Evaluation of degeneration retinal ganglion cell loss in acute glaucoma model rats using syringe pump technique

Siti Hajar1,2, Dessy Rakhmawati Emril3*, Nurwasis4, Zinatul Hayati5, Dedy Syahrizal6

INTRODUCTION
Glaucoma, recognized as the foremost cause of irreversible blindness on a global scale, encompasses a diverse array of neurodegenerative disorders characterized by the gradual functional decline and ultimate demise of retinal ganglion cells (RGCs) and their axons.1-3 According to a recent study, it is projected that around 76.0 million and 111.8 million individuals will be impacted by glaucoma in 2020 and 2040, respectively.4 Present therapeutic strategies rely on either clinical or surgical interventions aimed at lowering elevated intraocular pressure (IOP), a highly persistent risk factor contributing to the onset and advancement of glaucoma. Despite these efforts, around 45% of patients exhibit unresponsiveness to these approaches, leading to continued disease progression.5 Hence, comprehending the intricate and multifaceted pathophysiological aspects of glaucomatous neurodegeneration is imperative for devising effective therapies aimed at preventing or attenuating the degenerative process. These treatments should operate independently of or in conjunction with intraocular pressure (IOP) reduction. Glaucoma stands as the foremost cause of permanent blindness worldwide.1,5 Employing laboratory animals to model glaucoma is a well-established strategy for uncovering disease mechanisms and advancing the development of novel neuroprotective interventions. Within the array of rodent models of glaucoma, those induced by either acute or chronic ocular hypertension (OHT) closely mirror the condition observed in humans. The experimentally induced unilateral OHT offers advantages over genetic glaucoma models, providing superior control over optic nerve and retinal pathology, along with the availability of the fellow eye for use as an internal control. Furthermore, the larger size of the eye globe and associated anatomical structures in rats contributes to the suitability of this model.

METHODS
This research was an animal-experimental study in animal model adult male Wistar rat (Rattus norvegicus), taken in May - October 2023 in an integrated laboratory Faculty of Veterinary Medicine Universitas Syiah Kuala. Total samples used in this research were 10 rats, divided into two categories which are control group (A) and treatment group (B) were given a pressure of 250 mmHg pressure in the right eye for 60 minutes. Apoptosis and degeneration of retinal ganglion cells after 8 weeks of elevated intraocular pressure of 250 mmHg in the right eye for 60 minutes were observed by the HE staining.

Result: A significant difference in IOP was noted between control group rat and treatment group rat. All rats that received treatment experienced an increase in IOP (100%). The RGC density decreased significantly in the treatment group rat (mean: 11.72 cell/field of view or lower 46.88%) compared with the control group (p =0.005).

Conclusion: A pressure of 250 mmHg for 60 minutes has been proven to cause degeneration of the RGCs up to nearly 50% within a week. We tried a technique to induce increased IOP in rats as a model of acute glaucoma. Syringe pump technique can effectively induce acute ocular hypertension in rats, which causes progressive loss of retinal ganglion cells.
we present a novel and straightforward approach for inducing ocular hypertension in rats, utilizing a syringe pump to control intraocular pressure and induce acute ocular hypertension (OHT). This method results in progressive glaucomatous neurodegeneration that persists beyond the period of elevated intraocular pressure (IOP). Notably, this technique is simple to execute, cost-effective, efficient, and leads to the loss of ganglion cells within a relatively short time following a single procedure.1

Glaucoma comprises a group of diseases characterized by optic neuropathy and visual field loss, primarily attributed to elevated intraocular pressure (IOP). The condition is defined by the degeneration of retinal ganglion cells (RGC), either with or without an increase in IOP.6-9 Elevated intraocular pressure triggers various mechanisms, including abnormal aqueous humor flow, resulting in reduced ocular perfusion, and heightened oxidative stress caused by reactive oxygen species (ROS).10 This increased oxidative stress plays a role in the death of retinal ganglion cells (RGC) in glaucoma. Additionally, decreased antioxidative concentrations are observed in the vitreous of glaucoma patients, contributing to factors such as DNA oxidative damage and altered trabecular meshwork.11

