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Puerarin enhances superoxide dismutase activity and inhibits RAGE and VEGF expression in retinas of STZ−induced early diabetic rats

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ABSTRACT

Objective: To investigate the effects of puerarin on the activity of superoxide dismutase (SOD), and expressions of advanced glycation end−product (AGE) receptor (RAGE) and vascular endothelial growth factor (VEGF) in retinas of streptozotocin (STZ)−induced early diabetic rats. **Methods:** Diabetic rat models were established by inducing diabetes via intra−peritoneal injection of STZ. Rats were randomly divided into normal (control), diabetic (DM), and DM+ puerarin groups. After intra−gastric administration of puerarin (500 mg/kg/day for 4 weeks), levels of SOD and malondialdehyde (MDA) were determined in serum and retina. mRNA and protein expression levels of RAGE and VEGF in retinas were determined by real−time polymerase chain reaction (RT−PCR) (mRNA) and Western blot analysis (protein levels). **Results:** There was significantly lower SOD activity and significantly higher MDA in serum and retinas of the DM group compared with the two other groups ($P<0.05$). After treatment with puerarin, SOD activity increased and MDA content decreased in this group ($P<0.05$). mRNA and protein expression levels of RAGE and VEGF in the DM group were significantly higher than those of the other groups ($P<0.05$), and decreased after puerarin treatment ($P<0.05$). **Conclusions:** Puerarin is able to enhance SOD activity, and inhibit RAGE and VEGF expressions in retinas of STZ−induced early diabetic rats.

1. Introduction

Diabetic retinopathy (DR) is one of the main causes of blindness worldwide, especially in adults (20−70 year old). Although the precise pathogenesis of DR remains unclear, evidence indicates that enhanced hyperglycemia−induced oxidative stress plays a key role in its progression. Hyperglycemia−induced oxidative stress leads to excessive production of reactive oxygen species. Activation of a series of the intracellular signaling pathways regulating gene expression and production of various related cytokines result in chronic diabetic vessel complications[1,2]. Activity

of the free radical scavenger, superoxide dismutase (SOD), is decreased in the diabetic retina, where its expression is downregulated[3,4]. Concentrations of malondialdehyde (MDA), well−known as a universal biomarker of lipid peroxidation (LPO), has been shown to be elevated in the diabetic retina, where it is attenuated by anti−oxidant treatments[5,6].

Many studies indicate that vascular endothelial growth factor (VEGF) plays a critical role in the occurrence and development of DR[7-9]. Experimental evidence further demonstrates that oxidative stress may induce down−regulation of retinal connexin and increase expression of VEGF, thus increasing vascular permeability[10,11]. A number of studies have been undertaken to identify the effects of antioxidants in the initiation and development of DR[12,13]. Advanced glycation end−products (AGEs) have been implicated in the etiology of DR. There is much

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evidence demonstrating that the coupling of AGE and its signal transduction receptor (RAGE) activates vessel inflammation and expression of related genes, thus leading to the development of DR[14–16]. RAGE is a multi–ligand receptor that not only mediates many or all of the sequelae of AGE–cell surface interactions, but also binds other ligands, *eg.*, 100/calgranulins, amphoterin/high–mobility group box–1, and amyloid fibrils[17]. Since RAGE appears to act as a central modulator in DR, it is reasonable to consider suppression of its expression to be a promising therapeutic target for DR[18–20].

Puerarin (8–beta–D–glucopyranosyl–4´,7–dihydroxy–isoflavone; $C_{21}H_{20}O_9$) is extracted from the root of the kudzu vines, *Pueraria lobata* (Willd.) Ohwi or *Pluteus thomsonii* Benth, both species of which belong to the family Leguminosae (Fabaceae) (pulse, pea, or bean) and both of which are used in traditional Chinese medicine. In China, puerarin has been clinically applied to the treatment of diabetes and its complications, *eg.*, diabetic peripheral neuropathy, nephropathy, and DR[21–23]. Recently, several studies have shown that puerarin, as an effective, natural free–radical scavenger, is capable of preventing the damage caused by free radicals and LPO[24,25]. Puerarin increases the activities of SOD and catalase during oxidative stress, and strengthens the response capability of islet cells against oxidative stress, which leads to cell apoptosis[21]. Puerarin had a significant inhibitory effect on the non–enzyme glycosylation reaction. Shen *et al* reported that puerarin had notable inhibition effects on RAGE levels in the kidney and aorta of diabetic rats[26,27]. However, whether puerarin is able to increase SOD activity and decrease RAGE and VEGF expressions in retinas of diabetic rats is not known. Thus, the aim of the present study was to investigate the effects of puerarin on SOD activity and on expressions of RAGE and VEGF in the retinas of streptozotocin (STZ)–induced diabetic rats.

