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ORIGINAL ARTICLE

Crocetin confers neuroprotection and is anti-inflammatory in rats with induced glaucoma

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Abstract

Background Crocetin is a bioactive ingredient in saffron, derived from the *Crocus sativus* stigmas of the Iridaceae family. As a chemically carotenoid derivative, crocetin exhibites effects like anti-inflammatory, antioxidant, neuroprotective, etc. However, the protective effect of crocetin on glaucoma and its mechanism remains unclear. The current study assesed the neuroprotective and anti-inflammatory effects of crocetin on retinal neurons in glaucoma rats which were induced by 0.3% carbomer injection into the anterior chamber.

Methods and results The pathological structures on the retina and optic nerve were observed and examined by H&E staining and transmission electron microscopy. Immunohistochemical staining was used to detect the expression of TNF-α, IL-1β, and IL-6 of the retina and the expression of a brain-derived neurotrophic factor (BDNF) in the primary visual cortex (PVC). Western blot was carried out to detect the expression of PI3K, Akt, and NF-κB in the retina. It was found that crocetin ameliorated the pathological changes of the retina and ON and reduced the number of apoptotic retinal ganglion cells. Immunohistochemical staining showed that crocetin could decrease the contents of TNF-α, IL-1β, and IL-6 and increase the contents of BDNF. Western blot showed that crocetin was found to suppress the expression of PI3K, Akt, and NF-κB.

Conclusion The results obtained in this study have indicated that crocetin showes neuroprotective effects on retinal ganglion cells in glaucoma rats and inhibits retinal dysfunction. Meanwhile, crocetin exerted an anti-inflammatory effect to protect the retina by inhibiting the expression of the PI3K/Akt/NF-κB signaling pathway. This work provides substantial evidence that crocetin may be a potential drug for the treatment of glaucoma.

Keywords Crocetin · Glaucoma · Neuroprotection · Anti-inflammation · PI3K/Akt/NF-κB

Introduction

Glaucoma, the leading cause of visual impairment globally, is a neurodegenerative disease characterized by the chronic and progressive apoptotic death of retinal ganglion cells (RGCs), leading to age-related vision loss owing to a gradual rise in the intraocular pressure (IOP) [1, 2]. By 2040, the number of glaucoma patients is expected to rise to 111.8 million [3, 4]. Glaucoma is commonly caused by intraocular hypertension, thus reducing IOP is considered a primary therapeutic strategy for glaucoma to minimize or mitigate eventual visual loss. On the other hand, people with normal IOP may have RGC malfunction and apoptosis [5]. The specific pathophysiology driving glaucoma progression is unknown. Until now, evidence has accumulated indicating neuro-inflammation as a major reason responsible for priming the glaucoma disease, resulting in retinal and optic

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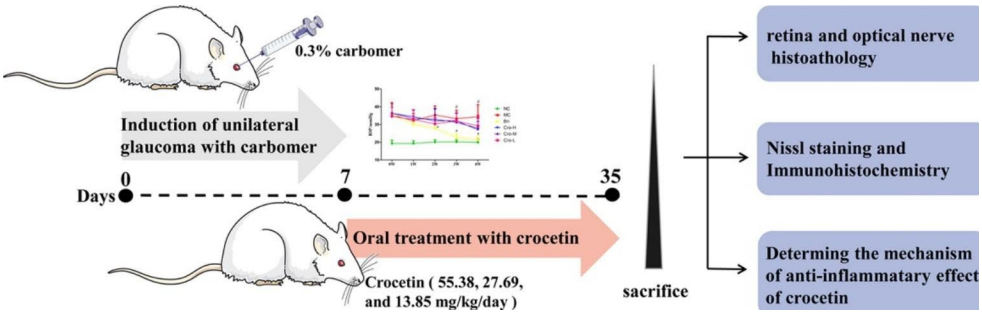

Fig. 1 Schematic representation of murine glaucoma model and experimental validation steps to confirm the effect of crocetin on glaucoma treatment

neuropathy, hence neuroinflammation is considered a critical process in glaucoma [6]. Inflammation has been reported as a common feature of clinical and experimental glaucoma, including the activation and proliferation of glial cells (astrocytes, Muller cells, and microglia), resulting in the release of a large number of inflammatory cytokines, pro-inflammatory cytokines, chemokines, and reactive oxygen species [7]. In patients and experimental glaucoma model animals, the expression of inflammatory factors was found to increase [8, 9]. Furthermore, glaucoma affects the retina and central visual pathways, resulting in pathological alterations upstream in the retina and downstream in the visual cortex [10]. Therefore, developing direct therapeutic strategies for neuroprotection is important in treating glaucoma.

Natural compounds with great pharmacological potential and minimal toxicity have gradually reached the public consciousness and have been considered novel therapeutic agents with emphasis on their research and development. According to historical records, saffron has been widely utilized in Chinese herbal medicine since the 16th century and remains in use today. Crocetin has been reported to possess multiple pharmacological actions like antidepressants [11], anticancer [12], liver protection [13], anti-oxidation [14], anti-inflammatory [15], and neuro-protection [16]. Crocetin is a bioactive element in saffron obtained from the stigmas of Crocus *sativus*, a member of the Iridaceae family and a chemically carotenoid derivative [17]. Crocetin is reported to exert a protective effect on RGC mortality in vitro and in vivo by suppressing caspase-3 and −9 activity [18]. It has also been found to protect against ischemia/reperfusion-induced retinal cell death, with the protective mechanism being a reduction in the phosphorylation levels of p38, JNK, NF-κB, and c-Jun [19]. It is further found to limit the increase in the number of TUNEL-positive cells caused by N-methyl-D-aspartate (NMDA) in the mouse retina by inhibiting the production of cleaved caspase-3 [20]. These findings showed that crocetin might be a novel neuroprotectant in glaucoma. On the other hand, crocetin's anti-inflammatory impact on glaucoma retinal protection, has not yet been studied.

