Effects of lycopene on number and function of human peripheral blood endothelial progenitor cells cultivated with high glucose

Yao-Chi Zeng[1], Gui-Ping Mu[2], Shu-Fen Huang[3], Xue-Hui Zeng[4], Hong Cheng[5] and Zhong-Xin Li[6§]

[1]Department of Clinical Nutrition, Shenzhen Traditional Chinese Medicine Hospital, China
[2]Department of Central Laboratory, Shenzhen Traditional Chinese Medicine Hospital, China
[3]Department of Health Education, Shenzhen Traditional Chinese Medicine Hospital, China
[4]Department of Clinical Laboratory, Shenzhen Traditional Chinese Medicine Hospital, China
[5]Department of Cardiovascular Medicine, Shenzhen Traditional Chinese Medicine Hospital, China
[6]Association of Traditional Chinese Medicine, Shenzhen Traditional Chinese Medicine Hospital, Shenzhen, 518033, China

BACKGROUND/OBJECTIVES: The objectives of this study were to investigate the effects of lycopene on the migration, adhesion, tube formation capacity, and p38 mitogen-activated protein kinase (p38 MAPK) activity of endothelial progenitor cells (EPCs) cultivated with high glucose (HG) and as well as explore the mechanism behind the protective effects of lycopene on peripheral blood EPCs.
MATERIALS/METHODS: Mononuclear cells were isolated from human peripheral blood by Ficoll density gradient centrifugation. EPCs were identified after induction of cellular differentiation. Third generation EPCs were incubated with HG (33 mmol/L) or 10, 30, and 50 μg/mL of lycopene plus HG. MTT assay and flow cytometry were performed to assess proliferation and apoptosis of EPCs. EPC migration was assessed by MTT assay with a modified boyden chamber. Adhesion assay was performed by replating EPCs on fibronectin-coated dishes, after which adherent cells were counted. *In vitro* vasculogenesis activity was assayed by Madrigal network formation assay. Western blotting was performed to analyze protein expression of both phosphorylated and non-phosphorylated p38 MAPK.
RESULTS: The proliferation, migration, adhesion, and *in vitro* vasculogenesis capacity of EPCs treated with 10, 30, and 50 μg/mL of lycopene plus HG were all significantly higher comapred to the HG group ($P < 0.05$). Rates of apoptosis were also significantly lower than that of the HG group. Moreover, lycopene blocked phosphorylation of p38 MAPK in EPCs ($P < 0.05$). To confirm the causal relationship between MAPK inhibition and the protective effects of lycopene against HG-induced cellular injury, we treated cells with SB203580, a phosphorylation inhibitor. The inhibitor significantly inhibited HG-induced EPC injury.
CONCLUSIONS: Lycopene promotes proliferation, migration, adhesion, and *in vitro* vasculogenesis capacity as well as reduces apoptosis of EPCs. Further, the underlying molecular mechanism of the protective effects of lycopene against HG-induced EPC injury may involve the p38 MAPK signal transduction pathway. Specifically, lycopene was shown to inhibit HG-induced EPC injury by inhibiting p38 MAPKs.

INTRODUCTION

Type 2 diabetes is characterized by a 2- to 4-fold increased risk of cardiovascular disease (CVD) [1]. This is generally attributed to the adverse effects of hyperglycemia and oxidative stress on vascular biology [2]. Endothelial progenitor cells (EPCs) play an important role in postnatal neovascularization and vascular repair [3,4]. As such, EPC dysfunction may be a cause of diabetic vascular complications [5]. In patients with type 2 diabetes, especially those with vascular complications, both the number and function of circulatory EPCs are known to be significantly reduced [6,7]. Furthermore, EPCs promote *in vivo* re-endothelization and are able to be incorporated into new vessels in animal models of hind limb ischemia [8]. EPCs are also involved in processes such as myocardial ischemia and infarction, wound healing, and endogenous endothelial repair [9,10]. Furthermore, *in vivo* animal model studies along with *in vitro* studies using EPCs from type 1 and type 2 diabetic patients have revealed a potential role for glucotoxicity in impairing EPC function [8,11-12]. In a clinical setting, high glucose (HG) conditions are usually accompanied by increased free fatty acid levels, which might have detrimental effects on

endothelial cells [13]. More importantly, HG has been shown to inhibit both the colony-forming ability as well as proliferation of EPCs, whereas it enhances senescence of early and late EPCs; HG also was shown to impair the migration and vasculogenesis capacity of late EPCs [14,15]. p38 mitogen-activated protein kinases (p38 MAPKs) are critical regulators of cell differentiation [16]. In addition, activation of p38 MAPKs has been implicated in induction of cell apoptosis [17]. p38 MAPKs coordinately regulate cellular proliferation and differentiation processes induced by a variety of cellular stresses. Specifically, HG conditions can activate the p38 MAPK pathway in many cell types, including endothelial cells [18]. As glucose has been reported to activate p38, it could be a potential effector in signaling mechanisms impairing the proliferation and differentiation of EPCs.

Lycopene, which is a naturally present carotenoid in tomatoes and tomato products, is the most potent singlet oxygen quencher among all natural carotenoids [19]. Epidemiological studies have shown an inverse relationship between intake of tomatoes, serum lycopene levels, or adipose tissue lycopene levels and incidence of coronary heart disease [20]. An *in vitro* study indicated that lycopene appears to be the most effective in reducing both human aortic endothelial cell (HAEC) adhesion to monocytes as well as cell surface expression of adhesion molecules among five carotenoids, suggesting an important role for lycopene in attenuating atherogenesis [21]. Lycopene was also reported to attenuate the phenotypic and functional maturation of murine bone marrow-dendritic cells [22]. Additionally, lycopene was shown to down-regulate the expression of co-stimulatory molecules and major histocompatibility complex type II molecules, as it was able to inhibit MAPKs such as extracellular signal-regulated kinase 1/2 (ERK1/2), p38 and c-Jun N-terminal kinase (JNK), and the transcription factor Nuclear Factor kappa B (NF-kB) [22]. Lycopene may modulate the MAPK signaling cascade by regulating phosphorylation [23]. However, the protective effects of lycopene against HG-induced EPC injury as well as its underlying mechanism are not well established yet.

Based on these considerations, we tested the hypotheses that HG-induced EPC dysfunction is initiated by activation of p38 MAPK, resulting in apoptosis, and that the underlying protective effects of lycopene on EPCs involve modulation of p38 MAPK signaling pathways.

