Effect of “Xiaoke Tongbi granule” on the proliferation, migration and tubule-forming ability of rat endothelial progenitor cells under high glucose conditions

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Abstract

Purpose: To investigate the effect of Xiaoke Tongbi granule (XTG) on the proliferation, migration and tubule-forming ability of endothelial progenitor cells (EPCs) of rats under high glucose conditions.

Methods: Six specific pathogen-free (SPF) and twenty-four healthy rats (mean weight = 200 ± 20 g) were used in this study. Twenty-four (24) healthy rats were treated with graded concentrations of XTG (0.75 – 2.25 g/mL) for 7 days, and were thereafter euthanized to obtain serum which was later used to treat EPCs isolated from bone marrow of SPF rats. The EPCs were seeded in culture plates pre-coated with human fibronectin, and cultured at 37 °C for 72 h in a humidified atmosphere of 5 % CO₂ and 95 % air. Cell viability and apoptosis were assessed using 3 (4,5 dimethyl thiazol 2 yl) 2,5 diphenyl 2H tetrazolium bromide (MTT), and flow cytometric assays, respectively. The morphology of isolated EPCs was assessed by immunofluorescence.

Results: The isolated EPCs exhibited normal morphology, and were CD34-positive. Proliferation and migration of EPCs, and number of tubular structures formed were significantly suppressed under high glucose conditions, but were significantly and concentration-dependently promoted by XTG treatment (p < 0.05). Treatment with XTG also significantly improved the morphology of isolated EPCs (p < 0.05). Apoptosis was significantly promoted by high glucose conditions, but was significantly and concentration-dependently reduced by XTG treatment (p < 0.05). The incidence of tubule formation in high glucose group was 0.63 %, but was progressively increased from 1.37 to 1.52 % after treatment with graded concentrations of XTG.

Conclusion: These results indicate that XTG reverses the effect of high glucose environment on EPC proliferation, migration and tubule-forming ability.

Keywords: High glucose conditions, Xiaoke Tongbi granule, Endothelial progenitor cells, Proliferation, Tubule formation

INTRODUCTION

The cellular and molecular processes that control vascular injury responses after percutaneous coronary intervention involve a complex interplay between vascular cells and progenitor cells, a process which controls arterial remodeling, neointimal proliferation, and re-re-
endothelialization. The re-endothelialization of injured vascular endothelium and angiogenesis of the ischemic lesion play important roles in the repair of vascular injury caused by diabetes mellitus (DM) and cardiovascular diseases (CVDs). Diabetes mellitus- and CVD-induced damages to endothelial cells are restored by two processes: endothelial repair and angiogenesis [1,2].

Xiaoke Tongbi granule (XTG) is used in Traditional Chinese Medicine (TCM) to facilitate the migration and homing of EPCs to ischemic muscle tissues and nerves, thereby contributing significantly to angiogenesis. It also facilitates blood supply to injured limbs via the upregulation of vascular endothelial growth factor (VEGF), hypoxia-inducible factor 1 (HIF-1) and PI3K/Akt protein expressions in ischemic limb muscles, thereby reinforcing the angiogenic effect. The present study investigated the effects of XTG on the proliferation, migration and tubule-forming ability of EPCs under high glucose conditions.

EXPERIMENTAL

Drugs and reagents

Xiaoke Tongbi granule (XTG) was obtained from the Preparation Center of the Affiliated Hospital of Shaanxi University of TCM. Fetal bovine serum (FBS), M199 medium and trypsin solution were products of Gibco (USA). Vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and epithelial cell growth factor (EGF) were obtained from Peprotech (USA), while EBM-2 high glucose medium was purchased from Invitrogen (USA). Annexin V-FITC apoptosis kit and CD34-FITC monoclonal antibody were products of BD Biosciences (USA); FLK-1-PE monoclonal antibody was purchased from Sigma-Aldrich (USA), while phosphate-buffered saline (PBS) and MTT assay kit were products of Solarbio (China).

This research was approved by the Animal Ethical Committee of Affiliated Hospital of Shaanxi University of Traditional Chinese Medicine, Xianyang, Shaanxi Province, China (approval no. 201822142), and performed according to "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [3].

Animals

Six SPF and twenty-four healthy rats (mean weight, 200 ± 20 g) were purchased from the Animal Experimental Center of Xi’an Jiaotong University School of Medicine (No. scxk (Shaan) 2013-003). The ratio of male to female rats was 50:50.

Rat grouping and treatment

Twenty-four healthy rats were randomly assigned to four groups of six rats each: control group, 0.75 g/mL XTG group, 1.5 g/mL XTG group, and 2.25 g/mL XTG group. XTG was administered orally using gavage. The control group rats received 2 mL of normal saline consecutively for 7 days. At the end of the treatment period, the rats were anesthetized with pentobarbital sodium, and blood was collected through the abdominal aorta 1 h after the last administration. After 24 h incubation at 4 °C, the blood was centrifuged at 3000 rpm for 15 min to obtain serum. The serum was sterilized using 0.22 μm microporous membrane filter, and refrigerated at -20 °C until use.

