Decorin inhibits glucose-induced lens epithelial cell apoptosis via suppressing p22phox-p38 MAPK signaling pathway

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Abstract

Purpose
To determine the effect of decorin on oxidative stress and apoptosis of human lens epithelial (HLE) cells under high glucose condition.

Methods
HLE cell line (HLEB3) was incubated in normal glucose (5.5 mM) or high glucose (60 mM) medium. Decorin (50 nM) was applied 2 hours before high glucose medium was added. Apoptosis detection was executed by flow cytometry and western bloting (analysis of bcl-2 and bax). Oxidative stress level was measured by the generation of reactive oxygen species (ROS), glutathione peroxidase (GSH) and superoxide dismutase (SOD). P38 mitogen-activated protein kinase (MAPK) phosphorylation, the expression of p22phox of HLE cells and human lens anterior capsules were detected by western bloting. Small interfering RNA transfection to p22phox and p38 MAPK was also carried out on HLEB3.

Results
High glucose caused HLE cells oxidative stress and apoptosis exhibiting the increase of apoptotic cells and ROS production and decrease of bcl-2/bax ratio, GSH/GSSG ratio and SOD activity. P22phox and phospho-p38 MAPK were upregulated in high glucose treated HLEB3 cells. Knocking down p22phox or p38 by siRNAs can reduce high glucose induced cell apoptosis and oxidative stress level. Silencing p22phox by siRNA can downregulate the phosphorylation of p38 MAPK. Decorin can inhibit the apoptosis, oxidative stress level and the induction of p22phox and phospho-p38 of HLEB3 induced by high glucose. Furthermore, the expression of p22phox and p38 were found significantly increased in lens anterior capsules of diabetic cataract patients compared to that of normal age-related cataract patients.
Conclusions

Results showed that p22<sub>phox</sub>-p38 pathway may be participated in high glucose induced lens epithelial cell injury, decorin may inhibit the high glucose induced apoptosis and oxidative stress injury by suppressing this pathway in part.

Introduction

Diabetic cataract is one of the most important complications of diabetes [1]. Oxidative stress induced by high glucose plays a pivotal role in the mechanism of diabetic cataract. Oxidation and aggregation of protein in lens epithelium cells, which led to lens opacity, are caused by high glucose [2]. Apoptosis and oxidative stress, which participated in the formation of diabetic cataract, occurred when human lens epithelial (HLE) cells exposing for 24 h to the condition of high glucose [3].

Decorin, which is a small leucine-rich proteoglycan, has been found to negatively regulate a variety of cellular functions when binding to extracellular matrix components or cell surface receptors [4, 5]. Overexpression of decorin could restrain angiogenesis mediated by tumor cell by suppressing the production of vascular endothelial growth factor (VEGF) [6]. Administration of decorin into corneal stroma could inhibit neovascularization of cornea in rabbit model [7]. In the post-traumatic brain injuries (TBI) rat cerebrum, it is reported that decorin increased the activity of superoxide dismutase (SOD), glutathione peroxidase (GSH) and prevented oxidative stress injury and apoptosis [8, 9]. Preliminary data of our team showed that decorin can inhibit retina pigmentosa epithelial (RPE) barrier disruption under diabetic condition through suppression of p38 mitogen-activated protein kinase (MAPK) activation [10]. However, the influence of decorin on diabetic cataract has not been studied yet.

In the current study, the effect and potential mechanism of decorin on high glucose induced oxidative stress and apoptosis in HLE cells were investigated. Apoptosis, reactive oxygen species (ROS), SOD and GSH were determined. Besides, p38 MAPK phosphorylation and the expressed level of p22<sub>phox</sub> of HLEB3 and human lens anterior capsules were evaluated. Furthermore, the role of p22<sub>phox</sub> and p38 MAPK were evaluated in mediating the oxidative stress caused by high glucose.

