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ORIGINAL PAPER

Activation of Nrf2/HO-1 pathway protects retinal ganglion cells from a rat chronic ocular hypertension model of glaucoma

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Abstract
Objective The objective of this work was to find out the effects of nuclear factor erythroid 2-related factor/heme oxygenase-1 (Nrf2/HO-1) pathway on retinal ganglion cell (RGC) injury in glaucoma.
Methods The chronic ocular hypertension (COH) rat models of glaucoma were constructed, and intraocular pressure (IOP) and RGC numbers were detected at different time points. Additionally, rats were divided into normal group (normal control rats), model group (COH model rats), and model + tBHQ group (COH model rats treated with Nrf activator, tBHQ). RGC apoptosis was detected by using TUNEL staining, and the expressions of Nrf2/HO-1 were detected by qRT-PCR and western blotting.
Results COH model rats showed significant IOP elevation and the increased mRNA and protein expressions of Nrf2 and HO-1 from 1 to 6 weeks after operation, with the evidently decreased RGC numbers at 4 weeks and 6 weeks after operation (all $P < 0.05$). Besides, rats in the model group had increased apoptosis index (AI) of RGCs and the elevated mRNA and protein expressions of Nrf2/HO-1 with remarkably reduced RGC numbers when compared with normal control rats, but the model rats treated with tBHQ exhibited an apparent decrease in AI of RGCs, as well as remarkable increases in RGC numbers and the mRNA and protein expression of Nrf2/HO-1 (all $P < 0.05$).
Conclusion Activation of Nrf2/HO-1 pathway significantly reduced the apoptosis and injury of RGCs in rats with chronic ocular hypertension (COH), thereby protecting RGCs in glaucoma, which could be a promising clinical target to prevent RGC degeneration in glaucoma.

Keywords Glaucoma · Nrf2/HO-1 pathway · Retinal ganglion cells · Apoptosis

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Introduction

Glaucoma, a neurodegenerative disease caused by multiple factors, constitutes the second leading common blindness-causing disease after cataract [1], which will influence approximately 79.6 million people by 2020 [2]. Optic atrophy, visual field defect, and loss of retinal ganglion cells (RGCs) were the common manifestations of glaucoma, while the pathological elevation of intraocular pressure (IOP) was one of the most important risk factors for glaucoma [3, 4]. Although a variety of factors, like

mechanical injury, glutamate receptor activation-induced excitotoxicity, neurotrophin deprivation, stress response, and so on, may directly or indirectly influence the mitochondrial dysfunction to induce the death of RGCs, the specific mechanism of RGC apoptosis in glaucoma has not been clearly elucidated [5–7]. Currently, the optical nerve damage of glaucoma has become the focus and hotspot of glaucoma research [8].

The function of retina was previously described to be sensitive to the dynamic alterations in reactive oxygen species (ROS) production, whereas the oxidative stress from mitochondrial dysfunction could also play a causal role in glaucoma pathogenesis [9, 10]. Nrf2 [Nuclear factor (erythroid-derived 2)-like 2], is an important transcription factor from CNC (“cap ‘n’ collar”) subfamily with a basic leucine zipper (bZIP) structure, playing as a crucial mediator in oxidative stress [11]. To realizing its antioxidant effect, Nrf2 binds to the antioxidant responsive element (ARE) in its promoter to protect cells and tissues from oxidative damage, via initiation of the transcription of various genes, like antioxidative proteins and phase II metabolic enzymes [12, 13]. Notably, Heme oxygenase-1 (HO-1), as the phase II metabolic enzyme, is a well-known rate-limiting enzyme with antioxidant, antiinflammatory, anti-apoptosis, and cytoprotective properties, which catalyzes the conversion of heme to bilirubin, free iron, and carbon monoxide [14, 15]. More importantly, Nrf2/HO-1 pathway has been found to be widely involved in the oxidative stress-induced injury of many tissues and organs, including heart, brain, liver, neural system, and so on, and it constitutes one of the most important endogenous antioxidant defenses systems [16, 17]. For instance, according to the study by Xu et al. [18], the knockout of Nrf2 can aggravate the injury of RGCs in ischemia–reperfusion. Also, Pan et al. [19] revealed that sulforaphane can activate Nrf2/HO-1 antioxidant pathway to attenuate the RGC injury induced by retinal ischemia–reperfusion. Therefore, we guessed that Nrf2/HO-1 signaling pathway may be important in glaucoma.

As such, the objective of this work was to discuss the impact of Nrf2/HO-1 pathway on the RGC injury in a rat chronic ocular hypertensive (COH) model of glaucoma and to provide a promising method concerning glaucoma treatment in the future.

Materials and methods

Ethics statement

All experiments followed the local principles for the management and use of laboratory animals and complied with the *Guide for the Care and Use of Laboratory Animals* formulated by the National Institutes of Health (No. 85-23, 1996 revised) in the current work [20].

Construction of the rat COH model of glaucoma

A total of 40 male SD rats (purchased from Shanghai SLAC Laboratory Animal Co., Ltd) weighing 200–300 g were obtained, and the COH model was constructed following a procedure described previously in detail [21]. The rats were anesthetized with an intramuscular injection of ketamine hydrochloride (25 mg/kg) and xylazine hydrochloride (10 mg/kg) for general anesthesia and further anesthetized locally with oxybuprocaine chloride 0.4% eye drops (Benoxil, Santen pharmaceutical CO., Ltd, Osaka, Japan) for local anesthesia. Under the ophthalmic operating microscope (OPMI VISU 140, Carl Zeiss, Jena, Germany), conjunctival sac was separated along the edge and the eyeground was exposed after turning the eye ball with forceps clamping the conjunctival sac. Next, three main veins were separated from the sclera of the right eye and ligated with 10-0 nylon sutures separately to block the venous reflux in the eye and increase the intraocular pressure (IOP). The vascular forceps were used to probe into the venous reflux in ligated veins to ensure that the blood vessel was indeed blocked, and then the conjunctiva and the inner eyelid were sutured. After surgery, chlortetracycline eye ointment was used on the surface of the eye to prevent infection or corneal damage. The portable tonometer TonoLab (Mento O&O, USA) was used to detect the IOP of right operated eye and the left control eye before operation (0 day) and at 1 week, 2 weeks, 4 weeks and 6 weeks after operation. The detection was performed at 9–10 o’clock in the morning to avoid the interference caused by the diurnal fluctuation of IOP.

