

## Effects of QDTMT on Anoxic Human Umbilical Vein Endothelial Cells\*

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**Abstract: Objective** To find the protective mechanisms of Qidantongmai tablet (QDTMT) to anoxic vascular endothelial cells. **Methods** Cells were got from infant umbilical cord for primary culture, then they were identified by morphologic characteristics. The prepared serum of QDTMT were added to passage HUVEC. According to different treatment factors, endothelial cells were divided into three groups: normoxic control, hypoxia control and QDTMT group. The proliferation of HUVEC was observed in MTT. The apoptosis rate of HUVECs was observed in FCM assay. The change of cell ultrastructure was also investigated with transmission electron microscope. Using immunofluorescence microscope, the cellular distribution of VEGFR2 was examined in hypoxic HUVECs. **Results** There were the characteristic factor VIII positive signal in cultured HUVECs. The MTT showed that QDTMT promoted the proliferation of HUVEC in hypoxic condition, ( $P<0.05$ ). The FCM assay showed that the apoptosis rate of hypoxic HUVECs was higher than that of normoxic control, but the apoptosis rate was higher in normal saline group than that of QDTMT group ( $P<0.05$ ). It was found that the early endosome structure in QDTMT group. The VEGFR2 expression level of HUVECs was higher in QDTMT group than that of normal saline group. **Conclusion** In hypoxia condition, improving the expression of VEGFR2 may be one of the endothelial protective effects of QDTMT.

**Key words:** traditional Chinese medicine; QDTMT; VEGFR2; endothelial cells

### INTRODUCTION

Traditional Chinese medicine(TCM) has received much attention regarding its potential therapeutic use in cardiovascular diseases<sup>[1-2]</sup>. Vascular endothelial growth factor receptor-2 (VEGFR-2, also known as Flk-1/KDR) is the key receptor regulating mitogenic, chemotactic, permeability and survival signals in vascular endothelial cells (EC) in response to its ligand, vascular permeability factor/VEGF (VPF/VEGF)<sup>[3-4]</sup>. Some researchers show that traditional Chinese medicine exerts cardioprotection through VEGF expression and secretion<sup>[5-6]</sup>. While we have learned much about the study on traditional Chinese medicine regulation of VEGF, we known comparatively little about the TCM regulation of KDR in EC. Previously, we had described Qidantongmai tablet (QDTMT) protected Vascular endothelial cell (VEC), increased the VEGF expression in ventricles of myocardial ischemia rats and improved the microvessel density(MVD) in the ischemia zones<sup>[7]</sup>. Recent studies have demonstrated that QDTMT have a two-way regulation effect on VEGF concentration in a hypobaric hypoxia-induced rat model<sup>[8]</sup>. However, the effects of QDTMT on KDR expression and endothelial cells in hypoxic condition are not known.

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The present study addresses this issue.

## MATERIALS AND METHODS

### 1. Materials

Drug QDTMT composed of radix astragali, salvia miltiorrhiza, safflower, angelica and ramulus cinnamomi, was made into the solution. It was with the herb extract concentration of 0.326 g/ml. Reagents: collagen II was purchased from Sigma Co., USA. Medium 199 (M199) and FBS (Fetal Bovine Serum) were from Gibco Co. USA, factor VIII antibody was from Zhongshan Co. China, KDR antibody was from R & D Co. USA.

### 2. HUVECs Cultivation

HUVECs were isolated from fresh human umbilical veins as described with some modifications<sup>[9]</sup>. Briefly, human umbilical veins were flushed with phosphate-buffered saline (PBS), then filled with PBS containing 0.2% collagen II and incubated for 20min at 37°C. The HUVECs were removed from the vein by PBS wash. The isolated primary HUVECs were maintained in 2% gelatin-coated tissue culture plates in complete growth medium 199 supplemented with 20% fetal bovine serum, 10 µg/ml endothelial cell growth factor (Gibco Co. USA), 10mg/ml heparin, and penicillin-streptomycin (50 mg/ml each) at 37°C in a 5% CO<sub>2</sub> incubator. HUVECs from passages 3-5 were used in this study. For the observation of cell imaging, cells were cultured onto acid-washed, fibronectin-coated glass coverslips and allowed to adhere overnight. Umbilical cords were obtained with written consent, with approval from the local ethics committee.

