Research Article

Quercetin protects against diabetic retinopathy in rats by inducing heme oxygenase-1 expression

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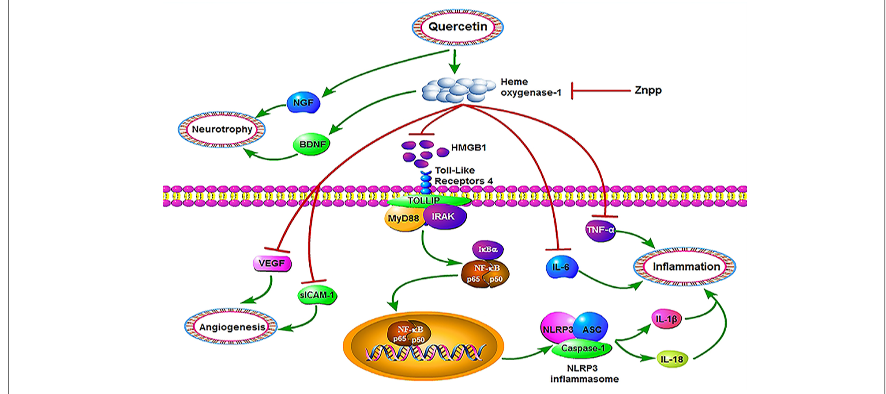
Graphical Abstract *Therapeutic role of quercetin in diabetic retinopathy is based on its anti-inflammatory, anti-angiogenesis and neurotrophic effects*



Abstract
Quercetin is a widely-occurring flavonoid that protects against cancer, and improves memory and cardiovascular functions. However, whether quercetin exhibits therapeutic effects in diabetic retinopathy remains unclear. In this study, we established a rat model of streptozocin-induced diabetic retinopathy. Seventy-two hours later, the rats were intraperitoneally administered 150 mg/kg quercetin for 16 successive weeks. Quercetin markedly increased the thickness of the retinal cell layer, increased the number of ganglion cells, and decreased the overexpression of the pro-inflammatory factors interleukin-1β, interleukin-18, interleukin-6 and tumor necrosis factor-α in the retinal tissue as well as the overexpression of high mobility group box-1 and the overactivation of the NLRP3 inflammasome. Furthermore, quercetin inhibited the overexpression of TLR4 and NF-κBp65, reduced the expression of the pro-angiogenic vascular endothelial growth factor and soluble intercellular adhesion molecule-1, and upregulated the neurotrophins brain-derived neurotrophic factor and nerve growth factor. Intraperitoneal injection of the heme oxygenase-1 inhibitor zinc protoporphyrin blocked the protective effect of quercetin. These findings suggest that quercetin exerts therapeutic effects in diabetic retinopathy possibly by inducing heme oxygenase-1 expression. This study was approved by the Animal Ethics Committee of China Medical University, China (approval No. 2016PS229K) on April 8, 2016.
Key Words: angiogenesis; diabetic retinopathy; flavonoid; heme oxygenase-1; inflammation; neurotrophin; quercetin; repair

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Introduction
Despite the continuous development of modern medicine, a substantial proportion (up to 8.8%) of the global population is still afflicted by diabetes mellitus (Ogurtsova et al., 2017). Diabetic retinopathy (DR), the most common vision-threatening complication of diabetes mellitus, severely impacts the patient's quality of life and is a great burden on society. Given that early intervention is key to successful treatment, novel preventive and early-stage therapeutic drugs with high efficacy are urgently needed.

The pathogenesis of DR is a complex pathophysiological process involving inflammation, neo-angiogenesis and apoptosis of retinal neurons (Martinez and Peplow, 2019; Catalani and Cervia, 2020). Previous work has revealed local inflammation and neovascularization as two major factors that influence the pathogenesis of DR (Rübsam et al., 2018; Mendonca et al., 2020). Sustained hyperglycemia can evoke a local low-grade inflammatory response and metabolic abnormalities in the very early stage of DR, which are regarded as key initiating events in the disease.

High mobility group box-1 (HMGB1), a conserved nucleoprotein, is an important initiating factor in chronic tissue inflammation. It is widely expressed by eukaryotic cells and has many functions, such as stabilizing nucleic acid structure and regulating transcription and gene expression (Yu et al., 2015). Our previous research showed that high glucose induces overexpression of HMGB1, and that inhibiting HMGB1 reduces damage to retinal cells in DR (Jiang and Chen, 2017). Other studies have reported an involvement of HMGB1 in the inflammatory response in DR, particularly on HMGB1

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receptor advanced glycation end products and Toll-like receptor 2/4 (TLR2/4) (Sohn et al., 2016). The NLR family pyrin domain containing 3 (NLRP3) inflammasome, a multi-protein scaffold composed of NLRP3, apoptosis associated speck like protein containing a card (ASC), and a caspase-1 precursor, has recently been identified as a downstream effector of HMGB1. After HMGB1 binds to TLR4, the activity of nuclear factor-kappaB (NF-κB) increases and induces the synthesis of the NLRP3 inflammasome. The NLRP3 inflammasome can sense metabolic abnormalities and promote the maturation of caspase-1, interleukin (IL)-1β and IL-18, which further aggravate inflammation (Kim et al., 2018).