Fluorogold (FG) retrograde tracing and RGC counting

The anesthetized rats were fixed on the stereotaxic apparatus 4 days before killing. The microinjector was used to take 5% FG (Fluorochrome, Denver, CO, USA) which was then injected into one side of superior colliculus from the bone porosity 6.3 and 6.8 mm to the bregma, by 3.6 mm and 4 mm depth from the top of the skull and with the dose of 0.8 μ. At each time point, eight rats were killed for eyeball reperfusion and fixation, and slide samples of retina was prepared and observed under the inverted fluorescence microscope. Each retinal quadrant under observation was classified into central region, middle region, and peripheral region under observation, which was 1, 2, and 3 mm from the optic disk, respectively. Labeled RGCs were counted in microscopic fields of 200 × 250 mm^2, with 200 magnifications in 4 central regions, 8 middle regions, and 12 peripheral regions in the four quadrants of the retina [5]. Finally, the cell counting was also applied to corresponding regions from all groups.

Rat grouping and treatment

Another 24 SD rats were randomly assigned into three groups with eight rats per each group: normal group (normal control rats), model group (COH model rats), and model + tBHQ group (COH model rats treated with Nrf activator, tBHQ). Rats in the model + tBHQ group were given tert-Butylhydroquinone (tBHQ, Sigma-Aldrich), a well-known Nrf2 activator, by oral gavage at the dose of 12.5 mg/kg once daily from 1 week after IOP elevation. Rats in the normal group and model group received equal volumes of vehicle (saline) with the same schedule. After administration for 6 week, those rats were killed to take out their eyeballs for fixation and section making.

HE staining

Hematoxylin and eosin (HE) staining was performed on sample sections as follows. First, sections were dewaxed in xylene, dehydrated with gradient alcohol, stained with hematoxylin for 5–15 min, stained with 0.1–0.5% eosin for 1–5 min, and diaphonized with xylene for 10 min. Then, the sections were mounted with neutral resin for microscopic examination. The pathological changes of retinal tissues were observed under a microscope (Leica, Wetzlar, Germany).

TUNEL staining

After routine dewaxing and hydrazination, sections were washed with PBS buffer and the tissues were treated with 100 μl of 20 μg/ml Proteinase K for 15 min before the addition of 100 μl of TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) mixture (Promega Company, USA) for 1 h reaction at 37 °C in a dark and wet box. Then, sections were incubated with 100 μl of streptavidin-HRP (horseradish peroxidase) (1:500 dilution by PBS buffer) was added for 30 min of incubation, followed by the addition of DAB for development, and dehydration with gradient alcohol hyalinization with xylene for 1 min × 2 times, then the sections were mounted with neutral resin. After that, sections were observed and photographed under a microscope. The number of positive cells and total RGCs were calculated by using the high-power binocular microscope (× 400), and the mean values were obtained after calculation. The apoptosis index (AI) was calculated according to the formula: AI (%) = number of apoptotic cell/number of total cells × 100%.

qRT-PCR (quantitative reverse transcription-PCR)

The retinal tissues of rats were ground with normal saline, and total RNA was extracted with the extraction kit (Omega, USA), which was determined for RNA purity and concentration with a UV spectrometer (UV-1800, Japan) and observed for RNA intactness by agarose gel electrophoresis. Nrf2 and HO-1 primers were designed by software Primer 5.0 and synthesized

Table 1 Primer sequences of Nrf2 and HO-1 used in the current study

Gene	Primer sequences
Nrf2	F:5′-CCATTTACGGAGACCCAC-3′
	R:5′-CTTATTTCAACGGCGAGT-3′
HO-1	F:5′-CCTCACTGGCAGGAAATCATC-3′
	R:5′-CCTCGTGGAGACGCTTTACATA-3′
GAPDH	F:5′-GAAGGTGAAGGTCGGAGTC-3′
	R:5′-GAAGATGGTGATGGGATTTC-3′

by Sangon Biotech (Shanghai) Co., Ltd. (Table 1). The reverse transcription of total RNA into cDNA was realized by using the Primescript^{TM} RT reagent Kit (Takara, Japan). SYBR® Premix Ex Taq TM kit (Takara Biomedical Technology (Dalian) Co., Ltd.) was used for qRT-PCR. The relative expression of Nrf2 and HO-1 was calculated using $2^{-\Delta\Delta Ct}$ method with GAPDH as the internal reference gene.

Western blotting

The retinal tissues of rats were added with lysate by 100–200 μl for each 20 mg of tissues in glass homogenizer. After 15 min of centrifugation at the rate of 12,000 rpm, the supernatant was collected and analyzed with SDS-PAGE electrophoresis. The proteins separated after electrophoresis were transferred to the nitrocellulose (NC) filter and blocked for 1 h at temperature with 5% skimmed milk powder–PBS buffer. Next, Nrf2 (ab137550) and HO-1 (ab13248) antibodies and GAPDH (ab8245) (purchased from Abcam) were added for overnight incubation at 4 °C. The NC filter was washed with PBS buffer (3 times) and then incubated at room temperature for 1 h with HRP-conjugated secondary antibody (purchased from CWBiotech, Beijing, China). Subsequently, the NC filter was washed again with PBS buffer before development with enhanced chemiluminescence (ECL). The gray value ratio of target bands to the reference band was regarded as the relative expression level of proteins, with GAPDH as the internal reference protein.