### 3. Optical Microscope Observation of Cell Growth

Using phase-contrast microscopy, the growth of HUVECs was observed and photos were taken.

### 4. Immunofluorescence Test of Factor VIII

After the cover glass was taken out and fixed with 4% formamine for 30min, the factor VIII primary antibody (with the dilution of 1:100) was drop on the glass for staining, incubate at 37°C for 1h. Then the primary antibody was removed by washing 3 times in 0.1 mol/L ice-cold PBS for 5 minutes. Then GFP labeled secondary antibody (with the dilution of 1:1000) was added in to cells, and they were vibrated and washed for 20 minutes, incubated at 37°C for 1 h, and flushed with PBS. Cells were stained with DAPI for 5 minutes. Then, seal the cover glass with glycerin and carry sheet glass and send it for immunofluorescence test.

### 5. Preparation of Serum Containing QDTMT

The normal SD rats were administered with QDTMT decoction at a dose of 2ml/100g rat weight (which was equal to 10 times the human dose in clinic) 2 times per day for 4 days. Two hours after the last administration, the sera were collected from the rats, mixed, and inactivated at 56°C for 30 minutes. The control sera were collected from the normal rats without taking the drug.

According to different treatment factors, endothelial cells were divided into three groups: normoxic control, hypoxia control and QDTMT group. For cell culture experiments, cells were either exposed to normoxic (21% O<sub>2</sub>, 5% CO<sub>2</sub>) or placed in a hypoxic incubator with 1% O<sub>2</sub>, 94% N<sub>2</sub>, 5% CO<sub>2</sub>.

### 6. Measurement of Cell Viability

The viability of HUVECs cell was measured by a colorimetric MTT assay. Briefly, HUVECs were seeded in 96 well plates at 3×10<sup>4</sup> cells/well in 200 µl medium containing 10% FBS. The cells were allowed to attach for 24 h, the medium was changed with fresh medium supplemented with 10% serum containing QDTMT and cells were transferred to a standard incubator or a hypoxia chamber for 24 h. MTT was added to the cultures at a terminal

concentration of 1 mg/ml and incubated for another 4 h at 37°C. After the medium had been removed, the dye crystal was dissolved in 150 µL DMSO. Finally, the optical density (OD) of each well was immediately measured on ELISA micro-plate reader (Metertech) at 490nm to represent cellular viability. The OD of control cell was taken as 100% viability.

### **7. Fluorescence-activated Cell Sorting Analysis (FACS)**

The cells were harvested at  $5 \times 10^5$  cells/well, washed with 2.5 ml ice-cold PBS, and resuspended with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI), according to the protocol of the annexin V-FITC kit (Miltenyibiotec). The final mixture was then applied to a FACS caliber flow cytometer and CellQuest software for analysis. The results were interpreted as follows: cells negative for both PI and annexin-V-FITC staining were considered as live cells; annexin-V-FITC-positive cells were considered early apoptotic cells; PI and annexin-V-FITC-positive cells were considered late apoptotic cells.

### **8. Cell Ultrastructure Observation**

The cells were harvested at  $1 \times 10^6$ , fixed with 30 ml/L glutaraldehyde for 2 h, 10 g/L osmic acid for 1 h, washed with 0.1 mol/L ice-cold PBS 3 times, dehydrated with acetone, embedded with resin, and ultrathin sections were observed with transmission electron microscope.

### **9. Immunofluorescence Test of KDR**

HUVECs were seeded onto coverslips at a density of  $2 \times 10^4$  cells/ml, grow overnight, then subjected to hypoxia or normoxic conditions in the presence or absence of serum containing QDTMT for 48 h. Cells grown on coverslips were rinsed and fixed in 4% paraformaldehyde for 30 min. The cell slides were washed in PBS, and permeabilized in 0.2% Triton X-100 in PBS for 5 min. The cells were washed again in PBS, pre-incubated with 3% H<sub>2</sub>O<sub>2</sub> for 10 min and blocked for 15 min in calf serum at room temperature. The KDR primary antibody (1:40 dilution) was then added. After were washed for 3×3min in PBS, the cells were away from light and incubated with GFP tagged secondary antibody (1:200 dilution) at 37°C for 120min. The cells were washed for 5×3min in PBS and were mounted over glycerine. A series of images was taken after observation with fluorescence microscope.