2. Materials and methods

2.1. Animals

Healthy male Sprague–Dawley rats (*n*=30) (8–10 weeks old, 200–230 g) were purchased from the Experimental Animal Center of Zhejiang Province. Animals were treated in accordance with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. This study was approved by the Animal Ethics Committee of Yangzhou University. All rats were maintained on a 12–h alternating light (08:00–20:00)/dark (20:00–08:00) cycle, at a temperature of 22–25 ℃ and humidity of 55%–60%. Rats were divided randomly into diabetic and control groups. Diabetes was induced by intra–peritoneal injection of a freshly prepared solution of STZ (Sigma, St Louis, MO, USA) in citrate buffer (pH 4.5), at a dosage of 60 mg/kg body weight (BW), as previously descibed[28]. Non–diabetic control rats (*n*=10) received a

volume of 0.5 mL citric buffer only. Animals with blood glucose levels >250 mg/dL 72 h after administration of STZ were considered to be diabetic. Diabetic rats were randomly divided into two groups: the DM+ puerarin group (*n*=10) received intra–gastric puerarin (Zhengda Tianqing Pharmaceutical Company, Nanking, China; purity = 99.8%) at a dosage of 500 mg/kg/day[26] starting on the day of successful diabetes induction; the DM group (*n*=10) was treated similarly but with normal saline only. Treatment was performed once each day every ~24 h. All animals were allowed to eat and drink freely. BW and blood glucose levels were measured weekly. After 4 weeks of treatment, animals were sacrificed by systemic anesthesia 3 h after final drug administration. After sacrifice, serum, eyeballs, and retinas were harvested for further testing.

2.2. Determination of MDA level and SOD activity

Following 4 weeks of puerarin treatment, six rats from each group were anesthetized with an intra–peritoneal injection of 2% pentobarbital sodium. Blood samples were obtained by cardiac puncture. Blood was centrifuged at 2 500 *g* for 10 min, and the serum retained for later analysis. Rats were sacrificed with an overdose of pentobarbital sodium. Eyeballs were then removed, and the retinas stripped under an anatomical microscope and stored at 4 ℃.

According to the weight of the retina, as determined with an electronic balance, pre–cooled normal saline was prepared (1:99 ratio). After the retina was shattered using an ultrasonication meter (60 s for homogenate) and centrifuged at 2 500 r/min for 10 min, the supernatant obtained was analyzed for MDA and SOD. MDA was measured using thiobarbituric acid colorimetric methods; SOD activity was tested for using the xanthine oxidase method (Jiancheng Agent Company, Nanking, China), according to the manufacturer´s instructions.

2.3. Real–time polymerase chain reaction (RT–PCR)

Neurosensory retinas, which had been stripped and stored in liquid nitrogen, from four rats from each group were used for RT–PCR and Western blot analyses. The retina of one eye of each rat was used for RT–PCR and that of the other eye for Western blot analysis. For RT–PCR, RNA was isolated from retinas for cDNA synthesis. The PCR method (ABI Prism 7500; PE Biosystems) was utilized to determine elevated gene expression levels of RAGE and VEGF. The primers (Bioneer, Inc, Korea) for RAGE were 5´–AGGCTCTGTGGATGGGTCTGG–3´ (sense) and 5´–CATGGATCATGTGGGCTCTGG–3´ (antisense), and those for VEGF were 5´–GTGGACATCTTCCAGGAGGAGTA–3´ (sense) and 5´–CTCTGAACAAGGCTCACAGT–3´ (antisense).

All reactions were performed according to standard procedures. Amplification conditions were as follows: 50 ℃ for 2 min, with an initial denaturation at 95 ℃ for 10 min, followed by 40 cycles of denaturation at 95 ℃ for 15 s, and annealing at 60 ℃ for 1 min. Each reaction

was run in duplicate, three times each, and the results expressed as the mean±SD. β−actin was used as a loading control and internal standard. The primers for β−actin were 5′−GCACCGCAAATGCTTCTA−3′ (sense) and 5′−GGTCTTTACGGATGTCAACG−3′ (antisense). The final results were normalized by comparing them with those obtained from controls; the control group was set as 1.0.

