

## Original Article

# Effects of resveratrol on the Nrf2 and HO-1 expression in diabetic vascular endothelial cells

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**Abstract:** Oxidative stress plays an important role in the pathogenesis of diabetes. High concentration of glucose can increase the generation of reactive oxygen species (ROS). Excessive generation of ROS can activate multiple signaling pathways of vascular disease, resulting in the damage of cells. Herein we aim to characterize the protective effect of resveratrol on diabetic rat vascular endothelium, and to investigate the influence of resveratrol on retinal nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase 1 (HO-1) genes, which are associated with elimination of ROS. In diabetic rat, body weight, blood lipid, blood glucose, capillaries density, basement membrane thickness and ratio of capillaries to muscle fibers were examined. Levels of transcripts and protein expression of Nrf2 and HO-1 genes in retinal tissue were detected by fluorescence quantitative PCR, Western blot and immunohistochemistry. Our results showed that resveratrol significantly decreased the concentration of blood glucose and triglycerides, and largely increased body weight and capillaries density. In addition, in our study the levels of transcripts and translation of Nrf2 and HO-1 genes gradually returned to normal from high points. In conclusion, resveratrol can decrease blood glucose, blood lipid and protect vascular endothelial cells from injury caused by diabetes, and can regulate the Nrf2/HO-1 pathway, which is firmly related to maintain redox state balance of retina.

**Keywords:** Diabetes, vascular endothelial cells, resveratrol, nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1)

## Introduction

Diabetes mellitus (DM) is a group of metabolic diseases in which there are high blood sugar levels over a prolonged period. Diabetes can cause many complications, like diabetic vasculopathy and diabetic vasculopathy is the major factor causing death [1]. Symptoms of diabetic vasculopathy include retinopathy, coronary heart disease, cerebral infarction, cerebral hemorrhage or atherosclerosis and plaque [2]. Oxidative stress and oxidative stress defense system play an important role in diabetic macroangiopathy [3]. Reactive oxygen species (ROS) can active transcripts of antioxidant genes through generating protective proteins induced by antioxidant response elements (ARE) to mitigate injury of cells resulting from ROS [4, 5]. Interaction of nuclear factor erythroid 2-related factor 2 (Nrf2) with ARE can promote the expression of Phase II detoxification enzymes and anti-oxidant protein, result-

ing in the decrease of ROS [6]. In Nrf2 knock-out mice, expression of anti-oxidant protein decreased significantly and oxidative stress markedly increased, indicating that Nrf2/ARE pathway plays an important role in regulating redox state [7]. Heme oxygenase 1 (HO-1) is an Nrf2 controlled gene that plays a crucial role in the prevention of oxidation, inflammation, apoptosis and damage caused by ROS [8].

Resveratrol (Res) is a kind of natural phenol extracted from several plants. It is a type of powerful antioxidant, which has a range of biological and pharmacological effects, such as protecting cardiovascular, regulating blood lipid, decreasing blood glucose, improving insulin resistance [9]. Res is able to inhibit the proliferation of vascular smooth muscles cells (VSMCs) by antioxidant [10].

Therefore, we deduce that Res may regulate the Nrf2/HO-1 pathway to attenuate the injury

of diabetic vascular. Thus, in streptozotocin (STZ) induced type-I diabetes rats we characterized the effects of Res on the growth of diabetes endothelial cells and the expression of retinal Nrf2/HO-1 genes, aiming to develop novel strategies of prevention and treatment of vascular complications of DM.