MATERIALS AND METHODS

Subjects

All subjects gave their informed consent, both verbally and in writing, after being informed about the study. Ethics approval for the trial was obtained from the ethical committee of the Guangzhou University of Traditional Chinese Medicine, China (Reference # KC08HISV0351), and the study was performed in accordance with the principles of the Declaration of Helsinki. The study enrolled 15 healthy medical personnel. Of these, eight were male and seven were female. The age range was from 29-52 years (mean 38.3 ± 12.1 years). All controls had no clinical history of diabetes or hyperlipidemia, and they showed normal fasting blood glucose (FBG) levels of < 7 mmol/L. They under-

went a normal physical examination and had not received any medication. Exclusion criteria for participation were use of cigarettes, coffee, recent trauma, surgery, or dermal ulcers, as these all inhibit EPC proliferation, migration, vasculogenesis capacity, and survival rate [24-26].

Chemicals and materials

Lycopene (purity $\geq$ 90%, from tomato), human fibronectin (FN), FITC-labeled Ulex, 1,1-dioctadecyl- 3,3,3,3-tetramethylin-docarbocyanine (DiI), labeled acetylated low-density lipoprotein (acLDL), and SB203580 were purchased from Sigma-Aldrich (St. Louis, MO, USA). M199 medium and fetal bovine serum were obtained from Hyclone (USA). Lydroxypropylmethyl Cellulose was purchased from Tianjin Haoyang Biological Manufacture Co., Ltd. China (Haoyang, China). RrhVEGF, recombinant human basic fibroblast growth factor (rhbFGF), epidermal growth factor (EGF), and insulin-like growth factor 1 (IGF-1) were purchased from Pepro Tech (USA). Annexin V-FITC Apoptosis Detection Kit was purchased from Jingmei Biotech Co., Ltd. (Shanghai, China). Boyden chamber was purchased from Haimen City Qilin Medical Instrument Factory. Antibodies against p38 MAPK (product code 9212) and phosphorylated p38 MAPK (product code 9216) were purchased from Cell Signaling Technology (Danvers, MA, USA). Goat Anti-mouse IgG/HRP (product code ZDR-5307) was purchased from Zhongshan Golden Bridge Bio-technology Co., Ltd. (Beijing, China). All other chemicals were obtained from common commercial sources and were of reagent grade. Pure lycopene was dissolved in tetrahydrofuran (THF), stored at 70℃, protected from light [27], and diluted with medium to the desired concentration before use.

Isolation and cultivation of EPCs

Fasting peripheral venous blood sample (15 mL) was isolated from healthy medical personnel to obtain mononuclear cells by density gradient centrifugation following treatment with sodium heparin anticoagulant (within 1 h of delivery) [28,29].

The obtained mononuclear cells were cultured in fibronectin-coated 6-well culture plates. Then, 0.5 mL of M199 medium containing 20% fetal bovine serum (FBS), 10 μg/L of recombinant human vascular endothelial growth factor (rhVEGF), 2 μg/L of rhbFGF, 10 μg/L of EGF, 12 μg/L of IGF-1, 1×10^5 U/L of penicillin, and 1×10^5 U/L of streptomycin were added. The cell solution was cultured in a 5% CO_2 incubator with saturated humidity at 37℃ for 4 days. Non-adherent cells were removed by washing with phosphate-buffered saline (PBS), after which attached early EPCs appeared. Adherent cells were incubated in fresh medium for another 4 days. After non-adherent cells were rinsed with PBS, the medium was changed for another 7 days of culture. Then, non-adherent cells were removed by PBS, and adherent cells were identified. Cultured cells were co-cultured with 2.4 mg/L of DiI-Ac-LDL at 37℃ for 1 h, followed by fixation with 20 g/L of paraformaldehyde for 10 min. After rinsing with PBS, 10 mg/L of FITC-labeled Ulex europaeus agglutinin I was added to the cell samples, which were cultured at 37℃ for 1 h. Under a laser scanning confocal microscope, Ulex lectin FITC-I and DiI-Ac-LDL double-stained positive cells were identified as differentiating EPCs [28,29]. Cell numbers were viewed with an inverted fluorescent microscope (Olympus).

Experimental grouping and procedures

Following culture in M199 medium without FBS, adherent cells were divided into six groups: normal control group (only M199 medium), mannitol group (30 mmol/L of mannitol and M199 medium, osmotic control), HG group (33 mmol/L of glucose) [30], and three lycopene groups according to dosage (each cultured with 33 mmol/L of glucose followed by lycopene administration at doses of 10, 30, and 50 μg/mL in M199 medium). Medium in each group was changed every 8 h, and the culture process lasted for 72 h. EPC proliferation and apoptosis were measured by MTT assay and flow cytometry at 12, 24, 48, and 72 h, respectively. Cells were incubated on the day of isolation with glucose/mannitol or lycopene; medium was not changed for the early EPC study, whereas cells under passage three were used for the late EPCs study. As above, EPCs were divided into six groups: normal control group, HG group, three lycopene groups according to dosage (each cultured with 33 mmol/L of glucose followed by lycopene administration at doses of 10, 30, and 50 μg/mL), and SB203580 (p38 MAPK inhibitor, 1 μmol/L) with 33 mmol/L of glucose group. The culture process lasted for 24 h. EPC migration, adhesion, vasculogenesis capacity, as well as phosphorylated and non-phosphorylated p38 MAPK protein expression levels were measured.

EPC proliferation and apoptosis

Freshly isolated early EPCs were used for treatment. The cells were treated with HG and/or different concentrations of lycopene throughout the 7-day experiment. Additional cells cultured with mannitol served as a control. The inert substance mannitol was used to maintain osmotic pressure. Cell proliferation and viability on day 7 were determined by MTT assay. After digestion with 0.25% trypsin and culture in M199 medium, the mononuclear cell suspension at a density of 1×10^4 cells per well was placed into fibronectin-coated 96-well plates, with each well containing a volume of 200 μL. Culture plates were placed into a 5% CO_2 incubator with saturated humidity at 37°C for 24 h. Then, 20 μL of a 5 g/L MTT solution was added to each well for continuous culture for 4 h. The supernatant was then removed, after which 150 μL of dimethyl sulfoxide (DMSO) was added to each well. A micro oscillator was used for 10 min to fully dissolve the crystals. The OD value was measured using an ELISA reader at 490 nm. The measurement was repeated three times, and the average values of absorbance were calculated.

Apoptotic EPCs were measured using an Annexin V-FITC Apoptosis Detection Kit. The cell concentration was adjusted to 10^9 cells/L following digestion and rinsing with PBS twice. The cell suspension (100 μL) was placed into a flow tube and mixed with 5 μL of AnnexinV/FITC and 10 μL of 20 mg/L propidium iodide (PI). The mixture was incubated at room temperature in the dark for 15 min and then diluted with 400 μL of PBS. According to flow cytometry, Annexin-V$^+$PI$^-$ cells were considered to be early apoptotic cells, Annexin-V$^+$PI$^+$ were apoptotic and necrotic cells, Annexin-V$^-$PI$^+$ were damaged cells, and Annexin-V$^-$PI$^-$ were living cells. Early apoptotic rate = number of apoptotic cells/total number of cells measured × 100%.

