Quercetin inhibited epithelial mesenchymal transition in diabetic rats, high-glucose-cultured lens, and SRA01/04 cells through transforming growth factor-β2/phosphoinositide 3-kinase/Akt pathway

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Abstract

Diabetic cataract (DC), an identified life-threatening secondary complication of diabetes mellitus, has proven to be a dilemma because of its multifactorial caused and progression. An increasing number of studies have shown that in addition to the maillard reaction, enhanced polyol pathway, and oxidative insults, epithelial mesenchymal transition (EMT) is related to the prevalence of DC. Quercetin, a classic flavonoid with multiple pharmacological effects has been reported to possess therapeutic efficacy in the management and treatment of this disease. However, the mechanism underlying its therapeutic efficacy in EMT of lens epithelial cells (SRA01/04) and contribution to resolving DC remains a mystery. Therefore, in this study, we investigated the effects of quercetin on EMT of SRA01/04 and high-glucose (HG)-induced lens opacity accompanied by lens fibrosis induced by type-1 diabetes. Furthermore, we sought to clarify the specific mechanisms underlying these effects. At week 14 after streptozotocin (STZ) intraperitoneal administration, diabetic rats showed lens opacity accompanied with diminished antioxidant function, enhanced polyol pathway activity, and oxidative stress. Western blotting confirmed EMT in rat SRA01/04 cells with significantly increased α-smooth muscle actin (α-SMA) and decreased E-cadherin expressions. Treatment of the lens with quercetin ameliorated the oxidative stress, inhibited aldose reductase (AR) activation, reduced advanced glycation end product (AGE) production, and finally suppressed EMT in the early stages. Our in vitro results showed that high-glucose activated the transforming growth factor-β2/phosphoinositide 3-kinase/protein kinase B (TGF-β2/PI3K/Akt) signalling and EMT in SRA01/04 cells. Further, induced oxidative stress, activation of aldose reductase, and accumulation of advanced glycation end products were also involved in this process. Quercetin was potent enough to effectively ameliorate the high glucose (HG)-induced EMT of SRA01/04 cells by inhibiting the activation of TGF-β2/PI3K/Akt, enhancing the antioxidant capacity, inhibiting AR activity, and reducing AGE production. From the whole animal to tissues, and finally the cellular level, our results provide considerable evidence of the therapeutic potential of quercetin for DC. This might be due to its inhibition of EMT mediated through inhibition of the TGF-β/PI3K/Akt pathway.

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1. Introduction

Hyperglycaemia is a risk factor for numerous diabetic complications including diabetic cataract (DC), DC, which is characterized by lens opacity, is one of the earliest secondary complications of diabetes mellitus associated with hyperglycaemia (Pollreisz and Schmidt-Erfurth, 2010; Albulescu and Zolog, 2011). Chronic hyperglycaemia induces oxidative insults, osmotic damage, and
non-enzymatic glycation processes in optical lens, which are the primary reasons for DC progression (Pollreisz and Schmidt-Erfurth, 2010). Thus, the decreased activity of antioxidant enzymes and reducing substances, excessive activation of aldose reductase (AR), and the accumulation of advanced glycation end products (AGEs) were considered as signs of the occurrence of DC in previous studies (Sadi et al., 2012; Richer et al., 2015; Kandarakis et al., 2014). Current evidence supports the view that there is a close relationship between cataract and epithelial and mesenchymal transition (EMT), which is the transdifferentiation of epithelial cells into mesenchymal cells. The EMT of lens epithelial cells (SRA01/04) describes a series of events during which epithelial cells lose several epithelial characteristics, such as E-cadherin, and acquire properties typical of mesenchymal cells, such as α-smooth muscle actin (α-SMA) (Kim et al., 2012). Although, growing evidence has implicated this process as a major pathway leading to the generation of cataract, it is not yet known if the prevalence of diabetic cataract is related to EMT of lens epithelial cells.

Many factors could lead to EMT including the inclusion of transforming growth factor-β (TGF-β), which has been recognized to be one of the strongest cell cytokines involved in the regulation of lens epithelial EMT (Katsuno et al., 2013). Among the three homologous isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3), TGF-β2 has the highest expression with the highest activity in the orbital tissue. Moreover, TGF-β2 can induce abnormal proliferation, differentiation, and apoptosis in lens epithelial cells, which also contributes to cataract formation (Meng et al., 2013). Phosphoinositide 3-kinase (PI3K) is an important downstream signalling molecule of TGF-β, which is involved in the regulation of cell division, differentiation, and apoptosis. Protein kinase B (PKB), also known as Akt, plays an important role in regulating cell survival, and can be activated by PI3K (Zhang et al., 2013). Studies have shown that treatment of HLEB-3 human lens epithelial cells with TGF-β2 leads to morphological changes, inhibition of cell connexin, and fibro-nectin expression with increased binding protein expression, thereby leading to EMT. Activation of the PI3K/Akt pathway plays a key role in the TGF-β2-induced HLEB-3 EMT process (Yao et al., 2008). High blood glucose stimulates the production of TGF-β2 by releasing of reactive oxygen species (ROS) in the cell (Tang and Lai, 2012). Therefore, it is reasonable to propose that high glucose (HG)-induced EMT in lens epithelial cells is mediated by the TGF-β2/PI3K/Akt pathway, which can be activated by ROS (Abbond, 2012).

