Ganglion Cell Death in Rat Retina
by Persistent Intraocular Pressure Elevation

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Glaucoma is characterized by loss of retinal ganglion cells(RGCs) and their axons. Retrograde axoplasmic transport blockade and excitotoxicity were proposed to be a major cause of RGC apoptosis. We conducted this study to characterize the episcleral vessel cauterization glaucoma model in the rat with respect to decreased retrograde axoplasmic flow and subsequent apoptotic RGC death. After episcleral vessels were cauterized in Sprague-Dawley rats, Fluorogold was injected into their superior colliculi by stereotactic method. Retrograde axoplasmic flow and TUNEL-stained apoptotic dead cells were observed microscopically. Elevated intraocular pressure was maintained for up to 6 weeks during follow-up. Retrograde axoplasmic flow to the rat retina was significantly decreased. Apoptotic RGC was selectively TUNEL-stained in the retina, especially at the ganglion cell layers. We concluded that elevated intraocular pressure caused apoptotic RGC death through retrograde axoplasmic flow blockage. Further studies will elucidate the neuroprotection strategies in glaucoma patients.

Key words: apoptosis, glaucoma, retinal ganglion cell

INTRODUCTION

The loss of retinal ganglion cells (RGCs) is a well recognized feature of glaucoma. Elevated intraocular pressure (IOP) is considered a primary risk factor for the initiation and progression of glaucomatous optic neuropathy and preferentially kills RGCs without causing ancillary injury to the retina and ocular structures.[1-7] Various animal models have been proposed including optic nerve transsection and crush, ischemia and reperfusion, excitotoxic damage, epis-

cleral vein occlusion or hypertonic saline injection or trabecular meshwork transphotocoagulation, and spontaneous intraocular pressure elevations.[1-13] The cautery of two or three episcleral veins is the most commonly used procedure. We chose the vein cautery model because this method has the advantage that it is technically easier than other models and gives reliable long-term IOP elevation.

The underlying cause of glaucomatous cell death remains unknown but axonal transport blockade within the optic nerve has been demonstrated in experimental animal models.[8-10] The final common pathway of optic nerve injury is RGC death, and in most cases this seem to occur by apoptosis.[14-6] We used the In situ cell death detection kit (Roche, Mannheim, Germany) to detect and quantify apoptosis at the single cell level, based on labeling of

DNA strand breaks.

The objective of the present study was to demonstrate that retrograde axonal flow blockage by elevated IOP induced RGC apoptosis. Susceptibility of the retina or the optic nerve to glaucomatous damage, and the means to prevent it, requires a detailed understanding of the mechanism. The elucidation of the apoptotic pathways in glaucoma will introduce the era of neuroprotective strategies.

MATERIALS AND METHODS

1. Animals

Adult, male, albino Sprague-Dawley rats, weighing 200 to 250 g, were obtained from the breeding colony of the Catholic university for this study. The animals were treated according to the regulations of the Catholic Ethics Committee of the Catholic University of Korea, Seoul, which conform to all National Institute of Health(NIH) guidelines. Animals were anesthetized with 4% chloral hydrate (1ml/100 g body weight) intraperitoneally. Maintenance of anesthesia was obtained by repeated injections of smaller amounts of the same anesthetic. The pupil was dilated with 1% tropicamide eye drops and the intact retinal circulation was observed immediately after surgery. Animals were sacrificed by an overdose of chloral hydrate after 1, 3, 7, 14, 28 and 42 days.

