Effect of Hua Yu Xiao Zheng decoction on the expression levels of vascular endothelial growth factor and angiopoietin-2 in rats with endometriosis

ZHEN-ZHEN CHEN¹ and XIN GONG²

¹School of Traditional Chinese Medicine, Beijing Key Lab of TCM Collateral Disease Theory Research, Capital Medical University, Beijing 100069; ²Department of Gynecology, Dong Fang Hospital of Beijing University of Chinese Medicine, Beijing 100078, P.R. China

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Abstract. The aims of the present study were to investigate the effects of a traditional Chinese medicine, Hua Yu Xiao Zheng (HYXZ) decoction, on surgically induced endometriosis in a rat model and to determine the possible underlying regulatory mechanisms. A total of 108 female Sprague-Dawley rats were divided into the control group (n=12) and endometriosis group (EM group; n=96), in which endometriosis was surgically induced in model rats by autotransplantation of endometrial tissues and 72 rats survived. After 3 weeks, the EM model rats were randomly divided into four subgroups (n=18), including the untreated model group, and three groups administered 7, 14 or 21 g/kg HYXZ decoction. Following 28 days of treatment, the associated proteins and genes of ectopic endometrial tissues were analyzed using immunohistochemistry, western blotting and quantitative polymerase chain reaction to investigate the underlying mechanisms. Compared with the model group, the size of the endometriotic implants decreased significantly in the HYXZ-treated groups. Furthermore, the expression levels of vascular endothelial growth factor (VEGF) and angiopoietin-2 (Ang-2) were significantly decreased in HYXZ-treated groups compared with the model group. These results indicate that HYXZ affected the inhibition of angiogenesis and decreased the endometriotic implant volumes and histopathological scores. The effectiveness of HYXZ may be partially attributed to the decrease of VEGF and Ang-2 expression levels in the ectopic endometrium.

Introduction

Endometriosis is defined as the growth of functional endometrial tissue outside the uterine cavity, and is characterized by the presence of chronic pelvic pain and infertility in females of a reproductive age (1). This disease affects women in the 15-49 years age group and presents a serious clinical problem with reproductive consequences. Current treatments for endometriosis, including surgery and hormonal therapy, are often insufficient, and result in a high rate of relapse and various side effects, such as hepatic injury and osteoporosis (2). Furthermore, the pathogenesis of endometriosis has not yet been fully elucidated; however, the most widely accepted hypothesis involves retrograde menstruation, which was initially proposed by Sampson in 1927 (3).

Recent studies have demonstrated the importance of neovascularization within the peritoneal cavity for endometriosis pathogenesis. Endometriotic lesions require an adequate blood supply to survive in their ectopic sites (4). Therefore, angiogenesis has become a promising target candidate for endometriosis therapy. Vascular endothelial growth factor (VEGF) is a heparin-binding glycoprotein with potent angiogenic, endothelial cell-specific mitogenic and vascular permeability activities (5). The concentration of VEGF is increased in human endometrium, and it may be important in physiological and pathological angiogenesis (6). VEGF expression varies among different types of lesions, with the early and highly vascularized red lesions exhibiting a greater VEGF expression in comparison with the later-stage and more inactive black lesions (7). In eutopic as well as ectopic endometria, no significant cyclic variations were observed throughout the cycle (7). However, VEGF content was found to be higher in the eutopic glandular epithelium of women with endometriosis during the late secretory phase, possibly suggesting a greater chance of implantation (7). Following the attachment phase, the high VEGF levels may provoke an increase in the subperitoneal vascular network, facilitating implantation and viability in the retroperitoneal space (8).

Neovascularization is considered to be a key factor in the progression of endometriosis; this pathological condition has been defined as an angiogenic disease (9). The angiopoietin
The aqueous extract of herbs was controlled according to the Pharmacopoeia of Chinese Medicine (Beijing, China). The quality of the raw Department of Dong Fang Hospital of Beijing University of Preparing formula.

China; approval no. AEEI-2015-100). All rats were acclimatized for 7 days to ascertain health before the experiments were performed. All procedures were conducted in accordance with the guidelines for the care and use of laboratory animals.