This study investigated crocetin's neuroprotective and anti-inflammatory effects on retinal neurons in a glaucoma rat model, which is envisaged to provide a theoretical foundation for the therapeutic use of crocetin in glaucoma. A schematic representation of the experimental procedure is shown in Fig. 1.

Materials and methods

Preparation of crocetin

Crocetin from saffron was separated as previously described [13]. Saffron 100 g was mixed with 1.4 L 70% ethanol solution and extracted in an ultrasonic bath at 50 ℃ for three times and 1 h each time. The combined extract was eluted by macroporous resin D101 with water, 25% ethanol and 60% ethanol in turn. Collect 60% ethanol eluent. The solution was adjusted to a pH of 12 by adding 2 M NaOH and hydrolyzed at room temperature for 12 h. It was then adjusted to a pH of 2 using 1 M H_2SO_4 and precipitated overnight at 4 ℃. The precipitate was obtained by centrifugation. The precipitate was washed twice with 1 L methanol supplemented with 0.5% H_2SO_4 and purified with double-distilled water to obtain a pure crocetin (HPLC > 97%).

Animals

Specific Pathogen Free (SPF) *Sprague Dawley* (SD) (Male, n = 72, weight = 220–230 g) were provided by Shanghai Jihui experimental animal breeding Co., Ltd vide certificate No. 20,170,012,003,926. They were acclimatized for seven days with a 12 h light and dark cycle and free access to food and water. All animal studies were carried out per the Guidelines for the Care and Use of Laboratory Animals at the Zhejiang University of Technology in Hangzhou, China, provided vide ethical code No. 20,200,810,104. All animals were randomly divided into six groups i.e., the normal control (NC) group which did not get glaucoma induction; the rest were induced with glaucoma and were divided into model control (MC), brimonidine (Bri), crocetin high-dose (Cro-H), crocetin medium-dose (Cro-M), and crocetin low-dose (Cro-L), each with 12 rats/ group.

Induction of unilateral glaucoma with carbomer

Glaucoma was induced using the previously reported method as described by Kim and Hwang-Gyun [21]. The animals were induced with glaucoma using 0.3% carbomer injection into the anterior chamber, owing to its ability to cause loss of RGCs associated with the development of persistent IOP elevation.

The rats were anesthetized by inhaling isoflurane and placed on a constant temperature heating pad. After anesthesia, the rat conjunctivas were clamped with micro-tweezers in the left hand under the microscope to fix the eyeball. The syringe with a 29-gauge needle was used to puncture at 1 mm from the limbus of the cornea with the right hand. The needle penetrated through the cornea from the subconjunctival into the anterior chamber. It was worth noting that the needle must avoid contact with the anterior lens capsule, iris blood vessels, and corneal endothelium. During the puncture, a conjunctival and corneal tunnel that can be closed was created. After removing the needle, the aqueous humor flowed out, the anterior chamber became shallow, and the eyeball became soft. The rats were excluded from the experiment if the iris blood vessel was punctured to cause bleeding. Afterward, 20 µL of 0.3% carbomer was quickly injected with a needle that again passed the anterior chamber tunnel of the rat's eye and adjusted to the angle of the chamber,. At this time, the blood vessels of the iris appeared pale, and the anterior chamber seemed thicker, which was observed. While pulling out the needle, the assistant immediately clamped the conjunctival wound with toothless forceps for about 1 min. Since the volume of the carbomer in the anterior chamber directly determines the level of IOP after surgery, it is crucial to avoid the carbomer from flowing out of the anterior chamber in the experiment.

Following each treatment, levofloxacin eye drops were applied topically twice daily to the ocular surface for one week. Meanwhile, the contralateral eye did not receive any application.

Drug administration

After successful glaucoma induction in animals, NC group rats were given water intragastrically. In constract, MC group rats were gavaged 0.5% carboxymethylcellulose sodium (CMC-Na) at a dose of 1 mL/100 g body weight. The crocetin was adequately suspended in 0.5% CMC-Na before gavage administration. Similarly, the crocetin groups received crocetin oral dosage at a dose of 55.38 mg/kg (Cro-H group), 27.69 mg/kg (Cro-M group), and 13.85 mg/kg (Cro-L group), respectively. In Bri group, 50 µL of brimonidine tartrate eye drops were applied topically twice daily to the rat's ocular surface. The animals received their respective doses once a daily for 28 days.

IOP measurement

The measurements were performed with little anesthesia by inhaling 3% isoflurane (the gas flow rate was 0.6 L/min) using a tonometer designed for rodents (TonoPen AVIA Vet, Reichert, Depew, NY, USA). It is worth noting that the IOP measurement was performed continuously simultaneously in the afternoon. The Liquid Crystal Displays (LCD) show the cumulative number of applanations detected during the measurement. Once ten applanations were achieved, the LCD displayed the average value of IOP in millimeters of mercury (mm Hg), along with a statistical confidence indicator. The average value of IOP was recorded as IOP data when the statistical coefficient indicator was 95. The IOP was measured three days before glaucoma surgery as baseline IOP and again at one, three, and seven days. Subsequently, the rats were administered treatment, and IOP was measured once a week until the rats were sacrificed. In previous studies, intraocular pressure (IOP) was usually measured once a week [21, 22], twice a week [23], and once a day [24, 25] in animal models of carbomer-induced glaucoma. The IOP detection frequency in this study is once a week, which is similar to the previous study. Therefore, it could reflect changes in IOP to some extent.