2. Glaucoma model

A 2-mm long incision through the conjunctiva and Tenon's capsule was made on the limbal periphery within the dorsal, ventral, temporal or nasal quadrant of the eye. Two radial relaxing incisions were made and the tissue was recessed posteriorly, exposing the underlying episcleral vein. Care was taken to minimize the amount of blood loss and damage to the conjunctiva and underlying sclera. The episcleral veins were identified by their location (slightly deeper than the ocular muscles), relative immobility, larger caliber, branching pattern, the darker color of the blood contained and their communication with a limbal venous plexus. In contrast, the arteries traveled a relatively straight course, communicating with a single perilimbal artery. The veins traveled on the surface of the sclera, extending

from the venous limbal plexus and giving rise to four major venous trunks posteriorly in each eye, equidistant around the circumference of the globe and measuring 0.5 mm in diameter. By manipulating the anchored muscles, a hand-held ophthalmic cautery was applied to a point on a predetermined number of trunks, thereby blocking their corresponding area of venous drainage. There was no evidence of retinal damage and attenuation or alteration of blood vessels under operating microscopic observation. Antibiotic eyedrops and ointment were applied on the cornea to prevent corneal desiccation and infection. During recovery from anesthesia, the rats were placed in their cages.

3. IOP measurement in a chronic glaucoma model

IOP was measured with the Tonopen (Solan, Jacksonville, USA) in control and operated eyes both before and after episcleral vessel cauterization. Rats were anesthetized with intraperitoneal 4% chloral hydrate (1ml/100g body weight) and 1% proparacaine hydrochloride. To standardize the measurements, IOP was measured about 10 minutes after intraperitoneal injection so that the animals were deeply anesthetized. The mean IOP of three consecutive measurements was recorded. Intermeasurer variability was excluded by having one investigator perform all IOP measurements.

4. Stereotaxic surgery

After fixing the head of the anesthetized Sprague-Dawley rats in Stereotaxic apparatus (Stoelting, Wood Dale, USA), we incised the scalp with a #11 blade. The bleeding was controlled with H_2O_2. We searched the bregma and fixed that point with surgical apparatus. Fluorogold (Fluorochrome, Denver, USA) injections into the superior colliculi were made with the skull horizontal at the following coordinates: 6 mm posterior to the bregma, 1.2 mm lateral to the midline and 3.8 mm to 4.2 mm by the 0.2 mm from the top of the skull. Fluorogold is taken up by the axon terminals of the RGCs in the superior colliculi and transported retrogradely to the somas in the retina.

5. Retrograde labeling of RGCs

To identify RGCs, we applied the fluorescent

tracer Fluorogold (Fluorochrome, Denver, USA) to the superior colliculi, the main RGC targets in the brain. A soluble fluorescent tracer, Fluorogold is characterized as an intense fluorescent retrograde tracer that persists within RGC soma for periods of at least 3 weeks without apparent fading or leakage.

6. Estimation of RGC densities

The mean densities of FG-labeled RGCs in the ganglion cell layer of every retina were estimated by the following methods. Labeled RGCs were counted from printed fluorescent micrographs of 4 standard areas of each retina (1.5 mm from the optic disc). Each rectangular area measured 0.025 μm $\times$ 0.02 μm. The number of labeled cells in the photographs was divided by the area of the region to calculate the mean densities of labeled neurons/mm^2 for each retina. Cell counts were conducted by the same investigator in a masked fashion. The identity of the retinas that led to the micrographs was unknown until cell counts from different groups were complete. Care was taken to count only neurons with clearly visible nuclei in the enumeration of FG-labeled cells.

For statistical analysis, data from experimental and control groups after different periods of ischemia and at different time intervals were compared using the Student's t test.

7. Detection of apoptosis

Cleavage of genomic DNA during apoptosis may yield double-stranded, low molecular weight DNA fragmentation as well as single strand breaks in high molecular weight DNA. Those DNA strand breaks can be identified by labeling the free 3'-OH terminal with modified nucleotides in an enzyme reaction. The detection kits (Roche, Mannheim, Germany) are composed of 2 vials: vial 1, an enzyme solution that contains terminal deoxynucleotidyl transferase from calf thymus in storage buffer and vial 2, a label solution that contains nucleotide mixture in reaction buffer. At first we immersion-fixed the cryopreserved tissue with fixation solution (paraformaldehyde [4% in PBS, pH 7.44], freshly prepared) for 20 minutes. After washing with phosphate buffered saline (PBS) for 30 minutes, we centrifuged the microplate and permeabilized cells in 0.1% Triton X-100, 0.1% sodium citrate and finally incubated the slide for 2 minutes on ice (2-8°C). Two negative controls and a positive control were included in each experimental set up. We incubated the fixed and permeabilized cells in 50 μl/well label solution (without terminal transferase) instead of TUNEL reaction mixture for a negative control and incubated the fixed and permeabilized cells with micrococcal nuclease or DNase I, grade I(3000 U/ml –3 U/ml in 50 mM Tris-HCl, pH 7.5, 1 mg/ml BSA) for 10 min at 15-25°C to induce DNA strand breaks, prior to labeling procedures.

