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Effects of Age on Induced Glaucoma in an In Vivo Rat Model

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Glaucoma-Induced Cell Loss in the Retinal Ganglion Cell Layer in Young (3-6 months) versus Old (1 year) Rats in an *in vivo* Rat Model.

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Presented for fulfillment
of Honor’s Thesis.

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Abstract:

Previous in vitro studies from this lab have demonstrated that injection of a hypertonic saline solution into the episcleral vein of the rat’s eye mimics the effect of glaucoma. However, the age of the rat related to the amount of cell loss in the retina ganglion cell (RGC) layer is unknown. To address this issue, we propose to analyze the correlation of age and cell loss between 3-6 month and 1-year-old Long Evan rats. When inducing glaucoma in the Long Evan rats, the left eye in each rat was left untreated and used as an internal control. In the right eye, hypertonic saline (0.05 ml of 2 mM NaCl) was injected into the episcleral vein creating scar tissue in the trabecular meshwork of the eye, reducing aqueous drainage and leading to an increase of intraocular pressure; the primary risk factors associated with glaucoma. To determine if significant cell loss occurred in the RGC layer, the number of surviving cells in the RGC layer were counted 1 month after hypertonic injections. To count these cells, whole retinas were removed from euthanized rats and retinal flat-mounts were placed in 4% paraformaldehyde for 24 hours at 4°C. Following fixation, retinas were stained with Cresyl Violet to label nuclei in the RGC layer. The number of stained cells in specific regions of the retina was counted throughout the RGC layer using the scanning capabilities of a Zeiss 510 confocal microscope. The number of cells were counted under the untreated conditions and compared to the number of RGCs that survived under treated conditions in the periphery region of the retina. The results of these studies demonstrate that there is no significant difference in the number of labeled RGCs in control eyes between the two age groups and no significant difference in cell death obtained after hypertonic injections between the two age groups. The results of these studies suggest that it would be appropriate to
use adult Long Evans rats from 3 months to 1 year for any future studies involving analysis of retinal ganglion cells quantification.

Specific Aims:

Glaucoma is a neurodegenerative disorder characterized by the progressive death of retinal ganglion cells (RGCs) and degeneration of axons in the optic nerve (Quigley, 1998). The primary risk factor associated with glaucoma is an increase of intraocular pressure (IOP) (Quigley, 1998). It is likely that the increase of pressure initiates the degenerative process of neurons by putting stress upon the cellular structures of RGCs within the eye (Quigley, 1998), causing cells to die and release excessive amounts of neurotransmitters. Excessive release of the neurotransmitter, glutamate, has a toxic effect on cells containing glutamate receptors causing them to go into a self-destruct mechanism known as apoptosis (Buttke and Sandstorm, 1994). This toxic effect is called excitotoxicity and is the likely reason that glaucoma progresses even if issues concerning IOP are managed (Brooks et. al., 1997; Dreyer and Lipton, 1999). The damage due to the disease is permanent and results in the loss of vision. We have demonstrated that the injection of hypertonic saline into the episcleral vein of the rat’s eye mimics the effect of glaucoma. However, it is unknown if the age of the rat affects the loss of cells from the RGC layer under conditions designed to mimic glaucoma. To address this issue, an in vivo model of glaucoma in Long Evans rats was used to test the hypothesis that there is no significant difference in loss of cells from the RGC layer in animals between the ages of 3-6 months and 1 year.
Introduction:

