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Letrozole, Berberine, or Their Combination for Anovulatory Infertility in women with Polycystic Ovary Syndrome: Study Design of a Double-blind Randomized Controlled Trial

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Abstract

Introduction: Letrozole is being used as an alternative to clomiphene citrate in women with polycystic ovary syndrome (PCOS) requiring ovulation induction. Berberine, a major active component of Chinese herbal medicine rhizomacoptidis, has been used to improve insulin resistance to facilitate ovulation induction in women with PCOS but there is no study reporting the live birth or its potential as an complementary treatment to letrozole. We here aim to determine the efficacy of letrozole with or without berberine in achieving live births among 660 infertile women with PCOS in Mainland China.

Methods and analysis: This study is a multicenter randomized, double blind trial. The randomization scheme is coordinated through the central mechanism and stratified by the participating site. Participants are randomized into one of three treatment arms: A) letrozole and berberine, B) letrozole and berberine placebo, or C) letrozole placebo and berberine. Berberine is administered three times a day (1.5g/day) for up to 24 weeks, starting on day 1 after a spontaneous period or a withdrawal bleeding. Either letrozole or letrozole placebo 2.5 mg is given daily from day 3 to day 7 of the first 3 cycles and the dose is increased to 5mg/day in the last 3 cycles, if not pregnant. The primary hypothesis is that combination of berberine and letrozole results in a significantly higher live birth rate than letrozole or berberine alone.

Ethics and dissemination: The study was approved by the ethics committee of First Affiliated Hospital of Heilongjiang University of Chinese Medicine. Study findings will be disseminated through peer-reviewed publications and conference presentations.

Trial registration: ClinicalTrials.gov identifier: NCT01619930

1. Background

Polycystic ovary syndrome (PCOS) is characterized by anovulation, hyperandrogenism and polycystic ovaries (PCO) on scanning and is the most common endocrine disorder in women of reproductive age as it affects 5-10% of premenopausal women.[1] Insulin resistance has been implicated in the pathogenesis of anovulation and infertility in PCOS and abnormalities in insulin action have been noted in a variety of reproductive tissues from women with PCOS and may explain the pleiotropic presentation and multi-organ involvement of the syndrome.[2]

The first line medical treatment for ovulation induction in PCOS women is clomiphene citrate (CC), which can result in an ovulation rate of 60–85% but a conception rate of only about 20%.[3-6] Anti-oestrogenic effects on the endometrium and cervix mucus of CC are thought to cause the low conception rate.[7] Also, CC may have a number of side effect including hot flushes, breast discomfort, abdominal distension, nausea, vomiting, nervousness, sleeplessness, headache, mood swings, dizziness, hair loss, and disturbed vision.[6] Letrozole, an aromatase inhibitor, is traditionally applied for estrogen-dependent carcinoma, has been used for ovulation induction for about a decade.[8 9] The effectiveness of letrozole versus CC for ovulation induction has been reviewed by two meta-analyses.[10 11] In both meta-analyses that included six randomized controlled trials, it was concluded that even though letrozole was associated with a lower number of mature follicles per cycle, there was no significant difference in the ovulation rate per cycle or the pregnancy, multiple pregnancy or miscarriage rates between letrozole and CC. No difference was found in the live birth rate, although it was only assessed in one meta-analysis.[11]

Based on the above two meta-analyses, letrozole appears to be at least as effective as CC in ovulation induction with some potential advantages over CC. Although side effects reported by patients in the group receiving CC were higher, while no complication was noted in the group receiving letrozole, [12] large sample sized clinical trials are still needed. As far as we know, two large randomized multicenter studies, PPCOSII (The Pregnancy in Polycystic

Ovary Syndrome II) [13] and AMIGOS (Assessment of Multiple Intrauterine Gestations from Ovarian Stimulation) [14] are ongoing and can provide definitive evidence for pregnancy outcome with the use of CC versus letrozole.

Insulin sensitizing agents are commonly used as adjunctive medication for women with PCOS and metformin is widely chosen.[15] Although a newly published meta-analysis showed that there was no evidence that metformin improved live birth rates in combination with CC (pooled OR 1.16, 95% CI 0.85 to 1.56, 7 trials, and 907 women), the clinical pregnancy rates were higher for the combination of metformin and CC than CC alone (pooled OR 1.51, 95% CI 1.17 to 1.96, 11 trials, 1208 women) .[15] Further, metformin was associated with a higher incidence of gastrointestinal disturbances than placebo (pooled OR 4.27, 95% CI 2.4 to 7.59, 5 trials, and 318 women), which hampered its clinical compliance with high dropout rates.[16]

Recent studies suggest that several Chinese herbal medicines could be beneficial as an adjunct to conventional medical management of PCOS, but the evidence is limited due to the poor methodology of existing trials.[17] Berberine, the major active component of *rhizome coptidis*, exists in a number of medicinal plants and displays a broad array of pharmacological effects.[18] In Chinese medicine, berberine has a long history for its anti-diabetic effect. A recent meta-analysis compared different oral hypoglycaemics including metformin, glipizide or rosiglitazone with berberine, and found no priority over glycaemic control but a mild anti-dyslipidemic effect following berberine.[19] The mechanism of its hypolipidemic effect was studied using human hepatoma cells. Berberine acts differently from that of statin drugs as it could up-regulate low-density lipoprotein receptor expression independent of sterol regulatory element binding proteins, but dependent on extracellular signal-regulated kinases (ERK) activation.[20]

A series of basic research also implicated that berberine could have beneficial effects in women with PCOS. In insulin resistant theca cells, berberine increased glucose transporter (Glut)-4, decreased peroxisome proliferator-activated receptor (PPAR) -δ mRNA levels,

increased glucose uptake, and reduced insulin resistance. [21-23] These results indicate that berberine can improve insulin sensitivity in insulin resistant ovary theca cells. In ovary granulosa cell, berberine was found to reduce the ovarian estrogenic responsiveness by their insulin sensitizing effects similar to metformin, but different from thiazolidinedione.[24] Berberine could also alleviate the degree of insulin resistance and the androgen synthesis in cultured insulin resistant ovaries, indicating that berberine may have a favorable effect on fertility in women with PCOS.[25]

