Original Article

Neuroprotective Effect of Compound Anisodine in a Mouse Model with Chronic Ocular Hypertension

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Abstract

Background: Compound anisodine (CA) is a compound preparation made from hydrobromide anisodine and procaine hydrochloride. The former is an M-choline receptor blocker with the function of regulating the vegetative nervous system, improving microcirculation, and so on. The latter is an antioxidant with the activities of neuroprotection. This study aimed to investigate the potential neuroprotection of CA, which affects the degeneration of the retinal ganglion cells (RGCs) in an animal model with chronic ocular hypertension.

Methods: Female C57BL/6J mice ($n = 24$) were divided randomly into four groups: Normal control group without any treatment (Group A, $n = 6$); CA control group with feeding the CA solution (Group B, $n = 6$); microbeads (MBs) control group with injecting MB into the anterior chamber (Group C, $n = 6$); CA study group with MB injection and with feeding the CA solution (Group D, $n = 6$). Intraocular pressure (IOP) was measured every 3 days after MB injection. At the 21st day, neurons were retrograde-labeled by Fluoro-Gold (FG). Animals were sacrificed on the 27th day. Retinal flat mounts were stained immunohistologically by β-III-tubulin. FG-retrograde-labeled RGCs, β-III-tubulin-positive RGCs, and β-III-tubulin-positive nerve fibers were quantified.

Results: Mice of Groups C and D expressed the incidence of consistent IOP elevation, which is above the IOP level of Group A with the normal one. There is no significant difference in IOP between Groups A and B ($P > 0.05$). On the 27th day, there were distinct loss in stained RGCs and nerve fibers from Groups C and D compared with Group A (all $P < 0.001$). The quantity was significantly higher in Group D as compared to Group C (all $P < 0.001$) but lower than Group A (all $P < 0.001$). There was no significant difference in the quantity of RGCs and nerve fibers between Groups A and B (all $P > 0.05$).

Conclusions: These findings suggest that CA plays an importantly neuroprotective role on RGCs in a mouse model with chronic ocular hypertension.

Key words: Compound Anisodine; Glaucoma; Neuroprotection; Ocular Hypertension

Introduction

Glaucomatous optic neuropathy is a chronically neurodegenerative disease which is characterized with the progressive loss of retinal ganglion cells (RGCs), atrophy of optic nerve, and eventually irreversible visual injury or loss.[1,2] Clinically, reducing intraocular pressure (IOP) for glaucoma patient is proven as the main therapeutic method. However, even if IOP level is reduced to the normal, it is still difficult to slow the progression of glaucoma, and many glaucoma patients suffer from the damage of progressive optic nerve and the loss of eventual visual. The death of RGCs, the loss of axons, and the progression of visual loss are all caused by the residual effect of acute or chronic RGCs impairment.[3,4] The primary risk factors for the progression of glaucoma patients are the increased IOP and the blood supply shortage of optic nerve.[5] Therefore, both reducing the IOP of glaucoma patients, and recovering the blood supply of optic nerve are supposed to be effective approaches to slow the incidence and progression of glaucoma.

Compound anisodine (CA) is a compound preparation made from hydrobromide anisodine and procaine hydrochloride. The

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former is an M-choline receptor blocker with the function of regulating the vegetative nervous system, and with the effects of center sedation, anti-shock, anti-allergy, as well as the improvement of microcirculation.[6,7] The latter is an antioxidant with the activities of nceuroprotection and the improvement of visual acuity.[7] CA is capable to improve visual function through regulating the function of ocular vessels, relieving angiospasm, and increasing ocular blood flow.[6] Moreover, researches showed that CA is an antioxidant which is able to relieve vasospasm of the whole body and improve the blood supply of tissues as well as microcirculation.[7]

At present, all medicine for glaucoma aims to reduce IOP level, and there is no one for neuroprotection. Therefore, we investigated its effects of counteracting the progression of neurodegeneration induced by high IOP though improving the blood supply of optic nerve. The aim of this study was to evaluate the efficacy of orally administered CA on the neuroprotective effects on RGCs of the mice with chronic ocular hypertension.

Methods

Animals

Twenty-four Specific Pathogen Free (SPF) female C57BL/6J mice (8 weeks old, weighing 20–30 g) were included in this experimental study. The experimental protocols were approved by the University Institutional Animal Care and Use Committee of Capital Medical University, and it was consistent with the National Institute of Health Guide for the Care and Use of Laboratory Animals (National Institute of Health Publications, No. 80-23, revised 1996). All procedures were performed according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The laboratory animals were kept in the animal facility under the standardized conditions and the normal room temperature, and they were fed with food and water in a 12 h of day and 12 h of night circle.