2.4. Western blot analysis

Samples were treated using pre−cooled cell lysate at a 1:6 ratio (weight/volume) after weighing, and were homogenized at 4 ℃. After incubation on ice for 1 h, extracts were clarified by centrifugation at 20 000 *g* for 30 min. Equal amounts of supernatant were mixed with 2.5 mL of 2× sampling buffer and heated at 100 ℃ for 3−5 min. Protein concentrations were determined using the Bradford method. Proteins were electrophoretically separated on 10% sodium dodecyl sulfate−page gels, with each lane receiving 20 μg of sample. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane, using the whole wet−mount method. Membranes were blocked with 5% nonfat milk (1 h) and rabbit anti−rat RAGE (1:1 000 dilution) (Cell Signaling Technology, Beverly, MA. USA). VEGF antibodies (1:1 000 dilution) (Cell Signaling Technology) were added and incubated overnight at 4 ℃, after which they were incubated with goat anti−rabbit IgG/HRP (1:6 000) for 2 hrat room temperature. An enhanced chemiluminescence reaction agent was reacted with the membrane for 3 min, and sheeting used to expose the membrane for 2 min. The gray scale of the specific band was analyzed quantitatively using image analysis software.

2.5. Statistical analysis

All data are expressed as mean±SD. Analysis of variance and Dunnett′s *t*−test were performed using the SPSS 13.0 statistical software package. A level of *P*< 0.05 was considered statistically significant.

3. Results

3.1. Characteristics of experimental animals

Levels of blood glucose and BW were used to indicate the severity of diabetes. During experiments, control group rats were active, and had a normal diet, smooth fur, and obvious BW gain. Compared with control group rats, rats in the DM group moved slowly, and had a dull color pattern, increased blood glucose, and BW loss. Four weeks after the onset of diabetes, differences between the DM and control group rats, vis−a−vis fasting blood glucose levels and BW, were statistically distinct (*P*<0.05). Rats in the DM+ puerarin group fared better than those in the DM group, *ie.*, fasting blood glucose levels were lower than that those of DM group rats

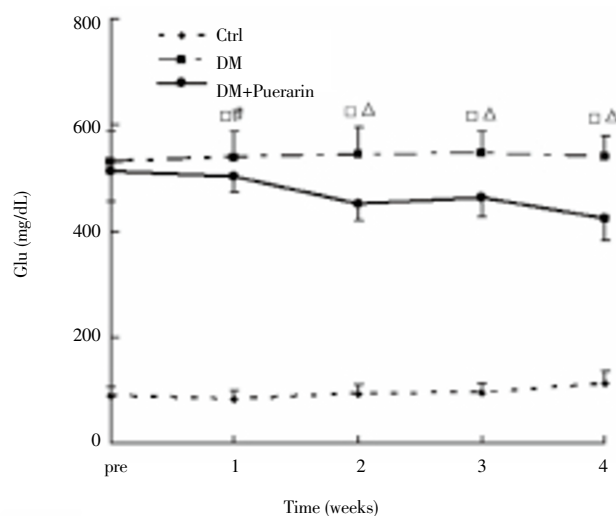(*P*<0.05), albeit still higher than those of control group rats (*P*<0.05) (Figures 1, 2).

Figure 1. Fasting blood glucose levels of rats in the various groups (*n*=10 in each).
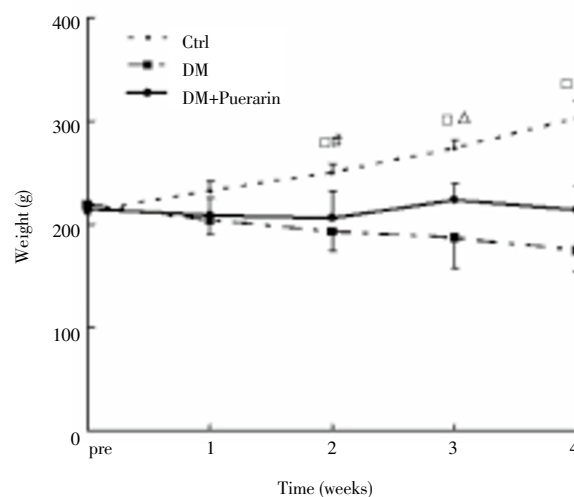□*P*<0.05, DM+Puerarin versus Ctrl; #*P*>0.05, DM+Puerarin versus DM; △*P*<0.05, DM+Puerarin versus DM.

Figure 2. Body weights of rats in the various groups (*n*=10 in each).
□*P*<0.05, DM+Puerarin versus Ctrl; #*P*>0.05, DM+Puerarin versus DM; △*P*<0.05, DM+Puerarin versus DM.

