Visfatin promotes angiogenesis of RF/6A cells through upregulation of VEGF/VEGFR-2 under high-glucose conditions

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Abstract. Visfatin is a type of adipocytokine that is highly expressed in the serum and vitreous of patients with diabetic retinopathy. The purpose of the present study was to investigate the effect and mechanism of visfatin on angiogenesis in RF/6A monkey chorioretinal retinal endothelial cells under high glucose (HG) conditions *in vitro*. RF/6A cells were randomly divided into four groups: Control group, under high glucose (HG) group (25 mM D-glucose), visfatin group 1 (10 nM visfatin + 25 mM D-glucose), visfatin group 2 (20 nM visfatin + 25 mM D-glucose) and visfatin group 3 (30 nM visfatin + 25 mM D-glucose). After 24 and 48 h, a Cell Counting Kit-8, wound-healing assay and Matrigel tube formation assay were used to detect cell proliferation, migration and cell tube formation, respectively. Subsequently, the expression levels of VEGF and VEGF receptor 2 (VEGFR-2) in cells of visfatin group 3 were observed by western blot and reverse transcription-quantitative PCR analyses. At 24 and 48 h, the cell proliferation and migration distance in the HG group were reduced compared with those in the control group ($P<0.05$). Compared with those in the HG group, the cell proliferation and migration distance in all visfatin groups were significantly increased ($P<0.05$), with the highest significance in visfatin group 3. Visfatin significantly promoted tube-like structure formation by RF/6A cells, particularly at the concentration of 30 nM. The protein and mRNA expression levels of VEGF and VEGFR-2 were significantly increased in the HG group as compared with those in the control group ($P<0.05$). Furthermore, compared with those in the HG group, VEGF and VEGFR-2 protein and mRNA expression levels were significantly increased in visfatin group 3 ($P<0.05$). Overall, visfatin promoted the proliferation, migration and tube formation of RF/6A cells under HG conditions, suggesting that visfatin has a potent effect on retinal neovascularization and its mechanism may be associated with the promotion of VEGF and VEGFR-2 expression under HG conditions.

Introduction

Diabetic retinopathy (DR) is an ophthalmic complication caused by diabetes mellitus that may lead to severe damage of vision and even blindness. In industrialized countries, DR has become the leading cause of blindness in the working population (1,2). In particular, when DR develops to the proliferative diabetic retinopathy (PDR) stage, it may cause severe complications, such as retinal neovascularization (RNV), vitreous hemorrhage, traction retinal detachment and neovascularization glaucoma (3), leading to severe vision impairment. RNV is one of the major pathological changes of PDR and its formation is a complex process, including the regulation of inflammatory factors, oxidative damage and various cytokines (4-7). Research has indicated that adipose tissue is not only a simple energy storage organ but also an endocrine organ. Adipose tissue synthesizes and releases various adipocytokines, such as leptin and resistin, which are widely involved in numerous pathophysiological states, such as obesity, insulin resistance and cardiovascular disease (8).

Visfatin is a novel adipocytokine that is synthesized and released primarily from visceral fat (9). Visfatin is able to bind to and activate the insulin receptor on specific loci to simulate an insulin-like effect to decrease blood sugar levels. It also participates in the inflammatory response, regulates lipid metabolism, promotes differentiation, participates in the formation of atherosclerosis and adjusts the maturity of vascular smooth muscle. A previous study has indicated that visfatin levels are elevated in serum and vitreous fluids in patients with PDR and that the plasma levels of visfatin are associated with vascular endothelial function in patients with

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diabetes (10). Shen *et al* (11) studied the effect of various doses of glucose on the proliferation of RF/6A cells in a time-course study performed for 24-72 h. The results from the MTT assay suggested that glucose promoted cell proliferation at all concentrations tested (10-50 mM) without any effect on their viability and at all time-periods tested (24-72 h). Another study indicated that the expression level of visfatin was increased in retinal tissues of diabetic rats (12). These results suggest that visfatin may be involved in the development of DR. However, whether visfatin promotes the formation of RNV in diabetic patients and its underlying mechanisms have remained elusive. Thus, in the present study, the effect of visfatin on angiogenesis in RF/6A monkey chorioretinal retinal endothelial cells under high-glucose (HG) conditions was investigated *in vitro*.

Materials and methods

Materials. RF/6A cells were purchased from The Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences. Human recombinant visfatin was purchased from Peprotech, Inc. The anti-VEGF (cat. no. BS91432) antibody and antibody-HRP (cat. no. BS6007MH) was obtained from Bioworld Technology, Inc. The anti-VEGFR-2 (cat. no. SC-393163) antibody, VEGFR2 inhibitor (SU1498) and GAPDH (cat. no. SC-166574) were purchased from Santa Cruz Biotechnology, Inc. RPMI-1640 was from Gibco (Thermo Fisher Scientific, Inc.). The other cell culture reagents were obtained from Beyotime Institute of Biotechnology.

