Original Article

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Yishen Huatan Huoxue decoction and quercetin ameliorate decidualization dysfunction in polycystic ovary syndrome: A comprehensive investigation combining clinical trial and experimental studies

Jing Wang[1,2,3,§], Lisha Li[1,2,3,§], Jing Zhou[1,2,3], Xinyao Pan[1,2,3], Qing Qi[1,2,3], Hongmei Sun[1,2,3], Ling Wang[1,2,3,*]

[1] Laboratory for Reproductive Immunology, Obstetrics and Gynecology Hospital of Fudan University, Shanghai, China;
[2] The Academy of Integrative Medicine of Fudan University, Shanghai, China;
[3] Shanghai Key Laboratory of Female Reproductive Endocrine-related Diseases, Shanghai, China.

SUMMARY Polycystic ovary syndrome (PCOS) is a common gynecological endocrine disorder characterized by a complex pathogenesis and limited treatment options. Yishen Huatan and Huoxue decoction (YHHD), as a traditional Chinese Medicine formula, has shown effectiveness in treating PCOS. However, the specific mechanisms by which YHHD exerts its therapeutic effects remain unclear. In this study, we performed to investigate the therapeutic effects of YHHD and quercetin on dehydroepiandrosterone-induced PCOS mice, and examine the effect of quercetin on the decidualization of T-HESCs under hyperinsulinemic conditions. The results showed that YHHD could reduce early miscarriage rates in PCOS patients and significantly improved glucose metabolism disorders, sex hormone levels, and the estrous cycles in PCOS mice. Quercetin could alleviate effect of high insulin levels and restore the low expression of insulin receptor substrate1/2 (IRS1/2) and glucose transporte 4 (GLUT4) in T-HESCs, demonstrating its potential to mitigate hyperinsulin-induced decidualization dysfunction *via* the GLUT4 signaling pathway mediated by IRS1/2. This study provides valuable molecular insights of YHHD and highlight the therapeutic potential of quercetin in treating decidualization dysfunction in PCOS.

Keywords Quercetin, polycystic ovary syndrome, decidualization, traditional Chinese medicine

1. Introduction

Polycystic ovary syndrome (PCOS) is a multifaceted clinical syndrome that mainly impacts women of reproductive age, resulting in endocrine abnormalities and infertility (*1-3*). This disorder is characterized by hyperandrogenism, ovulation dysfunction, polycystic ovarian changes, and insulin resistance (*4,5*). While ovulation dysfunction is the leading cause of infertility among PCOS patients, poor pregnancy success rates suggest that other pathologies may be involved, particularly in the endometrial microenvironment (*6,7*). Successful pregnancies heavily rely on decidual tissue, which facilitates blastocyst implantation, invasion, and communication between the mother and fetus (*8*). Despite the extensive studies on decidualization (DE) regulation mechanisms, especially those associated with the pathogenesis of PCOS, further research is necessary to identify additional factors.

Traditional Chinese Medicine (TCM) literature has described conditions similar to PCOS in chapters related to metrorrhagia, amenorrhea, abdominal pain, and infertility. The treatment regimen centers around rectifying the balance among kidney essence, Tiangui, Chongren, and uterus (*9*). Although various medications have been proposed for treating PCOS, they have limitations such as adverse effects, low compliance, low efficacy, and contraindications in certain instances (*10*). Given its effectiveness and safety, TCM could be an alternative or complementary treatment option for PCOS management (*11*). Yishen Huatan and Huoxue decoction (YHHD) is a TCM formulation that reportedly improves insulin resistance, regulates metabolism, increases blood flow, and eliminates blood stasis. This study has shown that YHHD has the potential to regulate ovarian haemodynamics, serum hormone levels, and

menstruation in PCOS patients, thereby normalizing endocrinology and improving menstrual irregularities, ovulation, and pregnancy rates. However, the biological mechanisms underlying the effectiveness of treatment options for PCOS are not fully understood. Quercetin (Que), a common flavonoid present in plant-based foods, contributes to glucose homeostasis regulation. However, the precise role of quercetin in the regulation of insulin action is not yet fully understood (*12*). This study aims to investigate how quercetin affects insulin-mediated glucose transporter translocation under hyperinsulinemic conditions and the specific molecular mechanisms underlying YHHD's promotive effect on decidualization. The study aims to evaluate the efficacy of YHHD in treating PCOS, shedding light on its underlying endocytic mechanisms and providing potential avenues for future research and treatment of the condition.

2. Materials and Methods

2.1. The clinical trial validates YHHD for PCOS

This retrospective trial was conducted at the Obstetrics and Gynaecology Hospital of Fudan University between 1 September 2018 and 30 September 2022. The study received approval from the hospital ethics committee (No. 2024-47) and followed the guidelines established in the Declaration of Helsinki for research involving human subjects (*13*). The diagnostic criteria of PCOS followed the Rotterdam criteria (*14*): *i* Occasional ovulation or anovulation; *ii* Clinical and/or biochemical indicators suggest hyperandrogenism; *iii* Ultrasonography of ovarian polycystic changes. The exclusion criteria included: *i* Uterine amenorrhea, primary amenorrhea, adrenal tumor, Cushing's syndrome, hyperprolactinemia, hyperthyroidism or hypothyroidism, Sheehan's syndrome, mental factors, emaciation, anorexia, and other hypothalamic amenorrhea, and sex chromosome abnormalities; *ii* Complications with genital malformation, congenital gonadal hypoplasia, tubal obstruction, and other organic diseases; *iii* Cardiovascular, pulmonary, and other serious primary diseases; *iv* Mental and neurological diseases such as hemorrhage, epilepsy, carbuncle, psychosis, and neurosis; *v* Those who are allergic to the test drug or have a severe allergic constitution; *vi* Those who have taken hormonal drugs such as contraceptives, ovulation promoting drugs, glucocorticoids, or related therapeutic drugs in recent months; *vii* Breastfeeding in the past 6 months.

According to the results of the two prior studies (*15,16*), the combined miscarriage rate was found to be 14.8% in the progestogen group, whereas the rate was 27.1% in the control group. Based on an 80% statistical power and a type I error rate of 0.05, a sample size of 200 women in each group was determined to be necessary. To account for potential drop-outs, our target was to recruit a total of 660 women, with 220 women in each group. The YHHD group received YHHD treatment obtained from the Fudan University Obstetrics and Gynaecology Hospital for a treatment duration of 14-28 days. The primary outcome of this study was the incidence of early miscarriage, which was defined as occurring before the 20th week of gestation.