Owing to the absence of symptoms in the early stages of the disease, approximately half of individuals affected by glaucoma are believed to be unaware of their condition. By the time vision loss becomes apparent, substantial and irreversible damage has already taken place. Glaucomatous visual field defects typically initiate in the peripheral vision and advance towards central vision, resulting in profound consequences for the patient’s overall quality of life. Despite achieving satisfactory control of intraocular pressure (IOP) in a significant percentage of patients, disease progression remains a common occurrence. It is estimated that one in eight patients will eventually experience blindness in at least one eye due to glaucoma progression, even in populations with access to the best available treatments. Therefore, the primary objective of glaucoma treatment should be the development of a therapy that prevents the death of retinal ganglion cells (RGC) and loss of optic nerve (ON) axons. Unfortunately, the mechanisms leading to RGC loss in glaucoma remain unclear. Given the complexity and multifactorial nature of glaucoma, it is likely that various molecular pathways converge, triggering structural and functional alterations.12

The irreversible damage to retinal ganglion cells (RGC) results in irreversible blindness, as indicated by previous research.8 The loss of RGC is recognized as the earliest sign of cell death in glaucoma patients, and the functional impairment of RGC is considered the pathogenic basis for visual field loss.13 Crucial corrective measures in glaucoma involve promoting the regeneration of axons, which can be achieved through the use of optimal neuroprotective agents specifically designed for glaucoma.14,15 Currently, there is no curative treatment. The only proven treatment is lowering intraocular pressure (IOP), the most important risk factor.16

Subsequent investigations should prioritize the enhancement of existing animal models, such as inducing glaucoma before implementing therapeutic interventions, and standardizing animal research practices to ensure increased reproducibility and reliability across diverse research groups.16 In contrast to human studies, animal models offer the advantage of a more invasive approach, such as enucleation of eyes for histological evaluation, and enable the monitoring of disease progression from its inception. Various types of animal models have been employed in the quest for novel treatment modalities. As a result, the majority of preclinical glaucoma models are designed to elevate intraocular pressure (IOP) to levels that induce degeneration of retinal ganglion cells (RGC) without adversely affecting the other intraocular structures.17

Rats are commonly utilized as laboratory animals, and various methods for increasing intraocular pressure (IOP) have been developed to induce a glaucoma mouse model. Consequently, having an animal model in which glaucoma can be consistently induced before implementing therapeutic interventions could prove beneficial for the preclinical validation of drugs. There are two distinct forms of glaucoma models: spontaneous (natural) and induced. Spontaneous models do not necessitate surgical intervention to initiate retinal ganglion cell (RGC) death. However, the severity, onset time, and comorbidities can vary among individual animals, potentially leading to an increased demand for additional animals due to heightened variability. Therefore, induced models might offer a more dependable method for generating glaucomatous eyes in animals.16 In general mice models are cost effective, molecular tools are readily available, they are easy to house, and genetic models are readily available.

The animal models of glaucoma are commonly used for the development of novel treatment modalities. Small rodents (e.g., mice and rats) are often used to study the animal models of glaucoma after increasing intraocular pressure (IOP) and to test drug candidates. We attempted to develop a model of acute glaucoma induced ocular hypertension and glaucoma in mice via the induction of reperfusion ischemia that reported to induce this pathology in rat Rattus norvegicus. Surprisingly, we found that induced ocular hypertension administration elevated IOP and RGCs degeneration in this study, we used a new method to establish a model of experimental acute glaucoma. Syringe pump technique can effectively induce acute ocular hypertension in rats. The increase in IOP in this model was stable and sustained for at least 8 weeks after acute ocular hypertension, leading to the loss of RGCs. Our model has the characteristics of strong operability.