Isolation and culture of EPCs

The SPF rats were anesthetized with 10 % chloral hydrate intraperitoneally at a dose of 0.35 mL/100 g. Then, rat knee joint and distal femur were excised and exposed, and two holes were thereafter drilled in them using a dental drill. Two syringes were separately attached: one was used to inject sterile PBS, and the other to extract bone marrow. The extracted bone marrow was dispersed into individual cells by subjecting it to density gradient centrifugation at 1500 rpm for 15 min at 4 °C using lymphocyte separation solution (a mixture of Ficoll and sodium diatrizoate) as the density gradient solution.

The bone marrow cells were subsequently resuspended in M199 medium containing 10 ng/mL VEGF, 10 % FBS, 5 ng/mL FGF and 5 ng/mL EGF. The resultant cell suspension was seeded in culture plates pre-coated with human fibronectin, and cultured at 37 °C for 72 h in a humidified atmosphere of 5 % CO₂ and 95 % air. The medium was changed every 2 days. The cells were observed under an inverted microscope, and those in logarithmic growth phase were selected and used in subsequent experiments.

Identification of EPCs

The isolated EPCs were stimulated with rhodamine-labeled CD34 antibody and incubated overnight at 4 °C. Then, 4', 6-diamidino-2-phenylindole (DAPI) was used for nuclear staining, and the EPCs were photographed using an inverted fluorescence microscope. Positive cells produced red fluorescence with blue nuclei.
Grouping of EPCs and treatment

The EPCs were randomly assigned to five groups: normal control group (C group), high glucose control group (G group), Z1 group, Z2 group and Z3 group. The cells were pretreated with phosphatidylinositide-3-kinase (PI3K) inhibitor, Wortmannin (0.2 μmol/L) or endothelial nitric oxide synthase (eNOS) inhibitor, L-NAME (200 μmol/L) for 30 min. Cells in C group were cultured in EBM-2 complete medium only, while in G group the medium was replenished with EBM-2 high glucose medium containing 30 mmol/L glucose. Cells in Z1 group were cultured in EBM-2 high glucose medium supplemented with 15-fold concentration of serum XTG; Z2 group cells were cultured in EBM-2 high glucose medium supplemented with 10-fold concentration of serum XTG; and cells in Z3 group were cultured in EBM-2 high glucose medium containing 5 times the concentration of serum XTG. Each group of cells was cultured at 37 °C. Morphological changes in the cells were assessed at intervals of 18, 36 and 72 h.

MTT assay

The proliferative ability of EPCs in the presence of XTG was assessed using MTT assay. The cells were seeded at a density of 1 x 10^6 cells/well in 96-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM) for 24 h. Then, serum containing graded concentrations of XTG (0.75 – 2.25 g/mL) was added to the cells and incubated for 72 h. At the end of the third day, 20 μL of MTT solution (5 mg/mL) was added to the wells, followed by incubation at 37 °C for 2 h. The medium was finally replaced with 150 μL of 0.1 % dimethyl sulfoxide (DMSO) to completely dissolve the formazan crystals formed. The absorbance of the samples was read in a microplate reader at 490 nm. The assay was performed in triplicate. Cell proliferation (P) was calculated as shown in Eq 1.

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P (%) = \frac{Ae-Ac}{100} \text{.............. (1)}
\]

Where Ae and Ac are the absorbance of experimental and control rats.

Apoptosis assay

The EPCs were seeded at a density of 2.5 x 10^6 cells/well in 6-well plates and cultured for 24 h. Then, serum containing graded concentrations of XTG (0.75 – 2.25 g/mL) was added to the medium and incubated for another 72 h. Thereafter it was washed with PBS, and thoroughly mixed with 200 μL binding buffer. The cells were then stained with 5 μL each of annexin V-fluorescein isothiocyanate and propidium iodide within 25 min at room temperature in the dark. Cell apoptosis was assessed using a flow cytometer fitted with argon laser operated at 485 nm.

**In vitro cell migration and invasion assay**

The cells (1 x 10^4 cells/well) were placed in Transwell chamber coated with substrate and cultured in serum-free medium. Medium containing 10 % FBS was added to the lower chamber. After 24 h, the cells that passed through the matrix gel membrane were stained with crystal violet after fixation with 4 % paraformaldehyde for 10 min, photographed and counted using an inverted microscope.

Lumen formation assay

The cells were seeded in 24-well plates (at a density of x 10^7 cells/mL, and 300 μL of matrigel was added to each well, followed by culturing at 37 °C for 60 min. Thereafter, graded concentrations of XTG in serum were added. After culturing for 20 h, tubular arrangement, and the number and integrity of tubular structures were examined under an inverted microscope using an Image-ProPlus 5.1 software so as to analyze the total tubule structure per field (× 40). The results were expressed as percentages relative to the control.

Statistical analysis

Data are expressed as mean ± SEM, and statistical analysis was performed using SPSS (version 19.0). Groups were compared using Student t-test. Values of p < 0.05 were considered statistically significant.