Table 1. Intraocular pressure(IOP) of eyes in which three limbal-derived veins were cauterized compared to the control eyes in Sprague-Dawley rats.

Time after surgery§	Intraocular pressure(mmHg)*		
	Mean (Range)	Standard deviation	P value
Control	21.1 (14-26)	3.3	< 0.001
Day 1	33.7 (22-50)	8.0	< 0.001
Day 3	36.1 (21-60)	9.2	< 0.001
Week 1	35.4 (25-44)	7.0	< 0.001
Week 2	30.8 (21-48)	7.7	< 0.001
Week 4	28.0 (23-32)	2.7	< 0.001
Week 6	27.0 (23-36)	5.1	0.009

*: *IOP was measured in anesthetized rats with Tonopen (Solan, Florida, USA).*
§: *The highest pressure measurements were obtained during the first week after surgery. Subsequently, IOP, although somewhat lower, remained statistically significantly elevated (unpaired Student's t test).*

After rinsing twice in PBS and air-drying, we added 50 *µl* of TUNEL mixtures. The samples were incubated in a dark room at 37°C for 60 minutes and then rinsed 3 times. Experimental results were evaluated using fluorescence microscopy with 450-500 nm wavelength. We used diaminobenzidine tetrachloride (DAB) after anti-POD (peroxidase) reaction in a dark room at 37°C for 30 minutes.

RESULTS

1. IOP elevation by episcleral vein cauterization

Three of the four to five major trunks formed by the limbal derived veins were exposed at the equator of the eye by incising the conjunctiva. IOP was elevated in male rats by cauterizing three episcleral vessels. On a given eye, 3-5 IOP readings taken directly from the display of Tonopen were recorded and averaged.

Tonopen readings showed 21.1 ± 3.3 mmHg (mean ± standard deviation) in normal eyes.

Episcleral vein cauterization produced marked elevation of IOP to 33.7 ± 8.0 mmHg at postoperative 1 day. Elevated IOP was maintained for 1 week, 35.4 ± 7.0 mmHg ($p < 0.001$), and for 6 weeks, at 27.0 ± 5.1 mmHg ($p = 0.009$) (Table. 1).

2. Decrease of retrograde axonal flow

The Fluorogold in the retina persists for at least 3 weeks without significant fading or leaking. By injection 1 week before sacrifice, we minimized the contribution of Fluorogold that had been taken up in the microglia from degenerationg RGCs. Compared with the control groups, the number of stained RGCs decreased significantly after operation (Figs. 1, 2). An RGC count decrease indicates the retrograde axonal transport blockage of various neurotrophic factors. Therefore retrograde axonal flow blockage can be an indirect evidence of RGC death.

