Contents lists available at ScienceDirect IF: 0.926

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi: 10.1016/S1995-7645(14)60173-5

Effect of Danzhi decoction on expression of angiogenesis factors in patients with sequelae of pelvic inflammatory disease

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ARTICLE INFO

Article history:
Received 24 September 2014
Received in revised form 10 October2014
Accepted 15 November 2014
Available online 20 December 2014

Keywords:
Danzhi decoction
Sequelae of pelvic inflammatory disease
Endometrial cells
Angiogenesis factor
Microenvironment

ABSTRACT

Objective: To investigate the effects of traditional Chinese medicine, Danzhi decoction, on the expression angiogenesis factors in human endometrial cells during the sequelae of pelvic inflammatory disease (SPID) and explore the role of Danzhi decction in improving the blood stasis microenvironment of SPID. **Methods:** A three-dimensional (3D) co-culture system including human vascular endothelial cells (VECs), endometrial stromal cells and glandular epithelial cells was established *in vitro* and treated with Danzhi decoction, sterilized water and aspirin respectively. A Milliplex multifunctional liquid chip technique was used to measure the expression levels of vascular endothelial growth factor (VEGF)-A/C/D, fibroblast growth factor -1/2, angiopoietin-2, epidermal growth factor (EGF), HB-EGF, bone morphogenetic protein-9, endoglin, endothelin-1, granulocyte colony stimulating factor, hepatocyte growth factor, interleukin-8, follistatin, placenta growth factor and leptin. The location of angiogenesis factors was monitored by immunofluorescence labeling and confocal laser scanning microscope 3D reconstruction. **Results:** Endometrial stromal cells and glandular epithelial cells were isolated and primary cultured for establishing a 3D co-culture system. The levels of VEGF-A/C/D in Danzhi decoction group and aspirin group were significantly lower than those in mock group ($P<0.05$), while there was no significant difference between Danzhi decoction group and aspirin group ($P>0.05$). Furthermore, the alterative location of VEGF-A/C/D was observed in the cytoplasm of endometrial glandular epithelial cells. **Conclusions:** Danzhi decoction may inhibit the expression of VEGF in the blood stasis microenvironment of SPID by targeting the cytoplasm of endometrial glandular epithelial cell.

1. Introduction

Sequelea of pelvic inflammatory disease (SPID), once called chronic pelvic inflammatory disease (CPID), is the legacy lesions of CPID. SPID is a common disease and frequently-occurring disease in the department of gynecology. Its clinical features include chronic pelvic pain and recurrent inflammation. SPID leads to infertility, tubal pregnancy and pelvic congestion syndrome, which seriously influences the health of women and the quality of life and increases the economic burden of family and society[1,2]. There is no definition of SPID in Chinese traditional medicine but some symptoms, such as abdominal pain, abdominal mass, irregular menstruation, infertility and morbid leucorrhea[3]. Chinese medical science considers that causes including cold and dampness damage uterus and uterine collateral and lead to Qi obstruction, stagnation of blood stasis, recurrent and refractory lingering. The stagnation of blood stasis is the main pathogenesis of SPID. Thus, promoting dredging the channel, circulation and removing stasis are principles for SPID treatment[4].

Chinese medicinal formulae, Danzhi decoction has a role in stasis-reducing and venation-flowing[5]. It is an effective medicine for improving the blood stasis microenvironment of SPID. In traditional Chinese medicine, promoting dredging the channel, circulation and removing stasis are principles for SPID treatment[5]. Danzhi decoction, a kind

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Foundation project: This study is supported by a grant from the National Natural Science Foundation of China (81373677).

