Grape seed proanthocyanidin extract protects the retina against early diabetic injury by activating the Nrf2 pathway

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Abstract. The present study aimed to investigate whether grape seed proanthocyanidin extract (GSPE) has a protective effect on diabetic retinal function. A total of 30 Wistar rats were randomly divided into three equal groups, including the control, diabetic and GSPE-treated diabetic groups. Retinal tissue was harvested and subsequently stained with hematoxylin and eosin. Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and methane dicarboxylic aldehyde (MDA) levels were evaluated using respective assay kits; whereas nuclear erythroid 2-related factor 2 (Nrf2) and heme oxygenase (HO)-1 expression levels were assessed by immunohistochemical and western blot analysis. Cell apoptosis in the retina was determined using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling method. The results showed that the structure of the retina was damaged in diabetic rats, as compared with the control rats. Notably, the structure of the retina improved in the GSPE-treated diabetic group, as compared with the diabetic group. SOD and GSH-Px activities were significantly increased in the retina of rats in the GSPE-treated diabetic group, as compared with the diabetic group (P=0.011 and P=0.001, respectively). Furthermore, a significant reduction in MDA was detected (P=0.013) and the expression levels of Nrf2 and HO-1 in the bladders of rats in the GSPE-treated diabetic group were significantly increased, as compared with the diabetic group (P=0.038 and P=0.043, respectively). Apoptosis of retinal cells was significantly increased in the diabetic group, as compared with the control group (P<0.001); a significant reduction was also detected in the GSPE-treated diabetic group, as compared with the diabetic group (P=0.014). These results demonstrate that GSPE administration may protect the retina against hyperglycemic damage, possibly by ameliorating oxidative stress-mediated injury via the activation of the Nrf2 pathway.

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Introduction

The prevalence of diabetic retinopathy (DR) in patients with established diabetes and newly diagnosed diabetes is 27.9 and 10.5%, respectively (1), and the incidence of DR is expected to increase substantially (2). DR is the leading cause of blindness in patients of working age (3), and one of the early manifestations of DR is persistent apoptosis of vascular and neural cells in the retinal tissue (4,5). Other consequences of DR include the breakdown of the blood retinal barrier, retinal edema, neovascularization and detachment and loss of vision (5). Although the pathogenesis of DR is complicated and has yet to be fully elucidated, hyperglycemia-induced oxidative stress, an imbalance in the production of reactive oxygen species (ROS) and ROS-induced damage have been demonstrated to serve a crucial function in the pathogenesis of DR (6).

Grape seed proanthocyanidin extract (GSPE), which is a potent antioxidant derived from grape seeds, provides a concentrated source of polyphenols (7). Previous studies have demonstrated that GSPE has an important role in antioxidation, anti-inflammation, radical scavenging and antitumor activity (7,8); and that the physiological benefits of GSPE are closely associated with its antioxidative and free radical scavenging properties. Furthermore, GSPE has been demonstrated to have a protective effect in DR by reducing the production of advanced glycation end products (9).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor involved in the Nrf2-antioxidant response element signaling pathway, which protects against oxidative stress; therefore, Nrf2 is crucially involved in the attenuation of inflammation-associated pathogenesis in numerous diseases (10). Previous studies have demonstrated that Nrf2 may have a protective role in the retina (11-13). Furthermore, it has been demonstrated that Nrf2 has a cytoprotective role for neurons and vasculature in the diabetic retina (12,14).

Scapagnini *et al* (15) found that modulation of the Nrf2 pathway was achievable using food polyphenols, which has since become a nutritional neuroprotective therapeutic strategy. To further understand the role of GSPE in the protection of DR and the mechanism of Nrf2 in the pathogenesis of DR, the present study investigated whether GSPE was capable of modulating the expression levels of Nrf2 and the downstream molecule, heme oxygenase (HO)-1, in the retina. Furthermore, whether GSPE administration could improve the structure and morphology of diabetic retinas was examined. The authors of the present study hypothesized that GSPE had a protective role in DR by modulating the Nrf2 pathway.

