Resveratrol Delays Retinal Ganglion Cell Loss and Attenuates Gliosis-Related Inflammation From Ischemia-Reperfusion Injury

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PURPOSE. Resveratrol has been shown to enhance the survival of retinal ganglion cells (RGCs) following ischemia-reperfusion (I/R) injury for glaucoma. However, the precise mechanisms for resveratrol’s protective effects are still unclear. The aim of this study is to determine whether resveratrol can inhibit RGC apoptosis, retinal gliosis, and inflammation, all of which are critical events in retinal degeneration following I/R injury.

METHODS. Right retinal ischemia was induced in adult male Sprague Dawley rats by increasing intraocular pressure to 110 mm Hg for 60 minutes, and the left eyes maintained at normal pressure serve as the control. Intraperitoneal injection of resveratrol or control buffer was performed continuously for 3 days from pre- to post-I/R injury and the protective effects were evaluated and compared. RGCs were retrogradely labeled with Fluoro-Gold by injection into superior colliculi. Apoptosis was detected by TUNEL staining. Western blotting and immunostaining for Bax, Bcl-2, and Caspase-3 were used to explore the Bax-associated apoptotic pathway. Gliosis was assessed by western blotting and immunostaining of retinal cross sections with anti-glia fibrillary acidic protein (GFAP) antibodies.

RESULTS. In this study, resveratrol treatment significantly reduced retinal damage and RGC loss as demonstrated by the relatively intact tissue structure in hematoxylin and cosin staining at day 7 and increased Fluoro-Gold labeling of RGCs at day 14, respectively. We found that resveratrol exhibited an anti-apoptotic effect as assessed by reduced TUNEL staining, inhibition of the early upregulated expression of the apoptosis-related protein Bax, and decreased subsequently cleaved caspase-3. However, it did not affect Bcl-2 levels. Moreover, in our I/R injury model, the combined response of reactive gliosis and related inflammation, which were demonstrated by an early induction of pro-inflammatory mediators and subsequently increased GFAP level, were significantly attenuated after resveratrol treatment.

CONCLUSIONS. These results demonstrate that resveratrol can prevent RGC death by blocking the Bax-caspase-3-dependent apoptotic pathway and suppressed gliosis-related inflammation in the retina after I/R injury. Together these results support the use of resveratrol as a possible therapeutic strategy for glaucoma.

Keywords: resveratrol, retinal ganglion cell, gliosis, ischemia-reperfusion, apoptosis

Glaucoma, a heterogeneous group of eye diseases characterized by progressive retinal ganglion cell (RGC) death and typical visual field defects, has become the primary cause of irreversible blindness worldwide. The main relevant risk factors for this disease are elevated intraocular pressure (IOP) and aging, which can lead to mechanical impairment including ischemia, gliosis, inflammation, excitotoxicity, and oxidative stress. Currently, lowering IOP is only partially effective for preserving visual function in glaucoma patients. Thus, developing new treatments to provide safe and effective RGC neuroprotection is critical.

RGC death in glaucoma pathogenesis occurs via the apoptotic programmed cell death pathway. Studies have demonstrated that the intrinsic apoptosis pathway induced by mitochondrial dysfunction is executed first, which ultimately leads to caspase-3 activation and RGC apoptosis. The principle regulator of this intrinsic mitochondrial apoptotic pathway, the pro-apoptotic factor Bax, acts as the “point of no return” in the apoptosis process and has been reported to be dysregulated after optic nerve crush injury. Additionally, the activity of anti-apoptotic Bcl-2 can counter the actions of Bax to release cytochrome c and thereby prevent intrinsic apoptosis. The expression level of Bcl-2 after retinal ischemia-reperfusion (I/R) injury is inconclusive, but the majority of studies show no significant change in expression level. Gliosis, another critical event contributing to glaucoma’s pathogenesis, is characterized as a hallmark of retinal degeneration. Reactive glia cells, such as astrocytes and Müller cell processes, respond to tissue injury by undergoing a differentiation program that results in the production of gliosis. Gliosis, in conjunction with apoptosis, is the major cause of irreversible blindness worldwide.
Resveratrol Protects RGCs, including antioxidant, anti-inflammatory, anti-apoptosis, and effective in preventing glaucoma-induced RGCs damage. We have previously reported that activated astrocytes in the optic nerve might lead to the induction of COX-2 and subsequent synthesis of PGE2, a pro-inflammatory mediator, which can lead to the death of RGCs by secondary injury. In addition, iNOS is another primary inducible enzyme that is related to inflammation and also plays a role in glaucoma RGC degeneration. Therefore, therapeutic strategies targeting these pathogenic mechanisms could be effective in preventing glaucoma-induced RGCs damage.