of Chinese medicinal formulae for disperses blood stasis and dredges collateral, is consisted with danshen root, mulberry twig, szechwan lovage rhizome, himalayan teasel root, weeping forsythia fruit, lychee seed, corydalis tuber and nutgrass galingale rhizome. It is an effective treatment for SPID and pelvic congestion syndrome. In these Chinese medicinal formulae, danshen root activate blood and resolve stasis. Mulberry twig dispel wind to free the collateral vessels. Szechwan lovage rhizome and himalayan teasel root promote Qi to activate blood. Weeping forsythia fruit and lychee seed are good at antipyretic detoxicate and reducing swelling. Compendium of Materia Medica has indicated that corydalis tuber have analgesic effects. Nutgrass galingale rhizome plays a role in regulation of painful menstruation. All these drugs have a role in activating blood circulation to dissipate blood stasis, antipyretic detoxicate, reducing swelling and analgesia. Modern pharmacological studies have found that dan-shen root expand the coronary artery and improve microcirculation[6]. Otherwise, it has a role in anti-bacteria and anti-tumorigenesis[6]. Mulberry twig exert anti-inflammatory effect by regulating mitogen-activated protein kinases signaling pathway[7]. Szechwan lovage rhizome increase blood flow in coronary artery and brain and improve blood circulation in heart and brain[8]. Himalayan teasel root can stop bleeding and alleviate pain[9]. Weeping forsythia fruit have a role in anti-bacteria and anti-viruses[10]. Lychee seed show functions in reducing blood glucose, blood fat regulation, anti-oxidant and anti-viruses[11]. Corydalis tubers not only have analgesic effects but reducing the fever[12]. Nutgrass galingale rhizomes have estrogen-like activity in regulation of painful menstruation[13].

In this study, the human endometrial cells were 3D cultured *in vitro*. Then we established 3D cultured model of endometrial stromal cells and glandular epithelial cells in the blood stasis microenvironment of SPID. The effects of Danzhi decoction on angiogenesis factors were observed in this model. Moreover, we investigated the role of Danzhi decoction in improving the blood stasis microenvironment of SPID. All these experiments are aimed to provide theory basis for treatment of SPID.

2. Materials and methods

2.1. Clinical samples

Endometrial samples were collected from patients, who underwent complete hysterectomy for simple uterine fibroids or received hysteroscopy for benign diseases in the Department of Gynecology, Oriental Hospital of Beijing University of Chinese Medicine. All patients with menstrual regularity were under 45 years old and did not receive preoperative sex hormone in 3 months. All tissues were confirmed to be proliferative phase of endometrium but not endometrial lesions by postoperative pathological examination. The uterine endometrium was got from the bottom of uterus using a curet under aseptic conditions. All samples were used after obtaining informed consent. The Oriental Hospital of Beijing University of Chinese Medicine Ethics Committee approved all protocols according to the Helsinki Declaration (as revised in Edinburgh 2000).

2.2. Danzhi decoction preparation

Danzhi decoction included 10 g danshen root, 10 g mulberry twig, 6 g szechwan lovage rhizome, 15 g himalayan teasel root, 10 g weeping forsythia fruit, 10 g lychee seed, 10 g corydalis tuber and 6 g nutgrass galingale rhizome. All the traditional Chinese medicine was obtained from the Dispensary of Traditional Chinese Medicine, Oriental Hospital of Beijing University of Chinese Medicine. The doses of these traditional Chinese medicines comply with the Chinese pharmacopoeia. Eight drugs were concentrated to 100 mL (77 g/100 mL) after 1 hour soaking and 1hours decocting. The administration concentration was confirmed as 5 mg/mL by CCK8 (Pep- tide Institute, Inc., Osaka, Japan) toxicity test.

2.3. Primary culture of endometrial stromal cells and glandular epithelial cells

The endometrium tissues were washed and divided into pieces (0.5-1.0 mm^3). Then tissues were mixed with 0.1% collagenase Ⅰ (Sigma, St-Louis, MO, USA) and 40 μg/mL Deoxyribonuclease I (Sigma). The cell suspension was subjected to Dynabeads® CD45 (DynalBiotech LLC, Brown Deer, WI) for removing white blood cell. Tissues were purified using CELLection Kit™ (Dynal, Oslo, Norway) Epithelial Enrich. Glandular epithelial cells were obtained by positive selection, while endometrial stromal cells were collected by negative selection. The cell suspension of endometrial stromal cells and glandular epithelial cells were incubated with vimentin and keratin, respectively. Flow cytometer was used to detect cell purity and the purity of them exceeded 90% indicated successful isolation of primary cell. After that, cells were cultured in 6-well NUNC UpCell plates (Nunc, Rochester, NY, USA) in a humidified containing of 5% CO_2 incubator at 37 ℃. When cell confluence was observed under inverted microscope, membranes were placed on the cell layer and incubated for 5-6 min at room temperature (20-25 ℃). Then cells were transferred from NUNC UpCell to membranes.

