

High glucose conditions suppress the function of bone marrow-derived endothelial progenitor cells via inhibition of the eNOS-caveolin-1 complex

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Abstract. The present study aimed to reveal how high glucose affects rat bone marrow-derived endothelial progenitor cells *in vitro*. Total mononuclear cells of bone marrow were obtained, cultured in endothelial cell growth medium-2 and identified by fluorescence microscopy. Using immunofluorescence, endothelial progenitor cells were identified by expression of VEGFR-2 as well as CD133 and were further characterized as those adherent cells which were double positive by Dil-Ac-LDL uptake and FITC-UEA-1 lectin binding. The attached cells were collected and glucose was added to the culture medium at various final concentrations (11.1, 33.3 and 55.5 mmol/l). Proliferation, migration and *in vitro* angiogenic ability of endothelial progenitor cells were measured. The change in mRNA and protein levels of caveolin-1 and endothelial nitric oxide synthase (eNOS) were examined; in addition, nitric oxide (NO) levels in the cell medium were measured. Based on the results, with increasing glucose concentration in the medium, proliferation, migration and *in vitro* angiogenic capacity of the cells were reduced, whereas mRNA and protein levels of caveolin-1 gene increased gradually. The mRNA levels of the eNOS gene did not differ, but the protein expression was reduced and NO levels in the culture medium declined. In conclusion, high glucose conditions are detrimental to the function of bone marrow-derived endothelial progenitor cells *in vitro*, probably by damaging the eNOS-caveolin-1 complex, which results in the reduction of NO synthesis, eventually leading to the impaired function of endothelial progenitor cells.

Introduction

Patients in the late stage of type 2 diabetes frequently suffer from macrovascular complications, including coronary heart disease, cerebrovascular disease and peripheral macrovascular complications. Amputation caused by peripheral macrovascular complications could significantly increase the risk of death and reduce patients' quality of life. Endothelial progenitor cells (EPCs) are precursor cells and have been identified in the bone marrow, umbilical cord blood and peripheral blood. EPCs have the characteristics of mobility, migration, homing to the ischemic tissue, secreting growth factors, differentiating into endothelial cells and involvement in angiogenesis in the ischemic areas (1). Recent studies have shown that EPCs have a reduced capacity for regeneration, adhesion and migration, and contribute to the occurrence and development of peripheral vascular disease in diabetic patients. Decreased activity of EPCs from peripheral blood and bone marrow may be related to hyperglycemia, hyperinsulinemia, duration of diabetes, high cholesterol and other factors in diabetes (2,3).

It has been established that endothelial nitric oxide synthase (eNOS) plays a key role in vascular relaxation by catalyzing cells to produce nitric oxide (NO). Moreover, caveolin is the surface marker and structure protein of caveolae, which are independent areas in the cell membrane. It is an essential molecule for the normal physiological function of the cardiovascular system. Caveolin-1 has been identified as one of the caveolin family members (4). One study has found that eNOS is situated in caveolae, combines with caveolin-1 and forms the complex of eNOS-caveolin-1, which is closely associated with the homing and angiogenetic properties of endothelial cells (5).

In this study, we investigated the effect of high glucose on bone marrow-derived EPCs and the function of the eNOS-caveolin-1 complex, and their relationship was further determined. Our results revealed a potential therapeutic target for diabetes with vascular complications.

Materials and methods

Animal experiments. Male SD rats, 8-10 weeks old and weighing 250-280 g, were used for the experiments. Animal

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experiments were approved by the Animal Ethics Committee of Zhejiang University.

Isolation of bone marrow-derived mononuclear cells (BM-MNCs) and EPCs culture. Rat bone marrow cells were isolated from the femora and tibiae. Mononuclear cells were separated by suspending the cells in Ficoll-Paque solution and centrifuged at 2000 rpm for 25 min. After being washed with phosphate-buffered saline (PBS), cells were resuspended with endothelial cell growth medium-2 (EGM-2, Lonza, Switzerland) and inoculated into 6-well plates. The medium was changed every 3 days (6).