However, there is only one study investigating the effect of berberine in women with PCOS.[26] Eighty-nine Chinese PCOS women with insulin resistance were randomized into one of three treatment groups: berberine+ cyproterone acetate (CPA) (n=31), metformin +CPA (n=30) and placebo +CPA (n=28) for three months. The author concluded that berberine showed similar metabolic effects on amelioration of insulin sensitivity and reduction of hyperandrogenemia, when compared to metformin. Berberine also appeared to have a greater effect on the changes in body composition and dyslipidemia. That study was limited by its small sample size, incomplete description of the methodology and use of surrogate outcomes (anthropometric measures and hormonal and metabolic value changes). There is also an ongoing trial (NCT01138930) testing efficacy of berberine on insulin resistance in women with PCOS as measured by a hyperinsulinemic-euglycemic clamp. Berberine was thought to be safe during clinical use.[19] The side effects were commonly gastrointestinal discomforts including constipation, diarrhea, nausea and abdominal distension. Constipation was one of the most common gastrointestinal complaints, but it is predictable since berberine had a long history used to treat diarrhea in China.

We aim to determine the efficacy of letrozole with or without berberine in achieving live births among women with PCOS seeking pregnancy in China mainland. The primary hypothesis is that combination of berberine and letrozole results in a significantly higher live birth than letrozole or berberine alone. We report herein the study design of our ongoing study.

2. Materials and methods

This is a multi-centre, randomized, double blind and controlled clinical trial. A total of 660 women with PCOS seeking pregnancy (or 220 per each treatment arm) will be enrolled at one of 18 participating sites and randomly assigned to three treatment arms.

The study was approved by the ethics committee of First Affiliated Hospital of Heilongjiang University of Chinese Medicine (2009LL-001-02). Written informed consent will be obtained from each patient prior her participation in the study. The trial is registered at clinicaltrials.gov (NCT01116167).

2.1. Primary and secondary hypotheses

The primary hypothesis is that combination of berberine and letrozole results in a significantly higher live birth than letrozole or berberine alone.

Secondary hypotheses are the adjunctive use of berberine to letrozole has an additive effect on the following:

1) Ovulation rate
2) Ongoing pregnancy rate at around gestation 8-10 weeks
3) Multiple pregnancy rate
4) Miscarriage rate: loss of an intrauterine pregnancy before 20 completed weeks of gestation.
5) Other pregnancy complications such as early pregnancy loss, gestational diabetes mellitus, pregnancy-induced hypertension and birth of small-for-gestational-age (SGA) babies.
6) Infant outcome
7) Changes in metabolic profile: glucose and insulin concentrations, cholesterol, triglycerides, high density lipoprotein (HDL-C) and low density lipoprotein (LDL-C)

8) Changes in hormonal profile: Follicle-stimulating hormone (FSH), Luteinizing hormone (LH), total testosterone (T), Sex hormone-binding globulin (SHBG) and Dehydroepiandrosterone sulfate (DHEAS)
9) Side effect.

2.2 Study population

Women with PCOS who desire pregnancy are eligible if they fulfill the following criteria.

2.2.1 Inclusion criteria

1) Age between 20 and 40 years.
2) Confirmed diagnosis of PCOS according to the Rotterdam 2003 criteria (2 out of 3):
    a. Oligo- or anovulation
    b. Clinical and/or biochemical signs of hyperandrogenism
    c. Polycystic ovaries and exclusion of other etiologies (congenital adrenal hyperplasia, androgen-secreting tumors, Cushing's syndrome)
3) At least one patent tube and normal uterine cavity shown by hysterosalpingogram, HyCoSi or diagnostic laparoscopy within three years.
4) Sperm concentration 15×10^6/mL and progressive motility (grades a* and b**) ≥40%.
*Grade a: rapid progressive motility (sperm moving swiftly, usually in a straight line).
**Grade b: slow or sluggish progressive motility (sperm may be less liner in their progression).
5) History of 1 year of infertility

2.2.2 Exclusion criteria

1) Use of hormonal drugs or other medications including Chinese herbal prescriptions in the past 3 months.
2) Patients with known sever organ dysfunction or mental illness.

3) Pregnancy, post-abortion or postpartum within the past 6 weeks.
4) Breastfeeding within the last 6 months.
5) Not willing to give written consent to the study.

Written informed consent will be obtained from each woman prior to the participation in this study.

2.3 Intervention

Eligible patients will be randomized into one of three arms: A) letrozole and berberine, B) letrozole and berberine placebo, C) letrozole placebo and berberine. Anovulatory patients will have a withdrawal bleed induced with a course of oral medroxyprogesterone acetate (MPA) before the initiation of study medication. Each subject will receive a medication package on a monthly basis that consists of a monthly supply of berberine capsules or placebo capsules and one or two package of pills (letrozole or letrozole placebo, one package per month for the first three months, and two packages per month for the last three months). Berberine or berberine placebo will be administrated orally at a daily dose of 1.5g for 6 months [26]. Patients will receive an initial dose of 2.5 mg (1 tablet) of letrozole or 1 tablet of letrozole placebo on days 3–7 of the first three treatment cycles and increased to 5 mg of letrozole (2 tablet) or 2 tablets of letrozole placebo on days 3–7 of the last three treatment cycles if not pregnant. Berberine and berberine placebo was produced by the Renhetang Pharmaceutical Co., Ltd., China. Letrozole and letrozole placebo were produced from Jiangsu Hengrui Medicine Co., Ltd., China.

2.4 Study specific visits and procedures

Each specific visit and measurement is summarized in Table 1. Baseline measures include fasting FSH, LH, total T, estradiol (E2), glucose and insulin concentrations, cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and height, weight, hip, waist measurements, and vital signs. Also, TCM syndromes of each patient will be accessed with the aim to associate the TCM diagnosis to the study endpoint outcome on baseline visit. Syndrome differentiation in TCM is the

comprehensive analysis of clinical information gained by the four main diagnostic TCM procedures: observation, listening, questioning, and pulse taking.[27] In PCOS, patients are empirically differentiated to be four categories diagnosed by the local Chinese doctors：1) spleen deficiency and phlegm-dampness syndrome, 2) kidney deficiency and liver qi stagnation syndrome, 3) kidney deficiency and blood stasis syndrome and 4) phlegm-dampness and blood stasis syndrome.[28] The TCM diagnosis will be made by an experienced TCM doctor in each participating site according to a standard questionnaire.

