• Basic Research •

Inhibition of viability of human retinal microvascular endothelial cells by vialinin A under high glucose condition

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Abstract

● **AIM:** To investigate the effects of vialinin A on viability of human retinal endothelial cells (HRECs) under high glucose condition and its potential mechanism.

● **METHODS:** The HRECs were divided into four groups: normal glucose control group (NG, 5 mmol/L D-glucose), high glucose group (HG, 30 mmol/L D-glucose), HG+1 μmol/L vialinin A group, and HG+5 μmol/L vialinin A group. The cell viabilities were measured with cell counting kit-8 (CCK-8) assay for proliferation, with scratch assay for migration, and tube formation, for evaluation of the impact of vialinin A on cellular behaviour. Real-time PCR and Western blotting were used to determine the expression level of vascular endothelial growth factor (VEGF).

● **RESULTS:** The proliferative capacity and migration of HRECs was reduced by 5 μmol/L vialinin A in high glucose environment (both $P<0.05$). Vialinin A also inhibited high-glucose-induced tube formation of HRECs. The expression level of VEGF and PI3K in HRECs was also significantly decreased by vialinin A ($P<0.05$).

● **CONCLUSION:** Vialinin A inhibits the cell viability of HRECs. It may serve as a potential target for anti-angiogenic therapy.

● **KEYWORDS:** vialinin A; vascular endothelial growth factor; human retinal endothelial cells; cell viability

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INTRODUCTION

Diabetes retinopathy (DR) is a serious impact on quality of life, and a problem with significant global health impact[1]. According to the data of Visual Loss Expert Group, 1.07% of blindness was associated with DR[2]. With the progress in early diagnosis and technology, the incidence of blinding eye diseases is gradually declining, but with the increase of the population base, rapid aging, and lifestyle changes, the number of diabetes patients will reach 700 million by 2045[3]. The latest research indicates that the global prevalence of DR is 22.27%, while three-quarters of diabetes patients who have been more than 15y will be affected, of which 20% will develop into proliferative DR after 25y of diabetes[3-5]. As is well known, neovascularization is the most typical pathological feature of proliferative DR[6]. These new blood vessels are very fragile, and if not treated in time, ultimately lead to blindness. Therefore, finding effective measures to control neovascularization remains an important and urgent task.

Although the underlying mechanism of proliferative DR abnormal angiogenesis is not fully understood, a series of evidence suggested that chronic hyperglycemia and high glycated haemoglobin levels were the main risk factors for DR[7-8]. More and more evidence suggested that hyperglycemia secrete various angiogenic cytokines and chemokines, such as vascular endothelial growth factor (VEGF), tumor necrosis factor-α, and fibroblast growth factor[9-11]. Among them, VEGF was considered the most important stimulating factor. It is closely related to angiogenesis, promoting migration, proliferation, and tubular formation[12].

At present, anti-VEGF is the main therapeutic target for angiogenesis. Although anti-VEGF therapy can significantly improve retinal hemorrhage and edema, there is a potential for retinal ischemia and hypoxia. Over time, the efficacy of anti-VEGF drugs decreases, leading to increased secretion of growth factors and leakage of blood vessels, resulting in recurrent bleeding and macular edema[13-15]. In addition, although many clinical trials had shown that anti-VEGF treatment improve the severity scale score of non-proliferative DR, further research is needed to assess whether this will have a positive impact on long-term vision outcome, and whether

the benefits of routine use in the real world outweigh the risks[16]. Therefore, further research on new drug targets to control DR is still urgently needed.

Vialinin A is an antioxidant compound isolated from edible mushrooms[17]. There were research reported that vialinin A is an effective inhibitor of ubiquitin-specific protease 5, tumor necrosis factor-α, and Sentrin/sumo-specific protease 1[18-20]. Despite its anti-inflammatory and anti-cancer properties, its specific role in neovascularization in DR is still uninformed. Therefore, we try to explore the role and possible mechanisms of vialinin A in neovascularization, to provide new ideas for the treatment of DR.

