

## Original Research Article

# Rutin prevents retinal ganglion cell death and exerts protective effects by regulating transforming growth factor- $\beta$ 2/Smad2/3Akt/P TEN signaling in experimental rat glaucoma

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### Abstract

**Purpose:** To investigate the protective effect of rutin against glaucoma in a rat model, and the mechanisms involved.

**Methods:** Sprague-Dawley rats were injected hypertonic saline in the limbal vein for elevation of intra-ocular pressure (IOP). Rats in the treatment group were administered rutin at doses of 12.5, 25 or 50 mg/kg orally and daily for 21 days.

**Results:** Rutin markedly ( $p < 0.05$ ) reduced IOP and prevented loss of retinal ganglion cells (RGCs). The expression of apoptotic pathway proteins, i.e., Bcl-xL, Bcl-2, Bad and Bax were significantly ( $p < 0.05$ ) regulated by rutin. Moreover, rutin caused a substantial decrease in TGF- $\beta$ 2 expression, and also down-regulated p-Smad2 and p-Smad3 dose-dependently ( $p < 0.05$ ). Raised levels of collagen I, fibronectin and elastin were effectively down-regulated. Rutin substantially up-regulated the Akt pathway involved in cell survival, and markedly improved the survival of RGCs subjected to hypoxia in vitro ( $p < 0.05$ ).

**Conclusion:** These results reveal that rutin exerts protective effect against glaucoma in a rat model via a mechanism involving regulation of the TGF- $\beta$ 2/Smad2/3Akt/P TEN signaling pathways. Thus, rutin has potentials for use in the management of glaucoma.

**Keywords:** Apoptosis, Glaucoma, Rutin, Retinal ganglion cell, Smad signaling, Transforming growth factor- $\beta$

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## INTRODUCTION

Glaucoma is a complex progressive optic neuropathic condition characterized by loss of

retinal ganglion cells (RGC), leading to visual impairment [1]. The principal risk factor implicated in glaucoma pathogenesis is abnormal elevation of intra-ocular pressure (IOP) [2]. The

RGCs are extremely susceptible to stress from aberrantly elevated IOP; increased resistance to aqueous humor (AH) outflow is associated with elevated IOP [3].

The trabecular meshwork (TM) is an optic tissue composed of layers of trabecular beams surrounded by elastic fibers, fibronectin and laminin. The trabecular cells secrete extracellular matrix (ECM) components, and are associated with ECM signals [4]. The trabecular meshwork (TM) exhibits several morphological and biochemical changes including decreased cellularity, ECM accumulation and changes in actin cytoskeleton in glaucomatous conditions, all of which contribute to increased resistance to AH outflow, thereby leading to high IOP [5].

Transforming growth factor- $\beta$  (TGF- $\beta$ ) is one of the major cytokines with wide range of functions. It is crucial for maintenance of optic tissue homeostasis and neovascularization in the optic tissues [6]. Isoforms of TGF- $\beta$ , on binding to their receptors, initiate intracellular signals via Smad proteins which have numerous subtypes [7]. The TGF- $\beta$  receptors phosphorylate Smad-2 and Smad-3. When phosphorylated, Smad-2 and Smad-3 bind to Smad-4, and the Smad-2-Smad-3-Smad-4 complex gets translocated from cytosol to nucleus, and activate transcription of target genes leading to ECM production. It is known that TGF- $\beta$ 2 induces expressions of ECM proteins such as collagen, elastin, fibronectin and proteoglycans, and also induces inhibitors of proteolytic degradation of ECM [8].

The PI3K/Akt pathway is a major signaling pathway associated with cell survival [9]. It has been reported that the AKT pathway promoted RGC survival in experimental animal models of glaucoma [10]. Phosphatase and tensin homolog (PTEN), the major negative regulator of the pathway, acts as a cofactor for Smad2/3 phosphatase and regulates TGF- $\beta$  signaling and ECM deposition. Phosphorylation of PTEN at Thr382, Thr383 and Ser380 reduces its activity and aids in activation of AKT signaling [11]. Understanding of cell signaling mechanisms by which TGF- $\beta$  induces excess ECM deposition gives an insight into the pathogenesis of glaucoma. Thus, compounds that target key pathways in glaucoma would be immensely valuable in its therapy.