Some natural Chinese medicine extracts that express little or no side effects have gradually been recognized by researchers as potential candidates in the prevention/treatment of diabetic cataract. Quercetin, 3,3’,4,5,7- pentahydroxy flavon (fl) 98% purity) used in the cell-based studies and streptozotocin (STZ) while the quercetin (≥98% purity) used in the animal experiments was purchased from Shaanxi Huike Plant Development Co., Ltd. The pirenoxine sodium (PS) eye drops were obtained from EBE Pharmaceutical Co., Ltd. The LY294002 used in cells was purchased from Selleck (St. Houston, TX, USA). T-superoxide dismutase (T-SOD) and GSH kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China) while the GSH/GSSG, catalase (CAT), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) kits were purchased from JieMei Bioengineering Institute (Shanghai, China). Tropicamide eye drops was purchased from Wuhan Wujing Medicine Co., Ltd. Medium 199 (M-199, M3769) was purchased from Sigma-Aldrich, (St. Louis, MO, USA). The E-cadherin, α-SMA, and TGF-β2 antibodies were purchased from Abcam (Cambridge, UK) while Akt, phosphorylated (p)-Akt, and β-actin antibodies were purchased from Bioworld Technology (St. Louis, MO, USA).

2.3. Experimental design

To detect the protective role of quercetin on DC, we established an in vivo STZ-induced DC Sprague-Dawley rat model, and in vitro experiments were performed in rat and human lens epithelial cells. The specific experimental groups and details are illustrated in Tables 1–3 (see Fig. 1).

2.4. Establishment of animal models and evaluation of cataract progression in diabetic rats

The rat lenses were examined using slit lamp microscopy and those without lens defect were included for induction of DC. Fifty
The development of rat cataract was examined using slit lamp observation by dilating the pupils with 0.25% tropicamide. At least 5 min later, the rats were anaesthetised by intraperitoneally injecting 10% chloral hydrate at a dose of 0.35 mL/100 g body weight. The cataract was scored on a scale of 1–V according to the description of Azuma et al. (1990). Grade I was a normal clear lens, grade II showed subcapsular opacity, grade III exhibited nuclear cataract, grade IV showed a strong nuclear cataract, and grade V exhibited dense opacity involving the entire lens. Relevant images were captured using a 66Vision Tech YZST900 imaging system.

2.5. Rat lens organ culture and analysis of lens opacity

The lens culture method used was previously described by Lu et al. (2014). Briefly, the lenses were incubated at 37°C under conditions of 95% air and 5% CO2 for 24 h. Then, the selected 36 transparent lenses were divided into the following six groups and treated as indicated: normal group (NC, 5.56 mmol/L glucose plus 44.44 mmol/L mannitol); HG group (HG, 50 mmol/L glucose plus 0.3% dimethyl sulphoxide, DMSO); low-, moderate-, and high-dose quercetin groups (QL, QM, and QH, administered 10, 20, and 40 μg/mL quercetin, respectively plus 50 mmol/L glucose each); and PS group (50 mmol/L glucose plus 0.5 μmol/mL PS eye drops, Table 2).

All the lenses were observed under an optical microscope and photographed on a black background. The photos were scanned into a computer and the ImageJ image processing software (National Institutes of Health, NIH, Bethesda, MD, USA) was used to calculate the grey value of the image and analyse the degree of lens opacity. The analytical method of lens opacity determination used was previously described by Olofsson et al. (2005). The photographic images were converted to grey scale, and each image pixel was automatically assigned one of 256 grey scale levels. The standard deviation (SD) and mean density (MD) of the values obtained for each pixel within a central circular 1/mm² area of the lens were calculated. The development of cortical cataract would show more grey and less black/white pixels and, thereby, lower the SD. The MD is only slightly affected by cortical cataract, but a nuclear opacification would act as a filter, making the pixels darker and the MD higher. The highest SD obtained for a clear lens in the material and the MD for a completely opaque lens were used to construct a formula to quantify the percentage lens opacification in arbitrary units (a.u.): (1-SD/59.4) × (MD/255) × 100.

2.6. Cell culture and cytomorphology

The cells were cultured in minimum essential medium (MEM, with 5.56 mmol/L glucose) containing 10% foetal bovine serum (FBS), supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified 5% CO2 atmosphere. Cells were grown in the normal medium until they were 60%–80% confluent. The cultured cells were divided into the following groups: NC, 5.56 mmol/L glucose plus 44.44 mmol/L mannitol; HG, 50 mmol/L glucose plus 0.5% DMSO; QL, QM, and QH 1, 2, and 4 μmol/L quercetin, respectively plus 50 mmol/L glucose each; PS, 50 mmol/L glucose plus 4 μmol/L PS eye drops; and PI3K inhibitor group (LY, 50 mmol/L glucose plus 20 μmol/L LY294002). After a 24-h incubation, the cell morphology was observed using an Olympus IX53 microscope (Tokyo, Japan) and the cells were harvested and stored at –20°C for subsequent analysis.

2.7. Analysis of lens epithelial cell E-cadherin and α-SMA expression using immunofluorescence microscopy

SRA01/04 cells cultured on glass coverslips were washed thrice with cold phosphate-buffered saline (PBS) and fixed with cold
methanol at –20°C for 20 min. After washing thrice with PBS, the cells were blocked with 5% bovine serum albumin (BSA) for 1 h at 25°C and incubated with anti-E-cadherin or anti-α-SMA antibodies for 2 h at room temperature, followed by incubation with a secondary antibody conjugated with DyLight 594 (Dylight, Millibrae, CA, USA) at 37°C for 1 h. Subsequently, the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI, Beyotime Institute of Biotechnology, Nantong, China) to detect the cell nuclei. The coverslips were mounted onto glass slides and the images were viewed using an Olympus BX43F fluorescence microscope (Tokyo, Japan).