2.4. Culture of human vascular endothelial cells (VECs)

Human VEC cell line (HUVEC), was purchased from the Cell Center of Peking Union Medical University and cultured in ECM complete medium (Gibco, Grand Island, NY, USA). Cells were divided into three groups: Danzhi decoction, aspirin and mock. 5 mg/mL Danzhi decoction and 4 mmol/mL aspirin were filtered by 0.22 μm filter membrane for degerming and treated cells for 24 hours. But cells in mock group were treated with sterile water. Treated cells were cultured in 6-well NUNC UpCell plates in a humidified containing of 5% CO_2 incubator at 37 ℃. When cell confluence was observed under inverted microscope, membranes were placed on the cell layer and incubated for 5-6 min at room temperature (20-25 ℃). Then cells were transferred from NUNC UpCell to membranes.

2.5. Establishment of a 3D co-culture system

Membrane-carried HUVEC cells were placed on the surface of NUNC UpCell culture plates for 30min at 37 ℃. Then HUVEC cells were seeded in NUNC UpCell culture plates. Endometrial stromal cells were seeded on the surface of HUVEC cells and glandular epithelial cells were placed on the top floor to establish a 3D co-culture system.

2.6. Detection of angiogenesis factor

Cells in 3D co-culture system were treated with cytokine, IL-17A/IL-17F (Sigma). The supernatant was collected and subjected to Milliplex multifunctional liquid chip technique (Millipore, Billerica, MA, USA) for angiogenesis factors, such as vascular endothelial growth factor (VEGF)-A/C/D, fibroblast growth factor (FGF)-1/2, angiopoietin-2, epidermal growth factor (EGF), HB-EGF, bone morphogenetic protein(BMP)-9, endoglin, endothelin (ET)-1, granulocyte colony stimulating factor (G-CSF), hepatocyte growth factor (HGF), interleukin (IL)-8, follistatin, placenta growth factor (PLGF) and leptin.

2.7. Immunofluorescence (IF)

Cells were fixed with 3% paraformaldehyde and permeabilized using 0.2% Triton X-100. Then the fixed cells were incubated with the VEGF (Cell Signaling, Danvers, MA, USA) (1:500) and keratin (Cell Signaling) (1:500) primary antibody. The secondary antibody is an Alexa Fluor-conjugated IgG (Invitrogen, Carlsbad, CA, USA). Fluorescence confocal images were captured using a LSM 5 Pascal Laser Scanning Microscope (Zeiss Germany, Oberkochen, Germany) using a×10 lens and Laser Scanning Microscope LSM PASCAL software (version 4.2 SP1)[14].

2.8. Statistical analysis

Results are expressed as Mean±SD. Significance was established, with the SPSS statistical package for Windows Version 16 (SPSS, Chicago, IL, USA), using one-way ANOVA and LSD-t or Tambane's $T2$ test when appropriate. Difference were considered significant when $P<0.05$.

3. Results

3.1. Isolation of endometrial stromal cells and glandular epithelial cells

The endometrial stromal cell shape was round under light microscope. After 30 min attachment, cells showed spindle-shaped like fibroblasts. The glandular epithelial cells showed tubular or grape-like growth. After 24 hours attachment and 3 days growth, cells were arranged in swirl (Figure 1A and 1B). Immunostaining revealed that endometrial stromal cells showed negative staining of keratin and positive staining of fibronectin. However, glandular epithelial cells showed positive staining of keratin and negative staining of fibronectin (Figure 1C and 1D). The flow cytometer indicated that the purity of endometrial stromal cell and glandular epithelial cell was 94% and 92%, respectively (Figure 2).

