Trimetazidine protects retinal ganglion cells from acute glaucoma via the Nrf2/Ho-1 pathway

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**ABSTRACT**

Acute glaucoma is one of the leading causes of irreversible vision impairment characterized by the rapid elevation of intraocular pressure and consequent retinal ganglion cell (RGC) death. Oxidative stress and neuro-inflammation have been considered critical for the pathogenesis of RGC death in acute glaucoma. Trimetazidine (TMZ), an anti-ischemic drug, possesses anti-oxidative and anti-inflammatory properties, contributing to its therapeutic potential in tissue damage. However, the role of TMZ in acute glaucoma and the underlying molecular mechanisms remain elusive. Here, we report that treatment with TMZ significantly attenuated retinal damage and RGC death in mice with acute glaucoma, with a significant decrease in reactive oxygen species (ROS) and inflammatory cytokine production in the retina. Furthermore, TMZ treatment directly decreased ROS production and rebalanced the intracellular redox state, thus contributing to the survival of RGCs *in vitro*. TMZ treatment also reduced the production of inflammatory cytokines *in vitro*. Mechanistically, the TMZ-mediated inhibition of apoptosis and inflammatory cytokine production in RGCs occurred via the inhibition of the nuclear factor erythroid 2-related factor 2/heme oxygenase 1/caspase-8 pathway. Moreover, the TMZ-mediated neuroprotection in acute glaucoma was abrogated when an HO-1 inhibitor, SnPP, was used. Our findings identify potential mechanisms of RGC apoptosis and propose a novel therapeutic agent, TMZ, which exerts a precise neuroprotective effect against acute glaucoma.

**CLINICAL PERSPECTIVE**

- Acute glaucoma jeopardizes normal vision due to substantially high intraocular pressure (IOP) and consequent retinal ganglion cell (RGC) death; currently, an attractive alternative for next-generation glaucoma therapy is to select an effective drug to prevent RGC apoptosis in IOP-induced retinal damage.
- This study demonstrated that trimetazidine (TMZ) significantly ameliorated high IOP-induced retinal damage and RGC apoptosis, by exerting therapeutic efficacy through its anti-oxidative and anti-inflammatory properties via the Nrf2/HO-1 pathway.
- Our findings suggested that TMZ can protect against acute glaucoma and emerge as a promising candidate in the treatment of acute glaucoma and other neurological disorders.
INTRODUCTION

Acute glaucoma is one of the main causes of irreversible visual impairment and blindness, especially among people of Asian descent [1,2]. Acute glaucoma is characterized by considerable high intraocular pressure (IOP), pain and vision loss [3]. The rapid increase and decrease of IOP causes retinal ischemic/reperfusion (I/R) injury, thus resulting in apoptosis of retinal ganglion cells (RGCs). Although the pathogenesis of RGC death is not fully understood, oxidative stress and neuro-inflammation have been considered critical for the pathogenesis for RGC death in retinal I/R injury during acute glaucoma [4]. Current treatments that lower IOP through medication and surgery cannot completely prevent the progressive death of RGCs in acute glaucoma. Therefore, safe and effective novel alternatives are needed for acute glaucoma treatment.

Trimetazidine (TMZ), a piperazine-derived antianginal agent [5,6], has traditionally been used as an anti-ischemic drug for coronary artery disease. Recently, studies have shown that TMZ possesses anti-oxidative and anti-inflammatory properties and plays cytoprotective roles in various tissues, including nervous tissue, pancreatic tissue and renal tissue [7,8]. Therefore, TMZ may be a potential therapeutic alternative for acute glaucoma treatment. However, the role of TMZ in acute glaucoma has not yet been explored. Moreover, the mechanisms by which TMZ mediates anti-oxidative and anti-inflammatory effects remain elusive. Thus, in this study, we investigated the therapeutic effect of TMZ on experimental acute glaucoma and the anti-oxidative and anti-inflammatory mechanisms of TMZ.
MATERIALS AND METHODS

Establishment of the retinal I/R model

Six- to eight-week-old C57BL/6J male mice were purchased from Guangdong Medical Laboratory Animal Center. The feeding and administration of mice strictly complied with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

The mice were anesthetized with 100 mg/kg pentobarbital sodium by intraperitoneal injection. Before operation, mice corneas were topically anesthetized with 0.5% Alcaine eye drops, and pupils were dilated with 1% Tropicamide. We then conducted cannulation into the anterior chamber of the right eye using a 30-gauge needle supplied with balanced salt solution to maintain the IOP at 70 mmHg for 60 minutes. Sham operation was performed in the contralateral eye without elevating the IOP to serve as the control. After 60 minutes, the IOP was normalized by withdrawing the needle, and then tobramycin ointment was used to prevent bacterial infection.

