Effect of You-Gui-Wan on endometrial angiogenesis in controlled ovarian hyperstimulation mice model

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ABSTRACT. Purpose: To explore the mechanism of You-Gui-Wan (YGW) in improving endometrial receptivity in mouse model of controlled ovarian hyperstimulation (COH).

Methods: Sixty female mice with two consecutive oestrous cycles were randomly divided into COH model group (n = 50) and normal control group (n = 10). The COH model was created by using GnRH-a + PMSG + HCG and further randomly divided into 5 subgroups of simple model, YGW high-dose, YGW medium-dose, YGW low-dose and aspirin control, 10 in each subgroup. YGW or aspirin or saline was administrated to the mice by gavage once a day for 11 days. Afterwards, blood and uterine samples were collected for examination of serum levels of estradiol (E2) and progesterone (P4), endometrial thickness and number of blood vessels as well as expressions of angiogenic factors of VEGF, Ang-1, Ang-2 and Tie-2 at transcription and translation levels by using H& E staining, ELISA, real-time qRT-PCR, and immunohistochemistry, respectively.

Results: COH per se increased the serum concentrations of E2 and P4, but decreased the number of endometrial blood vessels and the protein expressions of the angiogenic factors when compared to the normal control group (P< 0.05 or 0.01). YGW increased the mRNA expressions of all angiogenic factors and restored the effect of COH on number of endometrial blood vessels (P< 0.01), and the protein expressions of the angiogenic factors when compared to the COH simple model group (P< 0.05 or 0.01). In comparison, aspirin could only restore the number of endometrial blood vessels and the protein expressions of Ang-2 and Tie-2 (P< 0.05 or 0.01).
Conclusions: YGW can restore the side effect of COH on number of endometrial blood vessels and the expressions of angiogenic factors, thus improve endometrial receptivity in COH mice.

KEY WORDS: COH; YGW; Mice; Endometrial blood vessel; Angiogenic factors; Endometrial receptivity

INTRODUCTION

Infertility is a global public health issue that has already become the third most serious health problem secondary to cancer and cardiovascular disease (Inhorn MC, 2015). Infertility may result in being socially ostracized or divorced and have economic, mental, or other health implications.

Assisted reproductive technology, known as in vitro fertilization and embryo transfer (IVF-ET), brings hope to infertile patients. As one of important procedures in IVF-ET, controlled ovarian hyperstimulation (COH) is used to induce maturation and fertilization of multiple follicles by medication in a controlled range to obtain numbers of high quality embryos for implantation. With the increase of the number of ocytes, IVF has achieved to 75%, 90% success rates. However, the pregnancy rate remained only 20% ,30% due to low rate of embryo implantation (Huang H, 2003). The success embryo implantation is determined by the receptivity of the endometrium, which just might be impaired by COH (Koot YEM, 2016, Bourgain C, 2003, Bentin-Ley U, 2000).

In the view of western medicine, endometrial receptivity is closely related to endometrial angiogenesis in which vascular endothelial growth factor (VEGF) and angiogenin (Ang) play a key role (Du Y, 2014, Girling JE, 2009, Lu LR, 2015). Correspondingly, the theory of Traditional Chinese Medicine (TCM) believes that "kidney" governs reproduction for preservation" and invigoration of the "kidney" yang may improve the implantation rates (Lu LR, 2015).

You-Gui-Wan (YGW) is a classical TCM formula used to warm and invigorate the "kidney" yang, replenishing vital essence and nourishing the bone marrow according to TCM theory (ZHOU Min, 2015). Moreover, YGW has been clinically used to treat many Gynecological diseases in term of western medicine, such as female infertility, polycystic ovary syndrome, hypomenorrhagia, menopausal syndrome and other Gynecological diseases (Yang L, 2001).

To explore the mechanisms underlying the clinical effect of YGW, our previous studies demonstrated that YGW could increase the number of vaginal lamina blood vessels in ovariectomized rats by up-regulating VEGF and Ang (Hu X, et al, 2011, Yin QZ, et al. 2013). Based upon these clinical applications and experimental findings, we hypothesize that YGW may also influence VEGF and Ang expressions in endometrium to improve the endometrial receptivity that was impaired by COH.