Chronic inflammatory stimulation also perturbs vascular permeability, which may consequently induce microvascular occlusions and ischemic signaling (Kim et al., 2017). Vascular endothelial growth factor (VEGF) and soluble intercellular cell adhesion molecule-1 (sICAM-1) are two key pro-angiogenic factors in DR. An anti-VEGF agent was shown to be effective in DR treatment, but its high cost has impeded its widespread application (Zhao and Cheng, 2019). In addition, chronic inflammation also leads to a perturbation in the secretion of neurotrophins such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), which enhances neuronal apoptosis in the retina. Therefore, multifunctional and affordable drug interventions targeting these mechanisms should be ideal for the prevention and treatment of DR.

Quercetin, a commonly-occurring flavonoid with many biological activities, is widely distributed in plants. Quercetin is famous for its anti-oxidant properties (Cui et al., 2019). Recently, it has also been reported to have an anticancer action (Massi et al., 2017), and to improve memory (Babaei et al., 2018) and cardiovascular functions (Patel et al., 2018). Quercetin's effects may, in part, be attributed to its induction of heme oxygenase-1 (HO-1) (Liu et al., 2018). However, the role of quercetin in DR and the underlying mechanisms are still unclear. In this study, we investigate the therapeutic effect of quercetin and its mechanisms of action in DR.

Materials and Methods

Experimental animals

Forty healthy 8-week-old male Sprague-Dawley rats (specific-pathogen-free, weighing 200–220 g) were obtained from the Beijing Huafukang Biotechnology Co., Ltd., Beijing, China (license No. SCXK (Jing) 2019-008). Rats were all raised in the Research Center of Shengjing Hospital of China Medical University under standard conditions (room temperature: 18–22°C, humidity 50–60%). The experimental protocols were approved by the Animal Ethics Committee of China Medical University (approval No. 2016PS229K) on April 8, 2016. All experiments were designed and reported according to the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines.

Animal grouping and interventions

Forty rats were randomly divided into control, streptozocin (STZ), STZ + quercetin (Q), and STZ + Q + zinc protoporphyrin (Znpp) groups (n = 10 rats/group). Diabetes was induced by a single intraperitoneal injection of STZ (60 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) (Jiang and Chen, 2017). Rats were regarded as having undergone successful induction of diabetes when blood glucose (blood samples were extracted from the caudal vein) concentration was ≥ 16.7 mM at 72 hours after STZ injection (Jiang and Chen, 2017).

Rats with successful induction of diabetes at 72 hours after STZ injection were intragastrically administered quercetin (150 mg/kg; Sigma-Aldrich) at 9:00 a.m., once a day (Chen et al., 2017). An equal volume of saline (1 mL) was intragastrically administered to rats in the control and STZ groups. Znpp (30 mg/kg; Sigma-Aldrich) was intraperitoneally injected at 10:00 a.m. once every 2 weeks, starting 72 hours after STZ injection (Liu et al., 2018). An equal volume of saline (1 mL) was intraperitoneally injected in rats in the other three groups.

At 16 weeks after diabetes induction, all the rats were anesthetized with intraperitoneal pentobarbital (30 mg/kg; İ. E ULUGAY, Istanbul, Turkey), and their retinas were preserved for subsequent experiments. The right eyeballs were used for hematoxylin and eosin staining, while the left eyeballs were used for enzyme-linked immunosorbent assay (ELISA), real-time polymerase chain reaction assay and western blot analysis.

Histopathological analysis and detection of apoptosis

The right enucleated eyeballs were collected, fixed with 4% paraformaldehyde, and embedded with paraffin. Subsequently, the 3-μm sections were stained with hematoxylin and eosin for observation under a light microscope (Eclipse Ci, Nikon, Tokyo, Japan). Two independent observers were invited to examine the retinal tissue sections (blind method). NIS-Elements Basic Research Software (version 4.30, Nikon) was employed to capture five microphotographs for each tissue section at random (Sadikan et al., 2020), followed by analysis with ImageJ software (version 1.8.0, National Institutes of Health, Bethesda, MD, USA). Several parameters were used to assess the retinal changes, including the thickness of the inner nuclear layer (INL), outer nuclear layer (ONL) and space between the inner limiting membrane (ILM) and the outer limiting membrane (OLM), as well as cell count in the ganglion cell layer.

Apoptotic detection was conducted with a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit (*In Situ* Cell Death Detection Kit, Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. Using ImageJ software, we counted the TUNEL-positive cells in the INL and the ONL, as well as the total cell number in the tissue section. Then, the percentages of TUNEL-positive cells were calculated.