Preparation of Trimetazidine

TMZ with purity higher than 95% was purchased from Sigma Chemical Co. (St Louis, USA), dissolved in sterile PBS to a stock concentration of 0.1 mol/L, and stored at 4°C in the dark to be used within 2 days after preparation.

Experiment Grouping Design

The mice were randomly divided into five groups: normal control group (Normal), Tin protoporphyrin IX dichloride (SnPP) group, I/R group, TMZ (100 μM) group and TMZ + SnPP group. The mice in I/R, TMZ and TMZ + SnPP groups were subjected to experimental I/R. The normal control and SnPP groups had sham cannulation performed without elevating the IOP as described above. The eyes of mice in the SnPP or TMZ groups were intravitreally injected with 1 μL TMZ or SnPP solution, respectively, before the onset of reperfusion. The eyes of mice in the TMZ + SnPP group were intravitreally injected with 1 μL TMZ and SnPP solution together before the onset of reperfusion. The eyes of mice in the normal control
group were injected with 1 μL of sterile PBS into the vitreous cavity.

**RGC Labeling and Survival Quantification.**

Mice were anesthetized with 100 mg/kg pentobarbital sodium by intraperitoneal injection and placed in a stereotactic apparatus (Stoelting, USA). The skull was exposed and cleaned with PVP-J. Bilateral holes were drilled at the surface of superior colliculi. Approximately 1 μl of 4% Fluorogold (FG, hydroxystilbamidine; Fluorochrome, USA) solution was injected into both superior colliculi. After injection, the micro-syringe was kept still for 30 seconds and then slowly removed. Finally, the dissected scalp was sutured with topical application of tobramycin.

To ensure proper RGC labeling, the animals were allowed 7 days for retrograde transport of FG before sacrifice. FG-positive RGCs were identified with a fluorescence microscope (AxioImager; Carl Zeiss MicroImaging Inc., USA) in retinal flat mount. Surviving RGCs (green dots) were counted automatically using ImageJ (LOCI, University of Wisconsin-Madison).

**Histological examination**

Seven days after I/R treatment, mouse eyes were enucleated and embedded in paraffin. Every paraffin block was sectioned to 4-μm thickness through the optic nerve. Three sections of each eye were cut and stained with hematoxylin and eosin (H&E). The inner plexiform layer (IPL) thickness was measured within 1 mm to the optic nerve center to quantify retinal damage by Axiovision software (Carl Zeiss MicroImaging Inc.). The data from three sections per eye were averaged.

**Primary culture of RGCs**

Primary RGCs were obtained according to the protocol published by Alissa Winzeler and Jack T. Wang in 2013 [10]. In brief, retinal samples were separated from neonatal mice to prepare single cell suspensions. The retinal suspension was incubated in rabbit anti-mouse macrophage antibody-coated flasks (Cedarlane, USA) and goat anti-mouse macrophage
antibody-coated flasks (Jackson Immuno Research, USA) to remove the adherent macrophages. The non-adherent cells were transferred to Thy1.2 monoclonal antibody-coated flasks (Millipore Chemicon, USA) to collect adherent cells. The adherent RGCs were incubated at 37°C in 5% CO₂ with RGC Growth Medium containing supplements as described.

Mix culture with BV2 cells

Mouse microglia cell line BV2 (ATCC, USA) was co-cultured with primary RGCs in DMEM (High Glucose 4.5 g/ml, Gibco, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO₂ and 95% air. RGCs were placed on the upper permeable membrane of a transwell chamber, with BV2 cells grown in the lower well of the plate for 24 hours before treatment.

