

# c-Jun N-terminal kinase 3 expression in the retina of ocular hypertension mice: a possible target to reduce ganglion cell apoptosis

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## Abstract

Glaucoma, a type of optic neuropathy, is characterized by the loss of retinal ganglion cells. It remains controversial whether c-Jun N-terminal kinase (JNK) participates in the apoptosis of retinal ganglion cells in glaucoma. This study sought to explore a possible mechanism of action of JNK signaling pathway in glaucoma-induced retinal optic nerve damage. We established a mouse model of chronic ocular hypertension by reducing the aqueous humor followed by photocoagulation using the laser ignition method. Results showed significant pathological changes in the ocular tissues after the injury. Apoptosis of retinal ganglion cells increased with increased intraocular pressure, as did JNK3 mRNA expression in the retina. These data indicated that the increased expression of JNK3 mRNA was strongly associated with the increase in intraocular pressure in the retina, and correlated positively with the apoptosis of retinal ganglion cells.

**Key Words:** nerve regeneration; ocular hypertension; JNK3; retinal ganglion cell; glaucoma; laser photocoagulation; intraocular pressure; neural regeneration

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## Introduction

Glaucoma is a type of optic neuropathy and a major cause of blindness in the world. It is characterized by the vertical expansion of the cupping of the optic nerve head and the loss of retinal ganglion cells (Hatt et al., 2006). Over the years, there have been two accepted theories addressing the pathogenesis of glaucoma: the mechanical theory (Flammer et al., 1992; Halpern and Grosskreutz, 2002) and the hemorheological theory (Chung et al., 1999; Emre et al., 2004; Grieshaber and Flammer, 2005). The current deeper understanding of glaucoma pathogenesis suggests that the traditional mechanical pressure and vascular theories are insufficient to explain the pathogenesis of glaucomatous optic nerve damage. Other pathological mechanisms are likely to lead to the injuries to retinal ganglion cells and the optic nerve.

c-Jun N-terminal kinase (JNK) is in a class of serine/threonine kinases. The JNK signaling pathway plays an important role in cellular stress response, and can be activated by a variety of extracellular stress signals. It is also known as stress-activated protein kinase (SAPK) (Johnson and Na-

kamura, 2007). JNK is associated with the pathogenesis of many diseases, such as Parkinson's disease (Wilhelm et al., 2007), diabetes (Yang et al., 2007) and carcinogenesis (Sakurai et al., 2006). The relationship between JNK and apoptosis of retinal ganglion cells in glaucoma is still poorly understood.

This study explores a possible mechanism of action of JNK signaling pathway in glaucomatous optic nerve damage using a mouse model of chronic ocular hypertension and provides a theoretical basis for future research concerning this new target for the treatment of glaucoma.

## Materials and Methods

### Animals

A total of 44 clean male C57BL/6 mice aged 6 weeks and weighing 18–22 g were provided by the Chongqing Experimental Animal Center (License No. SCXK (Yu) 2007-0001) in China. All mice were fed with solid feed and clean water, and housed at 21°C in a 12-hour light/dark cycle in a specific pathogen-free environment. The protocols were approved by the Animal Ethics Committee of Kunming Medical University in China.

