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Investigation of Neuroprotective Effects of Ripasudil in Mechanic Optic Nerve Injury Model

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Abstract

Purpose: We aimed to create mechanic optic nerve injury model in rats and investigate the neuroprotective effects of ripasudil on retinal ganglion cells.

Study Design: Experimental study

Methods: Mechanic optic nerve injury model was created in the right eyes of male Wistar rats (n=15). Rats were divided into three groups: glaucoma model with sham treatment (group1) and 20 μM intravitreal ripasudil treatment (group 2) and 50 μM intravitreal ripasudil treatment (group 3). Treatment was applied intravitreally and rats were sacrificed at the end of 4 weeks. Glial fibrillary acidic protein (GFAP), Brn-3a antibody,anti- Iba1 was examined by immunohistochemistry.

Results: The number of Brn-3a positive RGC in the mechanical optic nerve injury model was 5.33 ± 2.08 (min: 3, max: 7) in sham group, 10.25 ± 2.63 (min: 8, max: 14) in 20 μM group and 16.75 ± 5.43 (min:9, max: 21) in 50 μM group ($p < 0.05$). GFAP positive RGC counts were recorded as 24.33 ± 2.08 (min: 22, max: 26) in sham group, 16.75 ± 1.70 (min: 15, max: 19) in 20 μM group and 13.00 ± 4.08 (min:10 , max: 19) in 50 μM group ($p < 0.05$). Ripasudil treatment also decreased Iba1 expression in the retina of mechanic optic nerve injury groups. In addition, ripasudil treatment prevented apoptotic cell death by increasing Bcl-xL protein expression and preserved Tfam protein expression in the retina.

Conclusions: Our experimental study has shown that ripasudil is neuropreotective in mechanical optic nerve injury model.

Keywords: Rat Glaucoma Model, Neuroprotection, Ripasudil, Retinal Ganglion Cell Number

Introduction

Glaucoma is a degenerative optic neuropathy and leading cause of irreversible blindness worldwide. Glaucoma patients suffer from progressive visual field loss due to deterioriation of optic nerve and retinal ganglion cell (RGC) loss [1]. Elevation of intraocular pressure (IOP), advanced age, family history,thin central corneal thickness, myopia are the most important risk factors for glaucoma [2]. The IOP elevation is major and clinically modifable risk factor responsible for the glaucomatous optic neuropathy involving death of RGCs and their axons. Currently, we know that glaucoma is a multifactorial disease, with several factors, including retinal vascular autoregulation, oxidative stress and free radical formation, alterations in local cytokines, glutamate exitotoxicity and irregular optic nerve perfusion pressure, having been implicated in its pathogenesis. Furthermore, ıt is shown that in some glaucoma patients, death of RGCs continues despite IOP reduction. Therefore, elevated IOP is now believed to be an important but not the only factor responsible for optic nerve damage [3].

The changing concepts in glaucoma pathophysiology allows to recognize new therapeutic goals such as neuroprotective and antioxidant therapies. The use of neuroprotective drugs in glaucoma has been intensively studied in recent years. Novel treatment strategies have been recently explored, such as protecting RGCs, reducing astroglial and microglial cell activation or increasing retinal-choroidal blood flow [4]. Although these new agents affect many factors among the pathogenesis of glaucomatous neuropathy, most of them have been shown in experimental studies and some of them have ongoing phase studies.

Rho-associated kinase (ROCK) controls multiple signaling pathways and mediates many cellular functions such as shape, actin cytoskeleton organization, cell adhesion, motility, secretion, proliferation, gene expression, inflammation. ROCK inhibitors have been examined for various diseases other than glaucoma, such as cardiovascular disease, diabetic nephropathy, stroke, and central nervous system diseases including Alzheimer disease [5,6,7].

The mechanism of the IOP-reducing effects of several ROCK inhibitors have been documented.ROCK inhibitors may alter the behavior of trabecular meshwork (TM) cells, increases conventional aqueous humor outflow, thus reducing IOP[8] [9]. Recent studies showed that ROCK inhibitors likely increase conventional aqueous humor outflow, accompanied by reorganization of the TM cells, increased giant vacuoles in Schlemm's canal endothelial cells and endothelial cell permeability and disrupting tight junctions [10,11].

