Int J Clin Exp Med 2015;8(11):19969-19981
www.ijcem.com /ISSN:1940-5901/IJCEM0008186

Original Article

Experimental study on apoptosis of TNFR1 receptor pro-endothelial progenitor cells activated by high glucose induced oxidative stress

Yong Liu[1*], Fei Xei[1,2*], Xiong-Fei Xu[1], Hong Zeng[1], Hu-Qiang He[1], Lei Zhang[1], Ying-Qiang Zheng[1], Yan-Zheng He[1]

[1]Deparyment of Vascular Surgery, Affiliated Hospital of Luzhou Medical College Luzhou, Sichuan, China; [2]First People's Hospital of Liangshan Yi Autonomous Prefecture, Liangshan, China. [*]Equal contributors.

Received March 18, 2015; Accepted June 3, 2015; Epub November 15, 2015; Published November 30, 2015

Abstract: Objective: To investigate whether high glucose in vitro activating TNFR1 and further promote rat marrow endothelial progenitor cells (EPCs) apoptosis. Methods: Rat morrow endothelial progenitor cells were cultured and identified by Confocal Microscopy; then were treated with high glucose (5.5, 15, 30, 60 mmol/L), mannitol (15, 30, 60, 90 mmol/L), high glucose + Tempol and high glucose+ MAB430. Apoptosis rate of the above cells were detected by flow cytometry. ROS and MDA level and anti-O_{2^-} were detected by colorimetric technique; the expression level of TNFR1 induced signal pathway related proteins were detected by Western blotting. Results: High glucose can induce endothelial progenitor cells apoptosis, which is mostly in the later stage (72 h-96 h) instead of the earlier stage (24 h-48 h); high glucose can also induce oxidative stress reaction and the produces ROS and MDA increase significantly in the later stage (after 72 h), but anti-O_{2^-} decrease significantly. TNF apoptosis signal pathway related protein expression level not increase in the earlier stage (before 24 h) but increase significantly in the later stage (after 72 h). Tempol and MAB430 down-regulate TNF apoptosis signal pathway related protein expression and reduce EPCs apoptosis. Conclusion: High glucose activates the TNFR1 of TPCs through oxidative stress reaction and further induces cell apoptosis.

Keywords: Endothelial progenitor cells, high glucose, apoptosis, oxidative stress reaction, TNFR1

Introduction

Endothelial progenitor cells (EPCs) are primarily generated and present in bone marrow cells, which are a class of directional stem cells between stem cells and vascular endothelial cells (VECs) with migratory characteristic and self-renewal proliferation and differentiation [1]. In 1997, Asahara et al [2] firstly isolated bone marrow-derived CD34 + and KDR + cells from the peripheral blood, named endothelial progenitor cells (EPCs). Undifferentiated EPCs were round; differentiated EPCs were fusiform or spindle-shaped, so it is difficult to separate EPCs from other cells by shape; most scholars rely mainly on CD34 +, AC133 +, vascular endothelial growth factor 2 (VEGFR-2) and other surface antigen markers to identify EPCs [3]. EPCs play an important role in angiogenesis and the reconstruction of damaged blood vessels [4, 5]. EPCs can differentiate into functional endothelial cells in vivo microenvironment to involve the angiogenesis in ischemic tissue; and they can be integrated into the neointimal of damaged vessel wall to be involved in re-endothelialization of vascular injury [6, 7].

Ischemic injury is the secondary symptom of many common and frequent diseases in clinical, including traumatic brain injury, myocardial infarction, cerebral infarction, and diabetes. Vascular disease secondary to diabetes is characterized by the severely impaired formation of collateral vessels [8], and the number of EPCs in peripheral blood of patients with diabetes was significantly reduced [9], and the function was severely impaired [10]. Therefore, in the mechanism research of impaired angiogenesis in diabetes, EPCs dysfunction-related factors have attracted extensive attention. Diabetic hyperglycemia environment allows EPCs apoptosis to increase and EPCs number to decrease.

The mechanism is: High blood sugar can induce the oxidative stress response of the cells, and induce cells to produce an excess of ROS; ROS regulates apoptosis gene to activate apoptotic receptor, and thus leading to a significant increase in apoptosis. Tumor necrosis factor ((TNF-α) is mainly involved in the regulation of apoptosis and inflammatory reactions; its receptor (TNFR1) plays an important role in the major pathway of apoptosis. Thus, under the conditions of oxidative stress caused by high glucose, ROS activates TNFR1 and then EPCs induces apoptosis, which may be an important factor in diabetic angiogenesis disorders. Therefore, in this study, whether high glucose in vitro activated TNF receptor by oxidative stress and thus affected the apoptosis in rat bone marrow-derived endothelial progenitor cells (EPC) was investigated to explore the effect of high glucose on endothelial progenitor cells.

Materials and methods

Materials

Experimental animals: Adult male Sprague-Dawley rats (4 months old) weighing 200 ± 59 were provided by the Experimental Animal Center attached to Luzhou Medical College; M199 culture solution medium was provided by the US Gibco company (Penicillin and streptomycin each 100,000 units were added and preserved at 4°C); Rat lymphocyte separation medium was purchased from Green day biotechnology Research Institute; quality fetal calf serum (FCS) was purchased from Gibeo company; trypsin were purchased from Shanghai Sangon biological Engineering technology Services Limited; type I rat tail collagen was purchased from R & D company; 4% paraformaldehyde and 3% H_2O_2 were purchased from Beijing Zhongshan biotechnology Co., Ltd.; FITC labeled UEA-I (FITC-UEA-I) were purchased from Sigma; Dil labeled acetyl LDL were purchased from Moleeula Probe company; AnnexinV/PI was purchased from Bender company; malondialdehyde assay kit, superoxide anion radicals and superoxide anion radicals generated test kit were purchased from Nanjing Jiancheng company; rabbit TNFR1 anti-rat, TRAF-2, TRADD, RIP, NF-kBp65, Caspase3 and β-actin monoclonal antibody were purchased from Sant-Cruz company; goat anti-rabbit IgG was purchased from Wuhan Boster Company.

Methods

Isolation, culture and identification of EPCs: Take femur, and flush out the bone marrow. Density gradient centrifugation methods was used to isolate mononuclear cells. M199 complete medium (containing 20% fetal bovine serum, VEGF 20 μg/L, bFGF4 μg/L penicillin 100 kU/L, streptomycin 100 kU/L) was used to resuspended cells. The mononuclear cells were seeded in the pre-coated with rat tail collagen type I 24-well and 6-well cultured plates at a density of 1×10^8/L. They were cultured at 37°C, 5% CO humidified incubator. After 4 days' cultivation the solution was changed and PBS was used to wash and remove non-adherent cells. Adherent cells and 2.4 μg/mL DiI-acLDL were incubated at 37°C ambient for 1.5 h together. 2% paraformaldehyde was used to fix, and FITC-UEA-I was used to re-stained. Laser confocal microscopy was used to identify differentiating EPCs with FITC-UEA-I and DiI-ac-LDL double staining positive cells.

Analysis of apoptosis rate of EPCs after treatment with high glucose by flow cytometry: EPCs were cultured and grouped. In M199 complete culture medium high glucose control group the concentration of glucose was 5.5 mmol/L; In ECM complete culture medium high glucose group the concentrations of glucose were 15, 30, 60 mmol/L, respectively; In addition, 30 mmol/L high glucose + Tempol group and 30 mmol/L high glucose + MAB430 group were set. After cultured for 24 h, 48 h, 72 h, 96 h, the detection was performed. In mannitol control group M199 complete culture medium the concentration of mannitol was 15 mmol/L; in mannitol group M199 complete culture medium the concentration of mannitol were 30, 60, 90 mmol/L, respectively. After cultured for 72 h, the detection was performed. Annexin V-FITC/PI apoptosis detection kit was applied to detect apoptosis. The steps were as follows: 1. Marrow EPCS with different stimulation and collect the cells by trypsin digestion. 2. Deionized water combine buffer with 1:4 dilution; 3. 4°C precold PBs was used to wash cells twice and the cells were resuspended with 250 uL binding buffer; Adjust its concentration to 1×106/ml; 4. Take 100 uL of cell suspension to 5 ml streaming tube, and add 5 mL AnnexinV-FITC and 10 mL, 20 mg/ml propidium iodide (PI) solution; 5. Set no Annexin V and PI blank control, only