Adhesion assay of EPCs

Third generation EPCs as target cells were pretreated for 24 h with different concentrations of lycopene and HG (500 μL) and then counted. Identical cells (1×10^5) were placed into fibronectin-coated 96-well plates and incubated for 30 min at 37°C. Non-attached cells were gently removed with PBS, adherent EPCs were fixed with 4% paraformaldehyde, and adherent cells were counted by independent blinded investigators in five random high-powered (× 200) microscopic fields in replicate wells. Three replicates were carried out, and the average was calculated.

EPC migration test

Migration of late EPCs was evaluated using a modified boyden chamber. In brief, isolated EPCs were detached using 0.25% trypsin, harvested by centrifugation, resuspended in 500 μL of M199 medium, and then counted. EPCs (2×10^4) were placed in the upper chamber of the modified boyden chamber. Vascular endothelial growth factor (VEGF) in M199 medium at 100 mg/L was then placed in the lower compartment of the chamber. After 24 h of incubation at 37°C, the lower side of the filter was washed with PBS and fixed with 2% paraformaldehyde. For quantification, cells were stained with Giemsa solution. Cells migrating into the lower chamber were counted manually in five random high-powered (× 200) microscopic fields in replicate wells. Three replicates were carried out, and the average was calculated.

In vitro vascular tube formation

Tube formation assay was performed on late EPCs to assess vasculogenesis capacity, which is believed to be important in new vessel formation [31]. In brief, ECMatrix gel solution was thawed at 4°C overnight, mixed with ECMatrix diluent buffer, and then placed in a 24-well plate at 37°C for 1 h to allow the matrix solution to solidify. EPCs were harvested as described above with trypsin/EDTA, after which 1×10^4 EPCs were placed on matrix solution containing M199 medium with HG or different concentrations of lycopene, followed by incubation at 37°C for 24 h. Tubule formation was inspected under five random inverted light microscope (× 100) fields in replicate wells. Three replicates were carried out, and the average was calculated. Tubule formation was defined as a structure exhibiting a length four times its width. Five representative fields were taken, and the average of the total area of complete tubes formed by cells was compared using the computer software Image-Pro Plus.

Western blot analysis

Following EPC pretreatment for 24 h with different concentrations of lycopene or HG, cells were washed with ice-cold PBS and lysed. The protein concentration was determined by the Bradford method. In brief, 30 μg of total proteins was loaded into each lane of a 9% sodium dodecyl sulfate-polyacrylamide gel, followed by electrophoresis and then transfer to a polyvinylidene difluoride membrane. The blots were probed with antibodies against phospho-p38 MAPK. Horseradish peroxidase-conjugated secondary antibodies were used in conjunction with an ECL chemiluminescence detection system

(Amersham, UK). Staining was quantified by scanning densitometry.

Statistical analysis

Data were analyzed using SPSS16.0 software (SPSS Inc., Chicago, IL, USA), and all measurement data were expressed as mean ± standard deviation ($\bar{x} \pm$ SD). Analysis of variance was used for group comparisons. Statistical analysis was performed using analysis of variance for mean comparison of multiple samples, LSD tests for paired comparison, and variance analysis of replicate measurements data for comparison of different experiment intervals. Absolute values, which were equal to differences in functional parameters between the lycopene plus HG group, were subjected to curve fitting analysis. The level of statistical significance for all analyses was set at $\alpha = 0.05$.

RESULTS

Identification of EPCs

The isolated mononuclear cells formed several cell masses from days 3-4, and some spindle cells sprouted from the edge of the cell mass from days 5-6. By day 7, adherent cells had increased in number, and some aggregated to form a "Blood Island". Laser confocal microscopy showed that Dil-Ac-LDL (red, excitation wavelength 543 nm) and Ulex lectin FITC-I (green, excitation wavelength 477 nm) double-stained positive cells were differentiating EPCs [10] (Fig. 1).

Effects of lycopene on EPC proliferation

As shown in Table 1, dramatic reduction of proliferation was observed following exposure of EPCs to HG for 12, 24, 48, and 72 h compared to the control group ($P < 0.05$) at all time points

during the experiment. In contrast, osmotic control with mannitol had no effect on proliferation of EPCs ($P > 0.05$). Lycopene significantly increased the proliferative capacity of EPCs compared to that of the HG group. Absorbance values of the three lycopene groups were higher than that of the HG group ($P < 0.05$). Compared with the control group, the absorbance value of EPCs from the 30 μg/mL lycopene group was the highest, and differences were statistically significant at three time points (12, 24, and 48 h; except 72 h). In the repeated measures analysis of variance, $F_{time} = 129.241$, $P = 0.000$; $F_{time*group} = 24.425$, $P = 0.000$; and $F_{groups} = 920.137$, $P = 0.000$. Lycopene was the main effect factor responsible for increasing the absorbance value and protecting the proliferative capacity of EPCs cultivated with HG (Table 1).

Effects of lycopene on EPC apoptosis

As shown in Table 2, dramatic elevation of apoptosis was observed following exposure of EPCs to HG for 12, 24, 48, and 72 h compared to the control and mannitol groups ($P < 0.05$) at all time points during the experiment. Mannitol had no effect on EPC apoptosis ($P > 0.05$). Lycopene significantly inhibited apoptosis of EPC compared to that of the HG group. Apoptotic rates of EPCs from the three lycopene groups were lower than that of the lycopene plus HG group ($P < 0.05$). Compared with the control group, the apoptotic rate of EPCs from the 30 μg/mL lycopene group was the lowest, but differences were not statistically significant at any time point. In the repeated measures analysis of variance, $F_{time} = 189.810$, $P = 0.000$; $F_{time*group} = 54.118$, $P = 0.000$; and $F_{groups} = 1741.180$, $P = 0.000$. Lycopene was the main effect factor responsible for inhibiting apoptosis of EPCs cultivated with HG (Table 2).
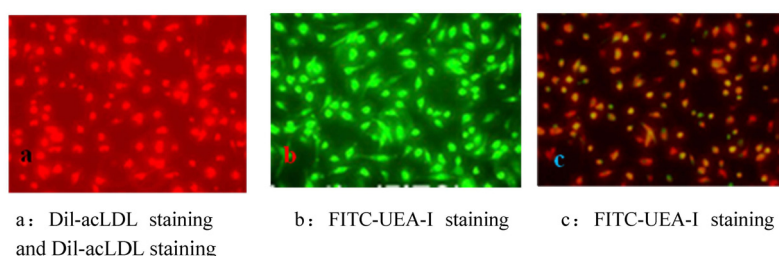

a：Dil-acLDL staining   b：FITC-UEA-I staining   c：FITC-UEA-I staining
and Dil-acLDL staining

