Influence of Heme Oxygenase-1 on rats with diabetic retinopathy through ERK1/2 signaling pathway

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ABSTRACT

The study aimed to investigate the influence of heme oxygenase-1 (HO-1) on the rats with diabetic retinopathy (DR) through the extracellular signal-regulated kinase (ERK) 1/2 signaling pathway. 40 rats were selected and divided into Control group (n=10), diabetes mellitus (DM) group (n=10), cobalt protoporphyrin (CoPP) group (n=10) and zinc protoporphyrin (ZnPP) group (n=10) according to weight. Streptozotocin (STZ) was intraperitoneally injected to establish the DM model in DM, CoPP and ZnPP groups, and CoPP and ZnPP solution was intraperitoneally injected in CoPP and ZnPP groups, respectively. Blood was drawn to determine fasting blood glucose. The changes in the protein and messenger ribonucleic acid (mRNA) levels were evaluated via Western blotting and polymerase chain reaction (qRT-PCR), respectively. Enzyme-linked immunosorbent assay (ELISA) was performed to measure antioxidant capacity and the levels of total reactive oxygen species (ROS),malondialdehyde (MDA),glutathione (GSH) and glutathione peroxidase (GPx). The weight of rats was notably higherin CoPP groupand lower inZnPP groupthan that in DM group ($p<0.05$). After induction of DM, compared with those in DM group, the protein expression levels of Nrf2 and pERK were considerably elevated in CoPP group ($p<0.05$), but declined remarkably in ZnPP group ($p<0.05$). The levels of total ROS and MDA were notably elevated ($p<0.05$) in DM and ZnPP groups, a lowered level of GPx and distinctlyelevated levels of MDA and total ROS ($p<0.05$). Moreover, the mRNA expression level of HO-1 in the retinas of rats was remarkably raised in DM group and CoPP group ($p<0.05$), but it declined markedly in ZnPP group ($p<0.05$). The red fluorescent aggregation of Nrf2 and pERK proteins was overtlyless in ZnPP group than that in DM group ($p<0.05$). HO-1 can affect the level of oxidative stress and intervene in retinopathy in DM rats through the Nrf2/ERK pathway.

Introduction

Diabetes mellitus (DM), a chronic metabolic disorder, is typically characterized by obvious increases in blood lipid and glucose and abnormality of glucose metabolism in bodies (1, 2) and occurs mainlybecause islet cells are destroyed, leadingtoaberrant secretion of insulin (3, 4). According to the statistics, the morbidity rate of DM is increasing annually (5). Diabetic retinopathy (DR) is attributed to the long-term rise in blood glucose, which is one of the most common diabetic eye diseases worldwide (6). The persistently high level of blood glucose in the body will undermine and impair small vessels in the retinas, resulting in diabetic macular edema, vitreous hemorrhage, retinal detachment and neovascular glaucoma (7). If not treated promptly, retinopathy will cause blindness to DM patients (8). Not only doesDRimpactpatients' quality of life, but it also has severe mental effects on patientsbecause of the limited autonomy,mobility and activities of daily living(9).

Heme oxygenase (HO) is a rate-limiting enzyme that mainly functions as the catalyzer of heme metabolism and plays a crucial role in mitochondrial function and oxidative stress (10). While red blood cells (RBC) are destruc-ted, hemes are released from hemoglobins and metabolized by HO (11). HO comprises two isozymes, namely HO-1 and HO-2. The expression of HO-1, an inducible antioxidant enzyme, can be induced by many stress factors, such as inflammation, hypoxia, oxidative stress and infection (12). HO-1 can decompose hemes into bilirubin, Fe and carbon monoxide (CO). Excessive hemes do harms to cells for the great effects of Fe-induced oxidation-promoting reactions on cells (13). Oxidative stress plays a key role in the pathogenesis of microvascular and macrovascular DMcomplications (14). Studies have demonstrated that the production of the signaling molecules reactive oxygen species (ROS),nitric oxide and CO is regulated by redox at the transcriptional level and associated with cellular signal transduction. CO, as the end product of cellular metabolism in cells and tissues, mainly comes from heme degradation catalyzed by HO-1.

Although HO-1 is related to various diseases in organisms, there are rare study reports on its influence on retinopathy in DM rats. By now, the exact mechanism of DR damaging retinal microvessels remains unclear. The present research intervened in HO-1 to investigate the influence of theextracellular signal-regulated kinase (ERK)1/2 signaling pathway on DR, thereby providing

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a certain experimental basis for the treatment of DM induced-retinopathy.

