

Original Research Article

Curcumin protects trabecular meshwork cells against hydrogen peroxide-induced oxidative stress and apoptosis via Nrf2-keap1 pathway

Yi Luo^{1,2}, Hongtao Ding^{3*}, Dujun Li⁴, Jihong Luo⁴

¹Department of Ophthalmology, Wuhan Forth Hospital, ²Department of Ophthalmology, Wuhan Puai Hospital, Tongji Medical College, Huazhong University of Science and Technology, ³Department of Internal Medicine, Wuhan No. 1 Hospital, Wuhan 430022, ⁴Department of Ophthalmology, Hubei Provincial Hospital of Traditional Chinese Medicine, Hubei Provincial Academy of Traditional Chinese Medicine, Hubei Provincial University of Traditional Chinese Medicine, Hubei 430065, China

*For correspondence: **Email:** Dinghttchb13@163.com

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Abstract

Purpose: To explore the effects and molecular mechanism of action of curcumin on trabecular meshwork cells (TMCs).

Methods: TMCs were isolated from pig eyes and NF-E2-related factor 2 (Nrf2) was knocked down by siRNA transfection. The effect of curcumin on intracellular reactive oxygen species (ROS) was measured by a ROS-specific dye. Annexin V-FITC/propidium iodide (PI) double labeling was applied to determine apoptosis. The expressions of apoptosis-associated proteins and Nrf2-Kelch-like ECH-associated protein 1 (Keap 1) pathway activation were assessed by western blotting, while target gene transcription was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR).

Results: Curcumin reduced the levels of intracellular ROS and apoptosis induced by H₂O₂, upregulated B-cell lymphoma-2 (Bcl-2), downregulated Bcl2-associated X (Bax), and activated caspase-3 and nine other proteins ($p < 0.05$). Curcumin reduced the expressions of Nrf2, HO-1, and NQO1, and increased Keap1 in H₂O₂-induced TMCs ($p < 0.05$). Moreover, Nrf2 knockdown partly reversed the effect of curcumin on ROS and apoptosis in TMCs induced by H₂O₂ ($p < 0.05$).

Conclusion: Curcumin inhibited oxidative stress and apoptosis by Nrf2-Keap1 activation in TMCs. Curcumin is therefore a potential therapeutic agent for the management of glaucoma.

Keywords: Curcumin, Oxidative stress, Apoptosis, Glaucoma, Nrf2-Keap1

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INTRODUCTION

Glaucoma, the second leading cause of blindness, affects more than 70 million people and is characterized by progressive degeneration of retinal ganglion cells (RGCs) [1]. Many risk factors for this disorder have been identified,

including elevated intraocular pressure (IOP), oxidative stress, apoptosis, alterations of cytokines, and autophagy [2-4]. Elevated IOP, a crucial factor related to RGC death which results from oxidative stress or apoptosis, is associated with biochemical changes in the trabecular meshwork (TM) [5]. The clinical application of

traditional Chinese medicines have resulted in improvements in treating glaucoma because it reduces IOP and modulates optic nerve nutrition and systemic conditions [6].

NF-E2-related factor 2 (Nrf2), a major protein of oxidative stress regulating, is sequestered by cytoplasmic Keap1 [7]. Numerous studies demonstrated that the Nrf2-Keap pathway attenuates oxidative and apoptotic responses *in vivo* and *in vitro* [8]. However, the specific effects of Nrf2 and its regulating manner in the treatment of glaucoma remain unclear. Curcumin, the major extract of turmeric, is derived from *Curcuma longa* and has been widely used in many countries for centuries [9]. Many studies have suggested that curcumin could be a therapeutic agent for ocular diseases [10].

However, the molecular mechanism of curcumin in treating glaucoma is unclear. In this study, we characterized the protective roles of curcumin on trabecular meshwork cells (TMCs) exposed to H₂O₂, and investigated the role of Nrf2-Keap1 in H₂O₂-induced oxidative stress and apoptosis.

EXPERIMENTAL

Chemical and reagents

Primary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). H₂O₂ was purchased from the Beyotime Institute of Biotechnology (Shanghai, China). Lipopolysaccharide was purchased from Sigma-Aldrich (St. Louis, MO, USA). Curcumin (> 98 % pure) was purchased from the National Institutes for Food and Drug Control (Beijing, China).