Immunofluorescence. When adherent cells reached 80-90% confluency, they were digested and passaged to 24-well plates. Once fully adhered, the immunofluorescence technique was performed using primary antibodies of vascular endothelial growth factor receptor 2 (VEGFR2; Cell Signaling Technology, USA) and CD133 (Cell Signaling Technology, USA), both of which were expressed on EPCs, and secondary antibody of fluorescein-conjugated anti-rabbit IgG (Cell Signaling Technology, USA). Cells were visualized using an inverted fluorescence microscope (7).

Dil-Ac-LDL/lectin staining. Dil-Ac-LDL/lectin staining was performed to further confirm the EPCs. Cells were incubated in 24-well plates using medium with 10 $\mu\text{g}/\text{ml}$ 1,1-dioctadecyl-3,3,3-tetramethylindocarbocyanine perchlorate-labeled acetylated low-density lipoprotein (Dil-Ac-LDL) Molecular Probes, Eugene, OR, USA) for 10 h. Cells were then fixed and counterstained with 10 $\mu\text{g}/\text{ml}$ FITC-labeled UEA-1 lectin (Vector, USA) for 1 h. After being washed three times with PBS, the cells were observed under fluorescence microscopy and images were captured (8).

Cell treatment. When the primary cells reached 80-90% confluence, the adherent cells were trypsinized, resuspended and randomly divided into four groups as follows: control group (cells were cultured in EGM-2 medium without additional glucose), and treatment groups (comprising 3 groups; glucose was added to the medium and calculated to final concentrations of 11.1, 33.3 and 55.5 mmol/l).

EPCs proliferation assay. The cells in each group described above were inoculated into 96-well plates at a density of 1×10^4 EPCs in 200 μl EGM-2 per well and cultured for 48 h. Then, 20 μl of MTT liquid was added to each well. The supernatant was removed and 150 μl dimethyl sulfoxide was added to each well 4 h later, followed by oscillations for 10 min. The OD values of each group were then analyzed at a wavelength of 490 nm (9).

EPCs migration assay. The migration of EPCs was evaluated by Millicell-PCF (Millipore, MA, USA) assay. EPCs ($n=5 \times 10^4$) in 200 μl EGM-2 were placed in the upper chamber of 24-well Millicell plates and 600 μl medium containing recombinant vascular endothelial growth factor (VEGF, 50 ng/ml) was placed in the lower chamber, followed by incubation for 24 h. The membrane was washed with PBS and fixed. The upper side of the membrane was wiped and the membrane was stained

using crystal violet solution. The magnitude of migration of EPCs was evaluated by counting the migrated cells in 6 random high-power microscopic fields (magnification, $\times 200$) (9).

EPCs tube formation assay. The tube formation assay was performed to assess the capacity for EPCs vasculogenesis. In brief, Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was mixed with serum-free EGM-2, and placed in a 96-well plate at 37°C for 30 min to allow solidification. EPCs ($n=1 \times 10^4$) in 100 μl EGM-2 were inoculated in the 96-well plate, and incubated at 37°C for 24 h. Representative fields ($n=6$) were taken and the average of the total area of complete tubes formed was compared using the Image-Pro Plus computer software. When a cell deformed to the extent that its length was 3 times greater than its width, it was considered as a small tube (10).

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR). After culturing for 48 h, the cells of each group were immediately homogenized and purified. Purified RNA was quantified and converted to cDNA. The amplification protocol was as follows: 95°C for 30 min, followed by 40 cycles at 95°C for 5 sec, 60°C for 34 sec, and 72°C for 30 sec. The data acquisition was performed at 72°C. PCR was performed using a gene-specific primer pair for rat GAPDH, eNOS and caveolin-1 (Sangon, Shanghai, China). The following primers were used: GAPDH forward, 5'-CAAGCCCAACAACAAGG-3', and reverse, 5'-TGGTTC CGCAATCACAT-3'; eNOS forward, 5'-CTGCTGCCCCAGAT ATCTTC-3'; reverse, 5'-CAGGTACTGCAGTCCCTCCT-3'; caveolin-1 forward, 5'-CAAGCCCAACAACAAGG-3'; reverse, 5'-TGGTTC CGCAATCACAT-3'. Gene expression was then determined by real-time RT-PCR and the absolute number of gene copies was quantified.

