Puerarin decreases apoptosis of retinal pigment epithelial cells in diabetic rats by reducing peroxynitrite level and iNOS expression

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Abstract: The purpose of this study was to investigate the protective effect of puerarin on retina pigment epithelial (RPE) cells of diabetic rats against apoptosis. One hundred and eight Sprague-Dawley (SD) rats were randomly divided into 3 groups: control group, streptozotocin (STZ) group and puerarin group. STZ and puerarin groups received 3 d of STZ injection (45 mg/kg per day, i.p.). Additionally, puerarin groups were treated with puerarin (140 mg/kg, i.p.) from the 4th day to the end of experiment. The rats from different groups were sacrificed on 20, 40 and 60 d after STZ injection for harvesting RPE cells. Western blot analysis, DNA laddering, RT-PCR and immunohistochemistry were used for determining the expression of nitrotyrosine (NT, the foot print of peroxynitrite), cell apoptosis, iNOS mRNA and Fas/Fas ligand (FasL) signal transduction in RPE cells, respectively. The results showed that control group maintained low apoptosis level and little NT, iNOS mRNA, Fas/FasL protein expressions, as well as normal blood glucose and body weight during 60 d of the experiment. Compared with control group, STZ group showed obvious apoptosis and higher NT, iNOS mRNA, Fas/FasL protein expressions from 20 d after STZ injection. Puerarin relieved apoptosis of RPE cells and decreased NT, iNOS mRNA, Fas/FasL protein expressions in puerarin group 20 or 40 d after STZ injection, compared with STZ group. These results suggest puerarin can decrease RPE cells apoptosis in diabetic rats by reducing peroxynitrite level and iNOS expression, thus being a potential therapeutic agent in controlling of diabetic retinopathy.

Key words: puerarin; retinal pigment epithelial cells; oxidative; cell signal; diabetes

Gruenferin通过下调过氧亚硝基阴离子水平和诱导型一氧化氮合酶表达来减少糖尿病大鼠视网膜色素上皮细胞凋亡

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摘要: 本研究旨在观察葛根素对糖尿病大鼠视网膜色素上皮(retinal pigment epithelial, RPE)细胞凋亡的影响及其机制。采用大鼠腹腔注射链脲佐菌素(streptozotocin, STZ)的方法建立糖尿病动物模型。108只Sprague-Dawley (SD)大鼠纳入实验，随机分为三组：对照组(腹腔注射生理盐水)、STZ组和葛根素组，每组n = 36。STZ组和葛根素组大鼠均接受3 d的STZ腹腔注射(每天45 mg/kg)。第4天起，葛根素组每天腹腔注射葛根素(140 mg/kg)，直至第60天实验结束。腹腔注射STZ后20、40和60 d分批处死动物，分离出RPE细胞。用免疫印记方法检测过氧亚硝基阴离子(peroxynitrite, ONOO−)的标志物——硝基酪氨酸(nitrotyrosine, NT)和Fas/FasL蛋白表达情况，用DNA laddering检测RPE细胞的凋亡情况，用RT-PCR检测iNOS的表达，用免疫组

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Diabetic retinopathy (DR) can cause irreversible blindness and is the severest complication in the eyes of patients with diabetic mellitus (DM). The identification of susceptibility factors contributing to development of DR is helpful for identifying susceptible patients and improving treatment efficacy. Retinal pigment epithelial (RPE) cells form a monolayer of cuboidal cells located between the photoreceptors of the neurosensory retina and the choroidal capillary bed. The RPE comprises an important blood-retinal barrier component and performs many important functions essential to the visual process[1–5]. It is well known that superoxide can interact with inducible nitric oxide synthase (iNOS)-produced nitric oxide and form the peroxynitrite (ONOO$^-$), a strong oxidant to induce oxidative stress. Moreover, ONOO$^-$ plays an important role in diabetic retinal degeneration[6, 7]. Puerarin, a major isoflavonoid derived from the Chinese medical herb radix puerariae, has been reported to be useful in the treatment of many diseases[8, 9]. We previously reported that puerarin was capable of down-regulating iNOS mRNA and protein in RPE and lens epithelial cells in a time-dependent manner[10, 11]. To better understand the protective effects of puerarin, we examined the effects of puerarin on iNOS and its products ONOO$^-$, as well as Fas, cell death surface receptor, and its ligand (FasL) signal pathways during streptozotocin (STZ)-induced apoptosis of RPE cells.