3.2. MDA level and SOD activity in serum and retina

Four weeks after onset of diabetes, MDA concentrations in serum and retinas of DM group rats were significantly higher than those of control group rats (*P*<0.05), while those of DM+ puerarin group rats were lower than those of DM group rats (*P*<0.05), with no significant differences compared with those of control group rats (*P*>0.05). SOD activity in serum and retinas of DM group rats were notably lower than those in the control group (*P*<0.05). Compared with DM group rats, SOD activity in serum and retina of DM+ puerarin group rats was significantly increased (*P*<0.05), with no significant

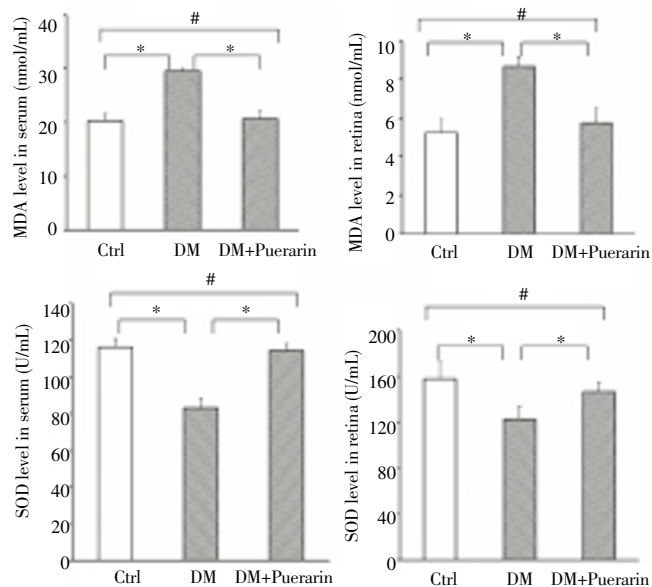differences when compared with control group rats (*P*>0.05) (Figure 3).

Figure 3. MDA concentration and SOD activity in serum and retina in the various groups (*n*=6 in each).
A: MDA concentration in serum; B: MDA concentration in retina; C: SOD activity in serum. D: SOD activity in retina. *P*<0.05; #*P*>0.05.

3.3. Expression of RAGE and VEGF mRNA in retina

RT–PCR data revealed expression levels of RAGE and VEGF mRNA in DM group rats to be higher than those in normal control group rats (*P*< 0.05). Expression of mRNA levels of RAGE and VEGF in the DM+ puerarin group were greatly decreased and showed significant differences compared with the DM group (*P*<0.05); no significant difference was found compared to the normal control group (*P*>0.05) (Figure 4).
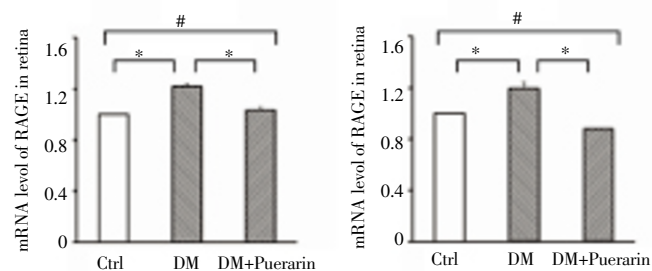

Figure 4. mRNA expression levels of RAGE and VEGF in the various groups (*n*=4 in each).
A: mRNA expression levels for RAGE; B: mRNA expression levels for VEGF. *P*<0.05; #*P*>0.05.

3.4. Protein levels of RAGE and VEGF in retina

Results of Western blot analysis indicated low expressions of RAGE and VEGF in retinas of normal control group

rats. Expressions of these proteins in DM group rats were significantly higher than those in normal control group rats (*P*<0.05). In contrast, expressions of RAGE and VEGF in DM+ puerarin group rats were decreased in comparison with those in DM group rats (*P*<0.05) and similar to those in normal control group rats (*P*>0.05) (Figure 5).
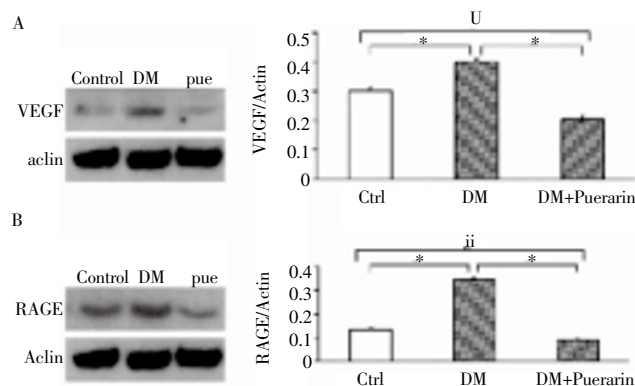

Figure 5. RAGE and VEGF protein levels in the various groups (*n*=4 in each).
A: Protein levels for RAGE; B: Protein levels for VEGF. *P*<0.05; #*P*>0.05.