**RESULTS**

Vascular EPC morphology

As shown in Figure 1, the isolated EPCs exhibited normal morphology. The shape was fusiform with a small number of dendrites. An increase in cell density led to cell-to-cell interaction and cross-linking.

Expression of CD34 in vascular EPCs

Results of immunofluorescence staining showed that the cells were highly purified. They were CD34-positive, with red fluorescence and blue nuclei (Figure 2).
Effect of XTG on morphology and proliferation of isolated EPCs in high glucose conditions

The proliferation of EPCs was significantly suppressed under high glucose condition, but this suppression was significantly and concentration-dependently reversed by XTG treatment ($p < 0.05$). Treatment with XTG also significantly improved the morphology of isolated EPCs ($p < 0.05$). These results are shown in Figure 3, Figure 4 and Figure 5.

Effect of XTG on apoptosis of EPCs

Apoptosis was significantly promoted by high glucose condition, but was significantly and concentration-dependently reduced by XTG treatment ($p < 0.05$; Figure 6).

Effect of XTG on extracellular lumen formation

Vascular endothelial cells in the control group were able to form distinct tubular structures. The number of tubular structures was significantly reduced under high glucose condition, but this was significantly and concentration-dependently increased after treatment with XTG ($p < 0.05$). The incidence of tubule formation in high glucose group was 0.63 %, but was progressively increased from 1.37 to 1.52 % after treatment with graded concentrations of XTG (Figure 8).
DISCUSSION

Diabetic macro- and microvascular complications result in vascular endothelial injury, accompanied by impaired or decreased number of EPCs. Thus, a possible way to treat vascular dysfunction and injury is by promoting the circulation of EPCs to regions of vascular injury and stimulating their differentiation into mature endothelial cells. In diabetic vascular disease, EPCs from different sources induce angiogenesis and indirectly reinforce neovascularization in ischemic tissues through division, proliferation and migration of the cells. Neovascularization stimulates the formation of functional microvascular networks capable of perfusion by red blood cells which serve as collateral circulation in response to local poor perfusion or ischemia. This enhances nerve function by promoting nerve regeneration and intraneuronal angiogenesis, thereby facilitating axonal extension, and promoting the survival of neurons, as well as satellite and Schwann cells [4-6].

Peripheral neuropathy (PN), a common complication of DM, refers to nerve damage caused by hyperglycemia. It is characterized by numbness, loss of sensation, and sometimes pain in the feet, legs, or hands. An understanding of its pathogenesis helps to unravel the relationship between fasting blood glucose variability and coronary artery collateral formation [7]. In TCM, the concept of “pulse and collateral - vascular system disease” is aimed at explaining the therapeutic effect of “replenishing qi and generating pulse” and “activating blood and generating pulse”. This provides basis for treatment of vascular lesions in TCM.

The results of this study indicate that XTG may promote qi, activate blood circulation and remove stasis, thereby facilitating recovery from vascular endothelial cell damage caused by diseases such as atherosclerosis, DM and peripheral vascular diseases [8].

Astragalus and Panax notoginseng accelerate the proliferation, migration and differentiation of EPCs, and promote endothelial cell repair under hyperglycemic conditions [9]. The bioactive compounds in Angelica, Salvia and Pueraria have been shown to activate blood circulation, removes stasis, and promote the proliferation, migration, adhesion and angiogenesis of EPCs, thereby reinforcing the repair of damaged endothelial cells and angiogenesis, both in vitro and in vivo [10-12]. A specially formulated drug for the treatment of diabetic PN, XTG consists of Astragalus, Pueraria, Angelica, Mulberry white, Clematis and Daphne giraldii Nitsche. It replenishes qi and activates blood circulation, while dredging collaterals and relieving pain. Studies have shown that when properly used, XTG accelerates the migration and homing of EPCs to ischemic muscle tissues and nerves in diabetic ischemic conditions; stimulates angiogenesis, and facilitates blood supply to the affected limbs. Vascular endothelial growth factor (VEGF) and HIF - 1 are highly expressed in peripheral nerves and blood vessels at the site of ischemic lesions, and they promote local endothelial cell proliferation, angiogenesis, and blood supply [13-17].

In this study, the proliferation and migration of EPCs, and number of tubular structures formed were significantly suppressed under high glucose medium, but were significantly and concentration-dependently promoted by XTG treatment. Treatment with XTG also significantly
improved the morphology of isolated EPCs. Apoptosis was significantly promoted by condition of high glucose, but was significantly and concentration-dependently reduced by XTG treatment. It is likely that the mechanism underlying the observed effects of XTG involves increased expression of PI3K/Akt/eNOS signal pathway-related proteins.

CONCLUSION

The results of this study suggest that XTG reverses the effects of high glucose condition on EPC proliferation, migration and tubule-forming ability.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Bifeng Gao conceived and designed the study, Youcui Yuan, Xiaoke Zhang, Huan Zhang, Bifeng Gao collected and analysed the data, while Youcui Yuan and Xiaoke Zhang wrote the manuscript.

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