To prove our hypothesis, in the present study, we first developed a COH mouse model and then treated the mouse model with various dosed of YGW to investigate whether or not YGW could restore the endometrial receptivity that was reduced by COH, focusing on endometrial thickness, number of endometrial blood vessels, and expressions of angiogenic growth factors such as VEGF, Ang-1, Ang-2 and receptor tyrosine kinase (Tie 2) at mRNA and protein levels. If YGW do have such effects, it might provide animal experimental reference for applying kidney tonifying herbal medicine as an adjuvant therapy to enhance success rate of ART.

MATERIALS AND METHODS

Animals

Sixty SPF grade female Kunming mice, aged 6-8week-old and weighting 25-30g were supplied by the Experimental Animal Center, Sichuan Provincial Academy of Traditional Chinese Medicine (experimental animal production license No. SCXK201319). The animals with 2 normal consecutive oestrous cycles confirmed by vaginal smear were randomly divided into model group (n = 50) and normal control group (n = 10). The animals in the model group were further randomly divided into 5 subgroups of simple model group, YGW high-dose group, YGW medium-dose group, YGW low-dose group, and aspirin control group, 10 animals in each subgroup. All animal in this study were fed and maintained in the animal experimental zone under Laboratory Animal Barrier System of Sichuan Provincial Academy of Traditional Chinese Medicine (experimental animal use license No. SYXK2013-100).

Development of COH mouse model

A COH mouse model was developed by using a hormone cocktail including gonadotropin-releasing hormone agonist (GnRHa), pregnant mare's serum gonadotrophin (PMSG) and human chorionic gonadotropin (hCG) as described previously (Fang Y, 2011, Li J, 2013). Briefly, we intraperitoneally injected 10μg/kg triptorelin (Ferring, USA) on the third day of oestrous cycle and once a day at 9 am for 9 days. 200U/kg of PMSG (Ningbo Second Hormone plant, China) was boosted on the 9th day followed by injection of 400U/kg of hCG (Livon, China) 48h later. The success of model was confirmed by significantly increased vaginal secretion and a large number of nucleated keratinocytes in secretion smear observed under the optical microscope.

Drug treatment and sample collection

For the YGW low-, medium- and high-dose subgroups, the COH mice were administrated with YGW (Tongrentang, Beijing) by gavage in doses of 1.5, 3 and 6g/kg in 0.4 ml, respectively. For the aspirin control subgroup, the COH mice were administrated with aspirin sodium carboxymethyl cellulose solution (Bayer, German) by gavage in a dose of 0.2g/kg. For the COH simple model subgroup, 0.9% sodium chloride solution (0.4 ml) was given by gavage. At the same time, equal volume of saline by gavage and intraperitoneal injection were given to the normal control animals. The drugs or saline were given once a day for 11days.

Blood samples were collected from post ocular venous plexus after 48h of Hcg injection for the COH model subgroups. Sera were obtained after centrifugation for 10 min at 1000 g and $4^{O}C$ for estradiol (E2) and progesterone (P4) determination. All animals were sacrificed by cervical dislocation after blood sampling. Uterus was removed and dissected, one side of uterus was frozen with liquid nitrogen for real-time qRT-PCR analysis and another side was fixed with formalin for H&E and immunohistochemical examinations. Meanwhile, blood and uterine samples were also collected for the same purpose as above from the normal control group which ovulation status was monitored by using the vaginal smear method.

Measurement of serum estradiol and progesterone

Serum concentrations of E2 and P4 were determined by using commercial kits purchased from Cayman Chemical Co. (USA) according to the manufacturers' instructions. All serum samples were run in duplicate and randomly distributed on the plates. The ELISA was performed in a blinded manner.

Examination of endometrial thickness and number of uterine vessels with H&E staining techniques

Uterine tissues were paraffin-embedded, sectioned, dewaxed to hydration and stained with H&E. The area of endometrium and the blank area encompassed by endometrium were measured to calculate the arithmetic mean of endometrial thickness and the number of vessels across the entire cross-section were counted in each specimen under low power microscope (10×10)

Real-time RT-PCR analysis

0.2g uterine tissues from each animal were placed in 1 ml of Trizol. RNA was extracted by Total RNA Extractor (Sangon Biotech Co, Shanghai, China). RNA purity and integrity were tested by agarose gel electrophoresis. cDNA was generated by using a First Strand cDNA Synthesis kit (Thermo Scientific, UK). Quantitative real-time fluorescence PCR was performed using CFX96 Real-time PCR System with Sybr Green Master Mix (BioRad). Primer sequences for the real-time PCR target genes were listed in Table 1.