Evidence suggests that sufficient consumption of fruits and vegetables can lower the risk of cataracts in humans [12]. Flavonoids are a large family of phytochemicals that are widely distributed in fruits and vegetables. Rutin (3, 3', 4', 5, 7-pentahydroxyflavone-3-rhamnoglucoside)

is a flavonoid which is abundantly present in citrus fruits such as lime, oranges, lemons and grapefruits [13]. Rutin possess antioxidant activities [14], as well as neuroprotective [15], anti-diabetic [16], and anti-cancer effects [17]. Considering the wide pharmacological properties of rutin, the present investigation was carried out to study its effect on RGC survival and TGF- $\beta$ 2/AKT/PTEN/Smad pathways in experimental glaucoma.

## EXPERIMENTAL

### Chemicals and antibodies

Antibodies against transforming growth factors  $\beta$  (TGF- $\beta$ ), TGF- $\beta$ 2, PTEN, p-PTEN, Akt, p-Akt, GSK-3 $\beta$ , p-GSK-3 $\beta$ , and collagen I were procured from Cell Signaling Technology (Danvers, MA, USA), while antibodies against cleaved caspase-3, Bcl-2, Bcl-xL, Bad, Smad-2, Smad-3, phospho-Smad-2, phospho-Smad-3, fibronectin, elastin,  $\beta$ -actin and horseradish peroxidase-labelled IgG secondary antibodies used for expression analysis were purchased from Santa Cruz Biotechnology (Texas, USA). Dulbecco's modified Eagle's medium (DMEM)-F-12 was product of Thermo-Fischer Scientific. Rutin, buffers used in Western blotting analysis and chemicals used in the study were obtained from Sigma-Aldrich (St. Louis, MO, USA).

### Experimental animals

Male Sprague Dawley rats weighing 180 - 200 g (n = 60) from Qingdao University animal study facility were used. The study designed and the protocols involved in the study were approved by the Institutional ethical committee of the Qingdao University (no. XY246123). The procedures and animal handling were conducted in accordance with the National Guidelines of Animal Care Committee in line with the International guidelines for study of laboratory animals [18].

The rats were housed in sterile cages (3 animals per cage), and were maintained under 12-h day/12-h night cycle in controlled laboratory conditions at a relative humidity of 55 – 60 % and temperature of 22 – 23 °C. The rats were acclimatized to the animal house conditions for 5 days before the initiation of the study.

### Study design

The rats were randomly divided to 6 treatment groups (n = 12/group). For experimental induction of glaucoma, rats were anesthetized (ketamine 100 mg/mL) and xylazine (20 mg/mL) and a drop of proparacaine hydrochloride (0.5 %)

was applied onto each eye. Intra ocular pressure (IOP) was measured instantly following anesthesia.

Hypertonic saline solution was injected into the eyes of the animals to induce experimental glaucoma. A stable baseline IOP was documented prior injection of saline. Approximately 50  $\mu$ L of 2M hypertonic saline was injected into the limbal venous system as previously described [19]. Separate groups of rats (group III - V) received rutin at 12.5, 25 or 50 mg/kg dose orally via gavage per day starting 24 h after glaucoma induction for 3 weeks. The glaucoma group (Group II) received equal volume of saline following glaucoma induction. Control group (Group I): the group received no medication, although an equal volume of saline solution was injected into the right eyes for hypertonic saline. Group VI: animals were administered with rutin at 50 mg/kg and were not injected with hypertonic saline.

### IOP measurement

IOP was measured under anesthesia as mentioned above. A calibrated Tonolab tonometer was used for measurement (Colonial Medical Supply Company, Franconia, NH, USA). IOP was measured every week for consecutive 6 weeks as suggested by Moore *et al* [20].

### Retrograde labelling of RGCs and counting

The rats were deeply anesthetized as described previously and the superior colliculus on each side was injected with 2 mL Fluoro-Gold (FG, 5 % in PBS) as previously reported. Seven days after FG injection, the animals were euthanized and the eyeballs were enucleated and fixed for 2 h at room temperature in paraformaldehyde (4 %). The retinas were cautiously dissected and observed. RGC were counted in the inner and peripheral retina at 1.5 to 2.0 mm and 3.5 to 4.0 mm from the optic disc. The RGC were quantified at 200 x under laser scanning confocal microscope (TCSSP8, Hamburg, Germany). Average counts in eight microscopic fields of identical size were calculated and the density is presented as number of cells/  $\text{mm}^2$ .