Tissue preparations

The rats were anesthetized with isoflurane after four weeks of therapy. The head was quickly decapitated, the brain dissected, and the left brain was fixed in a 4% paraformaldehyde fix solution for 72 h. Six rats in each group had their right eyeballs removed and immersed in FAS ocular fixative. Furthermore, the right eyeballs of the remaining six rats in each group were enucleated and dissected to immediately remove the retina, which was stored at -80 °C till further testing. The optic nerve (ON) was excised around 5 mm below the eyeball and promptly repaired in FAS ocular fixative (n = 11). Furthermore, a random ON from each group was put in the electron microscope fixative for fluoroscopy.

H&E staining

The eyeballs were fixed with FAS ocular fixative for 72 h before removal. One-third of the eye was excised parallel to the ON direction, and the eye contents were removed. It was then dehydrated, fixed in paraffin, and sliced into 4 µm sections. The sliced sections were then dewaxed in xylene, rehydrated in ethanol at decreased concentration, and subjected to H&E staining. After staining, the slides were

dehydrated with increased ethanol concentration, made transparent with xylene, and sealed with neutral gum. The respective sections were then observed and photographed using a BX43 optical microscope (Olympus Corporation, Tokyo, Japan).

Nissl staining

The primary visual cortex (PVC) and the ON were stained using the Nissl staining solution method (Beyotime Biotechnology, China). The 4 μm-thick paraffin sections were dewaxed, rehydrated, and then stained with Nissl staining solution for 10 min. Following that, the sections were then dehydrated, made transparent, and sealed after staining. The samples were photographed and examined under an Olympus light microscope.

Transmission electron microscopy (TEM)

The ON was carefully detached from rats and promptly placed in the electron microscope fixing solution at 4 °C for 2–4 h, followed by osmic acid (1%, pH = 7.4) treatment at 20 °C for 2 h. The TEM of the ON was done as described by Sorkou [26]. The slices were examined at 80 kV in a HITACHI H-7650 transmission electron microscope (Hitachi, Tokyo, Japan), and respective sections images were taken using a camera (Model 830 SC200 CCD, Gatan, USA).

Immunohistochemistry for IL-6, IL-1β, and TNF-α of retina and BDNF of PVC

To eliminate endogenous catalase, the paraffin sections were dewaxed and rehydrated before being immersed in 3% H_2O_2 for 10 min in the dark. The antigen was then microwaved in citrate antigen retrieval solution and treated with goat serum for 1 h at 37 °C to block certain non-specific antigenic sites. The sections were incubated with the corresponding primary antibodies at 4 °C for 14 h. The specific primary antibodies used were rabbit anti-IL-6, rabbit anti-IL-1β, rabbit anti-TNF-α (66,146-I-Ig, 26048-1-AP, and 17,590-I-AP, 1:200; Proteintech Group, Inc., USA), and rabbit anti-BDNF (66,292-I-Ig, 1:200; Proteintech Group, Inc., USA). The sections were incubated in a wet box for 2 h with the secondary antibody, stained with DAB and hematoxylin, and finally dried and sealed with neutral gum on slides. The samples were viewed and photographed using an Olympus light microscope. Image-pro plus was utilized to examine the immunohistochemistry pictures.

Western blot

The retinas of each group were pulverized, and RIPA lysate was added to extract the protein. The protein concentration in the samples was determined using a BCA kit (Beyotime Biotechnology, China) and adjusted to be equal to the loading buffer. A moderate quantity of protein (50 μg) was extracted from each group's retinas for electrophoresis and membrane transfer. The membranes were blocked in 5% skim milk for 2 h before rinsing with TBST four times for 5 min each. The membranes were incubated with the matching primary antibodies for 14–16 h at 4 °C. The membranes were washed three times with TBST and treated for 2 h with an anti-rabbit antibody or anti-mouse antibody, depending on the primary antibody. ECL Western blot detection technique was used to identify antibody reactivity of PI3K p85, p-PI3K p85, Akt, p-Akt, and NF-κB, from which the absorbance value was calculated.

Statistical analysis

All obtained data were presented as mean ± S.D. One-way ANOVA was used to analyze statistical comparisons (IBM SPSS statistics version 19.0). Furthermore, p values less than 0.05 were deemed statistically significant. (#): $p < 0.05$ vs. NC group; (##): $p < 0.01$ vs. NC group; (*): $p < 0.05$ vs. MC group; (**): $p < 0.01$ vs. MC group.

Results

The results of intraocular pressure

After successfully raising IOP in rats, they were separated into groups and given different treatments. Figure 2 depicts the results of IOP measurements taken once a week. Statistical analysis of five IOP recordings revealed that the IOP of the MC group remained consistent within four weeks and was always roughly 10 mmHg significantly higher than the NC group ($p < 0.05$). Bri group was shown to have considerably lower intraocular pressure, which was significantly lower than the MC group in the second week and reverted to normal levels in the third and fourth weeks ($p < 0.05$). Though a dropping tendency in the IOP was observed following crocetin administration in the three different dosage groups, the difference was insignificant. The results suggested that 0.3% carbomer administration into the anterior chamber effectively elevates the IOP and sustains it for an extended period. Brimonidine tartrate treatment might successfully lower IOP and restore it to the normal level, but crocetin could only alleviate the model rat's IOP to a limited extent.