Table 1. List of Real Time PCR target gene detection primers

Biomarkers	Primer sequence	Amplified size
VEGF-F	5'-CAACTTCTGGGCTCTTCTCG-'3	
VEGF-R	5'-CCTCTCCTCTTCCTTCTCTT-'3	144
Tie-2-F	5'-AGGCTGATTGTTCGGAGAT-'3	
Tie-2-R	5'-ATAAACCCAGGAGGGCAA-'3	128
Angpt1-F	5'-GGAACCGAGCCTACTCAC-'3	
Angpt1-R	5'-GCATCCTTCGTGCTGAAATC-'3	145
Angpt2-F	5'-ATGAAGGAGCAGAAGGACGA-'3	
Angpt2-R	5'-GAAGGAGCGAGTTGTTGACC-'3	109
GAPDH-F	5'-AATGGTGAAGGTCGGTGTGAAC-'3	
GAPDH-R	5'-AGGTCAATGAAGGGGTCGTTG-'3	114

The reaction system was as follow: 10μl of SYB green MIX, 1μl of corresponding upstream primer, 1μl of corresponding downstream primer, 0.5μl of sample cDNA template and 7.5μl of ddH2O. The reaction conditions were a 3 minute for pre-denaturation at 95 °C, 10 second for denaturation at 95 °C, 10 second for annealing at 60°C and 10 second for extension at 72°C. Amplification was performed for 42 cycles. After the completion of the reactions, the Ct value was recorded. mRNA expression levels of the angiogenic factors were presented by relative quantitative analysis using 2-ΔΔCT method (Kenneth J, 2001).

Immunohistochemistry

The prepared uterus specimens were stained with commercial antibodies against VEGF, Ang-1, Ang-2 and Tie-2 immunohistochemical kits (Santa Cruz, USA) according to the manufacturers' instructions. Each specimen was randomly observed 10 fields under light microscopy (40×10). A light brown reaction in specimen was considered to be positive. HD pathologic images with immunohistochemical staining were obtained by BX63F microscopic image acquisition system (Olympus, Japan). Integrated optical density (IOD) and positive staining area (Area) were measured by IPP7.0 image analysis system (Media Cybernetics, USA).

Acute toxicity experiments of YGW

We used 10 mice with similar age and weight for this experiment. The animals were administrated with YGW decoction of 180 times of clinical dose. The general status of the animals such as feeding, and activity was continuously observed for 7 days.

Data analysis

Statistical analysis was performed using SPSS 13.5 software package (SPSS Inc., Chicago, IL, USA). Experimental data were expressed as mean ± standard deviation (SD). Differences in mRNA and protein expressions of the angiogenic factors among treatment groups were compared by using analysis of variance (AVOVA) and/or Student t test. $P < 0.05$ was considered as statistically significant difference.

RESULTS

Effect of COH on serum levels of E2 and P4, endometrial blood vessels and expressions of angiogenic factors

As shown in Table 2, COH dramatically increased the serum concentrations of E2 and P4 when compared to the normal control (20.2 ± 6.0 pg/ml vs. 4.3 ± 2.8 pg/ml for E2, and 5.37 ± 0.35 ng/ml vs. 1.10 ± 0.50 ng/ml for P4, $P < 0.01$). However, COH decreased the number of endometrial blood vessels when compared to the normal control (25.5 ± 7.0 per m2 vs. 38.5 ± 8.9 per m2, $P < 0.01$, Fig.1). COH did not show significant effect on mRNA expressions (Fig.2) but decreased the protein expressions of VEGF, Ang-1, Ang-2 and Tie-2 when compared to the normal control ($P < 0.05$ or 0.01, Fig.3). No significant effect of COH on uterine endometrial thickness was observed when compared to the normal control group (data did not show).

Figure 1. H & E staining of endometrial blood vessels: (A) is representative samples in normal control group, (B) simple modal group, (C) aspirin group, (D) YGW low-dose group, (E) YGW medium-dose group and (F) YGW high dose group. Original magnification: 100X. (G) Comparison of the numbers of endometrial blood vessels among the groups. The data are represented as mean + SD (n = 10). **, P < 0.01 when compared to the simple COH model group.

