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Anti-angiogenesis effect and mechanism study of Huangzhi Neiyi capsule in a rat endometriosis model

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Abstract

Objective: To investigate the effects and possible mechanism of Huangzhi Neiyi capsule (HZNY) on angiogenesis in rats with endometriosis.

Methods: Sixty Sprague-Dawley rats were used to establish an endometriosis model by autologous transplantation and were randomly divided into sham operation, model, gestrinone (0.25 g/kg), and HZNY (4.5 and 9 g/kg) groups. All drugs were administered orally for 28 days. At the end of the experiment, all rats were killed by cervical dislocation, and the expression of proliferating cell nuclear antigen (PCNA) and CD31 in ectopic endometrial tissue was assessed by immunohistochemistry. The level of vascular endothelial growth factor (VEGF) in peritoneal fluid was determined by enzyme-linked immunosorbent assay. The mRNA expression of *VEGF* and hypoxia inducible factor-1α (*HIF1A*) in ectopic endometrium was evaluated by reverse transcription-PCR.

Results: HZNY suppressed the expression of PCNA and CD31 in ectopic endometrium, reduced concentrations of VEGF in peritoneal fluid, and reduced mRNA expression of *VEGF* and *HIF1A* in ectopic endometrium.

Conclusions: HZNY suppressed angiogenesis in endometriosis rats, perhaps by inhibiting expression of *VEGF* and *HIF1A*.

Keywords

Huangzhi Neiyi capsule, endometriosis, angiogenesis, vascular endothelial growth factor, traditional Chinese medicine, hypoxia inducible factor-1α

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Introduction

Endometriosis (EMT) is the growth of endometrial tissue (glands and stroma) outside the uterus.[1] EMT is a common gynecological disease and its incidence has increased in recent years. It has a higher incidence in women of childbearing age than in older women.[2] The chronic pelvic dysmenorrhea and infertility caused by EMT seriously affect the physical and mental health and quality of life of women.[3] The pathogenesis of endometriosis has not been fully elucidated, but the most widely accepted theories are the theory of transvenous countercurrent implantation first proposed by Sampson, and the theory of endometrial determinism in situ proposed by Chinese scholars.[4] With the development of immunology and molecular biology, an increasing body of research shows that immune and inflammatory factors play an important role in the formation and development of EMT.[5–7] At the same time, a variety of signal pathways that regulate the occurrence and development of tumors are involved in regulating the malignant biological behavior of EMT,[8] which provides a broad scope for the pathogenesis and treatment of the disease.

At present, Western treatment for EMT mainly relieves symptoms by inhibiting the secretion of pituitary gonadotropin and lowering the level of estrogen. However, treatment is often stopped because of adverse reactions, and EMT recurs, thus affecting the clinical application of treatment.[9] In recent years, traditional Chinese medicine (TCM) for the treatment of EMT has been applied clinically and some studies have shown that it has a positive effect.[10]

Huangzhi Neiyi (HZNY) capsule, a TCM preparation, was made by Professor Xia Min at Chongqing Traditional Chinese Medicine Hospital to treat EMT in the clinic, and it was shown to have a good therapeutic effect.[11] Professor Xia notes that choroid blood stasis is a persistent pathological state of endometriosis. It is not only the cause of disease but also a pathological product. Blood stasis does not go away and new blood does not circulate in the vessels, which leads to a vicious cycle, aggravating the disease. Therefore, in view of the pathogenesis of blood stasis, the use of TCM to promote blood circulation and remove blood stasis can achieve the purpose of normal blood circulation in the vessels, without pain.

HZNY contains three traditional Chinese medicines. Leech (*Whitmania pigra* Whitman, 3 g) is used to treat the main symptoms of EMT and it can inhibit the action of thrombin on fibrinogen, having anticoagulant and antiplatelet effects.[12,13] Cooked rhubarb (*Rheum palmatum* L., 9 g) can not only stop bleeding but also accumulate and break blood stasis by promoting blood circulation.[14,15] Processed *Cyperus* (*Cyperus rotundus*, 6 g) regulates qi and relieves depression, regulates menstruation, and relieves pain.[16,17] Overall, HZNY has the benefits of promoting qi, activating blood circulation, removing blood stasis, and relieving pain. Thus, in the study, we investigated the effects and possible mechanism of HZNY on inhibiting angiogenesis in an endometriosis rat model.