METHODS

This research was an animal-experimental study that used adult male rats (Rattus norvegicus), taken in May - October 2023 in an integrated laboratory Faculty of Veterinary Medicine Syiah Kuala University. We prepare Tonometer SW 500, experimental animals’ cage, and syringe pump for the materials used in this study. The subjects in this research were grown-up white male rat (Rattus norvegicus) and these rats were feed with BR I. Inclusion criteria for the samples are white male rat (Rattus norvegicus), aged 3–4 months old, weighted 200–300 gram, and active with no physical defects. Meanwhile the
exclusion criteria are inactive and disabled rat, also dead rat during the research. Total samples used in this research are 10 rats, divided into 2 categories which are control group (A) and treatment group (B) are given a pressure of 250 mmHg pressure in the right eye for 60 minutes. Measurements of IOP were done immediately before and after the experimental eyes' procedure, then every day in up to 8 weeks after experimental eyes and retinal ganglion cells evaluated by HE staining.

**Induction of Ocular Hypertension**
Rats were deeply anesthetized through an intramuscular injection of a solution containing ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (5 mg/kg). After a 5-minute interval, the right eyelids were gently held apart using curved forceps, and the globe was softly pushed forward to expose the limbal region. This facilitated the application of topical anesthesia to the ocular surface using ophthalmic tetracaine hydrochloride 2%. The animal was then positioned in a slight lateral decubitus, and a 30G cannula was utilized to make a stab incision in the superotemporal limbal area, visualized through a loop at 3x magnification. After that, a syringe pump that was already connected with cannula that was already regulated in 250 mmHg and maintained the pressure in 250 mmHg for 60 minutes. After treatment, a drop of ophthalmic levofloxacin (10mg/mL) was applied. Intraocular pressure was measured preoperatively, after treatment, and daily until the end of the study.

**Measurement of Intra Ocular Pressure and Clinical Follow-up**
Rats were applied to the surface of the eye topically anesthetized with ophthalmic tetracaine hydrochloride 2%, and IOP was measured using a handheld tonometer (Rebound Tonometer SW-500). The tip of the tonometer touched the center of the cornea perpendicularly. Measurements were done immediately before and after the experimental eye procedure, then every day in up to 8 weeks after experimental eyes. IOP is generally assessed using non-invasive methods as the rebound tonometry. This technique allows to measure the IOP in awaken or anesthetized animal without surgical interventions and subsequent animal.

**Processing of the Optic Nerve and Hematoxylin-Eosin Staining**
Rats were anesthetized and perfused in sequence with saline and 4% neutral buffered formalin (NBF) transcardially. The right eyes were enucleated and further fixed in 4% PFA. The eyecups were embedded in an optimum cutting temperature compound. The retinas were anteroposteriorly sectioned around the optic disc into slices 6 μm thick using a Rotary microtome (CUT 4062, SLEE microtome, Germany) and mounted on gelatin-coated slides. The HE procedure for staining retinal ganglion cells (RGCs) is using the HE - Harris Method and Staining Protocol.

**Density Analysis of H-E Staining Retinal Ganglion Cells (RGCs)**
The preparations were examined and assessed using a 400-magnification microscope (Zeiss Primo star microscope, Germany), the retinal ganglion cell density was assessed based on the number of retinal ganglion cells per 1 mm in each preparation with an average of 5 large visual fields (LPB) selected.

**Statistical analysis**
Data RGCs were expressed as mean ± standard deviation, RGCs density was averaged in rats on the control group and compared with treatment group. Analysis for differentiate RGCs data using independent sample T-test after previously carrying out normality test using shapiro wilk test, which obtains normally distributed data result. If p value below 0.05 that will conclude a significant difference of RGCs between two groups.