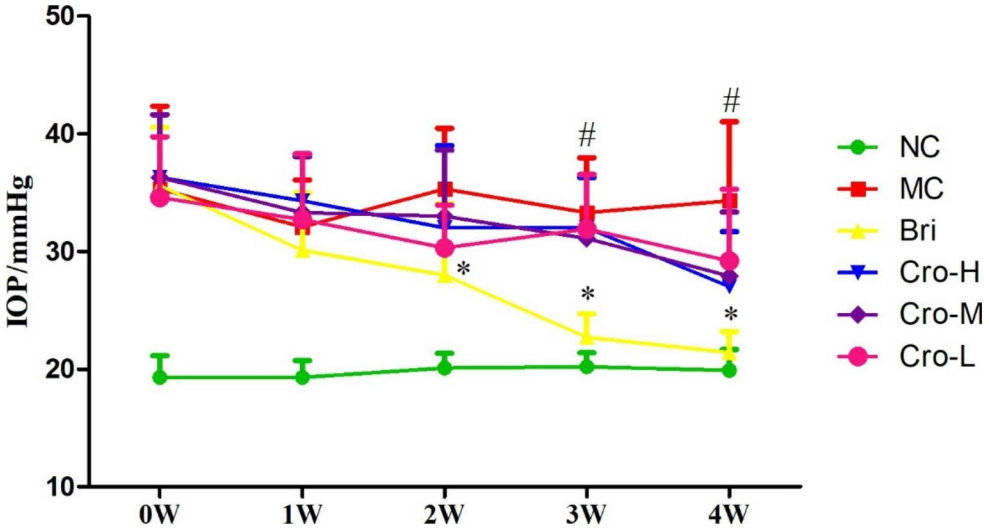

Fig. 2 A line graph indicates the level of intraocular pressure in rats

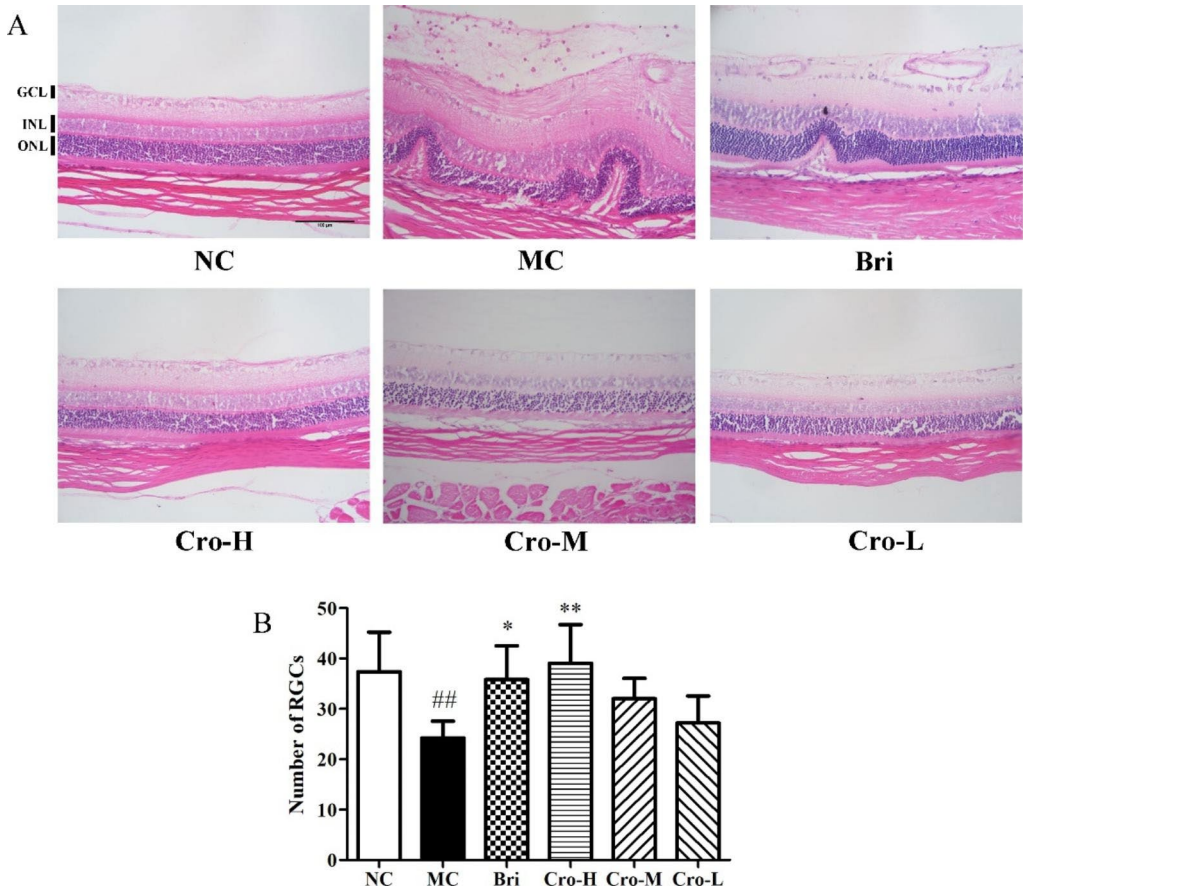

Fig. 3 Crocetin ameliorated pathological changes in the retina and reduced RGC loss during IOP elevation. (A) Representative pictures of H&E staining of rat's retina (400×). (B) The number of RGCs of GCL expressed by a histogram