Ripasudil hydrochloride hydrate (K-115), a small-molecule ROCK inhibitor developed for the treatment of glaucoma and ocular hypertension was approved for use in Japan in 2014 [12]. K-115 directly alters the extracellular matrix of the TM, Schlemm's canal endothelial cells, actomyosin contractility of the corneal endothelial cells [13,14].

Various studies demonstrated that ripasudil lowers IOP, however its role in neuroprotection must still be clarified. In this study we aimed to create anexperimental glaucomamodelin rats and investigate the neuroprotective effects of intravitreal ripasudil molecules on retinal ganglion cells and axons.

Materials and Methods

Animals

Male Wistar rats (250–300 g) were housed in covered cages, fed with a standard rodent diet and maintained on a 12-h light–dark cycle. The research followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the research was approved by the Gazi University Ethics Committee. All efforts were made to minimize the number of animals used and their suffering. There were 3 groups consisted of 5 animals per group, group 1 (sham treatment with mechanic optic nerve injury model), group 2 (20 μM intravitreal ripasudil treatment with mechanic optic nerve injury model), group 3 (50 μM intravitreal ripasudil treatment with mechanic optic nerve injury model). All procedures were performed to the right eyes of the animal. The left eye of each animal served as the control eyes. Four weeks following injection, the rats were euthanatized and eyes were enucleated.

Induction of Mechanic Optic Nerve Injury Model

The ratswere anesthetized by intramuscular injection ofketamine (100 mg/kg,Ketaset,Fort Dodge Animal Health, Fort Dodge, IA, USA) and xylazine (9 mg/kg, TranquiVed, Vedeco, Inc., St. Joseph, MO, USA). Eyes were also treated with 1 % proparacaine drops. An incision of the conjunctiva lateral to the cornea was made under an operating microscope (Zeiss-S5, Carl Zeiss, Inc., Göttingen, Germany). The retractor bulbs muscle was separated and the optic nerve was exposed beneath the external ocular muscle by blunt dissection. The Yasargil aneurysm clip was then placed with applying forceps (catalog no FT250T, Aesculap AG&Co.) ,2 mm behind the posterior eye pole for 60 seconds, and care was taken to avoid compromising the ophthalmic artery. After the injury, the rat fundus was examined by indirect ophthalmoscopy to confirm the patency of the central retinal artery[11].

Pharmacological Treatment

Ripasudil was dissolved in phosphate-buffered saline (PBS, pH 7.4) at two final concentrations which are 20 μM and 50 μM. Ripasudil solutions and PBS were sterilized with filtration method by using 0.22 μm cellulose acetate sterile syringe filter. After sterilization, 5 μL PBS only and 5 μL ripasudil solutions were administered to the vitreous via a stereotactically positioned 30-gauge needle attached to a 10-μl Hamilton syringe. The eye was examined ophthalmoscopically to check that the retinal vasculature was intact.

Western Blot Analysis

The retinas were immediately homogenized with homogenizer in RIPA lysis buffer (150 mM sodium chloride, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 1 mM dithiothreitol, 0.5% sodium deoxycholate, and 50 mM Tris-cl [pH 7.6]) containing complete protease inhibitors (Roche Biochemicals, Indianapolis, IN). Ten micrograms of pooled retinal protein from each group (n = 3 retinas per group) was separated by SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes. The membrane was blocked with PBS–0.1% Tween 20 containing 5% nonfat dry milk for 1 hour at room temperature and subsequently incubated with the primary antibodies for 16 hours at 4°C. The primary antibodies were rabbit monoclonal anti-GFAP an tibody (Cell Signaling, Danvers, MA), rabbit monoclonal beta-actin antibody (Cell Signaling, Danvers, MA), rabbit polyclonal anti-Tfam antibody (Cell Signaling, Danvers, MA), rabbit polyclonal anti-Bax antibody (Cell Signaling, Danvers, MA), and rabbit polyclonal anti–Bcl-Xl antibody (Cell Signaling, Danvers, MA). After several washes in Tween/PBS, the membranes were incubated with anti rabbit IgG HRP Linked Antibody (Cell Signaling, Danvers, MA) and film developed by using anti rabbit phototopeHRP western blot detection system (Cell Signaling, Danvers, MA). We used Image-J program for quantification.

Tissue Preparation

Enucleated globes were fixed in 10% buffered formalin. . To examine all the layers of the eye, alcohol was added to the fixation solution and intravitreal alcohol injection was performed. The globes were cut into two halves with horizontal sections from optic nerve to cornea. Tissue microarray samples were formed from the paraffin blocks.