Establishment of the Oxygen-glucose deprivation and reperfusion (OGD/R) model

The OGD/R model was established as follows: the culture medium was replaced with glucose-free DMEM (Gibco) after washing the cells twice with PBS, and then the cells were placed in a modular incubator chamber (Billups-Rothenberg, Inc., Del Mar, CA) filled with a gas mixture of 5% CO₂ and 95% N₂ at 37°C for 3 hours. The control cells were incubated in serum-free medium with 4.5 g/L D-glucose under normoxic conditions (5% CO₂ and 95% air) for the same duration. At the end of the exposure period, the cells were returned to normoxic conditions with glucose and incubated for 12 hours.

Cell treatment with TMZ

Primary RGCs were cultured in 6-well plates or transwell plates before treatment. TMZ was added at indicated concentrations into cell supernatants 6 hours prior to the onset of OGD/R or other assays. TMZ was diluted in DMEM and added at concentrations ranging from 0.1 to 100 μM.

Enzyme-linked Immunosorbent Assay (ELISA)

The cytokines TNFα and IL1β were measured in the culture supernatant of BV2 cells.
Immunochemical analyses were conducted using commercially available ELISA kits (eBioscience, Vienna, Austria). For measuring the level of 3-nitrotyrosine proteins in retinal homogenates, a commercial kit was used (Oxiselect Nitrotyrosine kit; Cell Biolabs, Inc., USA), following the manufacturer’s instructions.

Viability and Apoptosis Assays in RGCs

Cell viability was measured using the CCK8 Assay Kit (Beyotime Biotechnology, China) according to the manufacturer’s protocol. CCK8-reduction activity was presented as the percentage of the unexposed control cells (100%). Flow cytometry was also performed to measure OGD/R-induced apoptosis of RGCs by using a propidium iodide (PI) and Annexin V-FITC detection kit (BD Biosciences, New York, USA) according to the manufacturer’s instructions. Flow cytometric analysis using FlowJo 7.6.2 (FlowJo, LLC) was performed following standard protocols.

Inhibition of Ho-1 Activity

Ho-1 activity was inhibited in vivo through an intraperitoneal injection of SnPP (40 mol/kg, Tocris Bioscience, USA) 0.5 h prior to I/R and once daily for 3 days after I/R. SnPP was dissolved in 0.1 N NaOH and diluted with PBS (pH = 7.4).

Cells were seeded in a 6-well plate at approximately 70% confluency. Ten micromolar SnPP or vehicle was added to the culture medium for 6 hours. The cells were then treated with TMZ or vehicle for 6 hours and subjected to OGD/R.

Detection of Intracellular ROS Levels

Quantification of intracellular reactive oxygen species (ROS) accumulation was performed by fluorescence detection as well as flow cytometry using the fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA, KeyGEN, China). Primary RGCs were subjected to the appropriate treatments and then incubated for 20 minutes in the dark at 37°C with 10 µM DCFH-DA solutions. After incubation, the cells were analyzed within 30 minutes. Mean fluorescence intensity of ROS was measured using a fluorescence microscope, and flow
cytometry was performed using a Fortessa system (BD). The data were analyzed using ImageJ and FlowJo software.

**Analysis on MMP (JC-1 staining)**

The changes in mitochondrial membrane potential (MMP) in RGCs were explored using the 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine iodide (JC-1) probe as described by the manufacturer and then visualized using an inverted fluorescence microscope. The average result of three plates of cells or mice were obtained for each condition.

**Quantitative Real-time PCR**

Total RNA was extracted from the retinal samples and cultured cells by TRIzol reagent (Invitrogen, USA) following the manufacturer’s protocol. cDNA synthesis was conducted with PrimeScript RT Master Mix (TaKaRa, China). Quantitative analysis was performed with a Light Cycler 480 Real-Time PCR System. The expression level of target mRNA was measured and normalized to Gapdh. The primer sequences are as follows: GAPDH-forward primer CCGGGAAACTGTGGCGTGATGG; GAPDH-reverse primer AGGTGGAGGAGTGGGTGTCGTT; TNFα-forward primer GCACCACCATCAAGGACTCAA; TNFα-reverse primer TCGAGGCCCAGTGAATTCG; IL1β-forward primer GGGCCTCAAAGGAAAGAATC; IL1β-reverse primer CTCTGCTTGTGAGGTGCTGA.