All baseline measures will be repeated in all subjects at the end of visits. Safety tests will be repeated during monthly visit 3 and end of treatment visit. Blood samples will be collected and shipped to the core laboratory.

2.5 Randomization and Allocation concealment

The randomization will be performed through a web-based randomization system (http://210.76.97.192:8080/cjbyj) operated by an independent data center-Institute of Basic Research In Clinical Medicine, China Academy of Chinese Medical Sciences (IBRCM). Recruited subjects will be allocated randomly into one of the three groups in a ratio of 1:1:1. The identification code and random number, which are unique for each participant, will be given by a web-based system also produced by IBRCM. Subjects, investigators and physicians taking care of subjects will be blinded to the assignment.

2.6 Data Entry and Quality control of data

Case Report Forms (CRFs) have been developed for data entry and an electronic version is implemented in a Web-based data management system (http://210.76.97.192:8080/cjbyj).

Quality control of data will be handled at three different levels. The first level is the real-time logical and range checking built into the web-based data entry system. The investigators at

the participating sites are required to ensure the data accuracy as the first defense. The second is the remote data monitoring and validation that is the primary responsibility of the study data manager and programmer. The data manager will conduct monthly comprehensive data checks, as well as regular manual checks (within the database system). Manual checks will identify more complicated and less common errors. The data manager will query sites until each irregularity is resolved. The third level of quality control will be the site visits, where data in our database will be compared against source documents. Identified errors will be resolved between the data coordination center and clinical sites. The visits will assure data quality and patient protection.

2.7 Sample size calculation and Statistical analysis

The sample size calculation is based on the live birth rate. The previous study showed that the live birth rate of letrozole was 22%, [29] we hypothesis that combination of letrozole and berberine can increase the life birth rate to 30%. According to the sample size of the estimation formula [30]

$$n = (u_\alpha + u_\beta)^2 \times 2P \times (1-P)/(P_0 - P_1)^2$$

It is estimated that a sample size of 220 subjects per group will be required, considering 20% drop out. Intention-to-treat analysis will be applied to minimize bias due to dropouts. Primary efficacy analysis will be done by comparing the treatment groups with respect to the primary outcome of live birth using the Pearson Chi-square test.

For the secondary, supportive analysis, we will fit a logistic regression model to compare the treatment arms with respect to the primary outcome of live birth, adjusting for other factors such as randomization stratification of study site and prior exposure to study medications. The analysis of other secondary outcomes measured over time will entail the application of statistical methods that have been developed for correlated data since repeated observations will be made over time on each individual. For secondary outcomes such as hormone levels, a linear mixed-effects model will be fit where the main independent

variables will be treatment group, time, and their interaction as well as the designed randomization stratification factors as covariates. Cox proportional hazards models and a Kaplan-Meier method will be applied to compare time to pregnancy in the treatment groups. Adverse events will be categorized and percentage of patients experiencing adverse events and serious adverse events in this trial will be documented. Chi-square tests will be performed to examine differences in the proportion of total and categories of adverse events within each treatment arm. Unblinding of treatments will take place after all participants have delivered or reported final outcomes or when there are medical emergencies.

3. Summary

The present study has several distinctive features. To the best of our knowledge, this is the first clinical trial assessing reproductive effects of berberine on women with PCOS using the live birth rate as the primary outcome, instead of surrogate outcomes such as ovulation and pregnancy rates or metabolic index such as insulin resistance and gluco-lipid profiles. The present trial uses combination therapy of letrozole and berberine. Effects of berberine on the ovulation, corpus lutein, implantation, obstetrics complications as well as teratogenic effects at different stages of reproductive process will be assessed in the present study. Compared with metformin, berberine has less and milder side effects and ameliorating effects on lipid metabolism, which is also a stigma within women with PCOS.

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Contributors WXK, HLH, LJP and LY developed the study protocol. LY, KHY, SWJ, MHL and ZYH coordinated the study. WXK and HLH will oversee enrolment and data collection. LY draft the manuscript in collaboration with ESV and EHYN. All authors have read and approved the final version of the manuscript.

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Competing interests None

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Table 1. Overview of the study visits

	Screening Visit	Baseline Visit	Monthly Visit 1	Monthly Visit 2	Monthly Visit 3	Monthly Visit 4	Monthly Visit 5	End of Treatment Visit
Visit #	1	2	3	4	5	6	7	8
Sign Consent	×							
Urine Pregnancy Test	×		×	×	×	×	×	×
Physical Exam and History	×							×
Transvaginal Ultrasound	×				×			×
Semen Analysis	×							
Hysterosalpingogram or Sonohysterogram (SHG)	×							
Safety Eligibility Tests	×				×			×
Fasting Phlebotomy For Study Parameters		×			×			×
Progesterone Level			×	×	×	×	×	×
QoL Measures	×				×			×
Assess Adverse Events			×	×	×	×	×	×
Record Concomitant Meds	×	×	×	×	×	×	×	×

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Trial registration: ClinicalTrials.gov identifier: NCT01116167

1. Background

Polycystic ovary syndrome (PCOS) is characterized by anovulation, hyperandrogenism and polycystic ovaries (PCO) on scanning and is the most common endocrine disorder in women of reproductive age as it affects 5-10% of premenopausal women.[1] Insulin resistance has been implicated in the pathogenesis of anovulation and infertility in PCOS and abnormalities in insulin action have been noted in a variety of reproductive tissues from women with PCOS and may explain the pleiotropic presentation and multi-organ involvement of the syndrome.[2]

The first line medical treatment for ovulation induction in PCOS women is clomiphene citrate (CC), which can result in an ovulation rate of 60–85% but a conception rate of only about 20%.[3-6] Anti-oestrogenic effects on the endometrium and cervix mucus of CC are thought to cause the low conception rate.[7] Also, CC may have a number of side effect including hot flushes, breast discomfort, abdominal distension, nausea, vomiting, nervousness, sleeplessness, headache, mood swings, dizziness, hair loss, and disturbed vision.[6] Letrozole, an aromatase inhibitor, is traditionally applied for estrogen-dependent carcinoma, has been used for ovulation induction for about a decade.[8 9] The effectiveness of letrozole versus CC for ovulation induction has been reviewed by two meta-analyses.[10 11] In both meta-analyses that included six randomized controlled trials, it was concluded that even though letrozole was associated with a lower number of mature follicles per cycle, there was no significant difference in the ovulation rate per cycle or the pregnancy, multiple pregnancy or miscarriage rates between letrozole and CC. No difference was found in the live birth rate, although it was only assessed in one meta-analysis.[11]

Based on the above two meta-analyses, letrozole appears to be at least as effective as CC in ovulation induction with some potential advantages over CC. Although side effects reported by patients in the group receiving CC were higher, while no complication was noted in the group receiving letrozole, [12] large sample sized clinical trials are still needed. As far as we know, two large randomized multicenter studies, PPCOSII (The Pregnancy in Polycystic

Ovary Syndrome II) [13] and AMIGOS (Assessment of Multiple Intrauterine Gestations from Ovarian Stimulation) [14] are ongoing and can provide definitive evidence for pregnancy outcome with the use of CC versus letrozole.