### Immunohistochemistry

The eye sections (10  $\mu$ m) were incubated in 0.1 % Triton X-100 and bovine serum albumin (BSA; 3 % w/v) at room temperature for 30 min. The eye sections were incubated with primary antibodies (anti-cleaved caspase-3 and anti-survivin) at 4 °C overnight followed by incubation with fluorescein-conjugated secondary antibody

(Molecular Probes, Waltham, MA, USA) and Hoechst staining [21]. The sections were then visualized via confocal microscopy (Leica SP8, Hamburg, Germany).

### Western blotting

The retinal or the RGC cells were subjected to cell lysis and total protein were extracted using cell lysis buffer with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Total protein was quantified using BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Equivalent volumes of sample proteins (60  $\mu$ g) from different treatments were run on SDS-PAGE (10-12 %) and separated electrophoretically. The protein bands were blotted onto PVDF membranes (Invitrogen). The blotted membranes were blocked using TBST buffer (20 mM Tris -pH7.6; 137 mM NaCl; 0.1 % Tween 20) containing 5 % non-fat milk for 60 min at 37 °C. Membranes were then incubated overnight at 4 °C with specific primary antibodies (1:1000 dilution). Later, the membranes were then washed with TBST and incubated at 37 °C for 60 min with HRP-labelled secondary antibody (1: 2000 dilution). The positive bands were detected by enhanced chemiluminescence method (Millipore, USA) and analysed by ChemiDoc XRS imaging system (Bio-Rad, USA). The band intensities of the test proteins were normalised with expressions of  $\beta$ -actin that was used as internal control.

### Real-time (RT) PCR analysis

Total RNA from the retinal tissues were isolated using RN easy kit (Qiagen Inc. Valencia, CA) according to the manufacturer's protocol and total RNA content was estimated using RiboGreen fluorescent dye (Molecular Probes Inc. Eugene, OR). First strand of cDNA was synthesized using cDNA reverse transcription kit (Applied Biosystems, CA, USA). PCR was carried out by means of 7300 Real-Time PCR System (Applied Biosystems) with SYBR green fluorescence. The following primers were used for amplification- Smad2-forward, 5'-ATGTCGTCATCTTGCCATTC-3', reverse, 5'-AACCGTCCTGTTTTCTTTAGCTT-3'; Smad3-forward:5'-CATTACCATCCCCAGGTCAC-3', reverse,5' CGTAATTCATGGTGGCTGTG-3'; GAPDH- forward: 5'-CACCACCATGGAGAAGG C-3', reverse: 5'-CCATCCACAGTCTTC TGA-3'. The final PCR products were exposed to agarose gel electrophoresis (2 %). The bands were stained with ethidium bromide (0.05 %) and the intensities were analysed by Bio-Gel imagery apparatus (Bio-Rad, USA).

## RGC isolation and cell culture

RGCs were isolated from 3-day old Sprague-Dawley pups and cultured. The purity was also checked as described by Gao *et al* [22]. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-F-12 (Thermo-Fischer Scientific), supplemented with 4 mM glutamine, 10 % fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were cultured at 37 °C in the presence of 5 % CO<sub>2</sub>. Hypoxic conditions were induced by exposing RGCs to 200 µM cobalt chloride (CoCl<sub>2</sub>) for 48 h [23]. Fresh medium was replaced and varying concentrations of rutin (10, 20, 40, 80, 100 µM) was added and incubated for 48 h.

## Determination of RGC viability

Following exposure to CoCl<sub>2</sub> and rutin, cell viability was analysed using Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fischer Scientific, USA) and by TUNEL assay using TUNEL assay kit – BrdU-Red (Abcam, USA) according to manufacturer's instructions.

## Statistical analysis

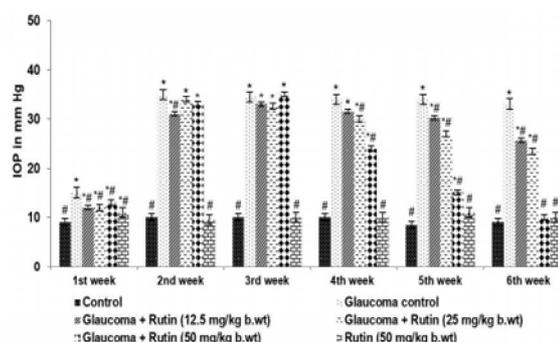
The data were subjected to statistical analysis using version 22.0 SPSS software (SPSS Inc, Chicago, USA). Multiple group comparisons were done using ANOVA (one-way analysis of variance) followed by Duncan's Multiple Range Test (DMRT). Values of  $p < 0.05$  were considered statistically significant.