Materials and methods

Antibodies and chemical agents

Mouse anti-bcl-2 (Abcam, Cambridge, UK), mouse anti-bax (Abcam, Cambridge, UK), mouse anti-β-actin (Proteintech, Chicago, Illinois, USA), rabbit anti-p38 (Abcam, Cambridge, UK), rabbit anti-phospho-p38 (Thr180/Tyr182, Abcam, Cambridge, UK), mouse anti-p22<sub>phox</sub> (Gentex, Irvine, USA), goat anti-mouse (Proteintech, Chicago, Illinois, USA) and goat anti-rabbit (Proteintech, Chicago, Illinois, USA) antibodies, recombinant human decorin (R&D Systems, Minneapolis, MN, USA), apoptosis detection kit (Beyotime Inst. Biotech, Haimen, China), Cell Counting Kit-8 (CCK-8, Dojindo, Japan).

Human lens anterior capsules

The human lens anterior capsular tissues were obtained from the eye-tissue bank. All procedures for collecting the anterior capsules followed the guidelines for the use of human materials. This study and the protocols used in the paper were approved by the Ethics Committee of
the First Affiliated Hospital of Zhengzhou University. All patients offered written reports of informed consent. Patients involved have not subjected to any specific clinical treatments. Twenty-six cataract patients (thirty eyes) with type 2 diabetes mellitus were included as the diabetic cataract group, and twenty-three cataract patients (thirty eyes) without any systemic or ocular disease were included as the senile cataract group (control group) (Table 1).

**Human lens epithelial cell cultures**

Human lens epithelial cell line (HLEB3) was a gift of Professor Hu in Henan University. This cell line was maintained in Dulbecco’s modified Eagle’s medium (DMEM with 5.5mM D-glucose) (Sigma, USA) containing 10% fetal bovine serum (FBS) (Genial, USA) and 1% antibiotics (penicillin G 110 U/ml, streptomycin 100μg/ml, solarbio, China) at 37˚C in the presence of 5% CO2. Cultured human lens epithelial cells were passaged when the cells reached 80%-90% confluency. Cells for 2 to 5 passages were used.

HLEB3 cells were seeded in 6-well plates with normal culture. When cell density reached about 50%, the cells were exposed to serum starvation (0.5% FBS) for 12 hours. Then the cells were divided into four groups: control group, high glucose group, decorin group and mannitol group. Cells in high glucose group were exposed to glucose (60 mM, Sigma, USA) for 72 hours. Cells in decorin group were exposed to recombinant human decorin (50 nM) for 2 hours [11] and then treated with 60 mM glucose for 72 hours. Mannitol group (5.5 mM D-glucose plus 54.5mM mannitol) was designed to eliminate potential deviations by osmosis.

**Cell viability assay**

CCK-8 was used to measure cell viability. HLEB3 cells were seeded in 96-well plates at a density of 1×10^4 cells per well for overnight. Then the medium was changed with medium (without FBS) containing 0, 50, 100 and 200nM of decorin. Plates were incubated for 24 h, then 10μl WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] solution was added to each well. After incubating for 4 hours at 37˚C, the plate was examined at 450nm using an iMark™ microplate absorbance reader (Bio-Rad, Hercules, USA). Number of living cells in each well was expressed as the value relative to the control.

**Small interfering RNA transfection**

Small Interfering RNA (siRNA) targeted to p22phox or p38 MAPK was purchased from San Cruz Biotechnology (Dallas, Texas, USA). The siRNA or scramble control siRNA was

| Table 1. Information of cataract patients. |
|-------------------------------------------|
|                                        | Diabetic Cataract Patients | Senile Cataract Patients |
|                                        | Mean ± SD | Range       | Mean ± SD | Range       |
| Sample size (M:F)                      | 26 (11:15) | 23 (11:12) |
| Eye number (R:L)                       | 30 (13:17) | 30 (14:16) |
| Diabetes, year                         | 9.58 ± 6.99 | 0.25–20    |
| Age, yr                                | 65.93 ± 7.84 | 46–89      |
| Cataract grade                         | 2.93 ± 0.68 | 1–4        |
| Hemoglobin A1c, %                      | 8.02 ± 0.68 | 7–10       |
| Receive treatment                      | 100%       |            |

SD, standard deviation; M, man; F, female; yr, year.
transfected to HLEB3 cells following the kit’s protocol. After incubating for 6 hours with 100 nM siRNA, the medium was changed with high glucose as described above.