Statistical methods

Data analysis was performed by using the statistical software SPSS 22.0. Measurement data were presented by mean ± standard deviation. Comparison between two groups was conducted by Student's *t* test, and differences among multiple groups were analyzed by one-way ANOVA. Results of $P < 0.05$ were regarded as the statistical significance.

Results

The changes of IOP and RGCs in rat COH model of glaucoma

No significant differences were discovered in IOP between the right and left eyes of rats before operation (all $P > 0.05$, Fig. 1), but the IOP in operated eyes (right) increased significantly from 1 to 6 week after operation, which was higher than that of the left control eye (all $P < 0.05$), presenting successfully established rat models of COH. According to the FG retrograde tracing and RGC counting, there was no significant difference in the number of RGCs between operated eyes and control eyes at 1 week and 2 weeks after operation (all $P > 0.05$), whereas at 4 weeks and 6 weeks after operation, the number of RGCs declined substantially (all $P < 0.05$), which suggested that RGC numbers would decrease with the continuous elevation of IOP.

The expression of Nrf2/HO-1 pathway in rat COH model of glaucoma

As displayed in Fig. 2, the mRNA and protein expressions of Nrf2/HO-1 pathway in rat COH models from 1 to 6 weeks after operation were detected by qRT-PCR and western blotting, and the result demonstrated that the mRNA and protein expressions of Nrf2 and HO-1 reached the peak at 1 week after operation, which gradually decreased but were still significantly higher than those of control eyes (all $P < 0.05$).

The morphological changes of retinal tissues observed by HE staining

In the normal group, each retinal tissue layer was clear and RGCs were arranged in single layer after HE staining. As for the model group, the ganglion cell layer (GCL) was loose with the thinning of the retinal inner plexiform layer (IPL). However, the retinal tissues in model + tBHQ group presented an increase in RGCs, orderly arrangement of single GCL, and apparently thickened IPL, as compared with the model group (Fig. 3).

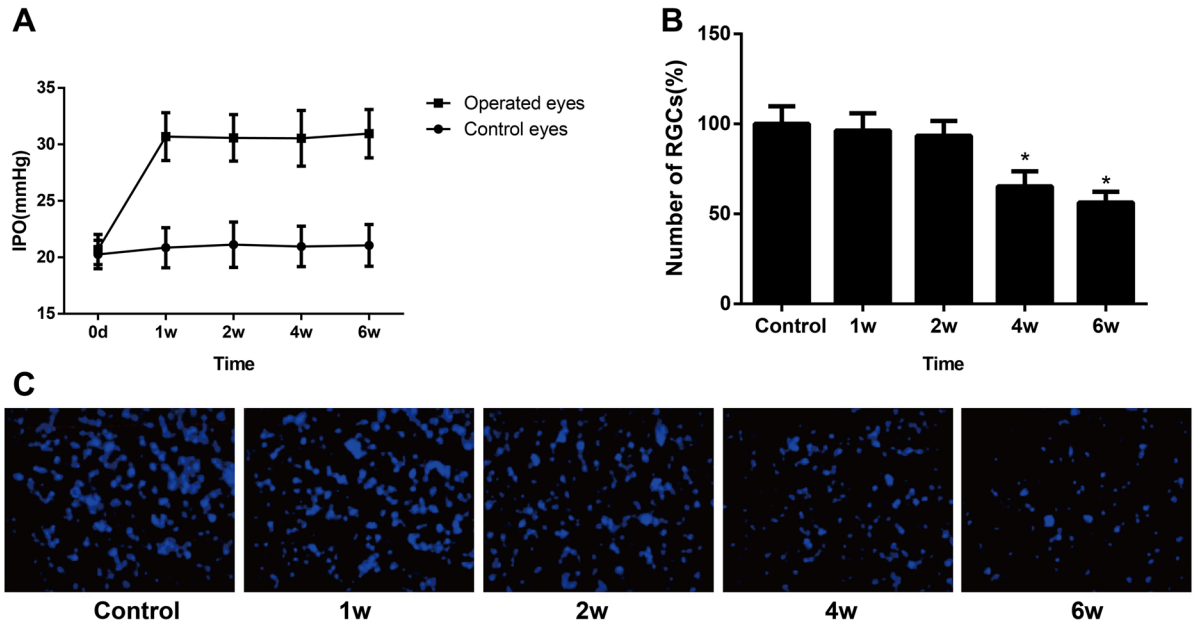

Fig. 1 The changes of IOP and RGCs in rat COH models after operation. *Note* **a**, **b** the changes of IOP (intraocular pressure) and RGC (retinal ganglion cell) numbers (**b**) in rat COH models after operation; **c** fluorogold (FG) retrograde tracing and RGC counting at different time points after operation. $^{*}P < 0.05$ compared with the control eyes