MATERIALS AND METHODS

Cell Culture Human retinal microvascular endothelial cell lines (HRECs) were purchased from Ya Ji Biological Technologies (Shanghai, China) and routine cultured in 5% CO_2 at 37℃ in Dulbecco's modified Eagle medium (Cyclone, Utah, USA) supplemented with 10% fetal bovine serum (Cölbe, Germany). Experiments on logarithmic growth phase of cells were conducted.

Cell Viability Assay Cells were seeded in 96-well plates at a density of 1500 cells/well overnight. Each experimental group was treated with 1, 5, and 10 μmol/L vialinin A. After treated for 24, 48, and 72h, cell viability was determined by cell counting kit-8 (CCK-8) assay. CCK-8 solution (Kumamoto, Japan) 10 μL was added to each well for 4h. The absorbance was recorded at a wavelength of 450 nm. Because the concentration of 10 μmol/L vialinin A affects cell growth. Based on previous research methods[21-23], the concentrations of 1 and 5 μmol/L vialinin A were chosen in the subsequent experiments and cells were divided into 4 groups: normal glucose group (NG, 5 mmol/L glucose), high glucose group (HG, 30 mmol/L glucose), HG+1 μmol/L vialinin A group, and HG+5 μmol/L vialinin A.

Cell Proliferation Assay The CCK-8 assay was used to observe the cell proliferation activity. First, cells were seeded into 96 well plates at a density of 1500 cells/well. Vialinin A needs to be dissolved with dimethyl sulfoxide (DMSO), so we added a DMSO control group. The cells were treated with 5 mmol/L glucose, DMSO, 30 mmol/L glucose, HG+1 μmol/L vialinin A, and HG+5 μmol/L vialinin A for 24h. The absorbance value at a wavelength of 450 nm was detected.

Wound-healing Assay The cells with logarithmic growth phase were selected, and inoculated into a 6-well plate with 20 000 cells per well. When the cell fusion rate reaches 80% wounded cells by a plastic pipette tip (200 μL tip) and rinsed with sterile phosphate buffer saline (PBS) three times to remove detached cells and incubated with serum-free medium containing different concentrations of glucose with or without vialinin A. The migration monolayer was photographed at 0 and 24h. Five fields were photographed for each well under the inverted microscope. The scratch repair rate was calculated as: Scratch repair rate=(0h scratch area−24h scratch area)/0h scratch area×100%.

Table 1 Specific set of primers of RT-PCR

Gene	Forward (5′-3′)	Reverse (5′-3′)
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
VEGF-A	TGGTCCCAGGCTGCACCCAT	CGCATCGCATCAGGGGCACA

RT-PCR: Reverse transcription-polymerase chain reaction.

Tube Formation Assay Capillary tube formation *in vitro* assay was performed as previously described with the manufacturer's instructions. Briefly, 60 μL Matrigel (Bedford, MA, USA) was added to a pre-cooled 96-well plate immediately and polymerized. The cells were seeded on the solidify Matrigel immediately at a density of 1.5×10^4 cells per well and cultured for 8h. The pictures were photographed and the numbers of formed meshes were qualitatively assessed by using an Angiogenesis analyzer (Image J, National Institutes of Health Bethesda, MD, USA).

Real-time Polymerase Chain Reaction Cells were harvested and total RNA was isolated using the RNeasy Mini Kit according to the manufacture's protocol. Reverse transcription (RT) of RNA was carried out in a 20 μL final volume using reverse transcription kit (Ominiscript RT Kit). The cDNA was stored at -20℃ before used in real-time polymerase chain reaction (PCR). RT-PCR was performed using a QuantiNova SYBR Green PCR kit (Qiagen) on an Applied Biosystems Stepone real-time PCR System to detect the expression of mRNA of VEGF. The primers sequences were summarized in Table 1. The relative abundances of VEGF were analyzed by $2^{-\Delta\Delta Ct}$ method and normalized with GAPDH, respectively.

