EXPERIMENTAL RESEARCH

The Effect of Xuefu Zhuyu Decoction (血府逐瘀汤) on in vitro Endothelial Progenitor Cell Tube Formation*

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ABSTRACT  Objective: To observe the effect of Xuefu Zhuyu Decoction (血府逐瘀汤)-containing serum (XFZYD-CS) on endothelial progenitor cell (EPC) tube formation in vitro. Methods: Mononuclear cells from rat bone marrow were prepared in a cell density gradient centrifuge, EPCs were separated by the differential attachment method, and observed with inverted microscope for the effect of XFZYD-CS on EPC tube formation. Results: After one day, EPCs exposed to the serum containing 5%, 10% and 15% XFZYD-CS formed typical tubes or vessel networks. The tube formation time was two days ahead of the control group and the size of most tubes in the serum groups was smaller than in the control group. Conclusion: XFZYD-CS could induce EPC angiogenesis and hasten tube formation, especially in capillary vessels. The study provides experimental evidence for the plausibility of Xuefu Zhuyu Decoction in the treatment of ischemic diseases. KEYWORDS  Xuefu Zhuyu Decoction, endothelial progenitor cells, tube formation

Endothelial progenitor cells (EPCs) are the precursor of angioblasts. In 1997 Asahara, et al reported that purified CD34+ hematopoietic progenitor cells from adult peripheral blood can differentiate into endothelial cells in vitro and incorporate into neovessels at sites of ischemia in vivo. Since then, evidence has accumulated that CD34 cells participate not only in embryonic but also in postnatal vasculogenesis. These results highlight the function of EPCs in angiogenesis, further developing current thinking about postnatal vasculogenesis and vascular injury repair and could lead to the development of new clinical therapies for ischemia diseases.

The herbal formula Xuefu Zhuyu Decoction (血府逐瘀汤, XFZYD, "Drive out stasis from the mansion of blood decoction") was first recorded in 1830 AD. in "Yi Lin Gai Cuo (医林改错, Corrections of Errors Among Physicians)" by WANG Qing-ren. It is composed of the following ingredients: Angelica sinensis (Oliv.) Diels 9 g, Rehmannia glutinosa Libosch. 9 g, Prunus persica 12 g, Carthamus tinctorius L. 9 g, Citrus aurantium L. 6 g, Paeonia lactiflora Pall. 6 g, Bupleurum chinense DC. 3 g, Glycyrrhiza uralensis Fisch. 6 g, Platycodon grandiflorum 4.5 g, Ligusticum Chuanxiong Hort. 4.5 g, and Cyathula officinalis Kuan 9 g. This herbal combination is considered to be a modification of an earlier formula, recorded in 1291 AD., known as Taohong Siwu Decoction (桃红四物汤), "Four-Substance Decoction with Safflower and Peach Pit" which was originally codified in Wang Haogu's "Yi Lei Yuan Rong (医垒元戎, Supreme Commanders of the Medical Ramparts).

METHODS

Preparation of XFZYD-containing Serum

The herbal material for XFZYD was supplied by the Fujian Institute of Traditional Chinese Medicine, Fujian University of Traditional Chinese Medicine and identified by Prof. QIU Song-ping. The herbs were prepared by water extraction twice in a manner consistent with common usage in China and therefore involved two decoctions. The extract was filtered and condensed to make the concentration equivalent to 1.3 g herb/mL, and then kept at 4℃.

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Twelve purebred Sprague-Dawley rats (lot No. 0037614, SPF grade), six weeks old, were obtained from Slac Laboratory Animal Company, Shanghai. Half were males and half females, all in good health and weighing $150 \pm 20$ g. They were raised in the animal center at Fujian University of Traditional Chinese Medicine and all animal procedures were performed according to the ethical principles for the care and use of laboratory animals. After three days of suitable feed, the rats were randomly distributed into two groups. In the first group, each of the six rats was orally administered the XFZYD solution at 13 mL/kg twice daily for seven days. The dosage of the medicine was equal to 10 times the human dosage. Within two hours of the last dosage of the XFZYD solution, blood from the rats was obtained from the arteria cruralis, centrifuged at 4,000 r/min for 30 min and the XFZYD-containing serum (XFZYD-CS) was collected. In the second group, rats were orally administered normal saline in the same protocol. Their serum was used as the control serum. Both the XFZYD-CS and control serum were inactivated by heating at 56°C for 30 min, filtered through a 0.22 μm filter, and stored at -20°C until use.

Isolation and Culture of EPCs

EPCs were isolated and cultured according to previously described techniques\(^{(7)}\). Briefly, total mononuclear cells (MNCs) were isolated from rat bone marrow by density gradient centrifugation using Ficoll separation (Sigma, USA). The MNCs were cultured at $1 \times 10^6$ cells/mL in the endothelial differentiation medium consisting of DMEM (Hyclone, USA) supplemented with 15% fetal bovine serum (PAA, Austria), 10 ng/mL recombinant human vascular endothelial growth factor (VEGF) (PeproTech, USA), 4 ng/mL basic fibroblast growth factor PeproTech, USA, and 4 μg/mL bovine pituitary extract (GIBCO, USA) on gelatin-coated dishes. After four days in culture, non-adherent cells were washed away with PBS, new media was applied and the culture was maintained for nine days, with the media changed every three days.

Tube Formation Assay

Attached cells were stimulated with XFZYD-CS (at final serum concentrations of 5%, 10%, 15% with DMEM) on the 8th day and the control group with control serum. The tube formation was observed daily and images were obtained using an Olympus 1 × 70 (Olympus, Japan) inverted fluorescence microscope equipped with a digital camera.

Immunohistochemistry

At the end of the culture, the cells attached to coverslips were fixed in 4% paraformaldehyde for 15 min at room temperature, and washed three times with PBS. The DAB method protocol was complide with the User Manual of MaxVision Kit (Maxim, China). The diluted concentrations of rabbit anti-rat von Willebrand factor (vWF, Maxim, China) and anti-VEGF receptor-2 antibodies (Abcam, USA) were 400× and 200×, respectively. Photomicrographs were taken with an Olympus PM-C35DX digital camera.

RESULTS

EPCs Morphological Change

EPCs separated from rat bone marrow are round. After 48-h culture, some cells adhered to the dish. On the 3rd day, these cells gathered to form colonies (Figure 1A) and on the 5th day the cells lined up in the typical EPC thready appearance (Figure 1B). Two days later most of the cells elongated into the spindle shape (Figure 1C) and on the 11th day formed tubes (Figure 1D).

EPCs Differentiation Analysis

In order to further analyze the state of EPC differentiation, the EPCs were cultured for 13 days to express more marker proteins. Immunohistochemistry results showed that EPCs spread out in flat or spindle

![Figure 1. EPC Morphological Change (×100)](image_url)

Notes: A: EPC colonies after two days culture; B: EPC linear array after four days culture; C: EPC spindle shape after six days culture; D: EPC tube formation after 10 days culture