### **10. Statistical Analysis**

Data were analyzed using a one-way ANOVA with SPSS13.0 software. The data represented means±S.E.M. and values of  $P < 0.05$  were statistically significant.

## **RESULTS**

### **1. Optical Microscope Observation**

The adherence of cultured primary HUVECs was seen in vitro. The adherent cells are relatively homologous in size, most of which are round or polygonal. The cells that are adherent within 48 h grow rapidly, and 6 days later, they cover 70%-80% area of the culture bottle. Cells were in the shape of cobble-stone shape after 8 days later. (Fig. 1)

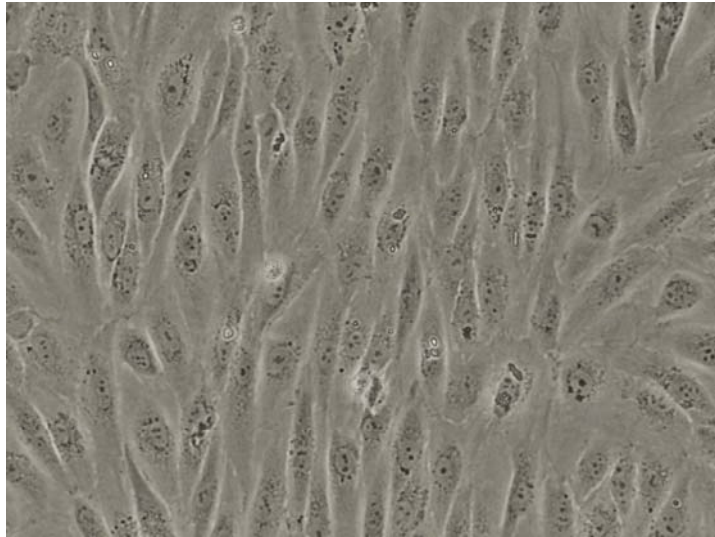
### **2. Immunofluorescence Result of Factor VIII**

The GFP labeling factor VIII can be seen clearly in HUVECs. (Fig. 2)

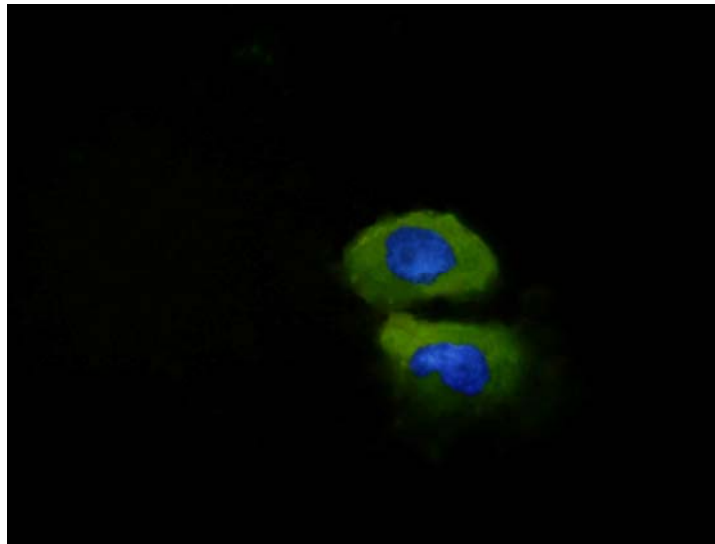
### **3. Influence of QDTMT on HUVECs Viability under Hypoxia**

As shown in MTT, HUVECs viability decreased in hypoxia, hypoxia resulted in a reduction in cell viability compared with normoxic control ( $P < 0.01$ ). The treatment with serum containing QDTMT improved cell viability

under hypoxia compared with hypoxia control ( $P < 0.05$ ). The cell viability of hypoxia control group was about 15%, however, the cell viability of QDTMT treatment group was about 22%. (Table. 3)