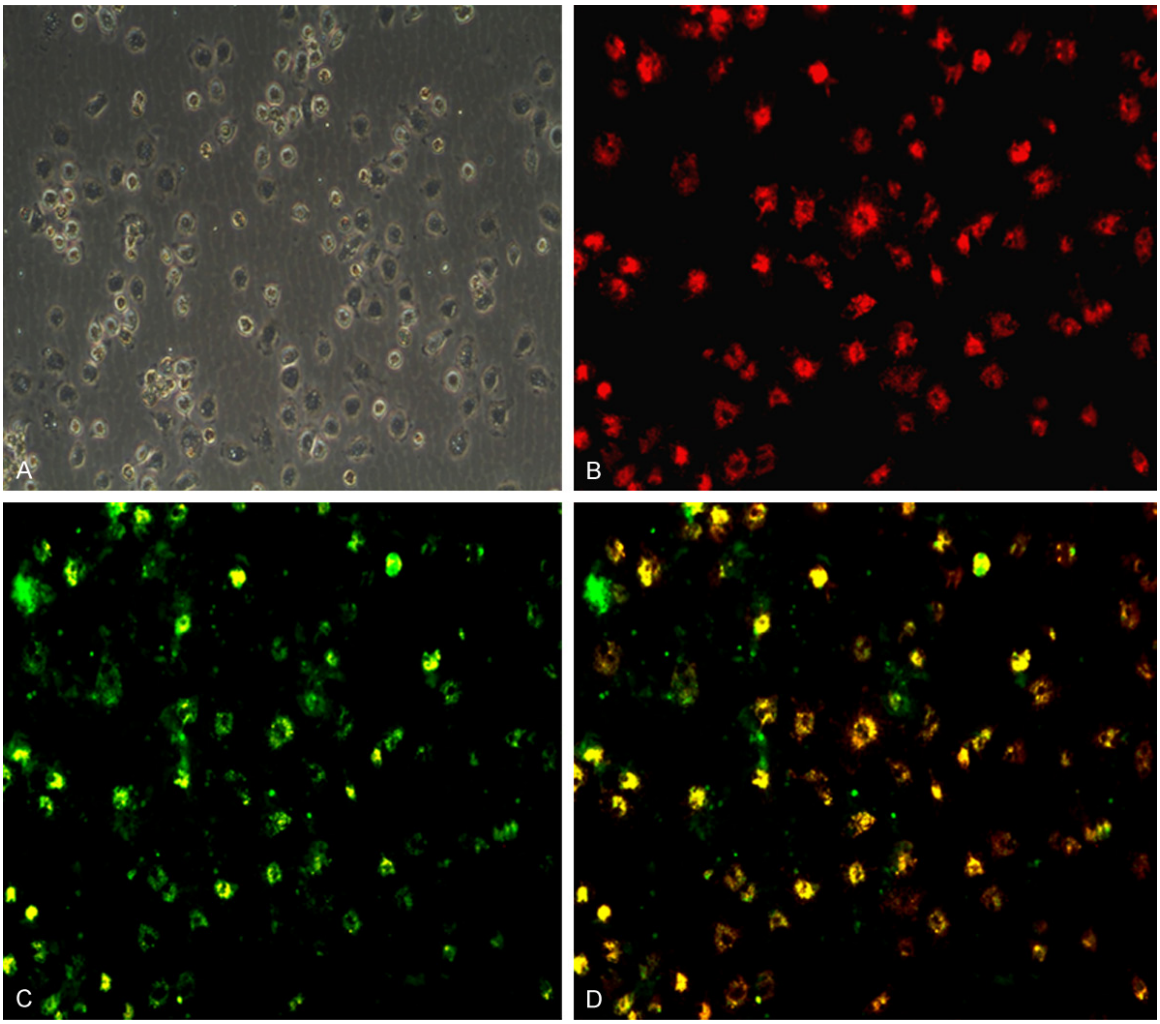
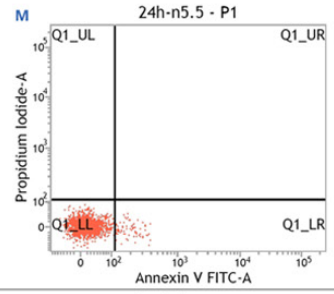


Figure 1. A: Appearance of primary EPCs (100×); B: DiI-acLDL positive cells; C: FITC-UEA-I positive cells; D: DiI-acLDL and FITC-UEA-I positive cells.

Annexin V contrast, the only PI control; 6. Mix the cells incubated in the dark at room temperature after several minutes; 7. Add 400 mLPBS to the reaction tube and perform machine analysis; 8. FACS preparation: light source was 488 nm laser. Flow-Check was used for optical path preset; 9. After the test is completed, using computer software to analyze the data and calculate the results of the apoptosis rate.

Measurements of oxidative stress products ROS, MDA content and anti O_{2^-} by colorimetric detection: EPCs were cultured and grouped; glucose concentration in M199 complete medium was 5.5 mmol/L in the control group, and 30 mmol/L in high glucose group; 30 mmol/L high glucose + Tempol group and 30 mmol/L high glucose + MAB430 group were set to study the effects of high glucose on ROS content; In M199 complete medium groups the concentration of glucose were 5.5 mmol/L (control group), 15 mmol/L, 30 mmol/L and 60 mmol/L respectively. They were used to study the high sugar to the content of MDA and anti-O_{2^-}. After cultured for 24 h and 72 h, trypsinized EPCs which were cultured board were transferred into the tube and stored in the refrigerator freezer at -20°C. They were retained one hour each time. Then they were kept at room temperature waiting for ice completely thawed and then placed in the -20°C freezer. Continued three times, the cells were lysed and make cell homogenates. They were stored at -20°C for spare. MDA detection test kit was used to detect the content of MDA following the instructions steps. MDA content (nmol/mL) = (absorbance of measurement tube

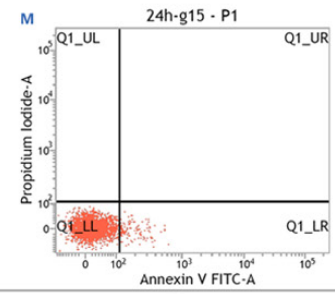
A



Statistics

Name	Events	% Parent	% Grandparent	% Total
24h-n5.5:All Events	10,000	***	***	100.00
24h-n5.5:Q1_UL	0	0.00	0.00	0.00
24h-n5.5:Q1_UR	0	0.00	0.00	0.00
24h-n5.5:Q1_LL	2,280	96.61	22.80	22.80
24h-n5.5:Q1_LR	80	3.39	0.80	0.80

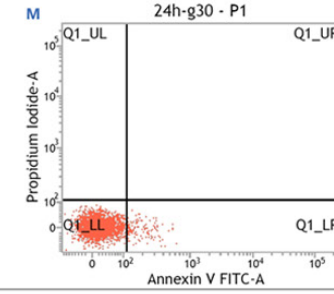
B



Statistics

Name	Events	% Parent	% Grandparent	% Total
24h-g15:All Events	10,000	***	***	100.00
24h-g15:Q1_UL	0	0.00	0.00	0.00
24h-g15:Q1_UR	0	0.00	0.00	0.00
24h-g15:Q1_LL	3,971	96.88	39.71	39.71
24h-g15:Q1_LR	128	3.12	1.28	1.28

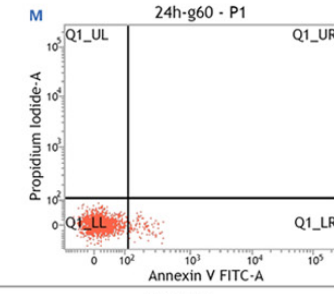
C



Statistics

Name	Events	% Parent	% Grandparent	% Total
24h-g30:All Events	10,000	***	***	100.00
24h-g30:Q1_UL	0	0.00	0.00	0.00
24h-g30:Q1_UR	0	0.00	0.00	0.00
24h-g30:Q1_LL	3,923	96.58	39.23	39.23
24h-g30:Q1_LR	139	3.42	1.39	1.39