### Establishment of mouse models of chronic ocular hypertension

The right eyes of C57BL/6 mice were used as experimental eyes, and the left eyes as untreated controls. At 30 minutes before surgery, the right eye of each mouse was administered mydriatic (atropine eye drops; Shenyang Xing Qi Pharmaceutical Co., Ltd., Shenyang, Liaoning Province, China) and compound tropicamide eye drops (Santen Pharmaceutical Co., Ltd., Osaka, Japan). After being weighed on an electronic scale (Wuxi Weigher Factory Co., Ltd., Wuxi, Jiangsu Province, China), the mice were injected intraperitoneally (i.p.) with 1.7 mL/kg 3% sodium pentobarbital. Simultaneously, each right eye was administered with three drops of oxybuprocaine hydrochloride (Santen Pharmaceutical Co., Ltd.). The behaviors of the mice were observed closely. When they did not react to a pinch of their dorsal skin, the surgery could begin. Under a slit lamp microscope (Chongqing Kanghua Medical Equipment Co., Ltd., Chongqing, China), a 0.3 mm × 13.0 mm needle punctured the anterior chamber from the corneal limbus below the nose to release aqueous humor. Thus, the anterior chamber became shallow and the anterior chamber angle closed. A 532-diode laser (Zeiss 532 laser machine, Jena, Germany) was directly used to perform right cornea edge 360° photocoagulation. The laser energy, illumination time, and light spot size were 100 mW, 0.05 seconds and 200 μm, respectively. The number of light spots was 93 ± 8. The whole procedure took less than 10 minutes (Aihara et al., 2003; Mabuchi et al., 2003). After the surgery, mice were administered chloramphenicol eye drops (Bausch & Lomb, Jinan, Shandong Province, China) and erythromycin ointment (Nanjing Baijingyu Pharmaceutical Co., Ltd., Nanjing, Jiangsu Province, China). The general condition of the mice and their level of consciousness were monitored. A total of 33 models of ocular hypertension were successfully established. Eleven were excluded, because one mouse died after general anesthesia, two mice suffered from corneal opacity, two mice developed cataracts, one mouse experienced atrophy of the eyeball, one mouse suffered from perforation of the eyeball, and intraocular pressure of a further four mice did not exceed 30% of preoperative intraocular pressure.

### Intraocular pressure measurement

We observed whether conjunctival hyperemia, corneal edema, or anterior chamber reaction appeared at 1 and 3 days after the surgery. A TONO-PEN AVIA pen tonometer (Reichert, Inc., Depew, NY, USA) was used to measure intraocular pressure at 3 days after the surgery. Subsequently, intraocular pressure was measured every 4 days. Using a previously accepted standard measurement (Aihara et al., 2003), intraocular pressure after laser photocoagulation increased by more than 30% compared with before laser photocoagulation, which indicated that the models were successful. That is, intraocular pressure (%) =  $100\% \times (\text{intraocular pressure}_{\text{after photocoagulation}} - \text{intraocular pressure}_{\text{before photocoagulation}}) / \text{intraocular pressure}_{\text{before photocoagulation}} \geq 30\%$ . At 3 days after photocoagulation, if intraocular pressure of the experimen-

tal eyes exceeded 30% of preoperative intraocular pressure, they were not treated again. If intraocular pressure of experimental eyes was lower than 30% of preoperative intraocular pressure, laser photocoagulation was performed again at 7 days.

### Sample collection and hematoxylin-eosin staining

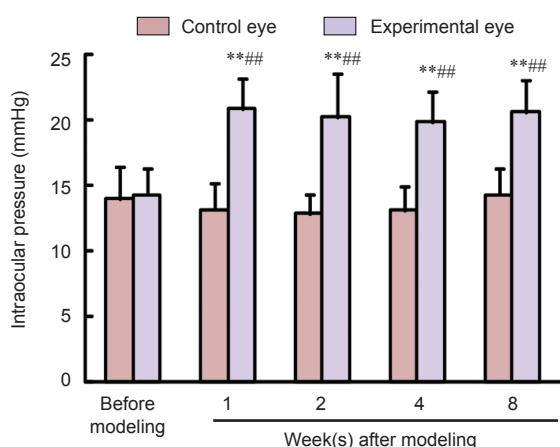
Eight mice were separately collected at 1, 2, 4 and 8 weeks after model establishment. All mice were intraperitoneally anesthetized with 3% sodium pentobarbital (1.7 mL/kg). Each eyeball was coronally incised along the pars plana. The anterior segment was discarded. The retina was carefully removed, dried with a filter paper, fixed, and stained with hematoxylin and eosin.

