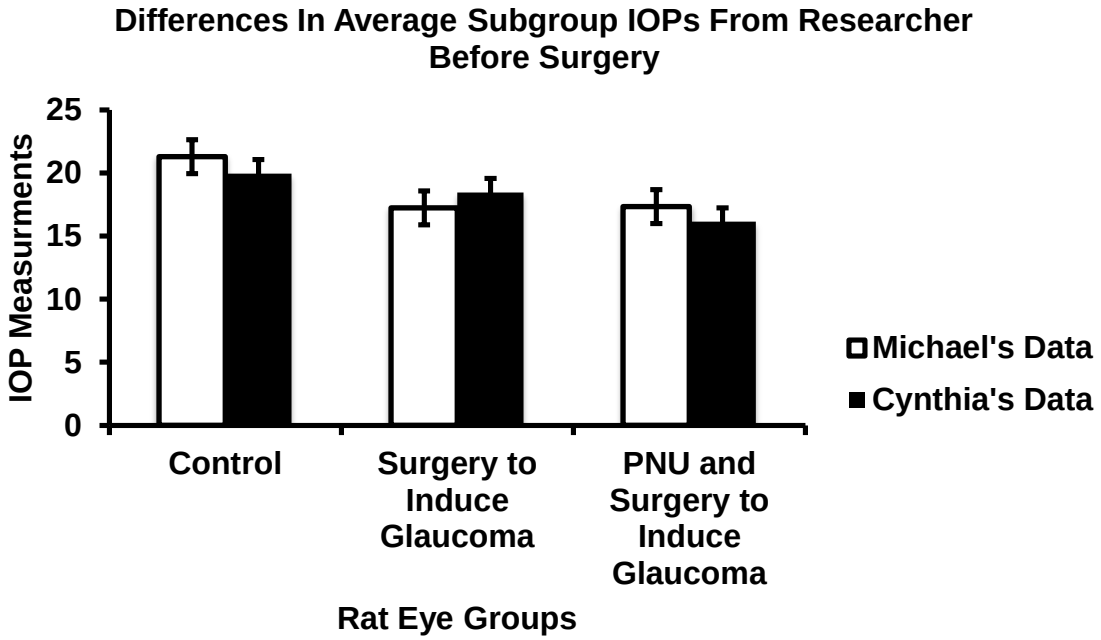


Figure 4: Average IOP measurements between Michael and Cynthia.

## Discussion

The results from this study were promising, but overall inconclusive in obtaining reliable IOP measurements through an *in vivo* Long Evans rat glaucoma model. The average IOP measurement in the control subject's right and left eye, respectively, was 22.11 and 18.93. Although the difference was found to be significant, the IOP measurements in control subjects should have been much more similar. Normal IOP measurements should be recorded between 13 and 18 mm Hg. These recordings obtained under the conditions described here did not routinely get measurements between 13 and 18. These IOP measurements may have been both higher and statistically significant since the rats were unfamiliar with the measurement technique. This graph's data was collected from two researchers and many more IOP measures were collected from the left eye than the right eye. This may have made the reason why the result was significant. Furthermore, these rats had never been out of their cage before the start of

this study and all successful readings from the Tono-Lab were recorded and then averaged in this graph.

Subjects that received surgery to induce glaucoma in the right eye had an average IOP of 18.6, one month after the surgery. The left eye of these subjects, which serves as an internal control, had an average IOP of 17.6. Lastly, rats that received 10mM PNU neuroprotective eye drops before the surgery to induce glaucoma had an average 15.11 left eye IOP and 18.63 in the right eye. Since these measurements were taken from rats that did receive surgery to induce glaucoma, all IOP measurements in these groups in the right eye should be higher than observed in the control measurements. Both of these groups had significant differences between the right and left eye as was expected.

Injecting hypertonic saline solution into the episcleral veins of the rat should have significantly increased the IOP in all right eyes of rats that received the surgery as demonstrated. Previous studies have shown that injection into the episcleral veins in rats increase scar tissue after surgery to decrease the aqueous drainage system and increase IOP (Morrison et al., 1997). However, although this change of IOP was found significant in this study, the control group also has significant difference between the right and left eye. Furthermore, the controls had overall higher IOP than the other two groups that received surgery to induce glaucoma.

In order to obtain the expected IOP results in the future, some alterations should be given consideration. Before the surgery, the rats should be fully desensitized to the IOP procedure. The rats we used in these studies were bred in the WMU animal facility and had never experienced being outside of their cages until the beginning of this study. In the future experiments using the Tono-Lab, it may help to habituate and adapt the animals for a minimum of 2 months to allow adequate time for the animals to get use to the researcher and being around

the Tonolab. During this 2-month time period IOP measurements should be attempted so that the animal becomes aware that the reading will not hurt or cause discomfort. Good behavior should be rewarded with a Cheerio similarly like this study.

As demonstrated in figure 4, the variation between researchers taking the IOP measurements were not significant. The slight difference in variation of measurement between the two researchers is likely due to subtle differences used when handling the rats, taking the measurements, difference in the smell or deepness of voice. However, the data suggests that future studies can utilize more than one researcher. Although, if more than one researcher is taking IOP measurements for one study, a uniform technique should be developed to achieve consistency when taking the readings.

Another option that would decrease the variability throughout the study should involve increasing both the amount of measurements and/or the number of days that measurements are taken. We found that it was typical for the rats to have higher IOP measurements at the beginning of the measurements each day and that the IOP measurements steadily decreased until they stabilized. Table 1 listed below suggests that a minimum of 10 readings should be taken until the IOP stabilize. This indicates that in the future, the first few IOP readings should be eliminated until it can be seen that the rats have calmed down. The averages created for this study utilized all data and only collected on average 5 readings in each eye. Table 1 suggests that the results of this study would look different if more measurements were collected until consistent.

| | IOP Measurements | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Rat 6, right eye (Surgery and PNU eye drops) | 28 | 18 | 16 | 13 | 14 | 15 | 16 | 16 | 16 | 16 |
| Rat 7, left eye (Surgery only) | 28 | 26 | 24 | 22 | 22 | 19 | 15 | 16 | 17 | 15 |

Table 1: Recent recordings that suggest IOP measurements stabilize after numerous attempts.

Lastly, to get more consistent IOP measurements, it is crucial that any researcher in future studies taps the transducer on the same spot of the cornea for each measurement. This is not an easy task, as the animal is fully awake and can move even if the animal has been habituated and is used to the procedure. The whiskers of all rats should remain trimmed. It was found that the Tono-Lab would gently brush the rat's whiskers and cause distress in the animal. Furthermore, an anesthetic eye drop may be applied in future studies to reduce the possibility of the rat's discomfort. Lastly, future researchers should alternate days between taking IOP measurements on the right and left eye. This would reduce confounding variables in the measurements, if the rats would be nervous in the early readings or restless in later readings on the other eye.

Ultimately, it is expected that the loss of RGCs that is demonstrated in the retina after surgery to induce glaucoma would correlate with an increase of IOP. Although, I was able to visualize labeled RGCs under the confocal microscope, I was not able to quantify any loss of RGCs in this study due to the lack of time. However, others in the lab intend to perform the quantification of RGCs. The correlation of RGC loss to changes of IOP measurements due to glaucoma-inducing surgery won't be relevant until consistent and true IOP measurements can be obtained.

In summary, the findings of this study reveal potentially vital evidence on how to precede in obtaining consistent IOP measurements through an *in vivo* Long Evans rat glaucoma model. Although the results of this study did give some promising results, it is overall inconclusive. A great deal was learned in the process of this study to make future studies more reliable in obtaining IOP measurements.

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