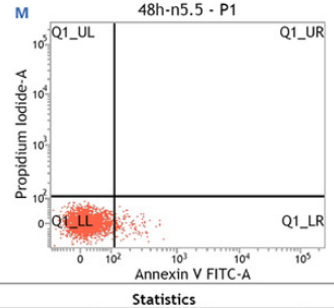
D



Statistics

Name	Events	% Parent	% Grandparent	% Total
24h-g60:All Events	10,000	***	***	100.00
24h-g60:Q1_UL	0	0.00	0.00	0.00
24h-g60:Q1_UR	0	0.00	0.00	0.00
24h-g60:Q1_LL	2,321	95.95	23.21	23.21
24h-g60:Q1_LR	98	4.05	0.98	0.98

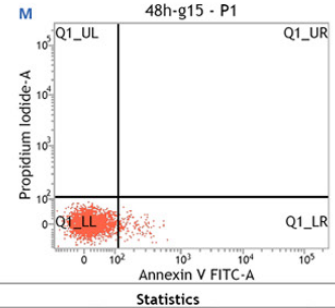
E



Statistics

Name	Events	% Parent	% Grandparent	% Total
48h-n5.5:All Events	10,000	***	***	100.00
48h-n5.5:Q1_UL	0	0.00	0.00	0.00
48h-n5.5:Q1_UR	0	0.00	0.00	0.00
48h-n5.5:Q1_LL	3,959	96.96	39.59	39.59
48h-n5.5:Q1_LR	124	3.04	1.24	1.24

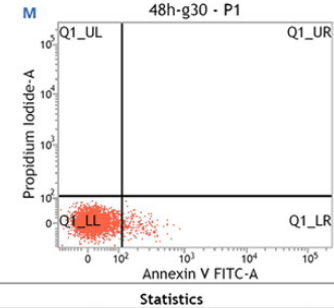
F



Statistics

Name	Events	% Parent	% Grandparent	% Total
48h-g15:All Events	10,000	***	***	100.00
48h-g15:Q1_UL	0	0.00	0.00	0.00
48h-g15:Q1_UR	0	0.00	0.00	0.00
48h-g15:Q1_LL	3,937	96.50	39.37	39.37
48h-g15:Q1_LR	143	3.50	1.43	1.43

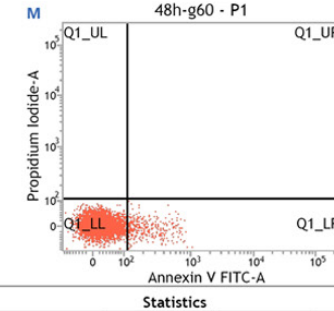
G



Statistics

Name	Events	% Parent	% Grandparent	% Total
48h-g30:All Events	10,000	***	***	100.00
48h-g30:Q1_UL	0	0.00	0.00	0.00
48h-g30:Q1_UR	0	0.00	0.00	0.00
48h-g30:Q1_LL	4,465	95.82	44.65	44.65
48h-g30:Q1_LR	195	4.18	1.95	1.95

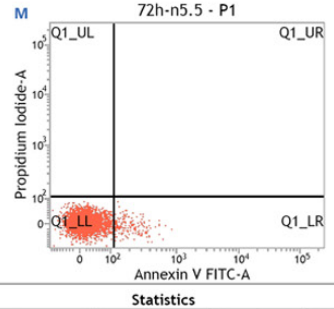
H



Statistics

Name	Events	% Parent	% Grandparent	% Total
48h-g60:All Events	10,000	***	***	100.00
48h-g60:Q1_UL	0	0.00	0.00	0.00
48h-g60:Q1_UR	0	0.00	0.00	0.00
48h-g60:Q1_LL	5,329	93.85	53.29	53.29
48h-g60:Q1_LR	349	6.15	3.49	3.49

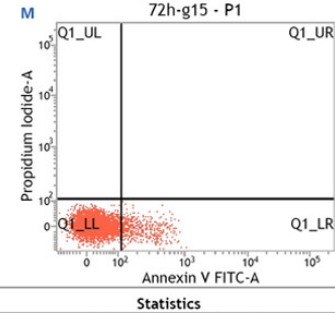
I



Statistics

Name	Events	% Parent	% Grandparent	% Total
72h-n5.5:All Events	10,000	***	***	100.00
72h-n5.5:Q1_UL	0	0.00	0.00	0.00
72h-n5.5:Q1_UR	0	0.00	0.00	0.00
72h-n5.5:Q1_LL	4,476	95.76	44.76	44.76
72h-n5.5:Q1_LR	198	4.24	1.98	1.98

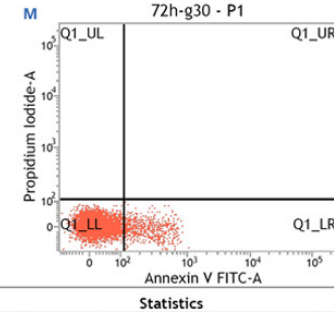
J



Statistics

Name	Events	% Parent	% Grandparent	% Total
72h-g15:All Events	10,000	***	***	100.00
72h-g15:Q1_UL	0	0.00	0.00	0.00
72h-g15:Q1_UR	0	0.00	0.00	0.00
72h-g15:Q1_LL	5,583	91.74	55.83	55.83
72h-g15:Q1_LR	503	8.26	5.03	5.03

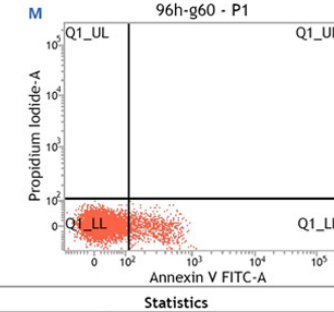
K



Statistics

Name	Events	% Parent	% Grandparent	% Total
72h-g30:All Events	10,000	***	***	100.00
72h-g30:Q1_UL	0	0.00	0.00	0.00
72h-g30:Q1_UR	0	0.00	0.00	0.00
72h-g30:Q1_LL	5,519	89.73	55.19	55.19
72h-g30:Q1_LR	632	10.27	6.32	6.32

L



Statistics

Name	Events	% Parent	% Grandparent	% Total
96h-g60:All Events	10,000	***	***	100.00
96h-g60:Q1_UL	0	0.00	0.00	0.00
96h-g60:Q1_UR	0	0.00	0.00	0.00
96h-g60:Q1_LL	6,331	88.29	63.31	63.31
96h-g60:Q1_LR	840	11.71	8.40	8.40