## RESULTS

### Rutin effectively reduced IOP in glaucoma-induced rats

There was a significant ( $p < 0.05$ ) increase in IOP 24 h after glaucoma induction by injection of hypertonic saline (Figure 1). The IOP remained steadily high in the animals treated with saline alone for over 4 weeks, following which a non-significant decrease was noticed. Rats treated with rutin (12.5, 25 or 50 mg) exhibited significantly ( $p < 0.05$ ) lower IOP than rats in group II throughout the period of study. The rats treated with rutin alone presented IOP close to normal levels throughout observation period, suggesting that rutin administration did not affect IOP. In glaucoma control rats, IOP increased from 15 mmHg in the 1<sup>st</sup> week to 34.5 mmHg in the 3<sup>th</sup> week, and to 34.0 mmHg at weeks 4 and 5. However, at week 6, the IOP slightly dropped to 33 mmHg, while it dropped from 35.5 to 15.0 mmHg on treatment with 50 mg rutin at week 5.

At week 6, the IOP levels were 10 mmHg in rats treated with 50 mg rutin. These observations indicate the potency of rutin in reducing IOP, which is of significant clinical value in treatment of glaucoma.

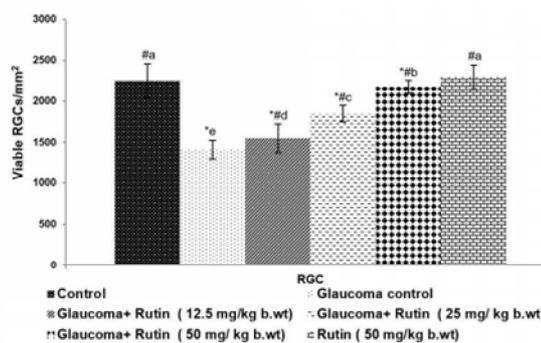


**Figure 1:** Effect of rutin on IOP levels following glaucoma induction

Data are presented as mean  $\pm$  SD ( $n = 6$ ). \* $p < 0.05$ , compared with control; # $p < 0.05$ , compared with glaucoma control; a - e represent mean values that differ from each other at  $p < 0.05$  as determined using one-way ANOVA and DMRT analyses.

### Rutin promoted RGC survival

The effect of rutin on RGC survival under glaucomatous conditions was assessed by retrograde FG labelling. Following induction of glaucoma, a significant reduction in RGC density was observed (Figure 2). Rutin treatment significantly ( $p < 0.05$ ) increased RGC survival as reflected by viable cell density. Rutin at a dose of 50 mg exerted maximal protective effects as shown by significant increase in RGC from 1412.1 cells/mm<sup>2</sup> to 2180 cells/mm<sup>2</sup>. Furthermore, there was no significant difference in density of RGCs in rats treated with rutin alone, when compared with normal control rats.

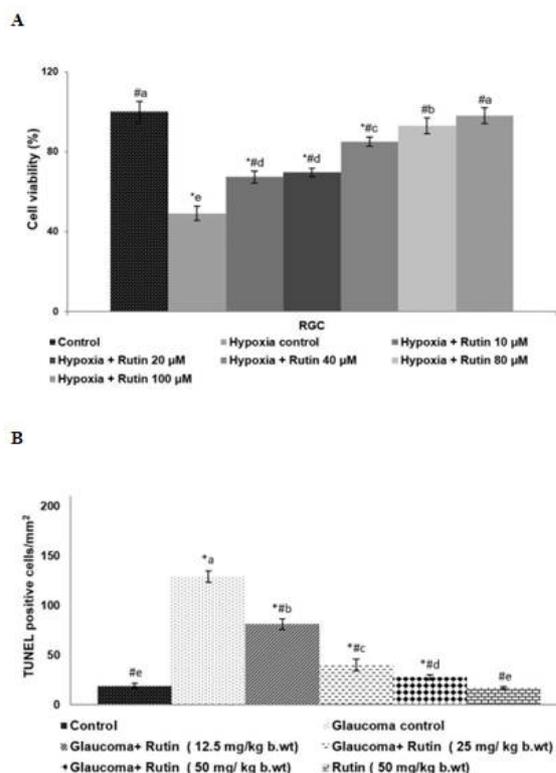


**Figure 2:** Effect of rutin on the viability of retinal ganglion cells

Data are presented as mean  $\pm$  SD ( $n = 6$ ). \* $p < 0.05$ , compared with control; # $p < 0.05$ ,