Materials and methods

Experimental design. A total of 30 Wistar rats, aged 8-10 weeks and weighing 230-250 g, were purchased from the Animal Center of Shandong University (Shandong, China; license number, SCXX20050015) and divided into three equal groups (10 rats/group): The untreated (control); untreated diabetic (DM); and diabetic treated with GSPE (DM + GSPE) groups. Animal care and handling in the present study was approved by the Ethics Committee of Shandong University.

Diabetes was induced in the DM and DM ± GSPE rats following 18 h of fasting by a single intraperitoneal injection with 65 mg/kg streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.1 M citrate buffer (pH 4.5). The control rats were administered a single intraperitoneal injection of isometric citrate buffer. The rats were maintained at 25±1˚C in a temperature-controlled room with a 12-h light/dark cycle and *ad libitum* access to food and water. Tail venous blood samples were harvested at 72 h after STZ treatment in order to measure blood glucose levels using a glucose monitoring system (cat. no. 1620368; Roche Diagnostics, Indianapolis, IN, USA). A total of 20 rats with serum glucose levels >300 mg/dl were included in the experiment. Following the induction of diabetes, 250 mg/kg GSPE (Tianjin Jianfeng Natural Product R&D, Co., Ltd., Tianjin, China) was administered per day in normal saline solution via oral gavage for 8 weeks.

Upon completion of the experiment, fasted rats were anesthetized with 80 mg/kg ketamine (Sigma-Aldrich), sacrificed by cervical dislocation, and their eyes were immediately removed. The right eyes were fixed in 4% paraformaldehyde (Sigma-Aldrich) for morphological analysis and apoptosis rate measurement; whereas the left eyes were harvested and stored at -80˚C for the evaluation of Nrf2 expression levels and determination of redox status.

Retinal morphology analysis. Retinal samples were cut into 4-μm sections, placed onto glass slides, deparaffinized in xylene (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and serially treated with 100, 96 and 70% ethanol. Subsequently, the slides were stained with hematoxylin and eosin (HE; Sangon Biotech Co., Ltd., Shanghai, China) and observed at x100-400 magnification under a light microscope (BX53F; Olympus Corporation, Tokyo, Japan). Morphological analyses were performed by two independent pathologists in a blinded manner.

Cytoplasmic and nuclear extraction. Using a nuclear extraction kit (cat. no. P0028; Beyotime Institute of Biotechnology, Beijing, China), each fresh isolated retina was homogenized in 200 μl ice-cold cytoplasmic extraction buffer for 15 min and centrifuged at 15,000 x g for 10 min at 4˚C, according to the manufacturer's protocol. The supernatant containing the cytoplasmic protein fraction was used to determine the activity levels of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), the quantity of methane dicarboxylic aldehyde (MDA) and the expression levels of HO-1. The remaining nuclear pellet was resuspended in 50 μl ice-cold nuclear extraction buffer for 10 min and centrifuged at 15,000 x g for 10 min at 4˚C. The supernatant containing the nuclear fraction was used for the quantification of Nrf2 in the nucleus. The Bicinchoninic Acid Assay kit (cat. no. P0012; Beyotime Institute of Biotechnology) was used to quantify the protein concentrations in the cytoplasmic and nuclear extracts.

Estimation of redox status in retinas. SOD and GSH-Px activity levels and MDA content were estimated using the Total Superoxide Dismutase Assay kit with WST-8 (S0101), the Lipid Peroxidation MDA Assay kit (S0131) and the Total Glutathione Peroxidase Assay kit (S0058), respectively (all from Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's protocols. Briefly, T-SOD activity was assessed based on the xanthine-xanthine oxidase system. GSH-Px activity was measured according the speed of enzymatic reaction, whereas MDA levels were determined by the thiobarbituric acid method.