**RESULTS**
In this research, a total of 10 rats divided into 2 categories, which are control group (A) and treatment group (B), are given a pressure of 250 mmHg pressure in
the right eye for 60 minutes. Rats were randomly assigned for induced acute ocular hypertension by syringe pump technique, all mice that received treatment experienced an increase in IOP (100%).

There were 10 rats which consisted of 5 rats of the control group and 5 rats of the treatment group during the process of the whole experimental period. The preparations were examined and assessed using a 400-magnification microscope (Zeiss Primo star microscope, Germany), the retinal ganglion cell density was assessed based on the number of retinal ganglion cells per 1 mm in each preparation with an average of 5 large visual fields (LPB) selected.

Density analysis of the loss of RGCs in rats with HE staining were used to evaluate the RGCs in the retinas of rats after induced acute ocular hypertension by syringe pump technique the density of RGCs shows in Table 1.

Comparison of RGCs values based on treatment in the control group (PN) and treatment group (PP) there were significant differences in value between the two groups. In the control group, the average RGCs value was 25.8±4.97 while in the treatment group the average RGCs values was 14.08±1.43 with the results of the different test the value of p = 0.005 was obtained which means that the RGCs values in the two groups were significantly different. The RGCs value for the treatment group was 11.72 (46.88%) lower than the control group.

Histology picture of RGCS expression in control and treatment group, the picture shows decrease of RGCs expression in treatment group shows in Figure 1.

Hematoxylin and eosin staining for RGCs marker demonstrated a significant decrease in the average number of RGCs within the ganglion cell layer in treatment group eyes.

Boxplots showing the distribution of estimated number of retinal ganglion cells (RGCs) at baseline in eyes of control and treatment group in Figure 2.

The median value of RGC loss was 25.8±4.97 cells in control group versus 14.08±1.43 cells in the treatment group, the degeneration of RGCs is commonly associated with a number of significant loss of RGCs and increased apoptosis.

**DISCUSSION**

The advancement of clinically relevant models for glaucoma research has significantly enhanced our comprehension of the pathobiological mechanisms underlying the disease and the potential identification of neuroprotective agents. Over recent years, rat and mouse glaucoma models have emerged as crucial tools, offering numerous advantages, including their susceptibility to experimental manipulation, cost-effectiveness, and ocular anatomy and biology that closely resemble that of humans. Pressure-dependent models of ocular hypertension typically target the primary risk factor, but the methods for inducing increased intraocular pressure (IOP) can vary significantly. Numerous research groups have successfully elevated IOP in rodents by obstructing the outflow of IOP in techniques such as laser photocoagulation, catarization of episcleral veins, injection of hypertonic saline, intracameral injections of microbeads, or viscoelastics.

Commonly employed experimental models of glaucoma are categorized as pre-trabecular, trabecular, or post-trabecular, with the trabecular meshwork (TM) serving as the anatomical reference point. However, multiple limitations have been reported for existing methods. First is intracameral injection of viscoelastic substances or microbead particles (pre-trabecular). This approach is invasive and carries a high risk of corneal damage, inflammation, and intraocular infections. Additionally, certain injected substances may diminish the transparency of the optic media, hindering functional analysis. Second, laser scarring of the TM and/or photocoagulation of the limbal vasculature and episcleral veins (trabecular). This method requires expensive equipment and is challenging to apply to pigmented animals due to the high and variable absorption of laser energy. Moreover, it often results in damage to the TM, corneal inflammation, and ulceration, which can impair functional assessment. Third is Morrison's procedure of injecting hypertonic saline into an episcleral vein (post-trabecular). This technique demands extensive training and results in highly variable intraocular pressure (IOP). Fourth, Shareef and Sharma's episcleral vein catarization (EVC). This invasive method requires a slow learning curve to access the deep episcleral veins behind the rectus muscles. It may lead to venous congestion because the targeted deep venous plexus includes the vortex veins, draining the entire globe, including the choroid. The period of ocular hypertension (OHT) is highly variable, ranging from 2 weeks to 6–7 months according to different authors.

