

## Original Article

# Pterostilbene impact on retinal endothelial cells under high glucose environment

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**Abstract:** Diabetic retinopathy (DR) has complicated pathogenic factors. Studies showed that DR belongs to chronic inflammatory disease, and retinal endothelial cells oxidation by free radicals is one of its mechanisms. Pterostilbene, as the homologous derivative of resveratrol, has obvious antioxidant effect. Its influence on the DR has not been studied. This study intended to investigate the effect and mechanism of pterostilbene on human retinal endothelial cells (hRECs) under high glucose environment to illustrate pterostilbene impact on DR and provide basis for DR clinical treatment. hRECs cultured in high glucose environment were treated by 1.0 mmol/L pterostilbene. MTT assay was applied to test cell proliferation. ELISA was used to detect inflammatory factor TNF- $\alpha$  and IL-1 $\beta$  content. Real time PCR and Western blot were performed to examine NF- $\kappa$ B mRNA and protein expression. ROS and SOD activities were analyzed. Under high glucose environment, hRECs proliferation increased, TNF- $\alpha$  and IL-1 $\beta$  expression elevated, and NF- $\kappa$ B protein level upregulated significantly. On the other side, ROS production increased and SOD activity decreased obviously ( $P < 0.05$ ). Pterostilbene can suppress hRECs over proliferation, decrease TNF- $\alpha$  and IL-1 $\beta$ , inhibit NF- $\kappa$ B protein expression, reduce ROS production, and increase SOD activity markedly compared with high glucose group ( $P < 0.05$ ). Pterostilbene may delay DR progress through alleviating inflammation and antioxidation to suppress hRECs over proliferation.

**Keywords:** Diabetic retinopathy, retinal endothelial cell, pterostilbene, inflammatory factor

## Introduction

Diabetic retinopathy (DR) is a serious complication of diabetes that caused great harm to patients with high incidence among acquired blinding disease [1, 2]. According to WHO survey, there are 360 million people suffered with diabetes, and it is expected to reach 1 billion in 2030 [3, 4]. It was found that DR was closely related to retinal microvascular system injury caused by high glucose environment [5]. DR is a chronic progressive inflammatory disease characterized as large amounts of leukocyte infiltration and adhesion. DR may change the structure of retina and cause metabolic dysfunction. Retinal microvascular endothelial cells (RECs) are responsible for supplying retina nerve nutritional requirements. They play key roles in vision protection by maintaining blood-retinal barrier, removing toxins and inflammatory factors [6, 7]. High blood glucose is an important contributing factor in DR develop-

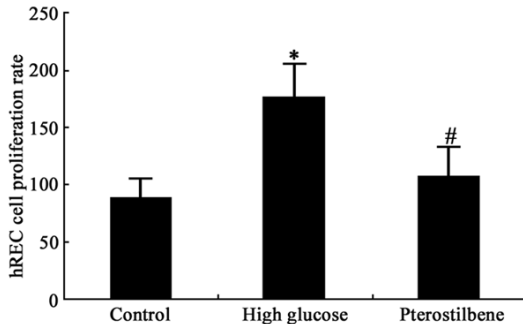
ment by damaging RECs resulting in retinal structure and function disorder. DR has complicated risk factors, and RECs oxidation by free radicals is one of its mechanisms. Thus, how can effectively remove oxygen free radical and maintain the balance between oxidation and antioxidation is one of research hotspots [8, 9].