**Fig. 1** HUVECs ( $\times 100$ ) cells was obviously changed like cobble-stone shape in after 8 days later



**Fig. 2** Immunofluorescence result of F factor ( $\times 200$ ) the GFP labeling of factor positive cells can be seen clearly in culturing HUVECs

**Table 3** Influence of QDTMT on HUVECs viability under hypoxia

Group	OD ( $\bar{x} \pm s, n=8$ )
Normoxic control	0.40 $\pm$ 0.05
Hypoxia control	0.15 $\pm$ 0.04
QDTMT group*	0.22 $\pm$ 0.05

Notes: As shown in MTT, HUVECs viability decreased in hypoxia, hypoxia resulted in a reduction in cell viability compared with normoxic control ( $P < 0.01$ ). At 24h, treatment with serum containing QDTMT improved cell viability under hypoxia compared with hypoxia control ( $P < 0.05$ ). The cell viability of hypoxia control group was about 40%, however, the cell viability of QDTMT treatment group was about 22%. \*  $P < 0.05$  vs Hypoxia control.

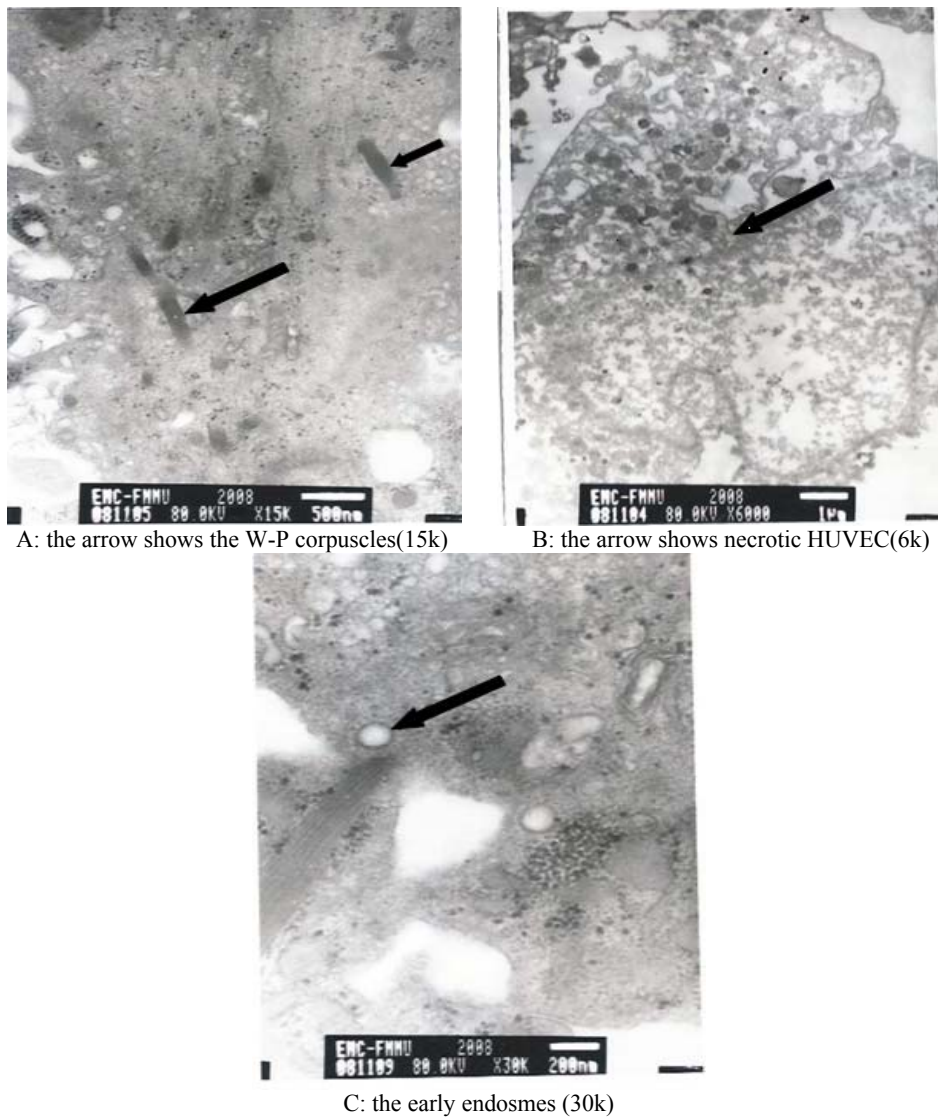
#### 4. Influence of QDTMT on Apoptosis of HUVECs under Hypoxia