**Flow cytometry**

Cell apoptosis was measured by flow cytometry with Annexin-V-FITC apoptosis detection kit. HLEB3 cells were collected and stained with Annexin-V-FITC for 10 min at room temperature. The cells were centrifuged and washed in PBS buffer. Then, the cells were resuspended in PBS containing propidium iodide (PI) and subjected to flow cytometer (BD™LSR II). Data were analyzed using Win MDI 2.9 software.

**Measurement of intracellular ROS**

The generation of intracellular ROS was stained with 2′, 7′-dichlorofluorescein diacetate (DCFH-DA, Beyotime Inst. Biotech, Haimen, China). Under oxidative stress condition, DCFH-DA was oxidized and converted to fluorescent dichlorofluorescein (DCF), which could be imaged by microsystem. Briefly, HLEB3 cells were incubated in diluted DCFH-DA for 20 minutes and then washed with serum-free DMEM. Then cells with fluorescence were counted and imaged in optical slice images with a fluorescence microscope (Leica Microsystem, German).

**Detection of SOD and GSH**

After harvested, cells were washed twice with PBS. Intracellular SOD activity and GSH/GSSG ratio were measured following the protocols of total SOD assay kit and GSH/GSSG assay kit (Beyotime Inst. Biotech, Shanghai, China). Then the plates were measured at 450nm and 412nm separately using an iMark™ microplate absorbance reader (Bio-Rad, Hercules, USA).

**Western blotting**

Western blotting analysis was performed as previously described [12]. Briefly, the equal amount of protein was separated in SDS-PAGE gel and transferred to polyvinylidene fluoride (PVDF) membrane. After incubating for 1 hour with 5% milk in TBST buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% triton X-20) at room temperature, membranes were incubated with primary antibodies overnight at 4°C. Washed in TBST buffer for three times, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody. The membranes were incubated with electrochemiluminescence (ECL) buffer and signals were exposed to X-ray film.

For the examination of human lens anterior capsules, the anterior capsules were collected from patients who underwent cataract surgery. In order to get enough protein for analysis, ten anterior capsules from each group were mixed together and subjected to immunoblotting.

**Statistical analysis**

Data from at least three independent experiments were represented as means ± standard error of the mean (SEM). Statistical analysis was carried out using SPSS 18.0 software. One-way ANOVA was used for comparisons among more than two groups. Student-Newman-Keuls test was used for comparisons between two groups. \( P \) value of < 0.05 was statistically significant.
Results

Cell viability assay

Cell viability of HLEB3 cells in different concentration of decorin (0nM, 50nM, 100nM and 200nM) showed no significant difference when examined by CCK-8 assay (Fig 1E). Moreover, morphological appearance of HLEB3 cells was observed by phase contrast microscopy. We did not find obvious morphological changes at different concentrations of decorin (Fig 1A–1D).

Decorin inhibits apoptosis of HLEB3 cells induced by high glucose

The influence of decorin on the apoptosis of HLEB3 cells are shown in Fig 2. The cells were distinguished into four groups (Fig 2A): unlabeled cells (viable cells), cells that stained with...
annexin V-FITC only (early apoptotic), cell that stained with PI only (necrotic), and cells that stained with both annexin V and PI (late apoptotic). The sum of late apoptotic cells and early apoptotic cells was defined as apoptotic cells. The apoptotic cell percentage was statistically analyzed. (C) Western blot analysis of bcl-2 and bax expression in HLEB3 cells. (D) Optical density ratio of bcl-2 to bax. Decorin reduced high glucose-induced apoptosis of HLEB3 cells. Con, Control; Mann, Mannitol; HG, high glucose. Data were expressed as mean ± SEM. *P < 0.05 vs the control group.