For immunohistochemical examination, BRN3A (1:500, clone: EP1972Y, EDTA, Genetex, Irvine, CA), Iba-1 (1:1000, clone: policlonal, EDTA,Synaptic Systems, Göttingen, Germany), GFAP (1:50-1:150, clone: GA-5, Citrat buffer,Genetex, Irvine, CA) were used.

One pathologist counting the RGCs was blind to the experimental procedures. Ten visual fields were sampled from the posterior portion of each retina using x 40 magnification objective.

Statistical Analysis

Statistical significance in cell number and proportion of cells in retinal layer between groups were determined by Mann-Whitney U test. Statistical analyses were performed with SPSS 18.0 for windows (SPSS Inc, Chicago, Illinois, USA). The level of statistical significance was set at $P<0.05$.

Results

Evaluation of Brn-3a positive Retinal Ganglion Cell

In our study, wedemonstratedthat 20 μM and 50 μM ripasudil treatment protects RGCs inexperimental glaucoma model. We determined RGC survival following treatment in rat retinas by immunohistochemistry using antibody raised against Brn3a.

In our study, the number of Brn-3a positive RGC in the mechanical opticnerve injury model was 5.33 ± 2.08 (min: 3, max: 7) in sham group, 10.25 ± 2.63 (min: 8, max: 14) in 20 μM group and 16.75 ± 5.43 (min:9 , max: 21) in 50 μM group. When we compared the groups for the protection of Brn-3a, there was statistically significant difference between sham group and 20 μM group- 50 μM group ($p < 0.05$) (Figure 1). There was no statistically significant difference between 20 μM and 50 μM treatment group.

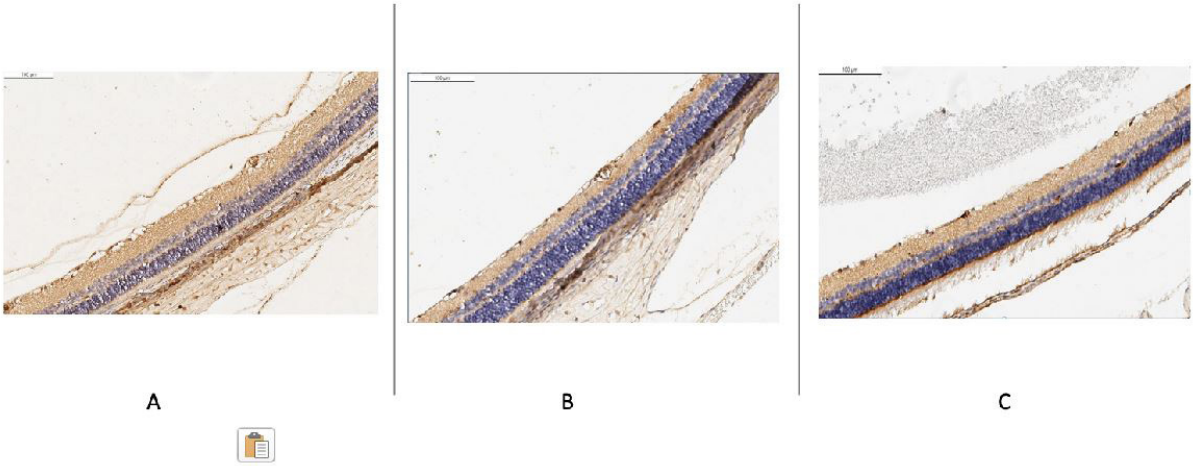

Figure 1: Brna 3a immunostaining, mechanic optic nerve injury model retinas: eye with sham treatment (A), with intravitreal 20 μM ripasudil treatment (B), with intravitreal 50 μM ripasudil treatment (C)

To determine the effect of ripasudil on the apoptotic cell death pathway we performed Western blot analysis using antibodies raised against Bax and Bcl-xL. *Bax protein expression was significantly increased in the retinas of sham treatment group compared with control eyes. 20* μM *Ripasudil treatment decreased Bax protein expression compared with the 50* μM and sham treatment group. In addition, 50 μM ripasudil treatment increased Bcl-xL protein expression in the mechanic optic nerve injury model when compared to 20 μM and sham treatment group (Figure 2).