2.2. Animal experimental protocols and testing

The study used female C57BL/6 mice of three weeks old, obtained from Zhejiang Viton Lihua Laboratory Animal Technology Co., with Specific Pathogen-Free (SPF) grade. The experimental procedures utilized in this study were approved by the Ethics Committee of the Obstetrics and Gynaecology Hospital of Fudan University. All the animals were acclimated under standard laboratory conditions (ventilated room, 25 ± 1°C, 60 ± 5% humidity, 12 h light/dark cycle) and had free access to standard water and food. After a one-week acclimation period, the mice, with an average body weight of 13.72 ± 2.42 g. The mice were randomly assigned to one of the four groups: Control, dehydroepiandrosterone (DHEA) (Aladdin Biochemical Technology Co., Ltd, Shanghai, China), DHEA + YHHD, and DHEA + quercetin (50 mg/kg) (Aladdin Biochemical Technology Co., Ltd, Shanghai, China). The DHEA-treatment was administered for 20 consecutive days. For the control group ($n = 6$), mice were administered a daily subcutaneous injection of 0.1 ml of sesame oil (Sigma-Aldrich Inc., UNITED STATES). The models of PCOS assigned to the DHEA group ($n = 20$) were given a daily subcutaneous injection of DHEA (6 mg/100 g) administered once daily. On day 21 of DHEA modelling, the mice in the DHEA group were screened for estrous cycle, hormone level, and body weight and were thereafter subjected to treatment with YHHD and quercetin, respectively. Their reproductive and metabolic functions were assessed 22 days after commencing treatment, and evaluations were continuous until the animals were euthanized. Additionally, their weight was monitored every other day during the treatment period. To ensure precise and consistent results, all samples were analyzed in triplicate.

2.3. Estrous cycle

To determine the stages of the estrous cycle, vaginal smears were obtained every day at 9 AM beginning from the 13th day following the initial DHEA injection and collected for seven successive days.

2.4. Glucose and insulin tolerance test

The mice fasted for 12 hours and had unrestricted access to water. The subsequent morning, tail vein

blood samples were obtained to determine fasting blood glucose (FBG). They were then administered 20% glucose solution until their stomachs were filled and blood glucose levels were measured at intervals of 15, 30, 60 and 90 minutes post-administration (Sinocare, China). The Oral Glucose Tolerance Test (OGTT) curve was generated, and the overall area under the curve (AUC) was calculated. Blood glucose and serum insulin levels were assessed, and the homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated using the following formula: fasting blood glucose [mmol/L] × fasting serum insulin [μU/mL]/22.5.

2.5. Hormonal analysis of serum

On day 21 of DHEA intervention and day 41 of treatment, mice were given anesthesia, and blood samples were collected from the retro-orbital plexus following an overnight fast. The serum concentrations of testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), testosterone (T), and insulin were measured using a mouse ELISA Kit (R&D Systems, USA).

2.6. Artificial decidualization

To induce artificial decidualization on the 4th day of pseudopregnancy, 20 μL of sesame oil was injected into one uterine horn, while the non-injected contralateral horn served as a control. On the 8th day of pseudopregnancy, the deciduoma was evaluated.

2.7. Molecular structure docking analysis

The two-dimensional structure of the active ingredient was obtained from the PubChem (*https://pubchem.ncbi.nlm.nih.gov/*) website and converted to a minimum free energy three-dimensional structure using the ChemBio3D software. The three-dimensional structures of the core targets were obtained from the PDB database, and proteins as well as drug components were converted to PDBQT format files using "Auto Dock Tools" software.

2.8. Treatment of T-HESCs

T-HESCs were obtained at the Institution of Obstetrics and Gynecology Hospital of Fudan University, and quercetin was dissolved in concentrations of 5, 10, and 20 μM for cell treatment. To simulate hyperinsulinemia seen in PCOS patients, T-HESCs were treated with insulin (Sigma-Aldrich Inc., UNITED STATES) at concentrations of 10, 50, 100, 200, and 500 nM for three days. To determine if the Insulin Receptor Substrate 1/2 (IRS1/2) inhibitor NT157 (TargetMol Chemicals Inc., Boston) could block the effects of quercetin, T-HESCs were pre-decidualized for three days and treated with 6 μM NT157 for one hour before being exposed to 50 nM insulin and quercetin treatment for three days. T-HESCs were then induced for decidualization *in vitro* by treating them with 8-Br-cAMP (50 mM, Sigma, MO, USA), progesterone (100 ng/ml, Sigma, MO, USA), and estradiol (10 ng/ml, Abcam, CA, USA) for a further three days.

2.9. Cells viability assays

The cells were incubated with a medium containing quercetin at concentrations of 0, 5, 10, 20, 50, 100, and 200 μM for 24, 48, and 72 hours. After the incubation period, the cells were subjected to a CCK-8 assay (Yeasen Biotechnology Co., Ltd.) and viable cells were identified and quantified by measuring absorbance at 450 nm.

2.10. Cell apoptosis assays

To analyze apoptosis, 3×10^3 cells were stained with Annexin V and propidium iodide (Biolegend Inc.), and the percentage of apoptotic cells was determined with flow cytometry (CytoFLEX; Beckman Coulter, Inc.).

2.11. Wound healing assays

To examine the impact of quercetin on cell migration, T-HESCs were exposed to serum-free Dulbecco's Modified Eagle Medium (DMEM) (GENOM BIO, Zhejiang, China) supplemented with 0, 5, 10, or 20 μM quercetin in 1.25% v/v Dimethyl sulfoxide (DMSO) (Sango Biotech Co.,Ltd.) (500 μL/well). A scratch was then made in the cell monolayer at 0 h, and fresh medium was added. The T-HESCs were then cultured for an additional 3 days while images were captured at consistent locations every 24 h over the 72-h period. The images were analyzed using Image J software to assess cell migration.

2.12. RT-qPCR

RNA extraction was carried out from the treated cells using the RNAiso Plus kit (Takara Biomedical Technology (Beijing) Co. Ltd.), and the cDNA was synthesized utilizing the PrimeScript™ RT kit (Perfect Real Time (Takara)). The list of target genes and primers used can be found in Table 1.

2.13. Statistical data analysis

The data that exhibited a normal distribution were presented as mean ± standard deviation (mean ± SD), and their statistical significance was evaluated using a one-way analysis of variance (ANOVA). The Student's *t*-test and ANOVA were employed to determine the significance of the outcomes, and these analyses were conducted utilizing GraphPad Prism v.8.

3. Results

3.1. YHHD reduces early miscarriage rates in PCOS patients

Between September 1, 2018, and December 30, 2022, a total of 660 patients diagnosed with PCOS were enrolled in the study after meeting the requisite inclusion and exclusion criteria. Baseline clinical and hormonal characteristics were similar across all groups (Table 2). The rate of early miscarriage rate was 12.19%, 24.44% and 16.62% in the groups receiving dydrogesterone (10 mg, Abbott Biologicals B.V) and YHHD, YHHD, and dydrogesterone alone, respectively. Notably, the combination of dydrogesterone and YHHD was found to be significantly associated with a lower rate of early miscarriage than YHHD alone ($P < 0.05$).