Western blot analysis. Nuclear extracts were used to detect the expression levels of Nrf2, whereas cytoplasmic extracts were used to analyze HO-1 levels. A Bicinchoninic Acid Assay kit was used to determine the protein concentration in the supernatant, and the samples were subsequently stored at -80˚C. Immediately prior to electrophoresis, loading buffer (Sangon Biotech Co., Ltd.) was added to the samples and heated at 95˚C for 4 min. Subsequently, 40 μg protein was added to each gel well and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Sangon Biotech Co., Ltd.). Separated proteins were electroblotted onto a 0.45-μm polyvinylidene fluoride (PVDF) membrane (Roche Diagnostics) using transfer buffer (Beyotime Institute of Biotechnology). Nonspecific binding was blocked by incubating the membranes in 5% fat-free milk for 1 h. PVDF membranes were incubated overnight at 4˚C with rabbit anti-rat Nrf2 polyclonal antibody (cat. no. ab31163), rabbit-anti-rat Lamin B polyclonal antibody (cat. no. ab13248), mouse anti-rat HO-1 monoclonal antibody (cat. no. ab16048) and mouse anti-rat β-actin monoclonal antibody (cat. no. ab8226; all 1:1,000; all Abcam, Cambridge, UK). Subsequently, the membranes were washed three times for 10 min each with Tris-buffered saline supplemented with Tween-20 (Sangon Biotech Co., Ltd.), prior to incubation with goat anti-rabbit secondary antibody (1:2,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 25˚C for 2 h. Chemiluminescence was detected using a Kodak Image Station 2000 MM (Kodak, Rochester, NY, USA). Grayscale analysis was performed using Scion Image analysis software 4.03 (Scion Corporation, Frederick, MD, USA).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. TUNEL staining of the

Table I. Body weight and blood glucose values in three groups.

Characteristic	Control	DM	DM + GSPE
Body weight (g)			
0 weeks	243.52±6.30	241.61±4.76	239.24±6.36
8 weeks	301.62±11.69	242.41±14.63[a]	251.85±12.14
Blood glucose (mg/dl)	94.53±9.03	451.2±18.74[a]	447.25±24.49

Data are presented as the mean ± standard deviation (n=10/group). [a]P<0.001 vs. the control group. DM, diabetes mellitus; GSPE, grape seed proanthocyanidin extract.

retinal sections on the glass slides was performed using a one-step TUNEL Apoptosis Assay kit (cat. no. C1089; Beyotime Institute of Biotechnology), according to the manufacturer's protocol. In order to stain the nucleus, 4'6'-diamino-2-phenylindole dihydrochloride was added for 10 min at room temperature. Following staining, slides were observed at a 550-nm excitation wavelength under an Olympus BX53F microscope. The cells with red fluorescence were defined as apoptotic.

Immunohistochemistry. Immunofluorescence techniques were performed to investigate the expression levels of Nrf2. Briefly, sections were blocked with 10% normal goat serum and 0.1 M phosphate-buffered saline (both Sangon Biotech Co., Ltd.) prior to incubation with rabbit anti-Nrf2 antibody (Abcam, Cambridge, UK) at 4˚C overnight. SP-9000 SP link Detection kits (cat. no. SP-9000-D; ZSGB-BIO, Beijing, China) were used according to the manufacturer's protocol. Slides were counterstained with hematoxylin for detection by light microscopy (BX53F; Olympus Corporation).

Statistical analysis. All data are expressed as the mean ± standard deviation (n≥6/group). Comparisons were performed using one-way analysis of variance for the different groups followed by Dunnett's post-hoc test for all pair comparisons using SPSS software, version 11.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

General characteristics. Despite consuming an increased quantity of food and water, compared with the control rats the DM rats gradually lost weight (242.41±14.63 vs. 301.62±11.69 g; P<0.001) by eight weeks after the induction of diabetes. Furthermore, the average overnight 8-h fasting serum glucose level of the DM rats was 451.2±18.74 mg/ml, which was significantly higher compared with the control group (94.53±9.03 mg/ml; P<0.001). No significant differences in glucose levels were detected between the DM + GSPE and DM groups (447.25±24.49 vs. 451.2±18.74 mg/dl; P=0.968) (Table I).