## Materials and methods

### *Animals and treatments*

A total of 60 healthy adult male and female Wistar rats were provided by the Experimental Animal Center of Luzhou Medical College. They were kept in standard conditions with free access to food and water. They were randomly divided into three groups: normal control group (n = 20), STZ group (n = 20) and Res group (n = 20). For the establishment of DM model, rats in STZ group were treated with a single intraperitoneal injection of STZ at a dose of 55 mg/kg body weight. Blood glucose level was measured at 72 h after STZ treatment and rats with blood glucose level > 16.7 mM were considered as DM rats. Rats in Res group were treated with STZ as rats of STZ groups and were then given Res at doses of 10 mg/kg, 20 mg/kg and 40 mg/kg body weight per day with gavages for 4 weeks. After 4 weeks, body weight and blood glucose of all rats were measured. Then, all of the rats were sacrificed. Blood was collected by cardiac puncture and processed for biochemical estimations such as glucose, TC (total cholesterol), TG (total triglyceride), LDL-C (low density lipoprotein cholesterol) and HDL-C (high density lipoprotein cholesterol). Retinal tissues were collected for immunohistochemical analysis. Skeletal muscle tissues were collected from the left lower limb. All animal experiments were conducted according to the ethical guidelines of Zhejiang University.

### *Characterization of the neovascularization of rats*

Skeletal muscle tissue was fixed in paraformaldehyde, embedded in paraffin and cut into 4  $\mu$ m tissue sections. Then the tissues were stained with haematoxylin and eosin. We randomly selected six microscope fields from each slice. The number of capillaries was counted at 200  $\times$  magnification with double-blind method. The thickness of capillary basement membrane was measured. The ratio of the number of capil-

laries to that of muscle fiber was calculated. Quantitative analysis of above measurements was performed using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

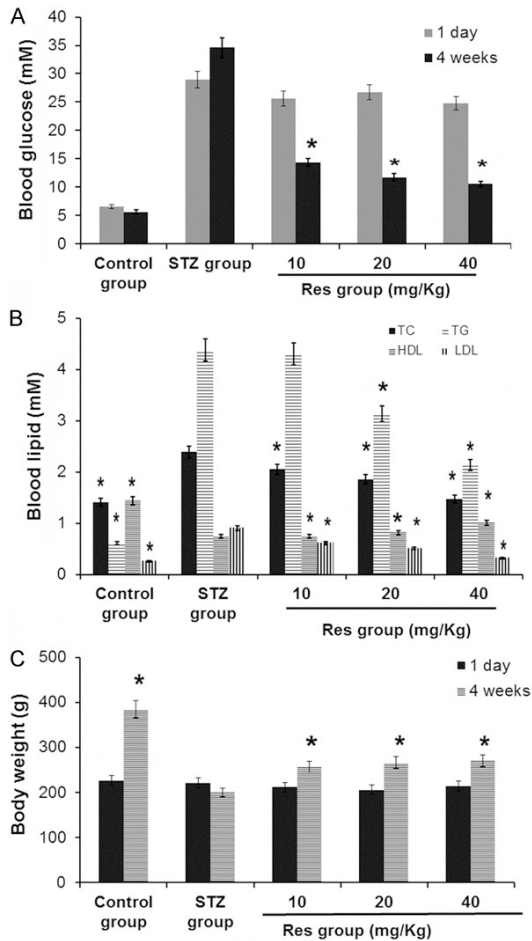
### *Quantitative real time PCR (qRT-PCR)*

Total RNA was isolated from retinal tissue following the procedure described previously [11]. Then RNAs were transcribed into cDNA according to the instructions provided by the Prime-Script Kit (TAKARA BIOTECHNOLOGY (DALIAN) CO., LTD. Dalian, Liaoning, China). Then, qRT-PCR was performed. The forward and reverse primers for Nrf2 and HO-1 were synthesized at Shanghai Sangon Biological Engineering Technology & Services, Shanghai, China. The forward and reverse primer sequences for Nrf2 were as follows: Nrf2 forward 5'CAAATCC-CACCTTGAACACA 3', and, Nrf2 reverse 5'CG-ACTGACTAATGGCAGCAG 3'. The forward and reverse primers for HO-1 were HO-1 forward 5'CAGAGTTTCTTCGCCAGAGG 3', and HO-1 reverse 5'TGAGTGTGAGGACCCATCG 3'. PCR reaction were carried out in a final volume of 25  $\mu$ l containing: 12.5  $\mu$ l of SYBR Premix Ex Taq II (Qiagen, Hilden, Germany), 1.0  $\mu$ l forward primer, 1.0  $\mu$ l reverse primer, 2  $\mu$ l template (about 120 ng) and 8.5  $\mu$ l dH<sub>2</sub>O. The PCR procedure were initial denaturation step at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. GAPDH was used as an internal control. The 2<sup>- $\Delta\Delta$ Ct</sup> method was used to calculate the relative expression levels of Nrf2 and HO-1.