2.8. Haematoxylin and eosin (H&E) staining and immunohistochemistry of rat lens epithelial cells

For the haematoxylin and eosin (H&E) staining, the rats were euthanised at the end of week 14, the lenses were harvested, and then fixed in 4% formaldehyde for more than 24 h after paraffin-embedding. Then lens were subsequently dehydrated using a fully automated dewatering machine, with the following sequential steps: 75% ethanol, 85% ethanol, 95% ethanol I, 95% ethanol II, 100% ethanol I, 100% ethanol II, xylene I, xylene II, paraffin I, paraffin II, and paraffin III, each run for 1 h. Next, the rat eyeballs of each group were embedded in paraffin and treated sequentially for 10 min each with xylene and xylene II dewaxing, followed by 100%, 95%, 80%, and 70% ethanol, and then after 5 min they were placed in distilled water. Finally, the lens were treated according to the following protocol: haematoxylin staining for 3 min; tap water rinse for 5 min, which was then change to distilled water; 1% hydrochloric acid alcohol treatment for 10 s; tap water wash for 5 min; 1% lithium carbonate Portland treatment for 1 min; 80% ethanol for 2 min; staining for 50 s; sequential dehydration, 95%, 95%, 100%, and 100% ethanol for 2 min each; xylene and xylene II dewaxing for 1 min; and then transparent neutral gum mounting.

For the immunohistochemistry, the rat lens samples were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (3-μm thick) were cut perpendicularly to the long axis of the lenses for immunohistochemistry. The paraffin embedded lens sections were deparaffinized in xylene, hydrated in graded concentrations of alcohol and water, and then subsequently placed in 3% H2O2 to eliminate endogenous peroxidase activity. Then, the sections were blocked with normal goat serum, followed by incubation with anti-E-cadherin and anti-α-SMA antibodies overnight at 4°C. The sections were stained using a polymer horseradish peroxidase (HRP) detection system (ZSGB-BIO, Beijing, China), counterstained with haematoxylin, and the examined using an Olympus BX43F microscope.

2.9. Determination of AGE levels in lenses using fluorospectrophotometry

Rats were euthanised at the end of week 14, their lenses were dissected, homogenized in pre-chilled 0.9% normal saline, the resultant homogenate was centrifuged at 4°C at 10,000 × g for 15 min, and then the supernatant and pellets were stored at –80°C until further analysis. The supernatant was collected to determine protein concentrations, AR activity, and GSH while the pellets were harvested to measure the AGEs levels. The levels of AGEs in the rat lens tissues and organs were determined using fluorospectrophotometry as previously reported (Bhattacharya et al., 2007) and expressed as the enzymatic activity of type I collagenase per milligram of protein (U/mg prot).

2.10. Measurement of lens AR activity using fluorospectrophotometry

The AR activity of the rat lenses and lens organs was detected using spectrophotometry according to Chaudhry (Chaudhry et al., 1983). The enzymatic reaction system adopted was in a 200-μL volume consisting of 135 mmol/L phosphate buffer, 100 mmol/L lithium sulphate, 150 μmol/L nicotinamide adenine dinucleotide phosphate (NADPH), 5 μmol/L lens homogenate, and 1.0 mmol/L DL-glyceraldehyde. The fluorescence was measured using a spectrophotofluorometer (Ex/Em=360 nm/460 nm, GloMax-MultiII, Promega Co., Ltd., USA), and expressed as the unit of AR enzyme activity per gram of protein (U/g prot).

2.11. Measurement of MDA, GSH/GSSG, CAT, GSH-Px and total SOD (T-SOD)

The levels of MDA, CAT, GSH-Px and GSH/GSSG in the tissues and SRAR01/04 cells were measured using a spectrophotometer using kits from JieMei Bioengineering Institute (Shanghai, China). The levels of T-SOD in the lenses, tissues, and SRAR01/04 cells were measured using a spectrophotometer using kits from JianCheng Bioengineering Institute (Nanjing, China).

Specific steps indicated in the kit manual were as follows. Sample pretreatment: rat lens tissue and rat lens organs/cells weight by weight (g) were added to 9 times their volume of 0.9% saline volume (ML) at a 1:9 ratio in an ice water bath. Then, the mechanical homogenate was prepared as a 10% homogenate, which was centrifuged at 2500 × g for 10 min, and the supernatant was measured. We mixed the test sample and kit reagents homogeneously, incubated the mixture in the 96-well microplate at the temperature indicated by the kit instructions, and then the OD was estimated using the microplate reader at different wavelengths for different indicators.