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Fig 2. Effects of decorin on cell apoptosis. (A) Quantitative analysis of HLEB3 cell apoptosis tested by flow cytometry with Annexin-V-FITC apoptosis detection kit. The upper right quadrants represented the late apoptotic cells, and the lower right quadrants represented the early apoptotic cells. (B) The sum of late apoptotic cells and early apoptotic cells was defined as apoptotic cells. The apoptotic cell percentage was statistically analyzed. (C) Western blot analysis of bcl-2 and bax expression in HLEB3 cells. (D) Optical density ratio of bcl-2 to bax. Decorin reduced high glucose-induced apoptosis of HLEB3 cells. Con, Control; Mann, Mannitol; HG, high glucose. Data were expressed as mean ± SEM. *P < 0.05 vs the control group.

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annexin V-FITC only (early apoptotic), cell that stained with PI only (necrotic), and cells that stained with both annexin V and PI (late apoptotic). The sum of late apoptotic cells and early apoptotic cells was defined as apoptotic cells. For the control group, the percent of apoptotic cells was 4.5% ± 0.6%. After treatment with high glucose, the apoptotic percentage increased to 9.9% ± 0.4% (P < 0.05 vs the control). When cells were treated with 50 nM decorin before high glucose administration, the apoptotic percentage decreased to almost normal level (Fig 2A and 2B). Additionally, the western blot results showed that high glucose make the protein expression level of bax increased and the protein expression level of bcl-2 of HLEB3 cells decreased. The densitometry quantitation indicated that the ratio of bcl-2 to bax decreased significantly
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in high glucose media compared to that of control. Decorin can reverse the expression ratio of bcl-2 to bax in high glucose-treated cells (Fig 2C and 2D).

Decorin inhibits the oxidative stress response of HLEB3 cells under high glucose condition

Results indicated that ROS production was significantly increased in high glucose condition (Fig 3A and 3B), and the ratio of GSH/GSSG (Fig 3C) and the SOD activity of HLEB3 cells were decreased significantly (Fig 3D). Decorin can reverse this phenomenon induced by high glucose (Fig 3).
Decorin inhibits the activation of p38 and p22_{phox} in HLEB3 cells induced by high glucose

Western blot analysis was used to examine the activation of p38 and p22_{phox}, which may participate in the effects of decorin on HLEB3 cells. Density analysis indicated that high glucose significantly increased the activation of p38 MAPK (Fig 4A and 4B) and the expression level of p22_{phox} protein (Fig 4C and 4D). When treated with 50 nM decorin, HLEB3 cells expressed decreased phosphorylation of p38 MAPK protein and the expression of p22_{phox} protein compared with high glucose group (Fig 4).

P38 MAPK is involved in regulating the oxidative stress and apoptosis of HLEB3 cells induced by high glucose

We do not know whether p38 MAPK was related to the high glucose-induced cell apoptosis and oxidative stress. To definite this, siRNA targeting p38 MAPK was used to transfecte with HLEB3 cells. Western blot was used to examine the expression level of p38 MAPK. Results showed that the expression of p38 MAPK was significantly reduced by p38 siRNA (Fig 5A and 5B). When cells were transferred with siRNA, flow cytometry analysis showed that the apoptotic percentage of p38 MAPK siRNA-transfected group was greatly reduced compared with control group (P < 0.05 vs the control) (Fig 5C and 5D). High glucose-induced expression level of bax and bcl-2 was reversed by p38 siRNA transfection (Fig 5E and 5F). Furthermore, transfection of p38 siRNA significantly reduced the ROS production, increased the ratio of GSH/GSSG and increased the SOD activity in HLEB3 cells treated with high glucose (Fig 6).

Fig 4. Effects of decorin on high glucose-induced activation of p38 and p22_{phox} in HLEB3 cells. (A-B) p38 MAPK phosphorylation was presented by western blot. (C-D) Western blot results of p22_{phox} expression. β-actin was regarded as an internal control. Decorin reduced the activation of p38 and the expression p22_{phox} in HLEB3 cells under high glucose condition. Con, Control; Mann, Mannitol; HG, high glucose. Data were means ± SEM. *P < 0.05 vs control group.