**Western Blot**

Total protein was isolated from the retinal samples and cultured cells according to standard procedures. Nuclear protein was extracted using ProteoExtract® Subcellular Proteome Extraction Kit (Merck Millipore, German) following the manufacturer’s protocol. Proteins were run on 12% polyacrylamide gels following standard protocol. The expression of total protein and nuclear protein was normalized to Gapdh and Histone H3, respectively, and then quantified using ImageJ.

The primary antibodies and dilutions used were as follows: anti-Nrf2 (CST, USA, 1:400), anti-Ho-1 (CST, 1:200), anti-cleaved-Caspase-8 (CST, 1:400), anti-Gapdh (CST, 1:400),
anti-Histone H3 (Abcam, UK, 1:400).

Caspase-8 Activity Assay

The Caspase-8 activity of retinal tissues and RGCs were performed using the CaspGLOW™ Fluorescein Active Caspase-8 Staining Kit (BioVision, Milpitas, USA) according to the manufacturer’s instructions. One microliter of FITC-IETD-FMK was added into each tube containing 300 µl of cell suspension and incubated for 1 hour at 37°C in 5% CO₂. Then, the cells were resuspended, and the fluorescence intensity was measured at Ex/Em = 485/535 nm.

Statistical Analysis

The data were presented as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed, followed by Bonferroni’s post hoc test using SPSS software, version 17 (SPSS Inc., Chicago, IL, USA). All statistical tests were two-tailed, and a p-value below 0.05 was considered statistically significant.
RESULTS

Neuroprotective effects of intravitreal TMZ in IOP-induced retinal damage

To evaluate the role of TMZ in acute glaucoma, TMZ was administered intravitreally before acute elevation of IOP. We first investigated the change in retinal thickness in three independent groups of mice. Retinas exposed to I/R displayed a significant decrease in IPL thickness compared with normal retinas 7 days after reperfusion. However, the damage to the retina, especially to the IPL, was significantly ameliorated by TMZ (Fig. 1A and 1B). The irreversible apoptosis of RGCs is another important indicator for the functional damage of the retina. FG analysis demonstrated that the number of RGCs was decreased in the experimental mice at 7 days after reperfusion compared with that observed in control mice (Fig. 1C and 1D). Intravitreal TMZ injections significantly decreased the severity of retinal damage and extent of RGC death (Fig. 1C and 1D). Previous studies have shown that over-production and accumulation of neurotoxic mediators, including ROS, TNF-$\alpha$ and IL-1$\beta$, play important roles in the primary and secondary waves of RGC apoptosis [11-13]. As shown in Fig. 1E, the production of nitrotyrosine (a protein derivative of ROS) was significantly increased in the whole retina at 24 hours after reperfusion compared with that in control retinas, and it declined in the retinas treated with TMZ. In addition, intravitreal injection of TMZ significantly suppressed the expression of TNF$\alpha$ and IL1$\beta$ mRNA (Fig. 1F and 1G). Collectively, these data show that TMZ exerts neuroprotective effects against IOP-induced retinal damage.

TMZ directly protects RGC apoptosis induced by OGD/R

Next, we performed a series of in vitro studies to explore whether TMZ is capable of directly inhibiting the apoptosis of RGCs and the underlying mechanisms. We first identified primary cultured RGCs by characteristic markers. Immunostaining results indicated that primary RGCs indeed expressed Brn-3a, $\beta$3-tubulin and RBPMS (Fig. 2A). CCK8 assay was used to test whether TMZ can influence cellular metabolism or proliferation. The results showed that cellular metabolism was affected when TMZ concentration reached 50 $\mu$M (Fig. 2B). Therefore, we selected the concentration range from 0.1 to 10 $\mu$M for subsequent in vitro

*Please note that the in vitro studies were performed at a concentration of 0.1 to 10 $\mu$M,*
studies. We then exposed primary RGCs to OGD/R to effectively mimic the IOP-induced I/R damage *in vivo* [4,14]. OGD/R decreased cell viability, and TMZ (0.1-10 μM) treatment significantly increased the number of surviving RGCs (Fig. 2C). The flow cytometry results showed that the number of apoptotic cells marked by PI and AnnexinV was increased after OGD/R exposure, whereas TMZ treatment significantly decreased the number of PI+Annexin+ cells (Fig. 2D and 2E). The TMZ concentration range from 1 to 10 μM was found to be the most effective in exerting cytoprotective effects against OGD/R. In addition, TMZ significantly decreased caspase-8 activation as measured by Western blotting and caspase-8 activity assay (Fig. 2F and 2G). These results demonstrate that TMZ decreases OGD/R-induced RGC apoptosis *in vitro*.