Insulin sensitizing agents are commonly used as adjunctive medication for women with PCOS and metformin is widely chosen.[15] Although a newly published meta-analysis showed that there was no evidence that metformin improved live birth rates in combination with CC (pooled OR 1.16, 95% CI 0.85 to 1.56, 7 trials, and 907 women), the clinical pregnancy rates were higher for the combination of metformin and CC than CC alone (pooled OR 1.51, 95% CI 1.17 to 1.96, 11 trials, 1208 women) .[15] Further, metformin was associated with a higher incidence of gastrointestinal disturbances than placebo (pooled OR 4.27, 95% CI 2.4 to 7.59, 5 trials, and 318 women), which hampered its clinical compliance with high dropout rates.[16]

Recent studies suggest that several Chinese herbal medicines could be beneficial as an adjunct to conventional medical management of PCOS, but the evidence is limited due to the poor methodology of existing trials.[17] Berberine, the major active component of *rhizome coptidis*, exists in a number of medicinal plants and displays a broad array of pharmacological effects.[18] In Chinese medicine, berberine has a long history for its anti-diabetic effect. A recent meta-analysis compared different oral hypoglycaemics including metformin, glipizide or rosiglitazone with berberine, and found no priority over glycaemic control but a mild anti-dyslipidemic effect following berberine.[19] The mechanism of its hypolipidemic effect was studied using human hepatoma cells. Berberine acts differently from that of statin drugs as it could up-regulate low-density lipoprotein receptor expression independent of sterol regulatory element binding proteins, but dependent on extracellular signal-regulated kinases (ERK) activation.[20]

A series of basic research also implicated that berberine could have beneficial effects in women with PCOS. In insulin resistant theca cells, berberine increased glucose transporter (Glut)-4, decreased peroxisome proliferator-activated receptor (PPAR) -δ mRNA levels,

increased glucose uptake, and reduced insulin resistance. [21-23] These results indicate that berberine can improve insulin sensitivity in insulin resistant ovary theca cells. In ovary granulosa cell, berberine was found to reduce the ovarian estrogenic responsiveness by their insulin sensitizing effects similar to metformin, but different from thiazolidinedione.[24] Berberine could also alleviate the degree of insulin resistance and the androgen synthesis in cultured insulin resistant ovaries, indicating that berberine may have a favorable effect on fertility in women with PCOS.[25]

However, there is only one study investigating the effect of berberine in women with PCOS.[26] Eighty-nine Chinese PCOS women with insulin resistance were randomized into one of three treatment groups: berberine+ cyproterone acetate (CPA) (n=31), metformin +CPA (n=30) and placebo +CPA (n=28) for three months. The author concluded that berberine showed similar metabolic effects on amelioration of insulin sensitivity and reduction of hyperandrogenemia, when compared to metformin. Berberine also appeared to have a greater effect on the changes in body composition and dyslipidemia. That study was limited by its small sample size, incomplete description of the methodology and use of surrogate outcomes (anthropometric measures and hormonal and metabolic value changes). There is also an ongoing trial (NCT01138930) testing efficacy of berberine on insulin resistance in women with PCOS as measured by a hyperinsulinemic-euglycemic clamp. Berberine was thought to be safe during clinical use.[19] The side effects were commonly gastrointestinal discomforts including constipation, diarrhea, nausea and abdominal distension. Constipation was one of the most common gastrointestinal complaints, but it is predictable since berberine had a long history used to treat diarrhea in China.

We aim to determine the efficacy of letrozole with or without berberine in achieving live births among women with PCOS seeking pregnancy in China mainland. The primary hypothesis is that combination of berberine and letrozole results in a significantly higher live birth than letrozole or berberine alone. We report herein the study design of our ongoing study.

2. Materials and methods

This is a multi-centre, randomized, double blind and controlled clinical trial. A total of 660 women with PCOS seeking pregnancy (or 220 per each treatment arm) will be enrolled at one of 18 participating sites and randomly assigned to three treatment arms.

The study was approved by the ethics committee of First Affiliated Hospital of Heilongjiang University of Chinese Medicine (2009LL-001-02). Written informed consent will be obtained from each patient prior her participation in the study. The trial is registered at clinicaltrials.gov (NCT01116167).

2.1. Primary and secondary outcomes

The primary outcome is that combination of berberine and letrozole results in a significantly higher live birth than letrozole or berberine alone.

Secondary outcomes are the adjunctive use of berberine to letrozole has an additive effect on the following:

1) Ovulation rate: Patients will take serum progesterone test at day 22 of each treatment cycle. Progesterone > 3 ng/mL will be considered as ovulation.
2) Ongoing pregnancy rate at around gestation 8-10 weeks. Pregnancy will be confirmed, if suspected, by measurement of serum hCG. Pregnancies will be followed by the serial rise of serum hCG and ultrasound will be utilized to determine location of the pregnancy and number of implantation sites. Participants who conceive will be followed through the study until the pregnancy has advanced to the point of determining the number of gestational sacs, their location, and fetal viability as determined by visualization of fetal heart motion by ultrasonography.
3) Multiple pregnancy rates.