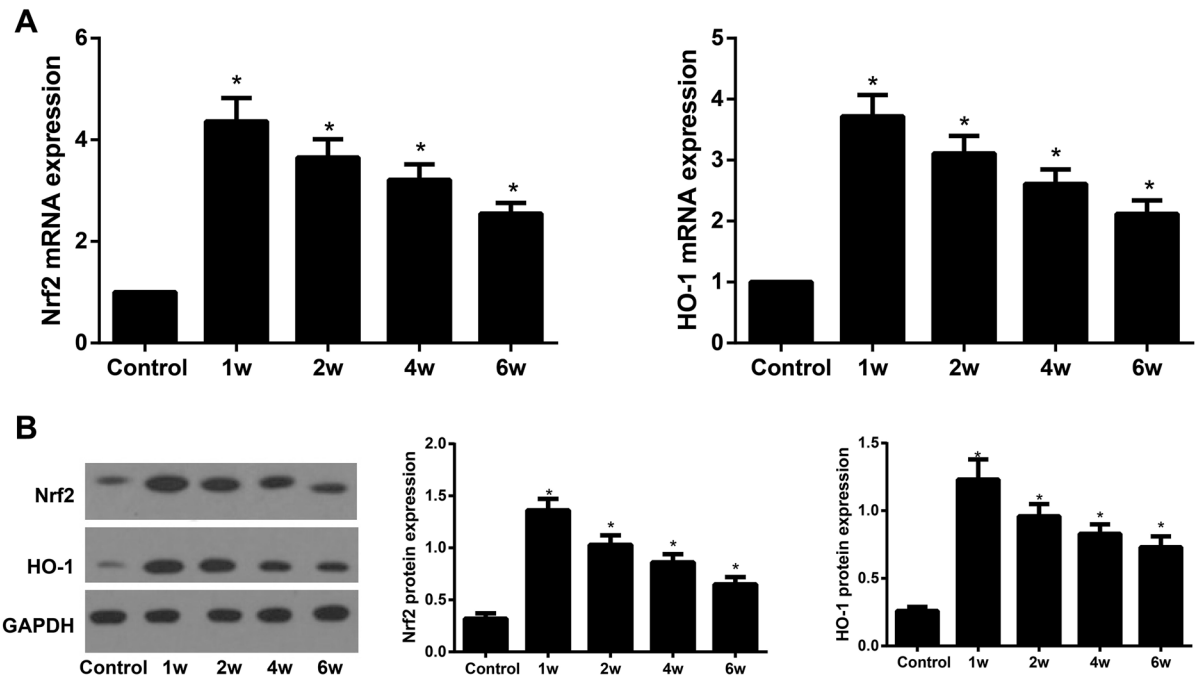

Fig. 2 The expression of Nrf2/HO-1 pathway in the retinal tissues of rats. *Note* **a** the mRNA levels of Nrf2 and HO-1 in rat COH models tested by qRT-PCR at different time points; **b** the protein expression levels of Nrf2 and HO-1 in rat COH models evaluated by western blotting at different time points; $^{*}P < 0.05$ compared with the control eyes

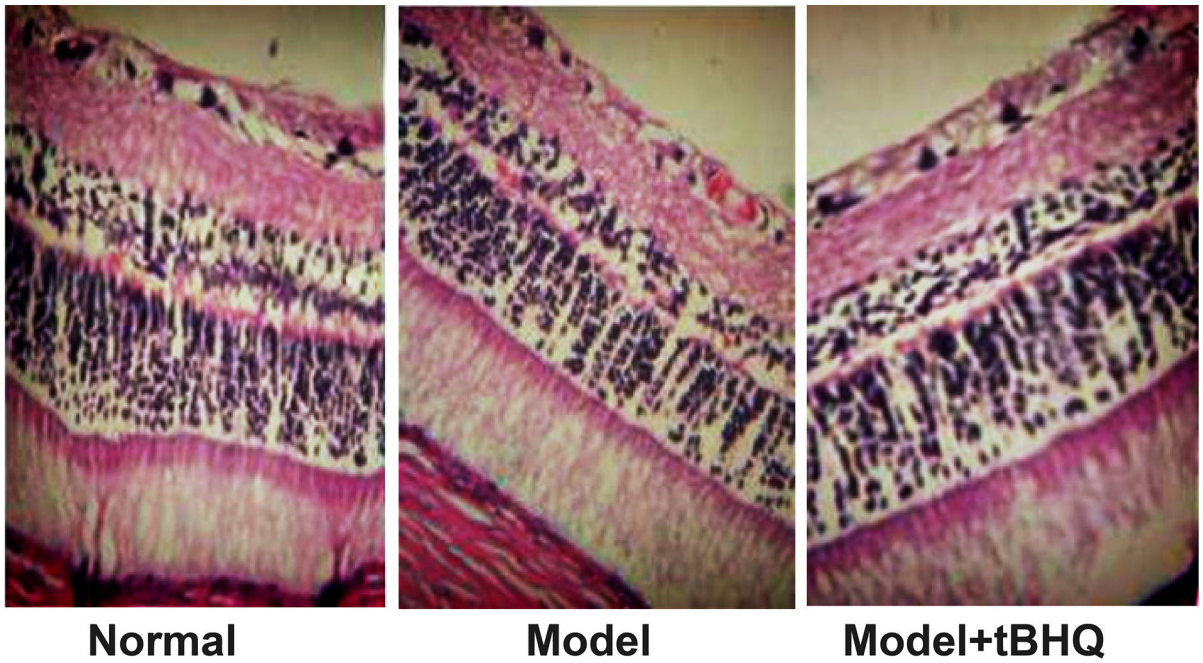

Fig. 3 The morphological changes of retinal tissues observed after HE staining

The number and apoptosis of RGCs of rats in each group

Rats in the normal group had few TUNEL-positive cells on the RGC layer with lightly stained nucleus, while rats in the model group had increased number of TUNEL-positive cells on the RGC layer with deeply stained nucleus and apparently higher AI (all $P < 0.05$). Meanwhile, rats in the model + tBHQ group had fewer TUNEL-positive cells, and the staining was significantly lighter with the lower AI when compared to the model group (all $P < 0.05$). As shown in Fig. 4, FG retrograde tracing demonstrated that rats in the model group had decreased number of RGCs compared with those in the normal group, while model + tBHQ group was dramatically higher than the model group regarding the number of RGCs (all $P < 0.05$).

The expression of Nrf2/HO-1 pathway-related proteins in retinal tissues of rats in each group

As illustrated by Fig. 5, the remarkably up-regulated mRNA and protein expression levels of Nrf2 and HO-1 were presented in the model group, as compared with the normal group (all $P < 0.05$). However, model + tBHQ group exhibited the higher mRNA and protein expression of Nrf2 and HO-1 than the model group (all $P < 0.05$).