Western blot analysis. In each treatment group, cells were cultured in 6-well plates for 72 h and then collected. Cells were lysed, and 40 μg total proteins was separated on SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. They were blocked for 2 h and incubated overnight at 4°C with primary antibodies. Primary antibodies used included rabbit anti-eNOS (1:200 in TBS-T, Santa Cruz, Santa Cruz, CA, USA), rabbit anti-caveolin-1 (1:1000 in TBS-T, Cell Signaling Technology, Danvers, MA USA) and rabbit anti- β -actin (1:2000 in TBS-T, Cell Signaling Technology) polyclonal antibody. Bound antibodies were visualized using secondary antibodies and enhanced chemiluminescence detection. The analytical gels contained control samples for internal signal correction (11).

NO levels. The cells treated as described above were seeded in 96-well plates at a density of 1×10^4 in 200 μl EBM-2 per well and incubated for 72 h. The concentration of nitrite/nitrate ions ($\text{NO}_2^-/\text{NO}_3^-$) in the supernatant was then analyzed and compared with the standard well to calculate NO secretion. Each group included three duplicate wells.

Statistical analysis. All results were expressed as mean \pm SEM. Statistical analysis was performed with the Student's t-test for comparisons between two groups and

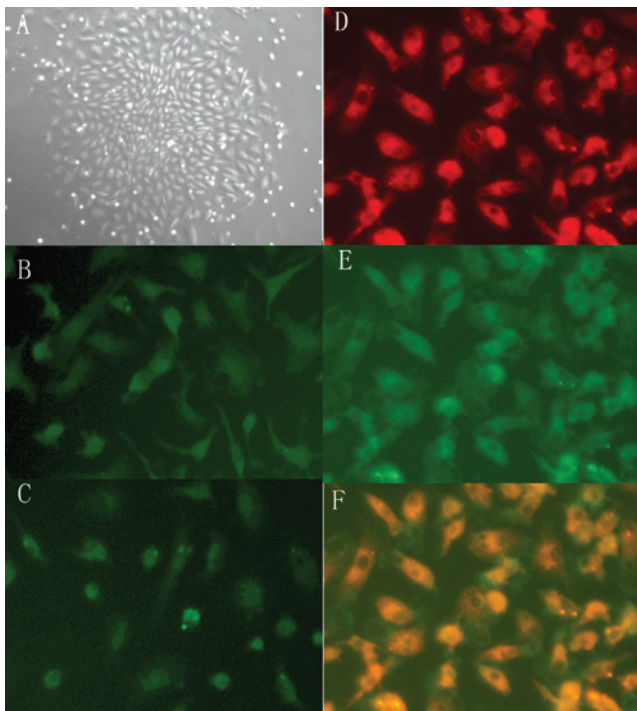


Figure 1. EPCs culture and identification. (A) Adherent cells were arranged in a spindle-like and colony-like manner. (B) Adherent cells expressed VEGFR-2. (C) Adherent cells expressed CD133. (D) Adherent cells were positive for uptake of Dil-Ac-LDL; red fluorescence was mainly noted in the cytoplasm. (E) Adherent cells exhibited positive binding of UEA-1-lectin; green fluorescence was mainly concentrated in the membrane. (F) Adherent cells were positive for uptake of Dil-Ac-LDL and binding of UEA-1-lectin. The double-positive cells were recognized as EPCs.