Resveratrol (RES), a polyphenol found in red grapes and peanuts, exhibits numerous potentially beneficial effects, including antioxidant, anti-inflammatory, anti-apoptosis, and anti-aging. In addition, studies have demonstrated that RES possesses neuroprotective properties in models of neurodegenerative disease such as Alzheimer’s disease and cerebral ischemia/reperfusion injury. Moreover, there have been no reports of apparent adverse effects to RES in both experimental animal studies and human studies, making it a superb candidate for therapeutic use. Several recent reports have shown that pretreatment or posttreatment with RES effectively protects injured RGCs in experimental glaucoma models. However, the precise mechanisms by which RES protects RGCs are still largely unknown.

Experimentally induced retinal I/R injury is an established model of glaucoma injury that ultimately leads to RGC apoptosis. In this study, we found that pretreatment and posttreatment with RES exhibited neuroprotective effects on I/R induced retina injury, and that this effect is related to the inhibition of Bax-caspase-3 dependent apoptosis of RGCs and reduction of gliosis-related inflammation in the retina after I/R injury. The neuroprotective effect of RES may be translatable to clinical applications, although further study may be warranted to investigate its possible effects on RGC axon protection and regeneration in other experimental glaucoma models.

**METHODS**

**Rat Model of I/R Injury and RES Administration**

All the experiments were performed on rats under general anesthesia in accordance with institutional protocol guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experiments were approved by the Nanchang University-Medical School Institutional Animal Use and Care Committee. The animals were housed under standard conditions and were maintained in temperature-controlled rooms on a normal 12-hour light/12-hour dark cycle. Sprague Dawley rats aged 2 to 3 months were used. Under anesthesia (chloral hydrate), transient, unilateral retinal ischemia was induced in rat eyes. The anterior chambers of both eyes were cannulated briefly, the IOP in posterior segments was fixed by immersion in 4% PFA in 0.1 M phosphate-buffered saline (PBS; pH 7.4). After perfusion, eyes were enucleated and the anterior segments were removed. The posterior segments were fixed by immersion in 4% PFA in 0.1 M PBS for 2 hours. After washing in PBS, the tissues were transferred to 70% ethanol overnight, then dehydrated and embedded in paraffin. The 5-μm-thick paraffin tissue sections were dewaxed and then rehydrated. Endogenous peroxidase activity was blocked by placing the sections in 3% H₂O₂ in methanol for 30 minutes. For antigen retrieval, the sections were heated in 10 mM sodium citrate buffer (pH 6.0) at a subboiling temperature for 10 minutes followed by cooling for 30 minutes. Then the tissue sections were incubated with specific primary antibodies (Table) diluted in 5% bovine serum albumin (BSA) in PBS overnight at 4°C. The sections incubated with PBS without primary antibody were used as negative controls. After several wash steps, the tissue sections were incubated with the secondary antibody Alexa Fluor 594 donkey anti-rabbit IgG (H+L) for 1 hour at room temperature. The sections were stained with 4′,6-diamidino-2-phenylindole (DAPI; Boster, Wuhan, China) and mounted with coverslips. The staining was repeated three or more times for each antibody and the results were consistent.

**Apoptosis Assay**

Apoptosis in retinal cells was performed as described before. Five micrometer-thick sections were prepared as described above, and apoptotic cell death was detected with a TdT-mediated dUTP nick-end labeling (TUNEL) assay kit (In Situ Cell Death Detection with Fluorescein; Roche Biochemicals, Mannheim, Germany) according to the protocol provided by the manufacturer. Tissue sections were examined with a microscope (Nikon, Tokyo, Japan) equipped with epifluorescence, digital images were obtained (SPOT; Diagnostic Instruments, Sterling Heights, MI, USA), and images were compiled using Photoshop versions 5.5 and 7.0 (Adobe Systems, San Jose, CA, USA).