Cell culture and treatment. RF/6A cells were incubated in RPMI-1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin solution at 37˚C with 5% CO_2 (all from Beyotime Institute of Biotechnology). The culture medium was replaced 24 h after seeding and different concentrations of visfatin and/or D-glucose were added to the medium. RF/6A cells were divided into 5 groups: Control group, HG group, visfatin group 1 (10 nM visfatin + 25 mM D-glucose), visfatin group 2 (20 nM visfatin + 25 mM D-glucose), visfatin group 3 (30 nM visfatin + 25 mM D-glucose) and the visfatin group 4 (HG + SU1498).

Cell counting kit-8 (CCK-8) cell proliferation assay. RF/6A cells were inoculated into 96-well plates at $5x10^3$ cells/well in 100 μl culture medium. Following incubation overnight in a 5% CO_2 incubator at 37˚C, the cells were treated according to their assigned group for 24 h. Subsequently, 10 μl CCK-8 solution was added to each well, followed by incubation for 4 h at 37˚C. This was repeated three times independently of each other. An ELISA plate reader at the wavelength of 450 nM was used to determine the absorbance and the optical density was recorded in order to calculate the cell proliferation rate.

Cell migration determined by cell scratch assay. A marker was used to draw three evenly spaced straight lines behind the 6-well plate as positioning lines. RF/6A cells were inoculated in 6-well plates in at $5x10^5$ cells per well. After 24 h of culture *in vitro* in a dish, a monolayer had formed and a 100-μl pipette tip was used to scratch the cells, perpendicular to the positioning line. The intersection point of the scratch and the positioning line were taken as the monitoring point. PBS was used to gently rinse the bottom of the culture well three times to remove any detached cells. Medium containing 10% fetal bovine serum (13,14) and different concentrations of visfatin and D-glucose were added according to the different group assignments. Under an inverted microscope, an image of the scratch width at the monitoring point was captured, which was considered as the 0-h time point. The cell culture was continued for 24 and 48 h and the width of the scratches at the monitoring points was recorded. ImageJ software version 1.7.0 [National Institutes of Health (NIH)] was used to calculate the width of the scratches. A total of five fields of view were selected for observation in each group. The independent experiments were repeated three times.

Cell tube formation detected by Matrigel assay. Matrigel was dissolved according to the manufacturer's instructions. Subsequently, 100 μl Matrigel was slowly added to each well of a 96-well plate. RF/6A cells were digested and diluted to $2x10^5$/ml with cell culture medium containing 10% fetal bovine serum. Cell suspension (50 μl) was added to each well and 50 μl visfatin and D-glucose were added according to the different group assignments. After incubation for 24 h, the cells were observed under a phase-contrast microscope, and images of 5 randomly selected fields were captured at a magnification of x100. ImageJ software version 1.7.0 was used to calculate the number of complete tubes formed. Each group was set up in triplicate wells. The independent experiments were repeated three times.

Expression of VEGF and VEGFR-2 detected by western blot analysis. RF/6A cells in each group were lysed using RIPA buffer (Beyotime Institute of Biotechnology). Nucleus proteins of RF/6A cells of each group were also obtained through using nucleus protein extraction kit (Beyotime Institute of Biotechnology). Cell lysate (30 μg protein per lane) was then separated by 12% SDS-PAGE. The separated proteins were transferred onto nitrocellulose membranes (Beyotime Institute of Biotechnology), which were then blocked with Tris-buffered saline containing Tween-20 with 5% non-fat milk. The membrane was incubated overnight with primary antibodies to GAPDH (1:1,000), VEGF (1:1,000) and VEGFR-2 (1:1,000), and then incubated with a horseradish peroxidase-conjugated secondary antibody (Beyotime Institute of Biotechnology; cat. no. P0239; 1:1,000) for 1 h at room temperature. The labeled bands were visualized and quantified using a chemiluminescence imaging system (Tanon 5000; CliNX). CliNX analysis software (version 1.7.0) was used to scan the gels and determine the gray value. The ratio of the target protein gray value to that of β-actin represented the relative expression levels of the target protein.

mRNA expression levels of VEGF and VEGFR-2 detected by reverse transcription-quantitative (RT-q)PCR. After total RNA was extracted with the TRIzol method, the RT reaction was performed according to the manufacturer's protoco. The complementary (c)DNA obtained was amplified on the fluorescence quantitative real-time PCR System (7300 Real-Time PCR System; Applied Biosystems; Thermo Fisher Scientific, Inc.). Using 1 μl cDNA as a template and GAPDH as the internal reference, the Power SYBR-Green PCR MasterMix kit (Beyotime Institute of Biotechnology) was used for PCR