ELISA

The supernatant of the retinal homogenate was collected after centrifugation at 4270 × *g* for 20 minutes. IL-1β (Dakewe Biotech Co., Ltd., Shenzhen, China), IL-18 (Dakewe Biotech Co., Ltd.), tumor necrosis factor-α (TNF-α; Jiancheng Biotech Co., Ltd., Nanjing, China) and IL-6 (Jiancheng Biotech Co., Ltd.) were chosen as inflammatory markers in the retinas. VEGF (Boster Biological Technology Co. Ltd., Wuhan, China) and sICAM-1 (Ruishuo Biotechnology Co., Ltd., Shanghai, China) were selected as pro-angiogenic factors. BDNF (R&D Systems, Minneapolis, MN, USA) and NGF (R&D Systems) are neurotrophic cytokines. They were detected according to the corresponding ELISA kit instructions.

Real time polymerase chain reaction

Isolation of total RNA from the retinal samples was conducted with Trizol (Invitrogen, Carlsbad, CA, USA), and then reverse-transcribed into complementary DNA (Takara Bio, Tokyo, Japan) following the manufacturer's instructions. An Applied Biosystems 7500 Fast Real-Time polymerase chain reaction system was used to perform amplification with the following thermocycling parameters: 95°C for 30 seconds; 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds; and 95°C for 5 seconds. The primer sequences are shown in **Table 1**. Expression levels were normalized to the control.

Table 1 | Primer sequences

Gene	Primer sequences (5′–3′)
HMOX1	Forward: TTG AGC TGT TTG AGG AGC TG
	Reverse: TGG CGA AGA AAC TCT GTC TG
HMGB1	Forward: CGA ATG TGT CTT TAG CTA GCC CTG T
	Reverse: CAG ACT GTA CCA GGC AAG GTT AGT G
NLRP3	Forward: TCT GAC CTC TGT GCT CAA AAC CAA C
	Reverse: TGA GGT GAG GCT GCA GTT GTC TAA T
ASC	Forward: GCA CAG CCA GAA CAG AAC AT
	Reverse: AGCACATTGCCATACAGAGC
Caspase-1	Forward: TGCAGCACAGACTTTCAACA
	Reverse: CTGCAGCAGCAACTTCATTT
β-Actin	Forward: AGCGCAAGTACTCTGTGTGG
	Reverse: AACAGTCCGCCTAGAAGCAT

ASC: Apoptosis-associated speck like protein containing a card; HMGB1: high mobility group box-1; HMOX1: heme oxygenase 1; NLRP3: NLR family, pyrin domain containing 3.

Western blot analysis

Total protein extracted from pulverized retinal samples was mixed with lysis buffer (radioimmunoprecipitation assay buffer containing 1% phenylmethylsulfonyl fluoride). The concentration of the extracted protein was quantified with the bicinchoninic acid protein quantification kit (Beyotime Biotechnology, Shanghai, China). The protein samples were denatured by heating, electrophoresed on 10 or 12% SDS-PAGE gels, and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk, and then incubated with primary antibodies (anti-HO-1 (1:500; rabbit monoclonal; Cat# ab189491, Abcam, Cambridge, UK), anti-NLRP3 (1:500; rabbit monoclonal; Cat# ab263899, Abcam), anti-TLR4 (1:500; rabbit polyclonal; Cat# ab13867, Abcam), anti-ASC (1:500; rabbit polyclonal; Cat# bs-6741R, Bioss, Beijing, China) or anti-caspase-1 (1:1000; rabbit polyclonal; Cat# bs-0169R, Bioss)) at 4°C overnight. Then, the blots were incubated with secondary antibodies labeled with horseradish peroxidase (1:10,000; ZSGB-BIO, Beijing, China), and thereafter subjected to chemiluminescent detection. Signal intensities were analyzed using ImageJ software and normalized to the normal control.

NF-κB transcription factor assay

Nuclear proteins were extracted from retinal tissue using the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA). The DNA binding activity of NF-κB (p65) was measured using the ELISA-based TransAM NF-κB Kit (Active Motif). Finally, the optical density was read at 450 nm wavelength and normalized to the normal control group.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software Inc., Carlsbad, CA, USA). For the analysis of mRNA and protein expression levels, two-way analysis of variance followed by Bonferroni's multiple comparison test was performed. One-way analysis of variance and the Kruskal-Wallis test were used for the other multiple comparisons. P < 0.05 was considered statistically significant.

Results

Quercetin markedly improves the histopathological changes in the retina in STZ-treated diabetic rats in a HO-1-dependent manner