Previous studies have shown that resveratrol featured as antioxidation, antibacteria, anticancer, regulating vasodilatation, inhibiting platelet aggregation, and regulating lipoprotein metabolism. It can improve the body's immune system [10, 11]. As the homologous derivative of resveratrol, pterostilbene belong to non-flavonoid polyphenol compound. Pterostilbene is rich in grapes, nuts, berries, dragon's blood and propolis. Pterostilbene is the 3, 5-dimethyl derivative of resveratrol that belongs to hydroxyl stilbenes compound, and the chemical structure is (E)-3, 5-dimethoxy-4-hydroxy-styrene [12, 13]. Same pharmacological effects with

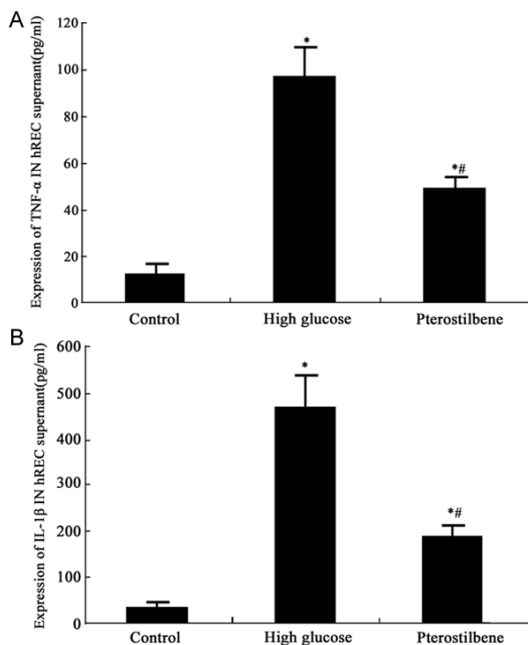
## Pterostilbene inhibits DR

**Table 1.** Primers used for PCR

Gene	Forward, 5'-3'	Reverse, 5'-3'
GADPH	AGTGCCAGCCTCGTCTCATAG	CGTTGAACTTGCCGTGGGTAG
NF-κB	CTCATCTAAGCGGAACAATGG	GCACATTCTCTCCGTAGCG



**Figure 1.** Pterostilbene effect on hRECs proliferation. \* $P < 0.05$ , compared with normal control; # $P < 0.05$ , compared with high glucose group.



**Figure 2.** Pterostilbene effect on TNF-α and IL-1β expression in hRECs. A. Pterostilbene effect on TNF-α expression in hRECs. B. Pterostilbene effect on IL-1β expression in hRECs. \* $P < 0.05$ , compared with normal control; # $P < 0.05$ , compared with high glucose group.

resveratrol, pterostilbene also has antifungal, anti-cell proliferation, prevention of oxidative stress, anti-inflammation, and reducing blood fat effects. It has been confirmed to have obvious effect in the treatment of Alzheimer's dis-

ease, cardiovascular disease, brain damage, tumors, and hypercholesterolemia [14, 15]. As the homologous derivative of resveratrol, pterostilbene has significant antioxidant effect and plays a role in preventing

and treating ischemia-reperfusion injury and tumor, but its influence on DR has not been studied. This study intended to investigate the effect and mechanism of pterostilbene on human retinal endothelial cells (hRECs) under high glucose environment to illustrate pterostilbene impact on DR and provide basis for DR clinical treatment.

### Materials and methods

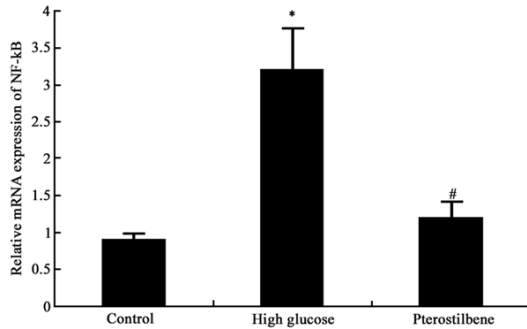
#### Reagents and instruments

hRECs were purchased from Angio-Proteomie (USA). Human microvascular endothelial cell medium was bought from Cell Applications (USA). Penicillin-streptomycin and EDTA were got from Hyclone (USA). Pterostilbene (purity 98%) was from Fuji co., LTD (Japan). DMSO and MTT were purchased from Gibco. Enzyme-EDTA was got from Sigma (USA). PVDF membrane was from Pall Life Science. TNF-α and IL-1β ELISA kits were bought from R & D (USA). RNA extraction kit and reverse transcription kit were from Invitrogen (USA). SOD activity detection kit was bought from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Western blot related reagents were got from Beyotime (Shanghai, China). ECL reagents were purchased from Amersham Bioscience. Rabbit anti-human NF-κB primary antibody and HRP-tagged goat anti-rabbit IgG secondary antibody were bought from Cell Signaling (USA). DNA amplifier was PE Gene Amp PCR System 2400 (USA). Other common reagents were purchased from Sangon (Shanghai, China).