Isolation and culture of TMCs

Primary porcine TMCs were obtained from fresh pig eyes, and cultured as previously described [11]. Briefly, the eyes were bisected along the equator. After lens, iris, vitreous, and ciliary body were removed from the anterior part, the trabecular tissue was scraped from the sclera. After digested by collagenase type IV and centrifuged, TMCs were cultured in low glucose Dulbecco's Modified Eagle's Medium (Gibco, Gathersburg, MD, USA) with 10 % fetal bovine serum, penicillin/streptomycin, sodium pyruvate, nonessential amino acids, L-glutamine, and amphotericin B. All reagents were purchased from Invitrogen (Carlsbad, CA, USA).

Intracellular ROS determination

The intracellular ROS of TMCs were measured by 2,7-dichlorofluorescein diacetate (DCF-DA).

TMCs or si-Nrf2 TMCs were placed into a dark 96-well plate and treated with different concentrations of curcumin at 20, 50 and 100 μM at 37 °C for 1 h, and exposed to H₂O₂ for another 24 h and stained by DCF-DA about 30 min in dark place. The DCF-DA fluorescence was measured with a flow cytometer (FACS Calibur) and the fluorescence was measured with a microplate reader (Thermo Fisher Scientific) with wavelength 488 nm (excitation) and 520 nm (emission).

Apoptosis assay

Quantitative assay of the apoptosis was evaluated by annexin V/FITC detection kit (BD Biosciences). TMCs or si-Nrf2 TMCs were seeded onto a black 96-well plate and treated with curcumin at 20, 50, and 100 μM at 37 °C for 1 h, and exposed to H₂O₂ for another 24 h and harvested for quantitation of apoptosis. The cells were blocked in binding buffer, stained with annexin V /PI for 15 min. The cells were then immediately analysed by fluorescence-activated cell sorting.

Protein extraction and western blotting

The treated cells were lysed in cold RIPA buffer with a protease and phosphatase inhibitor (Sigma-Aldrich) and centrifuged. Protein concentrations were measured by Pierce BCA Protein Assay Kit (Thermo Scientific) and diluted to equal concentrations with PBS and 5x SDS-PAGE sample loading buffer, then heated to 95 °C. Equal amounts of protein were loaded and separated on Invitrogen NuPAGE™ Bis-Tris gels (10 %), and transferred onto PVDF membranes (Millipore). The membranes were incubated with primary antibodies (1:1,000 dilution) and secondary antibody (1:10,000 dilution). Protein expressions were detected by an ECL system and quantified using Imaging Systems analysis software (VersaDocMp5000).

RNA extraction and RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies.). Equal amounts of RNA were synthesized to cDNA using GoScript™ (Promega). The target gene expression was detected by a SYBR Green PCR kit (Promega). The primers are shown in Table 1.

Nrf2 knockdown by transfection with small-interfering RNA (siRNA)

The siRNA against Nrf2 was synthesized by Sangon Biotech Co., Ltd.. Newly isolated TMCs were transfected with Nrf2-specific siRNA

(si-Nrf, 5'-GCUGAACUCCUUAGACUCATT-3') or negative control by Lipofectamine 2000 (Invitrogen).

Table 1: Primers used for RT-PCR

Gene	Primer	Sequence
Nrf2	forward	CAGTGA CT CGGAAATGGAA GAG
	reverse	AATGTGTTGGCTGTGCTTT AGG
NQO1	forward	AGAAGAGCACTGATCGTAC TGG
	reverse	CGTAATTGTAAGCAA CTCTCCTATG
HO-1	forward	GCTCTATCGTGCTCGCATG A
	reverse	AATTCCC ACTGCCACGGTC
Keap1	forward	GTGTGGAGAGGTATGAGC CA
	reverse	CTTCGTGTCAGCATTGGG
GADPH	forward	GTTCCAGTATGATTCCACC CACGGCAAGTT
	reverse	TGCCAGCCCCAGCATCAA A GGTAG AAGAGT

Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA) with Tukey's tests using Prism 6 software. Data are presented as mean \pm standard deviation (SD) and $p < 0.05$ was considered statistically significant.