In the microscopic observation of retinal sections stained with hematoxylin and eosin, those in the normal control group exhibited a normal retinal thickness as well as an ordered arrangement of retinal cells with a uniform distribution. Rats in the STZ-treated group showed pathological features of DR at 16 weeks after STZ-induced diabetes, including a marked decrease in the thickness of the INL and ONL, a reduction in the distance between the ILM and OLM, and a decrease in the number of ganglion cells in the ganglion cell layer (**Figure 1A**). These retinal histopathological changes were partially ameliorated by treatment with quercetin. Compared with the STZ group, the thicknesses of the INL (P < 0.01) and the ONL (P < 0.01), and the distance between the ILM and OLM (P < 0.01) were significantly increased in the retinas of the STZ + Q group (**Figure 1B–D**). Moreover, the reduction in ganglion cells was ameliorated by quercetin treatment compared with the STZ group (P < 0.01; **Figure 1E**). Downregulating HO-1 expression by intraperitoneal injection of Znpp partially blocked the neuroprotective effect of quercetin in the diabetic retina (**Figure 1A–E**). No significant differences were observed among the STZ, STZ + Q and STZ + Q + Znpp groups in blood glucose levels (**Figure 1F**). TUNEL staining showed that treatment with quercetin significantly decreased the percentage of apoptotic cells, and this effect of quercetin was weakened by Znpp (P < 0.01; **Figure 1G** and **I**). A large reduction in cell numbers was detected in the INL and ONL in the STZ group compared with the control group. Quercetin treatment markedly increased the cell numbers in these layers in these rats with DR (P < 0.01; **Figure 1H**).

Downregulation of HO-1 prevents the inhibition of pro-inflammatory cytokine secretion by quercetin in the rat retina

Because inflammation is an important part of the pathogenesis of DR (Rübsam et al., 2018), the influence of quercetin on pro-inflammatory factors was investigated to clarify the mechanisms underlying the retinal protection provided by the flavonoid. In comparison with the control group, levels of pro-inflammatory cytokines (IL-1β, IL-18, IL-6 and TNF-α) were remarkably increased in the STZ-induced diabetic rats. Treatment with quercetin significantly decreased the levels of pro-inflammatory factors in retinal tissue (**Figure 2A–D**). Synchronous downregulation of HO-1 blocked the reduction of IL-1β, IL-18 and TNF-α caused by quercetin (P < 0.05; **Figure 2A**, **B** and **D**). The slight difference in IL-6 between the STZ + Q and STZ + Q + Znpp groups was not statistically significant (**Figure 2C**).

Quercetin inhibits the expression of HMGB1 and NLRP3 inflammasome-related proteins by inducing expression of HO-1 in retinas of rats with STZ-induced diabetes

To gain insight into the mechanisms underlying quercetin's anti-inflammatory effect, we measured the mRNA and protein expression levels of HO-1 and several NLRP3 inflammasome-related factors. As shown in **Figure 3**, quercetin dramatically upregulated the expression of HO-1 in the retina, while intraperitoneal injection of Znpp effectively suppressed the induction of HO-1 by quercetin. In addition, STZ-treated rats showed significantly elevated retinal expression levels of HMGB1 and components of the NLRP3 inflammasome. Quercetin treatment effectively reduced the overexpression of HMGB1 and the hyperactivation of the NLRP3 inflammasome, which was reversed by Znpp. The mRNA and protein expression levels of HMGB1 and components of the NLRP3 inflammasome were increased in the STZ + Q + Znpp group compared with the STZ + Q group.

Quercetin suppresses the overexpression of TLR4 and the overactivation of NF-κB p65 caused by sustained hyperglycemia in a HO-1-dependent manner

We next sought to clarify the relationship between the reduction of HMGB1 and NLRP3 activation caused by

quercetin. Expression of TLR4, one of the major receptors of HMGB1 (Yu et al., 2015), was measured in the retina. As shown in **Figure 4A**, TLR4 levels were robustly increased in the STZ group compared with the control group (P < 0.05). Quercetin strongly suppressed the excessive expression of TLR4, and this effect of the flavonoid was diminished by simultaneous downregulation of HO-1 (**Figure 4A**). NF-κB acts downstream of HMGB1/TLR4 and plays an important role in the priming of the synthesis of NLRP3 inflammasome components (Dong et al., 2018). Consistent with our conjecture, rats treated with quercetin showed a reduction in NF-κB p65 activity in the retina compared with the STZ group (P < 0.05; **Figure 4B**). Similar to TLR4, NF-κB p65 activity was increased when HO-1 expression was inhibited (i.e., in the STZ + Q + Znpp group compared with the STZ + Q group; P < 0.05; **Figure 4B**).

Quercetin affects the expression of pro-angiogenic cytokines and neurotrophic factors in DR rats

In addition to the inflammation-related factors, we also detected pro-angiogenic cytokines and neurotrophic factors to further investigate the protective mechanism of quercetin in DR. VEGF and sICAM-1 are two characteristic pro-angiogenic factors in DR (Chen et al., 2017). We observed a notable increase in VEGF and sICAM-1 levels in the STZ group compared with the control group. Quercetin treatment inhibited the expression of VEGF and sICAM-1 in the retinas of STZ-induced diabetic rats (P < 0.05; **Figure 5A** and **B**). In the histopathological analysis of retinal tissue, we found that the loss of ganglion cells was significantly improved by quercetin (P < 0.01; **Figure 1E**). Considering that some neurotrophic factors are critical for the protection of retinal ganglion cells, we also measured the levels of the neurotrophins BDNF and NGF to explore the impact of quercetin on neuroprotection. Compared with the STZ group, quercetin upregulated BDNF and NGF in diabetic rats (P < 0.05; **Figure 5C** and **D**). Moreover, the upregulation of BDNF by quercetin was HO-1-dependent (P < 0.05; **Figure 5C**). However, the quercetin-induced increase in NGF was not affected by downregulation of HO-1 (P > 0.05; **Figure 5D**).