**The anti-oxidant effect of TMZ is involved in the TMZ-mediated neuroprotection in RGCs**

Primary RGCs in the OGD/R group display a greater extent of ROS production than the cells in the control group. TMZ was found to significantly attenuate the over-production of ROS in RGCs exposed to OGD/R as marked by the reduction in green fluorescence in a concentration-dependent manner (Fig. 3A and 3B). The diminished ROS production in primary RGCs by TMZ could also be observed by flow cytometry. The peak area occupied by FITC+ cells, representing cells with excess ROS, was found to be significantly decreased by TMZ treatment (Fig. 3C and 3D). OGD/R also resulted in a decrease in the MMP in RGCs, which was marked by fluorescence shift from red to green. However, TMZ treatment protected mitochondrial function by restoring the MMP (Fig. 3F and 3G). These results further support the anti-oxidant effect of TMZ on RGCs *in vitro*.

**TMZ conferred protection against apoptosis in RGCs via Nrf2/Ho-1 signaling**

The above results showed that the anti-oxidant effects of TMZ are actively involved in the TMZ-mediated protection of RGCs. Nrf2, a transcription factor, is regarded as a master regulator of cellular responses to oxidative stress. Thus, we next determined whether Nrf2 and its downstream signaling factor, Ho-1, is associated with the TMZ-mediated protection against RGC apoptosis. The Western blotting results showed that TMZ (10 μM) treatment
significantly induced the nuclear translocation of Nrf2 in RGCs under OGD/R conditions (Fig. 4A). TMZ treatment also increased Ho-1 protein expression compared with vehicle treatment (Fig. 4B). To further confirm the role of Nrf2/Ho-1 signaling in the TMZ-mediated neuroprotection of RGCs, SnPP, a Ho-1 inhibitor was used. Our results showed that SnPP treatment significantly but incompletely reversed the TMZ-mediated neuroprotection of RGCs (Fig. 4C and 4D). The reduction of intracellular ROS (Fig. 4E and 4F) and alterations in MMP (Fig. 4G and 4H) induced by TMZ were partially inhibited by SnPP. Taken together, these data suggest that TMZ confers neuroprotection through an Nrf2/Ho-1-dependent mechanism.

**Nrf2/Ho-1 signaling is essential for TMZ-mediated anti-inflammatory effects in vitro**

We also performed a series of *in vitro* experiments to further evaluate the anti-inflammatory effects of TMZ and to identify the underlying mechanisms. For this purpose, BV2 cells, an established mouse microglial cell line was cultured under OGD/R conditions. BV2 cells were plated in medium in the presence or absence of TMZ for 6 h before OGD/R. We used ELISA kits to examine the concentration of TNFα and IL1β in the supernatant of BV2 cells after OGD/R. The OGD/R-induced over-production of TNFα and IL1β could be inhibited by TMZ (Fig. 5A). The real-time PCR results further confirmed the anti-inflammatory effects of TMZ (Fig. 5B). Nrf2/Ho-1 signaling plays an important role in the TMZ-mediated neuroprotection of RGCs. Thus, we next assessed whether this pathway is also involved in the anti-inflammatory effects of TMZ. As shown in Fig. 5C and 5D, TMZ significantly induced the nuclear translocation of Nrf2 and increased the protein expression of Ho-1 in BV2 cells under OGD/R conditions. Our results demonstrated that SnPP significantly but incompletely reversed the TMZ-mediated inhibition of TNFα and IL1β production (Fig. 5E and 5F). In addition, the inhibitory effect of TMZ on caspase-8 activation was significantly decreased after SnPP treatment (Fig. 5G). These results indicate that TMZ is capable of regulating Nrf2/Ho-1 signaling to exert anti-inflammatory effects *in vitro*.