Materials and methods

Materials

Gestrinone was obtained from Beijing Zizhu Pharmaceutical Co. Ltd. (Beijing, China; batch no. 53110111253). Chloral hydrate, citric acid buffer solution (pH 6.0), and sodium citrate were purchased from the Chemical Reagent Company (Beijing, China) of state pharmacy group (batch nos. 20161222, 20161228, and 20161110, respectively). Rabbit anti-mouse platelet endothelial cell adhesion molecule (CD31) antibody (sc2112) and goat anti-rabbit immunoglobulin G antibody (sc0512,

Santa Cruz Biotechnology Inc.) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit anti-mouse proliferation cell nuclear antigen (PCNA) antibody (#2586) was purchased from Cell Signaling Technology (Danvers, MA, USA), and rat vascular endothelial growth factor (VEGF) ELISA kit (batch no. 284506) was from R&D Systems (Shanghai, China). TRIzol reagent (batch no. 14105) was from Invitrogen (Carlsbad, CA, USA), and first-chain synthesis kit of RT-qPCR (batch no. 00091602; Thermo Scientific Fermentas, Waltham, MA, USA) and Maxima SYBR Green/ROX qPCR Master Mix (batch no. 00093956) were from Thermo Scientific Fermentas. All primers were synthesized by Shanghai Jie Rui Bioengineering Co. Ltd. (Shanghai, China).

Preparation of HZNY

HZNY, containing 3 g of leech, 9 g of cooked rhubarb, and 6 g of processed *Cyperus*, was prepared and quality controlled by the Chongqing Traditional Chinese Medicine Hospital preparation room. To prepare HZNY, the leech was scalded in sand and then ground into a powder for use. Second, the cooked rhubarb and processed *Cyperus* were mixed, 10 times the volume of water was added; then, the mixture was decocted for 1 hour, filtered, and the liquid collected. Then, eight times the volume of water was added to the residue, the mixture was decocted for 1 hour, filtered, and the liquid collected again. Then, these two liquids were combined and concentrated to a thick paste. Finally, the leech powder was added to the paste, mixed, and dried, and the dried paste was crushed, loaded into capsules, and stored at 4°C for use.

Animals

Sixty female sexually mature Sprague-Dawley rats (body weight 200 to 220 g) were obtained from the Experimental Animal Center of Chongqing Traditional Chinese Medicine Hospital (Chongqing, China). Rats were kept in polyacrylic cages (six rats per cage) and quarantined for 1 week before the experiments. All animals were housed under standard controlled conditions (temperature: 24 ± 1°C, humidity: $50\% \pm 5\%$, and a 12-hour light/dark cycle), with free access to food and water, and received humane care according to National Institutes of Health Guidelines of the United States (National Research Council, 1996) and the related ethical regulations of Chongqing Traditional Chinese Medicine Hospital. Animals were fasted for 12 h before sampling of material.

Animal experiments were approved by the Committee on the Ethics of Animal Experiments of Chongqing Traditional Chinese Medicine Hospital (Chongqing, China) and were performed according to the guidelines for the ethical care of animals (approval no. SYXK 2018-0040).

Experimental design

Except for the 12 rats in the sham operation group (negative control), the rats were an established endometriosis model according to the method of Hu et al.[18] The rats were divided into four groups: the model group, gestrinone (0.25 g/kg) group, low-dose HZNY (4.5 g/kg) group, and high-dose HZNY (9 g/kg) group, with 12 rats in each group. Rats in the HZNY groups were administered the indicated dose orally once a day for 28 days. Rats in the gestrinone group were treated orally twice a week for 28 days. Rats in the negative control and model groups were given the same amount of double-steamed water once a day for 28 days. After treatment, all rats were killed by cervical dislocation, the ectopic endometrium was removed (normal endometrium was removed in the sham operation group), and each specimen was immediately

cut into two pieces for immunohistochemistry and reverse transcription (RT)-qPCR.