Materials and Methods

Laboratory animals and main reagents and instruments

A total of 40 healthy male Wistar rats weighing 200-220 g [Shanghai Super B&K Laboratory Animal Co., Ltd., certificate No.: SYXK (Shanghai)2008-0050] were adaptively fed in a suitable environment at $22\pm2°C$ in a 12/12 h light/dark cycle and had free access to food and water. Then they were randomly divided into blank control group (Control group, n=10), DM group (n=10), cobalt protoporphyrin (CoPP) group (n=10) and zinc protoporphyrin (ZnPP) group (n=10), and 1% streptozotocin (STZ) solution was prepared using sodium citrate buffer. After fasting for 12 h, the rats in DM, CoPP and ZnPP groups were intraperitoneally injected withSTZ at 60 mg/kg to establish the DM model.At 48 h after injection of STZ, blood was sampled from the tails of the rats, and the level of blood glucose was determined using a blood glucose meter. The rats with blood glucose >300 mg/L were considered to have DM and were employed for subsequent research. An equal volume of sodium citrate buffer was intraperitoneally injected in Control group. At 1 d after modeling, the rats in CoPP and ZnPP groups started to be administered with CoPP (5 mg/kg) and ZnPP (5 mg/kg), respectively, through intraperitoneal injection every other day, while those in Control and DM groups were intraperitoneally injected with the same volume of normal saline. After 12 weeks, venous blood was drawn from the tails of rats in each group to measure fasting blood glucose.

STZ (Sigma, USA), HO-1, nuclear factor erythroid 2-related factor 2 (Nrf2) and ERK monoclonal antibodies (Santa Cruz Biotechnology, Inc., USA), HO-1 activator CoPP and HO-1 inhibitor ZnPP (Sigma, USA), ribonucleic acid (RNA) extraction kit (Shanghai Sangon Biotech Co., Ltd.),confocal microscopeand polymerase chain reaction (PCR) amplification instrument.

Enzyme-linked immunosorbent assay (ELISA)

The retinas of rats were homogenized by the method in 1.2, and centrifuged at 2,000 rpm and 4°C for 10 min, and the supernatant was collected. The ELISA was performed according to the instructions of the kit. With the standard well, sample well and blank control well set, 50 μL of standard at a different concentration were added into the standard well, 10 μL of samples and 40 μL of sample diluent (namely the samples were diluted by 5 folds) into the sample well, and no reagent into the blank control well. Subsequently, the standard and sample wells were added with 100 μL of horseradish peroxidase (HRP)-labeled antibodies, and the plate was sealed for incubation at 37°C for 60 min. With fluid discarded, 300 μL of wash buffer was added, and the mixture was let stand for 2 min and patted dry using absorbent paper. After repeating for 5 times, incubation was conducted with the color development solution A (50 μL) and B (50 μL) in the dark for 15 min, followed by addition of 50 μL of reaction stop solution. Finally, the optical density (OD) value in each well was determined at 450 nm using a microplate reader. Standard and sample wells were set in triplicate, and the experiment was repeated for three times for statistical

analysis, with the levels of cytokines expressed as ng/mL.

Total RNA extraction and fluorescence quantitative PCR

Total RNA was extracted from the retinal tissues of rats using TRIzol reagent. Then the concentration and purity of the total RNAswere measuredusing an ultramicrospectrophotometer based on the ratio of absorbance (A)260/A280. Following measurement, the RNA samples were reversely transcribed into complementary deoxyribonucleic acid (cDNA). Quantitative reverse transcription (qRT)-PCR was performed using AceQ qPCR SYBR Green Master Mix kit under the following amplification conditions: 95°C for 5 min, 95°C for 10 s, 60°C for 30 s and 72°C for 1 min for 40 cycles in total, and 95°C for 10 min. The fluorescence quantitative PCR system comprised 5 μL of PCR Master Mix (2×), 1 μL of PCR reverse primer, 1 μL of cDNA, and 12 μL of ddH$_2$O. Finally, the results were obtainedbycalculating the value of $2^{-\Delta\Delta Ct}$. (Table I)