### Apoptosis of retinal ganglion cells as detected by TdT-mediated dUTP-biotin end labeling (TUNEL)

Frozen sections of mouse retina were fixed with 4% paraformaldehyde/0.01 M PBS (pH 7.4), washed with PBS for 5 minutes × 2, and then fixed with ethanol and acetic acid (2:1) at -20°C for 5 minutes in a coplin jar, followed by PBS washes for 5 minutes × 2. Sections were treated with a balanced solution (pH 7.4) at room temperature for 10 seconds, and incubated with TDT enzyme (Millipore, Boston, MA, USA) at 37°C in a wet box for 1 hour. Sections were shaken in a coplin jar containing a termination solution for 15 seconds, and incubated at room temperature for 10 minutes. The tissue was coated with anti-Dig 65 μL/5 cm<sup>2</sup> in a wet box at room temperature for 30 minutes, covered with peroxidase substrate at room temperature for 5 minutes, incubated in a series of coplin jars with double-distilled water at room temperature for 5 minutes, counterstained with 0.5% methyl green for 10 minutes, washed three times with double-distilled water and 100% butanol, incubated with xylene for 2 minutes, then mounted with resin and observed under a light microscope (Shanghai Optical Instrument Factory, Shanghai, China). Apoptotic nuclei were stained brown. The tissue was sliced into 5-μm-thick sagittal sections taking the optic axis as the center. Each eyeball was cut into two or three sections. Three fields were randomly selected from each section under a 400× objective lens. TUNEL-positive retinal ganglion cells were observed and quantified.

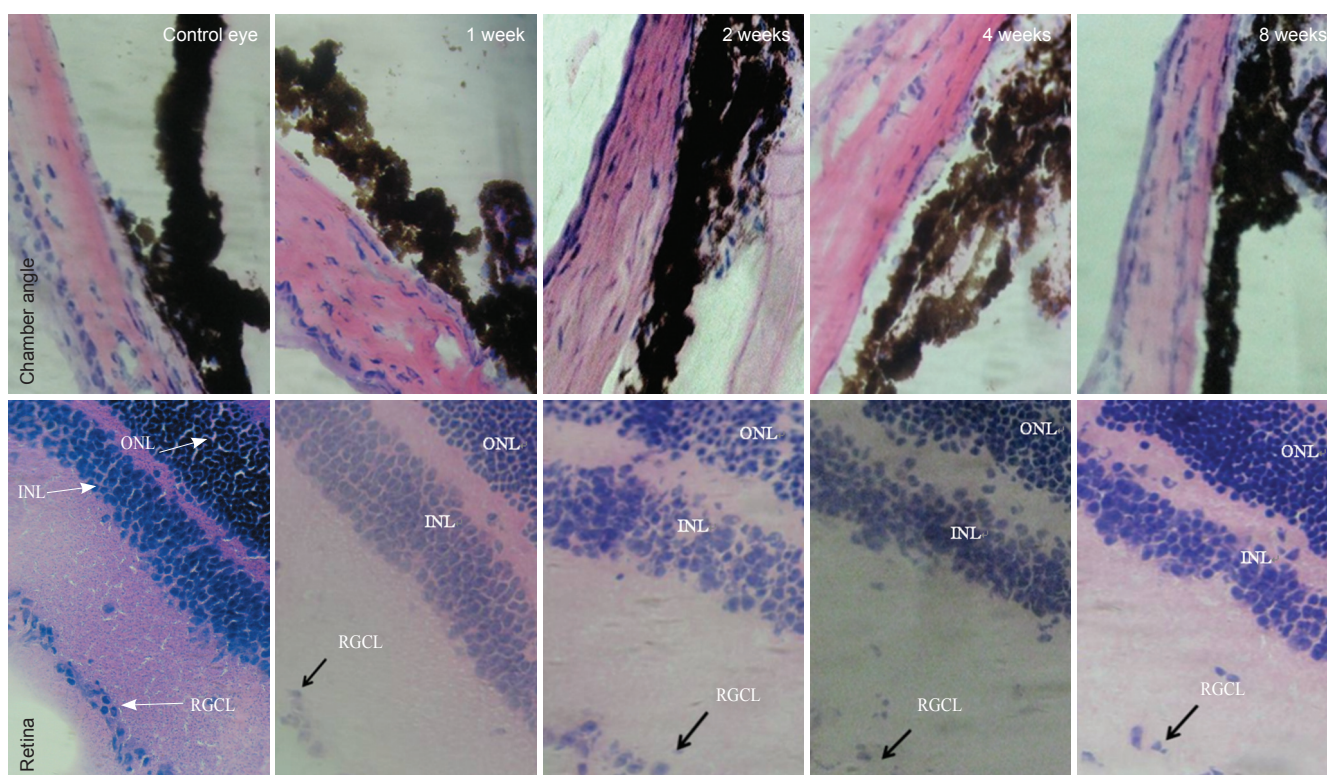
### JNK3 mRNA expression in the mouse retina as measured by real-time quantitative reverse transcription (RT)-PCR