All mice were examined by ophthalmoscopy to exclude ocularly abnormal mice before the study. The mice were subsequently divided randomly into three control groups and one study group: Normal control group (Group A, $n = 6$) without any treatment; CA control group (Group B, $n = 6$) with feeding the CA solution; microbeads (MBs) control group (Group C, $n = 6$) with injecting MB into the anterior chamber to induce chronic elevation of IOP; CA study group (Group D, $n = 6$) with injecting MB into the anterior chamber and with feeding the CA solution as well. The CA solution is prepared by the way of dissolving CA (CR Zizhu Pharmaceutical Co., Ltd, Beijing, China) with a dosage of 2 ml/kg daily in the fresh drinking water. The mice in Groups B and D were fed with CA solution 2 days ahead of MB injection process that needs 29 days totally. All mice were treated with water (8 ml for each mouse daily, taking the volume not consumed by the mice and minimal potential leakage from the water bottle into account).

For each group, mice served for obtaining full-thickness flat mounts of the retina, which would be retrograde-labeled of RGCs by 4% Fluoro-Gold (FG, Biotium Corporation, USA) at 21st day and then processed for immunochemistry at 27th day. Animals were killed at 27th day.

Intraocular pressure elevation of mice

Polystyrene MBs (diameter = 10 μm, Invitrogen, Carlsbad, CA, USA) were resuspended in phosphate-buffered saline (pH = 7.4) to a final concentration of 9.0×10^6 beads per milliliter. To control precisely the small volume (2 μl) of anterior chamber injection, we used a glass micropipette connected with a Hamilton syringe (Hamilton Company, Reno, Nevada, USA), which was linked with a 30-gauge needle at the end for easy entry. Two days after the first CA oral administration, mice in Groups C and D were anesthetized by intraperitoneal injection of 5% chloral hydrate (8 ml/kg, Sinopharm Chemical Reagent Co. Ltd., China), and then the pupils of left eyes were dilated with tropicamide phenylephrine eye drops (Mydrin-p, Santen Pharmaceuticals Co. Ltd., Osaka, Japan). Under a operating microscope, IOP was unilaterally elevated by injection of MB into the anterior chamber of the left eyes of mice. After the injection of 2 μl MBs and 2 μl air by using the micropipette within 2 min, MBs were found to be accumulated at the angle of the anterior chamber, with a big bubble on the top of anterior chamber. The big bubble, which prevents the aqueous humor from outflowing the wounded entry, would be self-absorbed in several hours. One drop of levofloxacin ophthalmic solution (Cravit, Santen Pharmaceuticals Co. Ltd., Osaka, Japan) was applied to the treated eye immediately after injection. What should be noted is that lens and iris must not be injured by the needle during the dispose of MB injection. The mice with surgical complications such as cataract, hyphema, or inflammatory responses (opaque cornea or iris exudation) were excluded from the study and replaced by other animals to keep six mice in each group for the whole experiment.

Intraocular pressure measurement

IOP in both eyes was measured every 3 days using a tonometer (TonoLab; Colonial Medical Supply, Espoo, Finland)[8] at the same time every morning to avoid diurnal variation. Mice were anesthetized by intraperitoneal injection of 5% chloral hydrate. The IOP measurement was initiated within 3 to 5 min when animals lost consciousness, which we cannot detect the forced movement and eye blinking. The tonometer is able to generate and display an average number of six measurements by internal software, and we considered this machine-generated average as one record. Six records were obtained for each surgery eye, and mean of the six records was calculated as the final IOP.

Retrograde labeling of retinal ganglion cells by Fluoro-Gold

In the 21st experimental day, 4% FG solution was inserted bilaterally over the SC of the anesthetized mouse. The mouse was placed in a stereotactic apparatus and the

skull was exposed. Based on the marked bregma, two holes (approximately 2 mm diameter) were drilled on the skull at the designated coordinates: 4.0 mm behind the bregma on the antero-posterior axis, ±0.5 mm lateral to the midline on both hemispheres. One microliter of 4% FG solution was slowly injected into SC bilaterally using Hamilton syringe (Hamilton, Reno, NV, USA). The tip of the syringe was perpendicular to the skull at an approximately 1.2 mm depth under the endocranium, and then the syringe was kept still for 5 min. Antibiotic ointment should be applied after suturing the skin over the wound.