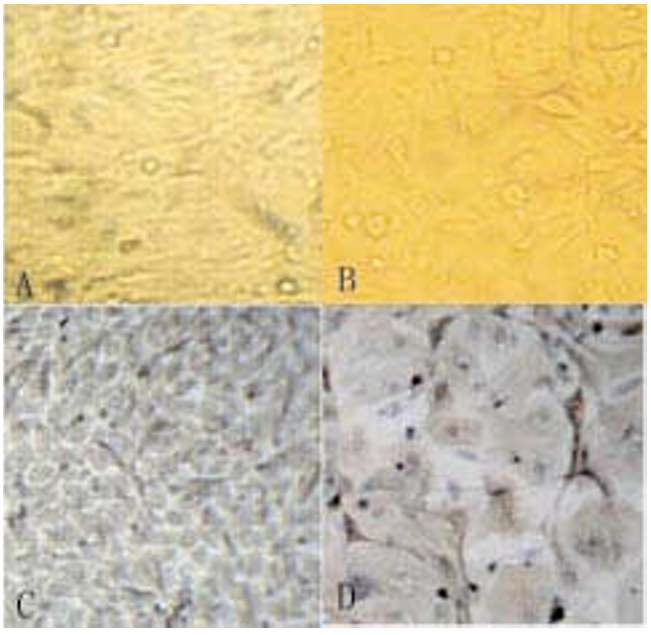

Figure 1. Immunostaining of endometrial stromal cells and glandular epithelial cells (original magnification×400).
A) The microscopic view of endometrial stromal cells. B) The microscopic view of glandular epithelial cells. C) Immunostaining of fibronectin in endometrial stromal cells. D) Immunostaining of keratin in glandular epithelial cells.

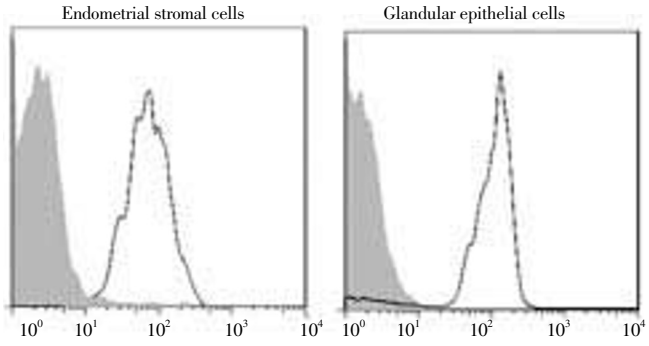

Figure 2. Purity of endometrial stromal cells and glandular epithelial cells.
The purity of endometrial stromal cells was analyzed by flow cytometry using a vimentin antibody. The purity of glandular epithelial cells was detected by flow cytometry using a keratin antibody. The dashed area showed control antibodies.

3.2. A 3D model of endometrium in vitro

Cells showed a 3D growth under the light microscope. Glandular epithelial cells showed a single columnar growth and endometrial stromal cells located at collagen matrix under the glandular epithelial cells. Under the electron microscope, we found that the surface of co-culture system was covered by glandular epithelial cells. These cells had polarity and microvillus on the plasma membrane. There was intercellular tight junction, intermediate junction and desmosome junction. Cytoplasm was rich in mitochondrion, ribosome, lipid droplet, big nuclear with obvious nucleolus (Figure 3).

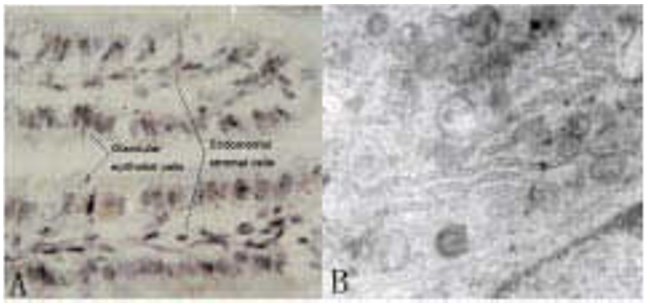

Figure 3. 3D model of endometrium *in vitro*.
A) The light microscopic view of the 3D model of endometrium (original magnification×400). B) The electron microscopic view of the 3D model of endometrium (original magnification×10 000).

3.3. Comparison of angiogenesis factor expression among three groups

As shown in Table 1, the levels of VEGF-A/C/D in Danzhi decoction group were significantly lower than those in mock group ($P<0.05$). Similarly, these three angiogenesis factors in aspirin group were obviously lower than those in mock group ($P<0.05$). However, there was no significant difference between Danzhi decoction group and aspirin group ($P>0.05$).