Eight mice were separately collected at 1, 2, 4 and 8 weeks after model establishment. After general anesthesia, each eyeball was coronally incised along the pars plana. The anterior segment was discarded. The retina was carefully removed, and dried with a filter paper. Total RNA was extracted in accordance with Trizol's method (Li et al., 2008). GeneQuantRNA/DNA analyzer (GE (General Electric), Coventry, UK) was used to measure the ratio of absorbance at 260 nm to absorbance at 280 nm of RNA samples. Simultaneously, samples were electrophoresed on a 1% agarose gel. The extent of



**Figure 1 Alterations in intraocular pressure of mouse models of ocular hypertension.**

Data are expressed as the mean  $\pm$  SD. Eyes from eight mice were used for each time point. The difference was compared using one-way analysis of variance followed by the least significant difference test. \*\* $P < 0.01$ , vs. control eye; ### $P < 0.01$ , vs. before modeling.

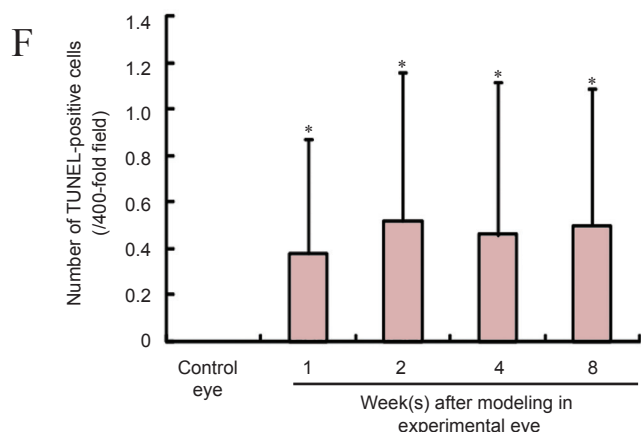
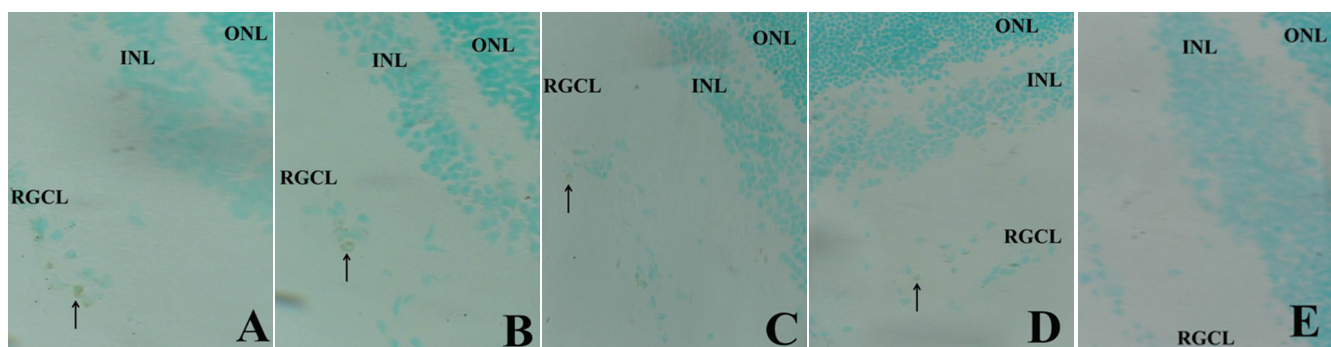