3. Detection of apoptosis by TUNEL reaction

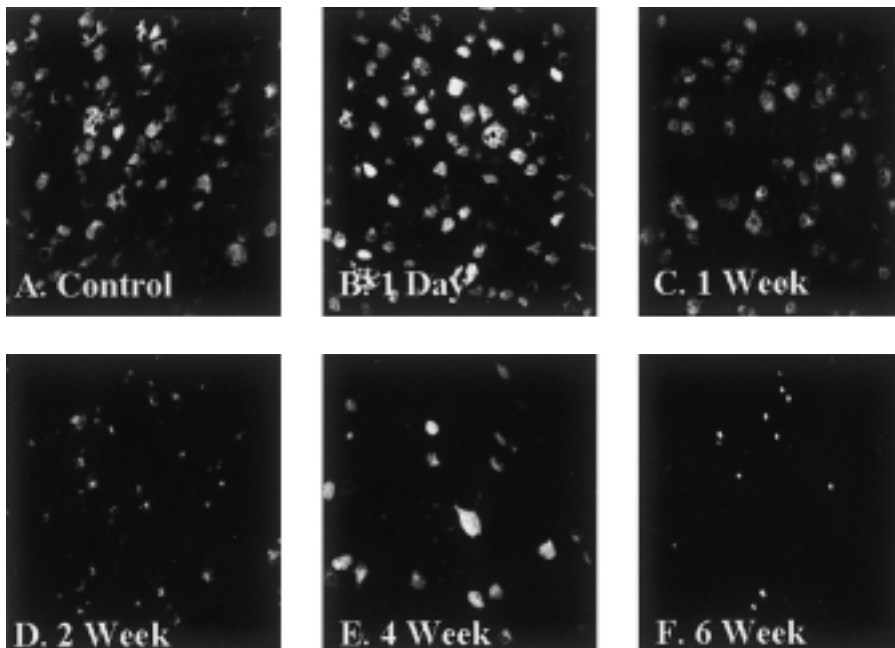In whole mount preparations, the episcleral vein

Fig. 1. Retinal ganglion cells (RGCs) in the control and cauterized eyes with chronic, moderately elevated IOP were labeled with Fluorogold. Fluorogold was microinjected into the superior colliculi of anesthetized rats in a stereotaxic apparatus (Stereotactic, Stoelting, USA). Retrograde axoplasmic flow to the RGCs decreased due to blockage.
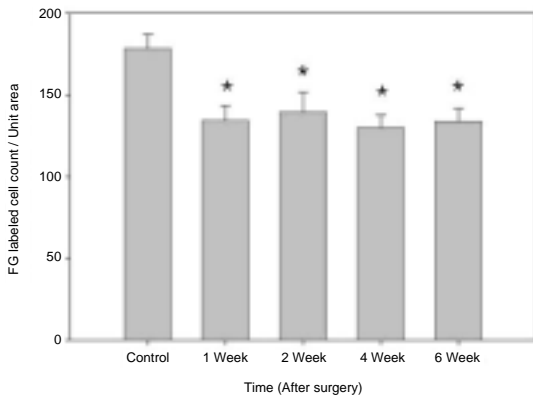
*: Significant decrease of Fluorogold labeled cells compared with control (P < 0.01)

Fig. 2. Bar histogram representing mean densities (cell/ unit area) of fluorogold labeled retinal ganglion cells (RGCs) for the experimental retina.
Densities were significantly decreased for 6 weeks. Error bar represents the standard deviation.

cauterized eyes showed many TUNEL positively reactive cells whereas the normal eyes had no TUNEL reactive cells (Fig. 3). TUNEL positive rates correlated with the duration of pressure increase and were dominantly observed at 6 weeks

after surgery. In cross-section studies apoptosis occurred selectively in RGC layers (Fig. 4), and the apoptotic cell count per 100 μm was significantly increased for 6 weeks (3.2 ± 0.48 [mean ± standard deviation] at week 1 and 7.3 ± 0.68 at week 6) (Table 2)