Pathological assessment of the retina

The retina's ganglion cell layer (GCL), inner nuclear layer (INL), and outer nuclear layer (ONL) were clean and well-demarcated in NC group, with normal cell shape and quantity. Retinal GCL separation and cavitation were detected in MC group, and the number of GCL cells was decreased (Fig. 3B). Brimonidine tartrate eye drops may have improved the diseased structure to some extent. However, GCL exhibited minor cavitation. Crocetin used orally may improve the abnormal structure of the retina in a dose-dependent manner. The retinal morphology in Cro-H group

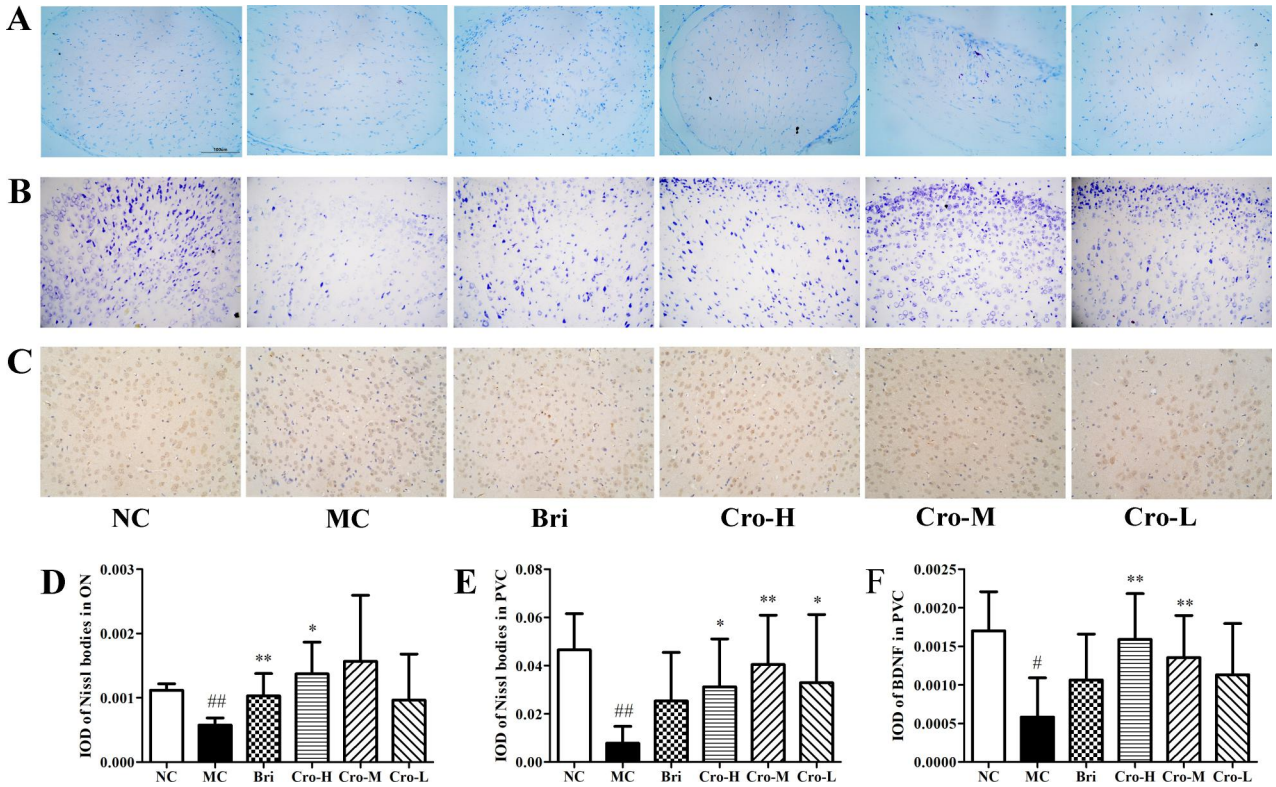

Fig. 4 Crocetin increased the content of Nissl bodies and BDNF in the body (A) Optic nerve Nissl staining (400×). (B) PVC Nissl staining (400×). (C) BDNF immunohistochemical staining in PVC (400×). (C) Immunohistochemically staining of BDNF in PVC (400×). (D) The histogram expressed the content of Nissl bodies in ON. (E) A histogram depicted the Nissl body content of PVC. (F) A histogram expressed the content of BDNF in PVC

was restored to normal, while the number of GCL cells in Cro-M and Cro-L groups decreased slightly.

Crocetin increased the content of Nissl bodies and BDNF

Nissl bodies are essential for synthesizing structural proteins, and enzymes required for synthesizing neurotransmitters, and neuromodulators of peptides. When neurons are injured or exhausted, their bodies shrink, dissolve, or vanish. Following detection, it was discovered that the content of Nissl bodies in the ON and PVC of rats in the MC group was significantly lower than in NC group ($p < 0.01$) (Fig. 4D, E). Compared to model rats, Cro-H group had considerably increased Nissl bodies in the ON of rats (Fig. 4D). Crocetin administration treatment enhanced it in the PVC, which has a highly significant impact at medium doses ($p < 0.01$) and a significant effect at high and low doses ($p < 0.05$) (Fig. 4E). Brain-derived neurotrophic factor (BDNF) is a protein with neurotrophic properties required for neuronal survival, differentiation, growth, and development, avoiding neuronal death from injury and ameliorating neuronal pathology. Compared to NC group, the expression of BDNF in PVC of MC group was considerably lower. Crocetin at high and medium dosages was found to significantly boost the BDNF expression in PVC ($p < 0.01$) (Fig. 4F).