Table I. Primer sequences.

mRNA/primer direction	Sequence
VEGF mRNA	
F	5'-GAGCCTTGCCTTGCTGCTCTAG-3'
R	5'-CACCAGGGTCTCGATTGGATG-3'
VEGFR-2 mRNA	
F	5'-GCGTGATTCTGAGGAAAGG-3'
R	5'-ATAAACAGTGGAGGCTATGTCG-3'
GAPDH	
F	5'-CGACAGTCAGCCGCATCTT-3'
R	5'-TCACCTTCCCCATGGTGTCT-3'

F, forward; R, reverse; VEGFR, VEGF receptor.

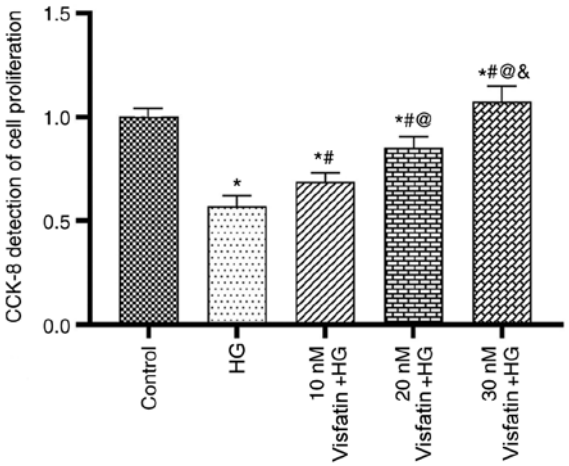

Figure 1. Visfatin promotes RF/6A cell proliferation. *P<0.05 vs. control; #P<0.05 vs. HG group; @P<0.05 vs. 10 nM visfatin group; &P<0.05 vs. 20 nM visfatin group. HG, high glucose; CCK-8, Cell Counting Kit-8.

amplification of gene fragments according to the manufacturer's protocol. Primer sequences are listed in Table I. The PCR reaction parameters were as follows: Pre-denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation for 15 sec at 95˚C and 60˚C for 1 min for annealing and extension, during which fluorescence signals were collected. The data were analyzed with 7300 System SDS software and the statistical $2^{-\Delta\Delta Cq}$ method was used to compare the mRNA expression in each group (15).

Statistical analysis. SPSS 22.0 software (IBM Corp.) was used for statistical analysis. Values are expressed as the mean ± standard deviation. The results obtained for all the tests were submitted to a normality test and groups were compared using parametric ANOVA and Tukey's multiple-comparisons test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of visfatin on RF/6A cell proliferation. The results of the CCK-8 assay indicated that various concentrations of visfatin influenced the proliferation of RF/6A cells (Fig. 1). At 24 and 48 h, the number of viable cells decreased in the HG group compared with that in the control group (P<0.05). Compared with that in the HG group, the cell proliferation in the three visfatin groups was significantly increased in a dose-dependent manner (F=47.13; P<0.05), with the most obvious increase in visfatin group 3.

Effect of visfatin on RF/6A cell migration. In a wound-healing assay, RF/6A cells were treated with visfatin at concentrations of 10, 20 and 30 nM under HG conditions and images of cell migration were captured and analyzed (Fig. 2). At 24 and 48 h, the cell migration distance in the HG group was increased compared with that in the control group (P<0.05). Compared with that in the HG group, the migration distance in the three visfatin groups increased significantly in a dose-dependent manner (F=571.67; P<0.05) and the migration distance in the visfatin group 3 was the largest. The results indicated that visfatin significantly promoted the migration of RF/6A cells.

Effect of visfatin on tube formation of RF/6A cells. On Matrigel, RF/6A cells were treated with visfatin at the three aforementioned different concentrations and the number of tube-like structures formed by RF/6A cells was analyzed after 24 h of cell culture (Fig. 3). The results suggested that, compared with that in the control group, the number of tubes formed by RF/6A cells was increased in the HG group (P<0.05). Compared with that in the HG group, the number of tubes formed in the three visfatin groups was significantly increased in a dose-dependent manner (F=253.78, P<0.05) and the amount of tube formation in the visfatin group 3 was highest. These results suggested that visfatin was able to significantly promote the formation of tube-like structures by RF/6A cells, especially at the concentration of 30 nM.

Expression of VEGF and VEGFR-2 in RF/6A cells. The protein expression levels of VEGF and VEGFR-2 were detected by western blot analysis (Fig. 4). The results suggested that the protein expression levels of VEGF and VEGFR-2 were significantly increased in the HG group as compared with those in the control group (P<0.05). In comparison with those in the HG group, the protein expression levels of VEGF and VEGFR-2 were increased in the 30 nM visfatin + HG group (P<0.05).