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A. Western blot analysis showing p38 and β-actin expression levels with Con, p38 siRNA, Con siRNA conditions.

B. Bar graph showing p38 expression levels with Con, p38 siRNA conditions.

C. Flow cytometry analysis showing PI and Annexin V-FITC staining with Con, HG, HG + con siRNA, HG + p38 siRNA conditions.

D. Bar graph showing apoptosis percentage with Con, HG, HG + con siRNA, HG + p38 siRNA conditions.

E. Western blot analysis showing Bax, Bcl-2, and β-actin expression levels with Con, HG, HG + p38 siRNA conditions.

F. Bar graph showing Bcl-2/Bax expression ratio with Con, HG, HG + p38 siRNA conditions.

G. Western blot analysis showing Bax, Bcl-2, and β-actin expression levels with Con, HG, HG + p38 siRNA, con siRNA conditions.

H. Bar graph showing Bcl-2/Bax expression ratio with Con, HG, HG + p38 siRNA, con siRNA conditions.
p22\textsubscript{phox}-p38 MAPK pathway may mediate apoptosis and oxidative stress of HLEB3 cells induced by high glucose

To determine whether there is any possible association between p38 MAPK and p22\textsubscript{phox} in the regulation of oxidative stress induced by high glucose, siRNA targeting p22\textsubscript{phox} was transfected with HELB3 cells. Western blot was used to examine the expression level of protein. Results

![Image](https://doi.org/10.1371/journal.pone.0224251.g005)

Fig 5. siRNA targeting p38 MAPK inhibited the apoptosis of HLEB3 cells induced by high glucose. (A-B) Cells transfected with p38 MAPK siRNA resulted in a significant reduction in expression level of p38 protein. Cells transfection with control siRNA did not affect the expression level of p38 protein. (C) Quantitative analysis of HLEB3 cell apoptosis was tested by flow cytometry with an Annexin-V-FITC apoptosis detection kit. The upper right quadrants represent the late apoptotic cells. The lower right quadrants represent the early apoptotic cells. (D) The sum of late apoptotic cells and early apoptotic cells was defined as apoptotic cells. The apoptotic percentage was statistically analyzed. (E, G) Western blot analysis of bcl-2 and bax expression. (F, H) Optical density ratio of bcl-2 to bax. Con, Control; HG, high glucose. Data were expressed as means ± SEM. *P < 0.05 vs control group, **P < 0.01 vs control group.

![Image](https://doi.org/10.1371/journal.pone.0224251.g006)

Fig 6. Alteration of intracellular oxidative stress after p38 MAPK siRNA transfection. (A-B) Fluorescent intensity with ROS level was examined by DCFH-DA staining with fluorescence microscopy. Scale bars = 20 μm. (C) GSH/GSSG ratio in the indicated groups. (D) The activity of SOD in the indicated groups. High glucose-induced increase of oxidative stress was nearly inhibited in cells transfected with p38 MAPK siRNA. Con, Control; HG, high glucose. Data were means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, compared to the control group respectively.
indicated that the expression of p22\textsuperscript{phox} was significantly reduced by p22\textsuperscript{phox} siRNA (Fig 7A and 7B). Transfection of p22\textsuperscript{phox} siRNA can decrease the phosphorylation of p38 in cells that under high glucose condition (Fig 7C and 7D). These results suggest that p22\textsuperscript{phox} regulates the activation of p38 MAPK and may mediate high glucose-induced apoptosis and oxidative stress of HLEB3 cells.

The increased expression of p22\textsuperscript{phox} and phospho-p38 in diabetic lens anterior capsules

Additionally, we examined the expression of phosphor-p38 MAPK and p22\textsuperscript{phox} in diabetic lens anterior capsules. Results indicated that the expression level of phospho-p38 was significantly increased in anterior capsules from diabetic cataract patients versus to that from simple senile cataract patients (Fig 8A and 8B). Similar results were also observed for the expression of p22\textsuperscript{phox} (Fig 8C and 8D). These results suggest that p22\textsuperscript{phox}-p38 pathway may participate in modulating the oxidative stress in diabetic lens.