Fig.1

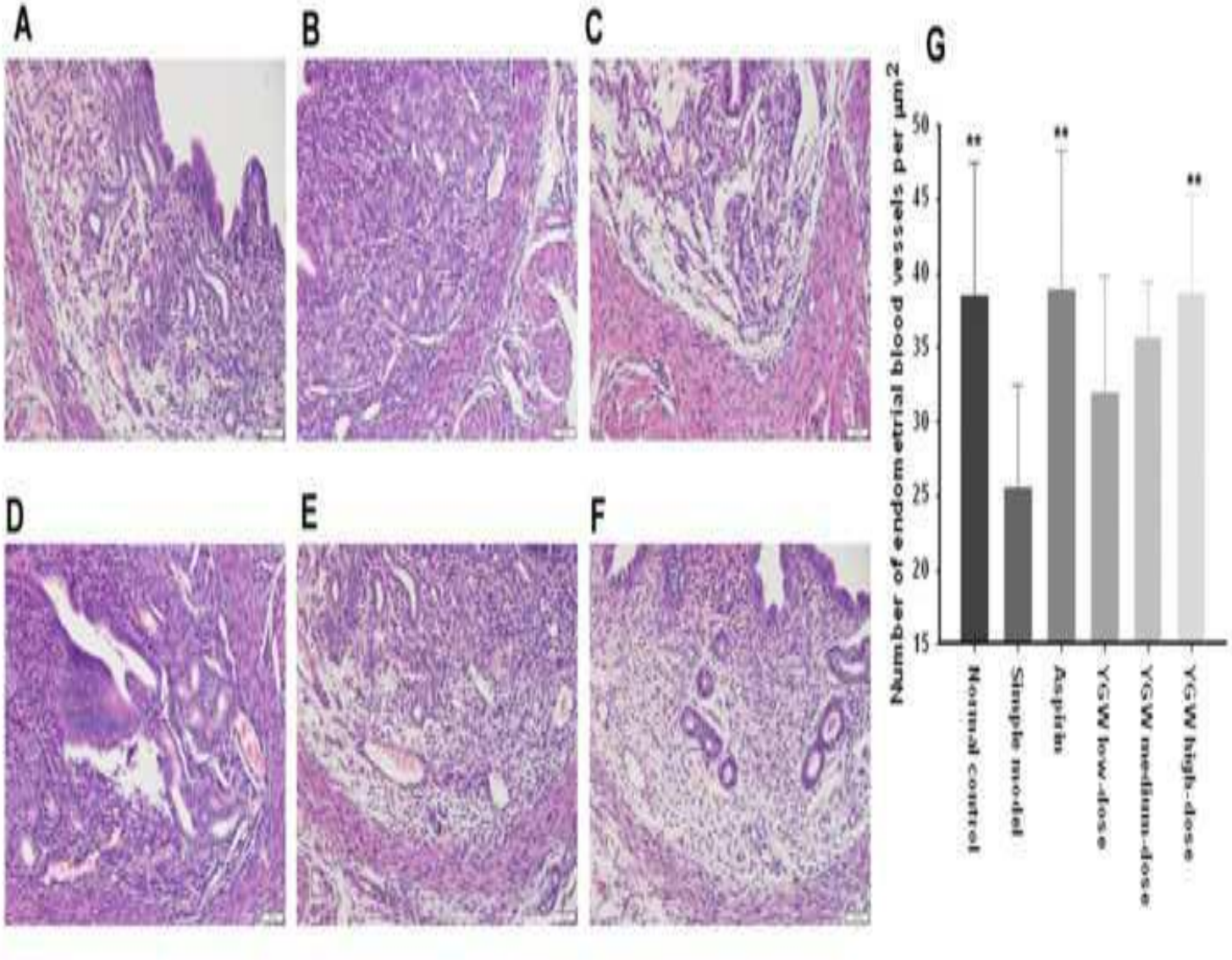

Figure 2. Effect of You-Gui-Wan on the mRNA expressions of VEGF (A), Ang-1 (B), Ang-2 (C)and Tie-2 (D) in endometrial tissues of COH mice. The results present fold changes relative to the normal control mice (2-ΔΔCt). Values present mean + SD (n =10). *, P < 0.05 and **, P < 0.01 when compared to the normal control group and simple COH model group.