2.12. Determination of TGF-β2, E-cadherin, and α-SMA expression and Akt phosphorylation using western blotting in HG-cultured human lens epithelial cells

Cultured SRAR01/04 cells were harvested in lysis buffer containing 50 mmol/L Tris (pH 7.6), 150 mmol/L sodium chloride (NaCl), 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1% NP-40, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L sodium orthovanadate (Na3VO4) and 20 mmol/L sodium fluoride (NaF0), and then lysed on ice for 30 min with vortexing every 10 min. The supernatant was decanted after centrifugation at 12,000 × g at 4°C for 15 min. The protein concentration was determined using the bicinechonic acid (BCA) protein assay kit (Pierce Thermo-Scientific, Rockford, IL, USA) according to the manufacturer’s instructions. For immunoblotting, an equal amount

Table 4

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Fasting blood glucose (mmol/L)</th>
<th>Week0</th>
<th>Week14</th>
</tr>
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<tbody>
<tr>
<td>NC</td>
<td>8</td>
<td>4.6 ± 2.1</td>
<td>6.3 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>DC</td>
<td>8</td>
<td>21.1 ± 3.0**</td>
<td>26.6 ± 3.3**</td>
<td></td>
</tr>
<tr>
<td>QL</td>
<td>9</td>
<td>20.1 ± 3.3</td>
<td>23.1 ± 7.1</td>
<td></td>
</tr>
<tr>
<td>QM</td>
<td>10</td>
<td>21.0 ± 5.5</td>
<td>27.2 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>QU</td>
<td>9</td>
<td>20.2 ± 4.9</td>
<td>21.9 ± 5.8</td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>9</td>
<td>20.1 ± 3.3</td>
<td>26.6 ± 3.7</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD, 8–10 rats in each group. **P < 0.01, compared with the NC group.
of protein (40–60 g) was separated using 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked in PBS containing 3% BSA for 1 h at room temperature, followed by incubation with primary antibodies overnight at 4 °C. The primary antibodies used were against E-cadherin, α-SMA, TGF-β2, and β-actin. The membranes were colorimetrically developed using the BCIP/NBT alkaline phosphatase color development kit (Beyotime Institute of Biotechnology, Nantong, China). The quantification involved measuring the signal intensity using the ImageJ software.

### 2.13. Statistical analysis

The data are presented as the mean ± standard deviation (SD), and the statistical analysis was performed using the statistical package for the social sciences (SPSS) software version 13.0 (SPSS, Inc., Chicago, IL, USA). Intergroup differences were compared using

#### Table 5

Effects of quercetin on the body weight of diabetic rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Weight (g) Week 0</th>
<th>Weight (g) Week 4</th>
<th>Weight (g) Week 8</th>
<th>Weight (g) Week 12</th>
<th>Weight (g) Week 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>8</td>
<td>191.2 ± 15.3</td>
<td>231.6 ± 21.4</td>
<td>260.2 ± 12.5</td>
<td>298.4 ± 12.1</td>
<td>305.2 ± 19.1</td>
</tr>
<tr>
<td>DC</td>
<td>8</td>
<td>195.2 ± 13.3</td>
<td>174.4 ± 20.8**</td>
<td>176.7 ± 27.3**</td>
<td>184.4 ± 37.5**</td>
<td>172.1 ± 37.5**</td>
</tr>
<tr>
<td>QL</td>
<td>9</td>
<td>197.0 ± 12.9</td>
<td>177.2 ± 12.7</td>
<td>174.9 ± 15.5</td>
<td>180.3 ± 24.4</td>
<td>164.7 ± 27.8</td>
</tr>
<tr>
<td>QM</td>
<td>10</td>
<td>199.9 ± 16.0</td>
<td>186.1 ± 21.8</td>
<td>182.0 ± 18.2</td>
<td>188.0 ± 33.1</td>
<td>177.6 ± 28.7</td>
</tr>
<tr>
<td>QH</td>
<td>9</td>
<td>201.4 ± 17.9</td>
<td>176.8 ± 28.4</td>
<td>180.1 ± 26.6</td>
<td>183.6 ± 41.1</td>
<td>163.5 ± 30.9</td>
</tr>
<tr>
<td>PS</td>
<td>9</td>
<td>211.8 ± 22.8</td>
<td>177.0 ± 11.4</td>
<td>176.9 ± 11.9</td>
<td>187.7 ± 23.4</td>
<td>173.1 ± 22.1</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD, 8–10 rats in each group. **P < 0.01, compared with the NC group.

### Fig. 2

Effects of quercetin on the lens opacity of diabetic rats (A) and high glucose cultured rat lens (B), effects of quercetin on the activities of AR in diabetic rats (C, n = 6) and high glucose cultured lens (D, n = 6) and effects of quercetin on the activities of AGEs in diabetic rats (E, n = 6) and high glucose cultured lens (F, n = 6). In all panels, data are presented as mean ± SD. **P < 0.01, compared with NC group, ##P < 0.01, compared with HG group. NC: normal control group; DC: diabetic cataract rats treated with 1% CMC-Na solution; HG: rat lens cultured in 50 mol/L glucose medium; QL: diabetic cataract rats treated with 30 mg/kg of quercetin, high glucose cultured lens treated with 10 μg/ml quercetin; QM: diabetic cataract rats treated with 60 mg/kg of quercetin, high glucose cultured lens treated with 20 μg/ml quercetin; QH: diabetic cataract rats treated with 120 mg/kg of quercetin, high glucose cultured lens treated with 40 μg/ml quercetin; PS: diabetic cataract rats treated with pirenoxine sodium eye drop twice a day, a drop at a time, high glucose cultured lens treated with 0.5 μmol/ml pirenoxine sodium eye drop.