RESULTS

Curcumin reduced H₂O₂-induced oxidative stress in TMCs

The intracellular ROS were measured by DCF-DA. As shown in Figure 1A, the level of intracellular ROS of H₂O₂ treated cells was about two times higher than control group ($p < 0.01$), which was inhibited by curcumin in concentration-dependent manner.

Curcumin reduced H₂O₂-induced cell apoptosis in TMCs

Hence, the anti-apoptosis effect of curcumin was confirmed by annexinV/PI staining. As shown in Figure 1B, the apoptotic cells (Q2 (late apoptosis), An⁺/PI⁺; Q3 (early apoptosis), An⁺/PI⁻) were increased in the H₂O₂-treated group (from 3.15 \pm 8.23 % in the control to 43.7 \pm 14.4 % in H₂O₂ group). When pre-treated with curcumin, apoptotic cells were remarkably decreased ($p < 0.01$) in a concentration-dependent manner. To further explore the mechanism of curcumin on apoptosis, the apoptosis-related proteins were measured by western blotting. As shown in Figure 1C, curcumin increased the expression of Bcl-2 (anti-apoptosis protein), and decreased the

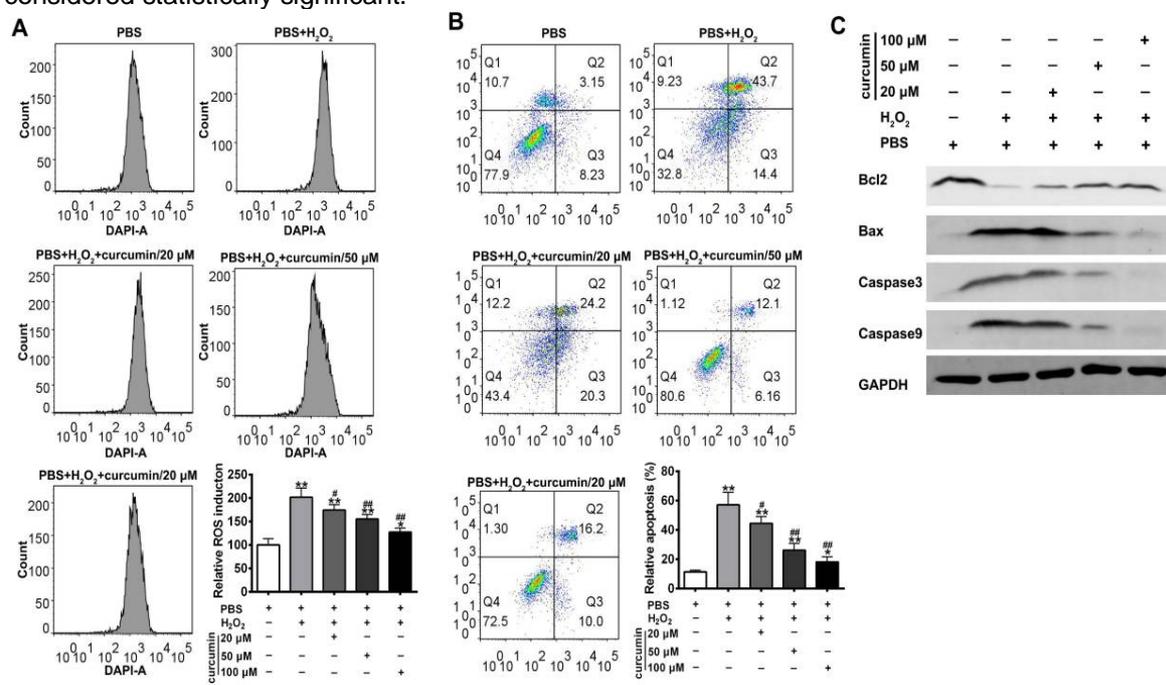


Figure 1: Effect of curcumin on the ROS and apoptosis in H₂O₂-treated trabecular meshwork cells (TMCs). (A) Flow cytometry analysis of ROS in TMCs exposed to 1 mM H₂O₂. Intracellular ROS were determined by 2,7-dichlorofluorescein diacetate. (B) AnnexinV/PI staining of TMCs. Quadrant Q1 ((An⁻/PI⁺)) represents necrotic cells, Q2 (An⁺/PI⁺) represents late apoptotic cells, Q3 (An⁺/PI⁻) represents early apoptotic cells, and Q4 (An⁻/PI⁻) represents viable cells. The percentage of apoptotic cells was shown in column chart, (C) Western blotting of apoptosis-related protein expression (Bcl-2, Bax, caspase-3, and caspase-9); * $p < 0.05$ and ** $p < 0.01$ vs. control; # $p < 0.05$ and ## $p < 0.01$ vs. H₂O₂ alone group