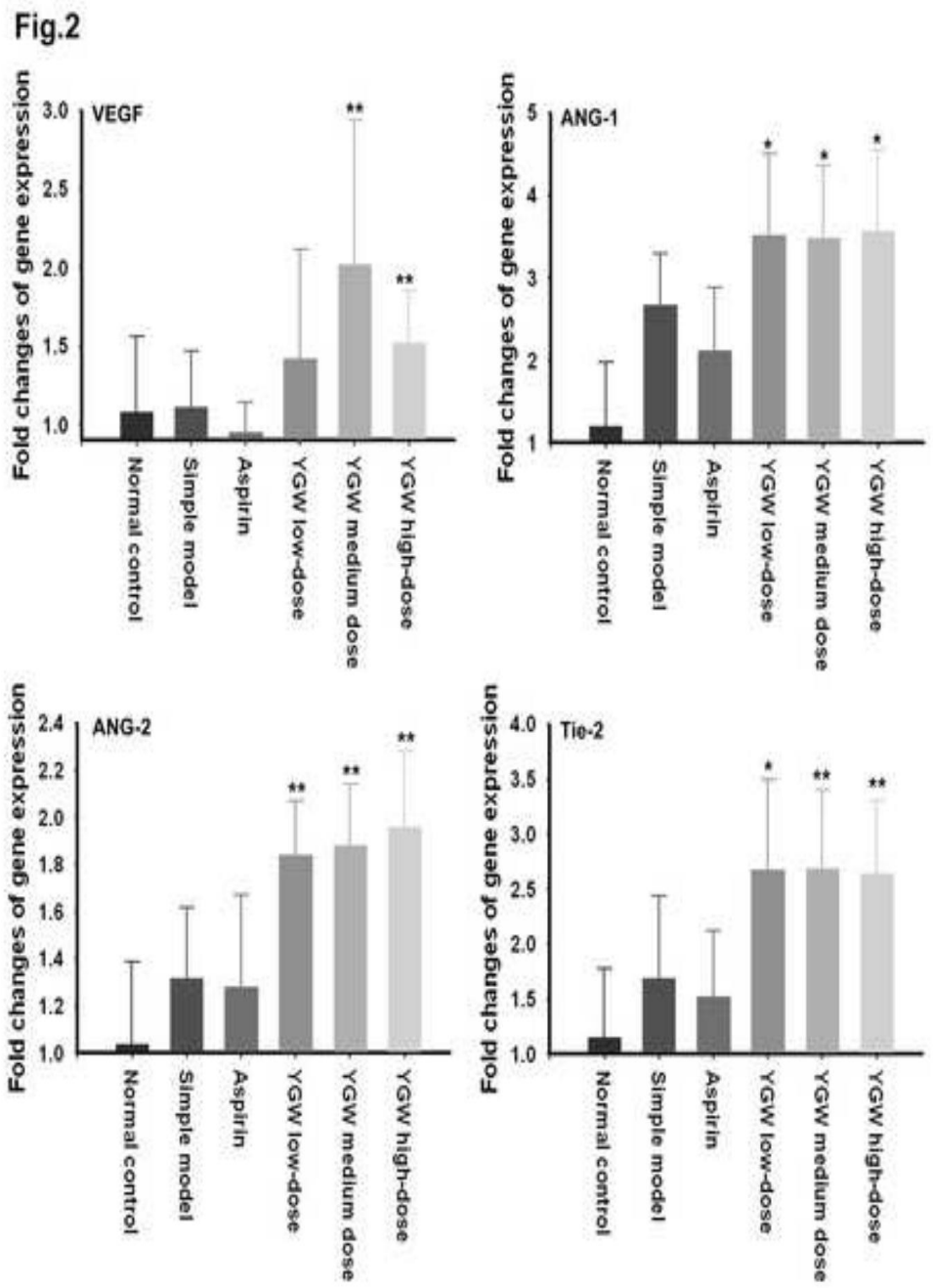

Figure 3. (A) are representative IPP 7.0 images of protein expressions of endometrial VEGF Ang-1, Ang-2 and Tie-2 among the groups of normal control, simple COH, aspirin and YGW high-dose. Original magnification: 100X. (B) Comparison of the protein expressions in these angiogenesis factors among the groups. Relative percentage changes in the expressions of the angiogenesis factors in the groups of simple COH, aspirin and YGW high-dose were compared to that in the normal control group (100%). The Values were presented as mean ± SD. *, $P < 0.05$ and **, $P < 0.01$.