**Figure 2 Changes in the anterior chamber angle and retinal morphology of experimental eyes of mouse models of ocular hypertension (hematoxylin-eosin staining,  $\times 400$ ).**

At 1, 2, 4 and 8 weeks after model establishment, narrow and closed chamber angle, loose interstitial tissue, and disordered inner nuclear layer were visible in experimental eyes. ONL: Outer nuclear layer; INL: inner nuclear layer; RGCL: retinal ganglion cell layer.

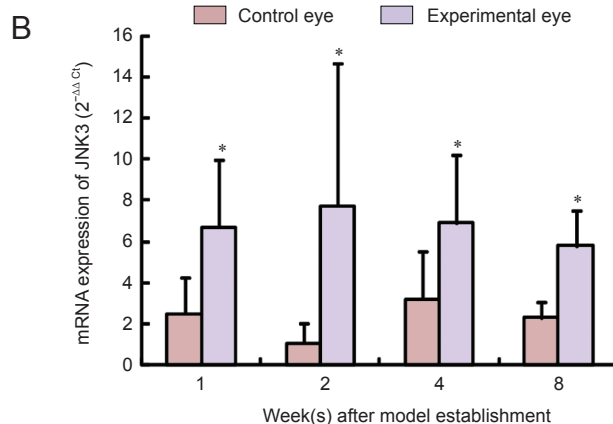
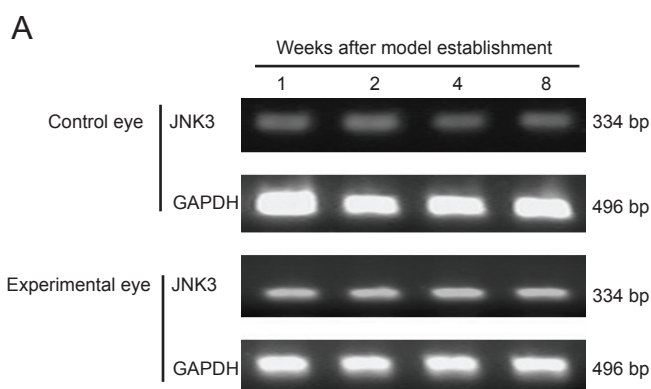
RNA degradation was measured. RNA was converted into cDNA using a cDNA synthesis kit (Fermentas, Pittsburgh, PA, USA). JNK3 primers were designed using Primer 5.0 software (PREMIER Biosoft, Palo Alto, CA, USA). The products of the above primers were correct after detection using Blast software (National Center for Biotechnology Information, Bethesda, MD, USA). An internal reference gene GAPDH was designed and produced by Fermentas. JNK3 primer sequence: upstream 5'-GAT GAC TCC GTA TGT GGT G-3', downstream 5'-GCT GGC TTT AAG TTT ATT GT-3', product of 334 bp. Internal reference gene GAPDH sequence:

upstream 5'-CAA GGT CAT CCA TGA CAA CTT TG-3', downstream 5'-GTC CAC CAC CCT GTT GCT GTA G-3', the product of 496 bp. The TaqMan probe was designed and synthesized by ABI (Grand Island, NY, USA). RT-PCR was performed in a 20- $\mu$ L reaction system with a 7300 Real-Time PCR System (ABI). Amplification conditions are as follows: 50°C for 2 minutes, one cycle; 95°C for 10 minutes, one cycle; 95°C for 15 seconds, one cycle; 60°C for 1 minute, 40 cycles. RT-PCR results could be revealed by their Ct values. The difference in gene expression was analyzed using the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001).