Discussion

DR is a common microvascular complication of diabetes mellitus. The pathogenesis of DR involves inflammation, neo-angiogenesis, and the irreversible death of retinal neurons. Currently, targeted therapeutic methods remain inadequate (Rübsam et al., 2018). In our present study, we investigated the protective effect of quercetin on DR. Quercetin alleviated the hyperglycemia-induced histopathological changes in the retinas of STZ-induced diabetic rats. Furthermore, we found that quercetin's retinal protection may be attributed to its inhibition of the HMGB1/TLR4/NF-κB/NLRP3 inflammasome/IL-1β/IL-18 axis, suppression of pro-angiogenic VEGF and sICAM-1 secretion, and upregulation of BDNF via the induction of HO-1. Our findings highlight the therapeutic potential of quercetin in DR based on its anti-inflammatory, anti-angiogenic and neurotrophic effects.

Quercetin, one of the most prominent members of the natural flavonoid family, has been reported to be beneficial in age-related ophthalmopathy (Bungau et al., 2019) and retinal ischemia-reperfusion injury (Arikan et al., 2015). Most previous studies mainly focused on its anti-oxidant properties. Thus, quercetin's protective effect on retinopathy was mainly attributed to its anti-oxidative, anti-inflammatory and anti-apoptotic effects in retinal cells (Bungau et al., 2019). Few recent studies have described quercetin's anti-angiogenic (Lupo et al., 2019) or neuroprotective effects (Ola et al., 2017). Furthermore, the mechanisms underlying the neuroprotective action remained unclear. In accordance with previous studies, we also found a protective effect of quercetin in the retinas of STZ-induced diabetic rats. The morphological observation of retinal tissues showed that quercetin partially ameliorated the chronic hyperglycemia-induced histological alterations. The high-glucose exposure accelerates the loss of retinal cells, thereby decreasing the thickness of the INL and ONL and the number of ganglion cells in the ganglion cell layer. These characteristic changes could be seen in retinas of the STZ group in our study. Treatment with quercetin significantly protected against the retinal changes caused by diabetes. It is noteworthy that concurrent inhibition of HO-1 dramatically diminished the protective effect of quercetin. These results hint at a pivotal role of HO-1 in quercetin-mediated neuroprotection in DR.

Recently, several studies showed that upregulation of the Nrf2/HO-1 axis protected against inflammation and oxidative stress in DR (Wang et al., 2020). Indeed, the Nrf2/HO-1 axis is an important component of the self-defense system against oxidative stress. Our previous study showed that HO-1 plays a critical role in quercetin-mediated protection against acute alcoholic liver injury (Liu et al., 2018). However, the link between quercetin and HO-1 in DR remained unclear. In the present study, we found that downregulation of HO-1 significantly reduced the retinal neuroprotection exerted by quercetin in DR—retinas in the STZ + Q + Znpp group showed a reduced thickness of the retinal cell layers and greater loss of ganglion cells.

Because HO-1 is a well-known anti-oxidative enzyme, it is not surprising that it may protect against oxidative stress in DR. Therefore, we investigated several other pathways to further explore the mechanisms of quercetin-mediated neuroprotection in DR. In recent years, the chronic tissue inflammatory response has been regarded as an important cause of DR. Tissue damage-induced HMGB1 release and NLRP3 inflammasome activation have been identified as critical to the immune inflammatory response. Quercetin was reported to be able to attenuate the expression of HMGB1 in sepsis (Cui et al., 2019), tumors and myocardial ischemia-reperfusion injury (Dong et al., 2018). However, their relationship is still unclear in DR. Similar to what we found here, many *in vitro* and *in vivo* studies have reported elevated levels of HMGB1 in DR. Compared with nondiabetic patients, patients with proliferative DR had much higher levels of HMGB1, especially those with retinal hemorrhage (El-Asrar et al., 2011). Overexpressed HMGB1 seemed to be associated with activating NF-κB, inducing breakdown of the blood–retinal barrier (Mohammad et al., 2019), and evoking oxidative stress (Abu El-Asrar et al., 2017). Our previous study showed that inhibiting HMGB1 with siRNA protected retinal cells from apoptosis and reduced tissue damage caused by hyperglycemia (Jiang and Chen, 2017). The priming role of HMGB1 in inflammation has been well established in many diseases. In the field of hyperglycemia-induced injury, Hu et al. observed tandem increases in HMGB1, TLR4, IL-1β and IL-18 in ganglion cells treated with different concentrations of glucose (Hu et al., 2017). These findings are in accordance with our results in the STZ group. In addition, HMGB1 also triggered the release of IL-1β in retinal endothelial cells and vascular smooth muscle cells (Kim et al., 2018). Consistent with these observations, we found similar changes in HMGB1 and inflammatory cytokines such as IL-1β, IL-18, TNF-α and IL-6. HMGB1-mediated activation of the NLRP3 inflammasome in the retina may underpin the heightened inflammatory response. This is supported by the fact that the mRNA and