Expression of PCNA and CD31 in endomembrane tissue detected by immunohistochemistry

The expression of PCNA and CD31 in endomembrane tissue was analyzed by routine immunohistochemistry analysis. Endomembrane tissues were fixed in 4% formaldehyde solution, dehydrated in a gradient ethanol, and embedded in paraffin. All specimens were cut into 3-μm-thick sections, deparaffinized, quenched, incubated with primary antibody, blocked with 10% goat serum, and incubated with the second horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody. The final positive signals were visualized by using 3,3′-diaminobenzidine (DAB)-H_2O_2. Images were magnified 200× (Olympus BX-50 Microscope, Olympus, Tokyo, Japan; Leica DMI, Leica Microsystems, Wetzlar, Germany).

Content of VEGF in peritoneal fluid detected by ELISA

After the rats were killed, the peritoneum was cut open, normal saline was injected into the abdominal cavity, and the rats were gently shaken. The peritoneal cavity was lavaged with normal saline to collect the lavage fluid, centrifuged, and the supernatant was used to detect the VEGF content. All steps were carried out according to the VEGF ELISA kit instructions (R&D Systems).

Detection of VEGF and HIF1A mRNA levels in endomembrane tissue by RT-qPCR

RNA samples were extracted for reverse transcription using Trizol (Invitrogen). The cDNA was synthesized according to the instructions of RT-qPCR first-strand cDNA synthesis kit. RT-qPCR was carried out according to the instruction of Maxima SYBR Green/ROX qPCR Master Mix kit, and *ACTB* was used as internal reference. The reaction conditions were 50°C for 2 minutes, 95°C for 10 minutes, one cycle at 95°C for 15 seconds, and 60°C for 60 seconds, for 40 cycles. The primer sequences were as follows: *VEGF* forward: 5′-AGCA GAGGAAAGAGGTAGCAG-3′, reverse 5′-CCCCAAAAGCAGGTCAGT-3′; *HIF1A* forward 5′-GACACCGCGGGCACCGATT C-3′, reverse 5′-TCGCCG AGATCGTGCT GCAT-3′; *ACTB* forward 5′-CACCCGCG AGTACAACCTTC-3′ reverse 5′-CCCATA CCCACCATCACACC-3′.

Statistical analysis

Data were expressed as mean ± SD, and all statistical comparisons were made by means of a one-way ANOVA followed by Dunnett's *t*-test. $P < 0.05$ and < 0.01 were considered statistically significant. All calculations were performed using SPSS software, version 19.0 (IBM Corp., Armonk, NY, USA).

Results

As shown in Figure 1, the endometrium in rats of the sham operation group was slightly thickened, with glandular cavity, intact and orderly arrangement of the middle membrane, and neatly arranged cells. The endometrium of rats in the model group was thicker, the glands were not obvious, the lumen was absent, and the cells were disordered. In rats of the gestrinone group, the ectopic endometrium was incomplete, the glandular cavity was irregular, the mesangial cells were contracted, and the cells were irregularly arranged. In rats treated with a high dose of HZNY, the ectopic endometrial lumen was intact but the shape was irregular, the endometrium was orderly, and the mesangial cells were arranged irregularly. In rats treated with a