4) Miscarriage rate: loss of an intrauterine pregnancy before 20 completed weeks of gestation.
5) Other pregnancy complications such as early pregnancy loss, gestational diabetes mellitus, pregnancy-induced hypertension and birth of small-for-gestational-age (SGA) babies.
6) Infant outcome: We will review pregnancy and birth records to document neonatal morbidity and mortality and the presence of fetal anomalies.
7) Changes in metabolic profile: Fasting glucose and insulin concentrations, cholesterol, triglycerides, high-density lipoprotein (HDL-C) and low-density lipoprotein (LDL-C). The blood sample for the tests will be draw at both the baseline visit and the end of treatment visit.
8) Changes in hormonal profile: Follicle-stimulating hormone (FSH), Luteinizing hormone (LH), total testosterone (T), Sex hormone-binding globulin (SHBG) and Dehydroepiandrosterone sulfate (DHEAS). The fasting blood sample for the tests will be draw at both the baseline visit and the end of treatment visit at menstrual cycle day 3-7.
9) Side effect: The patient will asked to record adverse event and reported to the coordinator during each visit.

2.2 Study population

Women with PCOS who desire pregnancy are eligible if they fulfill the following criteria.

2.2.1 Inclusion criteria

1) Age between 20 and 40 years.
2) Confirmed diagnosis of PCOS according to the Rotterdam 2003 criteria (2 out of 3):
    a. Oligo- or anovulation
    b. Clinical and/or biochemical signs of hyperandrogenism

c. Polycystic ovaries and exclusion of other etiologies (congenital adrenal hyperplasia, androgen-secreting tumors, Cushing's syndrome)

3) At least one patent tube and normal uterine cavity shown by hysterosalpingogram, HyCoSi or diagnostic laparoscopy within three years.

4) Sperm concentration 15×10^6/mL and progressive motility (grades a* and b**) ≥40%.

*Grade a: rapid progressive motility (sperm moving swiftly, usually in a straight line).

**Grade b: slow or sluggish progressive motility (sperm may be less liner in their progression).

5) History of 1 year of infertility

2.2.2 Exclusion criteria

1) Use of hormonal drugs or other medications including Chinese herbal prescriptions in the past 3 months.
2) Patients with known sever organ dysfunction or mental illness.
3) Pregnancy, post-abortion or postpartum within the past 6 weeks.
4) Breastfeeding within the last 6 months.
5) Not willing to give written consent to the study.

Written informed consent will be obtained from each woman prior to the participation in this study.

2.3 Intervention

Eligible patients will be randomized into one of three arms: A) letrozole and berberine, B) letrozole and berberine placebo, C) letrozole placebo and berberine. Anovulatory patients will have a withdrawal bleed induced with a course of oral medroxyprogesterone acetate (MPA) before the initiation of study medication. Each subject will receive a medication package on a monthly basis that consists of a monthly supply of berberine capsules or placebo capsules and one or two package of pills (letrozole or letrozole placebo, one package per month for the first three months, and two packages per month for the last three months). Berberine or berberine placebo will be administrated orally at a daily dose of

1.5g for 6 months [26]. Patients will receive an initial dose of 2.5 mg (1 tablet) of letrozole or 1 tablet of letrozole placebo on days 3–7 of the first three treatment cycles and increased to 5 mg of letrozole (2 tablet) or 2 tablets of letrozole placebo on days 3–7 of the last three treatment cycles if not pregnant. Berberine and berberine placebo was produced by the Renhetang Pharmaceutical Co., Ltd., China. Letrozole and letrozole placebo were produced from Jiangsu Hengrui Medicine Co., Ltd., China.

2.4 Study specific visits and procedures

Each specific visit and measurement is summarized in Table 1. Baseline measures include fasting FSH, LH, total T, estradiol (E2), glucose and insulin concentrations, cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and height, weight, hip, waist measurements, and vital signs. Also, TCM syndromes of each patient will be accessed with the aim to associate the TCM diagnosis to the study endpoint outcome on baseline visit. Syndrome differentiation in TCM is the comprehensive analysis of clinical information gained by the four main diagnostic TCM procedures: observation, listening, questioning, and pulse taking.[27] In PCOS, patients are empirically differentiated to be four categories diagnosed by the local Chinese doctors：1) spleen deficiency and phlegm-dampness syndrome, 2) kidney deficiency and liver qi stagnation syndrome, 3) kidney deficiency and blood stasis syndrome and 4) phlegm-dampness and blood stasis syndrome.[28] The TCM diagnosis will be made by an experienced TCM doctor in each participating site according to a standard questionnaire.

All baseline measures will be repeated in all subjects at the end of visits. Safety tests will be repeated during monthly visit 3 and end of treatment visit. Blood samples will be collected and shipped to the core laboratory.

2.5 Randomization and Allocation concealment

The randomization will be performed through a web-based randomization system (http://210.76.97.192:8080/cjbyj) operated by an independent data center-Institute of Basic Research In Clinical Medicine, China Academy of Chinese Medical Sciences (IBRCM). Recruited subjects will be allocated randomly into one of the three groups in a ratio of 1:1:1. The identification code and random number, which are unique for each participant, will be given by a web-based system also produced by IBRCM. Subjects, investigators and physicians taking care of subjects will be blinded to the assignment.

2.6 Data Entry and Quality control of data

Case Report Forms (CRFs) have been developed for data entry and an electronic version is implemented in a Web-based data management system (http://210.76.97.192:8080/cjbyj).

Quality control of data will be handled at three different levels. The first level is the real-time logical and range checking built into the web-based data entry system. The investigators at the participating sites are required to ensure the data accuracy as the first defense. The second is the remote data monitoring and validation that is the primary responsibility of the study data manager and programmer. The data manager will conduct monthly comprehensive data checks, as well as regular manual checks (within the database system). Manual checks will identify more complicated and less common errors. The data manager will query sites until each irregularity is resolved. The third level of quality control will be the site visits, where data in our database will be compared against source documents. Identified errors will be resolved between the data coordination center and clinical sites. The visits will assure data quality and patient protection.

2.7 Participation timeline

This trial was started on October 2009 and the first recruitment was on May 15th, 2010. The intervention will take up to 6 months and patient will come to the hospital to see doctor at least once a month to get progesterone test and medication.

2.8 Sample size calculation and Statistical analysis

The sample size calculation is based on the live birth rate. The previous study showed that the live birth rate of letrozole was 22%, [29] we hypothesis that combination of letrozole and berberine can increase the life birth rate to 30%. According to the sample size of the estimation formula [30]

$$n = (u_\alpha + u_\beta)^2 \times 2P \times (1-P)/(P_0 - P_1)^2 \quad (\alpha = 0.05, \beta = 0.1)$$

It is estimated that a sample size of 220 subjects per group will be required, considering 20% drop out. Intention-to-treat analysis will be applied to minimize bias due to dropouts. Primary efficacy analysis will be done by comparing the treatment groups with respect to the primary outcome of live birth using the Pearson Chi-square test.