Ultrastructural evaluation of ON by TEM

NC group rat's ON revealed tightly packed, well-myelinated nerve fibers, most of which were tiny in diameter (Fig. 5A). MC group had packed nerve fibers with greater diameters and lower axon density and aberrant axon expansion, distortion, swelling, and vacuolization (Fig. 5B). Furthermore, in MC group, distinct lamellae and whorls covering the myelin sheath were seen; and in Bri and Cro-L groups, slight lamellae and whorls covering the myelin sheath were seen (Fig. 5B, C, and **F**). The axoplasm was observed filled with cellular debris in Cro-M group (Fig. 5E). These results demonstrated that treatment with bromonidine and crocetin could ameliorate the ultrastructural damage of ON.

Effect of crocetin on IL-6, IL-1β, and TNF-α in the retina

TNF-α, IL-1β, and IL-6 levels in the retina in MC group were significantly higher than in NC group (Fig. 6G-I). Brimonidine significantly reduced the content of TNF-α

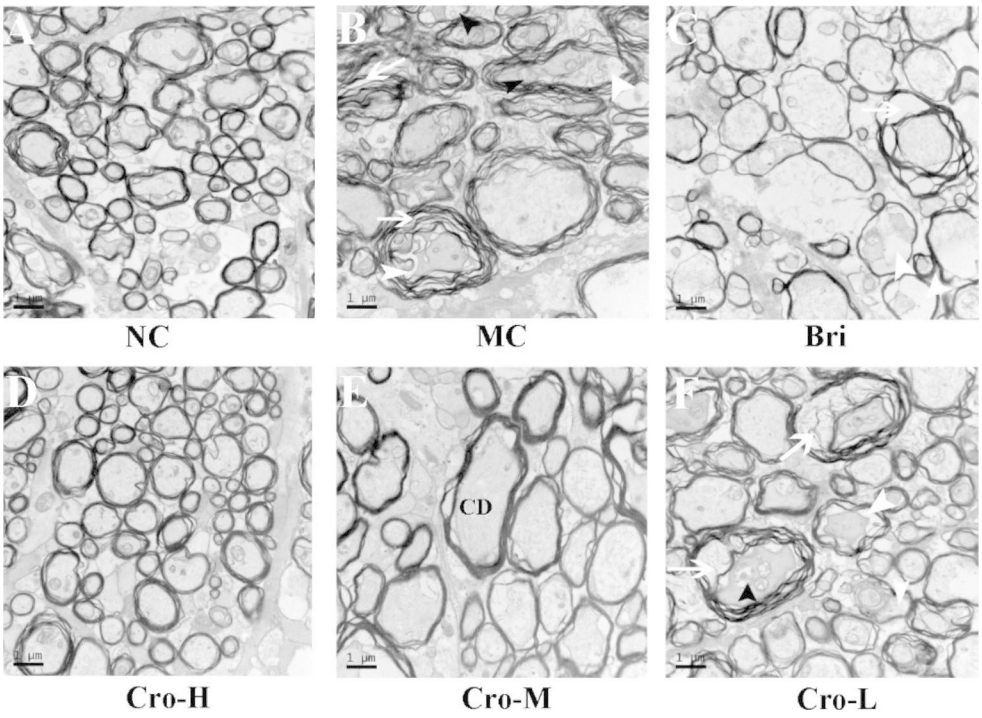

Fig. 5 Crocetin ameliorated the ultrastructure of the optic nerve. (A and D) myelin sheaths are densely packed, most of them small in diameter. (B) Decreased axon density, dilation, swelling, and vacuolization of axons (white arrowheads). (B, C, and E). The large-diameter myelin sheath was closely arranged. Separated lamellae (B and F) into whorls that covered myelin sheaths (white arrows) and watery degeneration (black arrowheads) (E). The axoplasm is filled with cellular debris (CD)

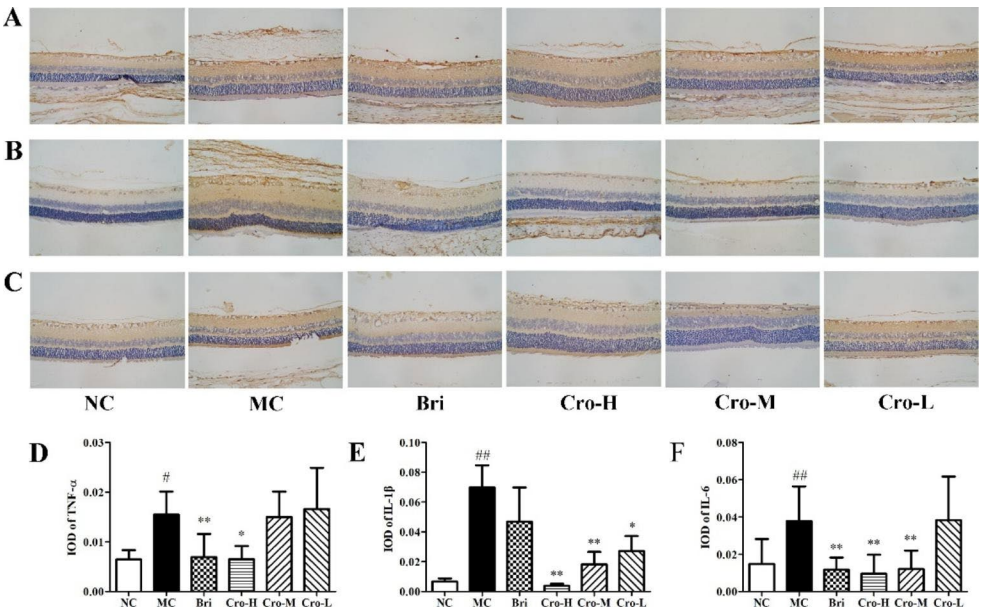

Fig. 6 Crocetin decreased the content of IL-6, IL-1β, and TNF-α in the retina. (A-C) Representative pictures of immunohistochemically staining of TNF-α, IL-1β, and IL-6 in the retina. (D-F) Histograms expressed their content in the retina

and IL-6 in the retina of rats ($p < 0.01$), but treatment with crocetin reduced the content of TNF-α, IL-1β, and IL-6 in the retina of rats in a dose-dependent manner. Crocetin at higher dosage though significantly decreased the expression of TNF-α ($p < 0.05$), IL-1β ($p < 0.01$), and IL-6 ($p < 0.01$).