### *Western blot*

Total proteins were extracted and separated by 12% SDS-PAGE. Then proteins were transferred onto nitrocellulose membrane. After blocking with non-fat milk, the membrane was incubated with the primary antibodies of rabbit anti-rat polyclonal antibody anti-Nrf2 (Beijing Biosynthesis Biotechnology, Co., LTD., Beijing, China) and rabbit anti-rat polyclonal antibody anti-HO-1 (Beijing Biosynthesis Biotechnology) at 4°C overnight. After washing, the membrane was then incubated with secondary antibody of goat anti-rabbit HRP conjugated antibody (Beijing Biosynthesis Biotechnology, Co., LTD., Beijing, China) at room temperature for 1 h. Finally, the membrane was developed by enhanced chemiluminescence plus reagent. The developed film was scanned using the Alphamager gel imaging systems (Alphama-

## Resveratrol on diabetic rats



**Figure 1.** Effects of Res treatment on blood glucose (A), blood lipid (B) and body weight (C) in diabetes rats. Control group: rats without STZ or Res treatment; STZ group: rats with STZ treatment; Res group: STZ group with Res treatment at doses of 10 mg/kg, 20 mg/kg and 40 mg/kg, respectively. The levels of blood glucose, blood lipid and body weight were measured at 1 day and 4 weeks of treatment. Compared with STZ group, \* $P < 0.05$ .

ger, Santa Clara, California, USA). And the Western blot images were analyzed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).  $\beta$ -actin was used as an internal control. The relative absorbance ratios of Nrf2 to  $\beta$ -actin and Ho-1 to  $\beta$ -actin were defined as the relative values of Nrf2 and HO-1, respectively.

### Immunohistochemistry

Immunohistochemistry of Nrf2 and HO-1 was performed using the streptavidin-biotin complex (SABC) method as described previously [12]. Briefly, retinal tissues were fixed in formal-

dehyde and embedded in paraffin. Sections were dewaxed, and rehydrated in graded alcohols. Then sections were incubated with 0.3% hydrogen peroxide to inactivate endogenous peroxidase activity. Antigen retrieval was achieved by incubating with sodium citrate (PH 6.0). After blocking, sections were incubated with the primary antibodies of rabbit anti-rat polyclonal antibody anti-Nrf2 (Beijing Biosynthesis Biotechnology, Co., LTD., Beijing, China) and rabbit anti-rat polyclonal antibody anti-HO-1 (Beijing Biosynthesis Biotechnology) at 4°C overnight. Then, sections were incubated with secondary antibody of goat anti-rabbit (Beijing Biosynthesis Biotechnology, Co., LTD., Beijing, China) at room temperature for 40 min. After incubating with SABC at room temperature for 40 min, sections were developed with DAB chromogenic reagent. Finally, sections were counterstained with haematoxylin. After mounting with neutral resin, sections were observed under microscopy (Leica DM4000B, Leica Microsystems, Wetzlar, Germany). Image-Pro Plus (Media Cybernetics, Inc., Rockville, MD, USA) was used to analyze the expression level of Nrf2 and HO-1.