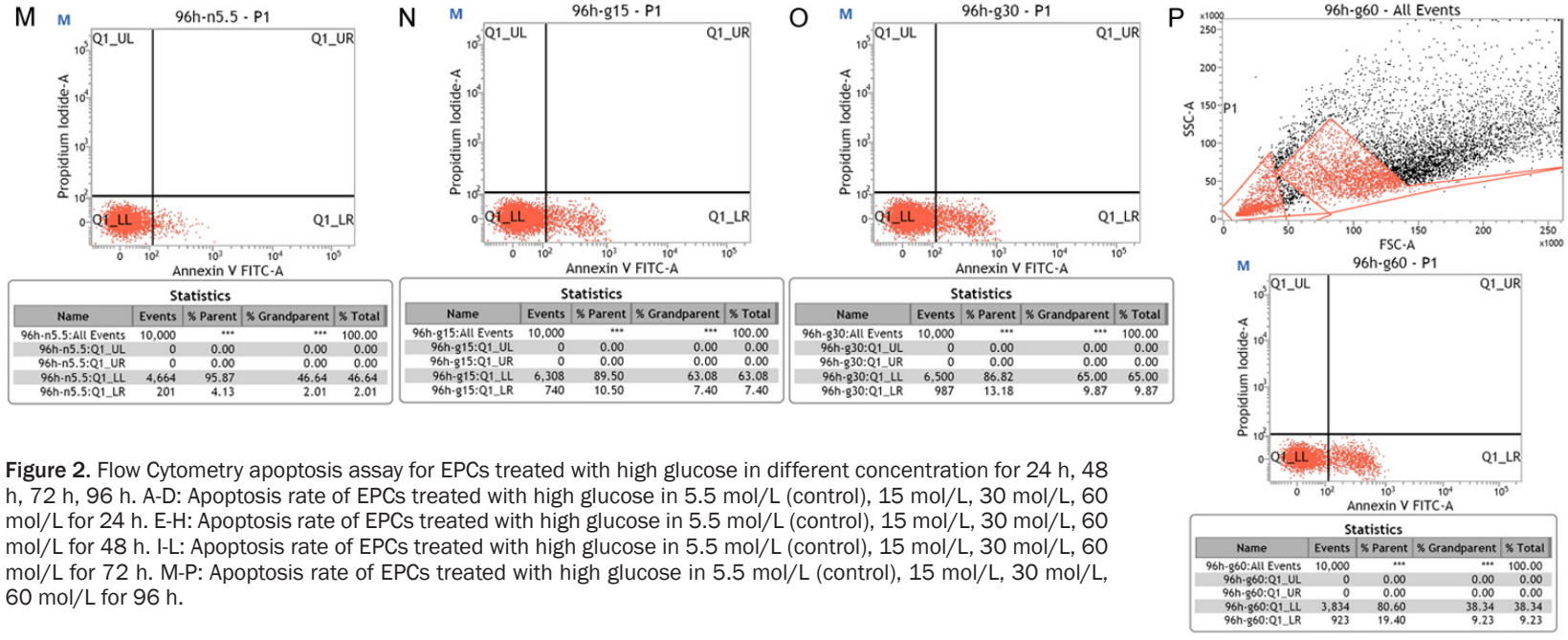


Figure 2. Flow Cytometry apoptosis assay for EPCs treated with high glucose in different concentration for 24 h, 48 h, 72 h, 96 h. A-D: Apoptosis rate of EPCs treated with high glucose in 5.5 mol/L (control), 15 mol/L, 30 mol/L, 60 mol/L for 24 h. E-H: Apoptosis rate of EPCs treated with high glucose in 5.5 mol/L (control), 15 mol/L, 30 mol/L, 60 mol/L for 48 h. I-L: Apoptosis rate of EPCs treated with high glucose in 5.5 mol/L (control), 15 mol/L, 30 mol/L, 60 mol/L for 72 h. M-P: Apoptosis rate of EPCs treated with high glucose in 5.5 mol/L (control), 15 mol/L, 30 mol/L, 60 mol/L for 96 h.

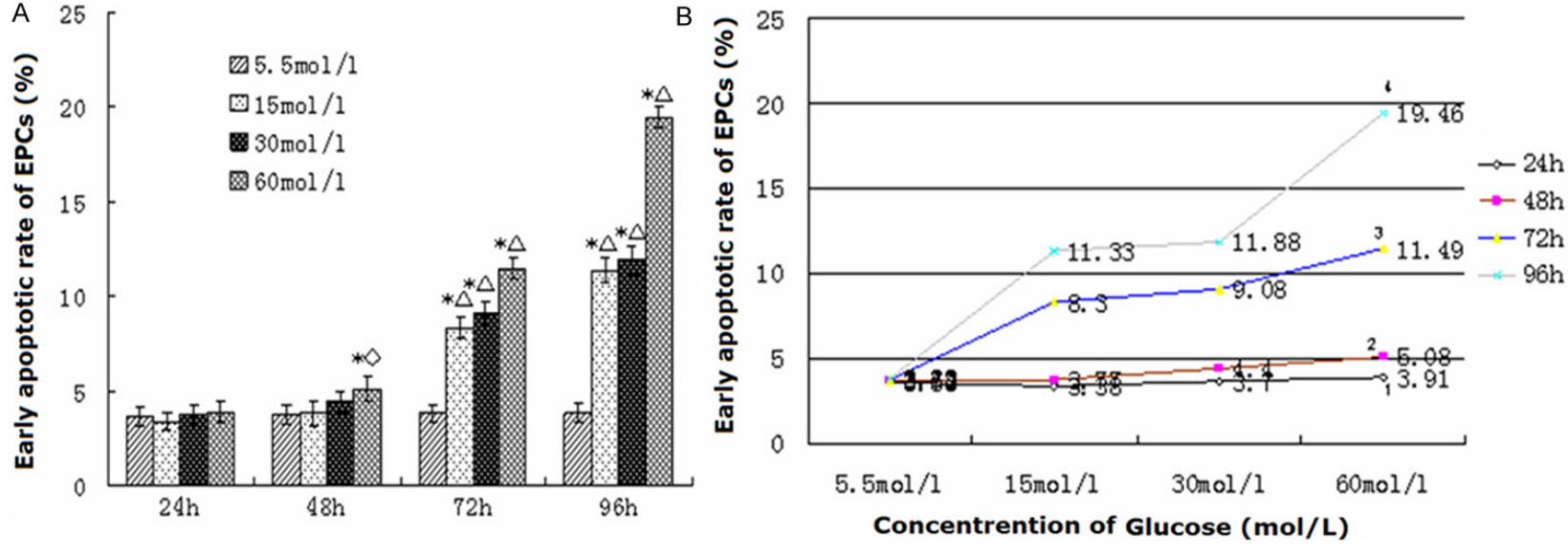


Figure 3. A: Effect of high glucose in different concentration for EPCs apoptosis; B: Apoptosis rate changes of EPCs treated with glucose with different concentration and in different time 1-4: Represent apoptosis rate changes of EPCs treated with 5.5 mol/L (control), 15 mol/L, 30 mol/L and 60 mol/L glucose for 24 h, 48 h, 72 h and 96 h. *P<0.01 represents very significant difference between control group and high glucose groups in the same time but with different concentration; ΔP<0.01 represents very significant difference between control group in 24 h and high glucose groups with the same concentration but in different time; ◊P<0.05 represents very significant difference between 60 mol/L high glucose.

- absorbance of tube blank)/(absorbance of standard pipe - absorbance of blank pipe) × standard sample concentration × test dilution before experiment. Anti-superoxide anion radical and generating superoxide anion radicals test kit were used to detect ROS content and anti-O_{2^-} capacity (expressed with anti-O_{2^-} energy units) according to the instructions steps. ROS content (nmol/mL) = (absorbance of measurement tube-absorbance of tube blank)/(absorbance of standard pipe - absorbance of blank pipe) × standard sample concentration × test dilution before experiment. Anti-O_{2^-} activity unit was calculated as follows: anti-O_{2^-} activity unit (U/L) = (absorbance of measurement tube - absorbance of tube blank)/(absorbance of standard pipe - absorbance of blank pipe) × standard concentration (0.15 mg/ml) × before 1000 ml × sample test dilution before experiment.

Western blotting analysis on the expression level of TNFR1 receptor mediated intracellular signaling pathways related proteins: EPCs were cultured and grouped. The concentration of glucose was 5.5 mmol/L in M199 complete medium control group; the concentration of glucose were 15, 30 mmol/L in M199 complete medium high glucose group; 30 mmol/L high glucose + Tempol group was set and cultured for 24 h and 72 h respectively to investigate the influence of high glucose on the expression level of endothelial progenitor cells TNFR1 receptor mediated intracellular signaling pathways associated protein TNFR1, TRAF-2, TRADD, RIP, NF-kBp65 and Caspase3. The concentration of glucose was 5.5 mmol/L in M199 complete medium control group; the concentration of glucose was 30 mmol/L in M199 complete medium high glucose group; 30 mmol/L high glucose + Tempol group + MAB430 group was set and cultured for 72 h. It was used to study the effect of oxidative stress antagonist Tempol, TNFR1 receptor antagonist MAB430 on the expression level of TNFR1 receptor mediated cell signaling pathway associated protein TNFR1, NFkBp65 and Caspase3. After the cells were washed with pre-cold PBS for twice, the cells were placed on ice operation. RIPA lysis buffer (50 mmol/L Tris-HCl, PH 7.5, 50 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L PMSF, 0.1 mmol/L NaVO4, 10 mg/mL LeuPePtin, 10 mg/mL aprotinin) 200 mL were used for cells lysed. Cells from different groups were collected in 1.5 ml Eppendorf tubes with cell scraper and allowed to stand for 30 min. In the intermediate intervals they were given sonication 15 s × 2 times; they were centrifuged at 12000 rpm 4°C for 30 min. The supernatant was the total protein. BCA method was used for protein quantification. After the measurement of total protein concentration by UV spectrophotometer, the protein concentration was adjusted to 5 μg/ul, and added a 5 × protein sample buffer. They were boiled at 95°C for 5 min, and then placed -70°C refrigerator for spare after repackaging. SDS polyacrylamide gel was prepared. 50 μg protein samples were added to each well, and then run electrophoresis with 10% polyacrylamide gel. The separated proteins were electro-blotted transferred to a nitrocellulose membrane. They were incubated overnight with rabbit anti-rat TNFR1, TRAF-2, TRADD, RIP, NF-kBp65, Caspase3 β-actin antibody. They were cultured with horseradish peroxidase labeled goat anti-rabbit secondary antibody for 1 hour, and then performed membrane washing, developing and fixing; Scanner was used to scan protein bands. Quantity one software was used to analyze the gray value of each protein band. With β-actin protein expression serving as internal control, the results were analyzed according to relative content of protein which was expressed by the ratio of bands grayscale value of target protein to β-actin.