Immunofluorescence assay

After the rats were sacrificed, the retinal tissues were taken out and prepared into frozen sections. The sections were let stand at normal temperature for 15 min, washed using phosphate buffered saline (PBS) for 3 times (3 min/time), treated with 0.3% Triton for 10 min, and washed again with PBS for 3 times (3 min/time). Subsequently, the resulting sections were blocked using goat serum at 37°C for 45 min, washed using PBS for 3 min, incubated with thediluted corresponding primary antibodies at 4°C overnight. After being washed with PBS for 3 times (3 min/time), the sections were incubated with thediluted corresponding secondary antibodies at 37°C for 1h and washed as above, followed by staining of cell nuclei with DAPI, PBS washing for 3 times (3 min/time), and sealing in 3% glycerol. Finally, the sections were observed under the laser scanning confocal microscope, and images were acquired.

Western blotting

The rats were killed to obtain the retinas, and then they were washed with PBS twice, ground in a pre-cooled mortar with liquid nitrogen, added with lysis buffer and let stand on ice for 1 h, followed by ultrasonic extraction of total protein. Subsequently, the total proteins extracted were centrifuged at 13,000 rpm for 10 min for the supernatant, and the protein concentration therein was determined by the BCA assay method. An equal amount of protein sample (30 μg) was loaded for 12% SDS-PAGE, and then the gels were transferred onto a 0.45 μm PVDF membrane. The membrane was blocked using 3% bovine serum albumin, washed using PBS with Tween 20 (PBST) twice (5 min/time), incubated with the corresponding primary antibodies diluted by the antibody diluent at 4°C overnight,

Table I. QRT-PCR primer sequences.

Primer	Sequence
HO-1	5'-TGCACATCCGTGCAGAGAAT-3'
	5'-CTGGTTCTGCTTGTTTCGC-3'
Nrf2	5'-CCGCCCTCCATATGATGGACTTGGA-3'
	5'-CTAAGAAATTAACTCGAGAGTTAAA-3'
GAPDH	5'-AGGTCGGTGTGAACGGATTTG-3'
	5'-TGTAGACCATGTAGTTGAGGTCA-3'

washed again with PBST for 3 times (5 min/time), and incubated with the HRP labeled-secondary antibodies diluted by the antibody diluent at 37°C for 1 h. Finally, the ECL fluorescence color development solution was added dropwise, andcolor development and quantification were conducted inthe ChemiDoc MP gel imaging system.

Statistical analysis

All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation ($\chi\pm s$). SPSS 16.0 software was adopted for statistical analysis of data. The results of intergroup comparisonswere represented as $\chi\pm s$,andthe intergroup comparisons were made via one-way analysis of variance. $p<0.05$ denoted statistically significant differences.
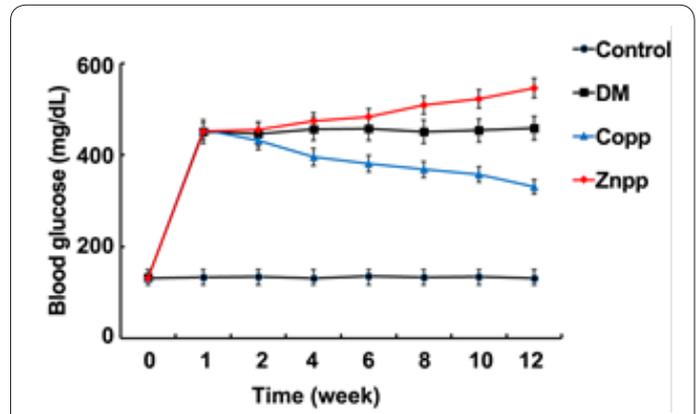
Results

Blood glucose and weight of rats

As shownin Figure 1, compared with those in Control group,the rats in DM, CoPP and ZnPP groups displayeda substantially raised level of blood glucose ($p<0.05$). However, at 12 weeks, the level of blood glucose in rats in CoPP group was notably lower than that in DM group ($p<0.05$), and the level of blood glucose in ZnPP group was markedly higher than that in DM group ($p<0.05$). At the beginning of the present study, the weight of rats in each group was similar (Figure 2), but in comparison with that in Control group, the final weight of rats in DM group declined by 49.39% ($p<0.05$). Besides,the weight of rats in CoPP group was overtlyhigher than that in DM group ($p<0.05$) and it was distinctlylower in ZnPP group than that in DM group ($p<0.05$).