The method described involves
inducing controlled intraocular pressure (IOP) elevation through a syringe pump, leading to ocular hypertension (OHT) and subsequent degeneration of the retina and optic nerve. This model closely resembles human glaucoma associated with heightened drainage pressure of the aqueous, resembling scenarios observed in cases of acute glaucoma. Furthermore, the transient rise in intraocular pressure (IOP) featured in our model is acknowledged to mimic the predominant aspects of acute glaucomatous disease progression. The current method offers a relatively straightforward, sturdy, and reproducible model of the disease by incorporating a pivotal modifiable risk factor along with distinct morphological and functional neurodegenerative markers. Therefore, it has the potential to contribute to uncovering both cellular and molecular mechanisms and testing novel therapies applicable to secondary degeneration. An optimal experimental glaucoma model should maintain the clarity of optical media while showcasing a chronic, progressive loss of retinal ganglion cells (RGCs). Moreover, it should be characterized by simplicity in operation and reasonable cost. Unfortunately, many of the current experimental glaucoma models do not fully embody these desired attributes.

In our study, we used a syringe pump for induced acute ocular hypertension in rat and to study acute degeneration of the RGCs in experimental glaucoma. Acute model of glaucoma based on controllable intraocular pressure with syringe pump effectively increased the IOP in rats. In the treatment group, IOP decreased quickly from the first measurement and remained relatively stable at the 8 weeks after the operation.

In acute IOP elevation, RGC loss was induced in the first week of injury but not thereafter, in an animal model of laser photocogulation-induced intraocular hypertension, the rate of RGC loss was 20–30% at 8 weeks and the rate of axon loss was 59% at 24 weeks. One study reported that the rate of RGC loss was 30% at 11 weeks and 8% at 12 weeks by another study in the model of episcleral vein cauterization and circumlimbal suture. Chen et al reported that the survival rate of RGCs was 37% in mice at 4 weeks of COH. In our study, we found that RGCs were gradually lost over time in the eyes after IOP elevation. The rates of RGC loss were 46.8% at 8 weeks of acute ocular hypertension. These animal models serve as valuable tools for unraveling the pathogenesis of glaucoma and identifying potential therapeutic targets. However, it’s important to note that each model captures only specific aspects of glaucoma rather than the entirety of the disease. Moreover, while efforts are underway to develop more sophisticated and applicable mouse models, the establishment of non-human primate models presents a challenge for future research. In summary, comprehending the strengths and limitations of individual disease models and employing them judiciously in advancing basic research will contribute to the generation of clinically relevant outcomes.

In this context, animal models have been created to investigate the impact of elevated intraocular pressure (IOP) on the optic nerve and the degeneration of retinal ganglion cells (RGC). Pertinent models for glaucoma involve inducing RGC and optic nerve damage through ocular hypertension. These animal models have significantly enhanced our understanding of the molecular mechanisms underlying this pathology by allowing comparisons of the effects of elevated IOP on ganglion cell size and death. Additionally, they have played a crucial role in advancing the development of new pharmacological interventions for glaucoma.

In this research, it is still limited in the treatment of experimental animals, differences are still needed in the application of pressure and observation time. Thus, modeling glaucoma in laboratory animals is becoming an established strategy for identifying disease mechanisms and developing new neuroprotective approaches.

CONCLUSION

Acute ocular hypertension induced through controlled intraocular pressure using a syringe pump presents a straightforward and replicable rat model of glaucoma. This model is distinguished by a sudden elevation in intraocular pressure (IOP), resulting in gradual structural impairment of the inner retina and optic nerve, accompanied by functional consequences. It offers opportunities for in vivo functional investigations and has the potential to enhance our understanding of glaucoma’s pathophysiology. Moreover, it may aid in the development of innovative therapeutic strategies for glaucomatous neurodegeneration.