Materials and methods

Animals. Specific-pathogen-free grade female, nonpregnant Sprague-Dawley rats (n=108; body weight, 190-230 g) were obtained from Vital River Laboratory Animal Technology Co., Ltd (Beijing, China; SCXK 2012-0001). Animals were raised at a constant temperature of 25˚C, 50% humidity and 12-h light/dark cycles. Standard rat feed and water were provided ad libitum.

Preparation of formula. All the medicinal plants used to prepare the HYXZ decoction were obtained from the Pharmacy Department of Dong Fang Hospital of Beijing University of Chinese Medicine (Beijing, China). The quality of the raw herbs was controlled according to the Pharmacopoeia of the People's Republic of China (18). The aqueous extract of HYXZ was prepared according to a previously described procedure (19). Briefly, the mixture of herbs listed in Table I was macerated in distilled water for 0.5 h. Following this, the herbs were decocted for 0.5 h, and the residue was then decocted again for 0.5 h. The final extract was subsequently filtered, combined and concentrated to 2.1 g/ml (crude dosage) by heating.

Rat model of endometriosis. The animal model was established by autotransplantation of endometrial tissues, as previously reported by Zhang et al (20). Briefly, 96 rats were anesthetized with intraperitoneal administration of 10% chloral hydrate (m/v) at a dose of 0.35 ml/100 g body weight. Prior to surgery, the abdominal skin was disinfected with 75% ethanol and the abdominal cavity was opened. The uterine vessel was ligated with 5-0 polypropylene sutures. The right uterine horn was removed and placed in a Petri dish containing phosphate-buffered saline (PBS) at 37˚C. Approximately 1 cm of the uterine horn was cut along the longitudinal axis, and then divided in half. Two squares of 5x5 mm open uterus were prepared. Each endometrium segment was fixed with 6-0 sutures to the peritoneal side of the bilateral abdominal wall with the endometrium facing the abdominal wall. Subsequently, the abdominal muscle and skin was closed using 4-0 polypropylene sutures. The control group underwent sham surgery, consisting solely of the unilateral hysterectomy without autotransplantation of endometrial tissues. Following surgery, all rats were treated with cephalexin (0.1 g; Tianjin Pharmaceutical Holdings Gencom Pharmacy Co., Ltd., Tianjin, China) by intraperitoneal injection for 3 days. After a recovery period of 21 days, the endometriosis model rats underwent a second exploratory laparotomy to examine whether the models of experimental endometriosis had been successfully established, which was determined by observation of red/brown color and cystic formation on the implant surfaces (21). In the present experiment, 72 out of 96 model rats survived, with a success percentage of 75%, and these rats were used in subsequent experiments.

Treatment. The 72 rats with successful endometriosis model were randomly divided into four groups as follows (n=18 in each group): Endometriosis (EM group), endometriosis + low-dose HYXZ (EM + L group), endometriosis + mid-dose HYXZ (EM + M group), endometriosis + high-dose HYXZ (EM + H group). Rats in the EM + L, EM + M and EM + H groups were administered with HYXZ at a dose of 7, 14 and 21 g/kg/day, respectively. The mid-dose group, which was similar to the dose administered to female patients, was calculated using the formula of dose translation among different species based on body surface area (22). Rats in the control and EM groups were administered with double-distilled water (10 ml/kg). The drugs and double-distilled water were administered daily by oral gavage for 28 days.

Specimen collection. After treatment for 28 days, the rats were sacrificed. Ectopic endometrial tissues were measured and dissected from the implant sites. Half of the lesion was immediately fixed in 4% paraformaldehyde and then embedded in paraffin for hematoxylin-eosin (HE) staining and immunohistochemical assays. The remaining half of the lesion was stored at -80˚C for analysis of the protein and mRNA levels of VEGF and Ang-2.
Table I. Composition of Hua Yu Xiao Zheng decoction.