For the secondary, supportive analysis, we will fit a logistic regression model to compare the treatment arms with respect to the primary outcome of live birth, adjusting for other factors such as randomization stratification of study site and prior exposure to study medications. The analysis of other secondary outcomes measured over time will entail the application of statistical methods that have been developed for correlated data since repeated observations will be made over time on each individual. For secondary outcomes such as hormone levels, a linear mixed-effects model will be fit where the main independent variables will be treatment group, time, and their interaction as well as the designed randomization stratification factors as covariates. Cox proportional hazards models and a Kaplan-Meier method will be applied to compare time to pregnancy in the treatment groups. Adverse events will be categorized and percentage of patients experiencing adverse events and serious adverse events in this trial will be documented. Chi-square tests will be performed to examine differences in the proportion of total and categories of adverse events within each treatment arm. Unblinding of treatments will take place after all participants have delivered or reported final outcomes or when there are medical emergencies.

3. Summary

The present study has several distinctive features. To the best of our knowledge, this is the first clinical trial assessing reproductive effects of berberine on women with PCOS using the live birth rate as the primary outcome, instead of surrogate outcomes such as ovulation and pregnancy rates or metabolic index such as insulin resistance and gluco-lipid profiles. The present trial uses combination therapy of letrozole and berberine. Effects of berberine on the ovulation, corpus lutein, implantation, and obstetrics complications as well as teratogenic effects at different stages of reproductive process will be assessed in the present study. Compared with metformin, berberine has less and milder side effects and ameliorating effects on lipid metabolism, which is also a stigma within women with PCOS.

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Contributors WXK, HLH, LJP and LY developed the study protocol. LY, KHY, SWJ, MHL and ZYH coordinated the study. WXK and HLH will oversee enrolment and data collection. LY draft the manuscript in collaboration with ESV and EHYN. All authors have read and approved the final version of the manuscript.

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Competing interests None

Ethics approval The study was approved by the ethics committee of First Affiliated Hospital of Heilongjiang University of Chinese Medicine (2009LL-001-02).

Provenance and peer review Not commissioned; externally peer reviewed.

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Table 1. Overview of the study visits

	Screening Visit	Baseline Visit	Monthly Visit 1	Monthly Visit 2	Monthly Visit 3	Monthly Visit 4	Monthly Visit 5	Monthly Visit 6	End of Treatment Visit
Visit #	1	2	3	4	5	6	7	8	9
Sign Consent	×								
Urine Pregnancy Test	×		×	×	×	×	×	×	×
Physical Exam and History	×								×
Transvaginal Ultrasound	×				×				×
Semen Analysis	×								
Hysterosalpingogram or Sonohysterogram (SHG)	×								
Safety Eligibility Tests	×				×				×
Fasting Phlebotomy For Study Parameters		×			×				×
Progesterone Level			×	×	×	×	×	×	×
QoL Measures	×				×				×
Assess Adverse Events			×	×	×	×	×	×	×
Record Concomitant Meds	×	×	×	×	×	×	×	×	×

Letrozole, Berberine, or Their Combination for Anovulatory Infertility in women with Polycystic Ovary Syndrome: Study Design of a Double-blind Randomized Controlled Trial

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Abstract

Introduction: Letrozole is being used as an alternative to clomiphene citrate in women with polycystic ovary syndrome (PCOS) requiring ovulation induction. Berberine, a major active component of Chinese herbal medicine rhizomacoptidis, has been used to improve insulin resistance to facilitate ovulation induction in women with PCOS but there is no study reporting the live birth or its potential as an complementary treatment to letrozole. We here aim to determine the efficacy of letrozole with or without berberine in achieving live births among 660 infertile women with PCOS in Mainland China.

Methods and analysis: This study is a multicenter randomized, double blind trial. The randomization scheme is coordinated through the central mechanism and stratified by the participating site. Participants are randomized into one of three treatment arms: A) letrozole and berberine, B) letrozole and berberine placebo, or C) letrozole placebo and berberine. Berberine is administered three times a day (1.5g/day) for up to 24 weeks, starting on day 1 after a spontaneous period or a withdrawal bleeding. Either letrozole or letrozole placebo 2.5 mg is given daily from day 3 to day 7 of the first 3 cycles and the dose is increased to 5mg/day in the last 3 cycles, if not pregnant. The primary hypothesis is that combination of berberine and letrozole results in a significantly higher live birth rate than letrozole or berberine alone.

Ethics and dissemination: The study was approved by the ethics committee of First Affiliated Hospital of Heilongjiang University of Chinese Medicine. Study findings will be disseminated through peer-reviewed publications and conference presentations.

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Recent studies suggest that several Chinese herbal medicines could be beneficial as an adjunct to conventional medical management of PCOS, but the evidence is limited due to the poor methodology of existing trials.[17] Berberine, the major active component of *rhizome coptidis*, exists in a number of medicinal plants and displays a broad array of pharmacological effects.[18] In Chinese medicine, berberine has a long history for its anti-diabetic effect. A recent meta-analysis compared different oral hypoglycaemics including metformin, glipizide or rosiglitazone with berberine, and found no priority over glycaemic control but a mild anti-dyslipidemic effect following berberine.[19] The mechanism of its hypolipidemic effect was studied using human hepatoma cells. Berberine acts differently from that of statin drugs as it could up-regulate low-density lipoprotein receptor expression independent of sterol regulatory element binding proteins, but dependent on extracellular signal-regulated kinases (ERK) activation.[20]

A series of basic research also implicated that berberine could have beneficial effects in women with PCOS. In insulin resistant theca cells, berberine increased glucose transporter (Glut)-4, decreased peroxisome proliferator-activated receptor (PPAR) -δ mRNA levels,

increased glucose uptake, and reduced insulin resistance. [21-23] These results indicate that berberine can improve insulin sensitivity in insulin resistant ovary theca cells. In ovary granulosa cell, berberine was found to reduce the ovarian estrogenic responsiveness by their insulin sensitizing effects similar to metformin, but different from thiazolidinedione.[24] Berberine could also alleviate the degree of insulin resistance and the androgen synthesis in cultured insulin resistant ovaries, indicating that berberine may have a favorable effect on fertility in women with PCOS.[25]