**Figure 3 Apoptosis of retinal ganglial cells in mouse models of ocular hypertension.**

Microphotographs of apoptotic retinal ganglial cells at 1, 2, 4 and 8 weeks after model establishment in experimental eyes (A–D); control eyes (E). Arrows show TUNEL-positive cells (TUNEL staining,  $\times 400$ ). (F) Number of apoptotic cells in mouse retina. Data are expressed as the mean  $\pm$  SD. Eight mice were detected at each time point. The difference was compared using one-way analysis of variance followed by the least significant difference test.  $*P < 0.05$ , vs. control eye. ONL: Outer nuclear layer; INL: inner nuclear layer; RGCL: retinal ganglion cell layer; TUNEL: TdT-mediated dUTP-biotinnick end labeling.



**Figure 4 JNK3 mRNA expression in the retina of mouse models of ocular hypertension.**

Data are expressed as the mean  $\pm$  SD. Eight mice were detected at each time point. The difference was compared using one-way analysis of variance followed by the least significant difference test.  $*P < 0.05$ , vs. control eye. JNK: c-Jun N-terminal kinase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

**Statistical analysis**

The data are analyzed using SPSS 11.5 statistics software (SPSS, Chicago, IL, USA), and expressed as the mean  $\pm$  SD. The difference was compared using one-way analysis of variance followed by the least significant difference test. A value of  $P < 0.05$  was considered statistically significant.

**Results**

**General conditions of mouse models of ocular hypertension**

At 1 day after the surgery, the experimental eyes of all mice suffered from conjunctival hyperemia and corneal edema.

Slightly turbid aqueous humor was visible in the anterior chamber of some eyes. These manifestations disappeared at 3 days, when the conjunctiva, cornea, and anterior chamber of control eyes were all normal.

**Changes in intraocular pressure of mouse models of ocular hypertension**

The intraocular pressures were similar between the left and right eyes of mice before model establishment ( $P > 0.05$ ). The intraocular pressures of the right eyes were significantly increased after model establishment ( $P < 0.01$ ) and were higher than the paired control left eyes ( $P < 0.01$ ); however, they did

not increase with prolonged time after modeling ( $P > 0.05$ ). The intraocular pressures of the control eyes did not alter significantly ( $P > 0.05$ ; **Figure 1**).

#### Alterations in retinal morphology in mouse models of ocular hypertension

Hematoxylin-eosin staining demonstrated that the chamber angle in control eyes was open. Retinal tissue was even and the structure of each layer was clear. The photoreceptors and pigment epithelium cells were tightly connected. The inner and outer nuclear layers were in clear bands, stained uniformly and the cells were of regular shape. The retinal ganglion cells were arranged neatly. At 1, 2, 4 and 8 weeks after model establishment, the experimental eyes displayed a narrow and closed chamber angle. Vacuoles were observed in the ganglion cell layer and inner nuclear layer. The interstitial tissue, nerve fiber layer and inner plexiform layer were more loosely arranged. Cells in the inner nuclear layer were disordered (**Figure 2**).

#### Apoptosis of retinal ganglion cells in mouse models of ocular hypertension

TUNEL assay displayed that brown, TUNEL-positive cells were seen in the ganglion cell layer of the experimental eyes but were not detected in the control eyes of the mice. The number of TUNEL-positive cells was significantly higher in the experimental eyes than in control eyes ( $P < 0.05$ ) but did not increase significantly after 1 day ( $P > 0.05$ ; **Figure 3**).

#### JNK3 mRNA expression in the retina of mouse models of ocular hypertension

JNK3 mRNA could be detected in the retina of normal mice and mouse models of ocular hypertension. Compared with control eyes, JNK3 mRNA expression was significantly higher in the retina of mouse models of ocular hypertension ( $P < 0.05$ ), but JNK3 mRNA expression did not increase further with time after modeling ( $P > 0.05$ ; **Figure 4**).