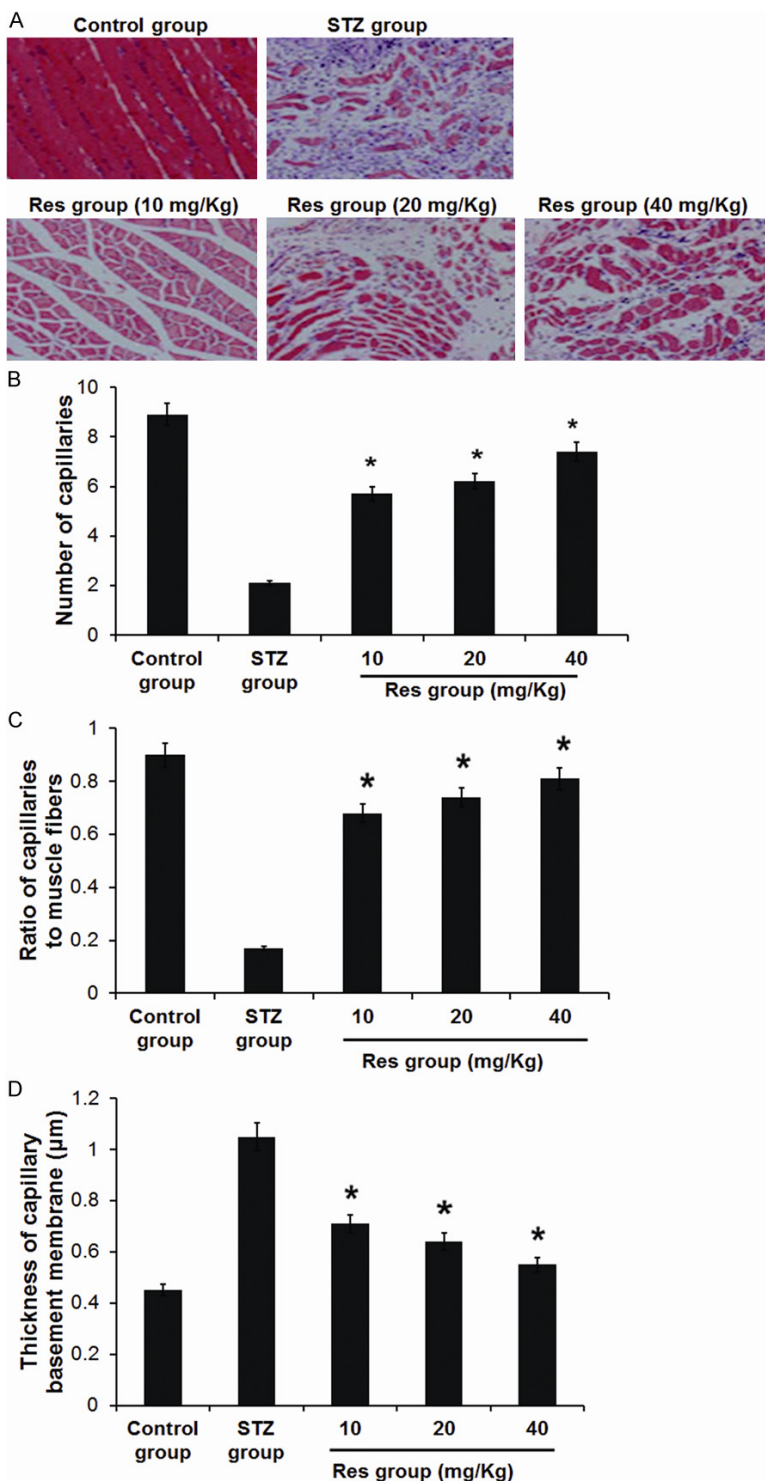
### Statistical analysis

Data were expressed as mean  $\pm$  standard deviation. SPSS 17.0 was used for statistical analysis. One-way analysis of variance was performed. SNK test was conducted to compare differences between groups. Non-parametric test was applied to data that did not meet the homogeneity of variance. Results were considered statistically significant when the  $P$  value was less than 0.05.