Protein expressions of HO-1, Nrf2, total ERK (tERK) and phosphorylated ERK (pERK) in the retinal tissues of DM rats

It was found through the Western blotting that almost no endogenous HO-1 was expressed in the retinas of normal rats (Figure 3). At 2 weeks after induction of DM, its expression was observed, and the expression level reached the peak value at 4 and 12 weeks. The expression level of Nrf2 started to rise in the rat retinas immediately after induction of DM, and the peak value was detected at 4 and 12 weeks. Meanwhile, there was no obvious difference in the level of tERK in retinas, but compared with that in Control group, the level of pERK rose dramatically in the retinas of rats immediately after induction of DM and reached the peak value at 4 weeks. (Table II)

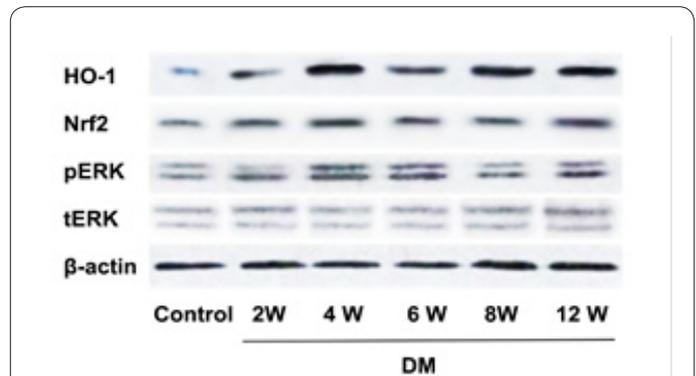### Impacts of CoPP and ZnPP on the oxidative stress in the retinas of DM rats

According to the ELISA results (Figure 4), compared with those in Control group, the total antioxidant capa-

Figure 1. Changes in blood glucose of rats in each group after induction of DM.
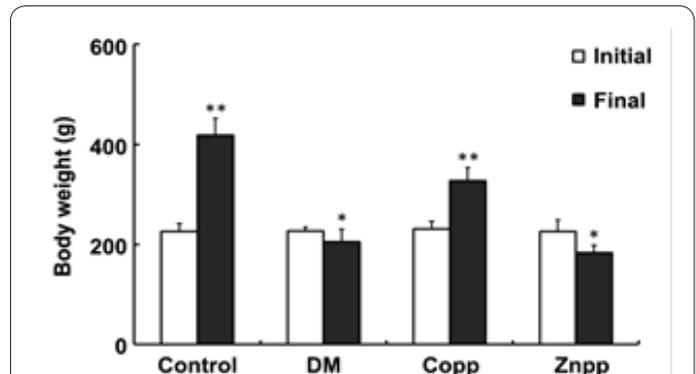
Figure 2. Changes in the weight of rats in each group after induction of DM.


Figure 3. Protein expressions of HO-1, Nrf2, tERK and pERK after induction of DM.

city and the levels of glutathione (GSH) and glutathione peroxidase (GPx) declined considerably ($p<0.05$), and those of total reactive oxygen species (ROS) and malondialdehyde (MDA) were notably elevated ($p<0.05$) in DM and ZnPP groups, while CoPP group exhibited no marked differences in the total antioxidant capacity and GSH level ($p>0.05$), a dramatically lowered level of GPx and subs-

Table II. Protein expressions of HO-1, Nrf2, pERK and tERK at different time points.

Protein	OD	Control	2 w	4 w	6 w	8 w	12 w
HO-1	Target protein/β-actin	0.10±0.24	1.46±0.44*	5.83±0.22*	2.18±0.55*	3.61±0.28*	4.77±0.91*
Nrf2	Target protein/β-actin	1.28±0.35	1.68±0.31	1.95±0.34*	1.54±0.47	1.68±0.41	1.98±0.27*
PERK	Target protein/β-actin	0.52±0.21	0.67±0.28	1.82±0.12*	1.77±0.10*	1.03±0.30	1.10±0.33
TERK	Target protein/β-actin	0.34±0.29	0.43±0.34	0.51±0.41	0.48±0.26	0.43±0.33	0.54±0.42