DISCUSSION

Glaucoma is characterized by progressive optic nerve head cupping, selective loss of RGCs and their axons, and loss of visual field. Elevated IOP is considered a primary risk factor for the initiation and progression of glaucomatous optic neuropathy.[1-7] It is recognized that mammalian RGCs whose axons are cut or severely injured along the nerve or tract generally do not survive, that elevated IOP blocks retrograde axonal transport, and that the interruption of the supply of an unknown factor to RGCs may contribute to cell loss in glaucoma.[8-10] Studies carried out over decades have therefore used the following variety of possible glaucoma models induced by different mechanisms in rodents: Crush/axotomy model, Ischemia/reperfusion model, Excitotoxic damage, Induction of elevated IOP(vein occlusion, laser, S-antigen) and spontaneous elevat-
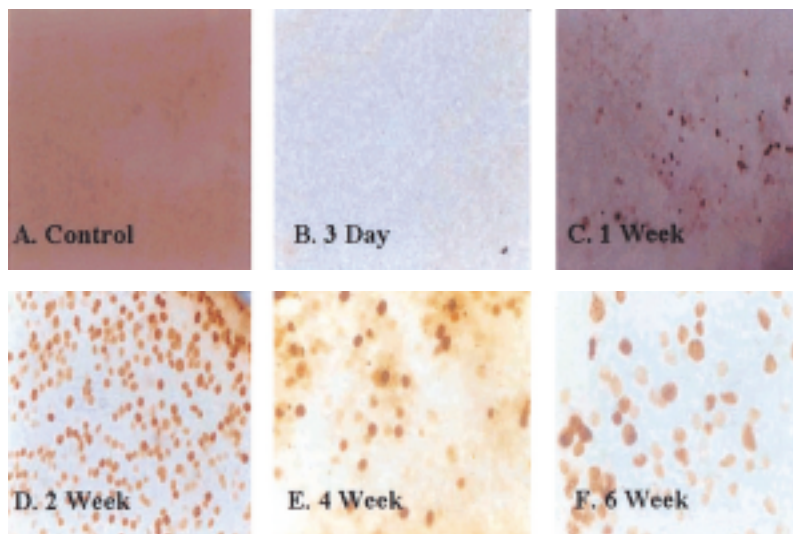

Fig. 3. TUNEL negative retinal ganglion cells (RGCs) in control eyes (A) and positive RGCs in the cauterized eyes (B-F) are visualized by confocal microscopy, Whole-mount(A-E, x 200, F. x 400). The TUNEL reaction preferentially labels DNA strand breaks generated during apoptosis. An RGC neuron with condensed chromatin clumping in the nucleus is shown. This allows discrimination of apoptosis from necrosis.
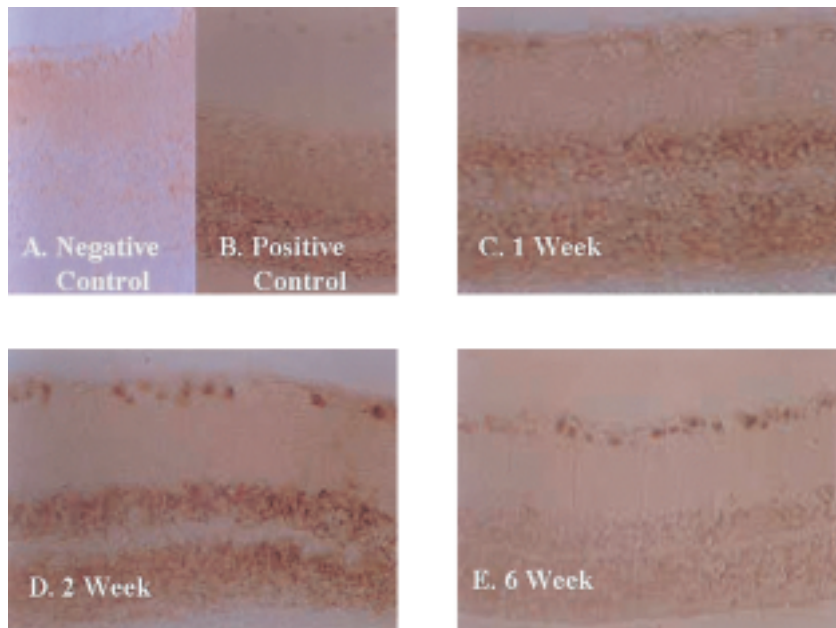
Fig. 4. TUNEL negative retinal ganglion cells (RGCs) in control eyes (A) and positive RGCs in the cauterized eyes (B-D) are seen; cross section. TUNEL labeling detects DNA fragments indicative of cellular apoptosis. Fluorescent apoptotic dead cells are shown chiefly on ganglion cell layers during postoperative 6 weeks.

Table 2. Count of TUNNEL-positive retinal ganglion cells (RGCs). The number of labeled cells with clearly visible nuclei per 100 μm was counted. The number of apoptotic ganglion cells increased significantly for postoperative 6 weeks.