Statistical analysis

SPSS13.0 statistical software was used for data processing. Measurement data were presented as mean ± standard deviation (uniform). t test was used to compare between the two groups. ANOVA was used to compare in multiple groups. P<0.05 was considered statistical significance among the groups.

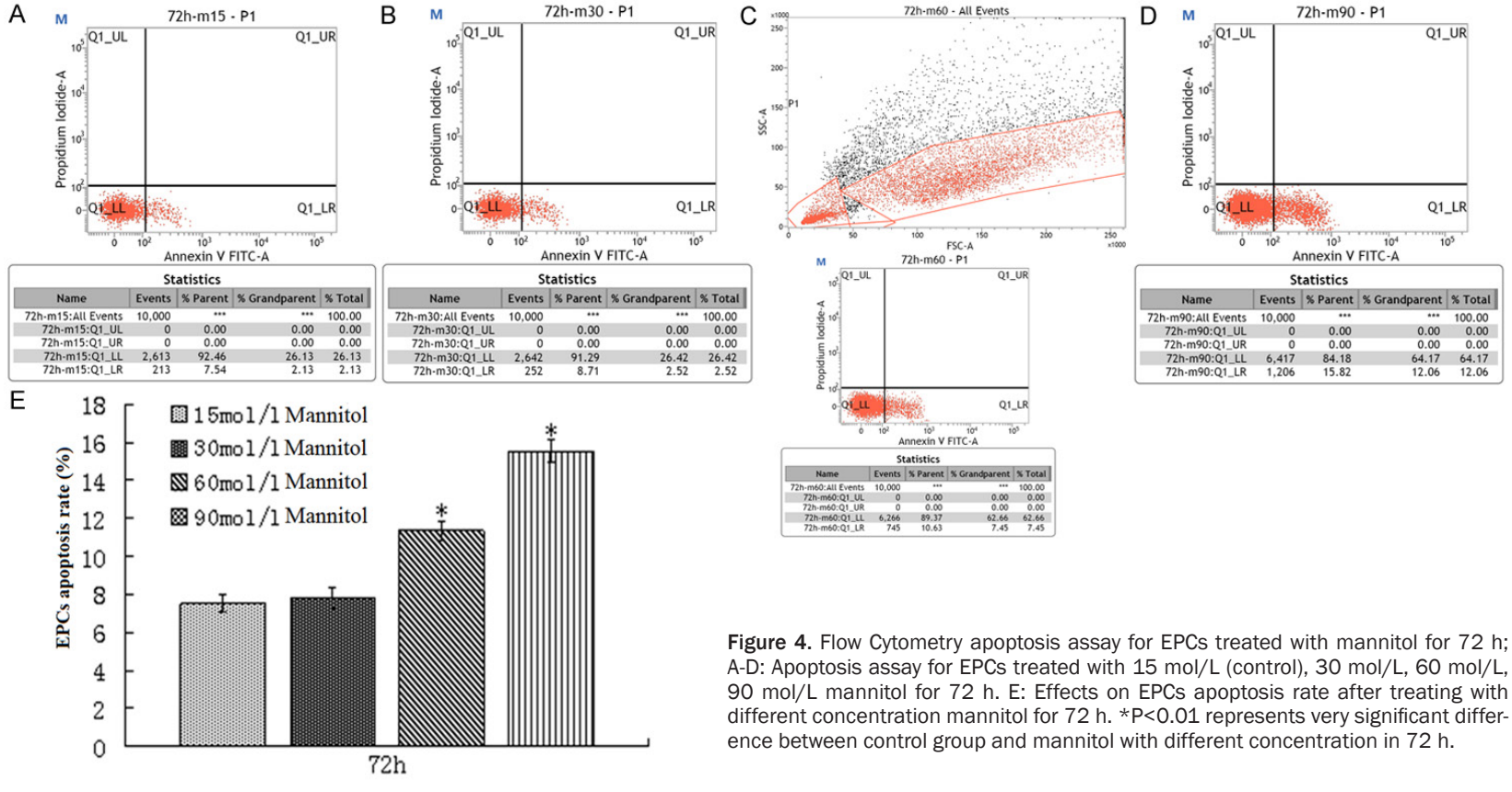


Figure 4. Flow Cytometry apoptosis assay for EPCs treated with mannitol for 72 h; A-D: Apoptosis assay for EPCs treated with 15 mol/L (control), 30 mol/L, 60 mol/L, 90 mol/L mannitol for 72 h. E: Effects on EPCs apoptosis rate after treating with different concentration mannitol for 72 h. *P<0.01 represents very significant difference between control group and mannitol with different concentration in 72 h.

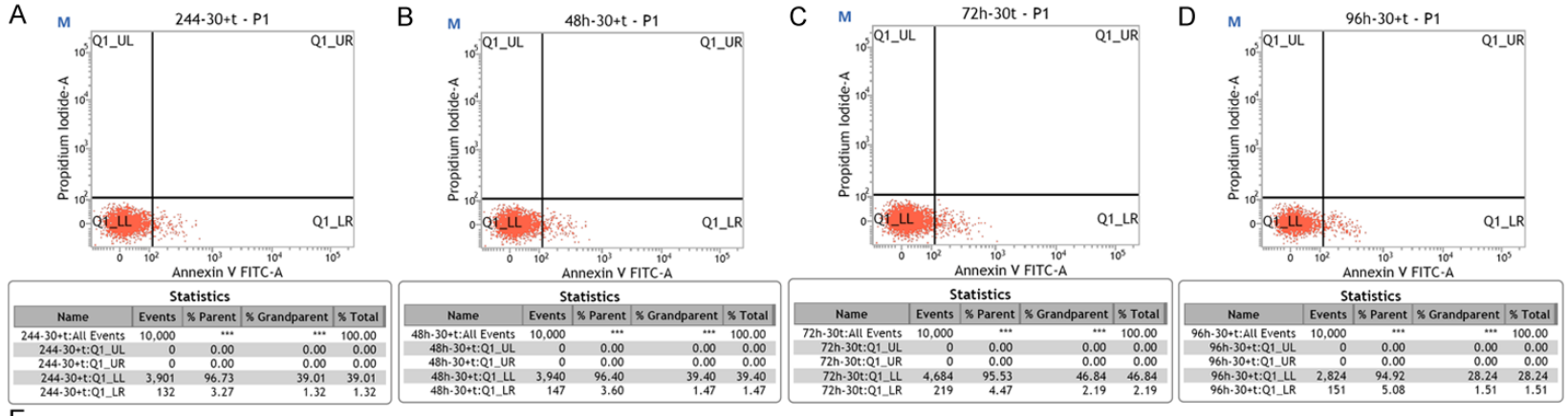


Figure 5. Flow Cytometry apoptosis assay for EPCs treated with high glucose + Tempol or EPCs + MAB430. A-D: apoptosis rate of EPCs treated with 30 mmol/L high glucose + Tempol for 24 h, 48 h, 72 h and 96 h. E: Effect of high glucose + Tempol on EPCs apoptosis rate; *P<0.01 represents very significant difference between high glucose + Tempol group and high glucose group. F-I: Apoptosis rate of EPCs treated with 30 mmol/L high glucose + MAB430 for 24 h, 48 h,72 h and 96 h.