Western blot detection

Western blot was used to explore the relationship between crocetin neuroprotective action and NF-κB, PI3K/Akt signaling pathways. The results are shown in Fig. 7. NF-κB expression was notably higher in the model rats than in normal rats. Brimonidine did not inhibit the expression of NF-κB when compared to the MC group, while crocetin significantly inhibited the expression of NF-κB ($p < 0.01$). Furthermore, PI3K and Akt expressions were significantly up-regulated in MC group. However, after treatment with brimonidine and different doses of crocetin, the expression of PI3K and Akt decreased ($p < 0.01$).

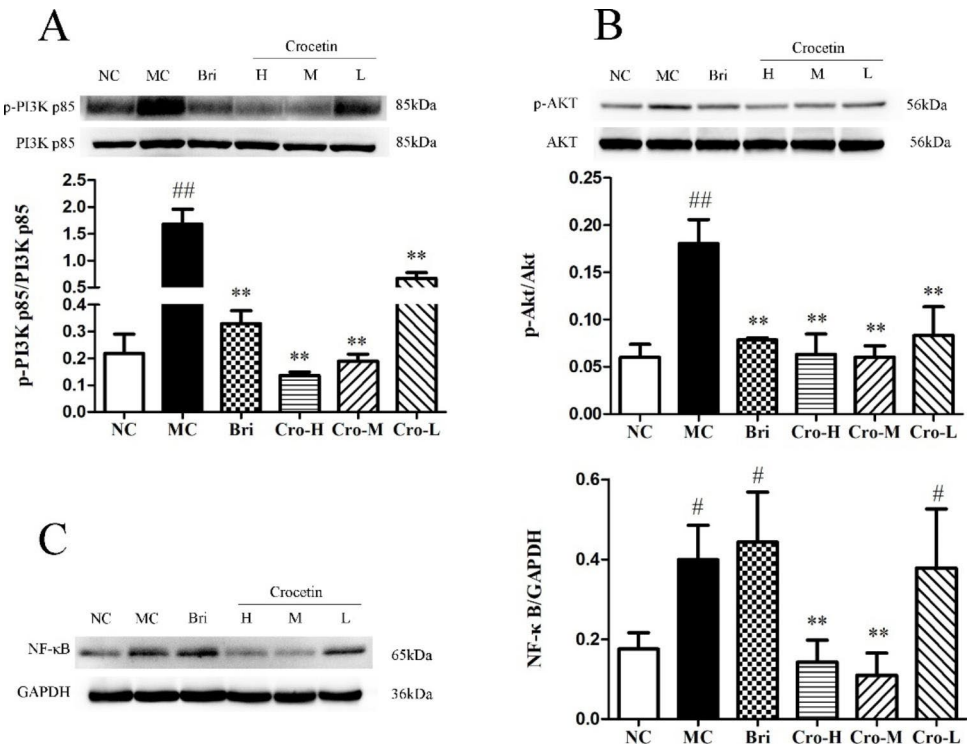

Fig. 7 The protein expression of PI3K, Akt, and NF-κB was notably higher in MC rats than in NC rats. Crocetin could inhibit the expression of PI3K, Akt, and NF-κB in the retina

Discussion

A 0.3% carbomer injection was administered into the anterior chamber to establish the glaucoma rat model. Crocetin may improve RGC survival in rats with experimental glaucoma. Crocetin boosted Nissl body expression in the ONs, improved ON structure, and raised Nissl body and BDNF expression in the brain's primary visual cortex. Furthermore, crocetin successfully inhibited the formation of inflammatory factors by inhibiting the expression of PI3K/Akt and NF-κB.

Glaucoma is a neurodegenerative eye disease defined by the loss of RGCs, leading to a gradual loss of visual function [27, 28]. The rat glaucoma model showed a reduction in retinal GCL cells (RGCs). On the other hand, crocetin may be able to prevent RGC decline.

Retrograde neurotrophic factor, interruption of axon transport [29, 30], additional signaling pathways induced by axonal injury, or direct damage to retinal cell bodies are all plausible causes of cell body death in glaucoma rats [31]. Following laser-induced intraocular hypertension, optic nerve axons swelled and vacuolated, with a 59% loss in axon number [32]. Superior and temporal optic axons are lost more selectively and significantly in mouse retinal ganglion cells following microbead injection-induced intraocular hypertension in mice [33]. According to our findings, crocetin and bromonidine could ameliorate the ultrastructure of optic neuron cells, reduce axonal vacuolization and myelin separation (Fig. 5). These findings showed that crocetin had a neuroprotective impact on retinal cells and ONs.