Eye Anatomy and Physiology

One needs a clear understand of normal eye anatomy and physiology to interpret mechanisms associated with glaucoma. The eye is a fluid-filled glob like structure enclosed by three tissue layers. Because the eye is an enclosed structure, it maintains its own hydrostatic pressure. From the outside to the inside, there are the sclera and cornea; the choroid, ciliary body and iris; and the retina (figure 1). The sclera is a tough layer of connective tissue that forms the white part of the eye and the cornea, which is a transparent part of the sclera that allows photons of light into the eye. The choroid is located underneath the sclera and contains high-pigmented containing blood vessels that nourish the retina. The pigmentation of the choroid prevents reflection and scattering of light photons that are not absorbed by the retina. The choroid because more specialized anteriorly and forms the ciliary body and iris. The ciliary body and iris are two types of smooth muscle. The ciliary body holds and adjusts the shape of a lens. The lens is a structure that refracts photons of light onto the retina. The lens also separates the eye into two different compartments called the anterior chamber and the posterior chamber. The ciliary body also produces a fluid called aqueous humor that fills the anterior chamber. The aqueous humor has a composition similar to blood and supplies nutrients to the avascular structures of the eye such as the cornea and lens. The fluid is constantly being produced at a rate of 5 ml/day. The aqueous humor drains at two locations found within the eye at a region known as the iridocorneal angle. The aqueous humor is then returned to the systematic circulation.
Drainage of aqueous humor is typically through the trabecular meshwork and the canal of Schlemm (figure 1). However, before draining through the Schlemm’s canal, the aqueous humor is filtered through a well-structured tissue called the trabecular meshwork (Avtar and Srvastava, 2006). This process is defined as the aqueous humor outflow. Glaucoma is associated with high intraocular pressures due to either over production of aqueous humor and/or resistance of outflow the trabecular meshwork can create. Some current pharmacological treatment options for glaucoma involve increasing aqueous humor.

Figure 1 depicts the anatomy of a normal human eye.
outflow. Prostaglandin analogs such as latanoprost, binatoprost, and travoprost have been found to increase uveoscleral outflow while $\alpha_2$-adrenergic agonists such as brimonidine and apraclonidine have been found to increase conventional outflow and also decrease aqueous humor production (Zimmerman, 1996; Rhee, 2001).

The iris is a thin-pigmented smooth muscle that can be seen through the cornea and gives the eye color. The iris adjusts its shape forming a variable sized round opening in the center, called the pupil, which allows a varied amount of light into the posterior part of the eye. With the help of the cornea and lens, light is focused onto the retina in the back of the eye, where photons of light are converted to electrical impulses. The retina is neural tissue and is the innermost layer of the eye, held in place by the pressure of a fluid that exists in the posterior chamber called the vitreous humor, and is located under the choroid. There are a total of seven layers within the retina. From the way light passes through the layers, they are the (1) optic nerve fibers; (2) ganglion cells; (3) inner plexiform layer; (4) horizontal, bipolar, and amacrine cells; (5) outer plexiform layer; (6) photoreceptor cells: rods and cones; and (7) pigment epithelium (figure 2). The pressure that holds the retina in place can be detrimental if it exceeds that of what is normal to that eye. In the retina, photons of light are absorbed and transduced into electrochemical signals that are transmitted to the brain for processing. This process of phototransduction can be compromised by glaucoma’s degenerative properties. These cells are damaged by glaucomatous activity and may die and no longer transduce information to the brain. If enough ganglion cells and optic nerve axons are destroyed, an individual will lose vision.
Phototransduction