Retinal morphology. Following HE staining, the retinas of the control group were highly organized with intact layers; whereas disorganized retinas with impaired layers were detected in the DM group. Retinal cells in the DM group were irregularly and loosely arranged and the nerve fiber and ganglion cell layers were narrower, as compared with the control and DM + GSPE groups. These results suggest that GSPE is able to attenuate the disorganization and impairment of the retinal layers associated with DM (Fig. 1).

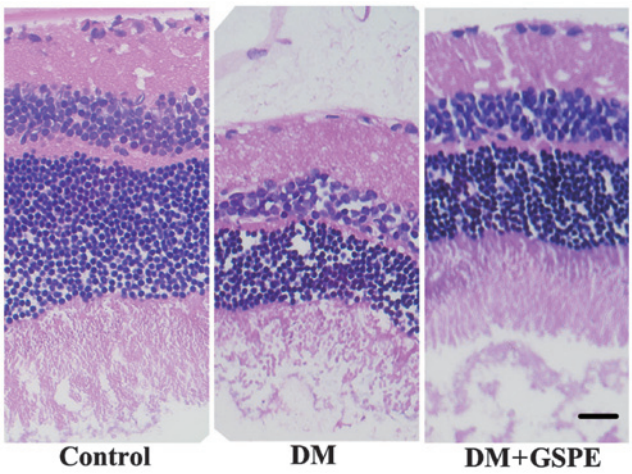


Figure 1. Retinal morphology in rats 2 months after the induction of DM and GSPE treatment. The retinas of the control and DM + GSPE groups were highly organized, with intact layers detected; whereas the DM group exhibited disorganized retinas with impaired layers (scale bar, 20 μm; stain, hematoxylin and eosin). DM, diabetes mellitus; GSPE, grape seed proanthocyanidin extract.

GSPE attenuates oxidative stress in diabetic retina. Table II presents the significant reductions in SOD (n=8; P=0.003) and GSH-Px (n=8; P=0.003) activity levels in the diabetic retina homogenates, as compared with the controls. Following GSPE administration, SOD (n=8; P=0.011) and GSH-Px (n=8; P=0.001) activity levels significantly increased in the DM + GSPE group, as compared with the DM group. Furthermore, MDA levels were significantly increased in the diabetic retina, as compared with the control group (n=8; P=0.002). MDA levels significantly decreased in the DM + GSPE group, as compared with the DM group (n=8; P=0.013) (Table II).

GSPE activates the Nrf2 pathway. Retinal Nrf2 expression levels were increased in the DM + GSPE group, as compared with the DM group (Fig. 2A). Nuclear Nrf2 expression levels were subsequently assessed by western blot analysis. The results demonstrated that Nrf2 protein expression levels in the nucleus were significantly increased in the retinas of the DM + GSPE group, as compared with the untreated DM group (n=6; P=0.038) (Fig. 2B). Furthermore, the expression levels of HO-1, which is the target gene of the Nrf2 pathway (14), were

Table II. Levels of the oxidative stress markers GSH-Px, SOD and MDA in the three groups (n=8 per group).

Marker	Control	DM	DM + GSPE
GSH-Px (U/mg)	18.42±3.38	12.12±2.47[a]	18.03±2.69[b]
SOD (U/mg)	16.63±3.27	10.80±1.54[a]	14.44±2.42[b]
MDA (nmol/mg)	7.09±2.03	16.86±3.97[a]	11.24±1.74[b]

Data are presented as the mean ± standard deviation. [a]P<0.05 vs. the control group; [b]P<0.05 vs. the DM group. GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; MDA, methane dicarboxylic aldehyde; DM, diabetes mellitus; GSPE, grape seed proanthocyanidin extract.