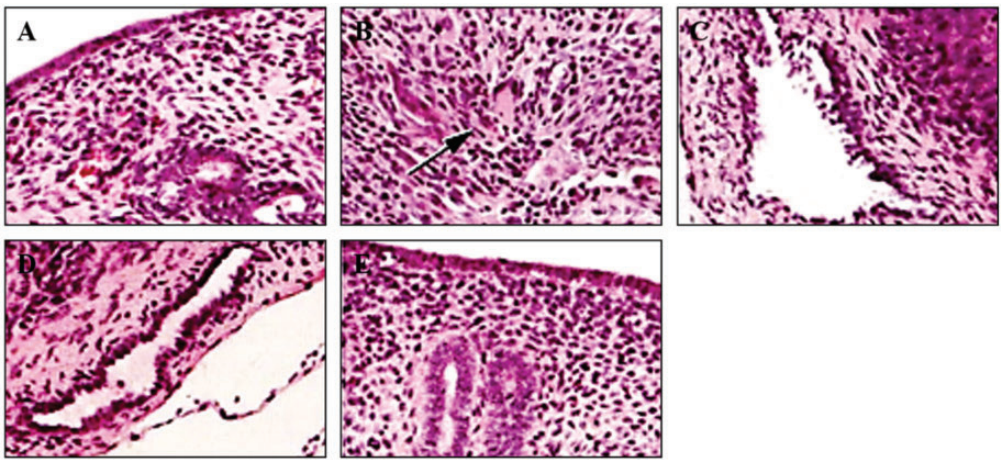

Figure 1. Morphology changes of uterus ectopic endometrium in rats of each group (H&E staining, 200×). A, Sham-operated group; B, model group; C, gestrinone (0.25 g/kg); D, low-dose HZNY (4.5 g/kg) group; E, high-dose HZNY (9 g/kg) group. The arrow in image B shows the absence of the glandular lumen and disordered cell arrangement in the model group. H&E, hematoxylin and eosin; HZNY, Huangzhi Neiyi.

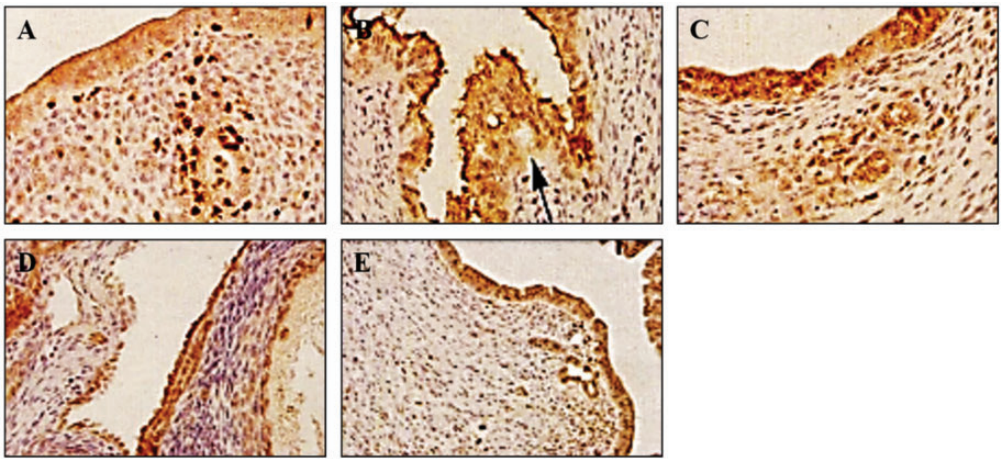

Figure 2. Effects of HZNY on protein expression of PCNA in ectopic endometrium (200×). A, Sham-operated group; B, model group; C, gestrinone (0.25 g/kg); D, low-dose HZNY (4.5 g/kg) group; E, high-dose HZNY (9 g/kg) group. The arrow in image B shows the brownish yellow staining granules of glandular epithelial cells in the model group. PCNA, proliferating cell nuclear antigen; HZNY, Huangzhi Neiyi.

low dose of HZNY, the ectopic endometrium was incomplete, the glandular cavity was irregular, the mesangial cells were contracted, and the cells were arranged irregularly.

PCNA is mainly expressed in endometrial vascular endothelial cell nucleus. As shown in Figure 2, significantly more PCNA-positive cells were found in the model group ($P < 0.01$) than in the sham

operation group. There were significantly fewer PCNA-positive cells in ectopic endometrium in the HZNY ($P < 0.05$) and gestrinone ($P < 0.01$) groups than in the model group, and this effect was dose-dependent in the HZNY groups. As shown in Figure 3, positive expression of CD31 was mainly located in endometrial endothelial cytoplasm and cell membrane. The number of CD31-positive cells in the model group was significantly higher ($P < 0.01$) than that in the sham operation group. After HZNY treatment, expression of CD31 was markedly decreased compared with that in the model group. The positive control (gestrinone) group showed decreased CD31 expression compared with the model group.