When a photon of light enters the eye through the cornea, it travels through the aqueous humor and is refracted by the lens. The lens focuses the light photon through the vitreous humor onto the retina where it travels through the five most inner layers and is absorbed by pigments in photoreceptors in the sixth layer. The seventh layer consisting of pigment epithelium will absorb any photons that were not absorbed by the photoreceptor cells. The effect of the pigmented layer ensures that photon will not reflect back onto photoreceptors creating the perception of false or skewed images. Photons of light are
absorbed by densely packed visual pigments found on the outer segments of rod and cone photoreceptors. When light hits the photoreceptors, they hyperpolarize and synapse onto bipolar cells using the neurotransmitter glutamate and are the first relay interneurons in the visual system. Bipolar cells can be distinguished by their different morphologies and connections with photoreceptors. Rod photoreceptors synapse with rod bipolar cells and cone photoreceptors synapse with cone bipolar cells (Ramon y Cajal, 1893; Daw et al, 1990). A total of 10 bipolar cell subtypes have been identified in the rodent retina (Haverkamp et al, 2003). However, there is only one type of rod bipolar cell. The circuitry of the rod pathway in the rat retina has been shown to be similar to that of other animals (Chun, et al, 1993). Bipolar cells receive signals from either photoreceptors or horizontal and synapse with amacrine cells (Daw et al, 1990) causing the excitation of cone “on” bipolar cells and inhibit cone “off” bipolar cells using glycine-mediated inhibition (Sassoe-Pognetto M et al, 1994). The response bipolar cells have on retinal ganglion cells are graded potentials meaning the membrane potential can be small to large. During dark phases, the photoreceptors are depolarized and release large amounts of glutamate onto bipolar cells. During light phases, the photoreceptors are hyperpolarized and release small amounts of glutamate onto bipolar cells. A decrease in release of glutamate from photoreceptors during light phases will reduce excitation of bipolar cells with excitatory glutamate receptors causing them to hyperpolarize. These are called “off” bipolar cells. Their responses are mediated through inotrophic glutamate receptor channels. A decrease in release of glutamate from photoreceptors during light phases will excite bipolar cells with inhibitory glutamate receptors causing them to depolarize. These are called “on” bipolar cells. “on:” bipolar cells inhibition is mediated
by metabotropic glutamate receptors that act through G proteins and a second messenger system. Both of these bipolar cells also release the neurotransmitter glutamate and they converge and synapse on retinal ganglion cells. Retinal ganglion cells contain ionotropic glutamate channels and upon stimulation calcium and sodium will influx causing retinal ganglion cells to depolarize (Feller et al, 2002). The depolarization of retinal ganglion cells sends action potentials along their optic nerve fibers (axons) to the lateral geniculate nucleus in the brain for processing and then to area one of the primary visual cortex for further processing. The visual cortex is located in the occipital lobe of the brain and is a higher order of processing that gives one the perception of vision.

*Glaucoma*

Glaucoma is one of the leading causes of blindness worldwide (Resnikoff, 2002). In America, it is estimated that over 4 million Americans have glaucoma but only half of those know they have it (*Prevent Blindness America*). Estimates put the total number of suspected glaucoma cases at around 65 million worldwide (Quigley, 1996). Glaucoma is a neurodegenerative disorder characterized by the death of RGCs and degeneration of the axons in the optic nerve (Quigley, 1996). The primary risk factor associated with glaucoma is an increase of intraocular pressure (Quigley, 1996). It has been hypothesized that the increase of pressure initiates the degenerative process of neurons by putting stress upon their cellular structures that triggers apoptosis (Quigley, 1996). The apoptotic effects on these cells cause them to die and release their neurotransmitters (Quigley, 1996). The excessive amounts of neurotransmitter allow too much calcium into the cells, which triggers apoptosis (Quigley, 1996). The damage due to the disease is permanent
and results in the loss of vision. The process is slow at first damaging cells that are responsible for a person peripheral vision and may go unnoticed, as pain is not normally associated with the most typical type of glaucoma. As the disease progresses, it will begin to damage cells that are responsible for central vision.

The disease has been nicked the silent blinder because one may not notice they have the disease until a substantial amount of damage has compromised their vision. The main means of treating the disease is to control intraocular pressures with medication or surgery. However, decreasing intraocular pressure is usually insufficient to prevent progression of visual field loss (Litcher P., 2002). Many previous students have suggested that glutamate-induced neurotoxicity may play an important role in glaucoma (Vivker et al., 1995; Brooks et al., 1997). As a result, high concentrations of glutamate have been used to induce excitotoxicity in a wide array of in vivo and in vitro preparations to mimic glaucoma (Luo et al., 2001). An in vivo model of glaucoma in the rat, based on the methodology first described by Morrison et al. (1997), was used in this study to experiment on possible neuroprotective treatments to counteract the effects of glaucoma. In this study we are interested in looking at the different age of rats and how self-induced glaucoma effects them. From this study we will be able to understand if age is a significant factor in RGC loss in the retina of Long Evan rats. With this knowledge we will know if we are able to use rats that are 1 year old for future experiments.
Materials and Methods:

Animals

Both male and female Long Evan rats were used in the study. A group of 3-month-old rats was used and a group of 1-year-old rats was used. Two 1-year-old rats were used and three 3-month-old rats were used for experimental treatment. The rats were also used to determine if the delivery method had any effect on glaucomatous RGC count. Animals were housed in WMU’s animal facility. All procedures and euthanasia were approved and in accordance with the Institutional Animal Care and Use Committees (IACUC) at WMU.

