

Evaluation of velvet antler total protein effect on bone marrow-derived endothelial progenitor cells

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Abstract. Lu Rong, velvet antler (VA), is a traditional Chinese medicine, which is used as a food supplement and therapeutic drug in China, Japan, Russia, New Zealand and Southeast Asia. The regenerative characteristics of VA have resulted in great research interest, particularly regarding the fields of organ grafting and stem cell differentiation. Various VA proteomic studies verified that proteins act as the primary bioactive components of VA. The present study aimed to investigate if VA proteins (VA-pro) influence endothelial progenitor cell (EPC) viability. Various methods have previously been used to investigate VA-pro, including freeze-drying technology, ultrasonic wave methods, high performance liquid chromatography-mass spectrometry, EPCs extraction and

culture. Results demonstrated that VA-pro promoted EPCs proliferation and migration, particularly at a concentration of 1 mg/ml. Furthermore, VA-pro increased the activation level of Notch1 intracellular domain and Hes1, and the level of phosphorylated-Akt and phosphorylated-mechanistic target of rapamycin. VA-pro may therefore affect EPC viability via regulation of the Notch and Akt signaling pathways. The present study revealed the effects and potential molecular mechanism of VA-pro on EPCs, and suggested an association between VA regeneration characteristics and the optimization of EPC viability. These findings may contribute to EPC transplantation research and aid in providing a novel treatment method for vascular diseases in the future.

Introduction

Endothelial progenitor cells (EPCs), also called angioblast, are precursor of endothelial cells. Under the stimulus of physiological or pathological factors, EPCs mobilize from bone marrow to peripheral blood for participating in the repair of damaged blood vessels. The EPCs play a crucial role in re-endothelialization and repairing the injured endothelium (1). They take part in many diseases, however, there are few EPCs in peripheral blood, and they do not proliferate readily even are dysfunctional (2), so expanding EPCs levels and enhancing EPCs viability may benefit patients. Furthermore, transplantation of EPCs is applied to the treatment of ischemic diseases, but how to optimize the EPCs proliferation and amplification is still a problem.

In the process of stem cell proliferation and differentiation, cell signaling pathways play a regulatory role, especially Notch signaling pathway and Akt signaling pathway. Akt signaling pathway has a close relationship with cell viability and migration. Some studies showed that it was involved in progesterone-induced EPC viability (3). Notch signaling pathway is highly conserved, and plays a key role in neovascularization and angiogenesis process. It participates in progenitor cells differentiation, proliferation (4) and tube formation (5). We

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Abbreviations: VA, velvet antler; VA-pro, velvet antler proteins; GO, Gene Ontology; KEGG, Kyoto encyclopedia of genes and genomes; pI, Protein isoelectric point; MW, proteins molecular weight; CMECs, cardiac microvascular endothelial cells; IH, ischemia hypoxia; MMP, mitochondrial membrane potential; CD31, Platelet endothelial cell adhesion molecule-1; vWF, von Willebrand factor; CVD, cardiovascular disease

Key words: velvet antler, endothelial progenitor cell, proliferation, migration, notch, Akt

assumed that the two signaling pathways were involved in the effect of velvet antler (VA) on EPCs.

VA is common and precious medicinal material of traditional Chinese medicine (TCM) which is called Lu Rong in China, and it has been widely used as food supplement or therapeutic drug generally in China, Japan, Russia, New Zealand and Southeast Asia. Many researches have been carried out to study the constituents as well as pharmacology of VA, however, like many TCM supplements, its mechanism has not been clearly interpreted from western viewpoint. VA has an inherent property that is the ability of regeneration that are structural and functional replicates (6,7). It can quickly grows into a tissue which has rich blood vessels, nerves, fur, flesh and blood within 60 days. Some research showed that VA regeneration is a stem cell-based epimorphic regeneration (8), so this property has drawn many scholars' attention and was related with organ grafting and stem cells differentiation (9), but there is rarely research in this filed.

Some studies have revealed that polypeptides and proteins are the main bioactive components of VA (10), promoting neurite outgrowth (11), the proliferation of epidermal cells and NIH3T3 cells (12), enhancing wound healing (13). Therefore, in this study we extracted VA proteins (VA-pro) using freeze-drying technology, water solvent and ultrasonic wave method, and determined the effect of VA-pro on rat bone marrow-derived EPCs, as well as the possibly molecular signaling pathway mechanisms.