However, there is only one study investigating the effect of berberine in women with PCOS.[26] Eighty-nine Chinese PCOS women with insulin resistance were randomized into one of three treatment groups: berberine+ cyproterone acetate (CPA) (n=31), metformin +CPA (n=30) and placebo +CPA (n=28) for three months. The author concluded that berberine showed similar metabolic effects on amelioration of insulin sensitivity and reduction of hyperandrogenemia, when compared to metformin. Berberine also appeared to have a greater effect on the changes in body composition and dyslipidemia. That study was limited by its small sample size, incomplete description of the methodology and use of surrogate outcomes (anthropometric measures and hormonal and metabolic value changes). There is also an ongoing trial (NCT01138930) testing efficacy of berberine on insulin resistance in women with PCOS as measured by a hyperinsulinemic-euglycemic clamp. Berberine was thought to be safe during clinical use.[19] The side effects were commonly gastrointestinal discomforts including constipation, diarrhea, nausea and abdominal distension. Constipation was one of the most common gastrointestinal complaints, but it is predictable since berberine had a long history used to treat diarrhea in China.

We aim to determine the efficacy of letrozole with or without berberine in achieving live births among women with PCOS seeking pregnancy in China mainland. The primary hypothesis is that combination of berberine and letrozole results in a significantly higher live birth than letrozole or berberine alone. We report herein the study design of our ongoing study.

2. Materials and methods

This is a multi-centre, randomized, double blind and controlled clinical trial. A total of 660 women with PCOS seeking pregnancy (or 220 per each treatment arm) will be enrolled at one of 18 participating sites and randomly assigned to three treatment arms.

The study was approved by the ethics committee of First Affiliated Hospital of Heilongjiang University of Chinese Medicine (2009LL-001-02). Written informed consent will be obtained from each patient prior her participation in the study. The trial is registered at clinicaltrials.gov (NCT01116167).

2.1. Primary and secondary outcomes

The primary outcome is that combination of berberine and letrozole results in a significantly higher live birth than letrozole or berberine alone.

Secondary outcomes are the adjunctive use of berberine to letrozole has an additive effect on the following:

1) Ovulation rate: Patients will take serum progesterone test at day 22 of each treatment cycle. Progesterone > 3 ng/mL will be considered as ovulation.
2) Ongoing pregnancy rate at around gestation 8-10 weeks. Pregnancy will be confirmed, if suspected, by measurement of serum hCG. Pregnancies will be followed by the serial rise of serum hCG and ultrasound will be utilized to determine location of the pregnancy and number of implantation sites. Participants who conceive will be followed through the study until the pregnancy has advanced to the point of determining the number of gestational sacs, their location, and fetal viability as determined by visualization of fetal heart motion by ultrasonography.
3) Multiple pregnancy rates.

4) Miscarriage rate: loss of an intrauterine pregnancy before 20 completed weeks of gestation.
5) Other pregnancy complications such as early pregnancy loss, gestational diabetes mellitus, pregnancy-induced hypertension and birth of small-for-gestational-age (SGA) babies.
6) Infant outcome: We will review pregnancy and birth records to document neonatal morbidity and mortality and the presence of fetal anomalies.
7) Changes in metabolic profile: Fasting glucose and insulin concentrations, cholesterol, triglycerides, high-density lipoprotein (HDL-C) and low-density lipoprotein (LDL-C). The blood sample for the tests will be draw at both the baseline visit and the end of treatment visit.
8) Changes in hormonal profile: Follicle-stimulating hormone (FSH), Luteinizing hormone (LH), total testosterone (T), Sex hormone-binding globulin (SHBG) and Dehydroepiandrosterone sulfate (DHEAS). The fasting blood sample for the tests will be draw at both the baseline visit and the end of treatment visit at menstrual cycle day 3-7.
9) Side effect: The patient will asked to record adverse event and reported to the coordinator during each visit.

2.2 Study population

Women with PCOS who desire pregnancy are eligible if they fulfill the following criteria.

2.2.1 Inclusion criteria

1) Age between 20 and 40 years.
2) Confirmed diagnosis of PCOS according to the Rotterdam 2003 criteria (2 out of 3):
    a. Oligo- or anovulation
    b. Clinical and/or biochemical signs of hyperandrogenism

c. Polycystic ovaries and exclusion of other etiologies (congenital adrenal hyperplasia, androgen-secreting tumors, Cushing's syndrome)

3) At least one patent tube and normal uterine cavity shown by hysterosalpingogram, HyCoSi or diagnostic laparoscopy within three years.

4) Sperm concentration 15×10^6/mL and progressive motility (grades a* and b**) ≥40%.

*Grade a: rapid progressive motility (sperm moving swiftly, usually in a straight line).

**Grade b: slow or sluggish progressive motility (sperm may be less liner in their progression).

5) History of 1 year of infertility

2.2.2 Exclusion criteria

1) Use of hormonal drugs or other medications including Chinese herbal prescriptions in the past 3 months.
2) Patients with known sever organ dysfunction or mental illness.
3) Pregnancy, post-abortion or postpartum within the past 6 weeks.
4) Breastfeeding within the last 6 months.
5) Not willing to give written consent to the study.

Written informed consent will be obtained from each woman prior to the participation in this study.

2.3 Intervention

Eligible patients will be randomized into one of three arms: A) letrozole and berberine, B) letrozole and berberine placebo, C) letrozole placebo and berberine. Anovulatory patients will have a withdrawal bleed induced with a course of oral medroxyprogesterone acetate (MPA) before the initiation of study medication. Each subject will receive a medication package on a monthly basis that consists of a monthly supply of berberine capsules or placebo capsules and one or two package of pills (letrozole or letrozole placebo, one package per month for the first three months, and two packages per month for the last three months). Berberine or berberine placebo will be administrated orally at a daily dose of

1.5g for 6 months [26]. Patients will receive an initial dose of 2.5 mg (1 tablet) of letrozole or 1 tablet of letrozole placebo on days 3–7 of the first three treatment cycles and increased to 5 mg of letrozole (2 tablet) or 2 tablets of letrozole placebo on days 3–7 of the last three treatment cycles if not pregnant. Berberine and berberine placebo was produced by the Renhetang Pharmaceutical Co., Ltd., China. Letrozole and letrozole placebo were produced from Jiangsu Hengrui Medicine Co., Ltd., China.