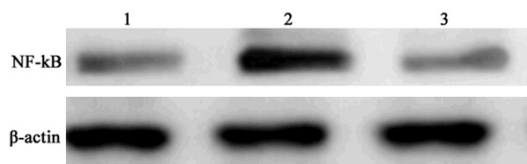
#### hRECs culture and grouping

hRECs in the 3<sup>rd</sup>-8<sup>th</sup> generation were cultured in the dish at  $1 \times 10^6/cm^2$  and maintained in DMEM supplement with 100 U/ml penicillin, 100 μg/ml streptomycin, and 5.5 mmol/L glucose in a humid atmosphere containing 5% CO<sub>2</sub> at 37°C. The cells were passaged every 2-3 days and randomly divided into three groups including normal control; high glucose group: hRECs were treated by microvascular endothelial cell medium supplement with 33 mmol/L glucose for 72 h; pterostilbene group: hRECs under high glu-

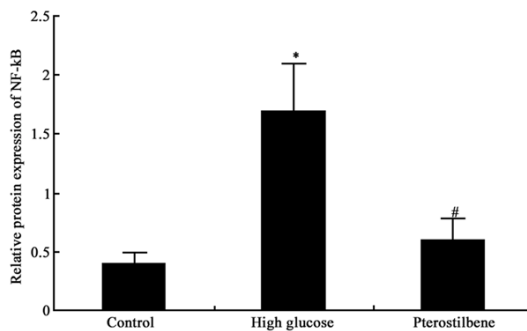
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**Figure 3.** Pterostilbene effect on NF-κB mRNA expression in hRECs. \* $P < 0.05$ , compared with normal control; # $P < 0.05$ , compared with high glucose group.



**Figure 4.** Pterostilbene effect on NF-κB protein expression in hRECs. 1, normal control; 2, high glucose group; 3, pterostilbene group.



**Figure 5.** Pterostilbene effect analysis on NF-κB protein expression in hRECs. \* $P < 0.05$ , compared with normal control; # $P < 0.05$ , compared with high glucose group.

cose environment was treated with 1.0 mmol/L pterostilbene for 72 h [16].

### MTT assay

hRECs in logarithmic phase were seeded in 96-well plate at 3000/well. The cells with five replicates in each group were cultured for 72 h. 20  $\mu$ l MTT at 5 g/L was added to each well. After 4 h incubation, the supernatant was removed and 150  $\mu$ l DMSO was added. The plate was read on microplate reader at 570 nm

after vibration for 10 min to calculate cell proliferation rate.

### ELISA

Cell supernatant was used to detect TNF- $\alpha$  and IL-1 $\beta$  expression. Major steps include: put 50  $\mu$ l diluted standard product into the corresponding reaction holes to prepare the standard curve. Add 50  $\mu$ l samples to each hole. After washing the plate for 5 times, 50  $\mu$ l enzyme reagent was added. The plate was washed for 5 times again after incubated at 37°C for 30 min. 50  $\mu$ l agent A and 50  $\mu$ l agent B were inserted to each hole and the plate was incubated at 37°C for 10 min. The reaction was terminated after adding 50  $\mu$ l terminates liquid. The plate was measured at 450 nm wavelength to get the absorbance value (OD value). The sample concentration was calculated according to the OD value and standard curve.