3.4. Glandular epithelial cell is sensitive to Danzhi decoction

Above results indicated that Danzhi decoction inhibited VEGF expression. Next we sought to investigate which cell was the therapeutic target of Danzhi decoction. Double IF for VEGF (green) and keratin or vimentin (red) revealed that the glandular epithelial cell was sensitive to Danzhi decoction (Figure 4). An obvious reduction of VEGF level in glandular epithelial cells with keratin staining was observed after

Table 1
Comparison of angiogenesis factor expression (pg/mL).

Angiogenesis factors	Danzhi decoction group	Aspirin group	Mock group	*F*	*P*
VEGF-A	145.23±75.25*	163.15±81.15*	227.56±62.24	2.470	0.010
VEGF-C	155.23±65.25*	167.64±64.67*	217.56±72.24	2.482	0.009
VEGF-D	158.25±68.23*	168.62±68.65*	218.55±77.25	2.370	0.015
FGF-1	52.88±16.80	49.77±17.60	50.89±16.64	0.524	0.595
FGF-2	49.63±17.86	50.89±16.60	49.36±20.11	0.242	0.824
Ang-2	195.23±68.25	198.32±75.52	201.85±76.67	0.598[a]	0.602
EGF	480.20±65.98	485.02±71.89	483.38±68.86	0.418	0.695
HB-EGF	368.58±141.25	378.85±136.52	375.57±135.53	0.624	0.525
BMP-9	33.27±8.10	34.72±8.45	35.53±9.01	1.624	0.090
Endoglin	219.95±42.24	215.59±52.42	222.51±54.65	0.612	0.595
ET-1	87.12±7.96	91.22±8.08	89.32±7.77	0.568	0.655
G-CSF	146.33±14.0	156.12±15.6	149.45±16.6	0.674	0.497
HGF	77.23±7.36	78.32±7.63	79.96±7.69	0.625	0.528
IL-8	26.70±4.01	25.90±3.88	26.30±3.29	0.254	0.799
Follistatin	126.39±8.97	126.95±7.45	122.74±9.58	0.608	0.538
PLGF	35.72±8.01	36.73±7.66	37.75±8.09	0.828	0.426
Leptin	13.30±3.55	13.45±3.99	13.84±3.70	0.142	0.898

* $P<0.05$ *vs.* mock group.

Danzhi decoction treatment (Figure 4).

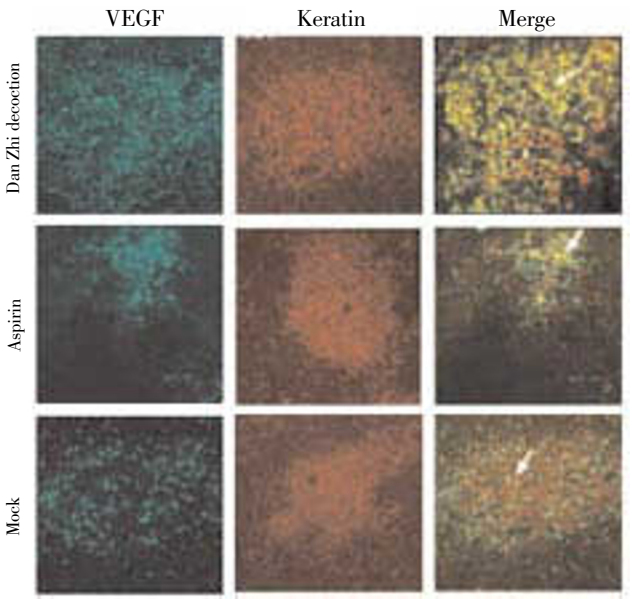

Figure 4. Double IF for VEGF and keratin in glandular epithelial cells (original magnification×100).
Cells from different group (Dan Zhi decoction, aspirin or mock group) were subjected to double IF for VEGF and keratin. These data showed that Danzhi decoction led to a prominent reduction of VEGF expression in glandular epithelial cells.