Retinal immunohistochemistry

At the 27th day, all the retrograde-labeled mice were cardio-perfused with 4% paraformaldehyde at a flow rate of 20 ml/min under the situation of deep anesthesia. The eyes were enucleated and fixed in 4% paraformaldehyde for 15 min, with marking the limbus at the 12 o'clock position. Anterior segment in every globe was then cut-off for removing the lens and subsequently leaving behind posterior eyecup. To prepare the retinal flat mount, the retina of the enucleated eye was detached from the underlying structures (choroid/sclera) and then divided into quadrants (superior, temporal, nasal, and inferior). Whole retinal flat mounts were then essayed for RGCs and nerve fibers counting. The whole procedure was processed on ice as to avoid the light. After fixed in 4% paraformaldehyde for 1 h, the retinal flat mounts were incubated with primary antibody, against RGCs specifically, β-III-tubulin (Tuj1; 1:50, Cell Signaling, USA) at 4°C overnight, followed by an Alexa Fluor 488-conjugated secondary antibody (1:1000, Cell Signaling, USA) at 37°C for 2 h.

The retinal flat mounts were then observed under a fluorescence microscope (LEICA DM 6000B, Germany). Six fluorescence micrographs (×400 magnification) were selected from each quadrant of the retina at a distance of 1 mm interval along the radius (0.09 mm^2) from the optic disc center. FG-positive and β-III-tubulin-positive RGCs were counted under different exciting light sets. RGCs counting (cells/mm^2) was performed by two observers respectively in a blind method and the results were averaged. To measure the interobserver variation, 20 fields were counted independently by two masked observers. RGCs survival rate was calculated by comparing the RGCs density of the surgical eye with that of the contralateral control eye.

Statistical analysis

Data were analyzed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Data of each group, with normal distribution conducted by Shapiro–Wilk test, were expressed as mean ± standard deviation (SD). Equal variance of sample means were tested by Levene test. IOP of animals in each group at different time points were analyzed by repeated measures analysis of variance; differences among FG-positive RGCs and β-III-tubulin-positive RGCs and nerve fibers were analyzed by randomized blocks analysis of variance with Bonferroni's posttest; the independent samples *t*-test and Pearson correlation were performed for comparing two groups. $P < 0.05$ was considered to be statistically significant.

Results

Microbead-induced elevation of intraocular pressure in mice

In this glaucoma model, after MB injection (Groups C and D), IOP was elevated significantly within 3 days after a single injection of MB, which was above that of the uninjected groups (Groups A and B) (all $P > 0.05$) [Table 1 and Figure 1]. At around the 15th day, when elevated IOP was reaching its peak mean value, 28.67 ± 2.88 mmHg (in Group C) and 29.67 ± 2.42 mmHg (in Group D), many MBs were observed to tightly block the angle of the anterior chamber under a light microscope.[9] The elevated IOP could still be maintained for the mean value, 15.83 ± 1.17 mmHg (in Group C) and 15.83 ± 1.47 mmHg (in Group D), with a few MBs still blocking the angle of the anterior chamber.[9] There is no significant difference for the mean IOP values between Groups A and B. Significant difference for the mean IOP values between Groups C and D was noted (all $P = 0.000$). Consequently, the results demonstrated that MB injection into anterior chamber effectively induced chronic and reversible IOP elevation of mice, whereas, CA had no effect on the IOP of mice.

Effects of compound anisodine on retinal ganglion cells survival

To quantify the density of RGCs, we performed retrograde labeling of RGCs by injecting FG into SC. The recorded quantity of RGCs in uninjected and injected groups is shown in Table 2. There was a statistical difference for the FG-positive RGCs densities among four groups ($F_{treat} = 3406.095$, $P = 0.000$; $F_{block} = 23.898$, $P = 0.000$). All *P* values among all groups were 0.000, with the exception of Groups A and B (*P* value is 0.443). Mean RGCs densities among Groups C and D were different from Groups A and B, moreover mean RGCs densities of former two groups were lower than that of Group A ($t = 63.606$, $t = 77.899$, respectively, both *P* were 0.000, independent samples *t*-test) [Figure 2].