4. Discussion

MDA, the end–product of oxidative stress–induced LPO, is also an index of LPO activity. Thus, MDA content can be employed as a measure of the severity of oxidative stress–induced damage. MDA concentrations were shown to be increased in the eyes of diabetic animals, and treatment with two different antioxidants, ebselen and lutein, restored MDA concentrations to control values[29]. SOD is one of the enzymes that are essential for combating oxidative damage; its activation allows scavenging of the superoxide radical. Its activity, therefore, is reflective of the anti–oxidative ability of a tissue. Accumulating evidence points to the excessive oxidative stress that results from increased free radical production as being a major underlying cause of diabetes–related ocular pathologies[30–34]. Therapies that inhibit development of retinopathy in diabetic rats, ie., amino guanidine and antioxidants, not only prevent diabetes–induced accumulation in retinal superoxide, but also thwart inhibition of SOD activity[4,35]. In the present study, we found that the levels of MDA in the DM group increased significantly and that SOD activity was notably lower, compared with the normal control group. Following treatment with puerarin, concentrations of both retinal and serum MDA decreased to normal levels, while SOD activities in both retina and serum increased to normal levels. These results indicate that puerarin improves SOD activity and attenuates LPO in early diabetic rat retina.

Studies have shown that antioxidants can prevent

VEGF up–regulation. Obrosova *et al*[36] reported that the antioxidants, taurine and DL–alpha–lipoic acid, significantly reduced early up–regulation of retinal VEGF in diabetic rats. Oral administration of the antioxidant calcium dobesilate also reduced retinal permeability in diabetic rats, as well as decreased both formation of AGEs and excessive expression of VEGF[11]. In our study, we also found that VEGF increased significantly at the protein and mRNA levels in diabetic rat retinas, consistent with the findings of Hammes *et al*[7,11] but decreased after puerarin intervention, which delayed the appearance of DR; this latter finding is consistent with those of Teng *et al*[37]. However, it was difficult for us to determine the mechanism(s) responsible for the reversal effect of puerarin on up–regulated VEGF levels. Our data indicate that although puerarin had some effects in decreasing blood glucose levels, these effects were not very strong. The fasting blood glucose levels of the puerarin treatment group at 4 weeks [(423.18±41.58) mg/dL] were still significantly higher than those of the normal control group [(113.76±9.00) mg/dL] ($P<0.05$). However, expression levels of VEGF and the marker for oxidative damage in the puerarin treatment group were reversed, approaching normal levels. Bebrevska *et al*[24] observed no lowering of blood glucose after treatment with puerarin at a daily dose of 50 mg/kg for 3 weeks, while plasma levels of MDA had decreased to normal. It appears that the antioxidant effects of puerarin are independent of its effect on blood glucose control. Therefore, we presume that the reversal of VEGF by puerarin in diabetic rat retina is not directly related to its effects on decreasing blood glucose, but is likely related to its antioxidant properties. Verification of this premise needs further investigation.

RAGE is a signal–transducing receptor for AGEs. Engagement of RAGE by AGEs elicits oxidative stress, induces vascular inflammation, and alters gene expression in retinal vascular wall cells, all of which point to its involvement in DR[14,15]. AGEs can increase VEGF expression of retinal cells[38]. The action of AGEs on RAGE was shown to be the source of oxidative stress in endothelial cells in retina; thus inhibition of RAGE expression might represent a potential target for DR treatment[16–18,39]. Puerarin has notable inhibitory effects on AGEs and RAGE levels in the kidney, and could be utilized for diabetic nephropathy therapy[27,40]. Another important finding of our study was the puerarin–mediated amelioration of up–regulated RAGE levels in diabetic retinas, which might suggest that puerarin ameliorates oxidative stress in early diabetic rat retinas via inhibition of retinal RAGE expression.

In summary, our study shows that puerarin enhances SOD activity, down–regulates expression levels of RAGE and VEGF in diabetic rat retina, and provides a foundation for further investigations into application of puerarin for the management of DR.

Conflict of interest statement

We declare that we have no conflict of interest.

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