Materials and methods

Velvet antler preparation. Fresh VAs were the fast-growing antlers of 4-year-old male deer (14) (*Cervus Nippon* Temminck), which was bred in Jilin Province of China. VAs were identified by Professor Chunsheng Liu, the director of the Department of Chinese Medicine Identification at the TCM Institute, Beijing University of Chinese Medicine. Blood was drawn from the VAs with a vacuum instrument for 6 h in 4°C. VAs were sliced and freeze-dried by lyophilizer (-60°C and 12 Pa, LABCONCO) until quality change was less than 0.1 g. With the superfine grinding technology, freeze-dried VAs were superfine comminuted in cryogenic environment. 54.08 g lyophilized VA powder was created, and the dehydration percentage of VA was 67.02%. The powder was stockpiled in sealed tubes and numbered XX-DGF01 to XX-DGF04.

Lyophilized VA powder (XX-DGF01) was mixed with ultrapure water (1:10) and blended through vortex oscillation (5 s/15 s). Using homogenate and immersion methods, the VA was ultrasonically extracted for 2 h in an ice-bath. The supernate was obtained in a centrifuge tube and the precipitate was removed by centrifugation (12,000 rpm, 30 min). The supernate was stored at -80°C for at least 12 h, and then get freeze-dried by the lyophilizer for 72 h. The precipitate was processed according to the above steps for 3 times. All freeze-dried VA-pro were mixed together and were numbered from XX-VApro01 to XX-VApro03, being stored at -80°C. The VA-pro mass concentration was 465.56 µg/mg, which was measured by a BCA protein assay kit (Thermo Fisher Scientific).

Nano LC-MS/MS analysis. We digested VA-pro with trypsin (30:1) for 24 h at 37°C, and the desalinization were taken with

C₁₈ column (Phenomenex Strata™). High performance liquid chromatography (HPLC) was performed and the desalted peptide mixture was separated with a reversed phase C₁₈ column (75 µm x10 cm, 5 µm, 300 Å, Agela Technologies) at 400 nl/min constant flow rate. Peptides were eluted with a gradient of 5-80% (v/v) and the acetonitrile in 0.1% formic acid over 65 min. The eluates were directly entered into a Q-Exactive Orbitrap Mass Spectrometer (Thermo Fisher Scientific), which was set in positive ion mode, the scan range was 350-2000 m/z and scan resolution was at 17,500. 1E+5 was the minimum signal threshold and isolation width was at 2 Da. Ion source voltage was set at 1800 V. 2 MS/MS acquisition modes and higher collision energy dissociation were employed to evaluate the performance of this MS on the iTRAQ-labeled samples (Fig. 1 The ensample of VA mass spectrometric patterns).

MS data analysis. After Nano LC-MS/MS measurement, the raw data were filtered by the PD (Proteome Discoverer 1.3, Thermo Fisher Scientific). The collected data files were sent to a MASCOT server (Version 2.3.0, Matrix Science) for peptide identification with the *Ruminantia* (Number of sequences: 93869) taxonomy constraint.

386 VA-pro were identified. Their molecular weight (MW) range and isoelectric point (pI) range were showed in Tables I and II.

EPCs primary culture. Bone marrow-derived mononuclear cells (BM-MNCs) were isolated from 2-week-old male SD rats femur by the density gradient centrifugation method, using lymphocyte separation medium (TBD Science). BM-MNCs were seeded on a 35 mm culture dish and incubated in complete endothelial basal medium (EBM-2 medium, Lonza) under standard cell culture condition. After 72 h, first medium change was performed, and non-adherent cells were removed, while the adherent cells were continuously cultured for 14 days, then the cells were identified by CD133 (Novus) and VEGFR2 (15) (Abcam).

Immunofluorescence. In order to characterize EPCs, cells were fixed for 15 min in 4% paraformaldehyde at room temperature. Then they were deal with 0.3% triton-100/PBS for 10 min, blocked with 3% BSA/PBS for 1 h. cells were incubated with primary antibodies overnight at 4°C, followed by immunolabelling with Alexa 488-conjugated secondary antibody (1:200, CST), for 1 h at room temperature in the dark. The nuclei were stained by DAPI for 10 min. The cells were imaged and counted under an Inversed Fluorescent Microscope (Nikon). Digital images were prepared using NIS Elements AR Analysis 4.10.00.

Cell proliferation assay. EPCs proliferation was detected with MTS kit (the Cell Titer 96® Aqueous One Solution Reagent). EPCs were cultured in 96-well plates with different treatment. Then each well was added 100 µl EBM-2 medium and 20 µl reagent and cells were cultured for 3 h. The optical density (OD) was measured by Microplate Spectrophotometer (Spectra MR).