Western Blotting Cells were harvested and lysed in 100 μL of lysis buffer with protease inhibitor cocktail for total protein extraction. The protein samples were separated by 10% SDS-PAGE and then electrophoretically transferred to a polyvinylidene fluoride membrane. Nonfat dried milk (5%) in PBS-T was used to block the membranes and then incubated with the rabbit anti-VEGF (Santa Cruz, California, USA, 1:500) and anti-phosphoinositide 3-kinase (PI3K; BioLegend, California, USA, 1:1000) antibodies at 4℃ overnight followed by the horseradish peroxidase-conjugated secondary antibodies (Beyotime Institute of Biotechnology, Shanghai, China) for 1h at 37℃. Immunoblots were visualized by chemiluminescence detection reagents (Cell Signing Technology, Danvers, MA, USA) and the picture was photographed by ChemiDocTM MP Imaging System (BIO-RAD, California, USA). The gray value of each protein band was determined by Image J software and β-actin (Beyotime Institute of Biotechnology, Shanghai, China) was served as an internal control.

Statistical Analysis SPSS 21.0 software (SPSS, Armonk, NY, USA) and GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) program were used for statistical analyses and graphic drawings. ANOVA was performed to assess statistical differences. P<0.05 was considered statistically significant.

RESULTS

Effect of Vialinin A on HRECs Effect of vialinin A on HRECs were analyzed by CCK-8. Vialinin A 5 μmol/L had no significant impact on the activity of HRECs in 24h (Figure 1). However, vialinin A 10 μmol/L reduced the growth ability of HRECs. Therefore, referring to previous study[24], 5 μmol/L vialinin A was used as an optimal level in the following studies.

Effect of Vialinin A on HRECs Proliferation Induced by High Glucose Compared with the NG group, DMSO had little effect on cell proliferation ability, and the difference was not statistically significant (P>0.05). Compared to HG, the proliferation of high-glucose-induced HRECs was inhibited by 5 μmol/L vialinin A (P<0.05; Figure 2).

Effect of Vialinin A on HRECs Migration Induced by High Glucose Compared to NG group, treatment with 30 mmol/L glucose significantly accelerated wound closure, whereas treatment of 5 μmol/L vialinin A inhibited high-glucose-induced cell migration (P<0.05; Figure 3).

Effect of Vialinin A on Tube Formation of HRECs As shown in Figure 4, capillary-like structures was significantly increased under HG conditions (P<0.05). However, high glucose-induced tube formation was significantly inhibited by 5 μmol/L vialinin A.

Effect of Vialinin A on VEGF Expression As illustrated in Figure 5, high glucose significantly increased VEGF, which was markedly suppressed by 5 μmol/L vialinin A (P<0.05).

Effect of Vialinin A on PI3K in High Glucose-induced HRECs Due to the downstream PI3K of VEGF, it could regulate the proliferation, migration, and sprouting of vascular endothelium, as well as the sensitivity of endothelial cells to fluctuations in PI3K[25-26]. To elucidate the underlying mechanisms of Vialinin A on the angiogenic of HRECs, we detected the protein of PI3K in each group. The levels of PI3K were significantly up-regulated in high glucose-treated cells, while these changes were partly reversed by the treatment of vialinin A in a dose-dependent manner (P<0.05; Figure 6).

DISCUSSION

Angiogenesis is an important process of growth and development, but due to the imbalance of various positive and negative vascular regulation in the vascular microenvironment, it causes dysfunction of endothelial cells, leading to disease progression[27]. Retinal endothelial dysfunction is a primary pathological mechanism of DR. As one of the most

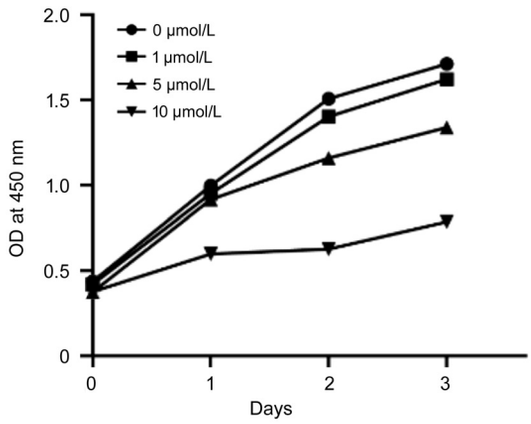

Figure 1 Influence of vialinin A on HRECs Cells were intervened with different concentrations of vialinin A. The absorbance was recorded at a wavelength of 450 nm. HRECs: Human retinal microvascular endothelial cell lines; OD: Optical density.