compared with glaucoma control; a - e represent mean values that differ from each other at  $p < 0.05$  as determined using one-way ANOVA and DMRT.

*In vitro* studies also revealed improved proliferation of RGC cells exposed to  $\text{CoCl}_2$  (Figure 3) on incubation with rutin (10 - 100  $\mu\text{M}$ ). The RGC cell viability increased to 98.1% from 49.2% on incubation with 100  $\mu\text{M}$  rutin. These results indicate the protective effects of rutin on RGCs under stress.

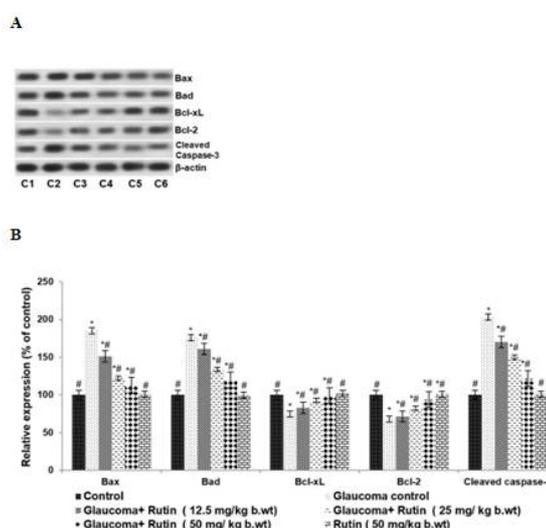


**Figure 3:** Effect of rutin on cell viability of retinal ganglion cells exposed to hypoxic conditions. (A) Rutin improved viability of RGC cells under hypoxic conditions *in vitro*. (B) Rutin reduced RGC apoptosis in glaucomatous rats, as determined by TUNEL assay. Data are presented as mean  $\pm$  SD,  $n = 6$ . \* $p < 0.05$ , compared to normal control; # $p < 0.05$ , compared to hypoxic control; # $p < 0.05$ , compared to glaucoma control; a-e represents mean values that differ from each other as derived using one-way ANOVA and DMRT

**Rutin down regulated cleaved caspase-3 and expressions of pro-apoptotic proteins**

The effect of rutin on extent of activation of caspase-3 under glaucomatous conditions was determined by assessing the expression levels of cleaved caspase-3 and apoptotic pathway proteins. Following glaucoma induction, marked ( $p < 0.05$ ) increase in cleaved caspase-3 expression and considerable decrease in survivin

levels were observed (Figure 4). These observations suggest that the saline injection raised the degree of apoptosis. Furthermore, there were significantly increased expressions of Bax and Bad, and marked reductions in the expression levels of Bcl-2 and Bcl-xL in the glaucomatous rats (Figure 4). However, the administration of rutin at all the three tested doses resulted in remarkably downregulated expressions of Bax and Bad, while Bcl-xL and Bcl-2 were significantly upregulated ( $p < 0.05$ ). Although 12.5 and 50 mg modulated protein expression, the higher dose of 50 mg brought more pronounced effects, which suggests that the effect of rutin was dose-dependent. These results indicate that rutin regulates the expressions of apoptotic pathway proteins.