Tubule formation assay. Tubule Formation Assay was determined with Matrigel (BD Biosciences). After thawing for 3 h

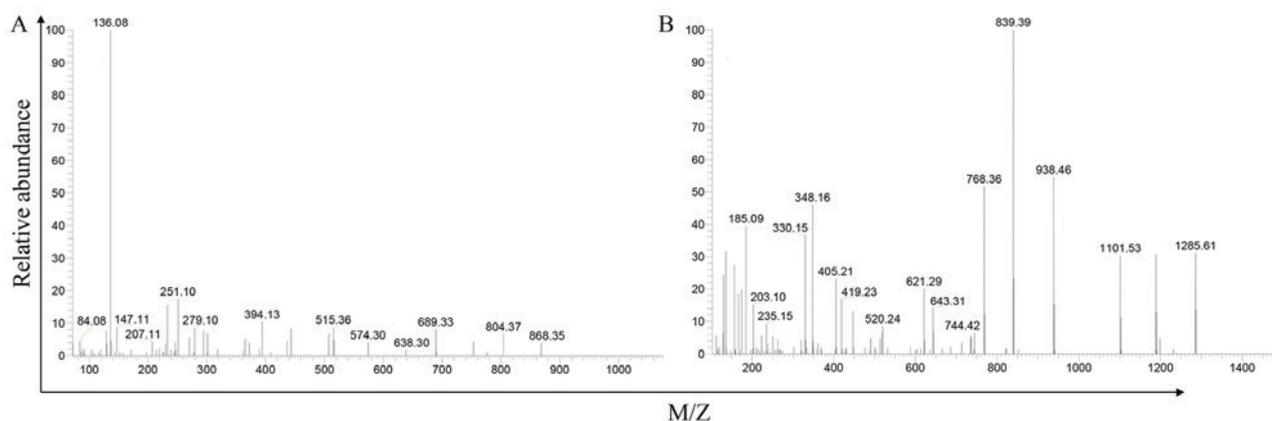


Figure 1. The sample of VA mass spectrometric patterns. (A) Heat shock protein 70.1 and (B) 14-3-3 protein β/α .

Table I. VA-pro molecular weight.

MW(kDa)	<10	10-20	20-30	30-40	40-50	50-60	60-70	70-80	80-100	100-200	>200
N	5	63	51	46	42	39	24	20	20	49	24

Table II. VA-pro isoelectric point.

pI	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12
N	50	128	84	29	49	20	16	7

at 4°C, 100 μ l Matrigel was added into every well of 48-well plate and was put into an incubator to solidify for 30 min. 2×10^4 EPCs were seeded on the top of the Matrigel layer and were treated with different concentrations of VA-pro. The net structure was observed under a bright field microscope (Leica Microsystems) within 24 h and it was calculated in four random fields using NIS Elements AR Analysis 4.10.00.

Wound healing assay. 4×10^5 EPCs were cultured in 12-well plates for 24 h. The wound was made by scratching confluent cell monolayers with a P200 pipette tip, three parallel 'wounds' were created in each well with floating cells being washed away. After cells were incubated with different treatment, the migrated cells were photographed and the mean distance between the two ends of the scratch was quantified by manual measurements with Prism 5.0 program (GraphPad Inc.).

Transwell assay. Cell transwell assay was performed using invasion chambers (BD Biosciences). 150 μ l cell suspension (1×10^5 cells/ml, FBS-free medium) was placed to the inserts, while medium containing VA-pro were added to the bottom chamber. After 24 h culture, the cells still in the upper side of inserts were cleared with cotton swabs and cells that had penetrated through the polyethylene terephthalate membrane were fixed and stained using mounting medium with DAPI (ZSGB-BIO). The cells were counted in 10 random microscopic fields.

Western blotting. Treated cells were washed and lysed with RIPA lysis buffer, containing PMSF (Beyotime) and

phosphatase inhibitors (sigma-Aldrich). Protein concentration was detected using a bicinchoninic acid protein assay kit (BCA, Thermo Fisher Scientific). Proteins were separated on 8% polyacrylamide gel SDS-PAGE for 110 min (Notch1, NICD, mTOR and p-mTOR) or 12% polyacrylamide gel SDS-PAGE for 50 min (Hes1, Akt and p-Akt), and they were transferred to a nitrocellulose membrane using 350 mA for 50 min (Hes1, Akt and p-Akt) or 3.5 h (Notch1, NICD, mTOR and p-mTOR). Membranes were blocked with 5% nonfat dry milk and washed with PBS. Then they were cut into 3 sections according to markers. Each section of membrane was incubated overnight at 4°C with the antibodies against Notch1, NICD, Hes1, Akt, p-Akt, mTOR, p-mTOR or β -actin (CST, 1:1,000). Membranes were then washed and further incubated for 1 h at 37°C with goat anti-mouse IgG or goat anti-rabbit IgG antibody (1:5,000, Santa cruz), which conjugated with HRP and was diluted in TBST containing 5% nonfat dry milk. Membranes were soaked with super ECL plus (1:1, Millipore) and wrapped with plastic wrap in the dark. The results were detected by an ECL luminescent detection system. Band density was analyzed using Image-Pro Plus 6.0 (Media Cybernetics).