## Results

### Positive effects of Res on blood glucose, lipid and body weight in diabetes rats

To determine the effects of Res on blood glucose, lipid and body weight, daily treatment of Res on rats was performed. At day 1, blood glucose level was up to 29.01 mM in STZ group. And blood glucose level was 25.61 mM, 26.72 mM and 24.78 mM in Res group at doses of 10 mg/kg, 20 mg/kg and 40 mg/kg, respectively (**Figure 1A**). It indicated that we were successful in constructing DM rat models. After treatment with Res for 4 weeks, blood glucose level in Res group at three doses all decreased



**Figure 2.** Effects of Res treatment on density of capillaries (A), the representative pictures of H&E staining (Magnification 200 ×). (B) The ratio of capillaries to muscle fibers (C) and the basement membrane thickness (D) in diabetes rats examine by microscope. At 4 weeks of treatment, these measures were performed. Compared with STZ group, \* $P < 0.05$ .

However, blood glucose level in Res group was still higher than that in normal control group.

Meanwhile, parameters, like blood lipid, TC and LDL, also presented the same trend with blood glucose (**Figure 1B**), especially after Res treatment of 20 and 40 mg/kg body weight per day. It was noteworthy that in STZ group, TG was up to 4.38 mM, which were 7 folds more than that of normal control group. But after 40 mg/kg Res treatment, the value of TG decreased to half of STZ group. In addition, quantification of blood lipid indicated that Res could decrease TC, TG and LDL, and increase HDL significantly ( $P < 0.05$ ) compared with normal control group. In addition, the levels of TC, LDL and HDL in DM rats were similar to those of normal control group after 40 mg/kg Res treatment.

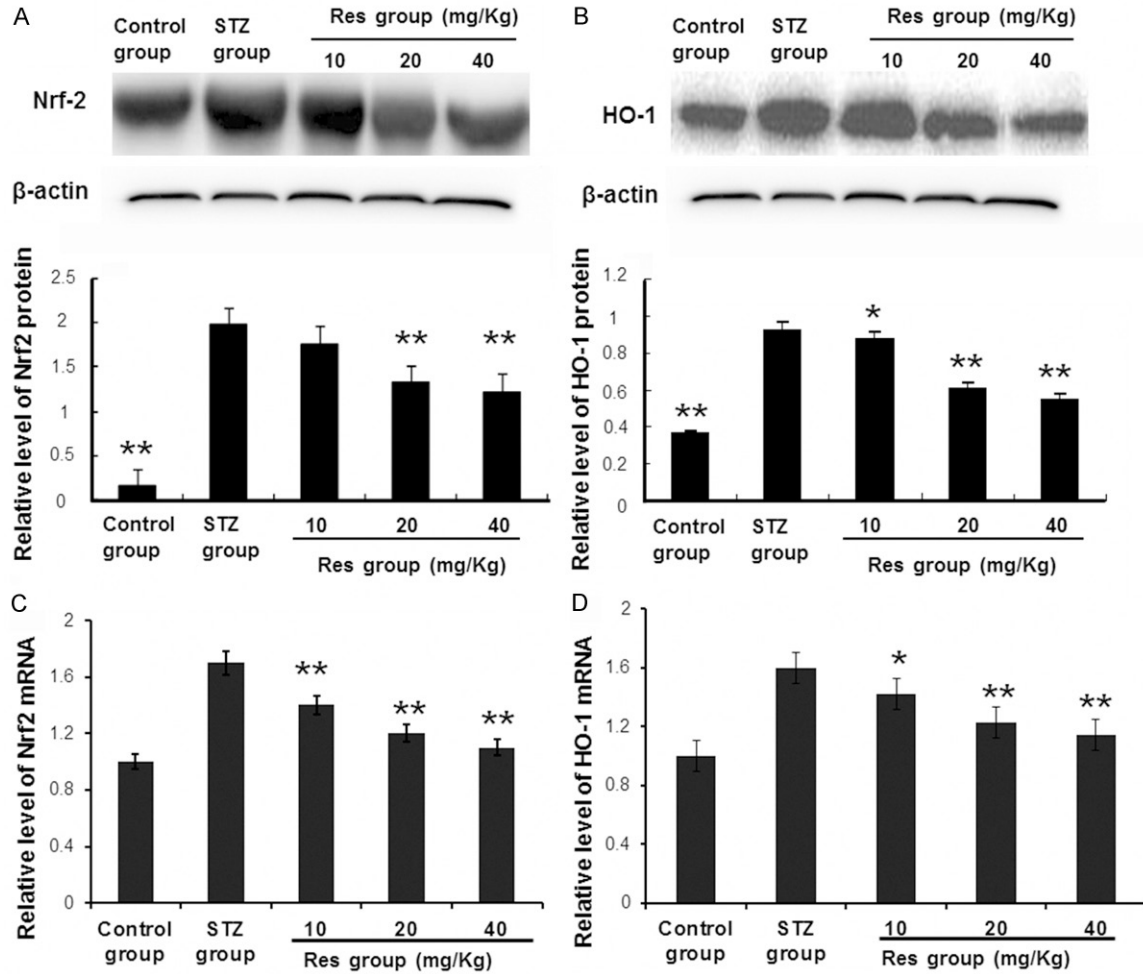
As for body weight, even though there was no obvious change in body weight with 40 mg/kg Res treatment for 1 day, a significant increase ( $P < 0.05$ ) appeared after 10 mg/kg Res treatment in diabetes rats for 4 weeks compared with Res untreated diabetes rats (**Figure 1C**). But there was not obvious difference in Res group at doses of 10 mg/kg, 20 mg/kg and 40 mg/kg, and the body weight of Res treated rats was still lighter than that of normal control group. These results indicate that Res is able to improve the weight of DM rats significantly.