Anesthesia

Rats were anesthetized by intraperitoneal injection of 1.0 ml/kg KAX standard rat cocktail, consisting of a solution 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. KAX was injected intraperitoneally into rats on the dorsal side, prior to hypertonic saline injections to induce glaucoma. To ensure that the rats were fully anesthetized, their feet and tails were pinched and pulled to check for any reflex activity. After a KAX injection and surgery, the rats were kept in the lab’s custody until they regained consciousness before returning to the animal colony.

Surgery to Induce Glaucoma

To induce glaucoma, rats were anesthetized with an intraperitoneal injection of KAX.
Two drops of pilocarpine, a local anesthetic, were applied to the right eye of each rat and a hemostat was used to pinch the tissue around the right eye to cause the eye to bulge out of its socket for viewing the episcleral vein. Under a dissection microscope, the episcleral vein of the right eye was located and injected with 50 microliters of 2M NaCl using a micro needle assembly. The left eye was always left as an internal untreated control. Once the micro needle had punctured the episcleral vein, the syringe was depressed and 2M NaCl was injected. Blanching inside the episcleral veins insured a successful injection every time. Following the injection, the hemostat was removed and a small amount of antibiotic ointment was applied over the injection site. The procedure was modified from the procedure originally developed by Morrison (1997).

**Micro needle Assembly**

There are four main components needed to make up the micro needle; (1) a 1 ml insulin syringe, (2) a 18 gage needle, (3) a flexible polyethylene tube, and (4) a 10 μL glass micropipette. Attaching the 18-gage needle to the 1 ml insulin syringe needle backfilled with 2M NaCl, and inserting the 18-gage needle into the flexible polyethylene tube assembled the micro needle. The 10 μL micropipette was inserted into the furthest end of the tube from the syringe. The micropipette was pulled Sutter micropipette puller into a long tapered end that was beveled by running it over a sharpening stone.

**Removal of Retina**

One month after the hypertonic saline injection, rats were euthanized by CO₂ asphyxiation and decapitated. Both the right and left eyes were removed and placed into
phosphate buffered saline solution. To access the retina, an eyecup was created from each eyeball by removing the cornea, iris, lens, and vitreous humor. The whole retina was then able to be peeled from the back of the eyecup, away from the choroid and pigmented epithelium. Care was taken to remove the retina in one piece after cutting the optic nerve. Once whole retinas were removed, four evenly spaced slits were made in the retina allowing the retinas to be flattened (figure 3). The retina was then pinned down to sylgard plates using cactus needles and fixed in 4% paraformaldehyde for 24 hours at 4°C. Following fixation, retinas were stained with Cresyl Violet to label nuclei in the RGC layer. Cresyl Violet is a Nissl stain that colors cell bodies a brilliant violet. The Cresyl Violet staining procedure involves soaking the reinal flatmount in the series of solutions designed to remove lipids using alcohol, stain cell bodies with cresyl violet, dehydrate tissue using alcohol, and to unstain parts of the tissue that are not cell bodies with a clearing agent.

**Confocal Microscopy and Analysis**

Once stained, the numbers of stained RGCs in specific regions of the retina were counted throughout the entire RGC layer using the scanning capabilities of a Zeiss 510 confocal microscope. Counting the number of RGCs was possible under the confocal microscope as each stained nuclei was opaque when viewed under the confocal microscope with a rhodamine filter. The cells were counted in the peripheral areas of the retina (400 μms from the optic nerve head, ONH) based on the nature of the disease as peripheral cell loss of RGCs is normally higher under glaucoma conditions compared to cell loss at more distal locations (Jampel, 2001). For each different aged rat, the different counts were
toted and quantified for statistical analysis. The areas counted were compared and contrasted between the right experimental eye and the left internal control eye in each rat.