3.2. YHHD ameliorated the symptoms in DHEA-induced PCOS mice

To investigate the potential therapeutic efficacy of YHHD in treating PCOS, we conducted an experiment to assess its impact on body weight, estrous cycles, histological changes, and serum hormone levels in three groups. According the protocol (Figure 1A), our findings demonstrated a significant increase in weight gain in PCOS mice when compared to the normal group, which was effectively reduced by YHHD treatment (Figure 1B). The estrous cycle of PCOS mice was closely monitored to confirm the efficacy of YHHD treatment, and our findings demonstrated that PCOS mice had disrupted estrous cycles, this disruption was characterized by prolonged metaestrous and diestrous (M/D) phases, as well as a reduction in proestrus and estrous phases (Figure 1C). Following administration of YHHD for two weeks, there was a gradual normalization of the estrous cycle. Compared with the normal control group, HE staining of ovarian tissue showed numerous vesicular follicles in PCOS group, with a significant reduction of granulosa cell layer. After YHHD treatment, a large number of luteal structures and all levels of growing follicles were seen in the ovarian tissue, and the normal ovarian structure was restored (Figure 1D). Moreover, YHHD treatment effectively reversed the abnormal hormone levels (Figure 1E). Compared to the control group (0.97 ± 0.73), the results showed a significant increase in HOMA-IR score in PCOS group (5.41 ± 0.62) (Figure 1F). Intriguingly, the HOMA-IR score decreased significantly (1.31 ± 0.68) ($P < 0.05$) in PCOS mice treated orally with YHHD. This indicates that YHHD has the potential to treat insulin resistance in PCOS mice. The control and YHHD group mice exhibited a peak increase in FBG levels prior to the 30-minute point of glucose loading, which then dropped to levels near-normal at 120 minutes. The oral administration of YHHD significantly reduced blood glucose levels in PCOS mice from 60 minutes onwards ($P < 0.05$) compared to PCOS mice (Figure 1F). This finding suggests that YHHD has the potential to improve glucose tolerance in PCOS mice by reducing insulin resistance. Overall, the results suggest that YHHD has a

Table 1. Primers and probes for real-time RT-qPCR

Gene	Primer sequence (5′–3′)	Product size (bp)
IGFBP-1	AGCACGGAGATAACTGAGGAGGAG GTTGGTGACATGGAGAGCCTTCG	129
PRL	GCAGATGGCTGATGAAGAGTCTCG GCAGTTGTTGTTGTGGATGATTCGG	130
FoxO1	TGTCCTACGCCGACCTCATCAC GCACGCTCTTGACCATCCACTC	96
IRS-1	AGTGGAGAGCAGCGGTGGTAAG AGTAGTAGGAGAGGACTGGCTTGTG	146
IRS-2	CTCTGCCTCGCTGGATGAATACAC GATGTCTCCGTAGTCCTCTGGGTAG	133
GLUT4	CCTTGGTCTCGGTGTTGTTGGTG AGGAGCAGAGCCACAGTCATCAG	109
GLUT12	GGTTGGAGTCGTCAAGGTCATTAGC GCCATCACAGAGGAGCCAATGC	99

Table 2. The comparison of the clinical data and pregnancy result of the patients with PCOS after treatment

Parameters	YHHD $N = 220$	Dydrogesterone $N = 220$	Dydrogesterone + YHHD $N = 220$	*P* values
Age median (years)	27.2 (5.2)	30.4 (2.1)	28.5 (5.3)	0.842
BMI (Kg/m^2)	23.8 (5.1)	25.9 (4.1)	22.9 (3.8)	0.604
FSH (mIU/mL)	6.56 (2.3)	5.86 (3.2)	6.32 (4.7)	0.674
LH (mIU/mL)	7.43 (4.6)	10.85 (5.3)	6.74 (3.2)	0.053
E2 (mIU/mL)	48.8 (22.3)	55.83 (33.4)	36.32 (42.6)	0.056
Testosterone (ng/mL)	0.56 (0.33)	0.86 (0.32)	0.32 (0.27)	0.042
SHBG (nmol/mL)	56.54 (15.3)	45.49 (23.2)	58.56 (24.5)	0.065
Glucose 0 minute (mmol/L)	5.1 (2.3)	6.1 (1.4)	4.4 (2.1)	0.063
Glucose 120 minute (mmol/L)	7.8 (2.1)	8.4 (3.2)	6.5 (4.3)	0.058
Insulin 0 minute (μU/mL)	10.53 (6.15)	12.11 (5.43)	9.56 (2.13)	0.051
Insulin 120 minute (μU/mL)	39.13 (14.15)	65.32 (24.34)	24.15 (23.13)	0.032
Early abortion rate (%)	24.44 (15.27)	16.62 (14.05)	12.19 (14.56)	0.042

All the data as expressed the mean.