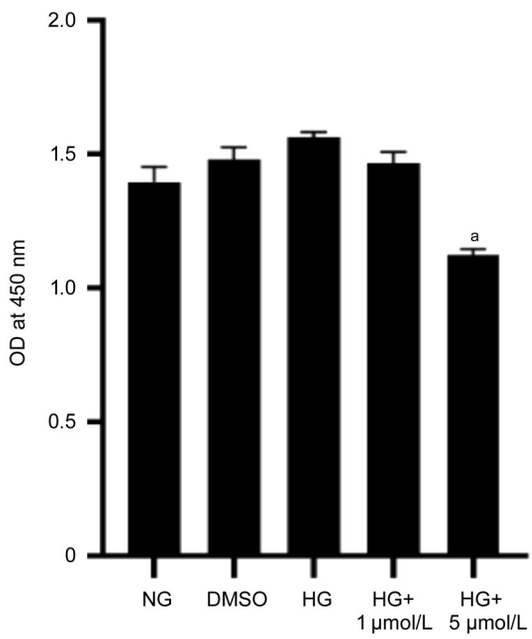

Figure 2 Effect of vialinin A on high-glucose-induced proliferation CCK-8 was used to detect cell proliferation. The data are repeated three times. [a]P<0.05 *vs* HG group. NG: Normal glucose; HG: High glucose; DMSO: Dimethyl sulfoxide; OD: Optical density.

metabolically active tissues, unstable blood glucose can lead to endothelial dysfunction in the retina[28]. Hyperglycemia can lead to a decrease in the transport rate of microvascular endothelial cells, resulting in an increase in intracellular polyol pathway flow and signaling pathways[29-30]. This makes retinal vascular endothelial cells the primary target of hyperglycemic injury, causing retinal endothelial cell damage and disruption of the blood-retinal barrier, leading to the accumulation of extracellular fluid within the macula, as well as thickening of the capillary basement membrane and resulting extracellular matrix deposition[31]. At present, inhibiting the expression of VEGF and its complex is still the hot-spot to treat diabetes retinopathy and cancer[32-33]. Therefore, it is significant to

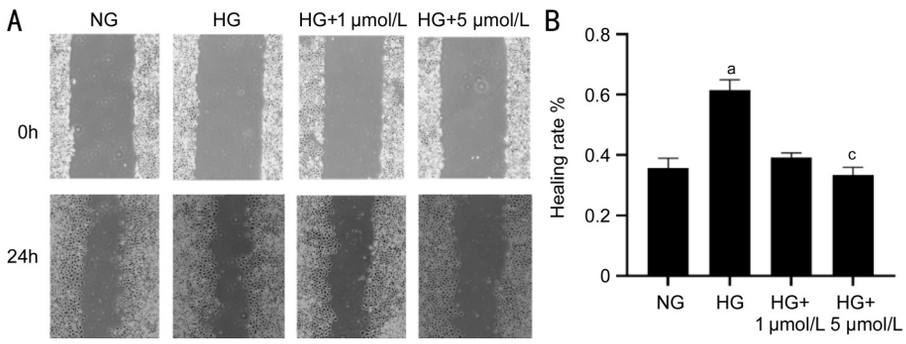

Figure 3 Effect of vialinin A on high-glucose induced migration A: Scratch experiments were used to analyze the migration ability of cells (×200); B: Statistical analysis of healing rates of each group. [a]*P*<0.05 *vs* NG group, [c]*P*<0.05 *vs* HG group. NG: Normal glucose; HG: High glucose; HRECs: Human retinal microvascular endothelial cell lines.