Hypoxia induced significant elevations of apoptosis ( $P < 0.01$ ), the early apoptosis rate was about 30%. Whereas serum containing QDTMT significantly attenuated cells early apoptosis compared with hypoxia control ( $P < 0.05$ ), the early apoptosis rate was about 16%. (Table. 4)

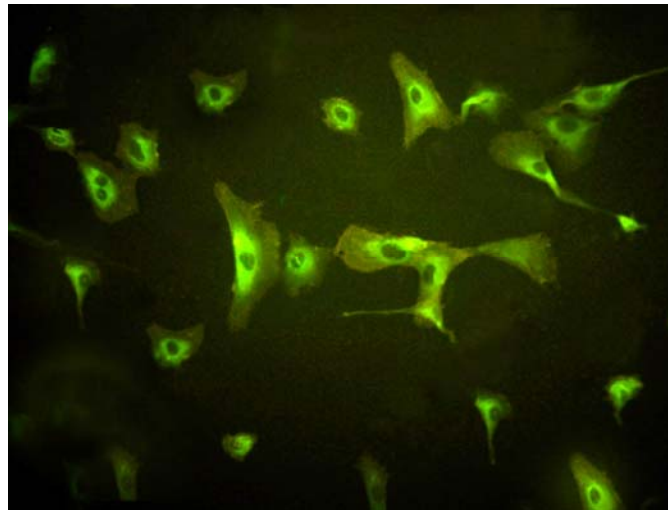
**Table 4 Influence of QDTMT on apoptosis of HUVECs under hypoxia**

Group	Rate of apoptosis
Normoxic control	$8.7 \pm 1.7\%$
Hypoxia control	$30.1 \pm 3.4\%$
QDTMT group*	$16.6 \pm 2.5\%$

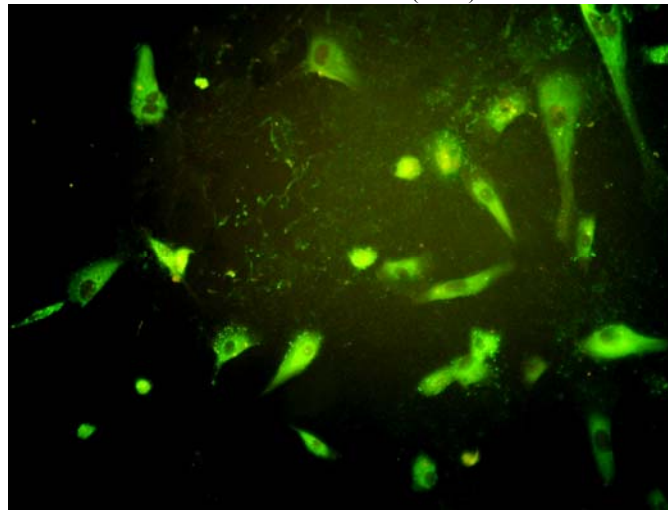
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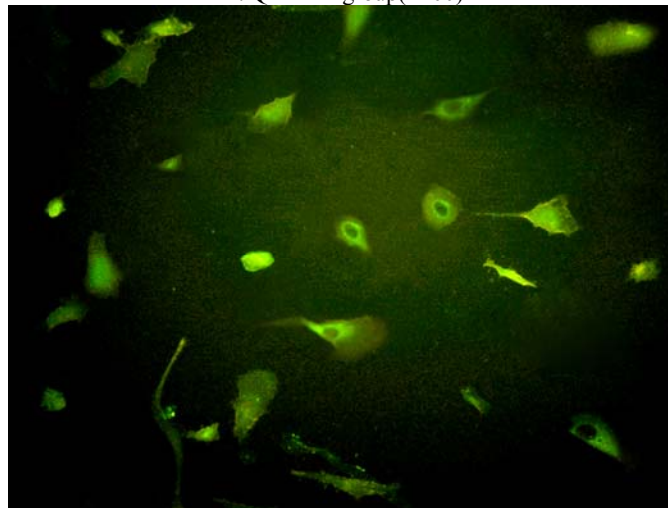
**Fig. 5 Influence of QDTMT on ultrastructure of HUVECs under hypoxia**