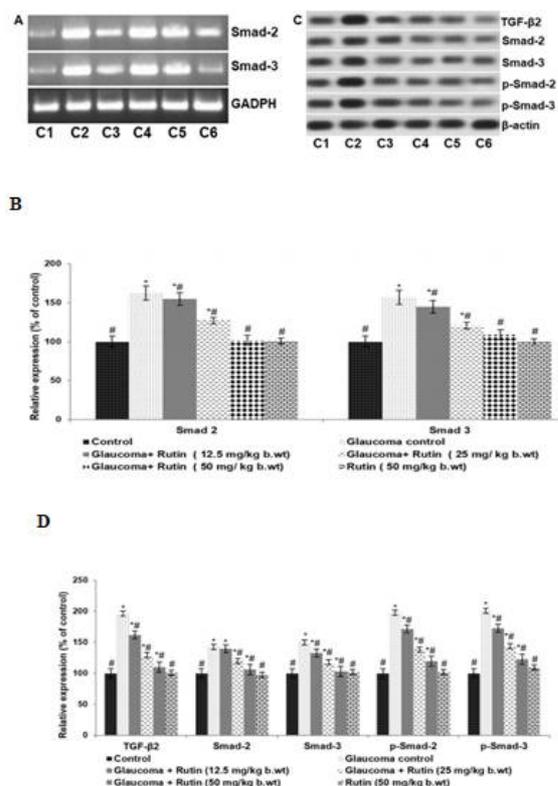


**Figure 4:** Effect of rutin on the expressions of apoptotic pathway proteins. (A) Representative immunoblot of proteins, and (B) relative expressions of proteins, with control expressions set at 100%. Data are presented as mean  $\pm$  SD ( $n = 6$ ). \* $p < 0.05$ , compared with normal control; # $p < 0.05$ , compared with glaucoma control. C1 = Control; C2 = glaucoma control; C3 = glaucoma+ rutin (12.5 mg/kg); C4 = glaucoma+ rutin (25 mg/kg); C5 = glaucoma + rutin (50 mg/kg); C6 = rutin (50 mg/kg)

**Rutin up-regulated TGF- $\beta$ 2 and Smad expressions**

The expression of TGF- $\beta$ 2 was markedly raised in the glaucoma rats, when compared to normal control (Figure 5). Enhanced levels of phosphorylated forms of Smad-2 and 3 were observed following saline injection, an indication of activation of Smad signaling. Moreover, there were significant increases in the mRNA and protein expressions of total Smad-2 and Smad-3 levels. Interestingly, rutin caused a substantial decrease in TGF- $\beta$ 2 expression, and also down-regulated p-Smad2 and p-Smad3 dose-

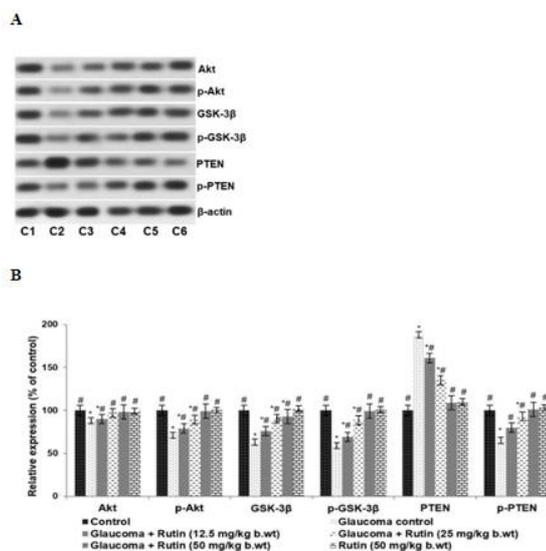
independently, while the expression of total Smad2 and 3 were brought back to near normal control levels. Rutin at 50 mg suppressed TGF-β2 more effectively than at the lower doses of 12.5 and 25 mg.



**Figure 5:** Effect of rutin on the expressions of Smad proteins. Rutin regulated the expressions of Smad-2 and Smad-3 both at mRNA (A & B) and protein levels (C & D). (A) SDS-PAGE showing Smad 2 and 3 mRNAs; (B) relative expressions of Smad 2 and 3 mRNA, with control levels set at 100%; (C) representative western blot results; (D) relative expression levels of proteins. Data are presented as mean ± SD (n = 6, \*p < 0.05, compared to normal control; #p < 0.05, compared to glaucoma control. C1 = Control; C2 = glaucoma control; C3 = glaucoma+ rutin (12.5 mg/kg); C4 = glaucoma+ rutin (25 mg/kg); C5 = glaucoma + rutin (50 mg/kg); C6 = rutin (50 mg/kg)