Statistics. Experiments were repeated at least three times. Quantitative Data were presented as means \pm SD and were analyzed using SPSS 17.0 software by ANOVA followed by Tukey's or Dunnett's posttest. $P < 0.05$ was considered as significant.

Results

EPCs characterization. EPCs were cultured from rat bone marrow for 7 days, the adherent cells were active and colonies were appeared (Fig. 2A, B). The cells potential to form tubes was tested (Fig. 2C). The stem cell marker CD133 and surface marker VEGFR2 were positive (Fig. 2D). All above manifested that BM-MNCs were induced into EPCs successfully.

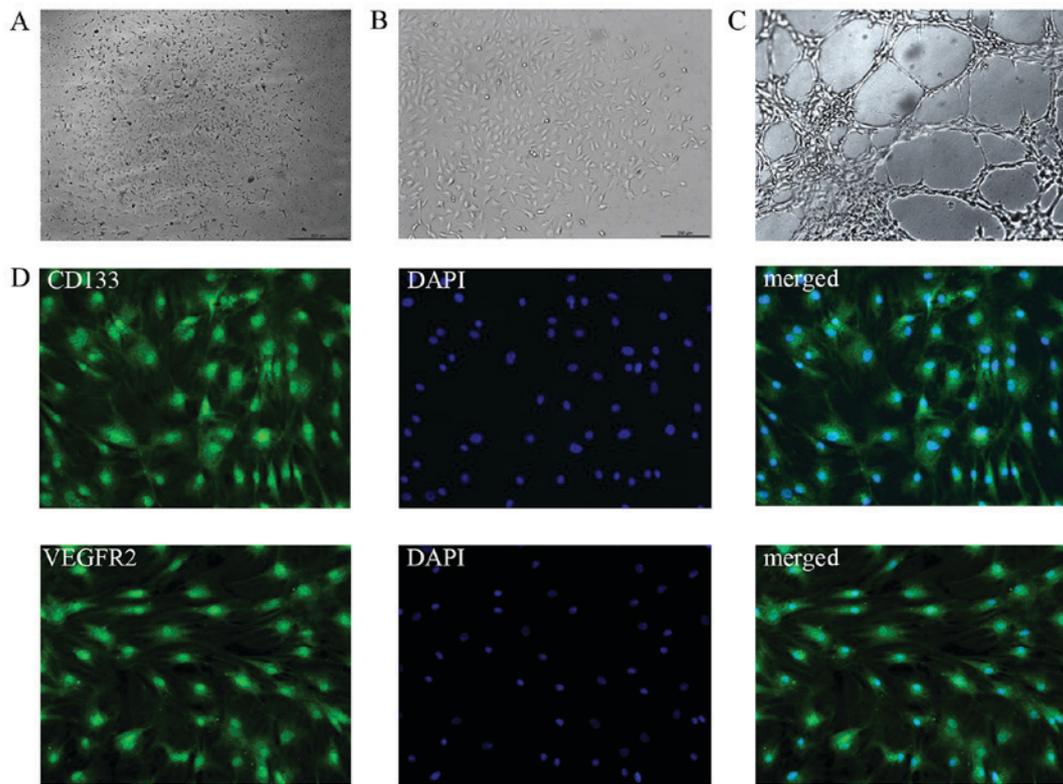


Figure 2. Characterization of EPCs. EPCs colonies appeared after 7 days culture (A, magnification: x50, Scale Bar: 500 μm; B, magnification: x100, Scale Bar: 200 μm). EPCs tube formation was assessed with Matrigel (C, magnification: x100, Scale Bar: 200 μm). Representative immunofluorescence staining of EPCs markers including CD133 and VEGFR2 were positive, cell nuclei were stained with DAPI (D, magnification: x200, Scale Bar: 100 μm).