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Collectively, compared with the DC group, the lenses of the QM, QH, reduced. Most of the lenses of the PS group were in grade in the proportion of grade III (55.56%) lenses, and those in grade V lenses of the QM group, those of the QH group showed an increase numerous lenses were also at grade V (40.00%). Compared with the rats were grade V and II (75.00 and 45.00%, respectively), but

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Cataract grades(percentage)</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>16</td>
<td>16 0 0 0 0 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DC</td>
<td>**</td>
<td>16 0 3(18.75%) 1(6.25%)</td>
<td></td>
<td></td>
<td>12(75.00%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QL</td>
<td>18</td>
<td>0 2(11.11%) 3(16.67%) 0</td>
<td></td>
<td></td>
<td>13(72.22%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QM</td>
<td>20</td>
<td>2(10.00%) 9(45.00%) 1(5.00%)</td>
<td></td>
<td></td>
<td>8(40.00%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QH</td>
<td>**</td>
<td>18 0 3(16.67%) 10(55.56%) 1(5.55%)</td>
<td></td>
<td></td>
<td>4(22.22%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>**</td>
<td>18 0 7(38.89%) 6(33.33%) 1(5.56%)</td>
<td></td>
<td></td>
<td>4(22.22%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD, **P < 0.01, compared with NC group, *P < 0.05, compared with DC group, *P < 0.01, compared with DC group.

a one-way analysis of variance (ANOVA) followed by the Dunnett's test. The cataracts were graded using the Wilcoxon signed-rank test, and the statistical significance was defined as P < 0.05.

3. Results

3.1. Effects of quercetin on FBG and body weight of diabetic rats

The FBG and body weight of the rats were examined after treatment with quercetin for 14 weeks. Compared with the rats in the normal group, the FBG level of the rats in the diabetic groups significantly increased, and treatment with quercetin had no effect (Table 4). In addition, measurement of the body weights showed a significant decline after STZ administration while quercetin administration had no effect on the body weights of diabetic rats (Table 5).

3.2. Quercetin delayed the development of DC in diabetic rats and high glucose cultured rat lens

The change in lens opacity is the most intuitive indicator of lens status. We used STZ-induced 14-week diabetic cataract rats as the in vivo model and HG-cultured rat lenses in vitro to evaluate the effect of quercetin on the lens opacity induced by HG. After the rats had been fed for 14 weeks, the eyes were examined using a slit lamp biomicroscope. The progression of lenticular opacity was assessed according to the description of Azuma et al. (1990). The slit lamp findings indicated that the lenses of the NC rats remained clear and transparent without any turbidity. In contrast, the rats in the DC group presented cloudy lenses with nuclear opacity within a range of grades III–V. Most of the lenses of the QL and QM group rats were grade V (75.00 and 40.00%, respectively), but numerous lenses were also at grade V (40.00%). Compared with the lenses of the QM group, those of the QH group showed an increase in the proportion of grade III (55.56%) lenses, and those in grade V (22.22%) reduced. Most of the lenses of the PS group were in grade II (38.89%), which was higher than the values of any other group. Collectively, compared with the DC group, the lenses of the QM, QH, and PS groups showed a significant improvement in lens opacity.

The in vivo model was used to examine the effect of quercetin on lens opacity, and the change in glucose-induced lens opacity was monitored microscopically. After 5 days, all the lenses in the NC group appeared clear while most lenses of the HG group rats were covered with opaque rings. After treatment with quercetin, the QM and QH group showed a significant reduction in HG-induced lens opacity levels compared with that of the HG group. Moreover, PS as the positive control also reduced the lens opacity (Fig. 2A and B; Tables 6 and 7).

AR and AGEs are two important indicators of DC. AR is a key enzyme in the polyol pathway, which can be activated in diabetic conditions and plays a crucial role in the pathogenesis of DC (Wilhelm et al., 2007). AGEs are generated by the non-enzymatic glycation reaction and accumulate in the lens, which leads to the opacification (Ding et al., 2007). The AR activities and AGE level of the DC-HG group were significantly increased compared with those of the NC group (P < 0.01). The diabetic rats in the QL, QM, and QH groups showed weaker AR activity, and lower AGEs levels than those in the DC group did (P < 0.01). PS therapy also significantly reduced the AR activity and AGE level of the DC rat lenses (P < 0.01). The administration of quercetin and PS decreased AR activity and AGE level to different degrees in vivo and in vitro. The above results suggest that quercetin significantly improved the internal DC crystal aldose reductase abnormalities and AGE accumulation in rats, whereas quercetin high-dose showed better effects than PS did (Fig. 2C–F).

3.3. Quercetin inhibited oxidative stress of lens epithelial in vivo and in vitro

An array of antioxidant defence mechanisms expressed by the cell plays a vital role in maintaining the physiological redox status and protecting the cells against the harmful effect of toxic drug metabolites and free radicals. These include GSH, GSSG, GSH-Px and ratio of GSH/GSSG, which reflect the oxidation-reduction state of cells, enzymatic antioxidants such as SOD and CAT, and the lipid peroxidation product MDA. A change in these indicators reflects the strength of the cellular antioxidant capacity and the degree of oxidative damage. SRA 01/04 cells and excluded lens were exposed to NC (5.56 mmol/L in cells, 2.40 mmol/L in the lens) or HG (50 mmol/L) for 24 h and 5 days respectively. The normal and STZ-induced DC rats were fed under specific pathogen-free (SPF) conditions for 14 weeks. The GSH and T-SOD were determined in HG-cultured lenses while the GSH/GSSG ratio, T-SOD, CAT, GSH-Px and MDA were detected in SRA 01/04 cells and 14-week STZ-induced DC rat lenses. The results revealed that HG down-regulated the levels of GSH in cells, lenses, and DC rats (Fig. 3A–C), GSH/GSSG in cells and DC rats (Fig. 3F and G), the activity of T-SOD in cells, lenses, and DC rats (Fig. 3J–L), GSH-PX and CAT in cells and DC rats (Fig. 3H, I, M and N) compared with the cells/lenses of the NC group. Conversely, increased levels of MDA and GSSG were observed in HG-cultured cells and diabetic rats (Fig. 3O, P, D and E). The administration of quercetin and PS increased the GSH level, GSH/GSSG ratio, and the T-SOD, GSH-Px and CAT activity under HG condition to different degrees in vivo and in vitro. Moreover, quercetin alleviated the accumulation of MDA and GSSG caused by HG in the HG-cultured cells and diabetic rats. Taken together, these data suggest that quercetin alleviates oxidative stress induced by HG in vivo and in vitro.