RT-qPCR was used to detect the mRNA expression levels of VEGF and VEGFR-2 in the cells (Fig. 5). The results suggested that the mRNA expression levels of VEGF and VEGFR-2 were significantly increased in the HG group as compared with those in the control group (P<0.05). Furthermore, as compared with those in the HG group, the mRNA expression levels of VEGF and VEGFR-2 were significantly increased in the 30 nM visfatin + HG group (P<0.05).

Functional experiments. The results of the CCK8 assay suggested that visfatin at different concentrations influenced the proliferation of RF/6A cells. At 24 and 48 h, cell proliferation in the control group was lower compared with that in the 30 nM visfatin group + HG. Compared with that in the HG + SU1498 (VEGF block) group, the cell proliferation in the 30 nM visfatin + HG group was significantly

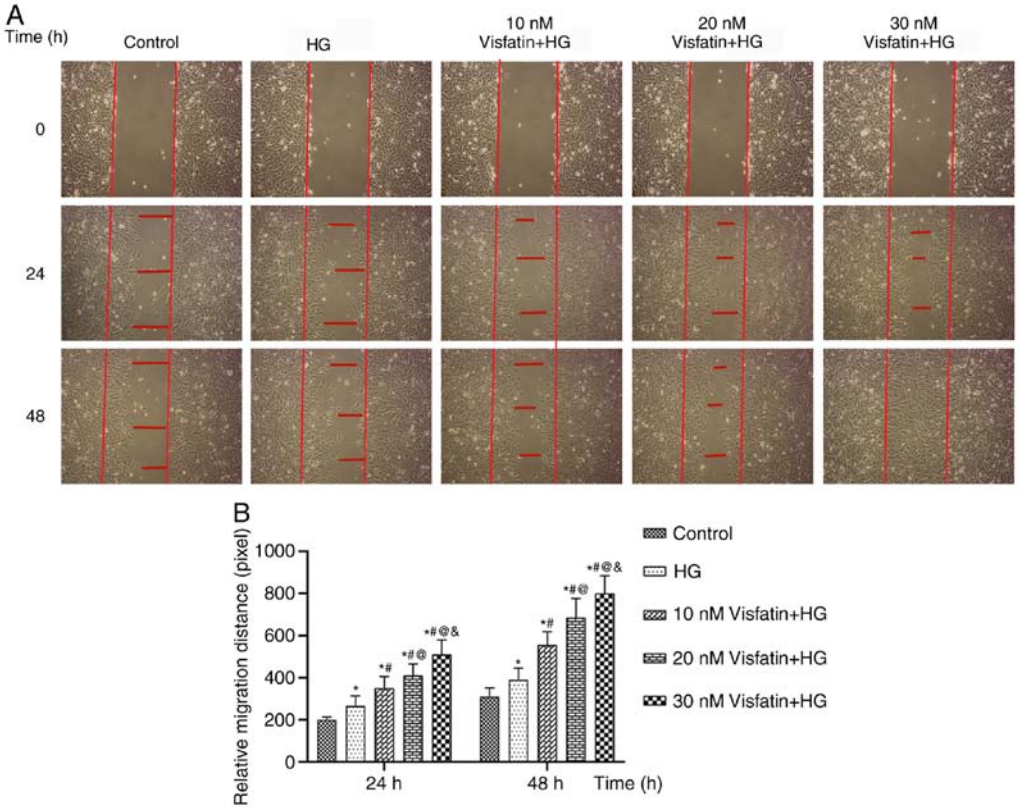

Figure 2. Effect of visfatin on RF/6A cell migration. (A) Image of RF/6A cell migration (magnification, x100). (B) Relative migration distance of RF/6A cells in each group. *P<0.05 vs. control; #P<0.05 vs. HG group; @P<0.05 vs. 10 nM visfatin group; &P<0.05 vs. 20 nM visfatin group. HG, high glucose.