**Western Blot Analysis**

Retinal tissues were dissected from the choroids and lysed in RIPA Lysis buffer containing phenylmethylsulfonyl fluoride (PMSF; Solarbio, China). The lysates were centrifuged, and supernatants were collected and subjected to western blot analysis. Protein concentrations were measured using the
Bradford assay (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer’s instructions. Protein samples (30 μg) were separated by 7.5% to 15% SDS-PAGE gels and transferred to nitrocellulose membranes. The blots were blocked in 5% nonfat dry milk in Tris-buffered saline/Tween-20 and incubated with the appropriate primary antibodies (Table) followed by incubation with peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA, USA). Finally, proteins on membranes were detected with enhanced chemiluminescence western blotting detection reagents (EMD Millipore, Burlington, MA, USA).

### Data Analysis
All quantified data represent an average of at least three samples. Statistical analysis of the data was performed by variance (ANOVA) followed by Tukey’s test using commercial graphing software (Graph Pad Prism 5.0; Graph Pad Software, San Diego, CA, USA). All data are expressed as mean ± SD. *P < 0.05 was considered significant.

## Results

### RES Reduced Loss of Retinal Tissue and RGCs After I/R Injury
RES has previously exhibited neuroprotective properties against the pathophysiological alteration of cerebral ischemia.25,26,29 To assess the effect of RES on retinal degeneration after retinal I/R injury, we first investigated the general histopathological changes of the retina at 7 days postinjury by hematoxylin and eosin (H&E) staining. Retinas from the control group appeared normal (Fig. 1A), and RES had no effect against the pathophysiological alteration of cerebral ische-

In addition, western blotting analysis also showed a markedly decreased expression of Brn3a in the I/R group, which was diminished in the I/R-plus-RES group (Fig. 2B). We also performed Brn3a immunofluorescence staining of the retina cross-sections prepared 7 days post-I/R injury, which was in agreement with FG labeling (Fig. 2C). Our results demonstrate that pretreatment plus posttreatment with RES is effective at reducing RGC loss as well as retinal tissue damage caused by retinal I/R injury.

### RES Inhibited Apoptosis of RGCs After Retinal I/R Injury
To assess the anti-apoptotic effects of RES in retinal I/R injury, fluorescent TUNEL staining was performed 1 day after I/R injury. As shown in Figure 3, notably more TUNEL-positive cells were observed in the ganglion cell layer (GCL), inner nuclear layer (INL), and outer nuclear layer (ONL) in the I/R group when compared to the control group. However, this increase in the number of apoptotic cells was attenuated by RES treatment (Fig. 3). We also analyzed expression levels of the apoptosis genes Bax, Bcl-2, and cleaved caspase-3. Western blotting showed that retinal I/R injury persistently upregulated Bax protein from as early as 1 day to 3 days (*P < 0.05; Fig. 4A), which was significantly prevented by RES intervention at day 1 after I/R injury (*P < 0.05; Fig. 4B). On the other hand, I/R injury did not significantly affect the levels of Bcl-2 (Fig. 4C), nor was the Bcl-2 protein level significantly altered by RES treatment at 1 day post-I/R injury (Fig. 4D). Immunofluorescence for Bax revealed a consistent effect of RES intervention with that shown by western blotting (Fig. 5).

**Table.** Primary Antibodies Used in the Study

<table>
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<tr>
<th>Antibody</th>
<th>Source</th>
<th>Catalog No.</th>
<th>Type of Ab</th>
<th>Dilution</th>
<th>MW</th>
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<td>Rabbit polyclonal</td>
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<td>155 kD</td>
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<td>TRANS</td>
<td>J10715</td>
<td>Donkey anti-mouse</td>
<td>1:1000 (WB)</td>
<td>55 kD</td>
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<tr>
<td>Caspase-3</td>
<td>CST</td>
<td>#1422</td>
<td>Rabbit polyclonal</td>
<td>1:1000 (WB)</td>
<td>17, 19, 35 kD</td>
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<tr>
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<td>#9661</td>
<td>Rabbit polyclonal</td>
<td>1:50 (WB)</td>
<td>17, 19 kD</td>
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CST, Cell Signaling Technology; IHC, immunohistochemistry; MW, molecular weight; WB, western blotting.
Together, these results indicate that RES can attenuate RGC apoptosis at least partially through modulating the expression of apoptosis-related proteins.