3.4. Quercetin inhibited morphological change and EMT of lens epithelial cell of diabetic rats and HG-cultured SRA01/04 cells

Under normal conditions, the SRA01/04 human lens epithelial cell morphology was regular and showed a typical cobblestone...
Fig. 3. Effects of quercetin on the level of GSH (A, B, C), GSSG (D, E) and MDA (O, P) and the activities of T-SOD (J, K), CAT (M, N) and GSH-Px (H, I) in diabetic rats (C, E, G, I, N, P) (n = 6), high glucose cultured lens (B, K) (n = 6), and HLECs (A, D, F, H, J, M, O) (n = 3). In all panels, data are presented as mean ± SD. *P < 0.05, compared with NC group. **P < 0.01, compared with NC group. ***P < 0.01, compared with HG group. NC: normal control group; DC: diabetic cataract rats treated with 1% CMC-Na solution; HG: rat lens or HLECs cultured in 50 mol/L glucose medium; QL: diabetic cataract rats treated with 30 mg/kg of quercetin; QM: diabetic cataract rats treated with 60 mg/kg of quercetin; QH: diabetic cataract rats treated with 120 mg/kg of quercetin; PS: diabetic cataract rats treated with pirenoxine sodium eye drop twice a day, a drop at a time, high glucose cultured lens treated with 0.5 μmol/ml pirenoxine sodium eye drop, high glucose cultured HLECs treated with 4 μmol/L pirenoxine sodium eye drop.
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**Fig. 4.** Effects of quercetin on the morphological change of high glucose cultured HLECs (A) and HE staining in rat lens epithelial cells (B). NC: normal control group; DC: diabetic cataract rats treated with 1% CMC-Na solution; HG: HLECs cultured in 50 mol/L glucose medium; QL: diabetic cataract rats treated with 30 mg/kg of quercetin, high glucose cultured HLECs treated with 120 mg/kg of quercetin; QM: diabetic cataract rats treated with 60 mg/kg of quercetin, high glucose cultured HLECs treated with 2 μmol/L quercetin; QH: diabetic cataract rats treated with 120 mg/kg of quercetin, high glucose cultured HLECs treated with 4 μmol/L quercetin; PS: diabetic cataract rats treated with pirenoxine sodium eye drop twice a day, a drop at a time, high glucose cultured HLECs treated with 4 μmol/L pirenoxine sodium eye drop.

Pattern. Cells cultured under HG conditions for 24 h showed a significantly elongated fibre-like form compared with the NC group cells. The shape of cells in the QH and PS groups were turned to regular and exhibited a cobblestone-like appearance. Furthermore, the results showed that HG induced SRA01/04 cell fibrotic changes while quercetin, which possesses a high PS content, improved the HG-induced cell fibrotic changes. This observation suggests that the PS content of quercetin improve the HG-induced human lens epithelial cell morphological changes (Fig. 4A). The HE staining also validated these results in vivo (Fig. 4B).

SRA01/04 cells were exposed to NC (5.56 mmol/L) or HG (50 mmol/L) for 24 h. The expression of the epithelial cell marker, E-cadherin, and the mesenchymal cell marker, α-SMA, was determined using western blotting and immunofluorescence. Western blotting demonstrated that HG down-regulated the expression of E-cadherin and up-regulated that of α-SMA compared with the NC cells (Fig. 5C and D). In correlation with the findings of the western blotting, decreased E-cadherin and increased α-SMA expressions were observed using immunofluorescence (Fig. 5A and B). In addition, each concentration of quercetin down-regulated the high expression of α-SMA in SRA01/04 cells exposed to HG. E-cadherin was prominently localized at the cell–cell junctions in cells cultured under NC conditions, but this distribution was not observed in the HG-cultured SRA01/04 cells. Treatment of these cells with quercetin induced the reformation of the cell-cell junction and decreased the expression of α-SMA in the cytoplasm compared with the HG group cells.

To confirm the effects of quercetin on HG-induced EMT, we used immunohistochemistry to detect the expression of E-cadherin and α-SMA in diabetic rats. Similar to the results of the SRA01/04 cell analysis, HG also induced EMT in diabetic rats in vivo, and quercetin alleviated this effect by increasing E-cadherin and decreasing α-SMA expressions, simultaneously (Fig. 6A and B). Taken together, these results support the hypothesis that treatment with quercetin reverses the EMT in HG-cultured SRA01/04 cells and diabetic rats.