Fig. 1. Characterization of EPCs under laser-scaning confocal microscope (× 200). Under the laser confocal microscope, Dil-Ac-LDL (red, excitation wavelength 543 nm) and Ulex lectin FITC- I (green, excitation wavelength 477 nm) double stained positive cells were differentiating EPCs

Table 1. Effects of lycopene on the proliferation of EPCs (n = 15, $\bar{x} \pm$ SD)

Group	A$_{490nm}$value			
	12 h	24h	48 h	72 h
Normal control group	0.207 ± 0.015$^{\Delta\blacktriangle}$	0.219 ± 0.007$^{\Delta\blacktriangle}$	0.216 ± 0.016$^{\Delta\blacktriangle}$	0.206 ± 0.013$^{\Delta}$
Mannitol group	0.204 ± 0.014$^{\Delta\blacktriangle}$	0.213 ± 0.009$^{\Delta}$	0.212 ± 0.007$^{\Delta}$	0.203 ± 0.006$^{\Delta}$
HG group	0.122 ± 0.015*$^{\blacktriangle}$	0.124 ± 0.007*$^{\blacktriangle}$	0.126 ± 0.008*$^{\blacktriangle}$	0.123 ± 0.015*$^{\blacktriangle}$
10 μg/mL lycopene group	0.137 ± 0.015*$^{\Delta\blacktriangle}$	0.166 ± 0.008*$^{\Delta\blacktriangle}$	0.162 ± 0.007*$^{\Delta\blacktriangle}$	0.159 ± 0.009*$^{\Delta\blacktriangle}$
30 μg/mL lycopene group	0.194 ± 0.009*$^{\Delta}$	0.208 ± 0.014*$^{\Delta}$	0.205 ± 0.018*$^{\Delta}$	0.203 ± 0.015$^{\Delta}$
50 μg/mL lycopene group	0.153 ± 0.007*$^{\Delta\blacktriangle}$	0.173 ± 0.015*$^{\Delta\blacktriangle}$	0.171 ± 0.014*$^{\Delta\blacktriangle}$	0.169 ± 0.014*$^{\Delta\blacktriangle}$
F	570.422	385.561	423.705	427.731
P	0.000	0.000	0.000	0.000

Compare with control, * $P< 0.05$; Compare with high glucose group, $^{\triangle} P< 0.05$; Compare with 30 μg/mL lycopene group, $^{\blacktriangle} P< 0.05$.

Table 2. Effects of lycopene on the apoptosis rate of EPCs (n = 15, $\bar{\chi} \pm$ SD)

Group	Apoptosis rate (%)			
	12 h	24 h	48 h	72 h
Normal control group	1.949 ± 0.230△	3.221 ± 0.449△	7.255 ± 0.334△	11.755 ± 0.738△
Mannitol group	1.986 ± 0.180△	3.361 ± 0.561△	7.329 ± 0.395△	12.822 ± 0.707△
HG group	7.125 ± 0.327*▲	9.656 ± 0.431*▲	24.745 ± 3.812*▲	28.708 ± 3.518*▲
10 µg/mL lycopene group	4.044 ± 0.333△▲	7.689 ± 0.469*△▲	16.212 ± 2.236*△▲	18.743 ± 3.838*△▲
30 µg/mL lycopene group	2.033 ± 0.165△	3.444 ± 0.374△	7.33 ± 0.329△	12.354 ± 0.855△
50 µg/mL lycopene group	4.524 ± 0.285*△▲	6.763 ± 0.544*△▲	19.902 ± 4.161*△▲	21.749 ± 3.709*△▲
F	951.530	497.320	140.116	90.913
P	0.000	0.000	0.000	0.000

Compare with control, * $P < 0.05$; Compare with high glucose group, △ $P < 0.05$; Compare with 30 µg/mL lycopene group, ▲ $P < 0.05$.

Table 3. Effects of lycopene on the functions of EPCs (n = 15, $\bar{\chi} \pm$ SD)

Group	Adhesion (cells/×200)	Migration (cells/×200)	Vasculogenesis (tubules/×100)
Normal control group	147.28 ± 11.13△▲	144.15 ± 15.48△▲	14.63 ± 2.87△▲
HG group	85.83 ± 9.53*▲	36.23 ± 9.50*▲	0.20 ± 0.41*▲
10 µg/mL lycopene group	91.46 ± 9.37*▲	70.51 ± 10.89*△▲	4.74 ± 2.71*△▲
30 µg/mL lycopene group	117.08 ± 12.02*△	112.86 ± 9.95*△	10.86 ± 3.43*△
50 µg/mL lycopene group	95.26 ± 8.78*△▲	103.25 ± 13.34*△▲	5.79 ± 1.87*△▲
SB203580 group	105.19 ± 13.75*△▲	111.75 ± 12.19*△	7.59 ± 2.30*△▲
F	77.34	198.13	62.32
P	0.000	0.000	0.000

Compare with control, * $P < 0.05$; Compare with high glucose group, △ $P < 0.05$; Compare with 30 µg/mL lycopene group, ▲ $P < 0.05$.

Effects of lycopene on EPCs migration, adhesion, and in vitro vasculogenesis capacity

As shown in Table 3, dramatic inhibition of migration, adhesion, and *in vitro* vasculogenesis capacity was observed following exposure of EPCs to HG for 24 h compared to the other groups ($P < 0.05$). The detrimental effects of HG on EPC function were significantly attenuated by treatment of EPCs with lycopene (10, 30, and 50 µg/mL) for 24 h compared with the 33 mmol/L glucose group ($P < 0.05$). EPC migration and adhesion were higher in the 30 µg/mL lycopene group compared to the 10 and 50 µg/mL lycopene groups ($P < 0.05$). In comparing the 30 µg/mL lycopene group with the signal inhibitor group (SB203580), there was no difference in migration, whereas adhesive capacity following lycopene treatment was higher than that of the signal inhibitor group ($P < 0.05$) (Table 3).