**Statistical Analysis**

Statistical analysis was done using software obtained from MS excel. T-test’s were used for comparison between the change from the internal control in the different age groups, p < 0.05 were considered as statistically significant. All data values are reported as the mean standard error of the mean (SEM).
Results:

Preparation of Retina

Figure 2 depicts a flat mounted rat retina that was removed from an eyecup preparation and pinned out in a sylgard dish with the RGC layer facing up. After labeling nuclei with cresyl violet, the tissue was viewed using confocal microscopy with a rhodamine filter and several images are taken through the RGC layer. In this study, it was imperative that the retina was removed from the eyecup intact so that distance from the ONH could be easily measured. Four sets of images were obtained from each retina in each of the 4 sections shown in figure 3 throughout the entire RGC layer. This results in good contrast between RGCs and background allowing for easy quantification. All the RGCs were counted after cresyl violet labeling under untreated and experimental conditions from each animal.
Photos obtained for quantification

Figure 4 depicts two photos obtained from flat mounted retina after Cresyl Violet staining’s. Figure 4A represents cresyl violet stained retinal section obtained from a rat’s left eye under untreated conditions. Figure 4B represents cresyl violet stained retinal section obtained from the rat’s right eye one month after surgery to induce glaucoma. Both sections were obtained from the same animal and images were taken under 200X magnification from the same location in the retina. The number of rat RGCs within the right experimental image were counted and compared to the left internal control. As seen in figure 3, there were significantly less number of nuclei in the RGC layer in the left-untreated image.

Left Eye (Control)  Right Eye (experimental)

Figure 4 depicts two photos taken in the peripheral location of the retina. Figure 4A was taken from the left internal control eye. Figure 4B was taken from the same location from the same animal from the right experimental eye. In comparison, the left internal control has considerably more nuclei stained compared to the right experimental eye. The arrowheads are demonstrating what a cresyl violet stained nuclei is, and the two sided arrows are showing where the axon tracts are. Axon tracts seen in the figure are bundles of axons from the RGCs that are heading for the ONH to leave the retina.
Figure 5 depicts two photos obtained from flat mounted retina after Cresyl Violet staining’s. Figure 5A represents cresyl violet stained retinal section obtained from a 1 year old rat’s left eye under untreated conditions. Figure 4B represents cresyl violet stained retinal section obtained from a 3-6 month year old rat’s left eye one month after surgery to induce glaucoma. Both sections were obtained from the same animal and images were taken under 200X magnification from the same location in the retina. The number of rat RGCs within the 1 year old rats left control image were counted and compared to the left internal control. As seen in figure 5, there were not a significantly less number of nuclei in the RGC layer in the left-untreated images from both ages of rats.

Left Eye (Control) 1 Year Old Rat       Left Eye (Control) 3-6 Month Old Rat

Figure 5 depicts two photos taken in the peripheral location of the retina. 5A was taken from the left control eye of a 1-year-old rat. Figure 5B was taking from the left control eye of a 3-6 month year old rat. From the picture, the internal controls obtained from the one year old rats didn’t look any different from the internal control rats obtained from the 3-6 month old rats.

Figure 6 depicts two bar graphs that show the typical amount of cell death that occurs due to the surgery, compared to control untreated conditions at 3-6 month old rats. Figure 5A depicts a bar graph that shows 100% survival for internal untreated control and a survival of only 71.7% (100% - 28.3%) survival of the 3-6 month old group. Figure 5B shows the
100% survival for internal untreated controls and a survival of only 66.87% for the 1 year old rats (100%-33.13%). From these graphs it shows the glaucoma effect on the loss of cells from the RGC layer.

![Graph 6A: 3-6 Month Old Rats](image)

![Graph 6B: 1 Year Old Rats](image)
Figure 6 depicts two bar graphs that show the typical amount of cell death that occurs due to the surgery, compared to control untreated conditions at 3-6 month old rats. Figure 6A depicts a bar graph that shows 100% survival for internal untreated control and a survival of only 71.7% (100% - 28.3%) survival of the 3-6 month old group. Figure 6B shows the 100% survival for internal untreated controls and a survival of only 66.87% for the 1-year-old rats (100%-33.13%).