#### *Influence of Res on capillaries of diabetes rats*

to levels below 16 mM. Compared with STZ group, the decrease was significant ( $P < 0.05$ ).

To determine the influence of Res on the capillaries of DM rats, the characterization of capil-

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**Figure 3.** Effects of Res treatment on the levels of retinal Nrf2 and HO-1 in diabetes rats. Levels of retinal Nrf2 and HO-1 protein were detected with Western blot.  $\beta$ -actin was used as an internal control. A. Protein level of Nrf2. B. Protein level of HO-1. Using GAPDH as internal control, the mRNA levels of Nrf2 and HO-1 genes were tested through qRT-PCR. Total RNA was extracted from retinal tissue in diabetes rats after 4 weeks of treatment. C. mRNA level of Nrf2. D. mRNA level of HO-1. Compared with STZ group, \* $P < 0.05$ , \*\* $P < 0.01$ .

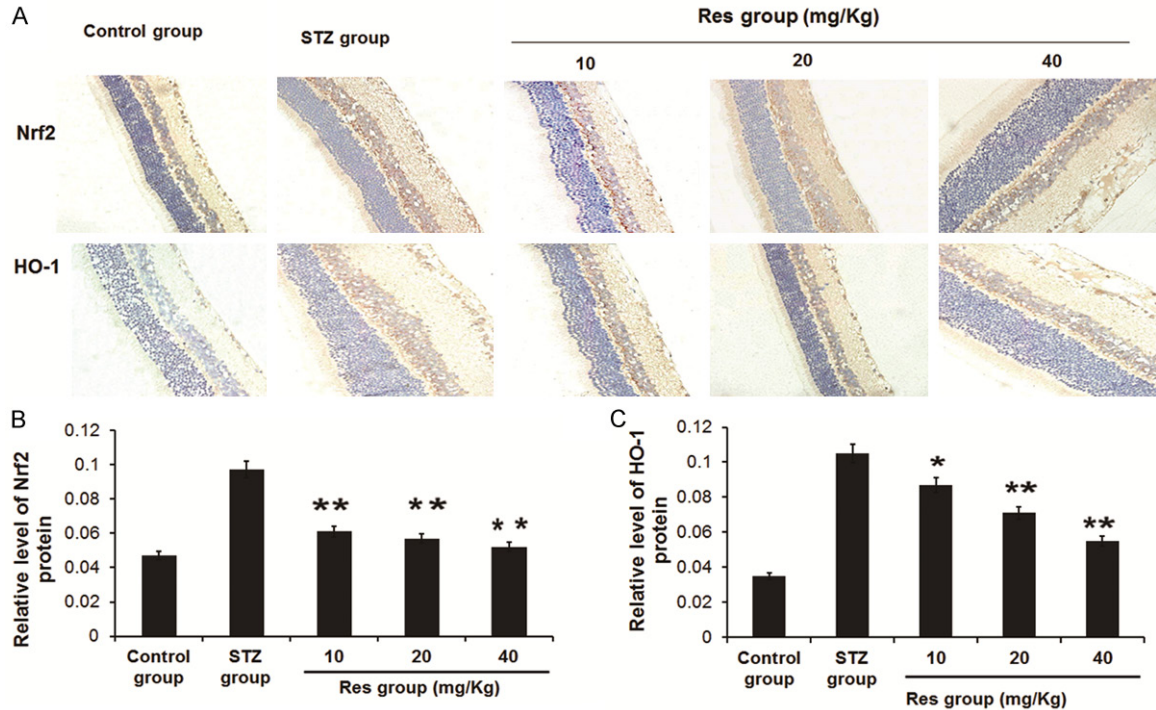
larities was analyzed. The representative pictures of H&E staining were shown in **Figure 2A**. Although there was no significant difference detected in diameter of capillaries in each group (data not shown), in STZ group both the number of capillaries and the ratio of capillaries to muscle fibers were only a quarter of that in normal control group. But when DM rats were treated with 10 mg/kg Res for 4 weeks, both of the number of capillaries and the ratio mentioned above increased by more than 2 folds (**Figure 2B** and **2C**), which were almost close to the normal level. In addition, the thickness of capillaries basement membrane of STZ group had increased to more than twofold of normal control group. Compared with STZ group, thickness of basement membrane in