**TMZ protects RGCs from IOP-induced damage via Nrf2/Ho-1 signaling**

Our *in vitro* studies showed that Nrf2/Ho-1 signaling plays a critical role in TMZ-mediated...
anti-oxidant and anti-inflammatory effects. Therefore, we determined whether Nrf2/Ho-1 signaling was also involved in the TMZ-mediated neuroprotection against IOP-induced retinal damage. The Western blotting results showed that TMZ treatment significantly induced the nuclear translocation of Nrf2 and increased the expression of Ho-1 in the retinas (Fig. 6A and B). SnPP was intraperitoneally injected (40 mol/kg) 0.5 h prior to I/R and once daily for 3 days after I/R. As shown in Fig. 6C-F, treatment with the Ho-1 inhibitor significantly blocked the TMZ-mediated neuroprotection against IOP-induced retinal damage. The Ho-1 inhibitor also significantly decreased the inhibitory effect of TMZ on ROS accumulation and inflammatory cytokine production (Fig 6G-I). These findings suggest that the upregulation of Nrf2/Ho-1 signaling may contribute, at least in part, to the TMZ-mediated neuroprotection against IOP-induced retinal damage.
**DISCUSSION**

Acute glaucoma jeopardizes normal vision due to substantially high IOP. The progressive loss of RGCs caused by rapid increase of IOP is mainly responsible for irreversible blindness in acute glaucoma [15]. Currently, selecting effective drugs to prevent RGC apoptosis in IOP-induced retinal damage has become an attractive alternative for next-generation glaucoma therapy. TMZ, which improves energy metabolism in ischemic myocytes, was first developed as an anti-angina drug [16]. However, a growing number of studies suggest that TMZ possesses numerous non-antianginal functions, including immunosuppression [17], anti-oxidation [18], anti-ischemia [19,20] and anti-apoptosis [21]. This study demonstrated that treatment with TMZ significantly ameliorated high IOP-induced retinal damage and RGC apoptosis, with a significant decrease in ROS and inflammatory cytokine production in the retina. TMZ primarily exerts its therapeutic efficacy through its anti-oxidative and anti-inflammatory effects. These results are consistent with those of previous studies, showing that TMZ treatment attenuates acute inflammatory responses and oxidative stress in experimental cardiac remodeling [22] and pancreatitis [23]. Next, we found that the anti-oxidative and anti-inflammatory effects of TMZ depended primarily on the Nrf2/HO-1 pathway. Interestingly, TMZ treatment in IOP-induced retinal damage resulted in an obvious activation of the Nrf2/HO-1 pathway, which plays a crucial role in TMZ-mediated protection against IOP-induced retinal damage. These findings suggest that TMZ exerts neuroprotective effects against acute glaucoma mainly through anti-oxidative and anti-inflammatory properties.

The transcription factor Nrf2 is a central regulator of cellular responses to oxidative stress stimulation [24]. Under normal conditions, Nrf2 is an inactive complex in the cytoplasm with Kelch-like ECH-associated protein 1. When cells undergo oxidative stress, the complex is disrupted, and, subsequently, Nrf2 translocates to the nucleus, leading to target gene transcription, including Ho-1, a key heme-degrading enzyme [25,26]. In addition to its well-known anti-oxidative properties, the Nrf2/Ho-1 signaling pathway also exhibits anti-inflammatory and anti-apoptotic properties [27,28]. In this study, we demonstrated that TMZ treatment promoted the nuclear translocation of Nrf2 and subsequently increased the
expression of HO-1 in primary RGCs and BV2 cells. Furthermore, HO-1 blockade significantly but incompletely reversed the TMZ-mediated anti-oxidative and anti-inflammatory effects. Importantly, the HO-1 inhibitor also blocked the TMZ-mediated neuroprotection in RGCs in acute glaucoma. Therefore, these compelling findings suggest that Nrf2/HO-1 signaling contributes, at least in part, to the TMZ-mediated neuroprotection in acute glaucoma.