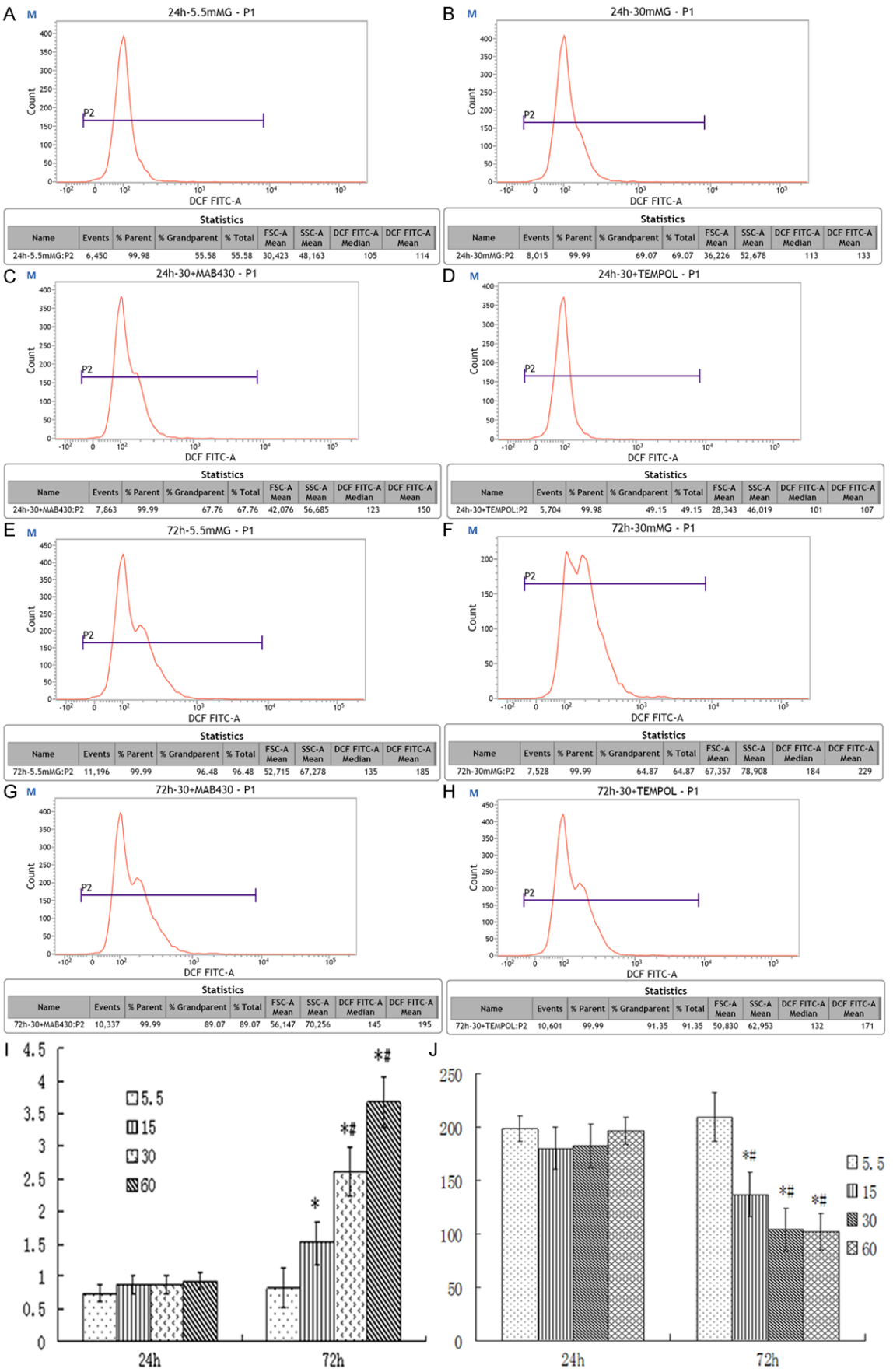

A
24h-5.5mMG - P1
Count
DCF FITC-A
P2
Statistics
Name | Events | % Parent | % Grandparent | % Total | FSC-A Mean | SSC-A Mean | DCF FITC-A Median | DCF FITC-A Mean
24h-5.5mMG:P2 | 6,450 | 99.98 | 55.58 | 55.58 | 30,423 | 48,163 | 105 | 114
B
24h-30mMG - P1
Count
DCF FITC-A
P2
Statistics
Name | Events | % Parent | % Grandparent | % Total | FSC-A Mean | SSC-A Mean | DCF FITC-A Median | DCF FITC-A Mean
24h-30mMG:P2 | 8,015 | 99.99 | 69.07 | 69.07 | 36,226 | 52,678 | 113 | 133
C
24h-30+MAB430 - P1
Count
DCF FITC-A
P2
Statistics
Name | Events | % Parent | % Grandparent | % Total | FSC-A Mean | SSC-A Mean | DCF FITC-A Median | DCF FITC-A Mean
24h-30+MAB430:P2 | 7,863 | 99.99 | 67.76 | 67.76 | 42,076 | 56,685 | 123 | 150
D
24h-30+TEMPOL - P1
Count
DCF FITC-A
P2
Statistics
Name | Events | % Parent | % Grandparent | % Total | FSC-A Mean | SSC-A Mean | DCF FITC-A Median | DCF FITC-A Mean
24h-30+TEMPOL:P2 | 5,704 | 99.98 | 49.15 | 49.15 | 28,343 | 46,019 | 101 | 107
E
72h-5.5mMG - P1
Count
DCF FITC-A
P2
Statistics
Name | Events | % Parent | % Grandparent | % Total | FSC-A Mean | SSC-A Mean | DCF FITC-A Median | DCF FITC-A Mean
72h-5.5mMG:P2 | 11,196 | 99.99 | 96.48 | 96.48 | 52,715 | 67,278 | 135 | 185
F
72h-30mMG - P1
Count
DCF FITC-A
P2
Statistics
Name | Events | % Parent | % Grandparent | % Total | FSC-A Mean | SSC-A Mean | DCF FITC-A Median | DCF FITC-A Mean
72h-30mMG:P2 | 7,528 | 99.99 | 64.87 | 64.87 | 67,357 | 78,908 | 184 | 229
G
72h-30+MAB430 - P1
Count
DCF FITC-A
P2
Statistics
Name | Events | % Parent | % Grandparent | % Total | FSC-A Mean | SSC-A Mean | DCF FITC-A Median | DCF FITC-A Mean
72h-30+MAB430:P2 | 10,337 | 99.99 | 89.07 | 89.07 | 56,147 | 70,256 | 145 | 195
H
72h-30+TEMPOL - P1
Count
DCF FITC-A
P2
Statistics
Name | Events | % Parent | % Grandparent | % Total | FSC-A Mean | SSC-A Mean | DCF FITC-A Median | DCF FITC-A Mean
72h-30+TEMPOL:P2 | 10,601 | 99.99 | 91.35 | 91.35 | 50,830 | 62,953 | 132 | 171
I
5.5
15
30
60
*
*#
*#
24h
72h
J
5.5
15
30
60
*#
*#
*#
24h
72h

Figure 6. A-D: ROS level in EPCs treated with 5.5 mol/L glucose(control), 30 mol/L glucose, 30 mol/L glucose + Tempol, 30 mol/Lglucose + MAB430 after 24 h; E-H: ROS level in EPCs treated with 5.5 mol/L glucose (control), 30 mol/L glucose, 30 mol/L glucose + Tempol, 30 mol/Lglucose + MAB430 after 72 h; I: Effect of high glucose on MDA level in EPCs. *P<0.05 represents significant difference between control group and other groups in the same time; #P<0.05 represents significant difference between the same group in 24 h and in other time. J: Effect of high glucose on anti- O_{2^-} level in EPCs; *P<0.05 represents significant difference between control group and other groups in the same time; #P<0.05 represents significant difference between the same group in 24 h and in other time.

Results

Morphology and identification of endothelial progenitor cells

As can be seen from **Figure 1A**, cultured EPCs were adherent cells, fusiform or spindle-shaped, and had a tendency to aggregate. The cells were cultured for 7 days, and then DIL-ac-LDL and FITC-UEA-I double fluorescent staining was performed; cells were observed under a confocal microscope; double positive cells with red and green fluorescence were considered EPCs (**Figure 1A-C**).