Res group dropped significantly ( $P < 0.05$ ) to a lower position similar to normal level even with only 10 mg/kg body weight per day for 4 weeks (**Figure 2D**). These results indicate that Res can increase capillaries density and the ratio of capillaries to muscle fibers, and decrease the thickness of capillaries basement membrane.

### *Effects of Res on mRNA and protein levels of retinal Nrf2 and HO-1 genes*

To determine the effect of Res on the mRNA and protein levels of retinal Nrf2 and HO-1, qRT-PCR and Western Blot were performed, respectively. As shown in **Figures 3** and **4**, the mRNA and protein levels of retinal Nrf2 and HO-1 were obviously higher in STZ group than

## Resveratrol on diabetic rats



**Figure 4.** Effects of Res treatment on the protein levels of retinal Nrf2 and HO-1 in diabetes rats tested by immunohistochemistry. At 4 weeks of treatment, immunohistochemistry was performed to detect Nrf2 and HO-1. A. Representative immunohistochemical staining results (Magnification: 40 ×). B. Relative level of Nrf2 protein. Compared with STZ group, \*P < 0.05, \*\*P < 0.01. C. Relative level of HO-1 protein. Compared with STZ group, \*P < 0.05, \*\*P < 0.01.

those in normal control group. However, Res treatment on STZ group significantly decreased the mRNA and protein levels of Nrf2 and HO-1 in retinal tissue ( $P < 0.05$ ) (Figures 3 and 4). In addition, at the dose of 40 mg/kg Res, the mRNA and protein levels of Nrf2 and HO-1 in Res group were similar to those in control group. These results indicate that Res can inhibit the expression of retinal Nrf-2 gene and HO-1 gene.