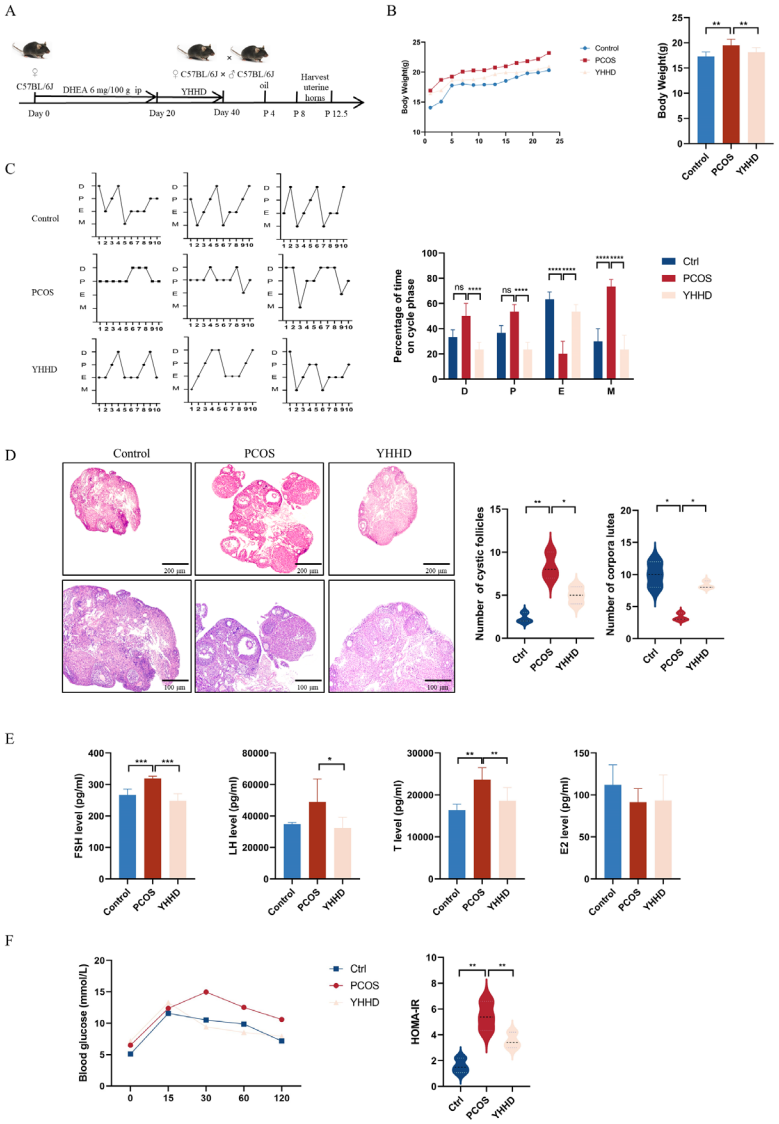


Figure 1. To depicts the effect of YHHD on DHEA-induced PCOS in mice. (A) The results showed the control groups and treatment schedule. **(B)** The effect of YHHD on body weight in PCOS mice ($n = 6$). **(C)** The representative estrous cycles and quantifies the changes in estrous cycle ($n = 6$). **(D)** The representative ovary tissue samples stained with H&E, and the quantification of cystic follicles and corpus luteum ($n = 6$). The scale bar represents 1mm. **(E)** The serum levels of sex hormone indicators including T, E2, LH, and FSH, which were determined using ELISA kits ($n = 6$). **(F)** Effect of YHHD on HOMA-IR and OGTT. The data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *vs.* the PCOS group.

significant therapeutic effect in relieving the endocrine abnormalities associated with PCOS.

3.3. YHHD improves decidualization dysfunction in PCOS mice

To investigate the impact of YHHD on decidualization in PCOS mice, we evaluated the artificially induced decidual response of different groups of female C57BL/6J mice. The control group showed a decidual response within 8 days of artificial stimulation, as demonstrated by the increase in the size of the decidual angle (Figure 2A, left). The YHHD group exhibited a decidual reaction compared to the PCOS group (Figure 2A, right). The outcomes demonstrated that the weight of stimulated uterine horns in the PCOS group was significantly lower than that in the control and YHHD groups, indicating that the decidual response was lower in PCOS mice. These findings suggest that YHHD treatment ameliorates the decidual response in PCOS mice, resulting in improved uterine wet weight gain (Figure 2A). Moreover, compared with the normal control group and YHHD group, the uterine thickness of PCOS group became thin, the glands was not tight and the number was significantly reduced, and the gland cavity was smaller (Figure 2B). The expression of decidual-related genes, bone morphogenetic protein-2 (*BMP-2*), *Prl8a2*, Heart And Neural Crest Derivatives Expressed 2 (*HAND2*), and Wnt Family Member 4 (*Wnt4)* was significantly reduced in the stimulated uterus of PCOS mice compared to that in control mice (Figure 2C). However, the expression levels of these decidual-

related genes were notably elevated in the YHHD-treated group compared to the PCOS group, suggesting amelioration of decidualization dysfunction in PCOS mice subsequent to YHHD intervention. These results suggest that YHHD has the potential to enhance the process of decidualization in PCOS mice by modulating the expression of decidual-related genes.

3.4. The therapeutic effect of quercetin on PCOS mice

The Table 3 displays the top three pharmacological components based on degree values, the top three compounds in the network, quercetin, daidzein, and kaempferol had 128, 62, and 53 targets, respectively, which are crucial for the treatment of PCOS. Therefore, we chose quercetin for the subsequent experiments. The mice were treated with quercetin for 2 weeks, and the effects on reproductive and metabolic parameters were evaluated (Figure 3A). Relative to the PCOS group, quercetin significantly reduced body weight in the PCOS mice ($P < 0.001$) (Figure 3B). The results showed that quercetin treatment substantially improved reproductive parameters, such as restoring estrous cyclicity (Figure 3C), promoting follicle maturation, and increasing the number of corpus luteum (Figure 3D). The results have found that quercetin can modulate DHEA-induced

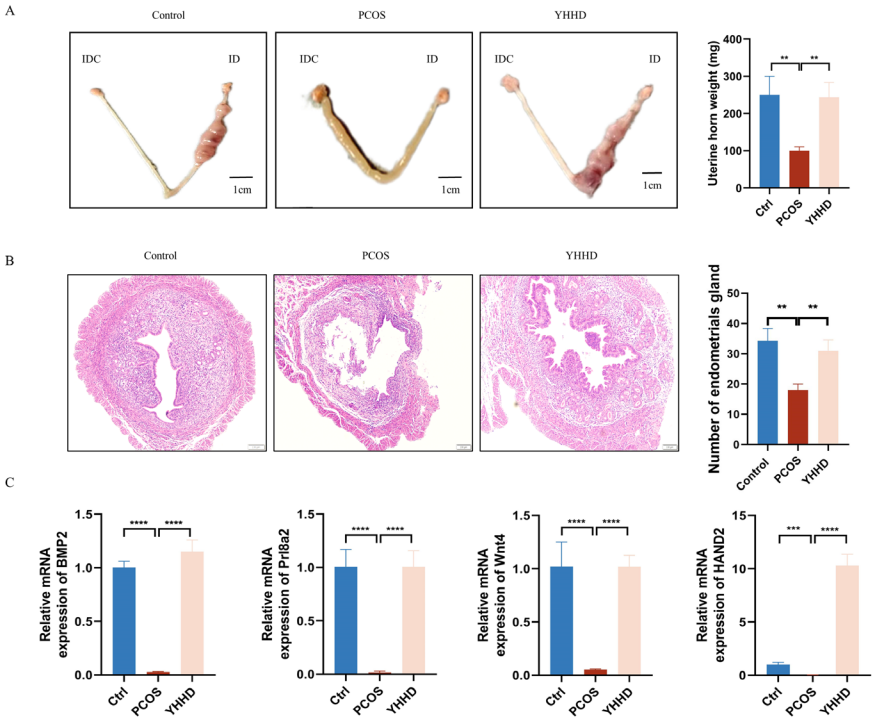
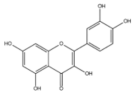
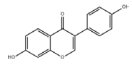
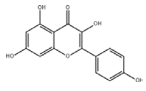


Figure 2. YHHD improves impaired decidualization in PCOS mice. (A) The gross morphology of the uterus was evaluated in all three groups of mice on day 5 after induction of artificial decidualization. The ratio of the mean weight of the stimulated uterine horns was measured in the three groups of mice ($n = 6$) and presented as a histogram. **(B)** Histological analysis of uterine sections from the three groups of stimulated mouse were performed using hematoxylin and eosin staining on day 5 after the induction of artificial decidualization. **(C)** The mRNA expression levels of the decidualization-related genes *BMP2*, *Prl8a2*, *HAND2*, and *Wnt4* were measured by real-time PCR in the uterus of the three groups of mice 5 days after oil infusion. The data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, $^{****}P < 0.0001$ *vs.* the PCOS group.