2.4 Study specific visits and procedures

Each specific visit and measurement is summarized in Table 1. Baseline measures include fasting FSH, LH, total T, estradiol (E2), glucose and insulin concentrations, cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and height, weight, hip, waist measurements, and vital signs. Also, TCM syndromes of each patient will be accessed with the aim to associate the TCM diagnosis to the study endpoint outcome on baseline visit. Syndrome differentiation in TCM is the comprehensive analysis of clinical information gained by the four main diagnostic TCM procedures: observation, listening, questioning, and pulse taking.[27] In PCOS, patients are empirically differentiated to be four categories diagnosed by the local Chinese doctors：1) spleen deficiency and phlegm-dampness syndrome, 2) kidney deficiency and liver qi stagnation syndrome, 3) kidney deficiency and blood stasis syndrome and 4) phlegm-dampness and blood stasis syndrome.[28] The TCM diagnosis will be made by an experienced TCM doctor in each participating site according to a standard questionnaire.

All baseline measures will be repeated in all subjects at the end of visits. Safety tests will be repeated during monthly visit 3 and end of treatment visit. Blood samples will be collected and shipped to the core laboratory.

2.5 Randomization and Allocation concealment

The randomization will be performed through a web-based randomization system (http://210.76.97.192:8080/cjbyj) operated by an independent data center-Institute of Basic Research In Clinical Medicine, China Academy of Chinese Medical Sciences (IBRCM). Recruited subjects will be allocated randomly into one of the three groups in a ratio of 1:1:1. The identification code and random number, which are unique for each participant, will be given by a web-based system also produced by IBRCM. Subjects, investigators and physicians taking care of subjects will be blinded to the assignment.

2.6 Data Entry and Quality control of data

Case Report Forms (CRFs) have been developed for data entry and an electronic version is implemented in a Web-based data management system (http://210.76.97.192:8080/cjbyj).

Quality control of data will be handled at three different levels. The first level is the real-time logical and range checking built into the web-based data entry system. The investigators at the participating sites are required to ensure the data accuracy as the first defense. The second is the remote data monitoring and validation that is the primary responsibility of the study data manager and programmer. The data manager will conduct monthly comprehensive data checks, as well as regular manual checks (within the database system). Manual checks will identify more complicated and less common errors. The data manager will query sites until each irregularity is resolved. The third level of quality control will be the site visits, where data in our database will be compared against source documents. Identified errors will be resolved between the data coordination center and clinical sites. The visits will assure data quality and patient protection.

2.7 Participation timeline

This trial was started on October 2009 and the first recruitment was on May 15th, 2010. The intervention will take up to 6 months and patient will come to the hospital to see doctor at least once a month to get progesterone test and medication.

2.8 Sample size calculation and Statistical analysis

The sample size calculation is based on the live birth rate. The previous study showed that the live birth rate of letrozole was 22%, [29] we hypothesis that combination of letrozole and berberine can increase the life birth rate to 30%. According to the sample size of the estimation formula [30]

$$n = (u_\alpha + u_\beta)^2 \times 2P \times (1-P)/(P_0 - P_1)^2$$ (α= 0.05, β = 0.1)

It is estimated that a sample size of 220 subjects per group will be required, considering 20% drop out. Intention-to-treat analysis will be applied to minimize bias due to dropouts. Primary efficacy analysis will be done by comparing the treatment groups with respect to the primary outcome of live birth using the Pearson Chi-square test.

For the secondary, supportive analysis, we will fit a logistic regression model to compare the treatment arms with respect to the primary outcome of live birth, adjusting for other factors such as randomization stratification of study site and prior exposure to study medications. The analysis of other secondary outcomes measured over time will entail the application of statistical methods that have been developed for correlated data since repeated observations will be made over time on each individual. For secondary outcomes such as hormone levels, a linear mixed-effects model will be fit where the main independent variables will be treatment group, time, and their interaction as well as the designed randomization stratification factors as covariates. Cox proportional hazards models and a Kaplan-Meier method will be applied to compare time to pregnancy in the treatment groups. Adverse events will be categorized and percentage of patients experiencing adverse events and serious adverse events in this trial will be documented. Chi-square tests will be performed to examine differences in the proportion of total and categories of adverse events within each treatment arm. Unblinding of treatments will take place after all participants have delivered or reported final outcomes or when there are medical emergencies.

3. Summary

The present study has several distinctive features. To the best of our knowledge, this is the first clinical trial assessing reproductive effects of berberine on women with PCOS using the live birth rate as the primary outcome, instead of surrogate outcomes such as ovulation and pregnancy rates or metabolic index such as insulin resistance and gluco-lipid profiles. The present trial uses combination therapy of letrozole and berberine. Effects of berberine on the ovulation, corpus lutein, implantation, and obstetrics complications as well as teratogenic effects at different stages of reproductive process will be assessed in the present study. Compared with metformin, berberine has less and milder side effects and ameliorating effects on lipid metabolism, which is also a stigma within women with PCOS.

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Contributors WXK, HLH, LJP and LY developed the study protocol. LY, KHY, SWJ, MHL and ZYH coordinated the study. WXK and HLH will oversee enrolment and data collection. LY draft the manuscript in collaboration with ESV and EHYN. All authors have read and approved the final version of the manuscript.

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Table 1. Overview of the study visits

	Screening Visit	Baseline Visit	Monthly Visit 1	Monthly Visit 2	Monthly Visit 3	Monthly Visit 4	Monthly Visit 5	Monthly Visit 6	End of Treatment Visit
Visit #	1	2	3	4	5	6	7	8	9
Sign Consent	×								
Urine Pregnancy Test	×		×	×	×	×	×	×	×
Physical Exam and History	×								×
Transvaginal Ultrasound	×				×				×
Semen Analysis	×								
Hysterosalpingogram or Sonohysterogram (SHG)	×								
Safety Eligibility Tests	×				×				×
Fasting Phlebotomy For Study Parameters		×			×				×
Progesterone Level			×	×	×	×	×	×	×
QoL Measures	×				×				×
Assess Adverse Events			×	×	×	×	×	×	×
Record Concomitant Meds	×	×	×	×	×	×	×	×	×