Note: *$p<0.05$ vs. Control group.
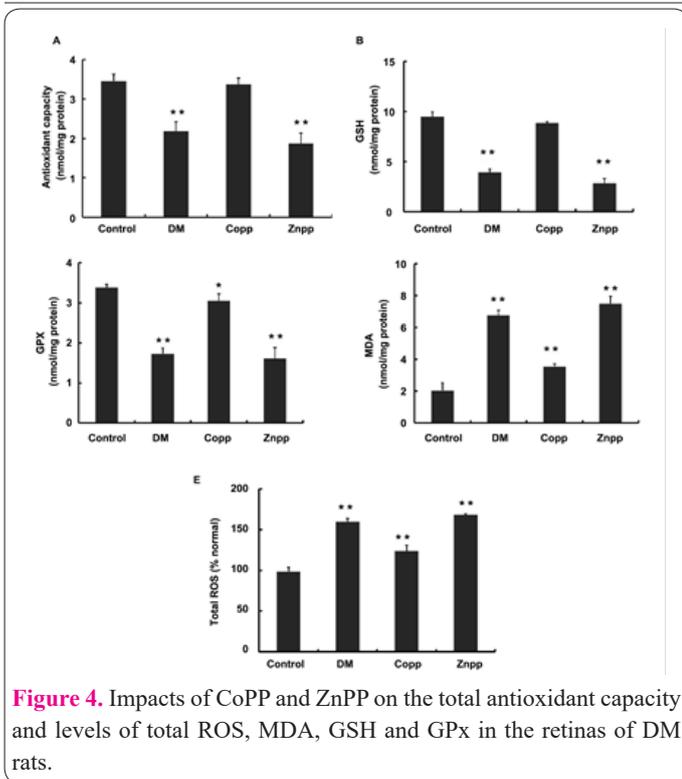
Figure 4. Impacts of CoPP and ZnPP on the total antioxidant capacity and levels of total ROS, MDA, GSH and GPx in the retinas of DM rats.

tantially elevated levels of MDA and total ROS (*p*<0.05).

Influences of CoPP and ZnPP on the mRNAs of Nrf2 and HO-1 in the retinas of DM rats

The results of the qRT-PCR revealed that compared with those in Control group, the mRNA expression level of Nrf2 in the retinas of rats was considerably elevated in DM, CoPP and ZnPP groups (*p*<0.05), and that of HO-1 in the retinas of rats was remarkably raised in DM group and CoPP group (*p*<0.05), but declined markedly in ZnPP group (*p*<0.05) (Figure 5).

Protein expressions of Nrf2, HO-1 and pERK in the retinas of DM rats detected via immunofluorescence assay

Based on the immunofluorescence assay results, an extremely small amount of HO-1 protein was detected in the retinas of normal rats, and after induction of DM in the rats, HO-1 protein was obviously expressed (Figure 6). Compared with that in Control group, DM and CoPP groups exhibited remarkably more green fluorescent aggregation of HO-1 protein (*p*<0.05), with no such aggregation observed in ZnPP group. Additionally, the red fluorescent aggregation of Nrf2 and pERK proteins in DM and CoPP groups was markedly more than that in Control group (*p*<0.05), and the red fluorescent aggregation in ZnPP group was substantially less than that in DM group (*p*<0.05).

Protein expressions of Nrf2, HO-1 and pERK in the retinas of DM rats detected via Western blotting