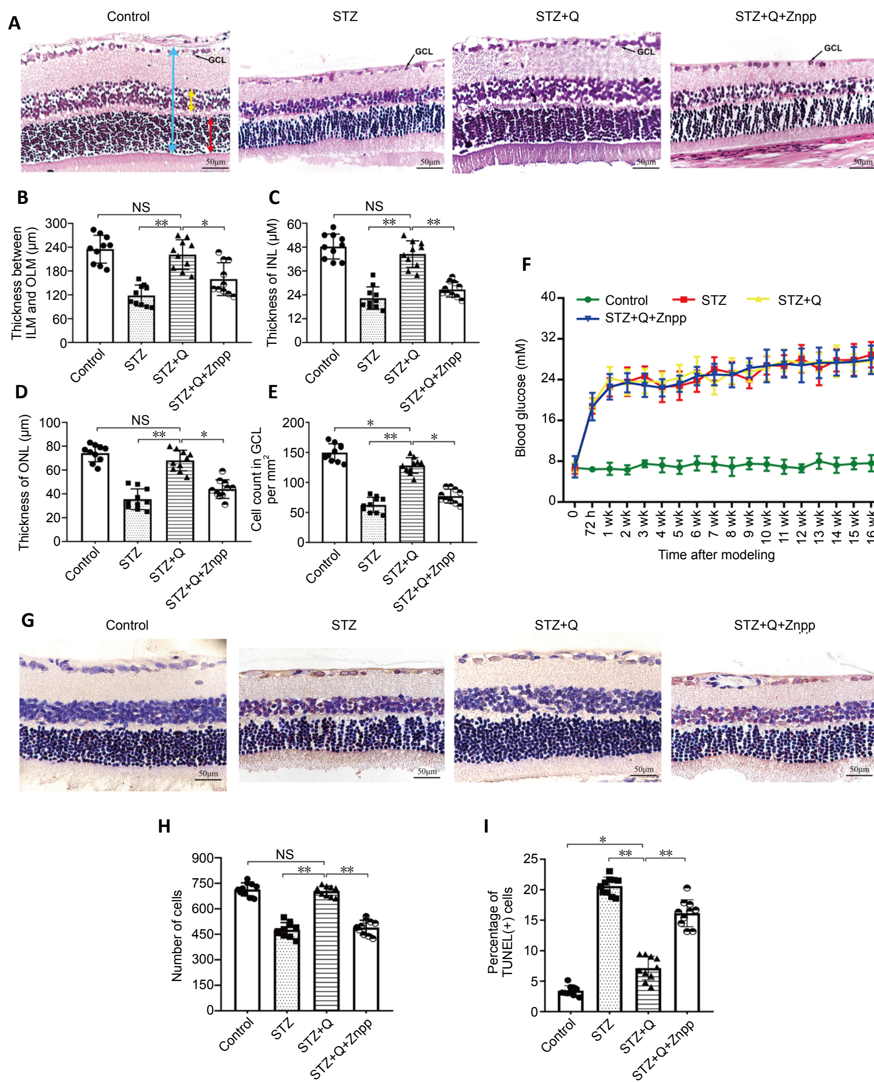

Figure 1 | Quercetin effectively alleviates the retinal histopathological alterations induced by STZ in diabetic rats.

(A) Histopathological observations of retinas (hematoxylin and eosin staining, original magnification 40×, scale bars: 50 μm). Normal control group exhibited normal thickness of retinas as well as an ordered arrangement of retinal cells with a uniform distribution. Rats in the STZ-treated group showed pathological features of DR at 16 weeks after STZ-induced diabetes, including marked reductions in the thickness of the INL and ONL, the distance between the ILM and OLM, as well as in the number of ganglion cells. These retinal changes were partially ameliorated by treatment with quercetin. Downregulating HO-1 expression by Znpp partially reversed the neuroprotective effect of quercetin in DR. (B) Quantitative analysis of the distance between the ILM and OLM (marked by blue arrow in A). (C) Quantitative analysis of the thickness of the INL (marked by yellow arrow in A). (D) Quantitative analysis of the thickness of the ONL (marked by red arrow in A). (E) Quantitative analysis of the number of cells in the GCL. (F) Blood glucose levels. (G) TUNEL staining of retinal sections (original magnification 40×, scale bars: 50 μm). The STZ-treated group showed more TUNEL-positive cells compared with the control group. Treatment with quercetin significantly decreased the percentage of apoptotic cells, and this effect was weakened by Znpp. (H) Quantitative analysis of total cell numbers in the whole section. (I) Quantitative analysis of the percentage of TUNEL-positive cells in the whole section. Data are expressed as the mean ± SD (n = 10). *P < 0.05, **P < 0.01 (one-way analysis of variance followed by the Kruskal-Wallis test). GCL: Ganglion cell layer; ILM: inner limiting membrane; ILN: inner nuclear layer; NS: not significant; OLM: outer limiting membrane; ONL: outer nuclear layer; Q: quercetin; STZ: streptozocin; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; Znpp: zinc protoporphyrin IX.