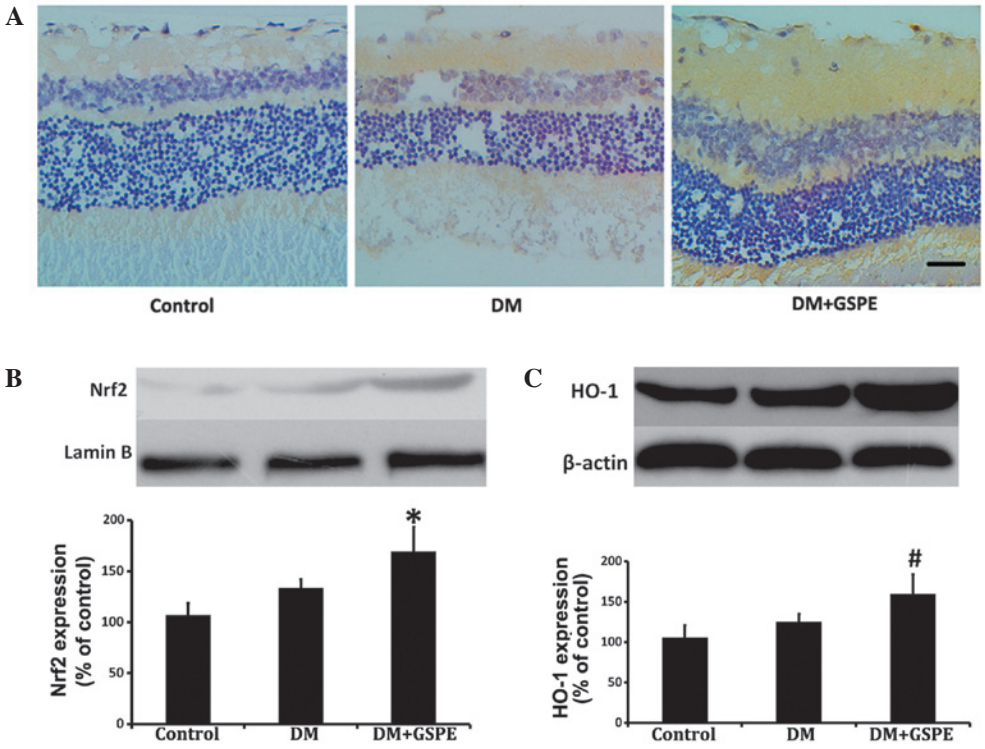


Figure 2. Expression levels of Nrf2 and HO-1 in the control, DM and DM + GSPE groups. (A) Immunohistochemical analysis demonstrated an increase in Nrf2 expression levels in the retinas of rats in the DM + GSPE group. Nrf2 was predominantly expressed in the nerve fibers, ganglion cells and inner plexiform layer of the retina (scale bar, 20 μm; stain, hematoxylin). (B) Western blot analysis showing increased nuclear Nrf2 expression levels in the DM + GSPE group, as compared with the DM group. (C) Western blot analysis showed increased cytoplasmic HO-1 expression levels in the DM + GSPE group, as compared with the DM group. *P=0.038 vs. the DM group; #P=0.043 vs. the DM group (n=6). DM, diabetes mellitus; GSPE, grape seed proanthocyanidin extract; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1.

significantly elevated in the DM + GSPE group, as compared with the untreated DM group (n=6; P=0.043) (Fig. 2C).

GSPE decreases cell apoptosis. The results of TUNEL staining demonstrated that the rate of apoptosis in retinal cells in the DM group was significantly increased, as compared with the control group (n=5; P<0.001). Apoptotic cells were predominantly detected in the nerve fiber, ganglion cell and inner plexiform layers of the retina. Treatment with GSPE significantly decreased the number of apoptotic cells (n=5; P=0.014) (Fig. 3).