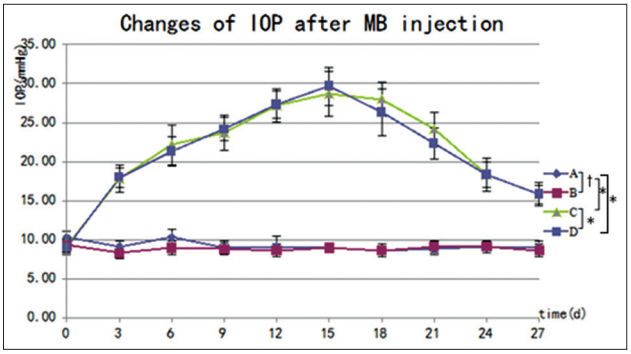

Figure 1: Changes of intraocular pressure after microbeads injection ([A] Normal control group; [B] Compound anisodine control group; [C] Microbeads control group drinking water; [D] Compound anisodine study group drinking water plus compound anisodine) *$P < 0.01$; †$P > 0.05$.

Table 1: IOP at different time points (mean ± SD, mmHg)

Groups (n = 6)	Before MB injection	3 days after MB injection	6 days after MB injection	9 days after MB injection	12 days after MB injection	15 days after MB injection	18 days after MB injection	21 days after MB injection	24 days after MB injection	27 days after MB injection
Group A	10.33 ± 0.82	9.17 ± 0.75	10.33 ± 1.03	9.00 ± 0.89	9.17 ± 1.33	9.00 ± 0.63	9.17 ± 0.75	8.83 ± 0.75	9.17 ± 0.75	9.00 ± 0.89
Group B	9.33 ± 0.82*	8.83 ± 0.75*	9.00 ± 0.89*	8.83 ± 0.75	8.67 ± 0.82*	9.00 ± 0.63*	8.67 ± 0.82*	9.17 ± 0.75*	9.17 ± 0.75*	8.67 ± 0.82*
Group C	9.17 ± 0.75	17.83 ± 1.72	22.17 ± 2.56	23.67 ± 2.25	27.17 ± 2.14	28.67 ± 2.88	28.00 ± 2.19	24.17 ± 2.14	18.33 ± 1.63	15.83 ± 1.17
Group D	9.00 ± 0.89	18.00 ± 1.26†	21.33 ± 1.86†	24.17 ± 1.47†	27.33 ± 1.75†	29.67 ± 2.42†	26.33 ± 3.01†	22.33 ± 1.97†	18.33 ± 2.16†	15.83 ± 1.47†

*P>0.05 versus Group A at different time points; †P<0.01 versus Group C at different time points except the time point before MB injection. F_{group}=317.812, P=0.000; F_{time}=142.121, P=0.000; $F_{interaction}$=54.420, P=0.000. IOP: Intraocular pressure; SD: Standard deviation; MB: Microbead.

Thus, MB-induced IOP elevation induces obviously glaucomatous RGCs degeneration in mice. Since mean RGCs density of Group D was distinctly higher than that of Group C (t = 28.187, P was 0.000, independent samples t-test), and there was a significant difference between RGCs survival rates of Groups C and D (48.63%, 66.23%, respectively, χ^2 = 350.33, P < 0.01), we can see the significantly protective role of CA on RGCs.

Next, we compared RGCs quantity by immunohistochemistry with β-III-tubulin, a marker of neuronal lineage cells which is highly expressed in the retinal ganglion cell layer (GCL). β-III-tubulin is commonly used as a marker for identifying RGCs in the retina in various optic nerve injury models.[10-15] There was also a statistical difference for β-III-tubulin-positive RGCs densities among four groups (F_{treat} = 261.627, P = 0.000; F_{block} = 3.745, P = 0.000). All P values among all groups were 0.000, with the exception of Groups A and B (P value was 1.000). Mean β-III-tubulin-positive RGCs densities among Groups C and D were different from Groups A and B, moreover mean β-III-tubulin-positive RGCs densities of former two groups were lower than that of Group A (t = 80.805, t = 71.270, respectively, both P values were 0.000, independent samples t-test) [Table 2 and Figure 2]. Thus, MB-induced IOP elevation leads to the glaucomatous RGCs degeneration in mice. Since β-III-tubulin-positive RGCs density of Group D was obviously higher than that of Group C (t = 22.049, P was 0.000, independent samples t-test), and there was a significant difference between β-III-tubulin-positive RGCs survival rates of Groups C and D (48.51% and 65.48%, respectively, χ^2 = 326.15, P < 0.01), we can see the significantly protective role of CA on RGCs.