Time after surgery	Number of TUNNEL-stained cell/100 μm	
	Mean ± standard deviation	*P value*
Week 1 (I)	3.2 ± 0.48	
Week 2 (II)	5.4 ± 1.24	0.002 (I & II)
Week 6 (III)	7.3 ± 0.68	0.008 (II & III)

ed IOP.[1-13] The crush/axotomy model can quantify the primary damage and demonstrate a secondary degeneration. The crush model mimics very closely the damage which occurs in traumatic optic neuropathy (TON). TON results from an indirect trauma to the optic nerve due to an impact to the head, causing a partial or complete loss of vision. These injuries appear to involve both mechanical (primary) and ischemia-induced (secondary) processes that include degeneration of nerve axons, loss of myelin, and, rarely, optic nerve hemorrhage. The crush model could be regarded as a highly acute glaucoma

model. The ischemia /reperfusion model mimics the possible chronic hypoxic stress that RGC might undergo in glaucoma. This leads to the shutdown of all energy dependent functions in the RGC with subsequent swelling and distinct disruptions of the axons. However, because it is not just specific damage to these cells but general injury to the whole retina, some neuroscientists use the model to study damage to the outer retina. This model imitates mostly the type of retinal damage that occurs in central retinal artery occlusion. In retinal ischemia elevated glutamate levels have been found in the vitre-

ous, which led to the development of an excitotoxic involvement. Excitatory amino acids or their analogues (NMDA, kainite and AMPA) induce RGC damage. But any neurological diseases can be associated with an altered state of excitatory amino acid turnover/metabolism and therefore it seems unlikely that this would be a unique feature of glaucoma. Spontaneous occurrence of primary and secondary glaucoma has been found. DBA/2NNia, DBA/2J and AKXD28/Ty inbred mouse strains have been found to spontaneously develop a form of secondary glaucoma in almost 100% of the individuals. These mouse systems will provide a powerful experimental system for drug studies. Elevated IOP is considered a primary risk factor for the initiation and progression of glaucomatous optic neuropathy. The development of rodent models with surgically induced, chronically elevated IOP to study the disease was the major aim. In both the S-antigen injection and the laser photocoagulation model inflammation and peripheral anterior synechia were found. Furthermore, multiple sessions were required in laser and hypertonic saline injection model. The vein cautery model has the advantage of being technically easier than the other models discussed above and is said to give a more reliable long term IOP elevation. Induced secondary glaucoma in rats mimics acutely increased IOP in humans following carotid cavernous fistula, cavernous venous thrombosis, orbital venous obstruction and obstruction of the superior vena cava. We choose the episcleral vein cautery model because it matches clinical glaucoma in IOP elevation range as well as pathophysiologically and has few indivisual variations. To exclude the possibility of venous occlusion in the intraocular vasculature, we performed fundoscopic examination.

Two distinct modes of cell death, apoptosis and necrosis, can be distinguished based on differences in morphological, biochemical and molecular changes of dying cells.[14-6] Apoptosis is a form of cell death designed to eliminate unwanted host cells through activation of a coordinated, internally programmed series of events effected by a dedicated set of gene products. Morphologic features such as cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and apoptotic bodies and phagocytosis of apoptotic cells or bodies, some best seen with the electron microscope, characterize cells undergoing apoptosis. Apoptotic cells usually exhibit a distinctive constellation of biochemical modifications such as protein cleavage, protein cross-linking, DNA breakdown and phagocytic recognition. Especially internucleosomal cleavage of DNA into oligonucleosomes in multiples of 180 to 200 base pairs by Ca- and Mg-dependent endonucleases are visualized by agarose gel electrophoresis as DNA ladders. The in situ cell death detection kit, fluorescein (Roche, Mannheim, Germany) labels TdT-mediated dUTP nick end by terminal deoxynucleotidyl transferase (TdT). Considering the extremely small fraction of apoptotic cells in control eyes, TUNEL positive cells suggest apoptotic ganglion cell loss by elevated IOP.