| Herb          | Component                        | Grams |
|---------------|----------------------------------|-------|
| Danshen      | *Salviae miltiorrhiza* Bunge, root | 15    |
| Bajitian     | *Morinda officinalis* How, root   | 10    |
| Sanqí        | *Panax notoginseng* (Burk.) F.H. Chen, root | 9     |
| Yiyiren      | *Cox lacryma-jobi* L. var. *ma-yuen* (Roman.) Stapf, seed | 25    |
| Zhebeimu     | *Fritillaria thunbergii* Miq., tuber | 10    |
| Xiuacuo      | *Prunella vulgaris* L., cluster   | 15    |
| Bianxu       | *Polygonum aviculare* L., overground part | 15    |
| Dahuang      | *Rheum palmatum* L., root         | 5     |
| Qumai        | *Dianthus superbus* L., overground part | 15    |
| Yanhusuo     | *Corydalis yanhusuo* W.T. Wang, tuber | 10    |
| Shuizhi      | *Whitmania pigra* Whitman, whole body | 3     |
| Puhuang      | *Typha angustifolia* L., pollen   | 6     |
| Xuejüe       | *Daemonorops draco* Bl., resin    | 2     |
| Total amount |                                  | 140   |

H&E staining. Both eutopic endometrium and ectopic endometria of rats were surgically detached. The endometriotic implants were first fixed in 10% formaldehyde solution, embedded in paraffin and cut into 4-μm sections. These sections were stained with H&E and examined under a light microscope. The morphology was captured with a digital camera (Nikon 4500; Nikon Corp., Tokyo, Japan).

Immunohistochemical analysis. The paraffin-embedded sections of ectopic endometrial tissue were subjected to antigen retrieval with citric acid buffer, and were then incubated overnight at 4˚C with the following primary antibodies: VEGF antibody (Ab46154; dilution, 1:200; Abcam, Cambridge, UK); and Ang-2 antibody (Ab180820; dilution, 1:200; Abcam). Subsequent to washing in PBS (pH 7.4) three times, the sections were incubated with secondary antibody (Ab205718; dilution, 1:50; Abcam) for 60 min at 37˚C. Following incubation, the slides were rewash with PBS and incubated with 0.01% 3,3-diaminobenzidine tetrahydrochloride hydrate for ~1 min. Sections were then washed thoroughly in PBS three times for 5 min each, and counterstained in hematoxylin for 20 sec. Finally, the tissues were dehydrated, rendered transparent and mounted prior to being analyzed under a microscope. Negative control tissues were treated with the same procedure, using PBS instead of primary antibody. Images of the tissues were captured with a digital camera (Nikon 4500). The positive area and optical density (OD) of positive cells were determined by measuring three randomly selected microscopic fields for each slide. The immunohistochemical index was defined as the average integral OD (AIOD), as follows: AIOD = positive area x OD/total area (23).

Western blot analysis. The tissues of the ectopic endometria were sectioned and lysed in radioimmunoprecipitation assay lysis buffer (C1053; Applygen Technologies, Inc., Beijing, China). The concentration of protein used for western blot analysis was first quantified by a bicinchoninic acid assay (P1511; Applygen Technologies, Inc.). Next, protein samples (20 µg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 1 h at room temperature in 5% skimmed milk. Subsequent to blocking, the membranes were incubated at 4˚C overnight with the following primary antibodies: VEGF (Ab46154; dilution, 1:2,000), Ang-2 (Ab180820; dilution, 1:2,000) and GAPDH (Ab181602; dilution, 1:20,000). All the primary antibodies were obtained from Abcam. Following rinsing in Tris-Buffered Saline and Tween-20 three times, the PVDF membranes were incubated with the secondary antibodies (C1633; dilution, 1:2,000; Applygen Technologies, Inc.) at room temperature for 1 h. Images were obtained using electrochemiluminescence (NCI4106; Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA), prior to being analyzed using Image J software v1.46r (National Institutes of Health, Bethesda, MD, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated from ectopic endometrial tissues using TRIzol reagent (15596-026; Thermo Fisher Scientific, Inc.), and then reverse transcribed into cDNA with a Reverse Transcription kit (D6130; Takara Bio, Inc., Otsu, Japan). The resulting cDNA was diluted 10-fold in sterile water, and aliquots were subjected to qPCR. The PCR primer pairs used in the analysis were designed and synthesized by Taihe Biotechnology Co., Ltd. (Beijing, China; Table II). PCR cycles were composed of one cycle of 95˚C for 5 min to denature all proteins, 40 cycles of 95˚C for 20 sec, and then 65˚C for 30 sec. The reaction was terminated at 72˚C for 5 min and quenched at 4˚C. qPCR reactions were performed with an ABI Prism 7700 sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The relative expression of each target gene compared with GAPDH was analyzed using the 2-ΔΔCq method (24).