**RES Attenuated the Reactive Gliosis Induced by Retinal I/R Injury**

To investigate the effect of RES on I/R-induced reactive gliosis, the expression of GFAP, a specific marker of glia activity, was explored. Western blotting analysis showed that expression of GFAP was markedly increased post-I/R injury with a peak at day 3 postinjury, and RES significantly attenuated the I/R-induced increase in GFAP (*\(P < 0.05\), **\(P < 0.05\); Fig. 6A). Consistently, as shown in Figure 6B, GFAP staining is typically localized to Müller cells and astrocytes, and I/R injury induced a strong upregulation of GFAP in the GCL, INL, and ONL in the form of threadiness at day 3, which was attenuated especially in the INL and ONL (the form of threadiness was more intermittent) by RES treatment.

**FIGURE 1.** Resveratrol treatment inhibited retinal tissue loss after I/R injury. (A) H&E staining of retinal cross-sections showed degeneration of RGCs and a decreased thickness of retinal tissue at 7 days after I/R injury, which was markedly inhibited by resveratrol treatment. Representative images from control group, control group plus resveratrol, 7-day I/R group, and 7-day I/R group plus resveratrol. Magnification, 40×. (B) Effects of resveratrol on retinal tissue after I/R injury. Retinal tissue thickness was assessed by H&E staining and Image J analysis. Data are shown as mean ± SEM (\(n = 5\) per group, *\(P < 0.05\), **\(P < 0.05\)).

**FIGURE 2.** Resveratrol inhibited RGCs apoptosis after I/R injury. (A) FG was injected into the bilateral superior colliculi at 7 days before I/R injury. Fluorescence microscopy analysis of flat-mounted retinas and FG-labeled cells was performed at 14 days after I/R injury. The remaining fluorochrome-labeled cells were quantified using image analysis and expressed as the mean number of cells ± SEM (\(n = 6\) per group, *\(P < 0.05\), **\(P < 0.05\)). Magnification, 20×. (B) The expression level of Brn3a was evaluated by western blot analysis. β-tubulin was used to ensure equal loading. Data are shown as mean ± SEM (\(n = 6\) per group, *\(P < 0.05\), **\(P < 0.05\)). (C) Rat retinas from different groups were harvested at 7 days after I/R injury and cross sections were subjected to immunostaining with Brn3a (arrows). Magnification, 40×.
RES Attenuated the Inflammation Induced by Retinal I/R Injury

Prior studies have shown that glia activation is linked to the production of pro-inflammatory mediators, which in turn promote the death of RGCs.\textsuperscript{15–18} We therefore tested the effects of RES on the release of pro-inflammatory factors in the retina after I/R injury. Western blotting showed a remarkable elevation of COX-2 at day 1 and iNOS proteins at day 3 post-I/R injury (\(\ast P < 0.05\), \#\( P < 0.05\); Figs. 7A, 7C), which was significantly attenuated by RES treatment (\(\ast P < 0.05\), \#\( P < 0.05\); Figs. 7B, 7D). Consistent results were observed in the immunostaining of the two proteins, and the modulation of the two proteins in retina was possibly related to the stress response of Müller cells and astrocytes (Fig. 8). Importantly, RES treatment alone had no effect on the levels of COX-2 or iNOS. Together, these results demonstrate that I/R injury leads to gliosis-associated inflammation in the retina, which can be prevented by RES treatment.