1 MATERIALS AND METHODS

1.1 Animals
Pathogen-free, male, Sprague-Dawley (SD) rats (5–6 weeks old) were used. All 108 animals were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the use of Animals in Ophthalmic and Vision Research in China.

1.2 Antibodies and reagents
Monoclonal mouse anti-nitrotyrosine (NT) antibody, Fas/FasL antibody, goat anti-mouse fluorescein isothiocyanate (FITC) antibody, and STZ, were purchased from Sigma Co. (St. Louis, MO, USA). Puerarin was the product of Kangenbei Pharmaceutical Limited Corporation, China. Dispase and MEM culture were from Invitrogen-Gibco (NY, USA).

1.3 Groups and animal model
Animals were divided into three groups: control, STZ and puerarin groups ($n = 36$ in each group). SD rats in STZ and puerarin groups were daily intraperitoneally (i.p.) injected with STZ (45 mg/kg) for 3 d to establish diabetic animal model. The rats in the control group received the same volume of saline. From the fourth day, the rats in puerarin group received daily injection of puerarin (140 mg/kg, i.p.) until 60 d after STZ injection. Animals were sacrificed on the days 20, 40 and 60 after STZ injection.

1.4 Harvesting of RPE sheets
RPE sheets were harvested from right eyes of the rats from different groups. The eyecup was isolated from freshly enucleated rat eye, then incubated with 25 U/mL dispase for 30 min and rinsed with CO2-free medium (CFM). With a circumferential incision into the subretinal space along the ora serrata, the RPE sheets were separated from the ocular tissue and placed on a slice of 50% gelatin. The gelatin film containing the RPE sheet was then incubated in a humidified atmosphere of 5% CO2 and 95% air at 37 °C for 5 min, allowing the gelatin to melt and encase the RPE sheet. Finally, the slice was kept at 4 °C for 5 min to solidify the liquid gelatin and then stored in CFM at 4 °C.

1.5 Western blotting
On days 20, 40 and 60 after STZ injection, three rats in each group and at each time point were sacrificed. RPE cells were prepared and homogenized in ice cold PBS, which contains phenylmethylsulfonyl fluoride (1 μg/mL), aprotinin (1 μg/mL), leupeptin (1 μg/mL), pepstatin A (1 μg/mL) and EDTA (1 mmol/L). The homogenate was centrifuged at 15 000 r/min at 4°C for 10 min,
then protein content of the supernatant was determined by the Bradford method [12]. After SDS-PAGE on 12% linear slab gel under reducing conditions, separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane using a semidy electrophoretic transfer cell (Trans-blot; Bio-Rad, Richmond, CA). Blots were incubated with monoclonal mouse anti-NT antibody (1:600) or monoclonal mouse Fas/FasL antibody (1:800) overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibody. Then the blots were developed using the enhanced chemiluminescence Western blot analysis detection system (ECL Plus; Amersham Pharmacia Biotech, UK). β-actin band was used as an internal reference.

1.6 DNA laddering analysis
On days 20, 40 and 60 after STZ injection, three rats in each group and at each time point were sacrificed. Apoptosis of RPE cells was detected by DNA laddering as described by Herrmann et al. [13].

1.7 Reverse transcription polymerase chain reaction (RT-PCR)
On days 20, 40 and 60 after STZ injection, three rats in each group and at each time point were sacrificed. Total RNA was extracted from the rat RPE cells in three groups, according to the kit manufacturer’s specifications (GeneAmp RNA-PCR kit; Applied Biosystems, USA). Equal amounts of the total RNA were used to detect the mRNA levels of iNOS by RT-PCR. The upstream and downstream oligonucleotide primers for rat iNOS were synthesized by Biological Engineering Corporation (Shanghai, China). The primer sequences are: iNOS (262 bp) upstream primer: 5’-CGCCCTTCCGCAGTTCT-3’; downstream primer: 5’-TCCAGGAGGACATGCAGC-3’. β-actin (420 bp) upstream primer: 5’-GAGACCTTCAACCCAGGC-3’; downstream primer: 5’-GCAGGATCGAACCAGCCTCA-3’. And 4 μg of RNA in a total volume of 20 μL (pH 8.3) were for synthesizing the cDNA. RT-PCR was first performed at 24 °C for 10 min, then at 42 °C for 15 min. The reaction mixture was heated at 99 °C for 5 min, and the RT product was mixed with DNA polymerase (AmpliTaq; Applied Biosystems, USA) and the primers in a buffer containing 20 mmol Tris-HCL, 50 mmol KCl, 2.0 mmol MgCl₂ (pH 8.3), and 50 mmol of each dNTP in a 100 μL volume. The mixture was then amplified by PCR using 29 cycles. The thermal cycle profile used in this study was as follows: an initial de-

2 RESULTS

2.1 STZ induced establishment of diabetic animal model
Compared with control group, STZ group showed higher blood glucose level and lower body weight on days 20, 40 and 60 after STZ injection, suggesting the successful establishment of diabetic animal model. Compared with STZ group, puerarin group showed lower level of blood glucose and higher body weight on days 20, 40 and 60 after STZ injection, suggesting ameliorating effect of puerarin (Table 1).