For the Matrigel network formation assay, EPCs were cultured on Matrigel for 24 h to form an extensive and enclosed microvascular network structure. HG impaired the ability of EPCs to form the microvascular network structure. Specifically, the total length of the tube network decreased while the tube network was severely disrupted under HG, resulting in an incomplete and sparse microvascular network ($P < 0.05$) (Table 3). Lycopene and signal inhibitor restored the ability of EPCs to form the microvascular network *in vitro*. There were also significant differences in vascular network capacity among the EPC groups according to the dosage level of lycopene. As shown in Table 3, lycopene improved EPC vasculogenesis capacity in the presence of 33 mmol/L of glucose in a dose-dependent manner, and maximal effect was observed at 30 µg/mL of lycopene. Compared with the other lycopene groups (10, 50 µg/mL) and signal inhibitor group, microvascular structures were more
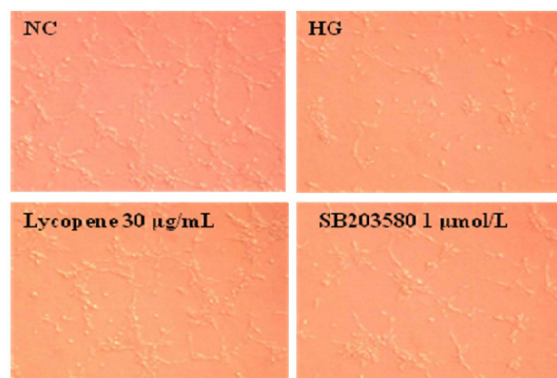

Fig. 2. Effects of lycopene on vasculogenesis activity of EPCs (× 100). Compared with the other dose lycopene groups(10,50 µg/mL)and signal blockers, microvascular structures of 30 µg/mL lycopene group were more complex and complete. Representative pictures of the tube formation of EPCs with different treatments are shown. NC: normal control group;HG:High glucose group; SB203580:p38 MAPK inhibitor .

complex and complete in EPCs treated with 30 µg/mL of lycopene. Representative pictures of tube formation in EPCs subjected to different treatments are shown (Fig. 2).

Effects of lycopene on MAPK activation in EPCs

To further characterize the mechanism underlying the protective effects of lycopene, activation of p38 MAPK was examined by Western blotting. As showed in Fig. 3, HG sharply increased the phosphorylation of p38 MAPK, and the increase was inhibited by lycopene. Total amounts of p38 MAPK were unaltered by either HG or lycopene. The protective effect
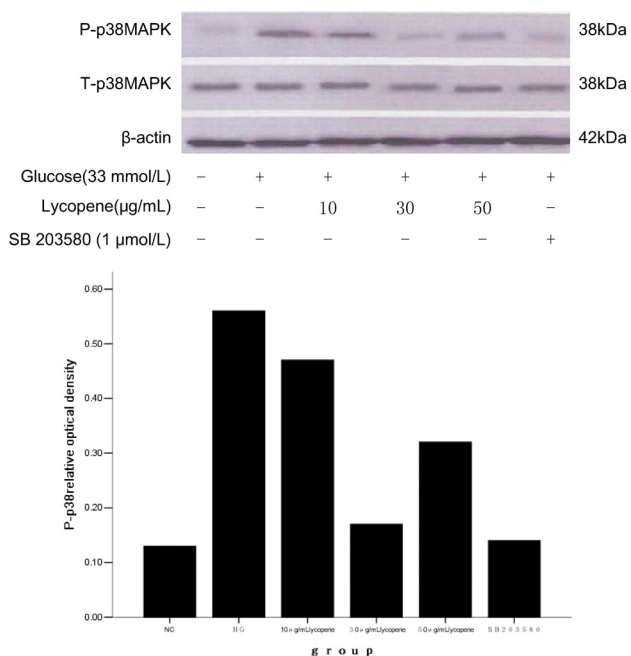
Fig. 3. Effects of lycopene on high glucose induced activation of p38 MAPKs in EPCs. Compare with NC, * *P* < 0.05; Compare with HG, △ *P* < 0.05; Compare with 30 μg/mL lycopene group, ▲ *P* < 0.05. NC: normal control group; HG: High glucose group; SB203580: p38 MAPK inhibitor.

observed in the 30 μg/mL lycopene group was greater than that in either the 10 or 50 μg/mL lycopene group. To confirm the causal relationship between MAPK inhibition and the protective effects of lycopene against HG-induced cellular injury, the effects of the MAPK inhibitor SB203580 on HG were further examined. The inhibitor similarly reversed the detrimental effects of HG (Fig. 3). These results indicate that inhibition of MAPKs is responsible for the protective effects of lycopene against HG-induced EPC injury.

Curve fitting analysis of effects of lycopene on EPC function
To explore the effects of lycopene to EPC functional variables, we carried out curve fitting analyses. Dose of lycopene was the independent variable, whereas the absolute number of differences in the various functional parameters (HG group

mean-lycopene groups value) was the dependent variable. The regression equation is shown in Fig. 4. Each regression equation's coefficient of determination R^2 was satisfactory, $P = 0.000$. Differences in EPC functional variables were detected within the range of lycopene doses (10-50 μg/mL) (Fig. 4).

DISCUSSION

EPCs are a kind of precursor cell that can differentiate into vascular endothelial cells. EPCs are not only involved in human embryonic angiogenesis but also in postnatal angiogenesis and endothelial repair, which is a major factor in maintaining dynamic equilibrium between endothelial injury and repair. Important risk factors for the occurrence and development of vascular complications due to diabetes mellitus are the number of EPCs and vascular endothelial dysfunction [32]. EPCs from patients with type 2 diabetes mellitus are characterized by reduced proliferative capacity as well as lower adhesiveness and ability to form capillary tubes *in vitro* [32]. It was recently shown that HG impairs the proliferation and function of early and late EPCs, including migration and capacity for vasculogenesis [14,33].