**3.5. Inhibitory effects of quercetin on TGF-β2/PI3K/Akt signalling-mediated EMT of SRA01/04 cells**

To investigate whether the TGF-β2/PI3K/Akt signalling pathway is involved in the EMT of SRA01/04 cells induced by HG, we examined the expression of TGF-β2 and phosphorylation of Akt at Ser473. Compared with the NC group, the expression of TGF-β2 was significantly increased in the HG group. Moreover, the phosphorylation of Akt (Ser473) was also elevated in HG-cultured SRA01/04 cells (Fig. 7A and B). Treatment with quercetin inhibited the HG-induced overexpression of TGF-β2 and phosphorylation of Akt in SRA01/04 cells, and increased E-cadherin and decreased α-SMA expressions (see Fig. 8).

Furthermore, administration of LY294002 (the PI3K inhibitor) ameliorated the HG-induced EMT, and the effect was comparable with that of intermediate doses of quercetin, based on the findings of the effects on E-cadherin and α-SMA using immunofluorescence (Fig. 7C and D). Collectively, these results indicate that TGF-β2/PI3K/Akt signalling was activated in the HG-mediated EMT of SRA01/04 cells. Furthermore, quercetin might prevent the EMT of human lens epithelial cell lines by inhibiting TGF-β2/PI3K/Akt signalling.

**4. Discussion**

EMT is a critical physiological process that exposes interactive
basement membrane epithelial cells to innumerable biochemical alterations and, thereby, induces a mesenchymal cell phenotype assumption. These phenotypic characteristics include invasiveness, enhanced migratory capacity, resistance to apoptosis, and increased extracellular matrix (ECM) component production (Kalluri and Neilson, 2003). Additionally, this transition is

Fig. 5. Distribution and expression of E-cadherin (A, D) and α-SMA (B, C) in HLECs cells through immunofluorescence and western blotting. In all panels, data are presented as mean ± SD, n = 3. NC: normal control group; HG: HLECs cultured in 50 mol/L glucose medium; QL: high glucose cultured HLECs treated with 1 μmol/L quercetin; QM: high glucose cultured HLECs treated with 2 μmol/L quercetin; QH: high glucose cultured HLECs treated with 4 μmol/L quercetin; PS: high glucose cultured HLECs treated with 4 μmol/L pirénexine sodium eye drop.

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associated with the down-regulation of epithelial cells biomarkers and the up-regulation of mesenchymal biomarkers (Lee et al., 2007). Various pathologies had been linked to this transition process, and DC, an end-stage diabetic complication that erodes the native integrity of eye lens, is involved in this scenario. We found that HG induces ROS generation and ultimately induces EMT-associated DC by activating the PI3K/Akt pathway. Furthermore, we proved that quercetin, a versatile flavonoid, has the potential to ameliorate DC through this pathway. Therefore, to buttress this point, we explored whole animal, tissue, and cellular experimental models using STZ-induced DC rats, HG-cultured rat lenses, and HLECs SRA01/04 cells.

First, we demonstrated that HG induced oxidative stress by evaluating the antioxidant enzyme system (the reduced form of GSH, GSH/GSSG ratio, GSH-Px, T-SOD, and CAT activities) and the content of lipid peroxidation products (MDA). The reaction between GSH and H2O2 catalysed by GSH-peroxidase (Px), which generates water (H2O) and GSSG can directly detoxify H2O2 and, therefore, the GSH/GSSG ratio of the lens depicts the level of its antioxidant capacity. In addition, antioxidant enzymes such as SOD, CAT, and GSH-Px play synergistic roles in the clearance of oxygen free radical (Ozmen et al., 2002). Therefore, it is obvious the antioxidant mechanisms that scavenge the deleterious oxidative insults are no operational.

Moreover, MDA is a key product of lipid peroxidation that binds to amino acid residues on the membrane, leading to the instability of cell membrane and cell death. Consequently, MDA is the most representative indicator of oxidative damage. Under HG conditions, GSH, GSH/GSSG ratio, GSH-Px, T-SOD, and CAT are down-regulated while the MDA content accumulates. This indicates that the antioxidant status of lenses in the diabetic condition is dysfunctional, which might increase the vulnerability of the cells to oxidative insults.

This observation suggests that quercetin protected the antioxidant status of the STZ-induced DC and HG-cultured lenses as well as the SRA01/04 cells. Quercetin also reduced the expression of aldose reductase and abated AGE production significantly. The key structural features of quercetin that might be responsible for its stability in fighting oxidative insults include the 8 ring α-dihydroxyl groups, the 4-oxo group in conjugation with the 2, 3-alkene, and the 3- and 5-hydroxyl groups (Hollman and Katan, 1997). These functional groups can donate electrons to the rings, which increases the number of resonance forms available in addition to those created by the benzene structure (Mariani et al., 2008).

The cellular platform of cataractogenesis is the pathological change in lens epithelial cells, and the exploration of their pathophysiological characteristics might facilitate the elucidation of the mechanism of cataract pathogenesis. Furthermore, this strategy could also provide further insight into the development of therapeutic options that could remedy the condition. EMT of lens epithelial cells is recognized as the pillar that supports the cataractogenesis machinery while the PI3K/Akt signalling pathway has been shown to be an important mediator of this process.

Previous studies have shown that HG-cultured rat lenses exhibit a significant increase in the expression levels of TGF-β2 mRNA and protein (Olofsson et al., 2005). In human lens epithelial cells, the PI3K/Akt signalling pathway is involved in TGF-β2-induced EMT (Wilhelm et al., 2007). Thus, TGFβ/PI3K/Akt signalling in the DC pathological process could invoke the onset and progression of EMT. Previous studies found that quercetin inhibits epithelial-mesenchymal transition of PC-3 cell lines in vitro by the epidermal growth factor receptor (EGFR)/PI3K/Akt signal transduction pathway (Hollman and Katan, 1997).