**DISCUSSION**

Glaucome is the most common blindness-causing eye disease resulting from irreversible RGC degeneration, for which safe and effective therapies are lacking.\textsuperscript{1} To help address this need,
we investigated the effects of RES on retinal I/R induced RGC death, an established model of acute glaucoma injury, as well as the related gliosis and inflammation induced by retinal I/R injury. Encouragingly, we found that pretreatment combined with posttreatment with RES directly prevented RGC death by inhibiting the Bax-cleavage caspase-3-dependent apoptotic pathway. In addition, our results showed that RES could attenuate reactive gliosis and decrease the concomitant production of pro-inflammatory mediators in the retina after I/R injury. Collectively, our data provide compelling evidence that RES may be a promising therapeutic intervention measure for glaucoma.

RES possesses anti-oxidant, anti-inflammatory, anti-apoptotic, and anti-aging properties and is neuroprotective in models of Alzheimer's disease and cerebral I/R injury.21–23 The neuroprotective effects of RES are notable because it shows production of pro-inflammatory mediators in the retina after I/R injury. Collectively, our data provide compelling evidence that RES may be a promising therapeutic intervention measure for glaucoma.

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Resveratrol Protects RGC

RES’s ability to cross the blood-brain barrier, which means it can also likely cross the blood-retinal barrier easily. Additionally, the superb safety profile of RES supports its potential to be a safe therapeutic intervention in numerous disease conditions.\textsuperscript{28,29} It has been reported that pretreatment or posttreatment with RES, primarily by oral administration, provides neuroprotection in glaucoma, but the underlying mechanisms were largely unknown.\textsuperscript{30–32} Considering that many studies have shown that oral bioavailability of RES was less than 1\%, which has possibly caused discrepancies between many in vitro and in vivo studies on RES, pretreatment plus posttreatment with RES has been performed in our retinal I/R injury models.\textsuperscript{38,39} We further investigated the mechanisms underlying RES’s protective effects on glaucoma.

Our histopathological observations indicate that I/R-induced retinal tissue degeneration was significantly attenuated and the structural integrity was markedly maintained with RES administration. It has been observed that Brn3a is primarily expressed in RGCs and becomes undetectable after cell death.\textsuperscript{36} The Brn3a protein level was markedly downregulated in the retina after I/R injury as observed by both western blotting and immunofluorescence, which was attenuated by RES treatment. This result was further corroborated by attenuation of the I/R-induced decrease in FG-labeled RGCs with RES treatment.

RGC death in glaucoma pathogenesis has been found to occur by the apoptotic programmed cell death pathway.\textsuperscript{2} In mammalian RGCs, apoptosis can be triggered extrinsically or intrinsically.\textsuperscript{40} The intrinsic pathway, which is involved in

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**Figure 7.** The effect of RES on the production of pro-inflammatory mediator. I/R injury markedly upregulated the expression of COX-2 at day 1 (A) and iNOS at day 3 (C), but resveratrol significantly inhibited the production of the two pro-inflammatory mediators (B, D). Data were shown as mean ± SEM (n = 3 per group, *P < 0.05, **P < 0.05).

**Figure 8.** The effect of RES on the expression location of pro-inflammatory mediator. I/R injury markedly upregulated the expression of COX-2 at day 1 (A) and iNOS at day 3 (B). Resveratrol significantly inhibited the production of the two pro-inflammatory mediators (n = 3 per group). Arrows: COX-2 and iNOS-positive staining. Magnification, 40X.
apoptotic stimulus induced mitochondrial dysfunction, starts with the release of mitochondrial cytochrome c into the cytoplasm and ultimately leads to an irreversible apoptotic cascade.3,4,8 Studies have demonstrated that the initial steps of the intrinsic apoptotic pathway converge at pro-apoptotic Bax, a key mediator of mitochondrial involvement.3 Anti-apoptotic Bcl-2 can counter the actions of Bax to prevent release of cytochrome c and thereby prevent intrinsic apoptosis.9 Caspase-3, a key mediator of apoptosis, is convened on by both the extrinsic and intrinsic apoptotic pathways. Therefore, Bax, Bcl-2, and caspase-3 have become a primary focus in studies of apoptosis.