Previous epidemiological studies have established that lycopene is strongly associated with reduced carotid intima-media thickness and lower incidences of myocardial infarction, ischemic heart disease, stroke, cancer, and diabetes. Intake of lycopene-rich foods can prevent the occurrence and development of these chronic diseases [20,34-38]. Among carotenoids, only lycopene has been shown to modulate adhesion molecule expression in human aortic endothelial cell cultures, which suggests a role in mitigating atherogenesis [21]. Some studies indicated that lycopene exerts a protective vascular endothelial function effect on a variety of endothelial dysfunctional processes [39-41]. EPCs can differentiate into vascular endothelial cells. In the present study, we observed the effects of HG on EPC proliferation, apoptosis, and function, as well as the potential mechanisms responsible for these effects. Lycopene has been shown to act as a pro-oxidant or antioxidant depending on its administrated dose [42,43]. In Lowe GM's study [42], lycopene was shown to only provide protection against DNA damage (induced by xanthine/xanthine oxidase) at relatively low concentrations (1-3 μM). However, at higher concentrations
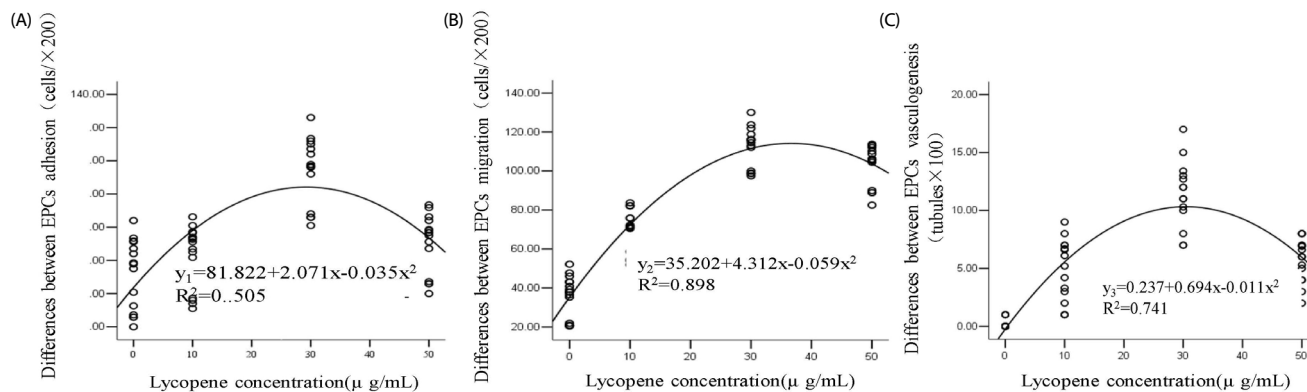

Fig. 4. Curve fitting figures of effects of lycopene on EPCs in high concentration of glucose

(4-10 µM), its ability to protect cells against such oxidative damage was rapidly lost. Another study using Hs68 cells demonstrated that lycopene can be either an antioxidant or pro-oxidant depending on the oxidants used, but it is unclear whether or not lycopene has pro-oxidant activity *in vivo* [43]. Our study suggests that apoptotic rate and phosphorylation of p38 MAPK are increased in EPCs cultivated with HG, whereas EPC proliferation, migration, adhesion, and *in vitro* vasculo-genesis capacity are attenuated. On the other hand, we found that lycopene inhibits the excessive phosphorylation of p38 MAPK, promotes proliferation, migration, adhesion, and *in vitro* vasculogenesis capacity, and reduces apoptosis of EPCs induced by HG. The results in Table 3 indicate that the proliferation, migration, adhesion, and *in vitro* vasculogenesis capacity of EPCs from the 30 µg/mL lycopene group were significantly elevated compared to the HG group and 10 and 50 µg/mL lycopene groups, whereas the apoptotic rate and phosp-horylation of p38 MAPK were significantly reduced. Therefore, whether or not the pro-oxidation phenomenon in the above experiments is observed with a higher dose of lycopene must be studied further. This study showed that the proliferation, migration, adhesion, and *in vitro* vasculogenesis capacity of EPCs were significantly higher in the three lycopene groups compared to the HG group, whereas apoptotic rate and phosphorylation of p38 MAPK were significantly lower. Our results indicate that lycopene can protect proliferative capacity and inhibit apoptosis of EPCs cultivated with HG. Further, lycopene not only can increase the number of EPCs cultivated with HG but also improve their adhesion, migration, and *in vitro* vasculogenesis capacity. Previous studies have shown that lycopene plays a role in the protection of endothelial cells [39-41]. In support of this, our study indicates that lycopene may promote endothelial repair by protecting the proliferative capacity and function of EPCs. HG-induced apoptosis of EPCs has been confirmed by many scholars, and its mechanism of action may promote oxidative stress [44], mitochondria-dependent and non-dependent caspase pathways [45], MAPK pathway, and p53 pathway [46,47].

Previous studies have demonstrated that HG accelerates the onset of EPC senescence via activation of p38 MAPKs [46] and increases oxidative stress [44]. It has been reported that glucose can activate the p38 MAPK pathway in EPCs [35]. Several studies demonstrated that blockage of p38 MAPKs is associated with increased angiogenesis [36-38]. Protein phosphorylation, induced by different kinases, plays a crucial role in triggering signal transduction pathways. MAPKs are a group of serine/threonine protein kinases comprising three subfamilies: p42/p44 ERKs, JNKs, and p38 MAPKs. MAPKs can be activated by various extracellular molecules and can induce downstream phosp-horylation of many key signaling molecules related to cell proliferation, inflammation, and apoptosis [48]. Studies have shown that p38 MAPK is a critical regulator of EPC differe-ntiation [47]. SB203580 can inhibit HG-induced senescence of EPCs, as well as improve proliferation, differentiation, and *in vivo* angiogenesis capacity [47]. In our results, proliferation and functional variables of EPCs exposed to HG were significantly reduced compared with the control group, whereas the apop-totic rate significantly increased. This can likely be attributed

to the increased phosphorylation of p38 MAPK.

Studies have shown that lycopene has a protective effect against a variety of exogenously-induced endothelial dysfunc-tional processes [39,40]. Recent studies also showed that lycopene significantly inhibits the effects of lipopolysaccharide (LPS) by suppressing a key inflammatory pathway related to MAPK, NF-κB, NO formation, and IL-6 expression in macrop-hages, and it has an anti-inflammatory effect [22]. Many independent lines of evidence suggest that different MAPKs can be activated in EPCs in response to HG [46,14]. Such results were confirmed in the present study. Specifically, lycopene significantly inhibited the phosphorylation or activation of p38 MAPK by HG, and total protein levels of MAPK remained unchanged. Therefore, lycopene blocked the activation but not the biosynthesis of MAPKs. Furthermore, we studied the effects of SB203580 on EPC functional variables. EPCs were treated with SB203580, a specific p38 kinase inhibitor. SB203580 reversed the inhibitory effects of HG on EPC function. These results suggest that the inhibitory effect of HG on EPCs is partially mediated through the p38 pathway.

To confirm the causal relationship between MAPK inhibition and the protective effects of lycopene against HG-induced cellular injury, we treated cells with SB203580. The inhibitor significantly inhibited the toxic effects of HG, and EPC adhesion, migration, and angiogenesis *in vitro* were markedly elevated. Therefore, p38 MAPK activation plays an important role in HG-induced EPC dysfunction, and lycopene can protect EPC function by blocking the activation of p38 MAPK.