Table 3. Networks analysis of major compound targets in YHHD and their topological properties

MOLID	Chemical composition	Degree	Molecular mass	Compound source	Structure
MOL000098	Quercetin	128	302.24	Huangbai Danpi Gouqizi Tusizi Xianlingpi Huangqi Chuanxiong Changpu Gegen	
MOL000390	Daidzein	62	254.24	Huangqi	
MOL000422	Kaempferol	53	286.24	Baishao Zhimu Danpi Tusizi Xianlingpi Huangqi Changpu	

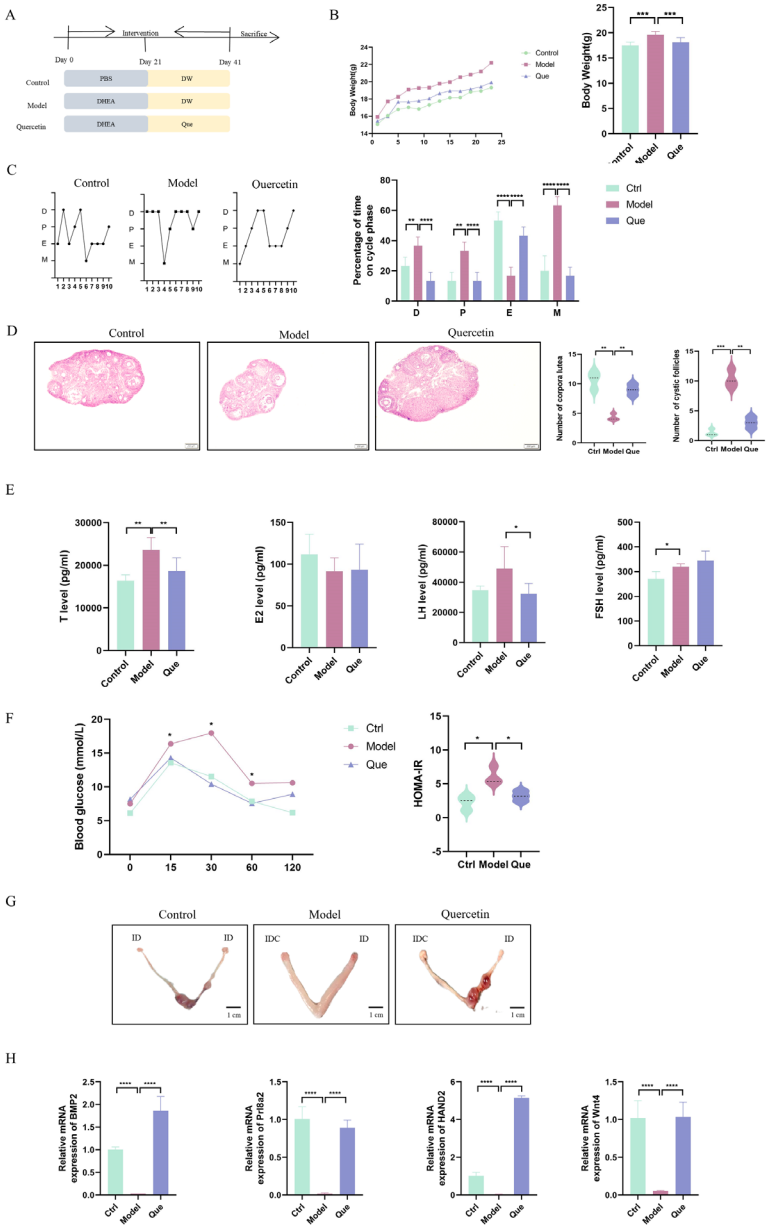


Figure 3. Effect of quercetin on DHEA-induced PCOS in mice. (A) The experimental plan was presented. **(B)** Quercetin was found to have an effect on body weight in PCOS mice ($n = 6$). **(C)** Representative estrous cycles were shown, and changes in estrous cycles were quantified ($n = 6$). **(D)** Representative ovarian tissue samples were stained with H&E, and the number of cystic follicles and corpus luteum was quantified ($n = 6$). **(E)** Serum levels of sex hormone markers, including T, E2, LH, and FSH, were measured using ELISA kits ($n = 6$). **(F)** Quercetin was found to have an effect on HOMA-IR and OGTT. **(G)** The gross morphology of the uterus was evaluated in all three groups of mice on day 5 after induction of artificial decidualization. **(H)** The mRNA expression levels of the decidualization-related genes *BMP2*, *Prl8a2*, *HAND2*, and *Wnt4* were measured by real-time PCR in the uterus of the three groups of mice 5 days after artificial decidualization. The data are shown as mean ± SEM, and statistical significance was observed when compared to the PCOS group as indicated by the '*' (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

changes in hormones, such as LH and T (Figure 3E). In addition, quercetin treatment also improved metabolic parameters, such as reducing the levels of insulin resistance (Figure 3F). The outcomes indicated that the mice in the quercetin group displayed a significantly more robust decidual reaction compared to the PCOS group (Figure 3G). The expression levels of decidual-related genes, including *BMP2*, *Prl8a2*, *HAND2*, and *Wnt4*, were assessed as indicators of decidualization within the stimulated uterus of PCOS mice. The expression of these genes was significantly reduced in the PCOS group when compared to the control group (Figure 3H). In contrast, the expression of these decidual-related genes in the quercetin group was significantly greater than that in the PCOS group, implying a reversal of the decidualization dysfunction observed in PCOS mice following quercetin treatment. Overall, the ability of quercetin to improve various hormonal parameters and metabolic markers suggests that it may have potential benefits in promoting successful pregnancy outcomes in

women with PCOS.