Additionally, we can detect the β-III-tubulin-marked nerve fibers as well [Figure 2]. There was also a statistical difference of the β-III-tubulin-positive nerve fibers densities among four groups (F_{treat} = 368.070, P = 0.000; F_{block} = 17.332, P = 0.002). All P values among all groups were 0.000, with the exception of Groups A and B (P value was 1.000). Similarly, mean β-III-tubulin-positive nerve fibers densities among Groups C and D were different from Groups A and B, moreover mean β-III-tubulin-positive nerve fibers densities of former two groups were lower than that of Group A (t = 20.843, t = 14.076, respectively, both P were 0.000, independent samples t-test) [Table 2 and Figure 2]. Therefore, MB-induced IOP elevation leads to the loss of nerve fibers in GCL. β-III-tubulin-positive nerve fibers density of Group D was higher than that of Group C (t = 31.286, P was 0.000, independent samples t-test), and there was a statistical difference between β-III-tubulin-positive nerve fibers survival rates of Groups C and D (46.71%, 71.27%, respectively, χ^2 = 36.94, P < 0.01). It is shown that CA has a distinctly protective role on nerve fibers expression in the GCL.

The Pearson correlation coefficient for the measurements obtained from the two examiners was 0.96 for the interobserver variability assessment.

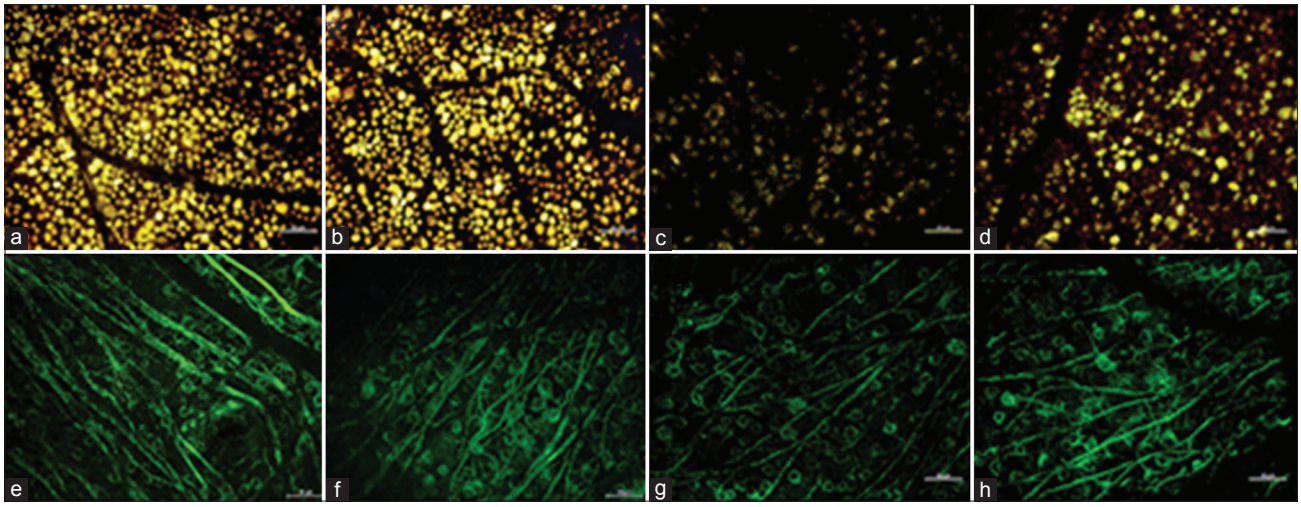

Figure 2: (a–d) Retrograde labeling of retinal ganglion cells by Fluoro-Gold; (e–h) β-III-tubulin immunohistochemistry. (a,e) Group A (normal control group); (b, f) Group B (compound anisodine control group); (c, g) Group C (microbeads control group); (d, h) Group D (compound anisodine study group). Scale bar: 50 μm.

Table 2: Quantity of RGCs and nerve fibers in uninjected and injected groups (mean ± SD)

Group (n=6)	FG labeled RGCs (cells/mm^2)	β-III-tubulin positive RGCs (cells/mm^2)	β-III-tubulin positive nerve fibers (fibers/mm^2)
Group A	5590 ± 392	5581 ± 344	292 ± 55
Group B	5630 ± 280§	5526 ± 278§	300 ± 57§
Group C	2669 ± 401*	2707 ± 371*	136 ± 22*
Group D	3670 ± 274†,‡	3618 ± 269†,‡	213 ± 29†,‡

*P<0.01 versus Group A, t=63.606, 80.805, and 20.843, respectively; †P<0.01 vs group A, t=77.899, 71.270, 14.076, respectively; ‡P<0.01 versus Group C, t=28.187, 22.049, and 31.286, respectively; §P>0.05 versus Group A, t=2.709, 2.319, and 1.987, respectively. FG-labeled RGCs: F_{treat}=3406.095, P=0.000; F_{block}=23.898, P=0.000; β-III-tubulin-positive RGCs: F_{treat}=261.627, P=0.000; F_{block}=3.745, P=0.000; β-III-tubulin-positive nerve fibers: F_{treat}=368.070, P=0.000; F_{block}=17.332, P=0.002. SD: Standard deviation; RGCs: Retinal ganglion cells; FG: Fluoro-Gold.