A: Normoxic control( $\times 100$ )



B: QDTMT group( $\times 100$ )



C: Hypoxia control( $\times 100$ )

**Fig. 6 Influence of QDTMT on KDR expression of HUVECs under hypoxia**  
Notes: The expression of KDR is higher in QDTMT group than that of hypoxia control.

### **5. Influence of QDTMT on Ultrastructure of HUVECs under Hypoxia**

By electron microscope, normoxic control showed some 0.1  $\mu\text{m}$ -5  $\mu\text{m}$  long Weible-Palade bodies in the ultrastructure of HUVECs. While cellular necrosis can be find in the ultrastructure of hypoxia control. We find the early endosomes in cells treated with serum containing QDTMT. (Fig. 5A, Fig. 5B, Fig. 5C)

### **6. Influence of QDTMT on KDR Expression of HUVECs under Hypoxia**

The expression of KDR is higher in QDTMT group than that of hypoxia control. (Fig. 6)

## **DISCUSSION**

Angiogenesis is controlled by a fine local balance between activating and inhibiting mediators. Physiologically, it is a very strictly regulated process, which results in a balance between stimulatory (angiogenic) and inhibitory (angiostatic) factors to control the correct development of blood vessels. It is well-known that VEGF ligands with either its receptor flt-1 or KDR. As positive signals of angiogenesis, the system of VEGF and its key receptor- KDR is a hot spot<sup>[10-11]</sup>. VEGF is reported to potently induce migration and proliferation of endothelial cells (ECs), enhance vascular permeability, and modulate thrombogenicity. The human endothelial vascular endothelial growth factor receptor 2 (VEGFR2, KDR/fetal liver kinase-1, Flk-1), as a tyrosine kinase receptor, is essential for VEGF-mediated physiological responses including endothelial cell proliferation, migration and survival. The pivotal role of KDR trafficking in proliferation and survival signals of ECs is also demonstrated in recent studies. It was showed that only 60% cellular KDR is present at the cell surface and available for interacting with VEGF in unstimulated cells, with the remaining 40% receptor present in an internal vesicular pool, the internal KDR pool is composed mainly of early endosomes and Rab4+ recycling endosomes, and VEGF stimulation provokes recycling of KDR to the cell surface<sup>[12]</sup>.

It has been reported that KDR is present on the plasma membrane, on endosomes, and in the perinuclear region of EC and colocalizes with early endosomal antigen (EEA1), caveolin-1, and dynamin-2<sup>[11]</sup>. VEGF is unable to fully mobilize this compartment, and the researcher speculated on the possible existence of stimuli that could do this, such mobilization would lead to a dramatic increase in the surface expression of KDR and presumably to a consequent increase in the magnitude of response to VEGF stimulation. So, it is possible that drug changes the distribution of KDR. Our in-vitro model utilized the “HUVECs”, well-known for studies on Vascular endothelial cell (VEC) activities and endothelium disorders. With the animal model of hypobaric hypoxia (HBH), we have investigated the regulation effects of QDTMT on VEGF-A level in serum<sup>[8]</sup>. In this study, serum containing QDTMT improved the HUVECs viability and suppressed cells apoptosis induced by hypoxia. We find the early endosomes in cells treated with serum containing QDTMT, and the expression of KDR was higher in QDTMT group than that of hypoxia control. We hypothesized that QDTMT might be involved in the VEGF stimulation and increase the recycling of KDR to the cell surface. It is important to recognize that there are limitations inherent in using the results of animal studies as pointers to the human situation. But, taken together, our data partly present the close relationship between the system of VEGF –KDR and QDTMT protection of hypoxic HUVECs.