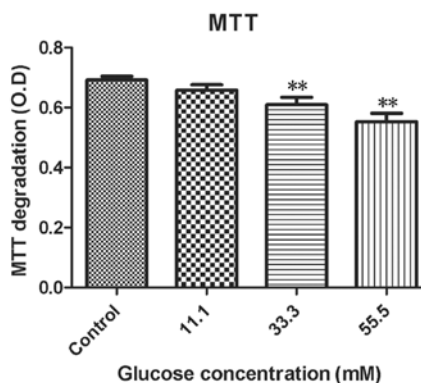


Figure 2. Influence of various glucose concentrations on the proliferation of EPCs. The greater the OD value, the stronger the cell proliferation. The OD value declined gradually in a manner dependent on the glucose concentration, indicating that the cell proliferation decreased. ** $p < 0.01$, significantly different from the control group.

ANOVA for more than two groups. $p < 0.05$ was considered statistically significant.

Results

EPCs culture and identification. In the case of the separated mononuclear cells cultured for 7-10 days, the majority of the cells were arranged in a fusiform, colony-like manner. As

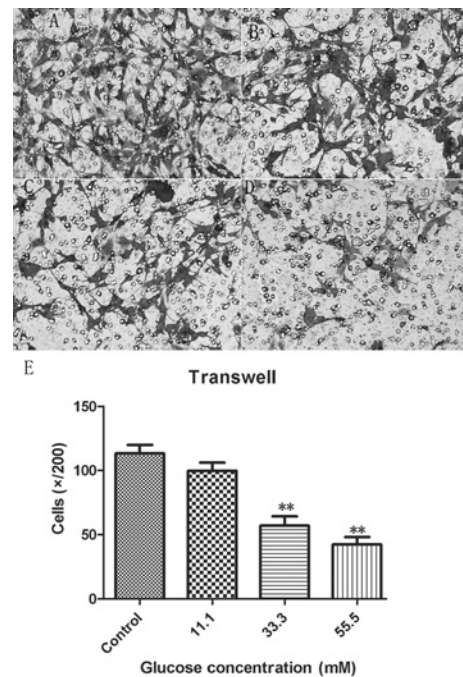


Figure 3. Influence of different glucose concentrations on the migration of endothelial progenitor cells (EPCs). These figures show migration of EPCs cultured in medium with various glucose concentrations: (A) control, (B) 11.1 mmol/l, (C) 33.3 mmol/l, (D) 55.5 mmol/l, respectively. (E) The graph shows the mean values \pm SEM of four groups. The migratory capability of EPCs decreased, dependent on the increasing concentration of glucose. ** $p < 0.01$, significantly different from the control group.

shown in Fig. 1 and identified by immunofluorescence, $>85\%$ of the cells expressed VEGFR-2 and $>80\%$ expressed CD133. Furthermore, using Dil-Ac-LDL and FITC-UEA-1 lectin double-fluorescence staining, the double-positive cells were revealed to be endothelial progenitor cells.

High glucose suppresses the proliferation of bone marrow-derived EPCs. MTT was used to analyze EPCs proliferation. As shown in Fig. 2, the proliferation of EPCs decreased, according to the increase in glucose concentration. Although the difference between 11.1 mmol/l glucose (0.6583 ± 0.01796) and the control group (0.6924 ± 0.01186) were not statistically significant, the proliferation of EPCs was significantly inhibited by 33.3 mmol/l (0.6099 ± 0.02417 versus control, $p < 0.01$) and 55.5 mmol/l glucose (0.5530 ± 0.02812 versus control, $p < 0.01$) compared with the control group.

High glucose suppresses the migration of bone marrow-derived EPCs. The modified millicell chamber assay was used to analyze EPCs migration ability. Fig. 3 shows that compared with the control group (113.3 ± 6.556), 11.1 mmol/l glucose (96.67 ± 6.433) did not significantly inhibit migration of EPCs, whereas 33.3 mmol/l (57.0 ± 7.312 vs. control, $p < 0.01$) and 55.5 mmol/l glucose (42.33 ± 5.852 vs. control, $p < 0.01$) significantly inhibited the migration of EPCs.