Discussion

DR remains the leading cause of blindness in adults of working age worldwide, and this condition may become a leading cause of visual impairment (1-3). Previous studies investigating DR have predominantly been focused on the identification of pathogenic molecules (14). However, the prevention and treatment of DR has been investigated (16) which is particularly relevant to patients with long-standing diabetes (17).

The results of the present study demonstrated that GSPE, which contains natural polyphenols, has a protective effect against DR. Following treatment with GSPE, the retinal morphology of STZ-induced diabetic rats was markedly improved. In particular, retinal cells in the GSPE-treated DM group were tightly arranged in a regular manner, as compared with the DM group, and the nerve fiber and ganglion cell layers increased in thickness. Furthermore, STZ-induced diabetic rats exhibited a reduction in body weight and treatment with GSPE increased the body weight of DM rats to a certain extent,

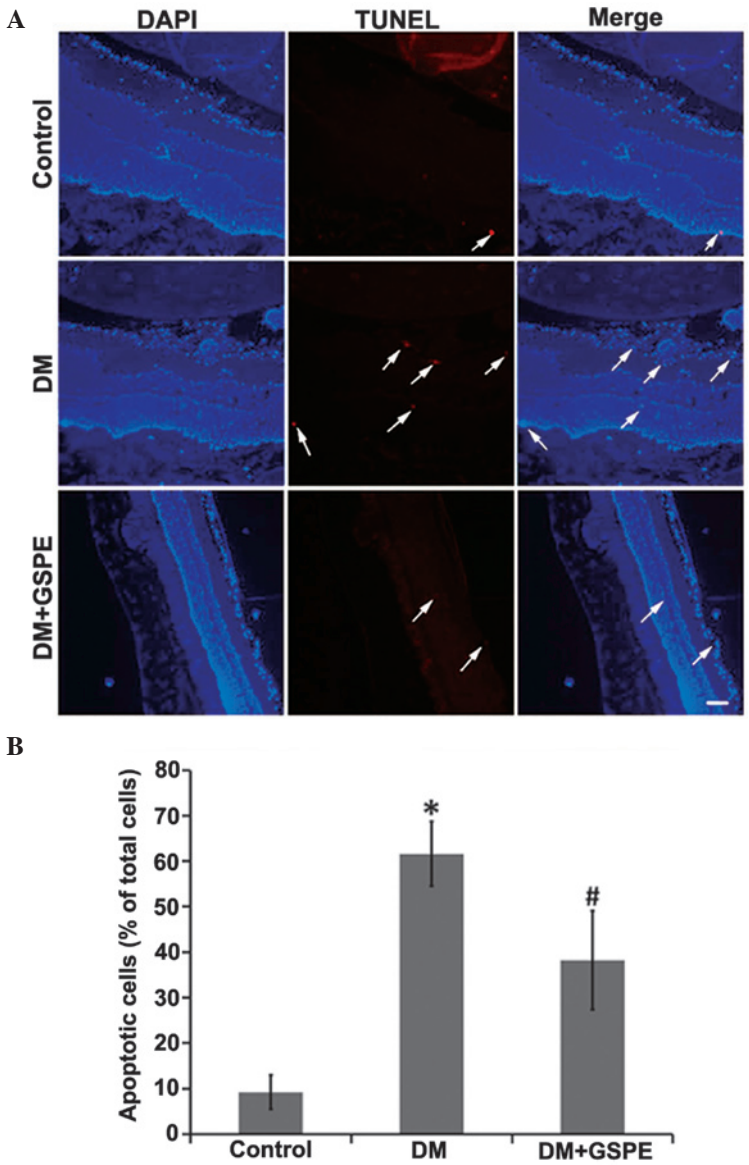


Figure 3. Apoptosis of retinal cells in the control, DM and GSPE-treated DM groups. (A) TUNEL staining demonstrated the apoptosis of retinal cells in the control, DM and GSPE-treated DM groups. DAPI (blue), arrow indicates apoptotic retinal cells (red; scale bar, 50 μm). (B) Following TUNEL staining, slides were observed at 550-nm excitation and cells with red fluorescence were defined as apoptotic. *P=0<001 vs. the control group (n=5); #P=0.014 vs. the DM group (n=5). DAPI, 4'6'-diamino-2-phenylindole dihydrochloride; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; DM, diabetes mellitus; GSPE, grape seed proanthocyanidin extract.

which has been controversial in previous studies (9,18,19). The results of the present study also demonstrated that GSPE was capable of attenuating oxidative stress in diabetic retinas. SOD and GSH-Px activity levels increased following GSPE administration, whereas MDA levels were decreased, which is consistent with previous findings (20).

Following this, the underlying mechanism of the protective effect of GSPE was investigated in diabetic retinas. Although GSPE has been extensively investigated due to its associations with cardiovascular system disease, nervous system disease, diabetic nephropathy, rheumatoid arthritis and human cancers (21,22), there has only been one previous study investigating the effects of GSPE in the retina (9). Li *et al* (9) found that GSPE significantly suppressed the vascular lesions of central regions and decreased capillary enlargements and neovascularization in diabetic retinas by reducing advanced glycation end products. Diabetes-associated increases in advanced glycation end products