### Discussion

Glaucoma is the second leading cause of blindness, following cataract. The difference is that glaucoma-induced decreased vision cannot be restored using current technology and therefore is particularly prevalent. The most dangerous factor for glaucoma-induced decreased vision is an increase in intraocular pressure. Current treatment is mainly preventative, aimed at reducing and stabilizing the intraocular pressure. Thus, it is important to establish a simple, economical and practical animal model of ocular hypertension for studying pathogenesis and the treatment of glaucoma and protection of the optic nerve. The anatomical structure of the mouse eyeball is similar to that of a human. The development cycle is relatively short and it is easy to perform transgenic manipulation in the mouse eyeball. It has been an ideal model for studying the molecular mechanisms of glaucoma pathogenesis, genetics and drug treatment. Mouse models of laser-induced ocular hypertension are most commonly used at present. Aihara

et al. (2003) first used glass microtubules to remove some aqueous humor from mice eyes, causing the anterior chamber to become shallower. A 532-diode laser (capability 200 mW, time 0.05 seconds, spot size 200  $\mu\text{m}$ , and number of spots  $64 \pm 6$ ) was then used for photocoagulation of mouse corneal limbus to induce an increase in intraocular pressure (Aihara et al., 2003). Among 22 NIH black Swiss mice, the intraocular pressure increased by more than 30% in 15 mice at 1 week after surgery, in nine mice at 4 weeks, and five mice at 12 weeks. The highest intraocular pressure reached 39.6 mmHg. Mabuchi et al. (2003) designed mouse models of ocular hypertension, and their intraocular pressure was mainly below 30 mmHg. Grozdanic et al. (2003) established mouse models of ocular hypertension by injecting indocyanine green in the anterior chamber and by photocoagulation of the trabecular meshwork using a diode laser, thus increasing the intraocular pressure of the experimental eyes. The highest intraocular pressure reached was 45 mmHg. The intraocular pressure persisted for 60 days. Shepard et al. (2007) confirmed human glucocorticoid-induced overexpression of the trabecular meshwork reactive protein mutants in mouse eyes using a viral vector, resulting in an evident increase in intraocular pressure. This study established mouse models of ocular hypertension by laser photocoagulation of the mouse limbus. The intraocular pressure increased by  $\geq 30\%$  in 75% of the experimental mice. Moreover, no obvious severe complications occurred for the duration of the experiment.

JNK is strongly associated with the occurrence and development of neurodegenerative diseases (Pan et al., 2007; Wang et al., 2013). Glaucoma is also a neurodegenerative disease, therefore the role of JNK is worth investigating to avoid the apoptosis of retinal ganglion cells. JNK3 mainly presents in the nervous system, and involves neuronal cell death (Bogoyevitch, 2006). The JNK signaling pathway mechanisms remain poorly understood, but numerous studies have shown their important effects. Initial studies found that  $\gamma$ -radiation can activate JNK1 and induce apoptosis (Chen et al., 1996).  $\gamma$ -Radiation can continuously stimulate JNK1 synthesis for a long time, and induces apoptosis of Jurkat cells (Chen et al., 1996). In the present study, a TaqMan probe of RT-PCR was applied to determine JNK3 mRNA expression. Our results verified that as long as the intraocular pressure remained high, there was an increase in JNK mRNA expression. Over time, there was an increased tendency for JNK3 mRNA expression. Ganglion cell apoptosis appeared and JNK3 mRNA expression increased in experimental eyes, but the above phenomena were not detectable in control eyes. These data suggested that JNK3 activation was directly or indirectly correlated with the apoptosis of retinal ganglion cells.

In summary, in the state of ocular hypertension, JNK3 was activated, which was directly or indirectly associated with the apoptosis of retinal ganglion cells. Our follow-up experiments will investigate whether retinal ganglion cells survive the state of ocular hypertension after inhibiting or knocking out JNK3.

**Author contributions:** YSY and YL designed the study. YH and SGZ implemented the study. JC participated in data analysis, statistics and image processing. HBL and JHG proofread this paper. YH wrote the paper and obtained the funding. All authors approved the final version of the paper.

**Conflicts of interest:** None declared.

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