Discussion

The IOP elevation has generally been credited as a key risk element for glaucoma, and thus, many scholars usually choose to induce IOP increase to construct a reliable animal model of glaucoma to know its underlying pathology [22, 23]. Through referring to a study by Ji et al. [21], we established the COH rat model of glaucoma in the present research, and then, we found the loss of RGCs with increased IOP, which was consistent with previous findings [5, 24], showing the successfully established models, which could simulate the process of increased IOP in the pathological apoptosis of RGCs. In addition, the Nrf2/HO-1 pathway was also detected in our experimental models, and the mRNA levels and protein expressions of Nrf2 and HO-1 were observed to be gradually decreased at 1 week after operation in the operated eyes of rats, although they were still higher than the nonoperated control eyes. It is now clear that glaucoma is a multifactorial pathology having a close

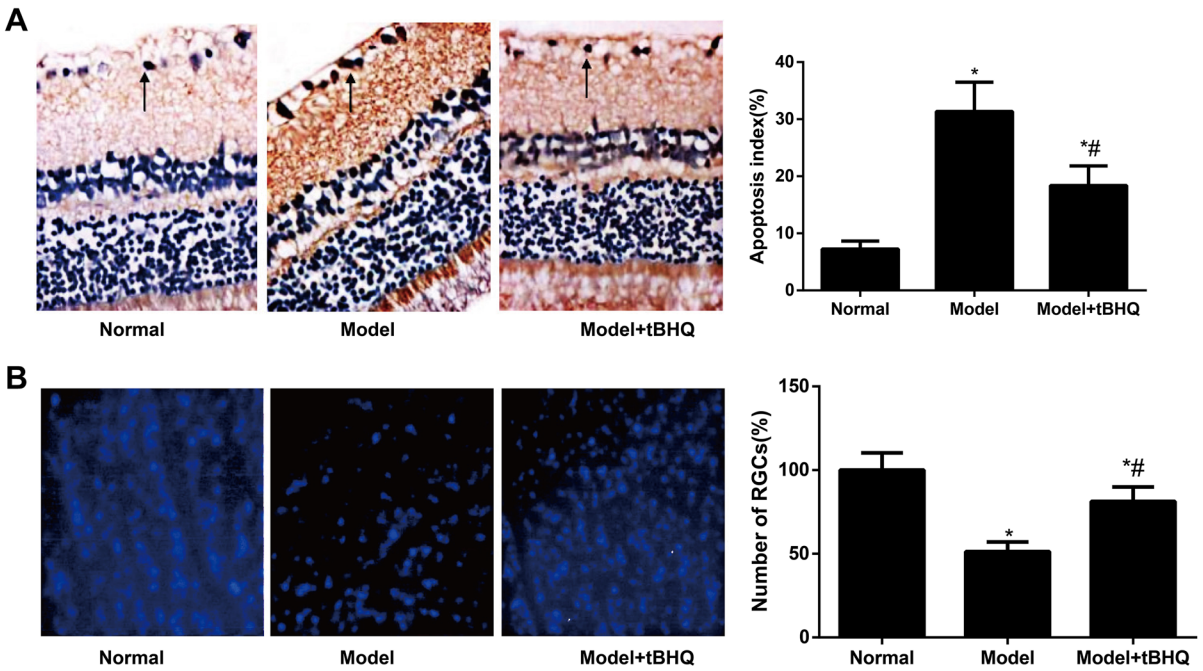

Fig. 4 The number and apoptosis of RGCs of rats in each group. *Note* **a** the apoptosis of RGCs in each group evaluated by using TUNEL staining; black arrows indicated the apoptotic cells; **b** the survival of RGCs labeled with fluorogold; $*P < 0.05$ compared with the normal group; $\#P < 0.05$ compared with the model group

relation with oxidative stress [25]. Under oxidative stress conditions, some specific transcription factors will be activated to exert protective functions, such as Nrf2, activated protein-1 (AP-1), nuclear transcriptional factor kappa B (NFκB), hypoxia inducible factor-1α (HIF-1α) [26]. Furthermore, electrophile will induce the conformational changes of Keap-1 protein to release Nrf2 or activate Nrf2 through signaling pathways like protein kinase C (PKC), mitogen-activated protein kinase (MAPK), and phosphatidylinositol-3 kinase (PI3 K), thereby promoting the formation of heterodimer by Nrf2 and Maf protein in nucleus, ultimately binding to ARE to initiate the transcription of HO-1 [27–30]. Therefore, we suggested that the up-regulation of Nrf2/HO-1 pathway in COH rats was very likely to be associated with oxidative stress. Similarly, in the study by Xu et al. [31], Nrf2 had an important protective effect on regulating the progression of diabetic retinopathy, since Nrf2 knockout aggravated the retinal neuron dysfunction. Besides, Wei et al. [32] revealed the significant up-regulation of Nrf2 in ischemic retinal tissues, which can modulate the paracrine of endothelial cells to promote vascular reconstruction, and thereby attenuating ischemic retinal damage. Moreover, inhibition of HO-1 led to the increased apoptosis and the decreased number of viable cells in retina after ischemia–reperfusion, as demonstrated by He et al. [33]. All of the above indicated that the up-regulation of Nrf2/HO-1 pathway may protect the retinal neurons in COH rats with glaucoma.