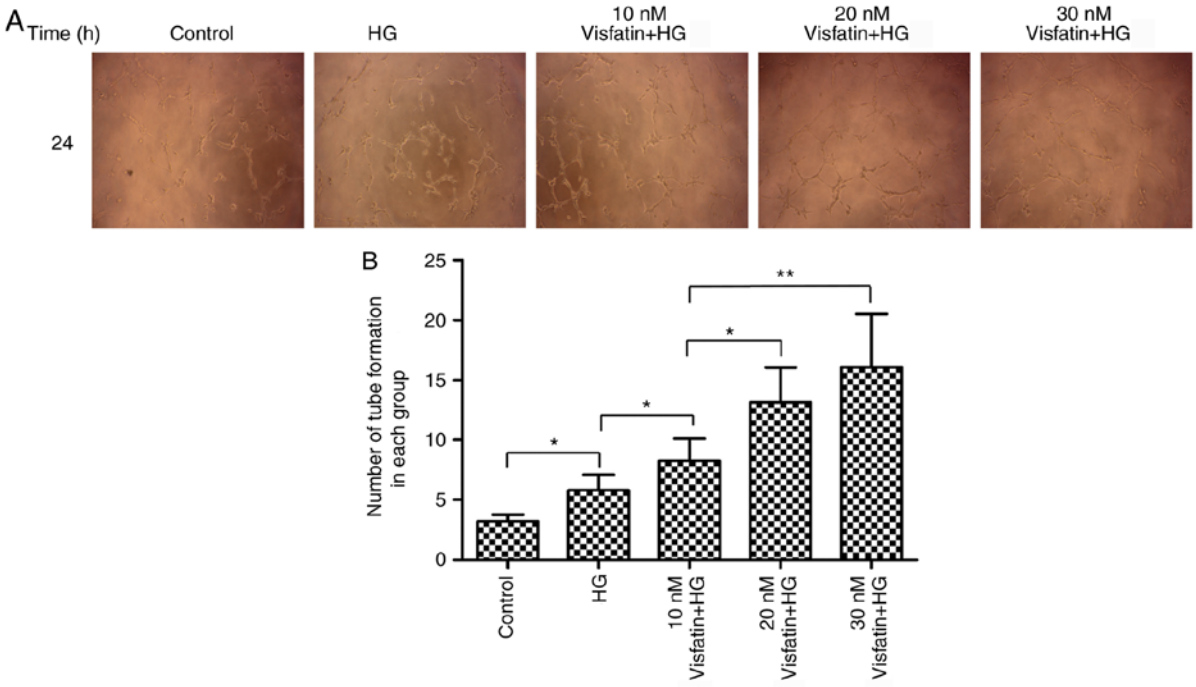

Figure 3. Effect of visfatin on the formation of tube-like structures by RF/6A cells. (A) Image of tube formation by RF/6A cells (magnification, x100). (B) Number of tubes formed in each group. *P<0.05; **P<0.01. HG, high glucose.

increased (P<0.05). Furthermore, compared with that in the 30 nM visfatin + HG group, the cell proliferation in the 30 nM visfatin group was significantly increased (F=15.14, P<0.05; Fig. 6).

The cell migration distance in the visfatin groups (30 nM visfatin and 30 nM visfatin + HG) at 24 and 48 h was increased compared with that in the control group (P<0.05) and the migration distance in the 30 nM visfatin + HG group was the

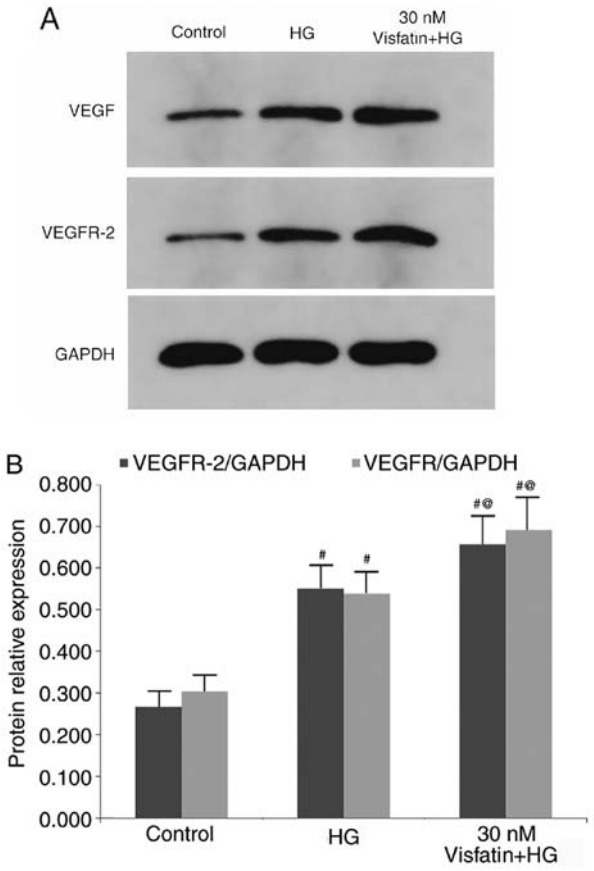

Figure 4. Protein expression levels of VEGF and VEGFR-2 in each group. (A) Detection of VEGF and VEGFR-2 in each group by western blot analysis. (B) Quantified expression levels of VEGF and VEGFR-2 protein in each group. #P<0.05 vs. control group; @P<0.05 vs. HG group. VEGFR, VEGF receptor; HG, high glucose.