### Real-time PCR

Total mRNA was extracted from hRECs by Trizol and reverse transcribed to cDNA (Table 1). Real-time PCR was used to detect target gene expression. PCR reaction contained 51°C for 1 min, followed by 35 cycles including 90°C for 30 s, 58°C for 50 s and 72°C for 35 s. Amplification curve and melting curve were verified for quality control. Gene expression levels were quantified relative to the expression of GAPDH using an optimized comparative Ct ( $2^{-\Delta Ct}$ ) value method.

### Western blot

After cracked on ice for 5-10 min, the cells were moved to EP tube and centrifuged at 10000 rpm and 4°C for 5 min. The supernatant was whole protein solution. The protein was separated by 10% SDS-PAGE electrophoresis and transferred to PVDF membrane. The membrane was blocked by 5% milk for 2 h and incubated with NF-κB antibody (1:1000) at 4°C overnight and secondary antibody (1:2000) at room temperature for 30 min. The bind detected by chemiluminescence and analyzed by Quantity One software. The experiment was repeated for four times (n = 4).

### SOD activity detection

Superoxide dismutase (SOD) activity was detected by kit according to the instructions. Cell protein was extracted, water bathed at 95°C for 40 min. Then it was cooled and centri-

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**Table 2.** Pterostilbene effect on oxidative stress markers in hRECs

Parameter	Normal control	High glucose	Pterostilbene
ROS	98 ± 12.19	167 ± 21.78*	109 ± 11.90#
SOD	167 ± 3.21	34 ± 1.0*	145 ± 2.75#

\* $P < 0.05$ , compared with normal control; # $P < 0.05$ , compared with high glucose group.

fused at 4000 rpm for 10 min. Ethanol-chloroform mixture (5:3, v/v 5:3) was used to extract the ethanol phase from the tissue to detect SOD activity.

### ROS content detection

Reactive oxygen species level in each group was detected. Cell protein was extracted, water bathed at 95°C for 40 min. Then it was cooled and centrifuged at 4000 rpm for 10 min. The protein was incubated in 2',7'-dichlorofluorescein diacetate (DCF-DA) for 15 min and then centrifuged at 10000 rpm for 15 min. Then the precipitation was resuspended by PBS and incubated at 37°C for 60 min. ROS level was determined by spectrophotometer.

### Statistical analysis

All statistical analyses were performed using SPSS16.0 software (Chicago, IL). Numerical data were presented as means and standard deviation ( $\bar{x} \pm S$ ). Differences between multiple groups were analyzed by one-way ANOVA.  $P < 0.05$  was considered as significant difference.

## Results

### Pterostilbene effect on hRECs proliferation

MTT assay was applied to detect the effect of different concentrations of pterostilbene on hRECs proliferation under high glucose environment. It was found that high glucose environment significantly promoted hRECs proliferation ( $P < 0.05$ ). Different concentrations of pterostilbene treatment can decrease hRECs proliferation obviously compared with high glucose group ( $P < 0.05$ ) (Figure 1). It suggested that pterostilbene can inhibit RECs proliferation under high glucose environment.

### Pterostilbene effect on TNF- $\alpha$ and IL-1 $\beta$ expression in hRECs

ELISA was used to test TNF- $\alpha$  and IL-1 $\beta$  expression in hRECs' supernatant. It revealed that inflammatory factors TNF- $\alpha$  and IL-1 $\beta$  expres-

sion increased significantly in hRECs supernatant under high glucose environment ( $P < 0.05$ ). Pterostilbene treatment inhibited TNF- $\alpha$  and IL-1 $\beta$  overexpression in hRECs cultured in high glucose ( $P < 0.05$ ). Compared with normal control, they were still high in pterostilbene group ( $P < 0.05$ ) (Figure 2). It indicated that pterostilbene can alleviate inflammation by reducing inflammatory factors secretion in hRECs under high glucose environment.