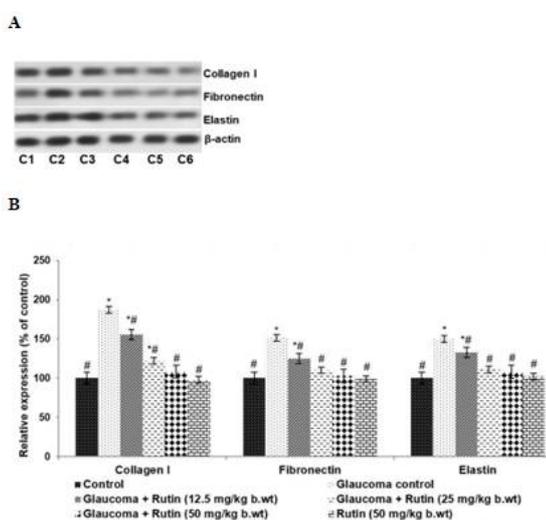
**Rutin regulated Akt/PTEN signaling**

Significantly raised PTEN expressions were seen in rats injected with hypertonic saline alone, relative to normal control rats (Figure 6). The expression levels of phosphorylated forms of Akt and GSK-3β were markedly (p < 0.05) decreased in glaucoma control, when compared with normal rats, indicating down-regulation of the pathway. However, rutin administration resulted in a significant (p < 0.05) up-regulation of p-PTEN, indicating the activation of Akt signaling. In line with raised p-PTEN levels, up-regulated expression levels of phosphorylated forms of Akt and GSK-3β were also noticed.



**Figure 6:** Effect of rutin on Akt signaling. (A) Representative immunoblot. (B) Relative expression levels, with control levels set at 100 %. Data are presented as mean ± SD, n = 6. \*p < 0.05, compared with normal control; #p < 0.05, compared with hypoxic control. C1 = Control; C2 = glaucoma control; C3 = glaucoma+ rutin (12.5 mg/kg); C4 = glaucoma+ rutin (25 mg/kg); C5 = glaucoma + rutin (50 mg/kg); C6 = rutin (50 mg/kg)

**Rutin downregulated the expressions of ECM proteins**



**Figure 7:** Effect of rutin on the expressions of ECM proteins in glaucoma. (A) Representative immunoblot. (B) Relative expression levels, with control levels set at 100 %. Data are presented as mean ± SD (n = 6). \*p < 0.05, compared to normal control; #p < 0.05, compared to hypoxic control. C1 = Control; C2 = glaucoma control; C3 = glaucoma+ rutin (12.5 mg/kg); C4 = glaucoma+ rutin (25 mg/kg); C5 = glaucoma + rutin (50 mg/kg); C6 = rutin (50 mg/kg)

The expression of ECM proteins i.e. collagen I, fibronectin and elastin were significantly raised (p

< 0.05) in rats following hypertonic saline injection, when compared with normal control (Figure 7). However, rutin administration resulted in down-regulation of collagen I expression. Furthermore, the levels of fibronectin and elastin were also significantly and dose-dependently ( $p < 0.05$ ) decreased, when compared to glaucoma control rats, with the 50 mg dose exerting the most pronounced effects. The expression of collagen I decreased from 187.15 to 108.41 % in rutin (50 mg)-treated rats.

## DISCUSSION

Glaucoma is one of the most common ocular diseases characterized by progressive loss of vision, RGC death, cupping and degeneration of the optic nerve head [24]. Accumulating experimental data suggest several mechanisms associated with pathogenesis of glaucoma and RGC loss. Elevated IOP, aberrant inflammatory responses and altered apoptotic pathways are implicated in glaucoma [25]. Prevention of the loss of RGCs is crucial in glaucoma treatment. Current therapies in glaucoma treatment are chiefly targeted at reducing elevated IOP levels. However, there is an inevitable need to develop novel candidate drugs that can reduce RGC loss as well as inflammatory responses.