In support of this mechanism, the Nrf2 activator (tBHQ) was used to treat COH model rats in our following experiments. As a result, the expressions of Nrf2 and HO-1 were enhanced, with the alleviated pathological damage of retina, increased RGC numbers, and declined AI. Coincidentally, activation of Nrf2 can significantly promote the expression of phase II metabolic enzymes and effectively reduce oxidative stress-induced death of RGCs, as suggested by Himori et al. [34]. Also, the activated Nrf2/HO-1 pathway can alleviate the RGC damage caused by oxidative stress in vivo and in vitro [35]. In general, the production of ROS in the retinal tissues of glaucoma went up and plenty of free radicals exceed the scavenging ability of antioxidant systems, which would induce the oxidative stress of tissues to impair the lipids, proteins, and DNA of retinal tissues, and eventually causing the

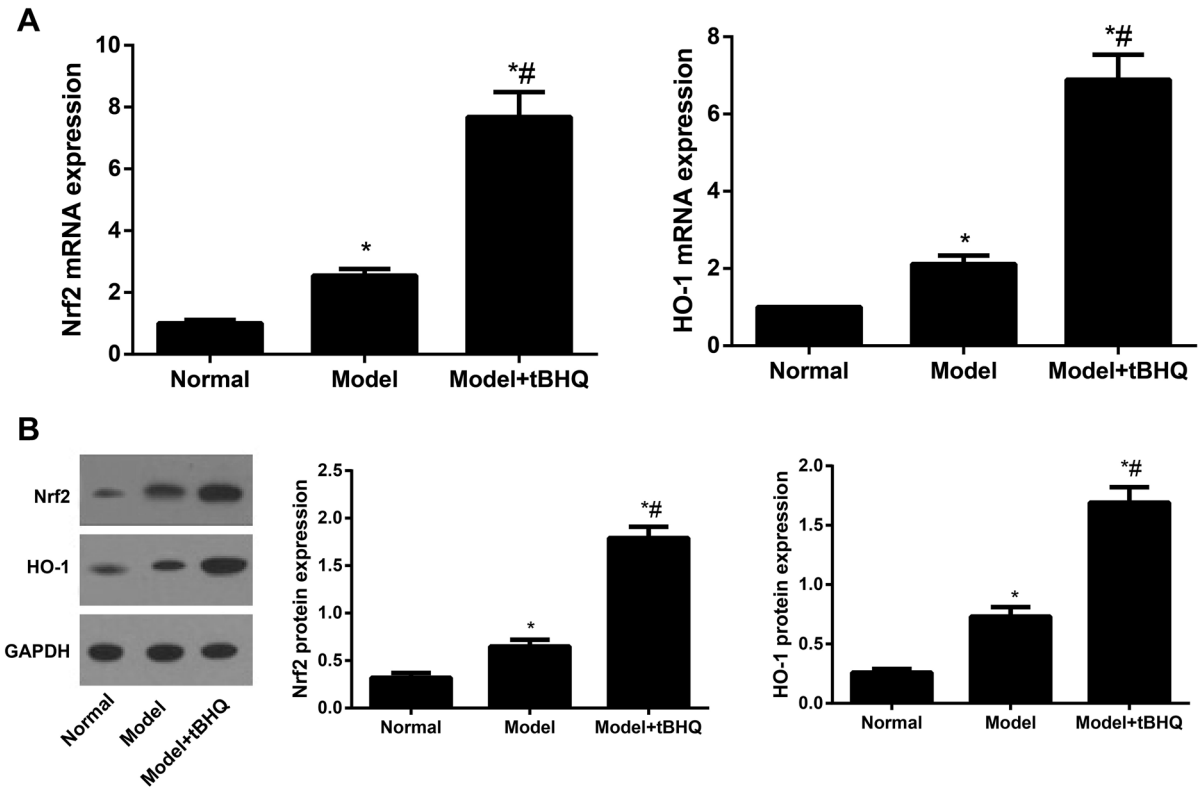

Fig. 5 The expression of Nrf2/HO-1 pathway-related proteins in retinal tissues of rats in each group. *Note* **a** the mRNA levels of Nrf2 and HO-1 of rats in each group determined by qRT-PCR at different time points; **b** the protein expressions of Nrf2 and HO-1 of rats in each group tested by western blotting at different time points; $*P < 0.05$ compared with the normal group; $\#P < 0.05$ compared with the model group

damage of RGCs [36, 37]. While p53 plays an essential part in the DNA damage caused by oxidative stress [38]. As a transcription factor, p53 can bind to the sequences of Bcl-2 and Bax promoters to advance the expression of Bax and inhibit the transcription of Bcl-2, thus breaking the Bcl-2/Bax balance and promoting cell apoptosis [39]. Moreover, ROS-induced mitochondrial dysfunction stimulated the released of cytochrome C from mitochondria into the cytoplasm, promoted the formation of apoptosomes (Apaf-1 and procaspase-9), and activated the caspases [40, 41]. Also, ROS can activate IκB kinase (IKK) and promote the phosphoprotein of IκBa, thereby inducing the dissociation of NF-κβ from the complex to the nucleus [42]. Meanwhile, activated NF-κβ can cause local inflammation and aggravate tissue ischemia to promote RGC apoptosis [43]. Consistent with the results in our study, Wan et al. [44] reported that Trimetazidine (TMZ) could reduce the ROS production and inflammatory cytokines via activating the Nrf2/HO-1 signaling pathway to alleviate the damage and death of RGCs in acute glaucoma, where this effect could be reversed by HO-1 inhibitor. Further, over-expressed HO-1 induced by drugs can enhance the activities of SOD-1 and Bcl-2, and reduce the HIF-1α, VEGF, and p53 expressions, thus effectively attenuating the apoptosis and damage of RGCs in diabetic retinopathy in rats, as indicated by Fan et al. [45]. From the study by Sun MH et al., we found that the high expression of HO-1 in RGCs of rats plays a protective role in retinal damage caused by ischemia–reperfusion injury via reducing p53, caspase-3, NF-κβ, iNOS, and MCP-1-mediated macrophage infiltration [46] suggesting that the downstream apoptosis-related factors, such as p53, caspase-3, and NF-κβ, might be inhibited by activation of Nrf2/HO-1 pathway to reverse the oxidative stress-induced damage and apoptosis of RGCs.