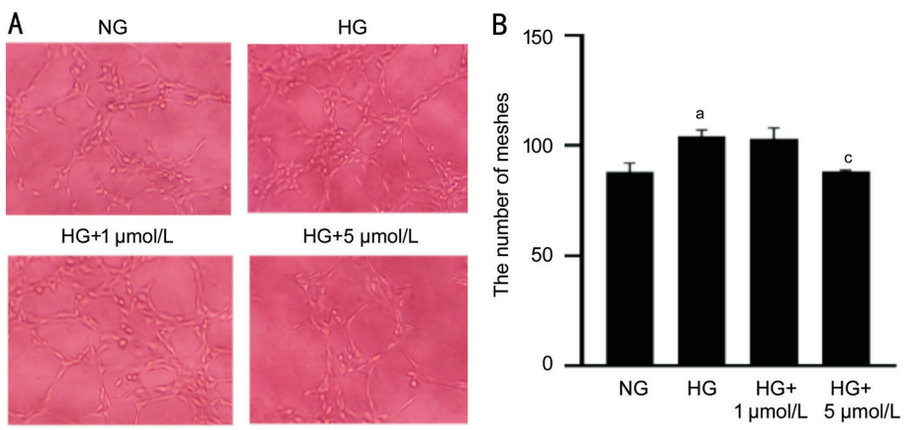

Figure 4 Effect of vialinin A on HG-induced tube formation A: Matrigel experiments were used to analyze the tube formation of HRECs (×200); B: Statistical analysis of vascular meshes of each group. [a]*P*<0.05 *vs* NG group, [c]*P*<0.05 *vs* HG group. NG: Normal glucose; HG: High glucose; HRECs: Human retinal microvascular endothelial cell lines.

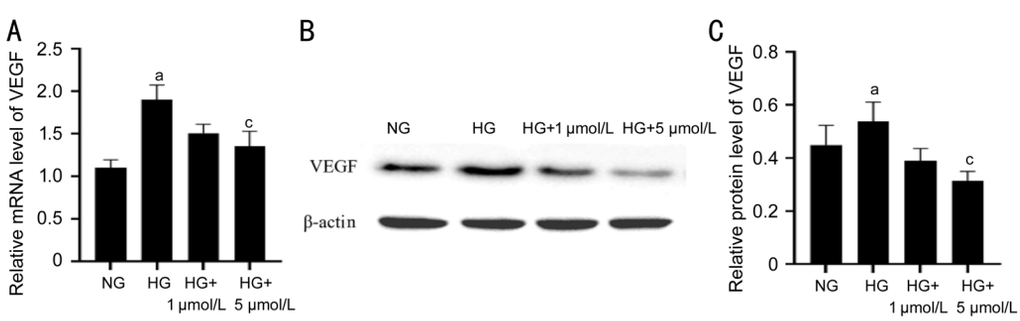

Figure 5 Effect of vialinin A on VEGF expression in HRECs A: VEGF expression by RT-PCR; B: VEGF protein expression by Western blotting; C: Ratios of VEGF to β-actin. [a]*P*<0.05 *vs* NG group, [c]*P*<0.05 *vs* HG group. NG: Normal glucose; HG: High glucose; HRECs: Human retinal microvascular endothelial cell lines; VEGF: Vascular endothelial growth factor; RT-PCR: Reverse transcription-polymerase chain reaction.

explore the function of retinal vascular endothelial cells in high glucose for the treatment of DR.

This study simulates the pathological state of DR *in vivo* using high glucose stimulation of HRECs. Similar to other studies, our work also indicates that high glucose promotes the viability of retinal vascular endothelial cells[34]. Our current work and previous research findings[22] indicated that high glucose can enhance the angiogenic activity of vascular endothelial cells.