As shown in Table 1, the level of VEGF in peritoneal fluid of the model group was significantly higher than that of the sham-operated group ($P < 0.01$, Table 1), whereas the level of VEGF in the peritoneal fluid of the HZNY groups decreased significantly after 28 days of treatment compared with the model group in a dose-dependent manner ($P < 0.05$ and $P < 0.01$ for the low and high doses of HZNY, respectively).

Figure 4 and Figure 5 show that the mRNA expression of *VEGF* and *HIF1A* in the model group was greater than that in the sham operation group ($P < 0.01$, Figures 4, 5). In rats treated with HZNY or gestrinone, expression of *VEGF* and *HIF1A* in ectopic endometrium was lower than that in the model group, in a dose-dependent manner ($P < 0.05$ and $P < 0.01$ for the low and high doses of HZNY, and $P < 0.01$ and $P < 0.05$ in the gestrinone group, respectively; Figure 4, 5).

Table 1. Effects of HZNY on the levels of VEGF in peritoneal fluid.

Group	VEGF (ng/L)
Sham-operated group	1.85 ± 0.56
Model group	$22.87 \pm 1.33^{\#\#}$
Gestrinone (0.25 g/kg)	$9.56 \pm 1.09^{**}$
HZNY (4.5 g/kg)	$15.05 \pm 1.02^{*}$
HZNY (9 g/kg)	$8.32 \pm 1.08^{**}$

$^{\#\#}P < 0.01$ vs. sham-operated group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. model group.
VEGF, vascular endothelial growth factor; HZNY, Huangzhi Neiyi capsule (n = 12 per group).

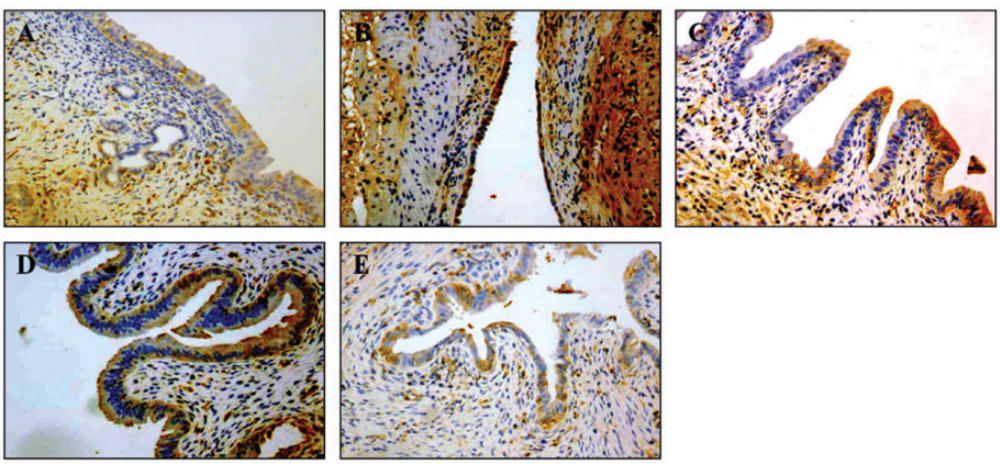

Figure 3. Effects of HZNY on protein expression of CD31 in ectopic endometrium (200×). A, Sham-operated group; B, model group; C, gestrinone (0.25 g/kg); D, low-dose HZNY (4.5 g/kg) group; E, high-dose HZNY (9 g/kg) group. HZNY, Huangzhi Neiyi.