3.5. Quercetin improves cell proliferation, migration and inhibits cell apoptosis

Within our study, we conducted a series of *in vitro* assays to explore the potential impact of quercetin on T-HESCs. We utilized flow cytometry to evaluate T-HESC apoptosis and identified no significant increase in apoptosis induction within the quercetin (5 μM, 10 μM, 20 μM) group when compared to the control group ($P > 0.05$) (Figure 4B). However, treatment with 50 μM and 100 μM quercetin resulted in an increase in the percentage of T-HESCs with positive Annexin V staining. Furthermore, we utilized CCK-8 proliferation assays to demonstrate that T-HESC cellular activity was inhibited in a dose-dependent manner following treatment with various concentrations of quercetin for 24, 48, and 72 hours (Figure 4C). The quercetin (100 μM, 200 μM) group showed a significant decrease in cell survival rate (CSR) at 24, 48, and 72 h. Quercetin (50 μM, 100 μM, 200 μM) showed significant growth inhibition after 72 h of intervention while the quercetin (10 μM, 20 μM) group did not show any effect on cell viability at the three time periods. Additionally, wound healing assays revealed that quercetin improved the cells' ability to migrate (Figure 4D). The percentage of wound closure values for cells treated with serum-free DMEM control, decidualization group, quercetin at 5 μM, 10 μM, 20 μM were 50.00%, 58.06%, 45.00%, 46.15%, and 28.57%, respectively. Compared to 8-Br-cAMP (0.5 mM), progesterone (100 ng/mL) and estrogen (10 ng/mL) (DE) for 72 h, treatment of 5, 10 and 20 μM quercetin increased the expression

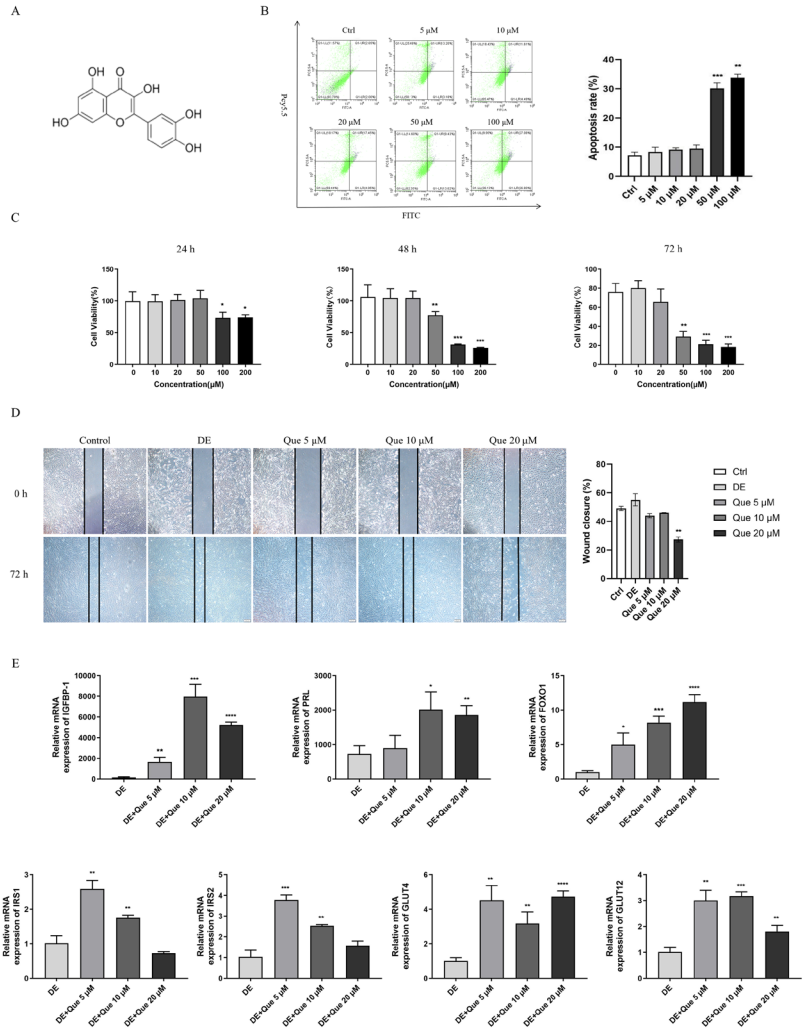


Figure 4. Effect of quercetin on the viability of T-HESCs. (A) Chemical structure of quercetin. **(B)** Apoptotic effects of quercetin, dot plots and percentage cell distribution of T-HESCs cells. Data are expressed as mean ± SD. **(C)** Cytotoxic effects of quercetin in T-HESCs were determined by CCK-8 assay at different concentrations for 24, 48, 72 h. **(D)** Quantitative analysis of the migration area reported as % wound closure. Representative images of T-HESC cells in a wound scratch assay. The images were taken immediately after the scratches had been made and then after 24, 48, 72 h in the presence and absence of quercetin. **(E)** Expression of decidualization markers IGFBP1, PRL, FOXO1 and IRS-associated and GLUT mRNA were determined using RT-qPCR analysis. Scale bars represent 200 μm. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. Three independent experiments were performed in at least triplicate and data are expressed as mean ± SD. Que, quercetin.

of decidualization markers (IGFBP-1, PRL, FoxO1) and IRS-1, IRS-2, GLUT4, GLUT12 during *in vitro* decidualization (Figure 4E).

3.6. Quercetin ameliorates the adverse effects of hyperinsulin on decidualization

In vitro decidualization experiments were conducted by incubating cells with varying concentrations of insulin (0, 10, 50, 100, 200, 500 nmol/L) for 72 hours. The results demonstrated that treatment with 50 nmol/L insulin significantly decreased the mRNA expression of IGFBP-1 and PRL (Figure 5A) while also significantly reducing IRS1/2 mRNA expression (Figure 5B). However, quercetin treatment was able to significantly restore IGFBP1 and PRL mRNA levels inhibited by 50 nmol/L insulin treatment (Figure 5C), indicating the possible involvement of insulin signaling pathways in decidualization. We also evaluated the effect of quercetin on insulin-mediated GLUT4 translocation and found that high levels of insulin led to reduced GLUT4 mRNA expression in T-HESCs. However, quercetin treatment was able to suppress this effect. Moreover, quercetin was found to inhibit the inhibition of IRS-1 and IRS-2 by hyperinsulin. These findings suggest that quercetin may have potential benefits in regulating insulin signaling pathways.