Vialinin A is a bioactive compound extracted from edible mushrooms[17]. Previous studies had found that vialinin A had effective anti-inflammatory and anti-cancer effects. Vialinin A exerted an anti-cancer role by inhibiting the production of reactive oxygen species[24]. Vialinin A reduced liver inflammation and fibrosis by inhibiting the Rheb/mTOR signaling pathway[35]. In addition, vialinin A also reduced the inflammatory response in the lungs by regulating T cells[36]. Vialinin A reduced neuronal apoptosis and protect against neurological dysfunction caused by cerebral ischemia-reperfusion injury[37]. In our study, vialinin A prevents the high glucose-induced vascularization process of HRECs. As a

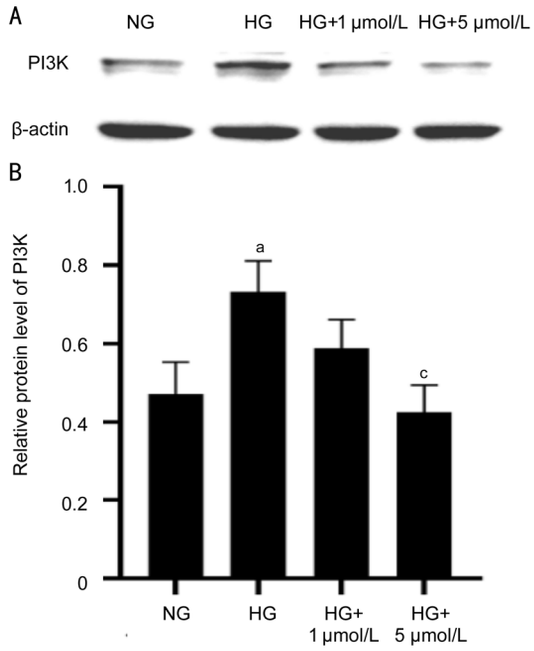

Figure 6 Effect of vialinin A on PI3K in high glucose-induced HRECs
A: Western blotting was used to determine the protein level of PI3K; B: Ratios of PI3K to β-actin. [a]*P*<0.05 *vs* NG group, [c]*P*<0.05 *vs* HG group. NG: Normal glucose; HG: High glucose; HRECs: Human retinal microvascular endothelial cell lines; PI3K: Phosphoinositide 3-kinase.

consequence, we believe that vialinin A may be a useful target for intervention in ocular angiogenesis.

At present, anti-VEGF therapy had become an effective weapon for treating neovascularization. In non-proliferative and proliferative DR patients, the elevated stimulation of nuclear factor κB may be associated with increased VEGF expression[38]. In addition, VEGF, as a pro-inflammatory molecule, can also promote macrophage inflammatory protein-1. The expression of monocyte chemoattractant protein-1, interleukin-8, and other proinflammatory cytokines, specifically blocking VEGF can reduce tumor necrosis factor α, intercellular adhesion molecules 1, and nuclear factor κB level in diabetes mice[39]. The increase in VEGF level caused by diabetes is considered a biological marker of DR severity, and the increase of VEGF content in vitreous has a significant correlation with DR severity[28]. Furthermore, PI3K is involved in the formation of neovascularization by stimulating the expression of various factors[40-43]. Some studies suggested that PI3K participates in the pathological and physiological processes of DR[44]. The VEGF-mediated pro-angiogenic signal may stimulate the anti-tumor formation of endothelial cells through PI3K[44]. In addition, blocking PI3K is considered a beneficial therapeutic strategy for treating proliferative DR[45]. Our research results showed that under high glucose conditions, HRECs induce significant activation of PI3K and VEGF. In addition, in high glucose-induced HRECs, vialinin A can inhibit VEGF secretion and PI3K activation, indicating that vialinin A may exert its anti-angiogenic role through inhibit VEGF and PI3K expression. Therefore, vialinin A can serve as a new direction for DR treatment.

There were several limitations. First, this research used a high glucose environment to simulate DR, which has certain limitations. These results need further confirmation *in vivo* experiments. Second, whether vialinin A may become a target for treating proliferative DR requires additional pharmacological data.

In summary, vialinin A inhibits the cell viability of HRECs. It may serve as a potential target for anti-angiogenic therapy.

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Conflicts of Interest: Chen ZG, None; **Liu GQ,** None; **Liu WM,** None; **Lu PR,** None.

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