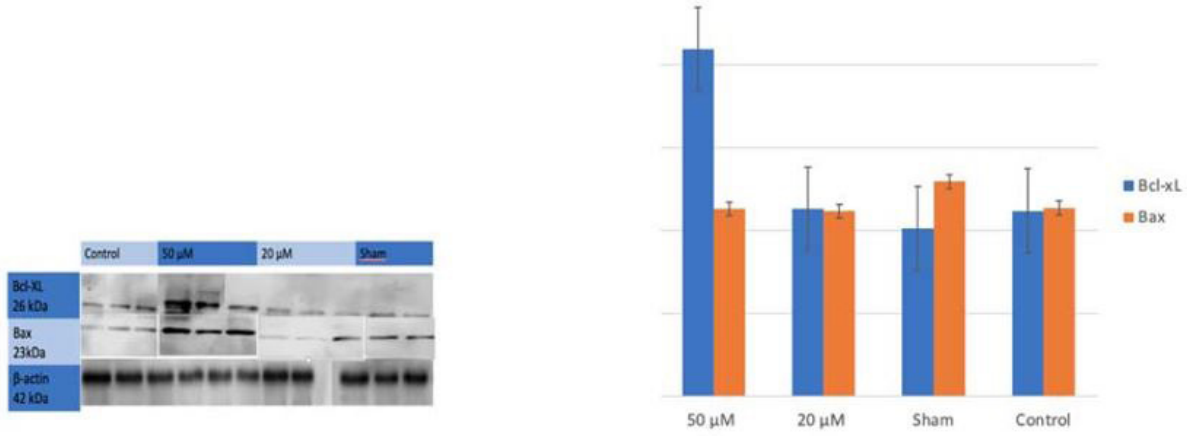

Figure 2: Bax and Bcl-xL protein expression in the mechanic optic nerve injury model

Expression of Glial Fibrillary Acidic Protein and Iba-1 antibody

RGC loss was accompanied by activation of astroglial and microglial cell as indicated by significantly increased GFAP and anti- Iba-1 expression.

In our study, GFAP immunohistochemistry was performed to evaluate the astoglial cell activation in response to mechanical optic nerve injury. GFAP positive astroglial cells counts were recorded as 24.33 ± 2.08 (min: 22, max: 26) in sham group, 16.75 ± 1.70 (min: 15, max: 19) in 20 μM group and 13.00 ± 4.08 (min:10 , max: 19) in 50 μM group. When we compared the groups, there was statistically significant difference between sham group and 20 μM group- 50 μM group ($p < 0.05$). There was no statistically significant difference between 20 μM and 50 μM group. (Figure 3).

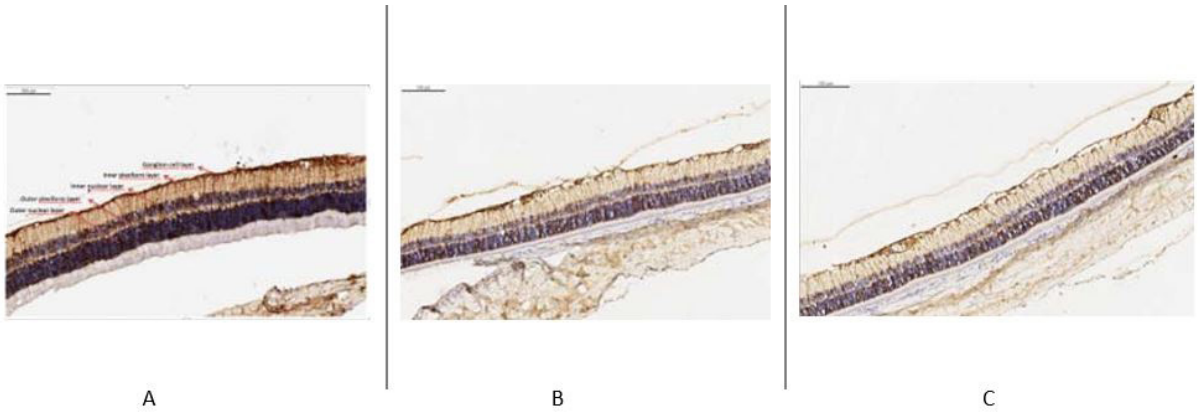

Figure 3: GFAP immunostaining, mechanic optic nerve injury model retinas: eye with sham treatment (A), with intravitreal 20 μM ripasudil treatment (B), with intravitreal 50 μM ripasudil treatment (C)

Anti Iba-1 positive cell counts were recorded as 89.33 ± 9.60 (min: 79, max: 98) in sham group, 67.0 ± 2.64 (min:64,max:69) in 20 μM group and 61.0±2.16 (min:59, max:64) in 50 μM group.When we compared 3 groups, herewas statistically significant decrease in ripasudil treatment group. More importantly, decrease in 50 μM group was significant when we compared with 20 μM group ($p < 0.05$) (Figure 4).