Discussion

IOP elevation is one of the major risk factors for the progression of glaucoma. In our study, the chronic elevation IOP of mice was induced by injecting MB into the anterior chamber, which was first applied by Sappington *et al*.[9,16] In this glaucoma model, a single injection of MB into the mouse anterior chamber induced IOP elevation within 27 days, with MB gradually accumulating in the angle of the anterior chamber, causing the outflow blockage of aqueous humor. This model has the advantage of avoiding overt damage to ocular structures, which will cause inflammatory responses, while it can effectively induce RGCs and axon degeneration that simulates the progression of glaucoma.[9]

In this study, the state of RGCs and axon degeneration was assessed by retrograde labeling with FG and β-III-tubulin immunohistochemistry. FG-retrograde labeling is a classic method that marked RGCs by placing FG into the SC, which is widely carried out in animal models of glaucoma to quantify the loss of RGCs.[17,18] However, the technique of retrograde labeling has a limitation that retrograde-labeling of RGCs depends on active axonal transport, a cellular function which is thought to be compromised under the incidence of elevated IOP.[19,20] Therefore, we also adopted a method of anti-β-III-tubulin immunolabeling for RGCs to evaluate the situation of RGCs survival.[9] Under the fluorescence microscope, the RGCs which were marked by β-III-tubulin showed round and intensely stained cell bodies in the RCL. Although anti-β-III-tubulin is known to label weakly a subpopulation of amacrine and bipolar cells, it has been commonly used for quantification of RGCs survival in optic injury models including glaucoma.[10,11] The present study showed[9] that counts of RGCs using both FG-labeling and anti β-III-tubulin immunolabeling had similar effects. In the glaucomatous mice in our study, it was found that densities of RGCs labeled by FG and RGCs with nerve fibers marked by β-III-tubulin were significantly lower in Group C as compared to Group D (all $P < 0.01$). The results suggested that CA may play a neuroprotective effect on inner retinal function with chronic IOP elevation.

Many clinical studies[5-7] showed that CA has multifunctions for treating ischemic optic neuropathy, retinopathy, choroidopathy, and so on. Moreover, CA has a unique efficacy for the treatment in various types of glaucoma, especially in the protection of the optic nerve in glaucoma. In previous clinical study, after application of CA, acuity, visual fields, and F-ERG of glaucoma patients had been improved.[21,22] It is indicated that CA might favorably influence ocular retinal function, and as an early treatment, it can moderately alleviate the incidence of glaucomatous damage. The exact mechanism of CA on RGCs neuroprotection in a chronic ocular hypertension might be an anti-oxidative characteristic[7] and an effective increase in ocular blood flow.[6] However, current studies on the effects of CA are primarily based on domestically clinical trials, lacking of

animal experiments, and foreign reports. Besides, the main limitation of our study is considered not to use the clinical administrative route, namely para-superficial temporal artery via subcutaneous injection. Another limitation is the lack of evaluation of different doses of CA to detect the dose effects of CA on neuroprotection. Further study should also focus on the long-term clinical benefit of CA in glaucoma and the different effects on RGCs under other dosages of CA. Last but not least, despite our results demonstrated that CA appeared to be effectively alleviate the RGCs loss caused by IOP elevation, the incidence of RGCs loss was still observed, and therefore, the neuroprotection duration time from CA might need to investigate. Therefore, we need to advance the experimental design in the future.

In conclusion, the current data suggest that oral administration of CA is able to protect the function of RGCs' bodies and axons by increasing the RGCs survival rates under the mice model with high IOP. Consequently, CA is proven to play a therapeutically neuroprotective role in the animal model of glaucoma. With broad therapeutic properties, especially the neuroprotective characteristic, CA is a promising medicine with the goal for archiving neuroprotection.

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The medicine used in this study, Compound Anisodine Hydrobromide Injection, was offered by CR Zizhu Pharmaceutical Co., Ltd, Beijing, China, and the sponsor had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of interest

There are no conflicts of interest.

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