4. Discussion

In order to investigate the mechanisms involved in Danzhi decoction improved blood stasis microenvironment of SPID, human endometrial cells in proliferative phase were 3D cultured *in vitro*. A blood stasis microenvironment of SPID model containing endometrial stromal cells and glandular epithelial cells was established *in vitro*. Milliplex multifunctional liquid chip technique was performed to determine the expression of angiogenesis factors, such as VEGF-A/C/D, FGF-1/2, angiopoietin-2, EGF, HB-EGF, BMP-9, endoglin, ET-1, G-CSF, HGF, IL-8, follistatin, PLGF and leptin. Compared with mock group, Danzhi decoction (5 mg/mL) inhibited VEGF-A/C/D expressions in human endometrial cells within blood stasis microenvironment of SPID. Previous studies have considered that the development of blood stasis microenvironment in SPID was correlated with vascular endothelium function[15]. Dysfunction of vascular endothelium is the key factor in the initiation and progression of blood stasis. However, VEGF is the only specific biomarker for vascular endothelium injury. VEGF stimulates VEC division and proliferation and subsequently changes microcirculation and leads to diseases. Previous studies reported that the Chinese medicinal formulae including lobed kudzuvine root, Chinese angelica, malaytea scurfpea fruit, prepared rhubarb and leech could prevent VECs from anoxia induced damage[16]. Lobed kudzuvine root increased physiological activity of VECs. Chinese angelica, malaytea scurfpea fruit, prepared rhubarb and leech had obvious protective effect on VECs. Another Chinese medicinal formulae including peach seed, safflower, pangolin scales, cowherb seed, blue turmeric rhizome, Chinese angelica, Chinese thorowax root, immature tangerine fruit, nutgrass galingale rhizome, common selfheal spike, kelp, chekiang fritillary bulb and loofah was applied to treat cyclomastopathy by reducing VEGF expression, suggesting that Chinese medicinal formulae with disperse blood stasis and dredge collateral function inhibits neovascularization by reducing VEGF expression[17]. Our studies indicated that Danzhi decoction decreased the level of VEGF expression in endometrial cells in the blood stasis microenvironment of SPID. Accordingly, it may improve endothelium function through regulating the endocrine function of VECs. Subsequently Dan Zhe decoction may exert positive effects on improving the blood stasis microenvironment of SPID. But the precise mechanisms need to be further investigated.

Modern medicine studies have considered that inflammation plays a critical role in the initiation and development of SPID[18, 19]. Several studies showed that most of SPID patients did not get bacterial infections, but illness had continued development[20]. Thus, bacteria-infective inflammation cannot explain the causes of SPID. Now, most of researchers consider that inflammatory injury is the key pathological change of SPID. Persistent activation of inflammatory factors and effusion of inflammatory cells lead to inflammatory autoimmune injury and subsequently indirectly or directly influences the initiation, progression and prognosis of SPID. Persistent inflammation results in blood stasis. In turn, blood stasis promotes inflammation. Prostaglandin epoxy enzyme inhibitor, aspirin, induces thrombosis in peripheral vessels by inhibiting platelet aggregation and accordingly increases the volume of blood flow. Recent researches have reported that aspirin is used to improve thrombosis in obstetrical and gynecological diseases. In this study, we chose aspirin (4mmol/mL) as a positive control for medicine treated SPID cell model. Our data indicated that the comparison of VEGF expression between Danzhi decoction and aspirin group was not statistically significant, suggesting that both Danzhi decoction and aspirin could evidently reduce VEGF expression in endometrial cell in the blood stasis of SPID. Danzhi decoction may repair injured VECs and improve local microcirculation to achieve the same clinical outcome of aspirin. Furthermore, double IF found that the reduction

of VEGF-A/C/D was observed only in the cytoplasm of glandular epithelial cell. These results provide a potential target for Danzhi decoction mediated VEGF reduction.

In conclusion, we establish a 3D endometrial cell model with the blood stasis of SPID via endometrial stromal cells and glandular epithelial cells *in vitro* culture. We find that Danzhi decoction improves the blood stasis microenvironment of SPID by reducing VEGF expression in glandular epithelial cells. All these data provide theoretic basis for the clinical treatment of SPID.

Conflict of interest statement

We declare that we have no conflict of interest.

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