Current evidence suggests that I/R injury induces Bax expression and caspase-3 activation in RGCs, and therapeutic interventions targeting Bax or caspase-3 have shown effective protection against RGC degeneration.3,7,8,43 However, the expression of Bcl-2 after retinal I/R injury remains inconclusive with the majority of studies showing no significant change.10-12 In our model, we detected an early increased expression of Bax in the retina 1 day after I/R injury, followed by subsequent upregulation of cleaved caspase-3 at day 3 post-I/R injury. In addition, the increased TUNEL staining demonstrates that the retinal damage does involve apoptotic induction of DNA breaks. However, we did not observe a significant change in retinal Bcl-2 protein level after I/R injury, which is consistent with another study.12 Additionally, our data showed that RES administration significantly inhibited DNA fragmentation, the expression of Bax, and cleavage of caspase-3, whereas it had no influence on the overall level of Bcl-2. Notably, a separate recent study observed another mechanism by which RES may inhibit RGC apoptosis, which was partly through downregulation of caspase-3 expression at 2 days after I/R injury.11 In our study, however, we did not observe RES to markedly decrease caspase-3 expression (Figs. 4E, 4F). These conflicting results could be due to species differences or the different time points analyzed. Therefore, our results indicate that RES can prevent the apoptosis of RGCs in I/R-injured retinas by blocking the Bax-cleavage induced, caspase-3 mediated pro-apoptotic pathway, although the role of Bcl-2 remains unclear.

Gliosis is another critical event in glaucoma pathogenesis that may play a degenerative role.15 Astrocytes and Müller cells, the major glial cells in normal retina, assume vital roles in maintaining the blood-retinal barrier and RGC health.16 In response to injury or disease, glial cells lose their physiological functions and exhibit a reactive phenotype, which is characterized by increased GFAP expression and expanded migration. This reactive gliosis response has been described in glaucoma.6,7 It has been demonstrated that reactive glia activation in the retina is linked to expression of various pro-inflammatory mediators, which, in turn, may promote the death of RGCs.15-18 Our results provide evidence that RES can significantly decrease I/R-induced reactive gliosis in the retina, as evidenced by the GFAP staining pattern, localized in both Müller cells and astrocytes, and the overall GFAP expression level, which were markedly increased 3 days after I/R injury but were significantly inhibited by RES treatment. Similarly, a recent study also found that RES can significantly attenuate the ischemia-induced increase in vimentin immunolabeling of Müller cells, also a marker of gliosis.19 As reversal of reactive gliosis has been demonstrated to be neuroprotective,15 the results presented in this study suggest that RES prevents RGC death at least partly by suppressing I/R-induced gliosis.

Our previous studies have reported that astrocytes exposed to stimuli rapidly produced COX-2, a pro-inflammatory mediator that could lead to RGCs’ secondary injury.18,19 In addition, iNOS is another primary inducible inflammatory enzyme that also plays a degenerative role in glaucoma.20 Our results demonstrate a remarkably increased expression of COX-2 at 1 day and iNOS at 3 days post-I/R injury, both of which were attenuated by RES treatment. Another recent study has reported that COX-2 immunoreactivity was upregulated in the retina as early as 6 to 12 hours after I/R injury.49 Consistently, we found that retinal COX-2 was induced at an early stage (1 day post-I/R injury), and that iNOS levels remained heightened at 3 days post-I/R injury. It is noteworthy that our immunostaining showed the modulation of these two pro-inflammatory cytokines was possibly related to glia cells. These results suggest that the neuroprotective effects of RES in I/R-injured RGCs might be due to both its prevention of Bax-caspase-3 dependent apoptosis as well as by counteracting reactive gliosis and the associated inflammation.

CONCLUSIONS

This study provides compelling evidence that RES treatment by intraperitoneal injection prevents RGC death, likely through inhibition of the Bax-caspase-3 dependent apoptotic pathway. In addition, our results demonstrate that the neuroprotective effect of RES might also be related to attenuation of the reactive gliosis and decreased production of pro-inflammatory mediators in the retina after retinal I/R injury. Together these results further suggest the potential therapeutic effectiveness of RES for treatment of glaucoma-induced I/R injury. Further studies are warranted to investigate its potential effects on RGC axon protection and regeneration in experimental glaucoma models in preparation for future human trials.

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