3.7. Quercetin ameliorates the adverse effects of insulin-induced decidualization dysfunction through IRS1/2 receptors

The impact of NT157 on T-HESC cells was explored, and cell viability was found to remain unaffected following treatment with various concentrations of NT157, ranging from 0 to 10 μM (Figure 6A). Molecular docking studies revealed that quercetin exhibited significant affinity for the insulin receptor substrates IRS1 (-8.0 kcal/mol) and IRS2 (-8.2 kcal/mol), indicating that it may contribute to promoting molt by interacting with these proteins as part of the insulin signaling pathway (Figure 6B). RT-qPCR analysis showed that during molt, treatment with 6 μM NT157 led to suppression of *IGFBP-1*, *PRL*, and *FoxO1* expression while reducing *IRS1*, *IRS2*, *GLUT4*, and *GLUT12* gene expression (Figure 6C). Additionally, NT157 inhibited the expression of decidualization-

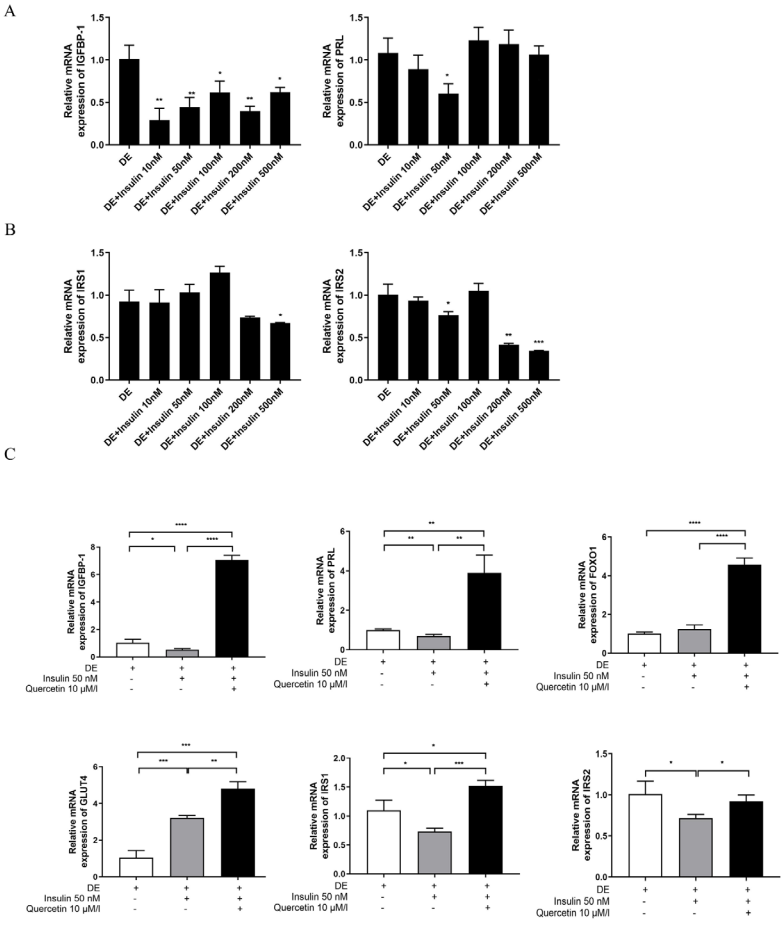


Figure 5. Quercetin reverses decidualization dysfunction due to high insulin levels. (A) Results of RT-qPCR conducted to analyze expression patterns of decidualization markers, IGFBP1 and PRL during in vitro decidualization with various concentrations of Insulin. **(B)** The expression of IRS1 and IRS2 during in vitro decidualization of different concentrations of insulin. **(C)** Quercetin reverses reduced decidualization indicators, IRS, GLUT-related gene expression due to high insulin levels. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

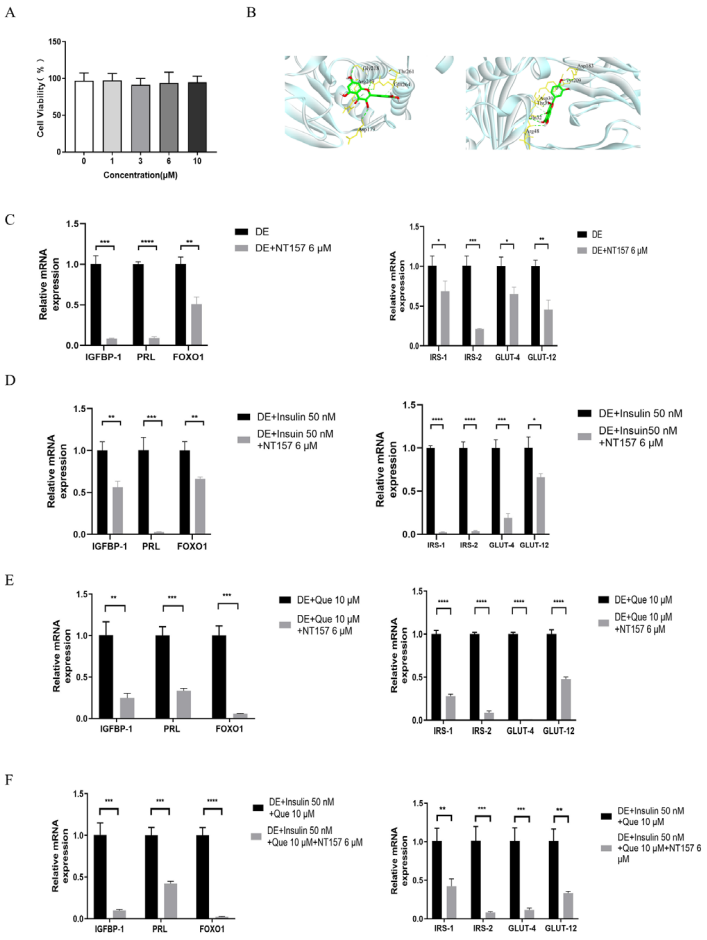


Figure 6. Quercetin reverses high insulin-induced decidualization dysfunction *via* IRS 1/2 receptors. (A) Cell viability of T-HESCs was determined by CCK-8 with or without NT 157 treatment (0, 1, 3, 6 and 10 μM) for 72 hours. **(B)** Molecular docking diagram of quercetin with IRS 1 and IRS 2. **(C)** Inhibition of decidualization markers and IRS-related receptors by NT 157. **(D-F)** Quercetin may act through the IRS 1/2 receptor to promote decidualization and alleviate insulin-induced decidualization dysfunction. Bars indicate the mean ± SD of at least three independent experiments and dots indicate the value for each experiment. **P* < 0.05, ***P* < 0.01, *****P* < 0.0001. Que, quercetin.

associated genes in high insulin intervention conditions (Figure 6D), while 10 μM quercetin had the opposite effect (Figure 6E), promoting the expression of *IGFBP-1*, *PRL*, *FoxO1*, *IRS*, and GLUT-related genes. The addition of quercetin was not capable of reversing the expression of decidualization-related genes under high insulin conditions. However, it may promote decidualization through the IRS1/2 receptor (Figure 6F).