Considering the above, the expression of Nrf2/HO-1 signaling pathway was found to be up-regulated in

the retina tissues of COH model rats in our study, and more importantly, activation of Nrf2/HO-1 pathway can protect RGCs in glaucoma through attenuating the apoptosis and damage of RGCs, which could be a promising clinical target to prevent RGC degeneration in glaucoma.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Pang Y, Wang C, Yu L (2015) Mitochondria-targeted antioxidant ss-31 is a potential novel ophthalmic medication for neuroprotection in glaucoma. Med Hypothesis Discov Innov Ophthalmol 4:120–126
2. Sun XB, Lu HE, Chen Y, Fan XH, Tong B (2014) Effect of lithium chloride on endoplasmic reticulum stress-related perk/rock signaling in a rat model of glaucoma. Pharmazie 69:889–893
3. Mossböck G, Weger M, Faschinger C, Schmut O, Renner W (2004) Primary open-angle glaucoma. N Engl J Med 363(9422):1711
4. Nickells RW (2007) From ocular hypertension to ganglion cell death: a theoretical sequence of events leading to glaucoma. Can J Ophthalmol 42:278–287
5. Park HY, Kim JH, Park CK (2012) Activation of autophagy induces retinal ganglion cell death in a chronic hypertensive glaucoma model. Cell Death Dis 3:e290
6. Orzalesi N, Rossetti L, Omboni S, Group OS (2007) Conproso: vascular risk factors in glaucoma: the results of a national survey. Graefes Arch Clin Exp Ophthalmol 245:795–802
7. Guo Y, Johnson E, Cepurna W, Jia L, Dyck J, Morrison JC (2009) Does elevated intraocular pressure reduce retinal TRKB-mediated survival signaling in experimental glaucoma? Exp Eye Res 89:921–933
8. McKinnon SJ, Goldberg LD, Peeples P, Walt JG, Bramley TJ (2008) Current management of glaucoma and the need for complete therapy. Am J Manag Care 14:S20–S27
9. Qu J, Wang D, Grosskreutz CL (2010) Mechanisms of retinal ganglion cell injury and defense in glaucoma. Exp Eye Res 91:48–53
10. Tezel G (2011) The immune response in glaucoma: a perspective on the roles of oxidative stress. Exp Eye Res 93:178–186
11. Sasaki H, Shitara M, Yokota K, Hikosaka Y, Moriyama S, Yano M, Fujii Y (2012) Increased NRF2 gene (NFE2L2) copy number correlates with mutations in lung squamous cell carcinomas. Mol Med Rep 6:391–394
12. Zhang DD (2006) Mechanistic studies of the Nrf2-Keap1 signaling pathway. Drug Metab Rev 38:769–789
13. Li W, Khor TO, Xu C, Shen G, Jeong WS, Yu S, Kong AN (2008) Activation of Nrf2-antioxidant signaling attenuates NFκB-inflammatory response and elicits apoptosis. Biochem Pharmacol 76:1485–1489
14. Shen X, Hu B, Xu G, Chen F, Ma R, Zhang N, Liu J, Ma X, Zhu J, Wu Y, Shen R (2017) Activation of Nrf2/HO-1 pathway by glycogen synthase kinase-3β inhibition attenuates renal ischemia/reperfusion injury in diabetic rats. Kidney Blood Press Res 42:369–378
15. Dunn LL, Midwinter RG, Ni J, Hamid HA, Parish CR, Stocker R (2014) New insights into intracellular locations and functions of heme oxygenase-1. Antioxid Redox Signal 20:1723–1742
16. Zhang H, Liu YY, Jiang Q, Li KR, Zhao YX, Cao C, Yao J (2014) Salvianolic acid a protects rpe cells against oxidative stress through activation of Nrf2/HO-1 signaling. Free Radic Biol Med 69:219–228
17. Guzman-Beltran S, Espada S, Orozco-Ibarra M, Pedraza-Chaverri J, Cuadrado A (2008) Nordihydroguaiaretic acid activates the antioxidant pathway Nrf2/HO-1 and protects cerebellar granule neurons against oxidative stress. Neurosci Lett 447:167–171
18. Xu Z, Cho H, Hartsock MJ, Mitchell KL, Gong J, Wu L, Wei Y, Wang S, Thimmulappa RK, Sporn MB, Biswal S, Welsbie DS, Duh EJ (2015) Neuroprotective role of Nrf2 for retinal ganglion cells in ischemia–reperfusion. J Neurochem 133:233–241
19. Pan H, He M, Liu R, Brecha NC, Yu AC, Pu M (2014) Sulforaphane protects rodent retinas against ischemia–reperfusion injury through the activation of the Nrf2/HO-1 antioxidant pathway. PLoS ONE 9:e114186
20. Bayne K (1996) Revised guide for the care and use of laboratory animals available. Am Phys Soc Physiol 39(199):111–208
21. Ji M, Miao Y, Dong LD, Chen J, Mo XF, Jiang SX, Sun XH, Yang XL, Wang Z (2012) Group I mGluR-mediated inhibition of Kir channels contributes to retinal muller cell gliosis in a rat chronic ocular hypertension model. J Neurosci 32:12744–12755
22. Yu S, Tanabe T, Yoshimura N (2006) A rat model of glaucoma induced by episcleral vein ligation. Exp Eye Res 83:758–770
23. Su W, Li Z, Jia Y, Zhuo Y (2014) Rapamycin is neuroprotective in a rat chronic hypertensive glaucoma model. PLoS ONE 9:e99719
24. Hong S, Kim CY, Lee WS, Shim J, Yeom HY, Seong GJ (2010) Ocular hypotensive effects of topically administered agmatine in a chronic ocular hypertensive rat model. Exp Eye Res 90:97–103
25. Izzotti A, Bagnis A, Sacca SC (2006) The role of oxidative stress in glaucoma. Mutat Res 612:105–114
26. Sagai M, Bocci V (2011) Mechanisms of action involved in ozone therapy: Is healing induced via a mild oxidative stress? Med Gas Res 1:29
27. Keum YS, Yu S, Chang PP, Yuan X, Kim JH, Xu C, Han J, Agarwal A, Kong AN (2006) Mechanism of action of sulforaphane: inhibition of p38 mitogen-activated protein kinase isoforms contributing to the induction of antioxidant response element-mediated heme oxygenase-1 in human hepatoma HepG2 cells. Cancer Res 66:8804–8813
28. Kang KW, Lee SJ, Park JW, Kim SG (2002) Phosphatidylinositol 3-kinase regulates nuclear translocation of