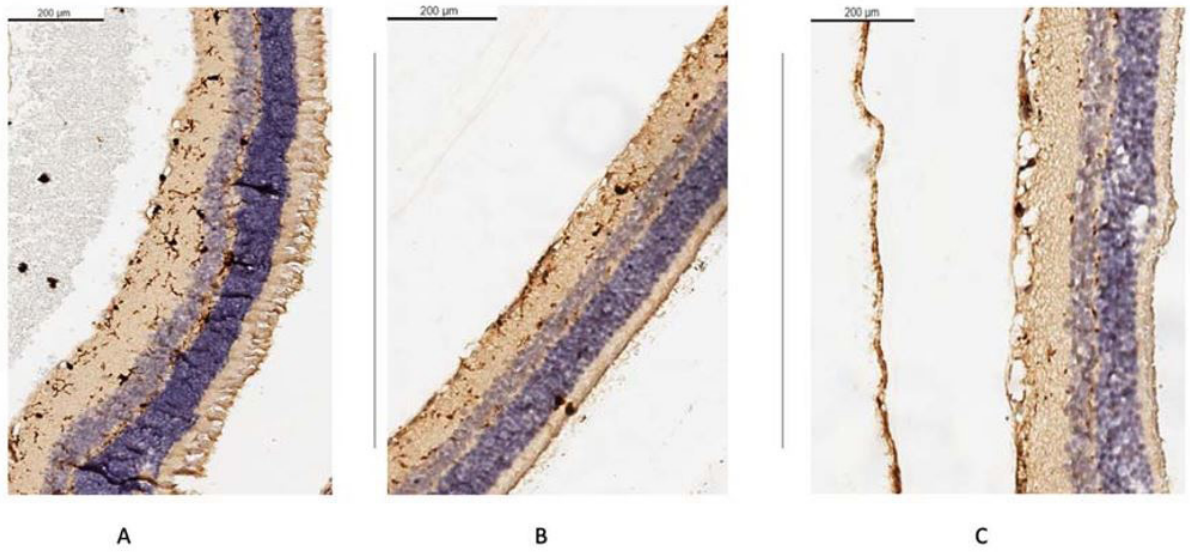

Figure 4: Iba-1 immunostaining, mechanic optic nerve injury model retinas: eye with sham treatment (A), with intravitreal 20 μM ripasudil treatment (B), with intravitreal 50 μM ripasudil treatment (C)

Expression of T fam

To determine whether oxidative stress induced by mechanic optic nerve injury alters Tfam and whether ripasudil treatment preserves this alteration in retina, Tfam expression was determined by Western blot analysis. We found that both 20μM and 50 μM ripasudil preserved Tfam protein expression compared with sham treatment group in mechanic optic nerve injury model (Figure 5).

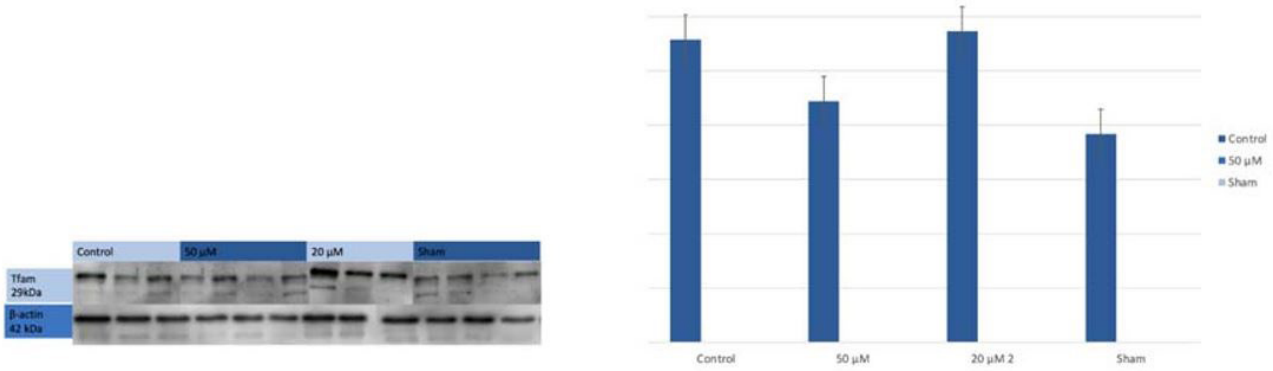

Figure 5: 20μM and 50 μM ripasudil preserved Tfam protein expression compared with sham treatment group in mechanic optic nerve injury model