4. Discussion

PCOS is a multifaceted gynecological disorder characterized by hormonal imbalances and metabolic dysfunction (*17-19*). Women with PCOS often experience reproductive disorders, including low pregnancy rates (*20,21*), low live birth rates (*22,23*), and high miscarriage rates (*24,25*). This suggests that many of the symptoms may be related to the endometrial microenvironment and anovulation (*26*). Pregnant women with PCOS have a higher incidence of gestational hypertension, pre-eclampsia, preterm birth, and meconium/placental alterations (*27,28*). Insulin resistance is commonly observed in patients with PCOS, leading to compensatory hyperinsulinemia, which amplifies the bioavailability of androgens and their secretions from ovaries and adrenal glands (*29,30*). This can lead to dysregulation of glucose metabolism in the endometrium and affect endometrial tolerance (*31,32*). High levels of plasma androgens and insulin can also affect the cyclical exfoliation of the endometrium and inhibit the production of IGFBP-1, leading to embryo implantation failure and an increased rate of miscarriage (*24,33*). An increase in insulin levels inhibits the production of IGFBP-1, suggesting that hyperinsulinemia dysregulates the endometrial function, leading to embryo implantation failure and an increased rate of miscarriage (*34,35*). While oral contraceptives (OCs) are a primary therapeutic option for menstrual irregularities in PCOS patients, they are not recommended for use during pregnancy (*36,37*). Insulin sensitizers can address hyperandrogenemia and lower insulin levels in PCOS patients, but they are often associated with gastrointestinal side effects (*38,39*). However, no single pharmaceutical preparation exists to fully manage all PCOS symptoms, and currently

available therapeutic agents can only target specific pathological aspects, presenting limitations (*40*). Given the complexity of PCOS and the challenges it poses, there is an urgent demand for a safe and efficient multi-targeted drug that can offer innovative strategies for treating this condition.

In China, TCM finds common usage as a treatment option for infertility and gynecological problems (*41,42*). TCM can effectively regulate ovarian hemodynamics, serum hormone levels, and menstruation in patients with PCOS, thereby, regulating endocrinology and improving menstrual irregularities, ovulation, and pregnancy rates (*43,44*). The primary pathogenesis of PCOS suggests that Chinese medicine treatment should aim to tonify the kidneys while concurrently addressing the liver, spleen, and heart. The use of Yishen, Huatan, and Huoxue to balance the YHHD formula represents a novel approach to treating PCOS. A preliminary clinical trial has shown that YHHD has demonstrated clinical efficacy in treating PCOS by decreasing LH levels, increasing FSH levels, improving ovarian cysts, promoting follicular development, increasing the number of mature follicles, enhancing ovulation and clinical pregnancy rates, and regulating glucose and lipid metabolism in human subjects. Quercetin as a candidate therapeutic agent for PCOS, which was further studied for its effect on decidualization. The underlying mechanisms through which quercetin and YHHD can provide therapeutic benefits in PCOS could potentially have far-reaching implications for improving maternal-fetal health outcomes and addressing the complexity of this disorder.

While the clinical trials conducted in our study provided substantial insight, they should be interpreted with caution as they were of preliminary and moderate quality. Further research is needed to validate the findings and confirm the effectiveness of quercetin and YHHD in treating PCOS. Specifically, our results indicate that the combined therapy of dydrogesterone and YHHD was associated with a lower rate of early pregnancy loss compared to either therapy used in isolation. This highlights the potential effectiveness of this Chinese and Western combination therapy in reducing the occurrence of early pregnancy loss. The clinical research has established the protective effects of YHHD in early pregnancies of PCOS patients. To further investigate the pharmacological mechanisms underlying YHHD and its primary active component, quercetin, we conducted both *in vitro* and *in vivo* studies to evaluate their effects. By utilizing a combination of network pharmacology, histopathology, and molecular biology analyses, we characterized the underlying mechanisms from a holistic perspective. Our network pharmacological analysis has revealed significant expression of hormone-related signaling pathways, including the cAMP signaling pathway and ovarian steroidogenic pathway. This suggests that YHHD may have a regulatory effect on a range of hormones. The network pharmacology-based predictions suggested that both YHHD and quercetin alleviate decidualization dysfunction by improving insulin resistance and the associated IRS1/2 pathway. Our experimental validation further demonstrated that YHHD and quercetin effectively ameliorate DHEA-induced decidualization dysfunction in PCOS mice and hyperinsulinemia-induced T-HESC.

Quercetin is a naturally occurring flavonoid that can be found in numerous fruits and vegetables (*45,46*). Studies have shown that quercetin has anti-inflammatory and antioxidant properties, as well as its ability to regulate insulin and glucose metabolism (*45,47*). Quercetin has the potential to become a novel treatment for PCOS, given its ability to intervene in multiple pathological pathways through a multi-target approach (*48*). It has been suggested that quercetin may be a promising therapeutic option for the management of PCOS. Consistent with previous research findings (*49,50*), the findings of this study demonstrate that a 20-day treatment with 50 mg/kg of quercetin produces estrogen-like effects, which improve the proportion of ovarian follicles in PCOS mice. Notably, this intervention has been shown to decrease the number of cystic follicles in individuals with PCOS, a marked increase in luteal and normal follicles, improved insulin sensitivity and glucose metabolism and a reduction in the levels of LH and LH/FSH ratios. However, the study didn't observe any significant improvement in the expression of E_2, which is inconsistent with previous research (*51*). Based on the pharmacological effects of YHHD and quercetin on the PCOS network, the primary therapeutic target appears to be insulin resistance. Thus, our upcoming research will concentrate on exploring insulin resistance. Our research has demonstrated that quercetin can enhance insulin signaling by increasing the expression of *IRS1* and *IRS2* genes. However, under high insulin conditions, its effects on decidualization-related genes were found to be limited, and NT157 was more effective in suppressing their expression, revealing that quercetin could only promote decidualization and improve insulin sensitivity under normal insulin conditions. Clinical studies have revealed that patients with hyperinsulinemia exhibit reduced embryo implantation capacity, which often leads to high rates of miscarriage after embryo implantation (*52*).

This study validates the potential therapeutic use of YHHD for PCOS treatment and uncovers its mechanism of action in regulating hormone levels, reversing insulin resistance, and promoting pregnancy, which have been validated in subsequent clinical trials and animal experiments. Additionally, quercetin, as a major compound in YHHD, has shown promising effects in reversing the negative impact of high insulin levels and restoring the expression of key proteins involved in decidualization dysfunction. These findings support the potential use of quercetin as a therapeutic intervention for PCOS-related decidualization dysfunction. Nonetheless,

further rigorous pharmacological and clinical studies are essential to confirm its therapeutic efficacy and safety.

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[§]These authors contributed equally to this work.

*Address correspondence to:
Ling Wang, Laboratory for Reproductive Immunology, Obstetrics and Gynecology Hospital of Fudan University, 419 Fangxie Road, Shanghai 200011, China.
E-mail: dr.wangling@fudan.edu.cn

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