### Pterostilbene effect on NF- $\kappa$ B mRNA expression in hRECs

Real time PCR was performed to detect NF- $\kappa$ B mRNA expression in hRECs cultured in high glucose. Compared with normal control, NF- $\kappa$ B mRNA expression level increased obviously under high glucose environment ( $P < 0.05$ ). Pterostilbene treatment suppressed NF- $\kappa$ B mRNA expression in hRECs markedly ( $P < 0.05$ ) (Figure 3).

### Pterostilbene effect on NF- $\kappa$ B protein expression in hRECs

Western blot was further used to determine NF- $\kappa$ B protein expression in hRECs. NF- $\kappa$ B protein level elevated obviously in hRECs culture in high glucose ( $P < 0.05$ ), while pterostilbene treatment reduced its level significantly ( $P < 0.05$ ) (Figures 4 and 5). It revealed that high glucose environment can increase NF- $\kappa$ B mRNA and protein expression, leading to RECs structure and function disorder. Pterostilbene act on hRECs under high glucose environment can downregulate NF- $\kappa$ B mRNA and protein expression to improve DR.

### Pterostilbene effect on oxidative stress markers in hRECs

ROS production and SOD content were detected. It was found that ROS production increased, while SOD content decreased markedly in hRECs under high glucose environment ( $P < 0.05$ ). Pterostilbene adding to hRECs under high glucose environment suppressed ROS production and increase SOD content significantly ( $P < 0.05$ ) (Table 2). It demonstrated that pterostilbene can protect RECs by regulating oxidative stress response to reduce hRECs abnormal proliferation.

## Discussion

DR is a common microvascular complication of DM that can lead to retinal microvascular progressive damage. It seriously affects the patients' physical and mental health, and brings heavy mental and economic burden to the society, patients and families [2]. Although medical technology progress continuously, the effect of DR treatment is still not satisfy. High blood glucose environment in DM patients may cause a serious of endocrine metabolism changes in RECs. High blood glucose is an important factor in DM complications occurrence and development, and further can lead to the retina and other organs abnormalities [17, 18]. In the chronic progress of DM, RECs structure and function changes is the most important cell mechanism of its pathological changes. DR belongs to chronic inflammatory disease, and RECs oxidation is one of its pathogenesis. High blood glucose can cause RECs disorder such as promoting cell proliferation and increase angiogenesis by regulating endothelin and vascular endothelial growth factor [19].

Pterostilbene is the metabolites of resveratrol with stronger pharmacological action than resveratrol. Pterostilbene not only has significant advantage in pharmacological effect, but also has high specificity. Pterostilbene presented high biological activity and selectivity but lower toxicity, suggesting that its application advantage is better than resveratrol. Pterostilbene plays an important role in anti-inflammation and regulating redox equilibrium [20, 21]. Therefore, this study confirmed that pterostilbene treatment can change hRECs abnormal expression in high glucose environment. NF- $\kappa$ B expression can activate the corresponding target genes including immune receptors, adhesion molecules, inflammatory cytokines, and acute reactive protein to regulate immune response and amplify inflammation [22]. This study verified that under high glucose effect, NF- $\kappa$ B expression in hRECs further promote inflammatory cytokines secretion. While pterostilbene can inhibit NF- $\kappa$ B expression and effectively reduce TNF- $\alpha$  and IL-1 $\beta$  abnormal increase in hRECs caused by high glucose environment. Increased ROS production can cause the body's tissues and organs oxidative damage. SOD was one of the important antioxidant enzymes in clearing the oxygen free radicals

that plays a vital role in balancing oxidation and anti-oxidation. Its vitality indirectly reflected the ability of organism removal of oxygen free radicals [23]. This study confirmed that ROS production increased and SOD expression decreased in hRECs under high glucose environment, suggesting redox system imbalance in DR development. And pterostilbene can effectively reduce ROS generation and increase SOD expression to scavenge free radicals and retard DR progress.

To sum up, pterostilbene can regulate oxidation balance by alleviating inflammation, and further regulate hRECs over proliferation to delay DR progress.

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## Disclosure of conflict of interest

None.

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