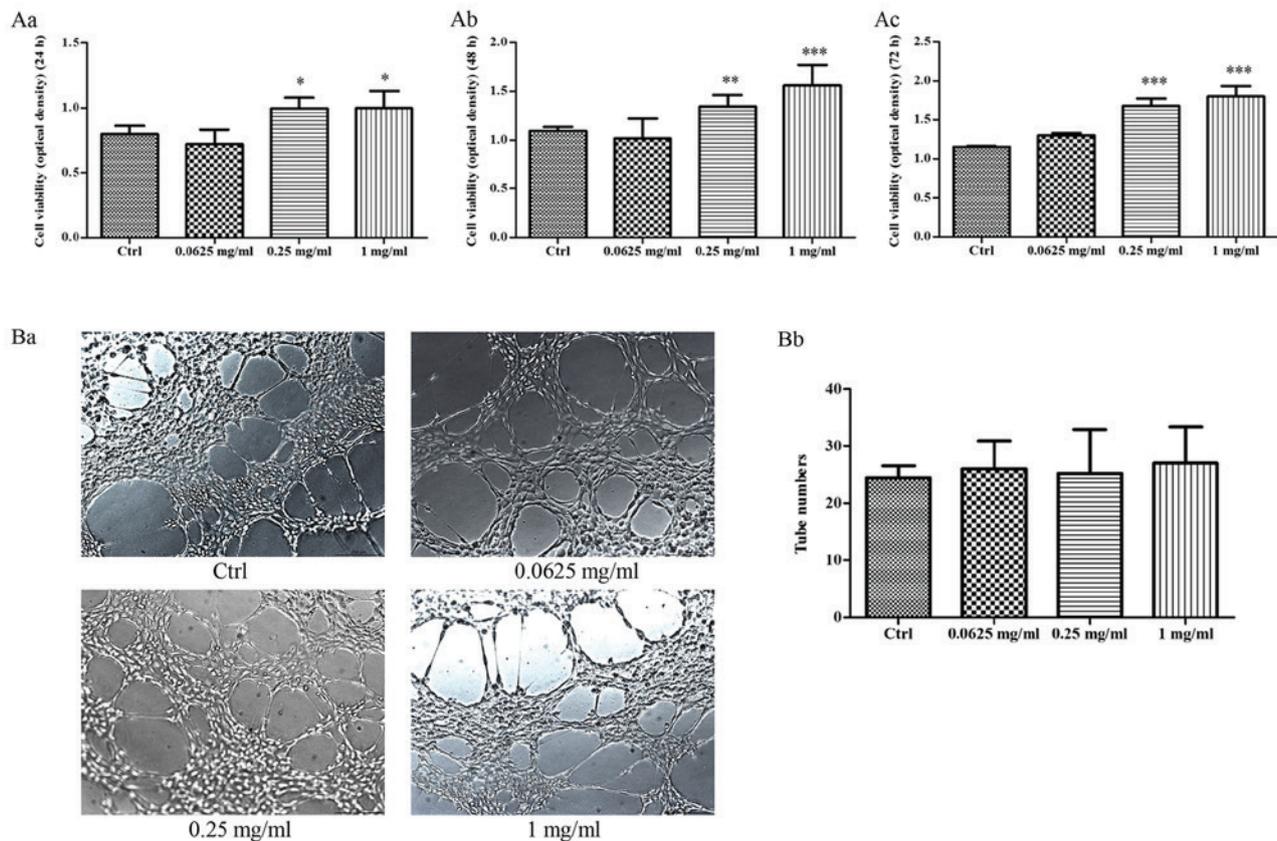


Figure 3. VA-pro promoted EPCs proliferation, but had no effect on EPCs ability of tube formation. Proliferation was detected using MTS kit at 24 h (Aa), 48 h (Ab) and 72 h (Ac), respectively. OD was measured by microplate spectrophotometer. The net structure of tube formation assay was observe under bright field microscope (Ba, magnification: x100, Scale Bar: 200 μm). Data were presented as mean ± SD, *P<0.05, **P<0.01 and ***P<0.001 compared to the control group.