### Discussion

The novel findings of the present study are: 1) parameters of diabetes rats including body weight, capillaries density, blood lipid and glucose came back to the levels similar to those of control rats after feeding them with Res, 2) Res downregulated expression of Nrf2 and HO-1 in retinal tissues of diabetes rats. Thus, we deduce that it is the Res-induced deduce of blood glucose and lipid that leads to the down-regulation of Nrf2, consequently followed by decrease of HO-1 in the retinal.

Diabetes is cogently associated with microvascular and macrovascular complications: cardi-

opathy, neuropathy, nephropathy, retinopathy, etc. [13-15]. High glucose promotes endothelial cell to die, resulting in damaging vascular endothelial [16]. Pathogenesis of microvascular is associated with microcirculatory disturbance, inflammation and abnormal oxygenation, retinal vascular dilation, increasing of advanced glycation end products (AGEs), changing of structure of blood vessels, thickening of blood vessels basement membrane, and narrowing of capillary luminal [17-19].

This study showed increasing of body weight and decreasing of glucose in diabetes rats with the help of Res, suggesting that Res has positive effect on diabetes. Further investigation on blood lipid showed that the concentrations of TC, TG and LDL decreased and HDL increased significantly. Meanwhile, there was no significant change in diameter of capillaries, while the density of capillaries and the ratio of capillaries to muscle fibers increased, but the basement membrane thickness decreased significantly. This indicates that Res has the function of vascular protection.

In endothelial cell, high glucose can lead to peroxide, oxygen free radicals increasing, lipid peroxidation, macromolecules depositing on a base film, damage of tight junctions, increasing permeability, decreases of myocardial capillary and basement membrane thickening by increasing production of cytokines [20-23]. Nrf2 and HO-1 play an important role in anti-oxidative stress [24]. In diabetes rat, production of ROS has an increasing trend, following the increase of Nrf2 expression to resist the stimulation of ROS [25, 26]. In Nrf2 knockout mice, the expression of anti-oxidative genes decreased evidently, but oxidative-stress damage increased significantly, indicating that Nrf2/ARE pathway plays a key role in the balance of redox state [27]. Activation of GSK-3 $\beta$ /Nrf2 pathway can induce the upregulation of antioxidant protein [28].

It has been reported that Nrf2 expressed in many kinds of cells, and the production of ROS in Nrf2<sup>-/-</sup> diabetes rats is higher than in Nrf2<sup>+/+</sup> [29]. Moreover, the expression of HO-1 is in type 2 diabetes mellitus, and it is positively associated with macroangiopathy [30]. Nrf2 can induce the transcripts of HO-1 gene to produce antioxidant effect [31].

Our study characterized the effect of Res on the mRNA and protein levels of Nrf2 and HO-1, and the results showed that diabetes rats expressed less Nrf2 and HO-1 protein with the help of Res than control group. The previous study demonstrated that Res upregulated Nrf2 expression followed by up-regulation of HO-1 [32]. However, herein when the blood glucose decreased, levels of the Nrf2 and HO-1 expression also came back to normal gradually. It suggests that Res can deduce the oxidation of diabetes rats, following the decreasing of Nrf2 and HO-1 expression, and Nrf2/HO-1 pathway is also responsible for maintaining glucose balance.

In conclusion, our study presents that Res is able to decrease glucose of diabetes rats and increase the number of capillaries, and reduce Nrf2/HO-1 expression, suggesting that Res may play a key role in the decreased expression of Nrf2 and HO-1, which provides a novel approach for prevention and treatment of diabetes.

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### Disclosure of conflict of interest

None.

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