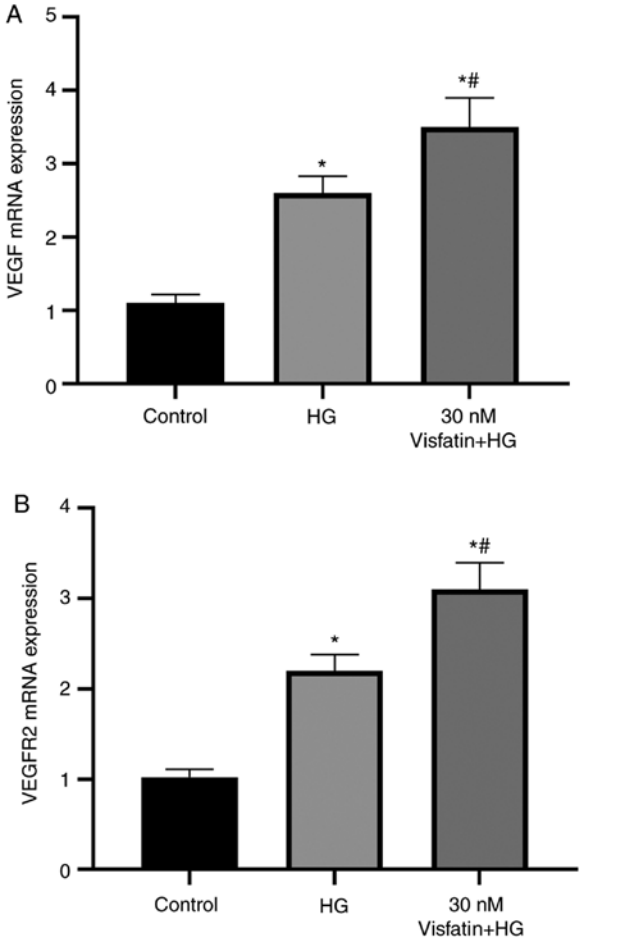

Figure 5. mRNA expression levels of VEGF and VEGFR-2 in each group. (A) VEGF and (B) VEGFR-2 mRNA expression in each group. *P<0.05 vs. control group; #P<0.05 vs. HG group. VEGFR, VEGF receptor; HG, high glucose.

largest (P<0.05). Compared with that in the visfatin + HG + SU1498 group, the migration distance in the 30 nM visfatin + HG group was significantly greater (F=177.89, P<0.05; Fig. 7).

The number of tubes formed by RF/6A cells in the 30 nM visfatin group was increased compared with that in the control group (P<0.05). Compared with that in the 30 nM visfatin + HG group, the number of tubes formed in the 30 nM visfatin and visfatin + HG + SU1498 groups was significantly decreased (F=13.50, P<0.05; Fig. 8).

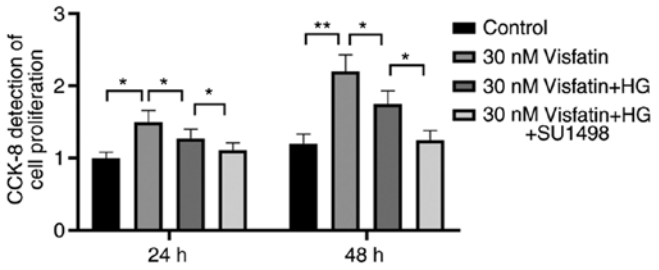

Figure 6. Visfatin promotes RF/6A cell proliferation. *P<0.05; **P<0.01. HG, high glucose; CCK-8, Cell Counting Kit 8.

Discussion

DR is one of the most common and serious microvascular complications in patients with diabetes and seriously affects their quality of life (16,17). While its pathogenesis remains to be fully elucidated, it is widely accepted that diabetes leads to damage to retinal capillaries, tissue edema, bleeding and capillary blockage, resulting in retinal ischemia and hypoxia, further leading to microaneurysms and other pathological changes, including the formation of new blood vessels (18). It has been indicated that cytokines secreted by adipose tissue may participate in the pathological changes in the eyes of patients with diabetes, such as the formation of new blood vessels and cell proliferation, which are closely associated with the onset of DR (19). The results of the present study suggested that visfatin is able to significantly promote the proliferation and migration of RF/6A cells under HG conditions. Among the concentrations tested, visfatin at 30 nM had the most obvious effect. Quantitative analysis of RF/6A cell tube-structure formation in a Matrigel assay indicated that after 24 h of culture, visfatin significantly promoted the tube-structure formation of RF/6A cells, particularly at the concentration of 30 nM. Visfatin and angiotensin II have been reported to influence each other: Angiotensin II is able to promote retinal angiogenesis by upregulating VEGF and higher angiotensin II levels increase insulin resistance, which in turn increases the production of visfatin (20). Similar