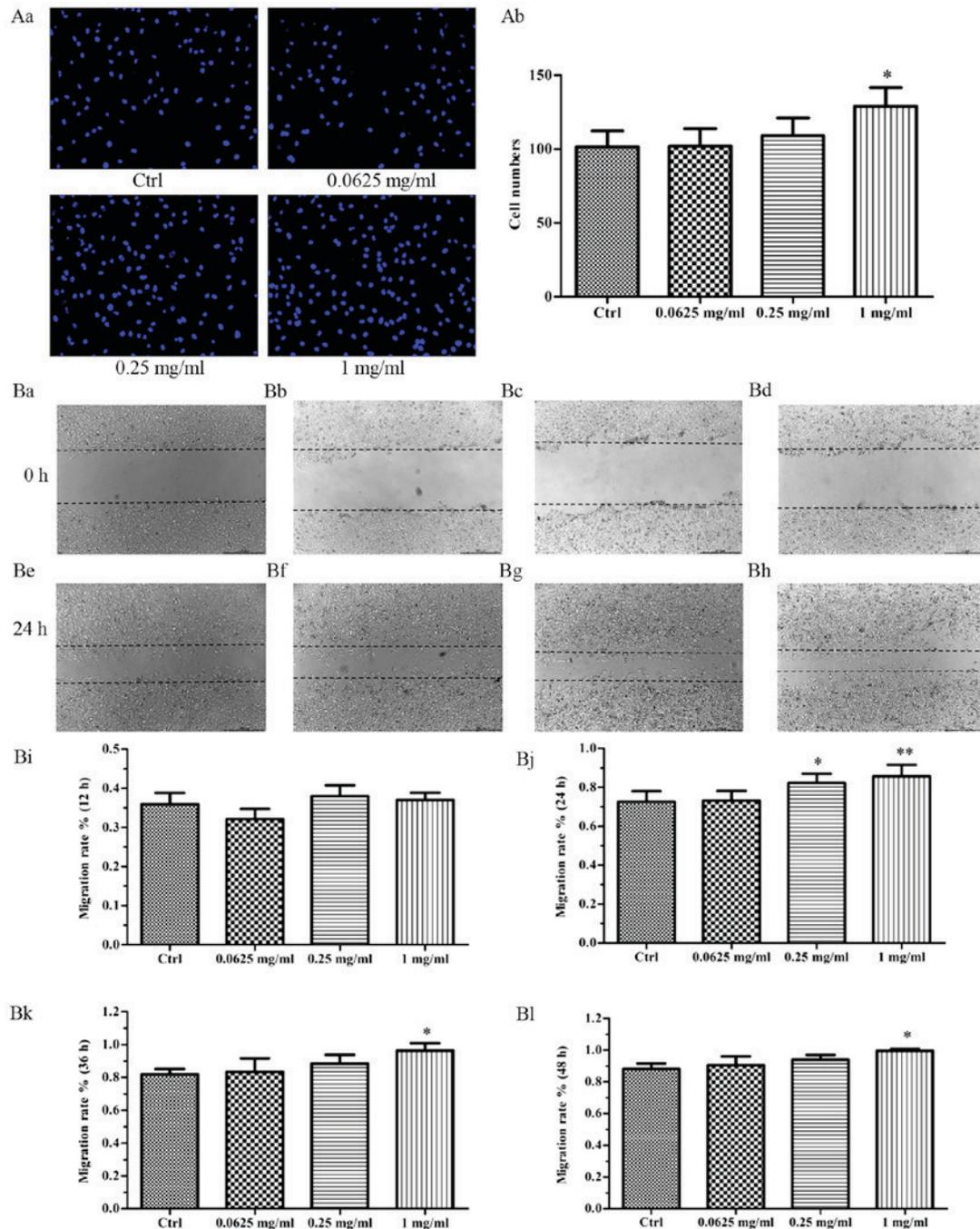


Figure 4. The migration of EPCs treated with 1 mg/ml VA-pro was different from the control group in transwell assay (A). Cells that had penetrated through the polyethylene terephthalate membrane were stained with DAPI and counted in 10 random microscopic fields (Aa, magnification, x200, Scale Bar: 100 μ m). After scratched, EPCs were photograph every 12 h, we choose the photos of 24 h to show here (Ba-h) (magnification: x50, Scale Bar: 500 μ m). The average width of scratches was measured and the EPCs migration rate was calculated, Data were presented as mean \pm SD, *P<0.05 and **P<0.01 compared to the control group.

The effect of VA-pro on EPCs proliferation, tube formation. EPCs of the control group were cultured in complete EBM-2 medium, while cells in other groups were cultured with 0.0625, 0.25 and 1 mg/ml VA-pro. We detected the cells proliferation at 24, 48 and 72 h, respectively. 0.25 and 1 mg/ml VA-pro treatment resulted in a promoting of EPCs proliferation in different degree at 3 time points, cell proliferation of 0.0625 mg/ml group has no difference compared with the control group (Fig. 3Aa, b, c). These data suggested that VA-pro can promote EPCs proliferation. A Matrigel tube formation assay was used

to evaluate the effect of VA-pro on EPCs tube formation. As shown in Fig. 3Ba-b, VA-pro had no effect on EPCs ability of tube formation.