High glucose suppresses the angiogenic ability of bone marrow-derived EPCs. Angiogenic ability of EPCs was examined by tube formation assay. As shown in Fig. 4, compared with the control group (28.67 ± 1.282), all treatment

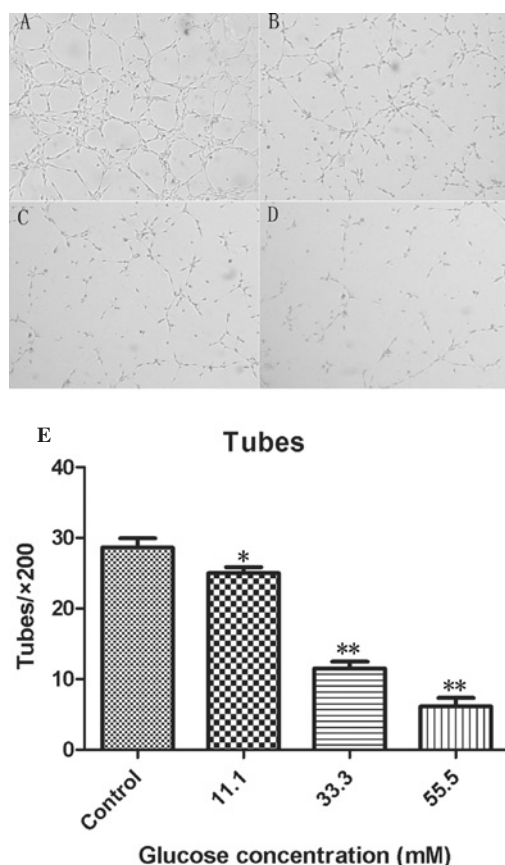


Figure 4. Influence of different glucose concentrations on the angiogenesis of EPCs. This figure shows the tube formation of EPCs cultured in medium with various concentrations of glucose: (A) control, (B) 11.1 mmol/l, (C) 33.3 mmol/l, (D) 55.5 mmol/l, respectively. (E) The graph shows the mean values \pm SEM of the control and treated groups. The tubes formed by EPCs decreased in number in a concentration-dependent manner. * $p < 0.05$, ** $p < 0.01$, significantly different from the control group.

groups significantly inhibited the angiogenic capacity of EPCs (11.1 mmol/l glucose, $p < 0.05$ vs. control; 33.3 mmol/l and 55.5 mmol/l glucose, $p < 0.01$ vs. control).

High glucose affects gene expression of eNOS and caveolin-1 in various degrees. As observed using PCR and Western blot analysis, with the increasing glucose concentration, the mRNA level of the caveolin-1 gene and its protein expression increased gradually (Figs. 5 and 6). However, the protein expression of eNOS gradually declined, although its mRNA levels showed no significant change.

High glucose suppresses the secretion of NO. As shown in Fig. 7, in the presence of various concentrations of glucose, the NO concentration in the medium declined gradually in a concentration-dependent manner, and the difference was statistically significant compared with the control group.

Discussion

Since Asahara *et al* successfully demonstrated the existence of endothelial progenitor cells in 1997, increasing attention has been paid to EPCs (1). EPCs, which express surface markers including VEGFR-2 and CD133 and differentiate