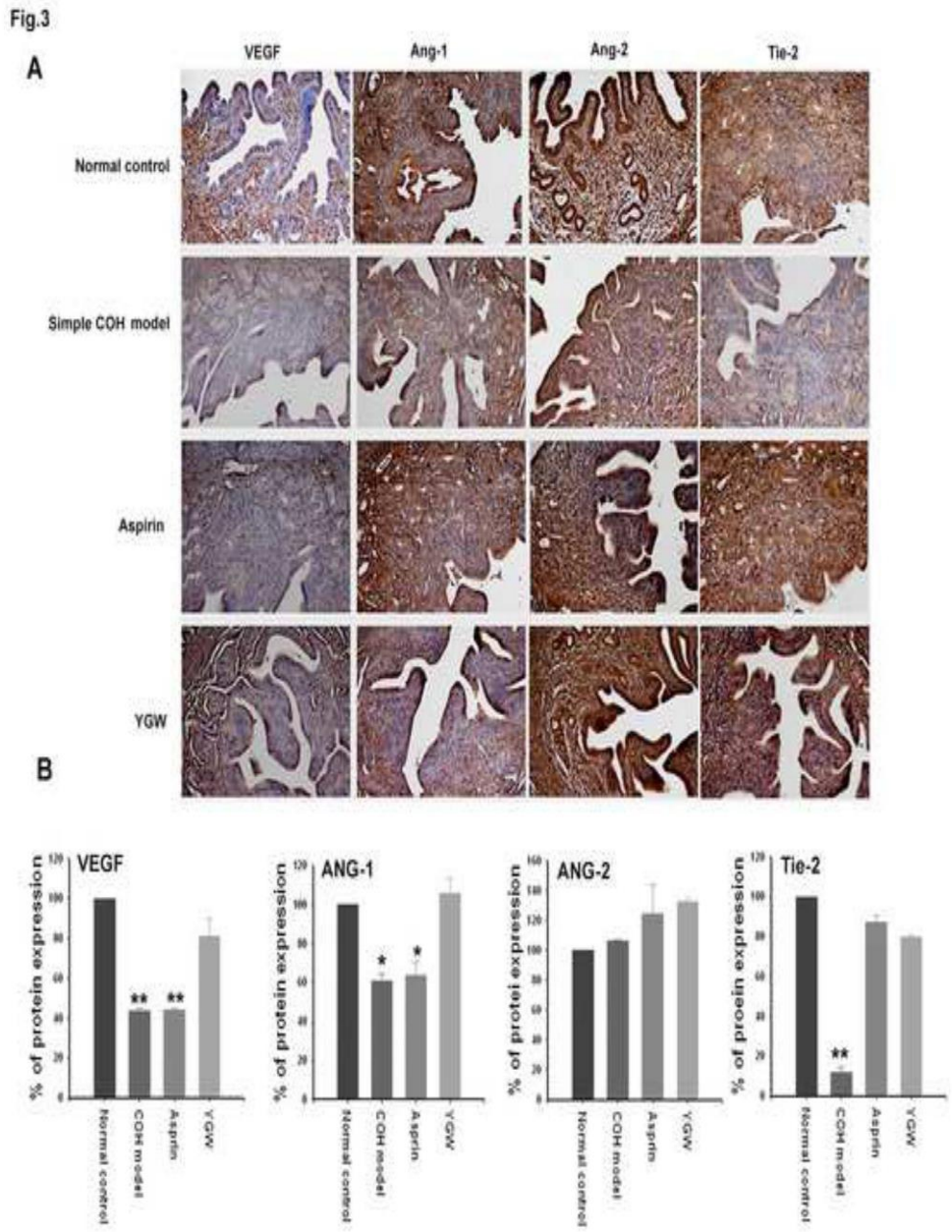

Table 2. Effect of YGW on the levels of serum E_2 and P in COH model mice (mean + SD)