Effects of high sugar on EPCs apoptosis

EPCs were respectively treated with 5.5 mol/L (control group), 15 mol/L, 30 mol/L and 60 mol/L glucose; the cells were collected and analyzed by flow cytometry at 24 h, 48 h, 72 h and 96 h; the results were shown in **Figure 2A-D** (24 h), **Figure 2E-H** (48 h), **Figure 2I-L** (72 h) and **Figure 2M-P** (96 h). Statistical analysis of the data was performed to analyze the effect of different concentrations of high glucose on EPCs apoptosis rate, and the results were shown in **Figure 3A**. As can be seen from **Figure 3A**, high glucose can induce apoptosis of EPCs, and the higher sugar concentration, the higher the EPCs apoptosis rate; the longer the induction time, the higher the rate of apoptosis o f EPCs; the trend was especially more obvious after 72 h of induction. The effect of glucose concentration and action time on the trend of EPCs apoptosis rate was analyzed and the results were shown in **Figure 3B**; as can be seen from **Figure 3B**, the higher the concentration of sugar and the longer the induction time, the higher the EPCs apoptosis rate. High glucose stimulation could induce the apoptosis of endothelial progenitor cells, and the apoptosis occurred mainly in the later stage of stimulation by high glucose (72-96 h) rather than the early stage (24-48 h); and the later apoptosis rate was more closely related with the stimulation time of high glucose.

Effect of mannitol treatment on EPCs apoptosis

EPCs were respectively treated with 15 mol/L (control group), 30 mol/L, 60 mol/L, and 90 mol/L mannitol; cells were collected after 72 h and flow cytometry analysis was performed (**Figure 4A-D**). Statistical analysis was conducted to compare the impact of different concentrations of mannitol treatment on EPCs apoptosis rate, and the results were shown in **Figure 4E**. **Figure 4E** showed that with the increase of the concentration of mannitol, apoptosis of EPCs increased.

Effect of high sugar + TEMPOL treatment on EPCs apoptosis

EPCs were treated with 30 mmol/L high glucose + TEMPOL; at 24 h, 48, 72 h, and 96 h, cells were collected to do flow cytometry (**Figure 5A-D**); the data were statistically analyzed to compare the effects of 30 mmol/L high glucose + TEMPOL treatment at different time points on EPCs apoptosis rate, and the results were shown in **Figure 5E**. **Figure 5E** showed that in the role of Tempol antagonist of oxidative stress, pro-apoptotic effect of high sugar was suppressed.

Effect of high sugar + MAB430 treatment on EPCs apoptosis

EPCs were treated with 30 mmol/L high glucose + MAB430; at 24 h, 48,72 h and 96 h, the cells were collected and analyzed by flow cytometry (**Figure 5F-I**).

ROS, MDA and anti-anti-O_{2^-} detection in EPCs

EPCs were treated with 5.5 mol/L glucose (control group), 30 mol/L glucose, 30 mol/L glucose + Tempol, and 30 mol/L glucose + MAB430; at 24 h and 72 h, the cells were collected and lysed; ROS content was determined by colorimetric method (**Figure 6A-H**). EPCs were treated with 5.5 mol/L glucose (control

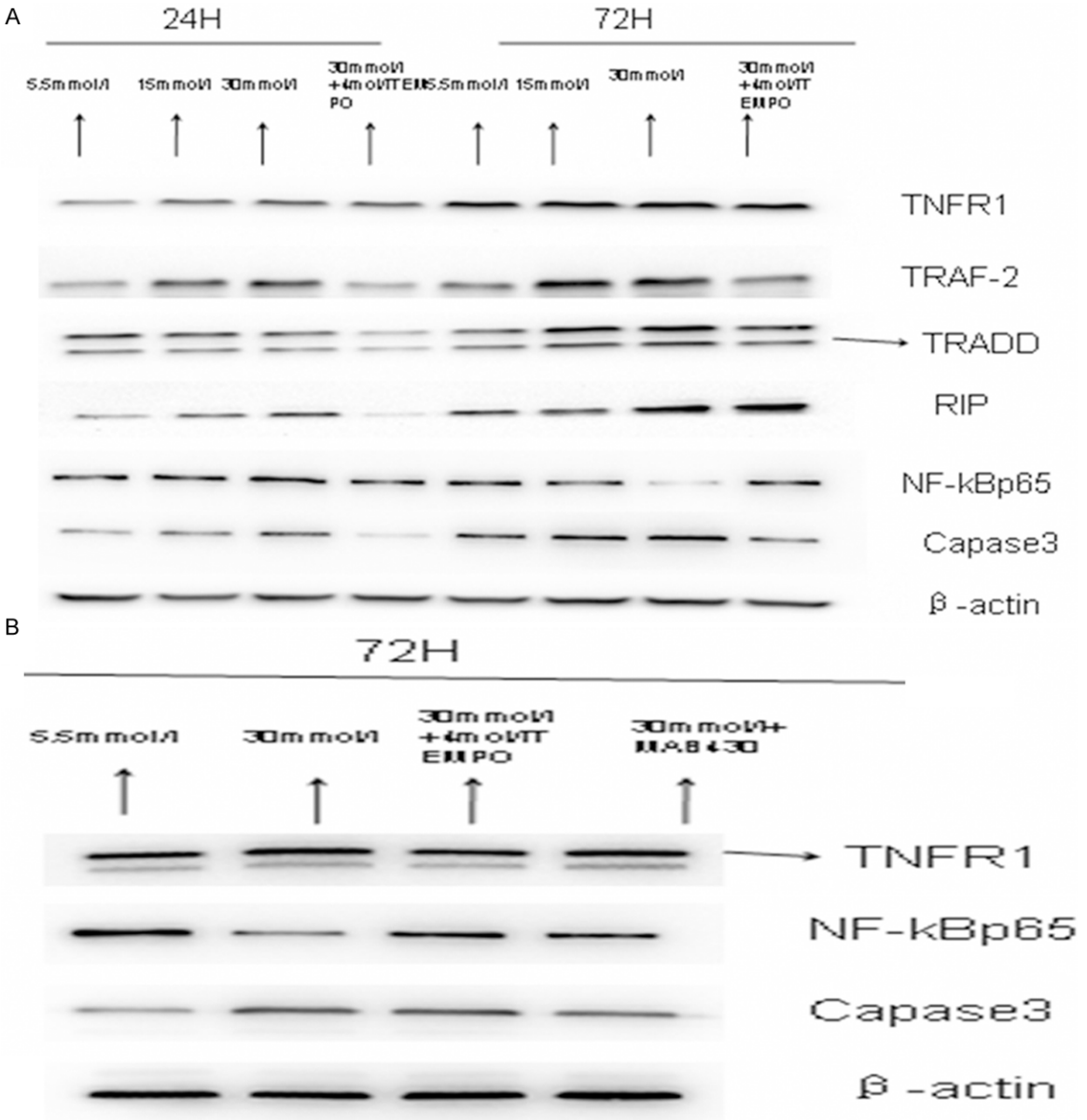


Figure 7. Effects of high glucose (A), Tempol and MAB430 (B) on TNFR1 induced signal pathway related protein expression.

group), 15 mol/L glucose, 30 mol/L glucose and 60 mol/L glucose; at EPCs 24 h and 72 h, the cells were collected and lysed; MDA and anti-O_{2^-} were measured colorimetrically, shown in **Figure 6I** and **6J**. **Figure 6A-I** showed that high glucose could induce oxidative stress response; oxidative stress products (ROS and MDA) had a significant increase in later stage after 72 h. **Figure 6J** showed that high glucose inhibited anti-O_{2^-} in EPCs, and the effect was particularly more significant at advanced stage (72 h).

Western blotting to detect the effect of high glucose on associated-protein expression in the intracellular signaling pathways EPCs mediated by TNFR1 receptor

Western blotting results showed a significant increase in protein expression; Oxidative stress antagonists, Tempol and TNFR1 receptor antagonist MAB430, can reduce the expression of related protein in TNF apoptosis signaling pathway in 72 h (**Figure 7A**). Oxidative stress antagonists (Tempol and TNFR1 receptor

antagonist MAB-430) can reduce the rate of apoptosis in the advanced stage of high glucose stimulation (**Figure 7B**).

Discussion

In a patient with diabetes, high blood sugar can cause endothelial dysfunction, thereby causing the occurrence and development of vascular complications in diabetic patients. This may be associated with high glucose-induced EPC dysfunction [11]; poor glycemic control in diabetic patients and long-term high blood sugar lead to a significant reduction in EPCs, EPC dysfunction, and weakened angiogenesis and angiogenesis. Pustovrh [12] has found the reduction in the number of EPCs, proliferation, adhesion and angiogenesis ability in patients with type I and type II diabetes. Yuan Hong et al [13] found that high sugar reduced the number of EPC in the peripheral blood in a dose and time dependent manner, indicating that EPCS plays an important role in the development of peripheral vascular disease in diabetes.