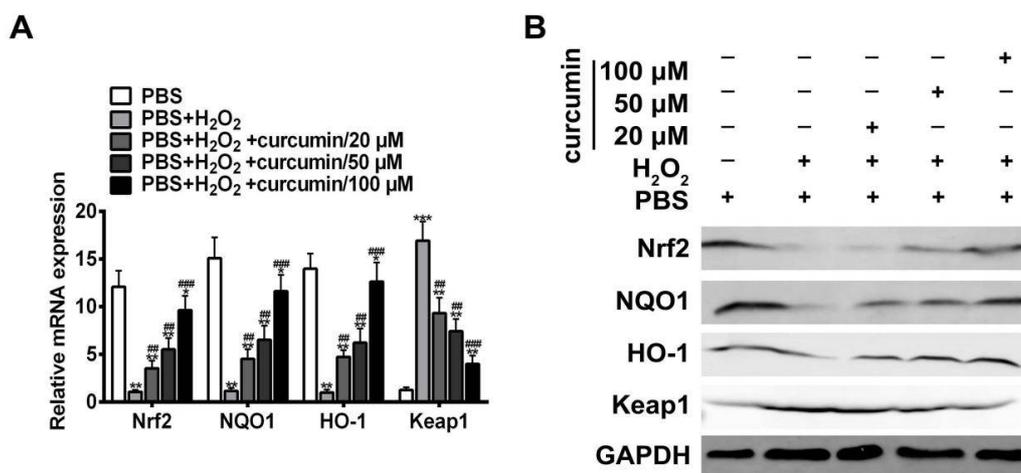


Figure 2: Effect of curcumin on Nrf2-related gene and protein expression in H₂O₂-treated TMCs. (A). RT-PCR analysis of *Nrf2*, *NQO1*, *HO-1*, and *Keap1* transcription in TMCs. (B) Western blotting of Nrf2-Keap1-related protein expression (Nrf2, NQO1, HO-1, and Keap1); Quadrant Q1 ((An⁺/PI⁺)) represents necrotic cells, Q2 (An⁺/PI⁻) represents late apoptotic cells, Q3 (An⁺/PI⁺) represents early apoptotic cells, and Q4 (An⁻/PI⁻) represents viable cells. The percentage of apoptotic cells was shown in column chart, (C) Western blotting of apoptosis-related protein expression (Bcl-2, Bax, caspase-3, and caspase-9); **p* < 0.05 and ***p* < 0.01 vs. control; #*p* < 0.05 and ##*p* < 0.05 vs. H₂O₂ alone group.

expressions of caspase-3, caspase-9, and Bax (pro-apoptosis protein), in concentration-dependent manner. The results demonstrate that curcumin reduced H₂O₂-induced apoptosis in TMCs.

Curcumin reduces Nrf2, NQO1, and HO-1 expression, and increases Keap1 in H₂O₂-induced TMCs

The gene and protein expressions of Nrf2, Keap1, NQO1, and HO-1 in H₂O₂-induced TMCs were investigated. As shown in Figure 2 A, gene expressions of *Nrf2*, *HO-1*, and *NQO1* were increased and *Keap1* was decreased, indicating activation of the Nrf2-keap pathway. Curcumin significantly inhibited H₂O₂-induced genetic transcription dysregulation in a concentration-dependent manner. In parallel, protein expression was consistent with the genetic transcription results (Figure 2 B).