Discussion

ROCKinhibitors havebeen widelyused for the treatmentofmanyneurodegenerative diseases such as Alzheimer's disease. spinal cord injuries,stroke, multiple sclerosis and glaucoma. The inhibitors of this pathway are still being used in the treatment of this disease and the newly discovered molecules of this group are promising for the future [15-19].

Previous studies reported that in the optic nerve head of patients with primary open-angle glaucoma there was a significant increase in the RhoA protein levels. That might lead to excessive activation of ROCK [20].ROCK inhibitors have been shown to reduce IOP in animal and human eyes. They directly modulate outflow by decreasing resistance in trabecular meshwork cells and tissue, this changes the behavior of Schlemm's canal endothelial cells and alters the production and pROCK inhibitors [8,11,13,21,22].

In addition to these two positive effects of glaucoma, it has recently been discussed that ROCK inhibitors may have neuron protective activities. Although the mechanism of this effect has not yet been fully elucidated, it may be directly related to ROCKinhibition. Studies have shown that targeting small Rho GTPase has a dose-dependent effect on regeneration of RGDs after injury to optic nerve damage.It has been reported that ROCK activity increased in the RGC layer after the axonal injury model [9,23,24].

In the previous neuroprotection and axonal regeneration studies, fasudil was used as the precursor molecule of this group.A recent study on fasudil has shown that after optic nerve injury model, fasudil has reduced apoptosis in cells and significantly increases axonal regeneration. ROCK inhibitor,Y-39983, suppresses ROCK expression in retinal tissues in a model of mouse optic nerve injury [25,26].

In this study, the neuron protective activity of ripasudil was investigated. It was reported that oral administration of ripasudil delayed RGC death in the optic nerve damage model [15]

It has been reported that ROCK inhibitors such as ripasudil can prolong the survival of RGC by suppressing oxidative stress in pathways involving the Nox1 family. In addition, this study suggests that ripasudil does not directly inhibit the production of reactive oxygen species (ROS), but by an indirect mechanism. [22] A recent study showed that the ROCK inhibitor L-F001 inhibits PC12 cell death by reducing the stress of the endoplasmic reticulum [16].

The last study about the neuroprotective mechnanism of ripasudil suggested that ripasudil-enhanced intra-axonal autophagy is at least partly involved in axonal protection. In that study TNF-induced optic nerve damage was created and study researched the effect of different ripasudil dose on optic nerve [17].

In another recent study, it has been shown that ripasudil applied in two different doses topically in normal tension glaucoma model decreases glaucomatous retinal degeneration besides IOP reduction [18].

Our study was the first study that investigate the neuroprotective role of two different intravitreal ripasudil doses in the mechanical optic nerve injury model and this activity was investigated by using immunohistochemical and western blot analysis. In this study, the effects of two different doses of intravitreal intravitreal ripasudil on RGC survival and glial cell reaction were investigated. At the end of the study, it was found that both 20 μM and 50 μM intravitreal ripasudil had a significant neuron protective effect on RGC density, resulting in reduced loss of RGC and reduced glial and microglial cell activation in mechanic optic nerve injury model.

Our study is valuable for investigating the efficacy of new molecules in glaucoma and for determining possible additional benefits. Although the importance of the hypotensive approach in the treatment of glaucoma is known, there are cases in which progression cannot be stopped only with IOP control. Therefore, neuron protective treatment approaches should be evaluated in glaucoma which is accepted as a neurodegenerative disease. Current data suggest that ROCK inhibitors and new molecules of this group are prominent in terms of IOP drop, ocular blood flowincrease, and possible neuron protective effects in which research is relatively new. Of course, research on different candidate molecules will also contribute significantly to the development of new treatment strategies in glaucoma.

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