NF-E2-related factor 2 through actin rearrangement in response to oxidative stress. Mol Pharmacol 62:1001–1010
29. Cullinan SB, Zhang D, Hannink M, Arvisais E, Kaufman RJ, Diehl JA (2003) Nrf2 is a direct perk substrate and effector of perk-dependent cell survival. Mol Cell Biol 23:7198–7209
30. McCubrey JA, Lahair MM, Franklin RA (2006) Reactive oxygen species-induced activation of the map kinase signaling pathways. Antioxid Redox Signal 8:1775–1789
31. Xu Z, Wei Y, Gong J, Cho H, Park JK, Sung ER, Huang H, Wu L, Eberhart C, Handa JT, Du Y, Kern TS, Thimmulappa R, Barber AJ, Biswal S, Duh EJ (2014) Nrf2 plays a protective role in diabetic retinopathy in mice. Diabetologia 57:204–213
32. Wei Y, Gong J, Xu Z, Thimmulappa RK, Mitchell KL, Welsbie DS, Biswal S, Duh EJ (2015) Nrf2 in ischemic neurons promotes retinal vascular regeneration through regulation of semaphorin 6A. Proc Natl Acad Sci U S A 112:E6927–E6936
33. He M, Pan H, Chang RC, So KF, Brecha NC, Pu M (2014) Activation of the Nrf2/HO-1 antioxidant pathway contributes to the protective effects of *Lycium barbarum* polysaccharides in the rodent retina after ischemia–reperfusion-induced damage. PLoS ONE 9:e84800
34. Himori N, Yamamoto K, Maruyama K, Ryu M, Taguchi K, Yamamoto M, Nakazawa T (2013) Critical role of Nrf2 in oxidative stress-induced retinal ganglion cell death. J Neurochem 127:669–680
35. Koriyama Y, Chiba K, Yamazaki M, Suzuki H, Muramoto K, Kato S (2010) Long-acting genipin derivative protects retinal ganglion cells from oxidative stress models in vitro and in vivo through the Nrf2/antioxidant response element signaling pathway. J Neurochem 115:79–91
36. Majsterek I, Malinowska K, Stanczyk M, Kowalski M, Blaszczyk J, Kurowska AK, Kaminska A, Szaflik J, Szaflik JP (2011) Evaluation of oxidative stress markers in pathogenesis of primary open-angle glaucoma. Exp Mol Pathol 90:231–237
37. Pinazo-Duran MD, Zanon-Moreno V, Garcia-Medina JJ, Gallego-Pinazo R (2013) Evaluation of presumptive biomarkers of oxidative stress, immune response and apoptosis in primary open-angle glaucoma. Curr Opin Pharmacol 13:98–107
38. Gallego-Pinazo R, Zanon-Moreno V, Sanz S, Andres V, Serrano M, Garcia-Cao I, Pinazo-Duran MD (2008) Biochemical characterization of the optic nerve in mice overexpressing the P53 gen. Oxidative stress assays. Arch Soc Esp Oftalmol 83:105–111
39. Duan P, Hu C, Butler HJ, Quan C, Chen W, Huang W, Tang S, Zhou W, Yuan M, Shi Y, Martin FL, Yang K (2017) 4-Nonylphenol induces disruption of spermatogenesis associated with oxidative stress-related apoptosis by targeting p53-Bcl-2/Bax-Fas/Fasl signaling. Environ Toxicol 32:739–753
40. Abu-Amero KK, Morales J, Bosley TM (2006) Mitochondrial abnormalities in patients with primary open-angle glaucoma. Invest Ophthalmol Vis Sci 47:2533–2541
41. Huang W, Fileta JB, Dobberfuhl A, Filippopolous T, Guo Y, Kwon G, Grosskreutz CL (2005) Calcineurin cleavage is triggered by elevated intraocular pressure, and calcineurin inhibition blocks retinal ganglion cell death in experimental glaucoma. Proc Natl Acad Sci U S A 102:12242–12247
42. Barakat DJ, Dvoriantchikova G, Ivanov D, Shestopalov VI (2012) Astroglial NF-κB mediates oxidative stress by regulation of nadph oxidase in a model of retinal ischemia reperfusion injury. J Neurochem 120:586–597
43. Tezel G, Yang X, Luo C, Cai J, Powell DW (2012) An astrocyte-specific proteomic approach to inflammatory responses in experimental rat glaucoma. Invest Ophthalmol Vis Sci 53:4220–4233
44. Wan P, Su W, Zhang Y, Li Z, Deng C, Zhuo Y (2017) Trimetazidine protects retinal ganglion cells from acute glaucoma via the Nrf2/HO-1 pathway. Clin Sci (Lond) 131:2363–2375
45. Fan J, Xu G, Jiang T, Qin Y (2012) Pharmacologic induction of heme oxygenase-1 plays a protective role in diabetic retinopathy in rats. Invest Ophthalmol Vis Sci 53:6541–6556
46. Sun MH, Pang JH, Chen SL, Han WH, Ho TC, Chen KJ, Kao LY, Lin KK, Tsao YP (2010) Retinal protection from acute glaucoma-induced ischemia–reperfusion injury through pharmacologic induction of heme oxygenase-1. Invest Ophthalmol Vis Sci 51:4798–4808