According to the Western blotting results (Figure 7), after induction of DM, the protein expression levels of Ho-1, Nrf2 and pERK were remarkably elevated (*p*<0.05), and following the activation of HO-1 by Copp, the protein expression levels of Nrf2 and pERK were notably higher than those in DM group (*p*<0.05). In addition, after HO-1 was inhibited by ZnPP, the protein expression levels of
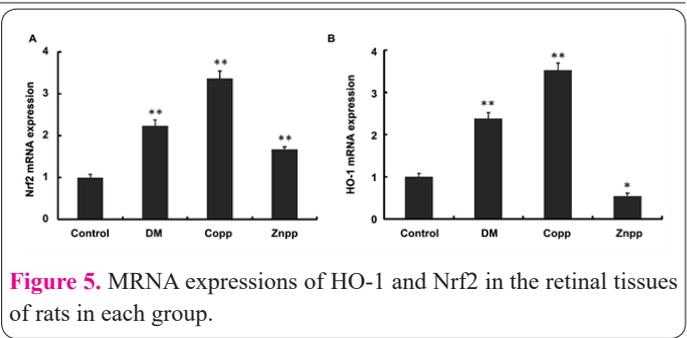
Figure 5. MRNA expressions of HO-1 and Nrf2 in the retinal tissues of rats in each group.
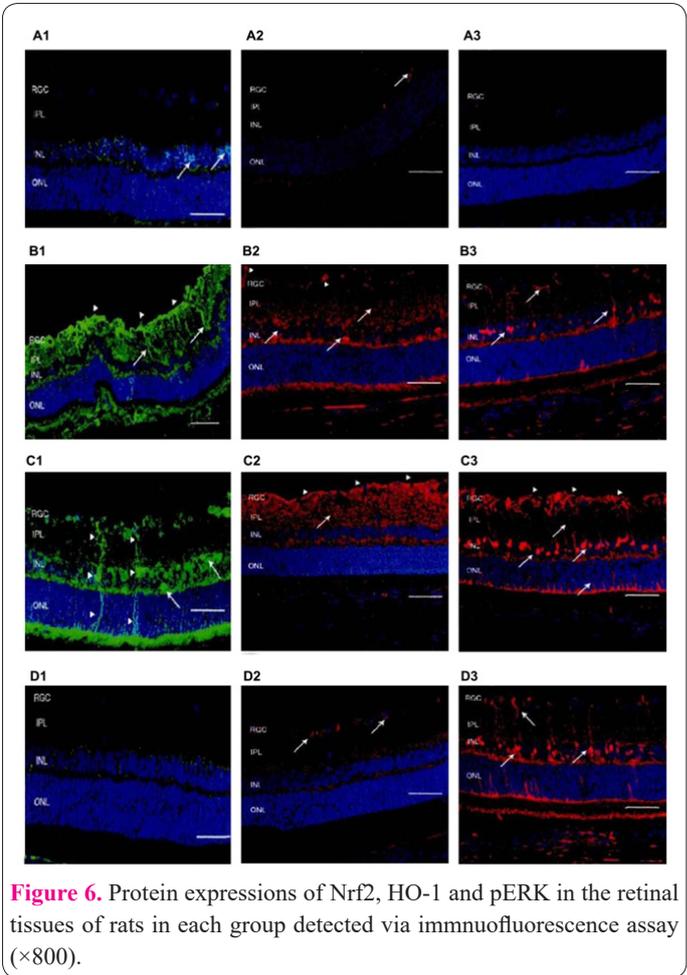
Figure 6. Protein expressions of Nrf2, HO-1 and pERK in the retinal tissues of rats in each group detected via immnuofluorescence assay (×800).
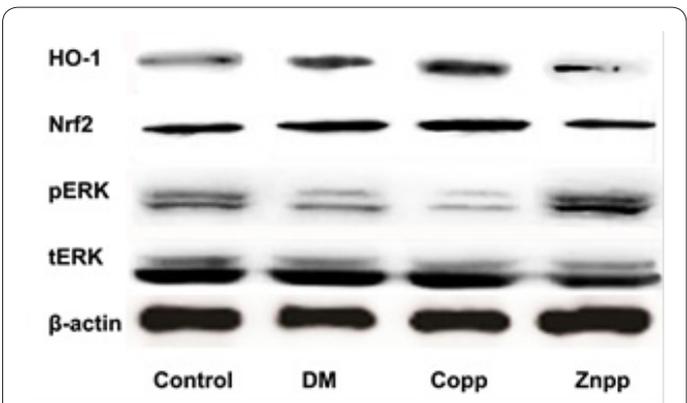
Figure 7. Protein expressions of Nrf2, HO-1 and pERK in the retinal tissues of rats in each group detected via Western blotting.

Nrf2 and pERK were remarkably lower than those in DM group (*p*<0.05). (Table III)

Discussion

DM is a chronic metabolic disorder that is one of the most common diseases. It can contribute to severe impair-

Table III. OD of protein bands of Nrf2, HO-1 and pERK in each group.

Protein	OD	Control	DM	CoPP	ZnPP
HO-1	Target protein/β-actin	0.90±0.24	1.97±0.37[a]	2.37±0.18[ab]	0.38±0.41[ab]
Nrf2	Target protein/β-actin	1.38±0.35	1.96±0.41[a]	2.51±0.24[ab]	1.02±0.36[b]
pERK	Target protein/β-actin	0.42±0.21	0.97±0.33[a]	0.46±0.31[ab]	1.56±0.12[ab]

Note: Control: blank control group, DM: DM model group, CoPP: CoPP group and ZnPP: ZnPP group. [a]$p<0.05$ vs. Control group, and [b]$p<0.05$ vs. DM group.