## **REFERENCES**

- [1] Guruvayoorappan C., Kuttan G. Anti-angiogenic effect of Biophytum sensitivum is exerted through its cytokine modulation activity and inhibitory activity against VEGF mRNA expression, endothelial cell migration and capillary tube formation. *J Exp Ther Oncol*, 2007, 6(3): 241-250.

- [2] Kojima-Yuasa A., HUA J. J., Kennedy D. O., et al. Green tea extract inhibits angiogenesis of human umbilical vein endothelial cells through reduction of expression of VEGF receptors. *Life Sci*, 2003, 73(10): 1299-1313.
- [3] Ferrara N., Gerber H. P., LeCouter J. The biology of VEGF and its receptors. *Nat Med*, 2003, 9(6): 669-676.
- [4] Khurana R., Simons M., Martin J. F., et al. Role of angiogenesis in cardiovascular disease: A critical appraisal. *Circulation*, 2005, 112(12): 1813-1824.
- [5] Ruixiang, Q., Jun, F., Jun M. The effect of xin mai tong capsules in protecting survival cardiac muscles of the patients with acute myocardial infarction. *J Tradit Chin Med*, 2005, 25(2): 139-142.
- [6] LIU C., JIANG C. M., LIU C. H., et al. Effect of Fuzhenghuayu decoction on vascular endothelial growth factor secretion in hepatic stellate cells. *Hepatobiliary Pancreat Dis Int*, 2002, 1(2): 207-210.
- [7] WANG W., WANG Z. R., ZHANG J. Z., et al. The experimental study on the effect of Qidantongmai tablet on ischemic myocardium in rats. *Journal of Emergency in Traditional Chinese Medicine*, 2007, 16(2): 190-191.
- [8] WANG B., MA J., LI J. H., et al. Regulation effect of Qidantongmai tablet on VEGF expression in rats. *Hypoxia Journal of Emergency in Traditional Chinese Medicine*, 2008, 17(11): 1563-1564.
- [9] Van Hinsbergh W. M., Draijer R. *Cultsure and Characterization of Human Endothelial Cells*. Oxford, England: Oxford University Press, 1996.
- [10] Bhattacharya R., Kang Decker N., Hughes D. A., et al. Regulatory role of dynamin- 2 in VEGFR-2/KDR -mediated endothelial signaling. *FASEB J*, 2005, 19(12): 1692-1694.
- [11] Shibuya M. Vascular endothelial growth factor (VEGF)-Receptor2: Its biological functions, major signaling pathway, and specific ligand VEGF-E. *Endothelium*, 2006, 13(2): 63-69.
- [12] Gampel, L. Moss, M. C. Jones, et al. VEGF regulates the mobilization of VEGFR2/KDR from an intracellular endothelial storage compartment. *Blood*, 2006, 108(8): 2624-2631.

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- [5] Johnson C. D., Kingsnorth A. N., Imrie C. W., McMahon M. J., et al. Double blind, randomised, placebo controlled study of a platelet activating factor antagonist lexipafant, in predicted severe acute pancreatitis. *Gut*, 2001, 48(1): 62-69.
- [6] Johnson C. D., Abu-Hilal M. Persistent organ failure during the first week as a marker of fatal outcome in acute pancreatitis. *Gut*, 2004, 53: 1340-1344.
- [7] Kong L., Santiago N., HAN T. Q., et al. Clinical characteristics and prognostic factors of sever acute pancreatitis. *World J Gastroenterol*, 2004, 10: 3336-3338.
- [8] Schmid S. W., Uhl W., Friess H., et al. The role of infection in acute pancreatitis. *Gut*, 2005, 45: 311-316.
- [9] Sawa H., Ueda T., Takeyama Y., et al. Expression of toll-like receptor 2 and 4 in intestinal mucosa in experimental severe acute pancreatitis. *Hepatology*, 2008, 55(8): 2247-2251.
- [10] Kumar R. S. Acute necrotizing pancreatitis: Current concepts. *Indian Journal of Surgery*, 2005, 67(2): 78-86.

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