In conclusion, our present study demonstrated that retrograde axonal flow blockage by elevated IOP induced RGC apoptosis. Apoptotic glaucomatous cell death is known as a secondary process rather than a direct cell body injury. Therefore, there is a time lags between axonal injury and cell death. Consequently, the idea to slow the process of neuronal degeneration and prolong the survival of neurons, that are otherwise destined to die within a short time frame, is particularly appealing. Although the main current therapy for glaucoma consists of the use of IOP-lowering drugs, new efforts are presently being directed towards the development of drugs that may halt or slow the progressive death of RGCs.

REFERENCES

1. Morrison JC, Fraunfelder FW, Milne ST, Moore CG. Limbal microvasculature of the rat eye. *Invest Ophthalmol Vis Sci*. 1995;36:751-756.

2. Valenzuela EG, Shareef S, Walsh J, Sharma SC. Programmed cell death of retinal ganglion cells during experimental glaucoma. *Exp Eye Res*. 1995;61: 33-44.

3. Sawada A, Neufeld AH. Confirmation of the rat model of chronic, moderately elevated intraocular pressure. *Exp Eye Res*. 1999;69:525-531.

4. Johnson EC, Deppmeier LM, Wentzien SK, Wentzien SK, Hsu I, Morrison JC. Chronology of optic nerve head and retinal responses to elevated intraocular pressure. *Invest Ophthalmol Vis Sci*. 2000;41:431-432.

5. Jia L, Cepurna WO, Johnson EC, Morrison JC. Patterns of intraocular pressure elevation after aqueous humor outflow obstruction in rats. *Invest Ophthalmol Vis Sci*. 2000;41:1380-1385.

6. Mittag TW, Danias J, Pohorenec G, Yuan HM, Burakgazi E, Redman RC, Pods SM, Tatton WG. Retinal damage after 3 to 4 months of elevated intraocular pressure in a rat glaucoma model. *Invest Ophthalmol Vis Sci*. 2000;41:3451-3459.

7. Bayer AU, Danias J, Brodie S, Maag KP, Chen B, Shen F, Podos SM, Mittag TW. Electroretinographic abnormalities in a rat glaucoma model with chronic elevated intraocular pressure. *Exp Eye Res*. 2001;72: 667-677.

8. Quigley HA, Nickells RW, Kerrigan LA, Pease ME, Thibault DJ, Zack DJ. Retinal ganglion cell death in experimental glaucoma and after axotomy occurs by apoptosis. *Invest Ophthalmol Vis Sci*. 1995;36:774-786.

9. Quigley HA, Mckinnon SJ, Zack DJ, Pease ME, Baumrind LA, Kerrigan FK, Mitchell RS. Retrograde axonal transport of BDNF in retinal ganglion cells is blocked by acute IOP elevation in rats. *Invest Ophthalmol Vis Sci*. 2000;41:3460-3466

10. Ko ML, Hu DN, Ritch R, Sharma SC. The combined effect of brain-derived neurotrophic factor and a free radical scavenger in experimental glaucoma. *Invest Ophthalmol Vis Sci*. 2000;41:2967-2971.

11. Goldblum D, Mittag T. Prospects for relevant glaucoma models with retinal ganglion cell damage in the rodent eye. *Vision Res*. 2002;42:471-478.

12. Levkovitch-Verbin H, Quigley HA, Keith R, 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.

13. Morgan JE. Retinal ganglion cell shrinkage in glaucoma. *J Glaucoma*. 2002;11:365-370.

14. Tatton NA, Tezel Z, Insolia SA, Nandor SA, Edward PD, Wax MB. In situ detection of apoptosis in normal pressure glaucoma: preliminary examination. *Surv Ophthalmol*. 2001;45;S268-S272.

15. Wang X, Tay SS, Ng YK. An immunohistochemical study of neuronal and glial cell reactions in retinae of rats with experimental glaucoma. *Exp Brain Res*. 2000:476-484.

16. Wein FB, Levin LA. Current understanding of neuroprotection in glaucoma. *Curr Opin Ophthalmol*. 2002;13:61-67.