may induce oxidative stress via various mechanisms, including enhancement of protein kinase C and hexosamine and polyol pathways fluxes (23). Nrf2 is an important protective factor which regulates the progression of DR as a part of the an important cellular pathway protecting against oxidative stress (12). Since it has previously been demonstrated that food polyphenols are capable of modulating the Nrf2 pathway (15), the present study investigated whether GSPE has a protective effect in DR by activating the Nrf2 pathway.

The results of the present study indicated that GSPE exerts protective activity in the retina via the activation of the Nrf2 pathway. The present study demonstrated that the expression levels of Nrf2 and its target gene, HO-1, were markedly increased in the retina following treatment with GSPE. Nrf2 was

predominantly expressed in the nerve fiber, ganglion cell and inner plexiform layers (Fig. 2A). It is well established that, as an antioxidation transcription factor, Nrf2 functions exclusively in the nucleus (14). Furthermore, treatment with GSPE significantly attenuated the apoptosis of retinal cells in the present study. These results suggested that GSPE may be capable of activating the Nrf2 pathway, which may protect diabetic retinal cells against apoptosis.

However, the precise mechanism underlying the anti-apoptotic effect of GSPE and the Nrf2 pathway remain unclear. A previous study has demonstrated that the protective effects of GSPE may be partially attributed to its ability to inhibit anti-death signaling mediated via proapoptotic transcription factors and genes, including c-Jun N-terminal kinase (JNK)-1 and c-Jun (24). Zou *et al* (25) have previously demonstrated that the activation of Nrf2 was capable of preventing oxidative stress-induced apoptosis by hydroxytyrosol in human retinal pigment epithelial cells via the JNK-p62/SQSTM1 pathways. Furthermore, Pehar *et al* (26) demonstrated that decreased Nrf2 expression and the downregulation of the enzymes associated with oxidative stress induces p75 neurotrophin receptor-induced motor neuron apoptosis (26). Furthermore, previous studies have indicated that activation of HO-1, which is the target gene of Nrf2, may protect diabetic retinal cells against apoptosis (27,28). Further studies are required in order to fully elucidate the anti-apoptotic effect of GSPE, and the underlying mechanisms.

In conclusion, the results of the present study suggested that early treatment with GSPE may protect diabetic retinal cells against diabetic retinopathy by attenuating oxidative stress-mediated cellular apoptosis, which may be associated with the activation of the Nrf2 pathway.

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