Caspase-8, a well-characterized initiator of apoptotic signaling, has been shown to have multiple non-apoptotic functions. Burguillos *et al.* reported that caspase-8 promotes microglial activation and neuroinflammation. Our recent study indicated that caspase-8 mediates the production of cytokines, including IL1β and TNFα in immunocytes, which play important roles in high-IOP-induced retinal damage [4]. In this study, we observed that TMZ inhibited caspase-8-mediated RGC apoptosis. Furthermore, TMZ reduced caspase-8-dependent inflammatory reactions both in *vivo* and in *vitro*. Therefore, combining our results on the protective role of TMZ in acute glaucoma with existing evidence enables us to propose a mechanistic model that is described in Fig. 7 Proposed model for the neuroprotective mechanisms of TMZ in acute glaucoma.

The unique pharmacological properties of TMZ enhance its efficacy and enable it to reach its peak concentration in less than 2 h. Timely treatment is closely associated with efficacy of neuroprotection [1,2]. Hence, TMZ could be used to halt the early stages of RGCs apoptosis in acute glaucoma, which is attributable to its fast mechanism of action. Due to its efficacy and safety, TMZ could be used a promising candidate to treat acute glaucoma and other related ocular diseases.

In summary, our study initially demonstrated the neuroprotective effects of TMZ on experimental acute glaucoma. Our results showed that TMZ inhibited RGC apoptosis and inflammation in *vitro* and in *vivo*, possibly by inhibiting caspase-8 signaling. Importantly, we further determined the neuroprotective mechanisms of TMZ by demonstrating that Nrf2-HO-1 signaling is essential for TMZ-mediated neuroprotective effects in *vitro* and in *vivo*. These findings provide compelling evidence that TMZ can protect against acute glaucoma and emerge as a promising candidate in the treatment of acute glaucoma.
References


Figure legends

Figure 1

A. Normal I/R TMZ

B. Mean thickness of IPN (μM)

C. Normal I/R TMZ

D. Mean thickness of IPN (μM)

E. Normal I/R TMZ

F. TNE mRNA (Fold change)

G. IL1 mRNA (Fold change)
Figure 1. Neuroprotective effects of intravitreal TMZ against IOP-induced retinal damage

A and B. H&E staining of retinal cross-sections showing that intravitreal TMZ injection significantly inhibited the attenuation of total retinal thickness and IPL thickness in response to I/R damage 7 days after reperfusion.

C and D. FG labeling was performed 7 days after reperfusion. The results indicated that TMZ significantly increased the number of surviving cells in the RGC layer compared with that in the untreated I/R group.

E. The production of nitrotyrosine was significantly decreased by TMZ treatment in the whole retina 24 hours after reperfusion compared with that in the untreated retinas.

F and G. As measured by real-time PCR, intravitreal injection of TMZ significantly suppressed the expression of TNFα and IL1β mRNA in retinas.

GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer. Scale bar = 50 μm.

The data represents the means ± SD (n=6). *p < 0.05, **p < 0.01.
Figure 2
A. Immunostaining results indicated that primary RGCs indeed expressed characteristic markers of retinal ganglion cells including Brn-3a (red), RBPMS (green, left) and β3-tubulin (green, right).

B. CCK8 assay showed that cellular metabolism was affected when TMZ concentration reached 50 µM.

C. CCK8 assay showed that cell survival was significantly increased by TMZ treatment after OGD/R.

D and E. The results of flow cytometry showed that the proportion of apoptotic cells was increased to 15.8% (± 3.1%) after OGD/R exposure. TMZ significantly decreased the proportion of PI+Annexin+ cells to an average of 2.93%.

F. Western blot analysis demonstrated that TMZ significantly suppressed the level of cleaved-caspase-8.

G. The reduction of caspase-8 activity was confirmed by caspase-8 activity assay.

Scale bar = 20 μm. The data represent the means ± SD (n=8). *p < 0.05, **p < 0.01.
**Figure 3**

**A**

TMZ 0, 0.1, 1, 10 μM

**B**

![Graph showing ROS Cell Count vs TMZ concentration](image)

**C**

Cell Count

![Bar chart showing Cell Count](image)

**D**

![Graph showing ROS Cell Count vs TMZ concentration](image)

**E**

![Images showing ROS Cell Count](image)

**F**

![Graph showing ROS Cell Count vs TMZ concentration](image)
Figure 3. The anti-oxidant effect of TMZ is involved in the TMZ-mediated neuroprotective effect on RGCs

A and B. After OGD/R exposure, primary RGCs were subjected to ROS over-production (green fluorescence). TMZ could significantly decrease ROS levels in RGCs.