Discussion

High glucose-induced oxidative stress is an important cause of diabetic cataract [2]. The human lens epithelial cell line (HLEB3) was widely used to do research [13–15]. Here, using the lens epithelial cell line, we found that high glucose can directly induce the oxidative stress
and cell apoptosis. P38 and p22 are important signaling factors that regulate the high glucose-induced oxidative stress and apoptosis, and p22 works upstream of p38 MAPK. The administration of decorin can inhibit the cellular oxidative stress and cell apoptosis induced by high glucose. Decorin can also inhibit high glucose-induced p22 expression and p38 MAPK activation. These results implied that decorin exhibits the potential therapeutic role to diabetic cataract.

P22 is a subunit of nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase (NOX) that is responsible for ROS production in mitochondria at oxidative stress condition [16]. p22 is upregulated in diabetic endothelial cells and enhance the injury of endothelial cells by high concentration of glucose[17]. The increase of p22 expression induces the oxidative stress injury via activation of p38 MAPK[18]. Our data showed that this signaling pathway also worked in diabetic lens epithelial cell injury. The supporting data are: 1) high glucose could directly induce ROS production, downregulate the ratio of GSH/GSSG and inhibit the SOD activity that lead to increase of the lens epithelial cell apoptosis; 2) high glucose can induce the expression of p22 and p38 MAPK activation. Knocking down p22 downregulates the high glucose-induced p38 MAPK activation, and knocking down p38 MAPK can inhibit high glucose-induced ROS production and cell apoptosis. The viewpoint that ROS is a key inducer of cell apoptosis or necrosis in divergent tissues has been widely accepted[16]; 3) additionally, we examined that the human lens anterior capsules from diabetic cataract patients had higher expression of phospho-p38 and p22 compared with that from senile cataract patients. These results suggested that p38 MAPK is downstream of p22 in the regulation of ROS production in lens epithelial cells at diabetic stress condition.

Decorin is a small leucine-rich proteoglycan constituting the extracellular matrix binding to collagen type I fibrils[4, 5]. In addition, decorin is a signaling molecule involving in regulating the angiogenesis, anti-apoptosis, tumor growth and inflammation[6–9]. Our data showed that decorin can inhibit the high glucose-induced oxidative stress in lens epithelial cells in

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**Fig 8. Expression of p22 and phospho-p38 in human lens anterior capsules.** (A-B) Western blot analysis of p38 MAPK expression. (C-D) Western blot analysis of p22 expression. The expression of phospho-p38 and p22 were both significantly increased in anterior capsules of diabetic cataract patients versus to that of simple senile cataract patients. SC, senile cataract; DC, diabetic cataract. β-actin was regarded as an internal control. Bars were means ± SEM. * P < 0.05 vs SC group.

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vitro. The administration of decorin decrease the ROS production, increase the ratio of GSH/GSSG and increase the expression ratio of bcl-2 to bax in cells treated with high glucose, which suggests decorin may inhibit the development of diabetic cataracts. Moreover, decorin can inhibit p22^{phox} induction and p38 MAPK activation of HLEB3 cells under high glucose condition. However, we only investigated the inhibitory influence of decorin on p22^{phox}-p38 MAPK pathway. We did not explore the other possible pathways and the downstream mechanism following the inhibition of p38 MAPK was not explored also. For financial reasons, we only used human lens epithelium cell line HLEB3 to study. Further research is needed to illuminate the other mechanisms of decorin on the oxidative stress of HLE cells in high glucose condition with primary cells.

In a word, our study proved that decorin can reduce the increment of oxidative stress injury and the apoptotic ratio of HLE cells significantly under high glucose condition in vitro. This effect was mainly regulated suppressing the activation of p22^{phox}-p38 MAPK pathway induced by high glucose. These findings suggested that decorin may have beneficial influence on preventing the development of diabetic cataract.

Supporting information
S1 Raw Images.
(TIF)

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