Nrf2 knockdown reverses the effect of curcumin on the Nrf2-Keap1 signaling pathway

To further characterize the role of Nrf2 in the regulation of curcumin on the expression of Nrf2-Keap1 pathway target proteins, Nrf2 siRNA was used to knockdown the expression of Nrf2 in TMCs. The transfection efficiency of the knockdown was confirmed by western blotting and RT-PCR analysis. The *Nrf2* mRNA and protein expression in the si-Nrf2 group were decreased when compared with the sc-Nrf2

group (Figure 3), and the expression of target genes and proteins changed accordingly. The expressions of Nrf2, HO-1, and NQO1 were much lower and Keap1 much higher of si-Nrf cells after treated by H₂O₂, which were reversed by curcumin in concentration-dependent manner. The protein expressions (Figure 3 B) were consistent with the genetic transcription results (Figure 3 A).

Nrf2 knockdown reverses the effect of curcumin on oxidative stress

The si-Nrf TMCs were also used to determine the effect of Nrf2 on curcumin against H₂O₂-induced oxidative stress. The levels of intracellular ROS in the si-Nrf2 group increased when compared with the sc-Nrf2 group (Figure 4 A), which was greatly enhanced by H₂O₂ in Si-Nrf cells. The intracellular ROS of H₂O₂-treated si-Nrf cells were decreased by curcumin in a concentration-dependent manner.

Nrf2 knockdown reverses the effect of curcumin on apoptosis

Si-Nrf TMCs were also used to determine the effect of Nrf2 on curcumin against H₂O₂-induced apoptosis. The amount of apoptotic cells of the si-Nrf group was higher than that of the sc-Nrf group, and greatly enhanced by H₂O₂ in si-Nrf cells. Curcumin inhibited H₂O₂-induced apoptosis of si-Nrf cells in a concentration-dependent manner. To further confirm the results of apoptosis, the apoptosis-related proteins,

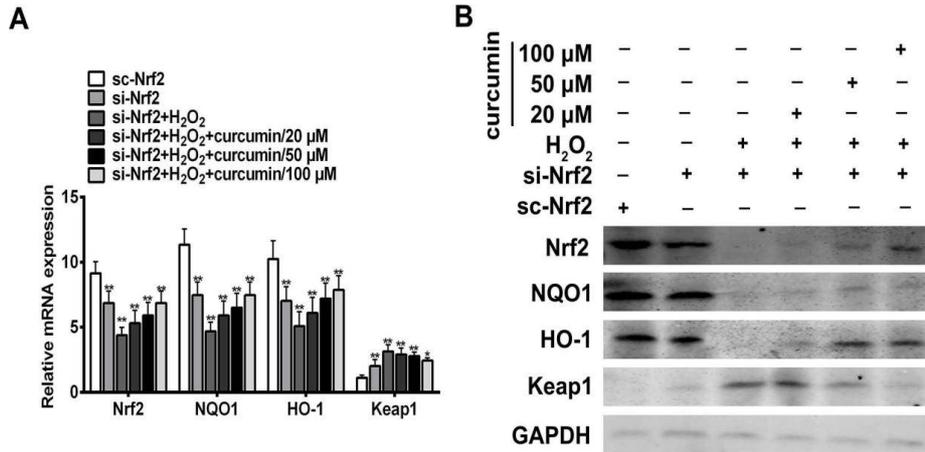


Figure 3: Nrf2 knockdown partly blocked the effects of curcumin on Nrf2 and related protein expression. (A) RT-PCR analysis of *Nrf2*, *NQO1*, *HO-1*, and *Keap1* transcription in Nrf2 knockdown (si-Nrf) trabecular meshwork cells (TMCs). (B) Western blotting of Nrf2-Keap1-related protein expression (Nrf2, NQO1, HO-1, and Keap1) in si-Nrf TMCs; (Nrf2, NQO1, HO-1, and Keap1); Quadrant Q1 ((An⁺/PI⁺)) represents necrotic cells, Q2 (An⁺/PI⁺) represents late apoptotic cells, Q3 (An⁺/PI) represents early apoptotic cells, and Q4 (An/PI) represents viable cells. The percentage of apoptotic cells was shown in column chart, (C) Western blotting of apoptosis-related protein expression (Bcl-2, Bax, caspase-3, and caspase-9); **p* < 0.05 and ***p* < 0.01 vs. control; #*p* < 0.05 and ##*p* < 0.01 vs. H₂O₂ alone group