2.2 Puerarin decreased NT expression in RPE cells
Control group showed a faint expression of NT, and the expression kept constant during 60 d of the experiment. The expression of NT exhibited time-dependent in-
crease in STZ group. But the expression of NT in puerarin group changed gradually from faint to strong at 40 d after STZ injection, then turned to weak at 60 d after STZ injection (Fig. 1). Quantitative analysis indicated that NT expressions in STZ and puerarin groups were higher than those in control group on 20, 40 and 60 d after STZ injection ($P < 0.05$ or $P < 0.01$). Compared with STZ group, puerarin group showed lower NT expressions from 20 to 60 d after STZ injection ($P < 0.05$) (Fig. 1).

2.3 Puerarin decreased apoptosis of RPE cells

There was no appearance of DNA ladder band in the control group, but there was distinct typical DNA ladder band in the STZ group on 20, 40 and 60 d after STZ injection ($P < 0.05$ or $P < 0.01$). Compared with STZ group, puerarin group showed lower NT expressions from 20 to 60 d after STZ injection ($P < 0.05$) (Fig. 1).

### Table 1 Effects of puerarin on blood glucose (mmol/L) and body weight (g) in diabetic rats

<table>
<thead>
<tr>
<th>Time after injection (d)</th>
<th>Control group</th>
<th>STZ group</th>
<th>Puerarin group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood glucose</td>
<td>Body weight</td>
<td>Blood glucose</td>
</tr>
<tr>
<td>20</td>
<td>3.69 ± 0.13</td>
<td>319.29 ± 2.22</td>
<td>20.99 ± 0.27$^{**}$</td>
</tr>
<tr>
<td>40</td>
<td>3.71 ± 0.19</td>
<td>390.11 ± 2.25</td>
<td>23.01 ± 0.29$^{**}$</td>
</tr>
<tr>
<td>60</td>
<td>3.68 ± 0.19</td>
<td>469.96 ± 2.55</td>
<td>24.56 ± 0.45$^{**}$</td>
</tr>
</tbody>
</table>

$^*P < 0.05$, $^{**}P < 0.01$ vs control group; $^*P < 0.01$ vs puerarin group. Means ± SD, $n = 12$.

2.4 Puerarin decreased expression of iNOS mRNA in RPE cells

There was little expression of iNOS mRNA in the control group during 60 d of the experiment. The level of iNOS mRNA exhibited time-dependent increase in STZ group. But the level of iNOS mRNA in puerarin group appeared gradual up-regulation from 20 to 40 d, then decreased at 60 d after STZ injection (Fig. 3). Quantitative analysis indicated that iNOS mRNA levels in STZ and puerarin groups were higher than that in control group on 20, 40 and 60 d after STZ injection ($P < 0.05$ or $P < 0.001$). Compared with STZ group, puerarin group showed lower iNOS mRNA level from 20 to 60 d after STZ injection ($P < 0.001$, Fig. 3).

2.5 Puerarin decreased Fas/FasL transduction in RPE cells

Immunohistochemistry staining revealed that the specific expression of Fas/FasL was yellow, brown-yellow or brown staining in the cell nucleus and cytoplasm of RPE cells. In the control group, a very faint yellow color could be observed during 60 d of the experiment. In STZ group, the staining ranged from yellow to brown-yellow, then to brown in the cell nucleus and cytoplasm. The staining could be observed to increase gradually on 20 to 40 d, then decreased at 60 d in the puerarin group (Fig. 4).

Western blot showed there was little expression of Fas/FasL in the control group during 60 d of the experi-
ment. The level of Fas/FasL expression exhibited time-dependent increase in STZ group. While the level of Fas/FasL expression in puerarin group appeared gradual up-regulation from 20 to 40 d, then decreased at 60 d.

Fig. 3. Expression of iNOS mRNA in RPE cells detected by RT-PCR. Lane M: marker. Lane 1: Control group. Lane 2–4: STZ group at 20, 40, and 60 d respectively. Lane 5–7: Puerarin group at 20, 40, and 60 d respectively. Means ± SD, n = 3. *P < 0.05, **P < 0.001 vs control group; #P < 0.001 vs puerarin group.