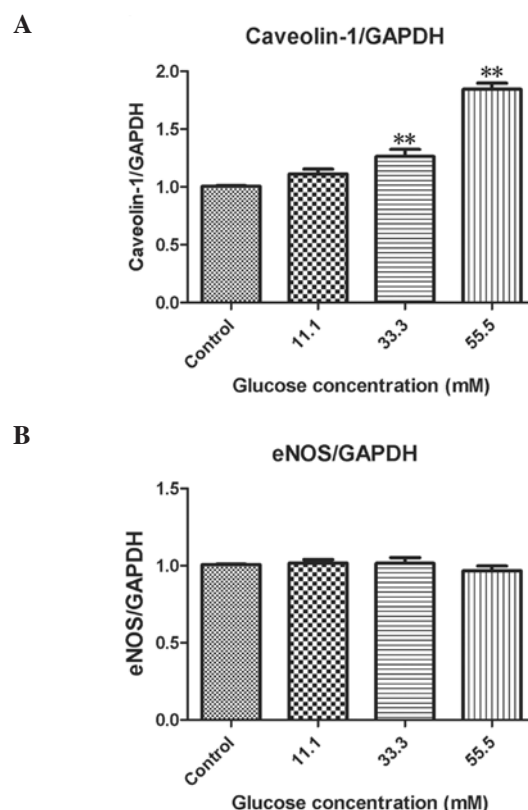


Figure 5. Caveolin-1 and endothelial eNOS mRNA expression in EPCs. mRNA levels of target genes were evaluated using real-time PCR, with GAPDH as internal control. Levels of target gene mRNAs were normalized to the level of GAPDH mRNA and the values were expressed relative to those of the control group. (A) In the presence of glucose, the mRNA level of caveolin-1 increased gradually in a concentration-dependent manner. (B) However, with regard to the eNOS mRNA levels, treated groups remained consistent as compared with the control group. ** $p < 0.01$, significantly different from the control group.

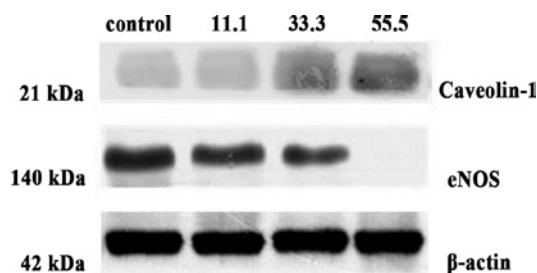


Figure 6. Caveolin-1 and endothelial nitric oxide synthase (eNOS) protein expression in EPCs. In response to increases in glucose concentration the protein expression of caveolin-1 gene increased whereas eNOS gene decreased gradually.

into endothelial cells, have the characteristics of mobility, migration, homing to the ischemic tissue, secreting growth factors and involvement in angiogenesis in ischemic areas. In 1999, Takeshita and Isner put forward the concept known as 'therapeutic angiogenesis', referring to the practice of complementing the number of EPCs or, through genetic modification of EPCs to improve their functions, rendering EPCs into the substrate of angiogenic factors and achieving more favorable angiogenic effects (12). EPCs transplantation in diabetic

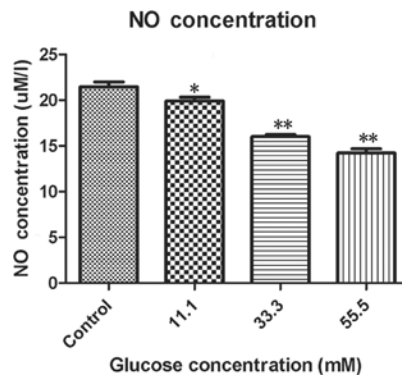


Figure 7. Nitric oxide (NO) levels in the medium. NO concentration in the medium decreased gradually in response to the rising level of glucose. The difference was statistically significant compared to the control group. * $p < 0.05$, ** $p < 0.01$, significantly different from the control group.

peripheral vascular disease, coronary heart disease, myocardial infarction and other ischemic diseases has shown marked potential for clinical application (13,14).