The effect of VA-pro on EPCs migration. For transwell assay, after cultured for 24 h, the migration of EPCs treated with 1 mg/ml VA-pro was different from the control group (Fig. 4Aa). These data indicated that VA-pro promoted the migration of EPCs, especially the 1 mg/ml concentration (Fig. 4Ab). For the wound healing assay, after scratched the confluent cell

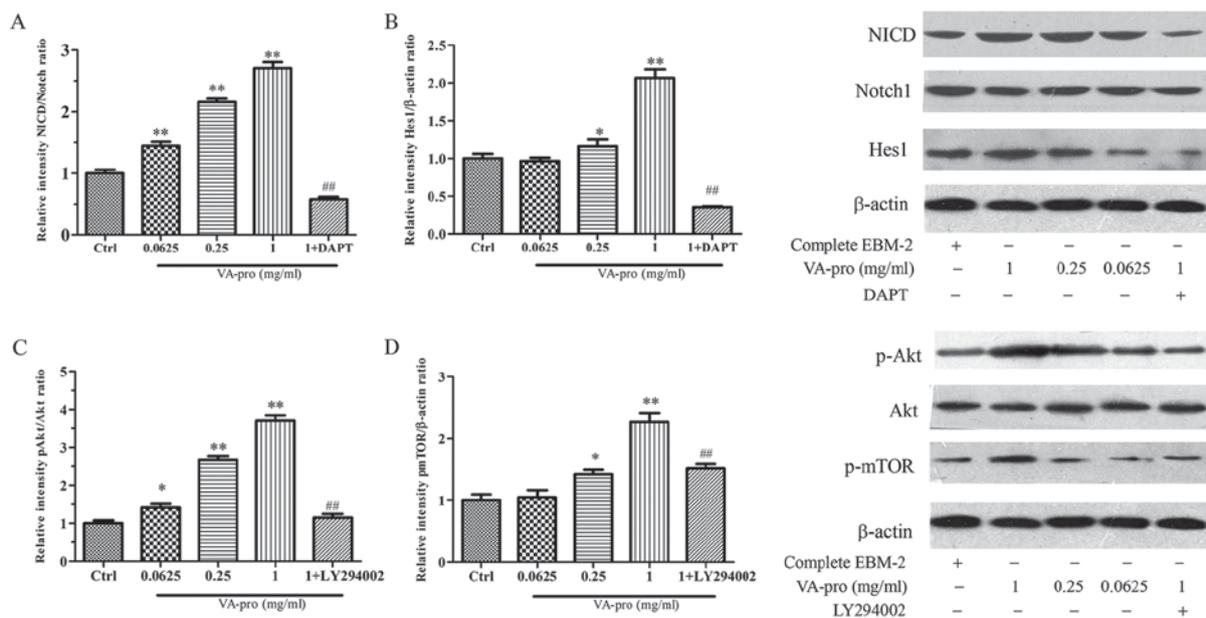


Figure 5. VA-pro can increase the activation level of Notch1 and Hes1 as well as the level of p-Akt and p-mTOR in EPCs. The activation level of NICD concentration-dependently increased along with VA-pro treatment (A), and Hes1 activation level also significantly increased (B). However, Notch1 pathway inhibitor (DAPT, 10 μ M) inverted the results. Compared to the control group, the level of p-Akt concentration-dependently increased along with VA-pro (C), and p-mTOR level also increased significantly (D). However Akt pathway inhibitor (LY294002, 10 μ M) suppressed the effects of VA-pro on p-Akt and p-mTOR expression. Data were presented as mean \pm SD, * P <0.05 and ** P <0.01 compared to the control group, ## P <0.01 compared to the VA-pro 1 mg/ml group.

monolayers, EPCs were photographed every 12 h, the average width of scratches was measured and the EPCs migration rate was calculated. Results showed that 1 mg/ml VA-pro treatment led to better cell migration at 24, 36 and 48 h compared to the control group. Besides, 0.25 mg/ml VA-pro caused a significant difference compared to the control group only at 24 h (Fig. 4Bi-l). We chose the photos at the time of 24 h to show the differences between groups (Fig. 4Ba-h).

Effect of VA-pro on the activation level of Notch and Hes1, and the phosphorylation level of Akt and mTOR in EPCs. Notch1 pathway inhibitor DAPT (10 μ M) and Akt pathway inhibitor LY294002 (10 μ M) were added to the medium containing 1 mg/ml VA-pro. As shown in Fig. 5, there was no obvious differences in the total amount of Notch1, but the activation level of Notch1 Intracellular Domain (NICD) significantly increased along with VA-pro stimulation concentration-dependently, and Hes1 activation level also significantly increased. However DAPT inverted all. The ratio of NICD to the total Notch1 and Hes1 to the β -actin were calculated to evaluate the activation of Notch1 and Hes1 (Fig. 5A, B).

Compared to the control group, VA-pro increased the phosphorylation level of Akt in a concentration-dependent manner, and mTOR phosphorylation level also significantly increased. However LY294002 inverted this outcome, it suppressed the effects of VA-pro on p-Akt and p-mTOR expression (Fig. 5C, D).