protein levels of NLRP3, ASC and caspase-1 decreased when quercetin downregulated HMGB1. Based on the parallel changes in HMGB1, TLR4, NF-κB and the NLRP3 inflammasome, we surmise that HMGB1 binds to TLR4, increases the activity of NF-κB p65, and upregulates the NLRP3 inflammasome and pro-inflammatory cytokines. In the present study, our *in vivo* experiments showed that quercetin effectively reduces the upregulation of the HMGB1/TLR4/NF-κB/NLRP3 inflammasome/IL-1β/IL-18 axis in a HO-1-dependent manner.

In regard to its anti-angiogenic action, quercetin was shown to decrease the expression of VEGF in some *in vitro* studies (Lupo et al., 2019). A previous study found that quercetin decreased levels of matrix metalloproteinase-9 and VEGF in the DR retina (Chen et al., 2017). Our results further show that quercetin suppresses the secretion of VEGF and sICAM-1 in a HO-1-dependent manner. In addition, HMGB1 was reported to be associated with VEGF expression in gastric cancer, breast cancer, and chronic cerebral ischemia (Da et al., 2019). Kim et al. (Kim et al., 2017) suggested that HMGB1 upregulates VEGF signaling as well as angiogenic factors in endothelial cells treated with HMGB1. However, whether quercetin's inhibition of VEGF is related to its inhibition of HMGB1 still needs to be elucidated.

Here, we also investigated the potential neuroprotective effect of quercetin, and we observed that quercetin successfully attenuated the loss of ganglion cells. Ola et al. (2017) reported that quercetin promotes the secretion of neurotrophic factors such as BDNF and NGF in DR. However, the underlying mechanisms remained unclear. Our current results are in agreement with those of Ola et al. (2017), who found elevated levels of BDNF and NGF in rats treated with quercetin. Furthermore, we show that quercetin stimulates the expression of BDNF in a HO-1-dependent fashion. In addition, the upregulation of NGF induced by quercetin was not significantly affected by HO-1 inhibition. This may be partially explained by Sun et al.'s (Sun et al., 2017) findings that NGF is an upstream regulator of HO-1. Further studies are needed to investigate their relationship in DR.

In summary, we identified a neuroprotective effect of quercetin against DR. The underlying mechanism involves the inhibition of the HMGB1/TLR4/NF-κB/NLRP3 inflammasome/IL-1β/IL-18 axis, the suppression of VEGF and sICAM-1 secretion, and the promotion of BDNF secretion via the upregulation of HO-1.

Author contributions: *Study design and manuscript preparation: GRC, SL; experiment implementation: GRC, HWY; manuscript revision: XLC. All authors read and approved the final manuscript.*

Conflicts of interest: *The authors declare that they have no conflict of interest.*

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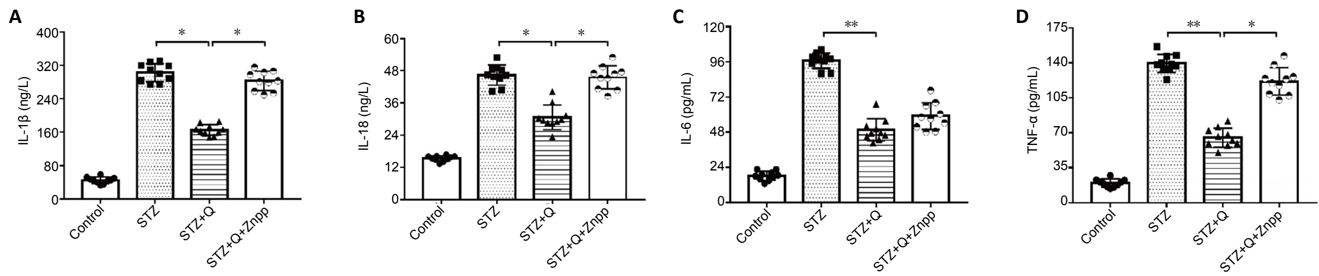

Figure 2 | Quercetin reduces pro-inflammatory cytokine levels in the retina of a STZ-induced diabetic rat.
(A–D) IL-1β (A), IL-18 (B), IL-6 (C), and TNF-α (D). Data are expressed as the mean ± SD (n = 10). *P < 0.05, **P < 0.01 (one-way analysis of variance followed by the Kruskal-Wallis test for multiple comparison). IL: Interleukin; Q; quercetin; STZ; streptozocin; TNF-α: tumor necrosis factor α; Znpp: zinc protoporphyrin IX.