It has been proposed that glaucoma damage endangers the central visual system. Glaucoma affects the entire visual system, and its visual impairment is caused by damage that spreads from the retina to the advanced visual center [34]. RGC atrophy and axon degeneration allow damage to extend to CNS optic neurons via synaptic degeneration [35]. Previous studies have proved that experimental injury to the lateral geniculate nucleus or primary visual cortex might trigger retrograde trans-neuronal degeneration of RGCs. The mechanism of action was considered to be due to a decrease in neurotrophic factors derived from the central nervous system [36]. BDNF is a neurotrophic factor that increases neuronal survival. Repeated intravitreal BDNF injections and topical BDNF eye drops have been shown to protect RGCs [37, 38]. Crocetin significantly inhibited the reduced expression of BDNF in PVC of glaucoma model rats (Fig. 4 C, F). Further damage to RGCs, on the other hand, would certainly result in a decrease in visual afferent impulses, exacerbating the disuse atrophy and degeneration of central neurons [39, 40]. MC group had considerably fewer Nissl bodies in the ON and PVC than the NC group ($p < 0.01$), and crocetin significantly enhanced Nissl bodies in ON and PVC (Fig. 4D, E). This vicious loop of RGC and central neuron damage might play a vital role in the progression of glaucoma visual impairment. Crocetin is thought to enhance the amount of Nissl bodies and BDNF production in rat optic cortex neurons, hence exerting a neuroprotective

effect and restoring their functions. Crocetin primarily restores the ON, the primary visual cortex of the brain and enhances the transfer of BDNF in axons, thereby reducing RGC loss. Crocetin's neuroprotective effect was validated further by assessing BDNF and Nissl body content and TEM of the ON.

Glaucoma is a complex multifactorial disease in which RGC mortality and axonal degradation are related to apoptosis, microglial activation, and inflammation [41]. Neuroinflammation associated with glaucoma can be seen in various locations, including the retina, ON, optic tract, and brain, as well as the periphery of blood, bone marrow, or other tissues [42]. It is worth mentioning that macrophages may be found in various tissues and organs throughout the body, including the eye. Pro-inflammatory cytokines released by macrophages in glaucoma include IL-1β, IL-6, and TNF-α [43]. Our study revealed that crocetin could decrease the contents of TNF-α, IL-1β, and IL-6 in rat retina, suggesting that crocetin may be involved in retinal protection in inflammatory diseases.

Saffron has neuroprotective and anti-inflammatory effects on open angle glaucoma (OAG) [44]. Crocetin, interestingly, reaches several tissues and organs in the body and can cross the blood-brain barrier [45]. As bridge molecules between extracellular signals and cellular response effects, PI3K and Akt play critical roles in regulating NF-κB pathway [46]. PI3K/Akt intracellular signaling pathway is an upstream factor of NF-κB pathway [47]. In this work, crocetin was found to suppress the expression of PI3K, Akt, and NF-κB (Fig. 7). Furthermore, recent research has indicated that NF-κB activation increases cellular production of inflammatory cytokines such as TNF-α, IL-1β, and IL-6, which is compatible with reducing the amount of internal inflammatory cytokines in our study. According to our findings, crocetin may exhibit anti-inflammatory effects in the glaucoma retina by reducing the activation of PI3K/Akt/NF-κB signaling pathway. However, the precise method by which crocetin controls the inflammatory process in the glaucoma retina remains unknown.

The results of this study showed that bromonidine had a good effect on lowering IOP, as well as anti-inflammatory and neuroprotective effects. Brimonidine is an α2-Adrenergic agonist that decreases the IOP ~14 – 21% by both lowering the aqueous production and increasing the outflow through the uveoscleral pathway [48]. Brimonidine tartrate is a rapid IOP-lowering drug, and some studies have shown that it reduces IOP relatively quickly [1; 49]. However, many experimental studies have also shown that Brimonidine gradually reduces IOP in experimental animals [48; 50–52]. The IOP results of this study are consistent with those of previous studies in that IOP showed a gradual downward trend. Bromonidine could prevent reduction in retinal GCL cells and decrease the contents of TNF-α and IL-6 in rat retina. Interestingly, the results of PI3K/AKT/NF-κB signaling pathway showed that the expression of NF-κB was significantly up-regulated in Bri group. Previous studies have shown that the three α2-Adrenergic receptors (α2-ARs) subtypes (α2A, α2B, and α2C) promote the interaction between PI3K and ERK1/2 in a subtype-specific way, leading to subtype-specific induction of transcription by NF-κB gene transcription [53], but does not affect the protein expression of PI3K and Akt. This may be the reason for the up-regulation of NF-κB in Bri group. However, the mechanism of activation of NF-κB by α2-ARs is still unclear, and further studies are needed.

Crocetin prevents retinal cell death generated by anterior chamber injection, and we assume this action is partly mediated by crocetin's neuroprotective and anti-inflammatory properties. According to current evidence, crocetin may be used as a preventive medication for retinal degenerative diseases.

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Author contributions Ping Wang and Suhong Chen designed the study, Qiaoqiao Li, Peishi Feng, Susu Lin, and Jiajing Zhao performed the research, Zijin Xu, Ziwei Chen, and Zirui Luo analyzed the data and interpreted the results, Qiaoqiao Li, Peishi Feng, and Yi Tao were involved in writing the manuscript. All authors discussed the results and revised the manuscript.

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Declarations

Declaration of competing interest The authors declare that they have no competing interests in this work.

Compliance with Ethical Standards All applicable international, national, and institutional guidelines for the care and use of animals were followed. All animal studies were carried out as per the Guidelines for the Care and Use of Laboratory Animals at the Zhejiang University of Technology in Hangzhou, China, provided vide ethical code No. 20200810104.

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