Statistical analysis. All statistical analyses were performed using the SPSS software, version 17.0 (SPSS, Inc., Chicago, IL,
Results

Treatment with HYXZ reduces the volume of the lesion. After the 28-day treatment with HYXZ, the longest lengths and perpendicular widths of the lesions were measured by a vernier calliper. The volume of the endometriotic lesion was calculated according to the following formula: \( \frac{1}{2} \times \text{width}^2 \times \text{length} \) (25). A comparison of the HYXZ-treated groups and the EM model group demonstrated a statistically significant reduction in the lesion size following the treatment (P<0.05). As the dose increased, the volume of the lesion size was further reduced (Fig. 1; Table III).

Light microscopy analysis. The H&E staining results demonstrated that the shape of the eutopic endometrium epithelial cells was columnar, and the glandular epithelial and stromal cells were normal in the control group. In the EM model group, H&E staining revealed that the ectopic endometrium was covered with connective tissues and that the glands and intercellular substances grew well and intensively. The intima was thick and the glandular and superficial epithelia formed a high column. Evident hyperplasia and angiopoiesis were also observed. In the EM + L, EM + M and EM + H treatment groups, the columnar shape was nearly natural, while the number of glands and glandular epithelial cells was almost normal (Fig. 2).

Expression levels of VEGF and Ang-2 as evaluated by immunohistochemistry. As illustrated in Fig. 3 and Table IV, the immunohistochemical scores of VEGF and Ang-2 were higher in the EM model group compared with the control sham-operated group (P<0.05). Following HYXZ administration (EM + L, EM + M and EM + H groups), the Ang-2 immunohistochemical score was significantly decreased compared with that of the EM group (P<0.05). Similarly, the VEGF immunohistochemical score was significantly decreased in the EM + H group compared with the EM group (P<0.05), but not markedly altered in the EM + L and EM + H groups.

Protein levels of VEGF and Ang-2. As shown in Fig. 4 and Table V, the protein levels of VEGF and Ang-2 in the EM group manifested a statistically significant increase in comparison with those in the control group (P<0.05). Compared with the
Figure 2. Pathological morphology of the eutopic (control) and ectopic (EM groups) endometrial tissues in each group after 28 days of treatment analyzed by hematoxylin-eosin staining and light microscopy (magnification, x40). The EM + L, EM + M and EM + H groups were treated with 7, 14 and 21 g/kg/day HYXZ, respectively. HYXZ, Hua Yu Xiao Zheng; EM, endometriosis.

Figure 3. Immunohistochemical analysis for the expression of (A) VEGF and (B) Ang-2 in the endometriotic lesions at the end of the treatment (magnification, x40). The EM + L, EM + M and EM + H groups were treated with 7, 14 and 21 g/kg/day HYXZ, respectively. HYXZ, Hua Yu Xiao Zheng; EM, endometriosis; VEGF, vascular endothelial growth factor; Ang-2, angiopoietin-2.
EM group, the protein levels of VEGF markedly decreased in all the HYXZ-treated groups (EM + L, EM + M and EM + H) in a dose-dependent manner (P<0.05). Furthermore, it was also observed that the protein levels of Ang-2 in the EM + M and EM + H groups were significantly decreased compared with those in the EM group (P<0.05).

Expression of VEGF and Ang-2 mRNA. Results of RT-qPCR demonstrated a pronounced difference in the expression levels of VEGF and Ang-2 mRNA in the EM model and HYXZ-treated groups when compared with the control group. As shown in Table VI, the expression levels of VEGF and Ang-2 mRNA in the EM group were significantly upregulated in comparison with the control group (P<0.05). Furthermore, the expression of VEGF and Ang-2 mRNA in the EM + M and EM + H treatment groups was markedly downregulated in comparison with the EM model group (P<0.05).