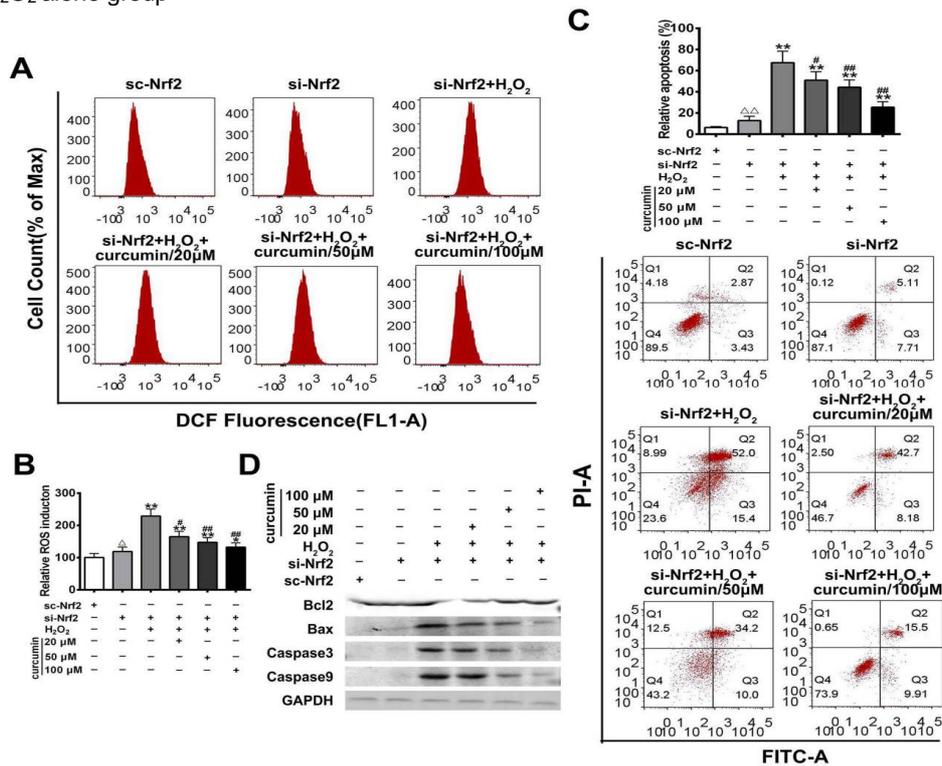


Figure4: Nrf2 knockdown reversed the effects of curcumin against oxidative stress and apoptosis in TMCs. (A) Flow cytometry analysis of reactive oxygen species (ROS) in si-Nrf2 TMCs exposed to 1 mM H₂O₂. Intracellular ROS were determined by 2, 7-dichlorofluorescein diacetate. (B) Flow cytometry analysis of si-Nrf2 TMC apoptosis by double staining with annexinV/propidium iodide. (C) Western blotting analysis of Bcl-2, Bax, caspase 3, and caspase-9; **p* < 0.05 and ***p* < 0.01 vs. control; #*p* < 0.05 and ##*p* < 0.05 vs. H₂O₂ alone group.

caspase-3, caspase-9, Bcl-2, and Bax were measured both in sc-Nrf and si-Nrf cells. The expressions of caspase-3, caspase-9, and Bax were increased and Bcl-2 decreased in the si-Nrf group compared with the sc-Nrf group, and the

effects were enhanced by H₂O₂ in si-Nrf cells. Curcumin decreased the expression of caspase-3, caspase-9, and Bax and increased Bcl-2 in a concentration-dependent manner.

DISCUSSION

Oxidative stress is one of the risk factors for glaucoma, and many studies have reported that local oxidative radicals are a determining factor in the pathogenesis of glaucoma [12]. Long-term oxidative damage may lead to the progressive loss of TMCs and glaucoma. Himori *et al* reported a close relationship between clinical ocular parameters and systemic oxidative stress parameters in 73 patients with normal-tension glaucoma [13]. Their study indicated that skin autofluorescence and 8-hydroxy-2'-deoxyguanosine, two markers of oxidative stress, may contribute to the pathogenesis of glaucomatous optic neuropathy.