ment of various physiological functions, such as renal failure, heart failure and blindness (15). The number of DM patients worldwide is about 415 million in 2015 and expected to rise to 642 million by 2040, and over 1/3 of DM patients have retinopathy(16), normally including vision-threatening DR or diabetic macular edema. Studies have demonstrated that DR is also a retinal neurodegenerative disease with the typical clinical pathological changes of microaneurysm, intraretinal microvascular abnormalities (capillary vessel, capillary occlusion and exudation), edema and tortuous vessels caused by intraretinal neovascularization. In the early asymptomatic stage, DR presentsno clinical characters in patients and then progresses into non-proliferative one, namely mild, moderate, severe and very severe DR, severe proliferative DR in cases of intraretinal neovascularization and macular edema, ultimately causing vision loss (17). The main causes of DR are degeneration of retinal pigment epithelial cells and choroidal neovascularization (18). Since new vessels are more fragile, the scars of tractional retinal detachment will be induced once leakage occurs (19). Another important cause of DR is the sudden episode of branch or central retinal vein occlusion (20).

As a rate-limiting enzyme catalyzing heme metabolism, HO plays a pivotal role in the regulation of oxidative stress and mitochondrial function (21). HO has two types of isozymes, namely inducible HO-1 and constitutive HO-2. Such factors as oxidative stress, infection, inflammation and ischemia can inducethe expression of HO-1, belonging to inducible antioxidant enzymes (22), and HO-1 can oxidize heme to produce biliverdin that generates bilirubin through bilirubin reductase and release Fe and CO at the same time(23). The former, an antioxidant, can resist oxidative stress, andthe latter, a crucial signaling molecule, is able to up-regulate cellular antioxidant responses (24). Moreover, HO-1 is involved in various stress responses, such as physical, chemical and biological stresses. Some studies have manifested that activating Nrf2 at the heme level can regulate HO-1. Many natural antioxidants can inhibit the production of ROS, thereby repressing HO-1. The ratio of intracellular GSH and GSSH is an important indicator for measuring oxidative stress, and under the action of ROS, GSH will decline in cells to induce oxidative stress and ultimately activate HO-1 (25). ROS, therefore, can activate HO-1.

In the present study, STZ was used to induce and replicate the DM rat model, and the HO-1 activator CoPP and its inhibitor ZnPP were employed, so as to explore the influence of HO-1 on the retinopathy in DM rats.After administration of drugs for 12 consecutive weeks, fasting blood glucose was detected, and it was found that STZ could induce the DM rat model well. However, after the activation of HO-1 by CoPP,the level of blood glucose exhibited adeclining trend compared with that in DM group, while the level of blood glucose in rats was raised and the

weight of rats was lowered after inhibition of HO-1 by ZnPP, suggesting that HO-1 can regulate the blood glucose and weight of DM rats. According to the findings in this study, at different time points after induction of DM,there was almost no endogenous HO-1 expressed in the retinas of normal rats, and it was expressed at 2 weeks after induction of DM. Besides, the expression level of Nrf2 started to rise in the rat retinas immediately after induction of DM. Meanwhile, the level of tERK in retinas showed no obvious difference, but compared with that in Control group, the level of pERK was elevated dramatically in the retinas of rats after induction of DM. The HO-1/Nrf2 pathway is closely associated with oxidative stress. The level of oxidative stress in rats was determined in this study, and it was discovered that HO-1 could effectively modulate the level of oxidative stress in organisms. Once HO-1 is inhibited in the retinas of DM rats, the expression of Nrf2 will accordingly decline, but the expressions of ERK1/2 will rise, illustrating that the ERK signaling pathway can be activated through inhibiting the antioxidant HO-1/Nrf2 pathway in organisms. The possible reason is that the ERK signaling pathway is activated to increase mitochondrial biosynthesis that is decreased after the HO-1/Nrf2 pathway is repressed, balancing exterior stimulation. Although the present study fails to elucidate all the related factors for DR and its complex molecular mechanism in detail, itlays an experimental foundation for subsequent in-depth research.

Availability of data and material
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
YZwrote the manuscript. YZ and XM were responsible for establishment of rat model. JL and KL worked on PCR and Western blot.All authors read and approved the final manuscript.

Ethics approval and consent to participate
The study was approved by the ethics committee ofWanzhou Aier Eye Hospital.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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