In our experiment, there was no significant difference between migration of EPCs from the 30 µg/mL lycopene group and the SB203580 group ($P > 0.05$). Adhesion and *in vitro* angiogenesis capabilities of EPCs from the 30 µg/mL lycopene group were superior compared to the SB203580 group. Moreover, the functional variables of EPCs from the 30 µg/mL lycopene group were higher than those of the 10 and 50 µg/mL groups. The exact molecular mechanisms responsible for the effects of lycopene on EPCs remain to be elucidated. Curve fitting (Fig. 4) and the regression equation demonstrated the effects of lycopene on the function of human peripheral blood EPCs cultivated with 10-50 µg/mL of HG, and the data provide a scientific basis for future experimental research.

In conclusion, a high concentration of glucose may attenuate the proliferation, migration, adhesion, and *in vitro* vasculo-genesis capacity of EPCs while increasing the apoptotic rate. On the other hand, lycopene may promote proliferation, migratory, adhesion, and *in vitro* vasculogenesis capacity as well as reduce apoptosis of EPCs cultivated with HG. These findings suggest that lycopene can improve the function of EPCs incubated with a high concentration of glucose by inhibiting the p38 MAPK pathway, which is one of the mechanisms responsible for protecting EPCs. The underlying molecular mechanisms of the p38 MAPK signal transduction pathway may involve the protective effects of lycopene against HG-induced EPC injury. Further, lycopene may be considered as a new strategy for preventing the macrovascular complications of type 2 diabetes.

REFERENCES

1. Almdal T, Scharling H, Jensen JS, Vestergaard H. The independent effect of type 2 diabetes mellitus on ischemic heart disease, stroke, and death: a population-based study of 13,000 men and women with 20 years of follow-up. Arch Intern Med 2004;164:1422-6.

2. Avogaro A, Fadini GP, Gallo A, Pagnin E, de Kreutzenberg S. Endothelial dysfunction in type 2 diabetes mellitus. Nutr Metab Cardiovasc Dis 2006;16 Suppl 1:S39-45.

3. Cubbon RM, Kahn MB, Wheatcroft SB. Effects of insulin resistance on endothelial progenitor cells and vascular repair. Clin Sci (Lond) 2009;117:173-90.

4. Kawamoto A, Tkebuchava T, Yamaguchi J, Nishimura H, Yoon YS, Milliken C, Uchida S, Masuo O, Iwaguro H, Ma H, Hanley A, Silver M, Kearney M, Losordo DW, Isner JM, Asahara T. Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia. Circulation 2003;107:461-8.

5. Fadini GP, Avogaro A. Potential manipulation of endothelial progenitor cells in diabetes and its complications. Diabetes Obes Metab 2010;12:570-83.

6. Reinhard H, Jacobsen PK, Lajer M, Pedersen N, Billestrup N, Mandrup-Poulsen T, Parving HH, Rossing P. Multifactorial treatment increases endothelial progenitor cells in patients with type 2 diabetes. Diabetologia 2010;53:2129-33.

7. Liao YF, Chen LL, Zeng TS, Li YM, Fan Y, Hu LJ, Ling Y. Number of circulating endothelial progenitor cells as a marker of vascular endothelial function for type 2 diabetes. Vasc Med 2010;15:279-85.

8. Schatteman GC, Hanlon HD, Jiao C, Dodds SG, Christy BA. Blood-derived angioblasts accelerate blood-flow restoration in diabetic mice. J Clin Invest 2000;106:571-8.

9. Dimmeler S, Zeiher AM, Schneider MD. Unchain my heart: the scientific foundations of cardiac repair. J Clin Invest 2005;115:572-83.

10. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 2003;348:593-600.

11. Fadini GP, Miorin M, Facco M, Bonamico S, Baesso I, Grego F, Menegolo M, de Kreutzenberg SV, Tiengo A, Agostini C, Avogaro A. Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus. J Am Coll Cardiol 2005;45:1449-57.

12. Loomans CJ, de Koning EJ, Staal FJ, Rookmaaker MB, Verseyden C, de Boer HC, Verhaar MC, Braam B, Rabelink TJ, van Zonneveld AJ. Endothelial progenitor cell dysfunction: a novel concept in the pathogenesis of vascular complications of type 1 diabetes. Diabetes 2004;53:195-9.

13. Creager MA, Lüscher TF, Cosentino F, Beckman JA. Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: part I. Circulation 2003;108:1527-32.

14. Chen YH, Lin SJ, Lin FY, Wu TC, Tsao CR, Huang PH, Liu PL, Chen YL, Chen JW. High glucose impairs early and late endothelial progenitor cells by modifying nitric oxide-related but not oxidative stress-mediated mechanisms. Diabetes 2007;56:1559-68.

15. Sheu ML, Ho FM, Yang RS, Chao KF, Lin WW, Lin-Shiau SY, Liu SH. High glucose induces human endothelial cell apoptosis through a phosphoinositide 3-kinase-regulated cyclooxygenase-2 pathway. Arterioscler Thromb Vasc Biol 2005;25:539-45.

16. Yoshizumi M, Abe J, Tsuchiya K, Berk BC, Tamaki T. Stress and vascular responses: atheroprotective effect of laminar fluid shear stress in endothelial cells: possible role of mitogen-activated protein kinases. J Pharmacol Sci 2003;91:172-6.

17. Uruno A, Sugawara A, Kudo M, Satoh F, Saito A, Ito S. Stimulatory effects of low-dose 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitor fluvastatin on hepatocyte growth factor-induced angiogenesis: involvement of p38 mitogen-activated protein kinase. Hypertens Res 2008;31:2085-96.

18. McGinn S, Saad S, Poronnik P, Pollock CA. High glucose-mediated effects on endothelial cell proliferation occur via p38 MAP kinase. Am J Physiol Endocrinol Metab 2003;285:E708-17.

19. Di Mascio P, Kaiser S, Sies H. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. Arch Biochem Biophys 1989;274:532-8.

20. Sesso HD, Buring JE, Norkus EP, Gaziano JM. Plasma lycopene, other carotenoids, and retinol and the risk of cardiovascular disease in women. Am J Clin Nutr 2004;79:47-53.

21. Martin KR, Wu D, Meydani M. The effect of carotenoids on the expression of cell surface adhesion molecules and binding of monocytes to human aortic endothelial cells. Atherosclerosis 2000;150:265-74.

22. Kim GY, Kim JH, Ahn SC, Lee HJ, Moon DO, Lee CM, Park YM. Lycopene suppresses the lipopolysaccharide-induced phenotypic and functional maturation of murine dendritic cells through inhibition of mitogen-activated protein kinases and nuclear factor-kappaB. Immunology 2004;113:203-11.

23. Chan CM, Fang JY, Lin HH, Yang CY, Hung CF. Lycopene inhibits PDGF-BB-induced retinal pigment epithelial cell migration by suppression of PI3K/Akt and MAPK pathways. Biochem Biophys Res Commun 2009;388:172-6.