Groups	No.	Dose (g/kg bw)	Estradiol (pg/ml)	Progesterone (ng/ml)
Normal control	10	/	4.3±2.8**	1.10±0.50**
Simple model	10	/	20.2±6.0	5.37±0.35
Aspirin	10	0.2	24.1±5.3	5.55±0.47
YGW Low dose	10	1.5	20.3±6.9	5.34±0.43
YGW Medium dose	10	3	24.3±5.7	5.39±0.47
YGW Medium dose	10	6	25.2±8.5	5.46±0.40

** $P<0.01$ when compared to COH model groups.

Effect of YGW on serum E2 and P4 levels, number of endometrial blood vessels and endometrial thickness in COH mice

Serum levels of E2 and P4 in the aspirin and YGW COH subgroups were significantly higher than that in the normal control group ($P < 0.01$) but not significantly different from that in the simple COH model group (Table 2).

Fig.1A-F showed represent images of H & E stained endometrial blood vessels. Fig.1G demonstrated that aspirin and YGW at high-dose restored the number of endometrial blood vessels from 25.5 ± 7.0 per μm2 of COH simple model group to 38.9 ± 9.3 per μm2 of aspirin group and 38.6 ± 6.7 per μm2 of YGW high-dose group ($P < 0.01$). Further, the numbers of endometrial blood vessels in the aspirin and YGW high-dose groups were not significantly different from the normal control group ($P > 0.05$).

There was no significant difference in endometrial thickness among the aspirin, YGW and simple COH model subgroup (data did not show). Moreover, there was no significant toxic effect of YGW at very high dose was observed in the acute toxic experiment (data did not show).

Effect of YGW on expressions of angiogenic factors

YGW significantly increased the mRNA expressions of all angiogenic factors in endometrial tissues when compared to the normal control group and the simple COH model subgroup ($P < 0.05$ or < 0.01, Fig.2). The mRNA expressions of these genes in the simple CHO model subgroup and the aspirin subgroup were also different from the normal control group but not reach statistically significant ($P > 0.05$, Fig.2).

Fig.3A showed representative images of immunohistochemical detection of expressions of VEGF, Ang-1, Ang-2 and Tie-2 in endometrial tissues in the normal control group, simple COH model subgroups, aspirin subgroup and YGW high-dose subgroups, respectively. YGW at high and/or medium doses restored the COH inhibitory effect on expressions of VEGF, Ang-1 and Tie-2 (Fig.3B) when compared to the simple COH model group ($P < 0.05$ or 0.01). Fig.3B also showed that YGW increased the Ang-2 expression when compared to the simple COH model group ($P < 0.05$ and 0.01). Aspirin was showed to increase the protein expression of Ang-2 andTie-2 when compared to the simple COH model group ($P < 0.01$, Fig. 3B).

DISCUSSION

In present study, we established a COH mice model by using GnRHa + PMSG + HCG as described by previous study to mimic the COH in the assisted reproductive technology treatment. The COH mice model showed the characteristics of increased serum levels of E2 and P4, but decreased number of endometrial blood vessels. The results are similar to previously published studies in which COH might result in an inhibition of endometrial angiogenesis (Ullah K, et al. Rackow BW, et al. 2008). During the peri-implantation period, the process of angiogenesis is considered an important part of the endometrial decidualization. Therefore, inhibition of endometrial angiogenesis may directly reduce endometrial receptivity, leading to a decrease of pregnancy rate or abortion (Karizbodagh MP, 2017, Rockwell LC, 2002.).

To improve the abnormalities, aspirin has been widely used to improve endometrial receptivity (Li R, 2006). In the present study, aspirin showed to restore the number of endometrial blood vessels when compare to the normal control group and the simple COH model subgroup. Interestingly, YGW at high dose could also restore the number of endometrial blood vessels. The finding is like the effect of YGW on the number of blood vessels in the lamina propria of OVX rats observed in our previous study (Hu X, 2011).