Discussion

Endometriosis is a common gynecological condition with diverse clinical manifestations, as well as highly variable and unpredictable clinical course with decreased quality of life. Although endometriosis is a benign disorder, the process through which endometrial cells attach to and invade surface shares features of malignancy (26). Current treatments, including surgery and hormonal therapy, are often insufficient, as well as present a high rate of relapse and various side effects, such as weight gain, abnormal lipid metabolism, hepatic injury and osteoporosis. Therefore, it is necessary to explore novel medical strategies and treatment approaches to improve the treatment of endometriosis lesions.

TCM herbs have become popular for relieving endometriosis-associated symptoms, including dysmenorrhea, dyspareunia, menorrhagia and chronic pain, which typically require surgical or medical intervention (27,28). Chinese herbal formulas for endometriosis therapy are designed to alleviate blood stasis and nourish the kidney (29). The present study aimed to examine the therapeutic potential of HYXZ, determine its ability to reduce endometrial explants growth and determine the possible mechanism of action in EM model rats, in order to provide a theoretical foundation for endometriosis treatment. To the best of our knowledge, the current study demonstrated for the first time that a physiological dose of HYXZ (which is six times greater than the amount administered to humans) administered for 28 days inhibited the growth of endometriotic tissues in a dose-dependent manner. Furthermore, the results demonstrated that HYXZ did not suppress the growth of the eutopic endometrium inside the uterine cavity of the rat model. These findings indicate
that HYXZ specifically targets the endometriotic tissues with minimal side effects on normal endometrium.

The development of new blood vessels is a complex dynamic process, which is characterized by a coordinated sequence of humoral and cellular interactions. It is widely accepted that endometriotic lesions are typically characterized by a dense vascularization that occurs through the angiogenesis process (30,31). The establishment and development of an effective blood supply and angiogenesis are essential for the growth of endometriotic implants (32). Endometrial expression of VEGF, which is a potent vascular permeability-inducing, mitogen and morphogen agent, is thus an important mediator of angiogenesis (33). Furthermore, Ang proteins are potent angiogenic factors that are expressed in the normal human endometrium. Ang-2 activates in response to hypoxia and induces vessel destabilization upon binding the Tie2 receptor (34). VEGF and Ang-2 have been reported to independently induce angiogenesis (35). Furthermore, they have been demonstrated to act synergistically to induce endothelial destabilization, increase vascular branching, and increase angiogenesis (36). In addition, the two growth factors independently induce formation of endothelial branches in ex vivo aortic ring assays, suggesting an angiogenic effect on perivascular cells as well (37).

Endometriotic lesions are highly vascularized, and angiogenesis is considered as a critical step for the establishment of endometriosis (38,39). VEGF stimulates endothelial cell proliferation and migration, while elevated levels of VEGF have been reported in the peritoneal fluid of women with endometriosis (7), with the same increase observed for Ang-2. In the present study, the protein and mRNA expression levels of VEGF and Ang-2 were significantly higher in the ectopic endometrium as compared with those in the normal endometrium. Thus, these results suggest that VEGF and Ang-2 serve an important role in the pathogenesis of endometriosis. The findings of the present study offer a novel perspective on the underlying mechanisms of endometriosis and indicate that anti-angiogenic therapy may be a potentially promising treatment method for patients with endometriosis. Furthermore, the current study observed that HYXZ was able to significantly reduce the protein and mRNA expression of VEGF and Ang-2 in the ectopic endometrium. The results also indicated that inhibition of angiogenesis may lead to regression of the lesion size following HYXZ treatment. Compared with other TCM formulas previously reported, HYXZ reduced the volume of endometriosis lesions to a similar extent as Guizhi Fuling capsules (40), and also demonstrated a significant inhibition of the angiogenesis process.

Although the present study offered a unique evaluation of the effects of HYXZ decoction on the endometriosis model, the small number of animals in the groups is a limitation of the study. Larger prospective controlled clinical trials are required in order to further elucidate the findings.

In conclusion, the current study revealed that HYXZ decoction significantly reduced the size of the endometrial explants in endometriosis model rats by decreasing the protein and mRNA levels of VEGF and Ang-2 in the ectopic endometrium. HYXZ may be a promising TCM formula to treat endometriosis, however, further clinical trials are required to confirm its efficacy.

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