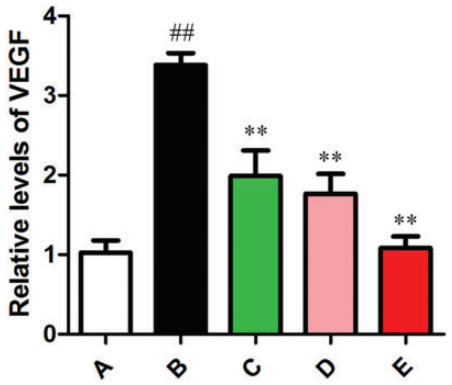

Figure 4. Effect of HZNY on mRNA expression of *VEGF* in ectopic endometrium (200×). A, Sham-operated group; B, model group; C, gestrinone (0.25 g/kg); D, low-dose HZNY (4.5 g/kg) group; E, high-dose HZNY (9 g/kg) group. $^{\#\#}P < 0.01$ vs. sham-operated group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. model group. HZNY, Huangzhi Neiyi; *VEGF*, vascular endothelial growth factor.

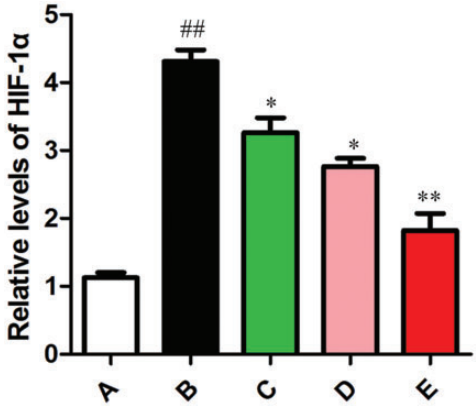

Figure 5. Effect of HZNY on mRNA expression of *HIF1A* in ectopic endometrium (200×). A, Sham-operated group; B, model group; C, gestrinone (0.25 g/kg); D, low-dose HZNY (4.5 g/kg) group; E, high-dose HZNY (9 g/kg) group. $^{\#\#}P < 0.01$ vs. sham-operated group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. model group. HZNY, Huangzhi Neiyi; *HIF1A*, hypoxia inducible factor-1α.

Discussion

During the development of endometriosis, neovascularization provides sufficient oxygen and nutrients for ectopic lesions, so angiogenesis is necessary for the implantation and proliferation of ectopic lesions.[19] Angiogenesis is a complex, multi-step process involving multiple cytokines and growth factors, of which VEGF is the most critical stimulator.[20,21] The level of VEGF in peritoneal fluid of patients with endometriosis is significantly higher than that of healthy women.[22,23] A positive correlation has been reported between the severity of endometriosis and the level of VEGF secreted in the peritoneal fluid of patients.[24]

In this study, the level of VEGF was significantly higher in the peritoneal fluid of the endometriosis model rats compared with rats in the sham operation group. After 28 days of treatment with HZNY, the level of VEGF in the peritoneal fluid decreased significantly. Consistently, the vascular density of the heterotopic lesions of rats in the HZNY groups was lower than that in model group, and cell proliferation was weaker than that in the model group. Furthermore, HZNY inhibited *VEGF* mRNA expression at the transcriptional level.

Ectopic endometrium is subjected to an anoxic environment during ectopic implantation. Hypoxia is a potent angiogenesis factor in the regulation of the HIF-1α/VEGF signaling pathway.[25] Under hypoxia conditions, HIF-1α binds to hypoxia-responsive elements of the VEGF promoter, thereby increasing the expression of VEGF.[26] HZNY significantly decreased the expression of *HIF1A* in ectopic lesions. Gestrinone (positive control group) is a hormone that is commonly used in the clinical treatment of endometriosis; it inhibits the growth of ectopic lesions mainly through an anti-estrogen effect.

Our results showed that both gestrinone and HZNY significantly reduced the number of PCNA-positive cells in ectopic endometrium and the expression of VEGF in ectopic endometrium. These results

suggest that both HZNY and gestrinone can be used to treat endometriosis.

HZNY may inhibit ectopic endometriosis by downregulating the expression of *HIF1A* and decreasing the level of VEGF in peritoneal fluid and ectopic lesions, thereby inhibiting the development of endometriosis. HZNY may also regulate the estrogen and immune system in the treatment of endometriosis, although the mechanism remains to be studied in depth.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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