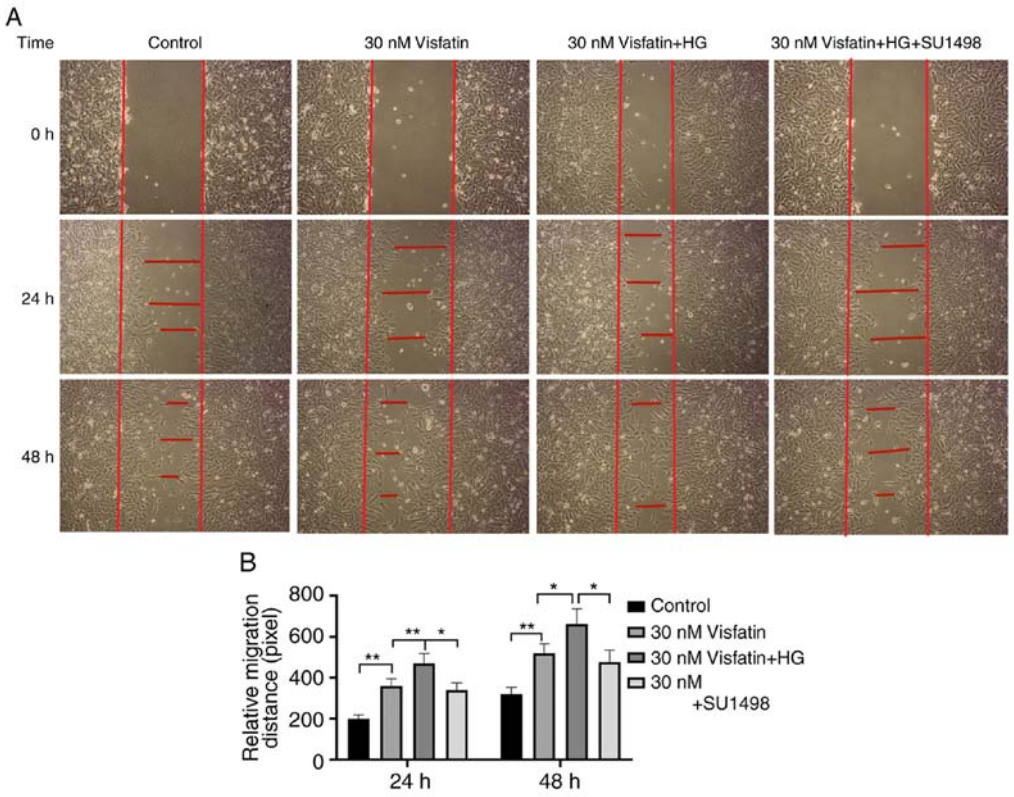

Figure 7. Effect of visfatin on RF/6A cell migration. (A) Image of RF/6A cell migration (magnification, 100). (B) Relative migration distance of RF/6A cells in each group. *P<0.05; **P<0.01. HG, high glucose.