These indicated that VA-pro affected EPCs possibly through activating the Notch pathway and Akt/mTOR pathway.

Discussion

EPC has been researched as a hotspot in recent years, not only has it been linked to the development of many diseases, but

plays a key role in treating certain diseases. EPCs have been engaged to replace damaged endothelial cells and increase neovascularization in many animal models (16,17).

Whether stem cells transplantation is more good than harm or not has been controversial, but some researches show that stem cells have good curative effects on the treatment of diseases. Better applications of this method are worth to be explored. Chen *et al* transplanted autologous EPCs into cerebral ischemia rabbits models, after 14 days, most of EPCs binded to UEA-1 lectin were incorporated into capillaries, resulting in an increase of microvascular density in the ischemia area, the infarction area reducing and brain function improving (18). Other research (19) has shown that for MODS pigs, autologous transplantation of EPCs migrated to the injured organs and induced angiogenesis for replying the blood supply of vital organs as well as improving their function. Furthermore, the clinical research about therapeutic angiogenesis by cell transplantation (TACT) reported that doctors injected the peripheral mononuclear cells which were collected from patient's right subclavian vein into local muscle for the treatment of ulcer causing by occlusive arterial sclerosis, the patient's general condition was not good and other treatment no curative effect on ulcer. 2 weeks postoperative, skin temperature increased. 1 month later, ulcer's blood flow improved and the ulcer completely healed by 6 months later. This suggested that transplantation may offer a suitable alternative rescue therapy for patients with critical limb ischemia (20). Earlier, a TACT program in Japan was also succeeded in 2003, the research about allogeneic EPCs transplantation also suggested that EPCs transplantation was very effective on vascular diseases. Meanwhile, it indicated that EPCs immunogenicity was not very high.

Now, how to optimize the EPCs proliferation and amplification is a great task for transplantation. Many scholars try

to make the EPCs culture system better. They have tried to use autologous serum (21), autologous culture system (22) and osteopontin (23). Therefore, this field is interesting and ponderable. In our experiment, we used the freeze-drying technology for better maintenance of proteins biological activity, VA-pro was extracted by water solvent and ultrasonic wave method (24). The experimental results provided that VA-pro can promote EPCs proliferation and migration.

Notch signaling pathway promotes proliferative signaling ways during neurogenesis and gets involved in heart development, neovascularization and angiogenesis process (25,26). The key link of Notch1 signaling pathway is the activation of Notch1 Intracellular Domain (NICD). Notch1 combines with the ligands, and then NICD is released into the cytoplasm and gets into nucleus, it binds to CSL (C-promoter binding protein-1, CBF-1) and a complex is formed to activate the genes of transcription inhibitory factor family, such as Hes, Hey or Herp (27). To be expected, it is related to EPCs proliferation, differentiation (4) and tube formation (5). EPCs activity reduces when Notch mRNA is inhibited (28). Hes1 is the direct downstream target of Notch signaling pathway and plays an essential role in the development of blood vessel and heart, etc. Our data revealed that the level of activatory NICD and Hes1 were increased after intervention with VA-pro, which suggested that Notch signaling pathway may be involved in the promoting effect of VA-pro on EPCs proliferation and migration. Akt/mTOR signaling pathway is directly related to cell proliferation, quiescence and longevity via regulating the cell cycle. One research showed that it was involved in progesterone-induced EPC viability (3). Interestingly, VA-pro elevated the phosphorylation level of Akt and mTOR in our research. Thus, the effect of VA-pro on EPCs may be associated with the Akt/mTOR signaling pathway.

Actually, there is much crosstalk between Notch and Akt signaling pathways (29,30). Previous studies on Notch and Akt signaling pathway suggested that Notch signaling pathway could stimulate the Akt signaling pathway by decreasing PTEN (phosphatase and tensin homology deleted on chromosome 10) levels (31). PTEN can stop all downstream effects of Akt signaling pathway by reducing the AKT activation, and it affects a variety of cellular functions widely. Hes1 can inhibit PTEN effectively via binding to its promoter, PTEN transcription will increase when shRNA silence of Hes1 is performed (31,32).

Interactions between Notch and Akt signaling pathways provide the basis for the similar results of NICD, Hes1, p-Akt and p-mTOR affected by VA-pro in this study, Research of the signaling pathway in this study just remind a possible molecular mechanism of VA-pro on EPCs, but it assists our main purpose, which is to find the effective components of VA-pro preferably. We aim to prove that VA-pro really work on EPCs primarily, and then we confirm the effective components of VA-pro gradually, based on VA characteristic of regeneration and in the hope of subsequent artificial extraction or synthetic.

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