The present study investigated the protective effects of rutin in experimental glaucomatous rats. Rutin effectively prevented RGC cell death and improved viability of cells in *in vivo* model of glaucoma, and *in vitro* in hypoxia-induced RGC cells. Rutin regulated the expressions of proteins of the apoptotic pathway. The delicate balance between the pro-apoptotic proteins (Bad and Bax) and anti-apoptotic proteins (Bcl-2 and Bcl-xL) is crucial in cell cycle regulation [26]. Rutin-mediated up-regulation of expressions of Bcl-2 and Bcl-xL could have, in part, contributed to the increased RGC viability. Normally, Bcl-2 prevents the release of cytochrome c from the mitochondria, while Bax induces cytochrome c release [27]. The increase in levels of cytochrome c in the extracellular space subsequently leads to the activation of caspase 3 and also the downstream proteins of the caspase cascade, which leads to apoptosis [28]. The significantly reduced expressions of Bax, Bad and cleaved caspase-3 observed in this study following rutin treatment indicate suppression of apoptosis. This suppression of pro-apoptotic proteins and cleaved caspase-3 could have, in part, contributed to decreased apoptotic counts as observed in fluoro gold staining. These findings suggest that rutin exerts protective effects against glaucoma.

Furthermore, the marked reduction in IOP following rutin treatment is a reflection of the efficacy of rutin in glaucoma treatment. Elevated IOP is well documented in progression of glaucoma and in RGC loss [25]. The reduction in IOP by rutin could have contributed to the prevention of RGC loss and improved viability. It has been reported that ECM plays a major role in regulating IOP, and that TGF- $\beta$ 2 causes overproduction and deposition of ECM components fibronectin, collagen I and IV. Inhibitors of ECM proteases include plasminogen activator inhibitor-1 and tissue inhibitors of matrix metalloproteinases [29]. Alterations in ECM lead to impaired cell-cell signaling and increased resistance to AH outflow in TM, leading to elevated IOP and cellular apoptosis [29]. The observed up-regulation in TGF- $\beta$ 2 along with elevated levels of ECM components (collagen I, fibronectin and elastin) in the glaucomatous rats show that TGF- $\beta$ 2-induced ECM accumulation led to elevated IOP as reported previously [30]. It has also been reported that TGF- $\beta$ 2 increased FN, collagen I and IV in glaucoma [31]. Furthermore, the enhanced expressions of phosphorylated forms of Smad-2 and Smad-3 observed in the present study suggest that activation of TGF- $\beta$ 2/Smad-2/3 signaling caused increased expression of ECM components. It is known that activated TGF- $\beta$ 2-receptor complex phosphorylates and activates Smad-2 and Smad-3, leading to activation of the pathway [32]. The TGF- $\beta$ 2-induced fibrosis operates through the canonical Smad pathway via Smad-2 and Smad-3 [33]. In the rutin-treated rats, the significantly down-regulated expressions of TGF- $\beta$ 2, p-Smad-2 and p-Smad-3 indicate down-regulation of TGF- $\beta$ 2/Smad-2/3 signaling. Reduced collagen I, fibronectin and elastin levels also indicate the inhibition of TGF- $\beta$ 2-induced expressions of ECM components by rutin. The reduction in ECM components could have, in part, contributed to reduced IOP and RGC loss. These results reflect the potential efficacy of rutin in glaucoma therapy.

Furthermore, this study demonstrated markedly down-regulation of Akt signaling in glaucoma rats, as evidenced by enhanced PTEN levels and reduced Akt expression. The AKT signaling is a major pathway for the regulation of cell survival [34]. Thus, activation of the pathway plays a protective role in glaucoma.

Interestingly, rutin-mediated activation of the pathway, as evidenced by up-regulated expressions of p-Akt and GSK-3 $\beta$  along with substantial down-regulation of PTEN, the major negative regulator of AKT, may be responsible for the anti-apoptotic effects of rutin. The TGF- $\beta$ /

Smad-2/3 signaling is regulated by PTEN [24]. Thus, the down-regulation of PTEN by rutin could have caused downregulated expressions of TGF- $\beta$ /Smad-2/3. This may have contributed to the subsequent reduction in the expressions of ECM components. These observations suggest that rutin exerted substantial protective effects under glaucomatous conditions by significantly reducing IOP and decreasing RGC loss and deposition of ECM components, and by regulating major signaling pathways.

## CONCLUSION

The results of the study reveal that rutin effectively reduces RGC loss, and decreases excess production of ECM components and IOP via regulation of TGF- $\beta$ /Smad2/3/Akt/PTEN signaling pathway. These findings indicate the potential of rutin as a candidate drug for glaucoma therapy.

## DECLARATIONS

### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors have equally contributed to this study. Ying Li, Liang Ying and Dabo Wang designed this study, collected and analysed the data, and also prepared the manuscript. Leilei Qin and Hanguang Dong contributed to the experimental work and statistical analysis.

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