Fig. 4. Expression of Fas/FasL in RPE cells from different groups detected by immunohistochemistry. A faint expression of Fas/FasL could be seen in the control group. Expression of Fas/FasL turned to strong time-dependently in STZ group. But the expression of Fas/FasL in puerarin group changed gradually from faint to strong from 20 to 40 d, then turned to weak at 60 d after STZ injection. Scale bar, 100 μm. The arrows indicate layers of RPE cells.

Fig. 5. Fas/FasL protein expression in RPE cells from different groups detected with Western blotting. Lane 1: Control group. Lane 2–4: STZ group at 20, 40, and 60 d respectively. Lane 5–7: Puerarin group at 20, 40, and 60 d respectively. Means ± SD, n = 3. *P < 0.05, **P < 0.01 vs control group; #P < 0.05 vs puerarin group.
after STZ injection (Fig. 5). Quantitative analysis indicated that Fas/FasL expression levels in STZ and puerarin groups were higher than that in control group on 20, 40 and 60 d after STZ injection \((P < 0.01 \text{ or } P < 0.05)\). Compared with STZ group, puerarin group showed lower Fas/FasL level on 20, 40 and 60 d after STZ injection \((P < 0.05)\) (Fig. 5).

3 DISCUSSION

RPE cells are exposed to continual oxidative stress throughout lifespan. This oxidative stress arises from several sources including photoreceptor outer segment, phagocytosis, exposure to peroxidized lipid membranes, and photooxidative reactive oxygen intermediates, especially ONOO−, and oxidative stress developed from ONOO− are popular in other events[7,14–20]. Using RPE cells isolated from diabetic rat model, we previously tested the hypothesis that ONOO− may induce oxidative stress through up-regulation of iNOS and generation of ONOO− to mediate retinopathy in RPE cells[21,22]. In the present study, we further explored whether puerarin could protect RPE cells of diabetic rats through inhibiting iNOS up-regulation and ONOO− generation mediated through Fas/FasL signal pathway.

The results of the present study showed that the intensity of NT, DNA damage, iNOS mRNA and the number of Fas/FasL-positive cells increased in STZ group. So it could be believed that the deterioration of above-mentioned indexes was the expression evidence of the apoptosis on RPE cells. Yu et al.[23] have verified puerarin inhibits angiopoiesis of endometriotic tissue and regulates tumor-related gene expression of endometriosis, as well as reduces the occurrence of apoptosis and improves neurotrophic function of astrocytes. Similarly, puerarin relieved apoptosis of RPE cells and decreased NT, iNOS mRNA expressions in the present study, which indicates a protective role of puerarin on RPE cells of diabetic rats.

Strategies for preservation of vision that would interrupt the apoptotic signal require more understanding of the molecular events associated with apoptosis. This study investigated the susceptibility of RPE to Fas/FasL-dependent apoptotic pathways when challenged with different stimuli. The specific overexpression of Fas/FasL in RPE cells was correlated with the ONOO− accumulation in the RPE of diabetic rat model. Interaction of the death receptor and death ligand is one of the main ways to induce apoptosis, of which, the Fas/FasL system is considered as the major signal transduction pathway to mediate the apoptosis[24–27]. The occurrence and development of many eye diseases are related to the regulation imbalance of RPE cell’s apoptosis[28–31]. Oxidative stress-induced RPE cell apoptosis has been proposed as a major pathophysiological mechanism in age related macular degeneration (AMD)[32]. The puerarin can protect RPE cells from apoptosis in response to several different types of stimuli. We infer that puerarin may promote cell survival by phosphorylating and thereby inhibiting the proapoptotic proteins. This leads in turn to the inhibition of effector Fas/FasL. Puerarin inhibits apoptosis early in the Fas/FasL cascade, antagonizing the activation of initiator Fas/FasL. This inhibition may involve inhibition of Fas/FasL recruitment to the death domain receptors. This role in regulating initiator Fas/FasL is an entirely novel role and suggests a new mechanism by which puerarin promotes cell survival. Taken together, puerarin suppressed the oxidative stress events. Further studies are needed to provide more supports to this contention. These results highlight the cell-type specificity of therapeutic strategies to reduce apoptotic cell death. We hypothesize that puerarin may provide RPE cells additional protection from ONOO−-induced cell death. Although it is beyond the scope of the present work, studies are currently under way in our laboratory to test this hypothesis.

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