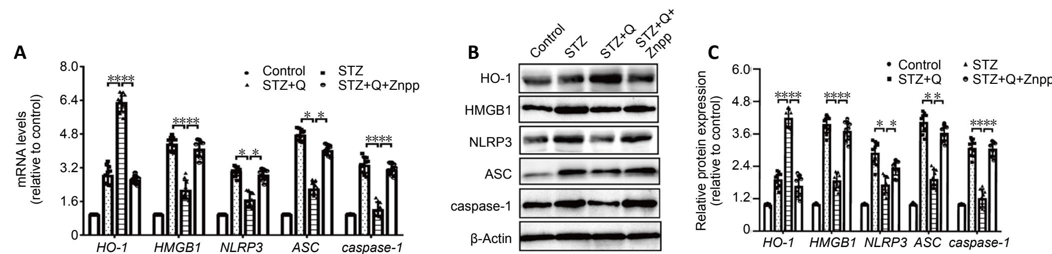

Figure 3 | Quercetin induces HO-1 expression, and inhibits HMGB1 expression and the activation of the NLRP3 inflammasome in the retina of STZ-induced diabetic rats.
(A) mRNA expression levels of *HO-1*, *HMGB1*, *NLRP3*, *ASC* and   in the retina detected by real-time polymerase chain reaction. (B, C) Western blot analysis of HO-1, HMGB1 and NLRP3 inflammasome-related protein expression in the retina. Data are expressed as the mean ± SD (n = 10). *P < 0.05, **P < 0.01 (two-way analysis of variance followed by the Bonferroni multiple comparison test). ASC: Apoptosis associated speck-like protein containing a card; HMGB1: high-mobility group box 1; HO-1: heme oxygenase-1; NLRP3: NLR family, pyrin domain containing 3; Q: quercetin; STZ: streptozocin; Znpp: zinc protoporphyrin IX.

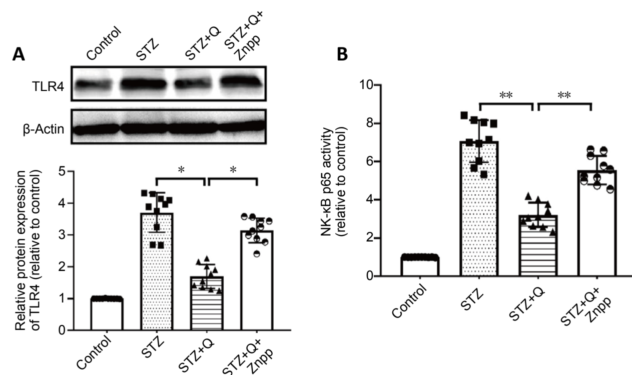

Figure 4 | Quercetin suppresses the overexpression of TLR4 and the overactivation of NF-κB p65 induced by sustained hyperglycemia in a HO-1-dependent manner.
(A) Western blot assay of TLR4 expression in retinal tissue. (B) Determination of NF-κB p65 activity in retinal tissue. Data are expressed as the mean ± SD (n = 10). *P < 0.05, **P < 0.01 (one-way analysis of variance followed by the Kruskal-Wallis test for multiple comparison). NF-κB: Nuclear factor-kappaB Q: quercetin; STZ: streptozocin; TLR4: Toll-like receptor 4; Znpp: zinc protoporphyrin IX.

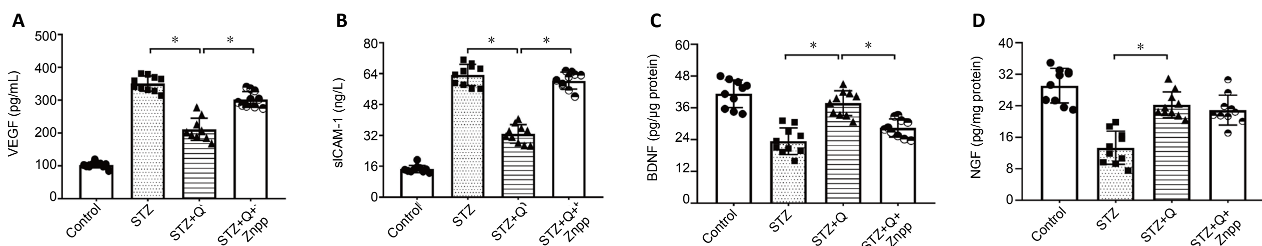

Figure 5 | Effects of quercetin on the expression of pro-angiogenic cytokines and neurotrophic factors in diabetic retinopathy detected by enzyme-linked immunosorbent assay.
(A, B) Pro-angiogenic cytokines VEGF (A) and sICAM-1 (B) in retinal tissue. (C, D) Neurotrophic factors BDNF (C) and NGF (D) in retinal tissue. Data are expressed as the mean ± SD (n = 10). *P < 0.05, **P < 0.01 (one-way analysis of variance followed by the Kruskal-Wallis test for multiple comparisons). BDNF: Brain-derived neurotrophic factor; NGF: nerve growth factor; Q: quercetin; sICAM-1: soluble intercellular adhesion molecule-1; STZ: streptozocin; VEGF: vascular endothelial growth factor; Znpp: zinc protoporphyrin IX.

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