Percent of cells in RGC layer

Figure 7 depicts bar graphs that summarize the average percent of labeled cells in the RGC layer that were lost after hypertonic injections of saline in 3-6 month old rats and in 1 year old rats. Each bar graph represents the average loss of cells from the RGC layer compared to the animal’s internal control. There is not a significant amount of RGC loss between the 3-6 month old rats and the 1-year-old rats. The 3-6 month old rats showed an average of 28.30% (SEM +/-9.3) change in RGCs and the 1-year-old rats showed an average of 33.13% (SEM +/-12.2) change in RGCs. This supports the hypothesis that experiments can be conducted on animals ranging from 3 months to 1 year as there was no significant difference in results was observed on data collected from these two ages.
Discussion:

In this study we have provided evidence that 3-6 month old rats and 1-year-old rats show ultimately the same amount of RGC loss in the peripheral location of the RGC layer after surgery to induce glaucoma. Using the Morrison model (1997), we have provided the evidence that loss of cells from the RGC layer can be induced in our *in vivo* rat model through the injection of hypertonic saline solution into the episcleral vein of the eye. This was done to mimic glaucoma-like conditions. In the Morrison model, the back flow of 2M NaCl into the episcleral vein causes scaring of tissue found within the trabecular meshwork. The scaring of the trabecular meshwork increases the resistance of aqueous humor outflow, which mimics glaucoma. The resistance of outflow initiates an increase in IOP that ultimately leads to cell death within the retina (Kim et. al., 1998). It has been hypothesized that the death of these retinal cells dumps their transmitter, increasing the

Figure 7 depicts the average percent change in RGCs in 3-6 month old rats and 1-year-old rats. Each average percentage was calculated by comparing the left-untreated internal control eye to the right-experimental eye. Results were obtained from three 3-6 month old rats and two 1-year-old rats. Error bars represent SEM.
extracellular glutamate concentration. The increase in glutamate leads to over excitation of retinal ganglion cells, programming them for apoptosis. It is these implications that suggest glaucoma is associated with glutamate excitotoxicity (Casson et al., 2006).

However, the injection of hypertonic saline into the episcleral vein varies a bit with each surgery. The amount that is injected and the amount of blanching, or “white,” that occurs in the eye after injection could result in higher RGC loss in the RGC layer. However, since our results show no significant difference in cell death obtained after hypertonic injections between the two age groups, it is likely that the effect of varied injections does not produce a significant impact on the survival of the cells. Future studies need to be conducted using even older rats between 1-year-old rats and 2-year-old rats to further investigate the issue of age as a factor in cell loss in the RGC cell layer. Normally, as rats age it is known that RGCs are lost due to the down-regulation of a cellular inhibitor of apoptosis during the maturation of rats (Kisiswa, 2010). There were two main concerns of this study. (1) To determine if there was a general loss of cells from the RGC layer associated with age and (2) to determine if the method we used to induce glaucoma effects the younger rats differently than the older rats we used (1 year).

Since our studies are only labeling nuclei, you cannot exactly tell which type of cells we are staining. This can be linked to Muller cell activity. Studies in avian models have shown that Muller cells (the gilia of the retina) have progenitor like qualities and can take the form of RGCs in times of stress (Fischer, 2003). Is it possible that Muller cells within the rat retina also posses this quality that hypertonic saline injection stimulates the Muller cells to form new RGCs? An experiment that could be done to examine the generation of rat ganglion cells from Muller cells is to monitor DNA
construction. Using the thymidine analog bromodeoxyuridine (BrdU), one can label newly formed neurons. This compound could be introduced to the retina following treatments of hypertonic saline injections, after which, histology could be performed at different time points to examine if BrdU has been incorporated in newly formed cells. These studies need to be preformed in the future. (Fischer, 2003).

However, the lab can now directly label RGCs and not just the nuclei of cells in the RGC layer. The Thy1.1 glycoprotein found only in the plasma membrane of RGCs in the retina can be labeled to distinguish RGCs from all other cells. The results of these studies that only label RGCs confirm that the cells that are lost in the RGC layer using the method by Morrison (1997) are, in fact, RGCs and not amacrine or glial cells.

In conclusion, we provided evidence that 3-6 month old rats and 1-year-old rats show ultimately the same amount of RGC loss in the peripheral location of the RGC layer. The comparison between the two different age groups of rats suggest that these studies can be done on adult rats between the age of 3 months and 1 year and age has no significant effect on these studies using animals between 3 months and 1 year.

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