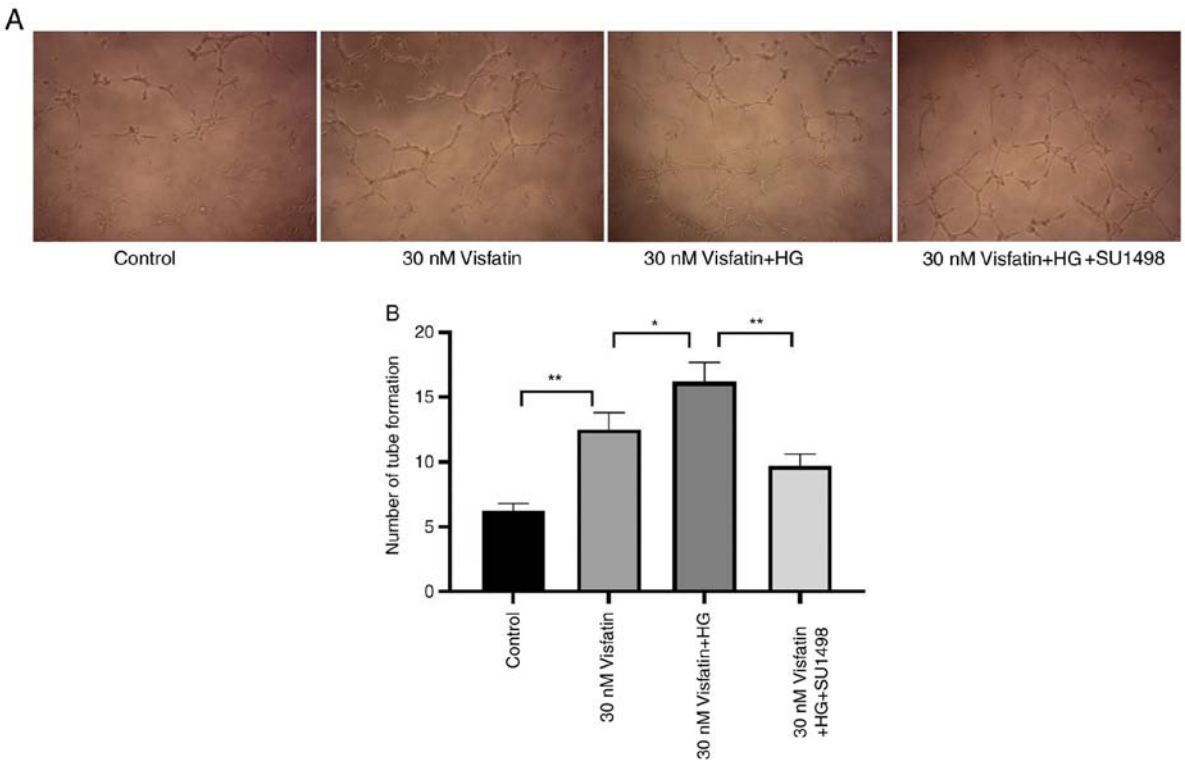

Figure 8. Effect of visfatin on the formation of tube-like structures by RF/6A cells. (A) Image of RF/6A cells with tube formation (magnification, x100); (B) Number of tubes formed in each group. *P<0.05; **P<0.01. HG, high glucose.

to VEGF, visfatin may promote the proliferation of retinal capillary endothelial cells and directly induce the occurrence of DR. Visfatin is able to interact with inflammatory factors and oxidative stress. Serum inflammatory factors are able to

upregulate the expression and secretion of visfatin. However, increases in visfatin were determined to further influence the protein kinase C signal transduction pathway, promote the synthesis of reactive oxygen species, aggravate oxidative stress and promote the occurrence of DR (21).

Chen (9) reported that the level of visfatin increased in the plasma of patients with DR; however, the precise mechanism of the effect of visfatin on angiogenesis in DR had remained to be fully elucidated. In the present study, RF/6A cells were treated with different concentrations of visfatin to observe its effect on VEGF and its receptor VEGFR-2. The results indicated that the protein and mRNA expression levels of VEGF and VEGFR-2 were significantly increased in the HG group as compared with those in the control group. Different concentrations of visfatin were able to significantly increase the expression of VEGF and VEGFR-2 in RF/6A cells cultured with HG. VEGF is a classical vascular growth factor, which may be produced by a variety of tumor cell and certain normal cell types. The main function of VEGF is to promote endothelial cell proliferation and induce the formation of new blood vessels (22,23). There are many factors promoting angiogenesis, such as VEGF, fibroblast growth factor, angiogenic nutrients, IL-1, IL-8, as well as some small molecules of lipids, nucleotides and vitamins, among which VEGF plays an important role in the occurrence of blood vessels (24-26). Under normal conditions, the expression level of VEGF is relatively low, while under pathological conditions such as hypoxia, both the gene expression and protein levels of VEGF are upregulated (27). VEGF is able to bind to its receptor VEGFR-2, which promotes endothelial cell proliferation and angiogenesis.

These results suggested that high levels of visfatin may help promote the process of RNV. Previous studies have indicated that visfatin is able to upregulate the mRNA expression and release of VEGF in endothelial cells (28). As a key regulator of angiogenesis, VEGF is able to significantly promote endothelial cell division, cell proliferation, migration and tube formation, which may be the mechanism of visfatin promoting neovascularization. The results of the present study suggested that visfatin may be an endogenous angiogenic molecule that promotes RNV. The effective concentration of visfatin used in the present study was similar to that reported in two previous studies (29,30). In addition, the current study indicated that the migration distance in the 30 nM visfatin + HG group was significantly greater compared with that in the 30 nM visfatin + HG + SU1498 group. SU1498 is an inhibitor of VEGF. Thus, in an HG environment, visfatin plays an important role in the angiogenesis process. FBS was used for the cell-scratch experiment, which contains components that promote cell proliferation and maintenance, this represents a limitation of the current study.

In conclusion, the present results suggested that visfatin is able to promote the proliferation, migration and tube formation of RF/6A cells under HG conditions, suggesting that visfatin has an important role in RNV and its mechanism may be related to the promotion of VEGF and VEGFR-2 expression under HG conditions.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DC: Study concepts and design, literature research, manuscript preparation, manuscript editing, statistical analysis; YW: Study concepts and design, literature research; ML: Contributions to analysis and interpretation of data, drafting the manuscript; JC: Participate in experimental design and literature research; ZL: Data analysis; YS: Manuscript preparation; JD, Guarantor of integrity of the entire study, study concepts and design. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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