In the present study, COH shows to have an inhibitory effect on the protein expressions of VEGF, Ang-1, Ang-2 and Tie-2 although it seems not significantly affect the mRNA expressions of the angiogenesis factors when compared to the normal controls. As we mentioned above, VEGF and Ang/Tie system play a crucial role in endometrial angiogenesis. Inhibition of VEGF can prevent estrogenic-induced uterine edema and embryo implantation (Rockwell LC, 2002). The Ang family is a growth factor that promotes angiogenesis and remodelling secreted by the vascular endothelium (Augustin HG, et al. 2009). Ang-1 and Ang-2 are important family members, both acting on the same receptor Tie-2 to play different roles. ANG1 activates the TIE2 receptors, whereas ANG2 antagonises the activation of TIE2. However, if VEGFA is present, ANG2 will enable endothelial cell migration and proliferation and therefore angiogenesis. If VEGFA is inhibited, ANG2 will lead to endothelial cell death and vessel regression (Gale NW, et al. 2002). Therefore, it may be proposed that one of molecular mechanisms of impaired endometrial receptivity by COH is to inhibit the expressions of angiogenesis factors as well as their interactions.

However, it is not clear yet why COH could inhibit the expressions of the angiogenic factors. Since supraphysiological levels of serum E2 are induced by COH, it might be hypothesized that high E2 levels or other hormonal alterations may probably contribute to the adversely affection on the endometrial angiogenic factors by COH. Nevertheless, further studies are required to verify the hypothesis.

In the present study, we show that YGW could increase or restore the mRNA or protein expressions of the angiogenic factors regardless of high serum E2 levels in COH mice. Thus, YGW probably directly acts on the regulation of the expressions of VEGF and Ang rather than through estrogenic receptor as proposed in our previous study in OVX rat (Yin QZ, 2013, et al.) The more deeply molecular mechanisms of YGW effect on the angiogenic factors under COH conditions need further studies in the future.

Nevertheless, TCM considers that infertility caused by poor endometrial receptivity falls into the category of "fetal is not solid" which is closely related to deficiency of “Kidney” function. Insufficient “Kidney” function would induce blood empty, uterus malnutrition, and sparsely venation, which makes implantation impossible. Therefore, tonifying “Kidney” by TCM should be an effectively alternative approach to improved embryo quality and endometrial receptivity during COH process. Several studies about TCM formulas to improve the endometrial receptivity through various mechanisms such as promotion of endometrial hyperplasia (Li XN, et al. 2013). reversion of leukemia inhibitory factor (LIF) and integrin μ3 expression (Yu N, 2011, Zhang M, 2008) and up-regulation of HOXA10 (Gao Q, 2015). Our study demonstrated that tonifying “Kidney” formula improves the endometrial receptivity in COH via reversion of endometrial angiogenesis by increased angiogenic factor expressions.

In the present study, we also showed that aspirin could restore the number of endometrial blood vessels but did not change the inhibition of COH on the expressions of VEGF and Ang-1. It has been reported that aspirin can improve endometrial receptivity by inhibiting uterine vasoconstriction and ovarian and platelet aggregation to improve the blood supply to the endometrium, thus, to improve endometrial receptivity (Li R, 2006, Zhu H, 2009). Recently, Chen et al reported that aspirin could improve the endometrial receptivity by also increasing the expression of integrin 3 (Chen XY, et al. 2015). In contrast, Carnovale et al. found that aspirin could inhibit prostaglandin in a dose-dependent manner, which is the main prostaglandin involved in cultivation and decidualization (Carnovale DE, et al. 2001).

Furthermore, it is also possible that YGW may act at multi-level and multi-target to improve the endometrial receptivity according to integral and comprehensive theory of TCM, although the present study only revealed its role in regulation of endometrial angiogenesis. Otherwise, the effect of aspirin is single, which only regulate the endometrial blood vessels. From this point of view, as well as previous studies of TCM formula and/or aspirin, it could be concluded that TCM tonifying “Kidney” formula such YGW might be superior to aspirin in the treatment of COH impaired endometrial receptivity.

In conclusion, the study has demonstrated that YGW could revise the endometrial angiogenesis through up-regulating the expressions of endometrial angiogenic factors. Although the endometrial receptivity is affected by a variety of factors such as endometrial angiogenesis, hormones, cytokines, immune factors and adhesion molecules, the current data may provide an explanation for the underlying mechanisms of YGW to improve the endometrial receptivity, as well as to provide an improved understanding of the potential health benefits of the herbal agents of YGW as an adjuvant treatment to enhance success rate of ART.

AUTHORS' CONTRIBUTIONS

QY and JZ participated in the design of the study and performed the data analysis. LL, YL, LH, and NN participated in experimental operation. The final manuscript was read and approved by all authors.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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