To confirm the occurrence of EMT under HG conditions in vivo and in vitro, we examined the expression of E-cadherin and α-SMA, and observed a significant decrease and increase in E-cadherin and α-SMA expression in rat lens epithelial cells through immunohistochemistry. (A) E-cadherin expression in rat lens epithelial cells through immunohistochemistry. (B) α-SMA expression in rat lens epithelial cells through immunohistochemistry. NC: normal control group; DC: diabetic cataract rats treated with 1% CMC-Na solution; QL: diabetic cataract rats treated with 30 mg/kg of quercetin; QM: diabetic cataract rats treated with 60 mg/kg of quercetin; QH: diabetic cataract rats treated with 120 mg/kg of quercetin; PS: diabetic cataract rats treated with pirenoxine sodium eye drop twice a day, a drop at a time.
Fig. 7. (A) Effects of quercetin on the expression of TGF-β2 in high glucose cultured HLECs (n = 3). (B) Effects of quercetin on the phosphorylation of Akt in high glucose cultured HLECs (n = 3). (C, D) The role of quercetin in PI3K/Akt pathway compared with PI3K inhibitor (LY294002). In all panels, data are presented as mean ± SD. **p < 0.01, compared with NC group. "p < 0.01, compared with HG group. NC: normal control group; HG: HLECs cultured in 50 mol/L glucose medium; QL: high glucose cultured HLECs treated with 1 μmol/L quercetin; QM: high glucose cultured HLECs treated with 2 μmol/L quercetin; QH: high glucose cultured HLECs treated with 4 μmol/L quercetin; PS: high glucose cultured HLECs treated with 4 μmol/L pirenoxine sodium eye drop; LY: high glucose cultured HLECs treated with 20 μmol/L LY294002.

Fig. 8. Activation of TGF-β/PI3K/Akt signalling promoted the progression of EMT and diabetic cataract, and the protective effects of quercetin might be related to inhibition of TGF-β/PI3K/Akt pathway.
α-SMA expressions, respectively in HG-cultured human lens epithelial cells. This observation was accompanied by cell morphological changes from a neatly arranged cobblestone type to the shuttle-shaped fibre type. The results showed that the HG environment promoted the occurrence of EMT in lens epithelial cells. In addition, TGF-β2 expression and Akt phosphorylation increased in HG-cultured cells, which suggests that HG induced the EMT of lens epithelial cells by activating TGF-β2/Pi3K/Akt signalling.

The administration of quercetin significantly improved E-cadherin expression, inhibited α-SMA, and reduced TGF-β2 expression and Akt activation, which inhibited the EMT of lens epithelial cells and, thereby, inhibited cataractogenesis. Probably, we could infer that quercetin had an effect on Pi3K inhibition because Pi3K is an upstream protein of Akt. There is evidence that quercetin and the inhibitor LY294002 are structural analogues and, therefore, are likely to have similar roles, including the inhibition of Pi3K/Akt signalling (Mariani et al., 2008).

In DC, a reduction in galactokinase activity and insulin deficiency provokes high blood glucose concentration, leading to increased intraocular aqueous osmotic pressure and swelling of the lens fibre, which eventually sets the stage for lens opacification, a critical hallmark of DC (Pollreisz and Schmidt-Erfurth, 2010). Long-term research studies have proved beyond reasonable scientific doubt that high concentration alone might not be sufficient to cause DC. These observations further suggest that HG likely acts in concert with other processes such as oxidative stress, abnormal polypeptide metabolism, non-enzymatic glycosylation, and alteration in lens epithelial to form a multiple factor that could induce a strong enough response to provoke the onset and progression of DC (Hedeg and Varma, 2005; Obrosova et al., 2010).

While some research findings have reported that HG concentration and oxidative stress are closely related twin factors that cannot be separated, other data suggest a mutual relationship between oxidative stress and non-enzymatic glycation. The specific target of quercetin might require further investigations; however, our present findings indicate that quercetin inhibited EMT-induced DC through the TGF-β2/Pi3K/Akt pathway.

5. Conclusion

Our results demonstrate that HG induced the generation of ROS, increased TGF-β2 expression and Akt phosphorylation and altered the biological markers of EMT to mediate the development of cataractogenesis. This observation clarifies that the occurrence of DC is driven by multiple factors and not just a single mechanism. Quercetin, an incredibly versatile flavonoid, was proven to have considerably more effects other than antioxidant activity. This was based on its amelioration of the opacification of lenses and cataractogenesis by the improvement of antioxidant status, down-regulation of TGF-β2 expression, reduction of Akt phosphorylation, down-regulation of α-SMA, and up-regulation of E-cadherin expressions. Therefore, the evidence supporting the anti-cataractogenesis potency of quercetin at the cellular, tissue, and whole animal levels, indicates that this compound could potentially qualify as a therapeutic breakthrough agent. Furthermore, it should be considered as nutraceutical that is worth further investigation as a candidate drug to combat this pathology. It was also interesting to report that its anti-cataractogenesis activity might be mediated by the inhibition of TGF-β2/Pi3K/Akt pathway-induced EMT. Therefore, the three experimental model perspectives of our research (whole animal, tissue, and cellular), quercetin was proven to be potent enough to ameliorate DC in the early stages, thereby presenting a potential therapeutic strategy for its treatment and management.

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