However, certain studies have found that diabetes reduces the number of circulating EPCs and impairs their function, weakening the adhesion, migration and regeneration of EPCs (2). In their study, which used EPCs transplantation to treat severe limb ischemia in mice, Capla *et al* found that this method was significantly more effective in mice with simple peripheral vascular disease than in those with diabetes mellitus and peripheral vascular disease (15). The impact of diabetes on EPCs may have a number of causes, due to the complicated internal environment of the diabetic, but hyperglycemia is undoubtedly a highly significant aspect. High glucose *in vitro* was therefore used in our study to examine the influence of high glucose on the functions of bone marrow-derived EPCs. We found that the proliferation, migration and angiogenesis of EPCs declined in response to the increase in glucose concentration. As glucose concentration increased, the proliferation of EPCs decreased. At the concentration of 11.1 mmol/l glucose, the proliferation was weaker than that of the control, but with no significant difference. However, the 33.3 mmol/l and 55.5 mmol/l groups were significantly different from the control group. High glucose also impaired angiogenic ability and migration of EPCs in a concentration-dependent manner. We therefore conclude that high glucose suppresses the proliferation, migration and angiogenesis of bone marrow-derived EPCs, and that this inhibition is concentration-dependent.

McGinn *et al* found that high glucose inhibits the proliferation of endothelial cells through the p38 mitogen-activated protein kinase (p38 MAPK) pathway (16). Kuki *et al* revealed that a high glucose concentration inhibits cell proliferation and promotes the phosphorylation of p38 MAPK, and the application of p38 MAPK inhibitor restores cell proliferation, indicating that the MAPK pathway may play a significant role in the damage caused by high glucose. However, increased glucose concentrations induce dysfunction of several intracellular signal transduction cascades, including modulation of protein kinase C, generation of reactive oxygen species, and accumulation of advanced glycation end product (17).

Therefore, there may be other manners in which high glucose influences EPCs.

eNOS is one of the most vital enzymes determining the endothelial function. eNOS catalyzes the production of NO, a relaxing factor, which determines the function of endothelial cells and is essential for vascular formation and migration of endothelial cells (18). Caveolae, independent areas in the cell membrane, are abundant in the cardiovascular system and are involved in various physiological processes. Caveolin is a surface marker protein and structure protein of caveolae. Caveolin-1 a member of the caveolin family is abundant in endothelial cells. In a study on caveolin-1 knockout mice, Wunderlich *et al* found that disruption of caveolin-1 led to enhanced nitrosative stress as well as systolic and diastolic heart failure, which demonstrated that caveolin-1 plays a crucial physiological role in the cardiovascular system (19). As mentioned above, eNOS is situated in the caveolar membrane and binds to caveolin-1 forming a complex. Caveolin-1 interacts with reductase domains of eNOS and inhibit NO synthesis (20). Therefore, alteration of caveolin-1 abundance and its interaction with eNOS impact eNOS function. Our study found that, caveolin-1 protein increased, whereas eNOS protein decreased gradually, in response to the rising glucose concentration; moreover, in response to the the increase in glucose concentration, mRNA of caveolin-1 gene showed a gradual increase, while the eNOS gene did not, indicating that the caveolin-1 gene performs its regulatory role at the transcriptional level, whereas eNOS may do so at the post-transcriptional level. As mentioned above, NO produced by eNOS is an essential factor in the regulation of endothelial function and blood flow. We speculated that the variation in eNOS protein expression led to the differences in the production of NO, eventually leading to the impaired function of EPCs. In our study, just as we expected, we found that the the level of NO in the medium decreased in a concentration-dependent manner in response to the increase in glucose concentration, whereas the proliferation, migration and angiogenic ability of EPCs *in vitro* were gradually inhibited. These results confirmed our hypothesis.

However, there were several limitations to our study. Our evidence that high glucose impairs the function of EPCs partly through regulating caveolin-1 and eNOS expression was indirect. Additionally, it is unclear whether the study would be equally effective if we treated the diabetic lower limb ischemic mice with EPCs that are caveolin-1 down-regulated or eNOS up-regulated. Further research on this subject is warranted.

In conclusion, to the best of our knowledge, our study was the first to demonstrate that, high glucose conditions suppress the proliferation, migration and *in vitro* angiogenic ability of bone marrow-derived EPCs via injury of the eNOS-caveolin-1 complex. This study may provide a new therapeutic target for diabetic patients with vascular complication.

Acknowledgements

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