Apoptosis is an important form of regulating the number of cells in the arterial wall; the incidence and development of diabetic vascular complications are closely related with vascular endothelial dysfunction. Hyperglycemia, insulin resistance and oxidative stress can cause functional and structural changes in endothelial cells, and thus lead to vascular damage [14]. This study found that high glucose can induce apoptosis in endothelial progenitor cells, and the higher the concentration of sugar, the more significant the apoptosis; moreover, apoptosis occurs mainly in the later stimulated period by high glucose (72-96 h); the latter apoptosis is more closely related with the action time of high glucose.

Reactive oxygen species (ROS) are a class of oxygen-containing free radicals or molecules with a high reactivity, widely involved in intracellular signal regulation, metabolism, survival and apoptosis. He extent of ROS accumulation can determine the survival state of the cells [15]; excessive accumulation of ROS can cause oxidative stress in cells, leading to cell damage and even death [16]. Ingram et al [17] reported that exogenous H_20_2 -induced EPC apoptosis was related to the apoptosis signal-regulated kinase (ASKI) signal pathway; apoptosis can be initiated by a downstream effector molecule JNK and p38 kinase after ASKI activation. In this study, we examined the oxidative stress-related products (ROS, MDA, anti-O_{2^-}), and found that ROS and MDA levels were significantly increased in EPCs, while the concentration of anti-O_{2^-} was decreased in a time and concentration-dependent manner to some extent. It indicated that under high glucose conditions, oxidative stress of EPCs was increased and anti-oxidative stress was significantly reduced, leading to dysfunction of EPCs. We also observed that under high glucose-induced high oxidative stress, early apoptosis of rat bone marrow-derived EPC increased, and with the effect time was prolonged, apoptosis was increased. Meanwhile, the addition of antioxidant Tempol and TNFR1 receptor to antagonist MAB430 can significantly reduce the rate of apoptosis, indicating that oxidative stress was closely related with EPC apoptosis; high sugar possibly may regulate and increase EPC apoptosis through oxidative stress, and further cause vascular damage.

Tumor necrosis factor (TNF-α) is a mononuclear factor mainly generated by mononuclear phagocytic cells, playing a regulatory role in apoptosis and inflammatory response. Dome P et al [18] found that TNF-α content in the peripheral blood of diabetic patients was negatively correlated with the number of EPCs. Chen Tugang et al [19] found that TNF-α can reduce iNOS and eNOS content in EPCs, and iNOS and eNOS content decreased with the increasing concentration and action duration of TNF-α. And iNOS and eNOS are closely related with the oxidative stress in EPCs under high sugar condition. Seeger et al [20] found that high glucose can induce the apoptosis of endothelial cells, and further aggravate TNF-α-induced apoptosis. This study found that at the early stage of high glucose stimulation (24 h), TNF apoptosis signaling pathway-related protein expression did not increase, but after 72 h of stimulation, related protein expression was significantly upregulated. Oxidative stress antagonists, such as Tempol and TNFR1 receptor antagonist MAB430, can reduce the expression of related protein in TNF apoptosis signaling pathway in 72 h. So we speculated that one of the mechanisms of EPC apoptosis induced by high glucose may be that high glucose-induced oxidative stress activated apoptotic receptor TNFR, further triggered programmed cell death.

Acknowledgements

This study was supported by National Natural Science Foundation of China (81241008/H0217).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yan-Zheng He, Department of Vascular Surgery, Affiliated Hospital of Luzhou Medical College, NO. 25#, Taiping Street, Jiangyang District, Luzhou, Sichuan, China. Tel: 86-02877655443; Fax: 86-02877655443; E-mail: heyanzhe77@163.com; Dr. Yong Liu, Department of Vascular Surgery, Affiliated Hospital of Luzhou Medical College, NO. 25#, Taiping Street, Jiangyang District, Luzhou, Sichuan, China. Tel: +86-0830-3165481; Fax: +86-0830-3165481; E-mail: lyong-7423@163.com

References

[1] Hao JS, Chen Z. Progress and application of functional endothelial progenitor cells. Xin Fei Xue Guan Bing Za Zhi 2009; 28: 288-290.

[2] Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; 275: 964-967.

[3] Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, Oz MC, Hicklin DJ, Witte L, Moore MA, Rafii S. Expression of VEGFR-2 and CD133 by circulating human CD34 (+) cells identifies a population of functional endothelial precursors. Blood 2000; 95: 952-958.

[4] Nakul-Aquaronne D, Bayle J, Frelin C. Coexpression of endothelial markers and CD14 by cytokine mobilized CD34+ cells under angiogenic stimulation. Cardiovasc Res 2003; 57: 816-823.

[5] Wang WD, Chen ZT. Hematopoietic and vascular development. Xin Fei Xue Guan Bing Za Zhi 2003; 22: 247-249.

[6] 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.

[7] Rauscher FM, Goldschmidt-Clermont PJ, Davis BH, Wang T, Gregg D, Ramaswami P, Pippen AM, Annex BH, Dong C, Taylor DA. Aging, progenitor cell exhaustion, and atherosclerosis circulation. Circulation 2003; 108: 457-463.

[8] Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; 275: 964-967.

[9] Badorff C, Brandes RP, Popp R, Rupp S, Urbich C, Aicher A, Fleming I, Busse R, Zeiher AM, Dimmeler S. Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardio-myocytes. Circulation 2003; 107: 1024-1032.

[10] Pesce M, Orlandi A, Iachininoto MG, Straino S, Torella AR, Rizzuti V, Pompilio G, Bonanno G, Scambia G, Capogrossi MC. Myoendothelial differentiation of human umbiliacal cord blood derived stem cells in ischemic limb tissues. Circ Res 2003; 93: e51-e62.

[11] Gaenzer H, Neumayr G, Marschang P, Sturm W, Lechleitner M, Föger B, Kirchmair R, Patsch J. Effect of insulin therapy on endothelium dependent dilation in type 2 diabetes mellitus. Am J Cardiol 2002; 9: 431-434.

[12] Pustovrh MC, Jawerbarm A, Capobianco E, White V, López-Costa JJ, González E. Increased matrix metalloproteinases 2 and 9 in placenta of diabetic rats at midgestation. Placenta 2005; 26: 339-348.

[13] Yuan H, Chen JZ, Wang XX. The effects of high glucose on endothelial progenitor cell number and function. PLA Medicine 2004; 29: 803-806.

[14] Stamm C, WestPhal B, Kleine HD, Petzsch M, Kittner C, Klinge H, Schümichen C, Nienaber CA, Freund M, Steinhoff G. Autologous bone marrow Steme Cell transplantation for myoeardial regeneration. Lancet 2003; 361: 45-4.

[15] Rankin EB, Giaccia AJ. The role of hypoxia-inducible factors in tumorigenesis. Cell Death Differ 2008; 15: 678-85.

[16] Fan LF. Diao LM, Chen DJ, Liu MQ, Zhu LQ, Li HG, Tang ZJ, Xia D, Liu X, Chen HL. Expression of HIF-1α and its relationship to apoptosis and proliferation in lung cancer. Cancer 2004; 21: 254-258.

[17] Ingram DA, Krier TR, Mead LE, McGuire C, Prater DN, Bhavsar J, Saadatzadeh MR, Bijangi-Vishehsaraei K, Li F, Yoder MC, Haneline LS. Clonogenic endothelial Progenitor cells are sensitive to oxidative stress. Stem Cells 2007; 25: 297-304.

[18] Dome P, Teleki Z, Rhlner Z, Peter L, Dobos J, Kenessey I, Tovari J, Timar J, Paku S, Kovacs G, Dome B. Circulating endothelial progenitor cells and depression: a possible novel link between heart and soul. Mol Psyehiatry 2009; 14: 523-31.

[19] Chen TG, Xie XD, Chen HJ. Tumor necrosis factor -α endothelial progenitor cell function and nitric oxide synthase content. Zhongguo Lao Nian Xue Za Zhi 2007; 9: 385-389.

[20] Quan Y, Jiang CT, Xue B, Zhu SG, Wang X. High glueose stimulates TNF-α and MCP-1 expression in rat microglia via ROS and NF-kB Pathways. Acta pharmacol Sin 2011; 32: 188-193.