24. Van Craenenbroeck EM, Conraads VM. Endothelial progenitor cells in vascular health: focus on lifestyle. Microvasc Res 2010;79:184-92.

25. Kondo T, Hayashi M, Takeshita K, Numaguchi Y, Kobayashi K, Iino S, Inden Y, Murohara T. Smoking cessation rapidly increases circulating progenitor cells in peripheral blood in chronic smokers. Arterioscler Thromb Vasc Biol 2004;24:1442-7.

26. Gill M, Dias S, Hattori K, Rivera ML, Hicklin D, Witte L, Girardi L, Yurt R, Himel H, Rafii S. Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. Circ Res 2001;88:167-74.

27. Lin CY, Huang CS, Hu ML. The use of fetal bovine serum as delivery vehicle to improve the uptake and stability of lycopene in cell culture studies. Br J Nutr 2007;98:226-32.

28. Azuma K, Kawamori R, Toyofuku Y, Kitahara Y, Sato F, Shimizu T, Miura K, Mine T, Tanaka Y, Mitsumata M, Watada H. Repetitive fluctuations in blood glucose enhance monocyte adhesion to the endothelium of rat thoracic aorta. Arterioscler Thromb Vasc Biol 2006;26:2275-80.

29. Wang H, Wang L, Li KZ, Ling R, Sun BH, Ma FC, Li XJ. Culture and identification of endothelial progenitor cells from human peripheral blood. Chin J Clin Rehabil 2006;10:47-9.

30. Wang HX, Zhao S, Li BG, Mao H, Shao SY. Effects of high-glucose on proliferation and apoptosis in endothelial progenitor cells of type 2 diabetes mellitus. Chin J Pathophysiol 2007;23:2210-3.

31. Kuwana M, Okazaki Y, Kodama H, Satoh T, Kawakami Y, Ikeda Y. Endothelial differentiation potential of human monocyte-derived multipotential cells. Stem Cells 2006;24:2733-43.

32. Tepper OM, Galiano RD, Capla JM, Kalka C, Gagne PJ, Jacobowitz GR, Levine JP, Gurtner GC. Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. Circulation 2002;106:2781-6.

33. Nakamura N, Naruse K, Kobayashi Y, Matsuki T, Hamada Y, Nakashima E, Kamiya H, Hata M, Nishikawa T, Enomoto A, Takahashi M, Murohara T, Matsubara T , Oiso Y, Nakamura J. High glucose impairs the proliferation and increases the apoptosis of endothelial progenitor cells by suppression of Akt. J Diabetes Investig 2011;2:262-70.

34. Gianetti J, Pedrinelli R, Petrucci R, Lazzerini G, De Caterina M, Bellomo G, De Caterina R. Inverse association between carotid intima-media thickness and the antioxidant lycopene in atherosclerosis. Am Heart J 2002;143:467-74.

35. Rissanen T, Voutilainen S, Nyyssönen K, Salonen JT. Lycopene, atherosclerosis, and coronary heart disease. Exp Biol Med (Maywood) 2002;227:900-7.

36. Rissanen TH, Voutilainen S, Nyyssönen K, Salonen R, Kaplan GA, Salonen JT. Serum lycopene concentrations and carotid atherosclerosis: the Kuopio Ischaemic Heart Disease Risk Factor Study. Am J Clin Nutr 2003;77:133-8.

37. Vaishampayan U, Hussain M, Banerjee M, Seren S, Sarkar FH, Fontana J, Forman JD, Cher ML, Powell I, Pontes JE, Kucuk O. Lycopene and soy isoflavones in the treatment of prostate cancer. Nutr Cancer 2007;59:1-7.

38. Ylönen K, Alfthan G, Groop L, Saloranta C, Aro A, Virtanen SM. Dietary intakes and plasma concentrations of carotenoids and tocopherols in relation to glucose metabolism in subjects at high risk of type 2 diabetes: the Botnia Dietary Study. Am J Clin Nutr 2003;77:1434-41.

39. Liu X, Qu D, He F, Lu Q, Wang J, Cai D. Effect of lycopene on the vascular endothelial function and expression of inflammatory agents in hyperhomocysteinemic rats. Asia Pac J Clin Nutr 2007;16 Suppl 1:244-8.

40. Balestrieri ML, De Prisco R, Nicolaus B, Pari P, Moriello VS, Strazzullo G, Iorio EL, Servillo L, Balestrieri C. Lycopene in association with alpha-tocopherol or tomato lipophilic extracts enhances acyl-platelet-activating factor biosynthesis in endothelial cells during oxidative stress. Free Radic Biol Med 2004;36:1058-67.

41. Suganuma H, Inakuma T. Protective effect of dietary tomato against endothelial dysfunction in hypercholesterolemic mice. Biosci Biotechnol Biochem 1999;63:78-82.

42. Lowe GM, Booth LA, Young AJ, Bilton RF. Lycopene and beta-carotene protect against oxidative damage in HT29 cells at low concentrations but rapidly lose this capacity at higher doses. Free Radic Res 1999;30:141-51.

43. Yeh S, Hu M. Antioxidant and pro-oxidant effects of lycopene in comparison with beta-carotene on oxidant-induced damage in Hs68 cells. J Nutr Biochem 2000;11:548-54.

44. Piconi L, Quagliaro L, Assaloni R, Da Ros R, Maier A, Zuodar G, Ceriello A. Constant and intermittent high glucose enhances endothelial cell apoptosis through mitochondrial superoxide overproduction. Diabetes Metab Res Rev 2006;22:198-203.

45. Tamareille S, Mignen O, Capiod T, Rücker-Martin C, Feuvray D. High glucose-induced apoptosis through store-operated calcium entry and calcineurin in human umbilical vein endothelial cells. Cell Calcium 2006;39:47-55.

46. Kuki S, Imanishi T, Kobayashi K, Matsuo Y, Obana M, Akasaka T. Hyperglycemia accelerated endothelial progenitor cell senescence via the activation of p38 mitogen-activated protein kinase. Circ J 2006;70:1076-81.

47. Seeger FH, Haendeler J, Walter DH, Rochwalsky U, Reinhold J, Urbich C, Rössig L, Corbaz A, Chvatchko Y, Zeiher AM, Dimmeler S. p38 mitogen-activated protein kinase downregulates endothelial progenitor cells. Circulation 2005;111:1184-91.

48. Adams RH, Porras A, Alonso G, Jones M, Vintersten K, Panelli S, Valladares A, Perez L, Klein R, Nebreda AR. Essential role of p38alpha MAP kinase in placental but not embryonic cardiovascular development. Mol Cell 2000;6:109-16.