Curcumin, a natural compound that has many biological properties including anti-inflammation, antiviral, antifungal, and anticancer activities, has been suggested to be an effective treatment for several ocular diseases, such as glaucoma [12]. Some studies have reported that curcumin exhibited antioxidant properties in neurodegenerative diseases, and was associated with activation of the Nrf2 cytoprotective pathway [14]. This study indicate that curcumin decreased the level of ROS in H₂O₂-induced TMCs and was associated with the activation of the Nrf2 signaling pathway, which were consistent with the results of previous studies.

Apoptosis is a physiological cell death mechanism that plays a crucial role in glaucoma. It is thought that the ultimate cause of irreversible blindness in this disease is RGC apoptosis [15]. The application of a new imaging technique called "detection of apoptosis retinal cells" (DARC) has shown that the level of RGC apoptosis is predictive of disease severity in patients with glaucomatous neurodegeneration [16]. Moreover, treatment with agents that inhibit RGC apoptosis attenuate glaucoma [17,18]. Apoptosis is initially characterized by a series of morphological changes, including cellular shrinkage, membrane blebbing, and nuclear chromatin condensation. Apoptotic programs are related to two pathways and involve the activation of a series of caspases [19]. The upstream caspase for the intrinsic pathway (also known as the membrane receptor-mediated pathway) is caspase-9 and the downstream effector is caspase-3 [20].

The intrinsic pathways are closely-regulated by the Bcl-2 family, which includes anti-apoptotic members (Bcl-2 and Bcl-Xl) and pro-apoptotic members (Bax and Bak)[21]. This results indicate that curcumin treatment prevent H₂O₂-induced

TMCs apoptosis, with downregulation of cleaved caspase-3, caspase-9, and Bax, and upregulation of Bcl-2 protein. More importantly, analyses of apoptosis showed that high concentrations of curcumin (50 and 100 μM) not only decreased the apoptosis of TMCs, but also of necrosis cells, indicating that curcumin did not cause secondary necrosis and immune responses.

Nrf2, which is sequestered by Keap1, is an important transcription factor under oxidative stress. Nrf2-Keap1 has been reported to play an important role in the regulation of oxidative stress and the apoptosis of RGCs[22]. *Lycium barbarum* polysaccharides protect the retina after ischemia-reperfusion, partially by activation of Nrf2 [23]. Berberine could ameliorate methotrexate-induced oxidative stress and apoptosis by activating the Nrf2/HO-1 pathway and PPARγ *in vivo* [24]. Curcumin has similar effects on oxidative damage. Dai *et al* reported that curcumin inhibited quinocetone-induced oxidative stress, apoptosis, and inflammation via Nrf2/HO-1 and the NF-κB pathway in human hepatocytes [25]. However, the Nrf2-mediated molecular mechanism of curcumin remains unclear.

In the present study, the results showed that curcumin activated the Nrf2-Keap1 signaling pathway and regulated target gene expression in H₂O₂-induced TMCs, resulting in alleviation of both oxidative stress and apoptosis in TMCs. Moreover, Nrf2 knockdown partly reversed the inhibitory effect of curcumin on ROS and apoptosis in H₂O₂-induced TMCs. These results strongly suggest that curcumin inhibit oxidative stress and apoptosis by Nrf2-Keap1 signaling pathway. Overall, the results indicate that curcumin has a protective effect against H₂O₂-induced oxidative stress and apoptosis of TMCs via the Nrf2-Keap1 signaling pathway.

CONCLUSION

The results of the present study provide the experimental basis for the possible clinical use of curcumin in the management of certainly inflammatory diseases, as well as the possibility of its development as a therapeutic agent for the treatment of glaucoma.

DECLARATIONS

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Conflict of interest

The authors declare that no conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the researchers listed in this article. All liabilities related with the content of this article will be borne by the authors. Hongtao Ding designed all the experiments and revised the paper. Yi Luo and Dujun Li performed the experiments, Jihong Luo wrote the paper.

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