C and D. As marked by the peak area of FITC+ population, the proportion of cells with excess ROS was significantly decreased from 80% (± 6.1%) to 30% (± 1.7%) in the OGD/R + TMZ RGCs.

E and F. OGD/R decreased the MMP, which was marked by a fluorescence shift from red to green in RGCs. TMZ pretreatment elevated the PE/FITC ratio.

Scale bar = 50 μm. The data represent the means ± SD (n=8). *p < 0.05, **p < 0.01.
Figure 4
Figure 4. TMZ protects RGCs from apoptosis via Nrf2/Ho-1 signaling

A and B. Western blot analysis demonstrated that TMZ elevated the nuclear translocation of Nrf2 and total expression of Ho-1 in primary RGCs under OGD/R conditions.

C and D. TMZ suppressed OGD/R-induced apoptosis in primary RGCs. SnPP treatment significantly but incompletely reversed TMZ-mediated cytoprotective effect on RGCs.

E and F. Flow cytometry assay of ROS accumulation (FITC+) in RGCs. SnPP resulted in an increase in the proportion of FITC+ cells and inhibited the TMZ-induced anti-oxidative effect.

G and H. The decreasing of PE/FITC ratio indicated significant disruption of the MMP in the SnPP-treated RGCs, which could not be rescued by TMZ treatment.

Scale bar = 50 μm. The data represent the means ± SD (n=8). *p < 0.05, **p < 0.01.
Figure 5
Figure 5. Nrf2/Ho-1 signaling is essential for the TMZ-mediated anti-inflammatory effects in vitro

A. As measured by ELISA, the concentration of TNFα and IL1β in supernatants was found to be significantly reduced by TMZ.

B. Real-time PCR analysis showed that TMZ decreased TNFα and IL1β mRNA in the TMZ group.

C and D. TMZ significantly induced the nuclear translocation of Nrf2 and increased the protein expression of Ho-1 in BV2 cells under OGD/R conditions.

E and F. SnPP significantly but incompletely reversed the TMZ-mediated inhibition on TNFα and IL1β production as measured by ELISA and real-time PCR.

G. SnPP treatment significantly decreased the inhibitory effect of TMZ on caspase-8 activation.

The data represent the means ± SD (n=8). *p < 0.05, **p < 0.01.
Figure 6
Figure 6. TMZ protects RGCs from IOP-induced damage via Nrf2/Ho-1 signaling

A and B. Western blot analysis demonstrated that TMZ dramatically induced the nuclear translocation of Nrf2 and increased the expression of Ho-1 in the retinas.

C and D. Treatment with the SnPP significantly blocked the TMZ-mediated neuroprotection in IOP-induced retinal attenuation, especially IPL thickness.

E and F. SnPP dramatically inhibited the TMZ-mediated neuroprotection in IOP-induced RGC apoptosis.

G. SnPP also significantly decreased the inhibitory effect of TMZ on nitrotyrosine production.

H and I. Real-time PCR results confirmed that SnPP significantly suppressed the TMZ-induced decrease in TNFα and IL1β mRNA levels compared with the TMZ only group.
Proposed Model for Neuroprotective Mechanisms of TMZ in Acute Glaucoma
**Figure 7. Proposed Model for the Neuroprotective Mechanisms of TMZ in Acute Glaucoma**

I/R damages the function of mitochondria by disrupting the MMP, which leads to the over-production of ROS. ROS pushes cells toward the apoptotic fate and stimulates neuro-inflammation. Nrf2/Ho-1 pathway can be activated by TMZ to confer protective effects by inhibiting the accumulation of ROS. Immunocyte activation during injury results in caspase-8-dependent inflammation by increasing TNFα and IL1β production, which also induces RGC apoptosis. Pro-inflammatory reactions can be effectively suppressed by TMZ. Therefore, RGC apoptosis can be attenuated by pretreatment with TMZ via anti-oxidative and anti-inflammatory mechanisms in acute glaucoma.

I/R, ischemia/reperfusion injury; RGC, retinal ganglion cell; MMP, mitochondrial membrane potential; ROS, reactive oxygen species.