A Pilot Study of the Effect of RECO-18 Containing Natural Plant Extracts on Infertile Women Undergoing in Vitro Fertilization-embryo Transfer

Clinical Study Protocol

Sponsor: Sun Yat-sen Memorial Hospital of Sun Yat-sen University

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Objectives:

To investigate the effect of RECO-18 pretreatment on pregnancy outcomes of IVF/ICSI in infertile women when compared with oral administration of multi-vitamins.

Design:

This study is a prospective, randomized, controlled and superiority clinical trial.

Participants:

Eighty infertile patients undergoing IVF/ICSI are randomized to either RECO-18 treatment or multi-vitamins with the ratio of 1:1.

Inclusion criteria

- female, 20 to 40 years old;
- the 1st or 2nd cycle of IVF/ICSI treatment;
- BMI≤30Kg/m2, 4) with bilateral ovaries;
- be eligible for IVF/ICSI treatment.

Exclusion criteria

- repeated implantation failures (with previous 3 or more IVF/ICSI failures);
- moderate to severe endometriosis;
- untreated hydrosalpinx;
- untreated endometrial disease;
- contraindications for assisted reproductive techniques or gestation;
- a history of ovarian surgery;
- expected poor ovarian response (POR) or previous POR;
- polycystic ovarian syndrome;
- participants in clinical trials of other drugs within one month prior to enrollment;
- hypersensitivity to follicle-stimulating hormone-α, FSH, human menopausal gonadotropin, LH or excipients;
- uncontrolled endocrine diseases (such as hyperthyroidism, hypothyroidism, adrenal gland disease, obesity, etc.);

- percutaneous epididymal sperm aspiration or testicular sperm aspiration.

Research procedure:

Eighty infertile patients undergoing IVF/ICSI are randomized to either RECO-18 treatment (treatment group, n=40) or multi-vitamins (control group, n=40).

The treatment group begins to take Reco-18 on the 1st to 5th day of menstruation with a dosage of 4 pills per day for the whole menstrual cycle, then perform ovulation induction on the second menstrual cycle, and continue to take Reco-18 until the day of oocyte retrieval.

The control group takes the multi-vitamins (Elevit, Bayer S.A.) with the dosage of one tablet per day as the same period in the treatment group.

Primary and secondary outcomes

The primary outcome is the ongoing pregnancy rate at 12 weeks' gestation;

The secondary indicators are the number of oocytes retrieved, normal fertilization rate, the high quality embryos rate, implantation rate, clinical pregnancy rate, and early miscarriage rate.

Statistical analysis:

Data are analyzed using SPSS 19.0 statistical software. Measurement data are expressed as mean ± standard deviation, and categorical variables are described as percentages. Data are compared using the independent sample t-test or chi-squared test. $P<0.05$ is considered statistical significance.

Plan of follow-ups:

	Screening and randomization	IVF/ICSI treatment		Follow-up		
	V1	V2	V3	V4	V5	V6
	Before IVF/ICSI treatment	IVF/ICSI treatment cycle	Thawed ET cycle	2 weeks after ET	4-5 weeks after ET	12 weeks of gestation
Informed consent	√					
Eligibility Criteria	√					
Personal information	√					
History	√					
Companied medication	√	√	√	√	√	√
Physical examination	√					
Vital signs	√	√	√			
Sex hormones	√	√	√			
Ultrasound	√	√	√		√	√
Complication and adverse events		√	√	√	√	√

1. Study protocol summary

How to improve the fertility of infertile women has become a hot topic in the field of reproductive medicine. Animal experiment has shown that RECO-18 significantly improved the female fertility in mice, and the specific mechanism was related to reducing follicular atresia, promoting follicle development and improving oocyte quality. Therefore we aim to conduct a pilot study to explore the effect of RECO-18 in infertile women undergoing in vitro fertilization-embryo transfer (IVF-ET). This study is a prospective, randomized, controlled clinical study. The treatment group takes RECO-18 while the control group takes the multi-vitamins. The primary indicator is the ongoing pregnancy rate at 12 weeks' gestation; the secondary indicators are the number of oocytes retrieved, normal fertilization rate, the high quality embryos rate, implantation rate, clinical pregnancy rate, and early miscarriage rate.

2. Research background

In recent years, the group of infertile women has gradually expanded with the effect of environmental pollution, diet and sleeping habits. The decline of female fertility is manifested with decreased ovarian reserve and poor oocyte quality. With the increasing education and social status improvement in female, more and more women have postponed their childbearing plans. It is reported that the average childbearing age of women is nearly 30 years old in our country. Moreover, the liberalization of two-child and three-child policy maybe further delays female reproductive age. Therefore, how to improve the female fertility has become a social and scientific problem in China, and it is urgent to develop new technologies and products to improve the fertility of infertile women.

In female mammals, a follicle is the basic functional unit of the ovary, consisting of an oocyte and single or multiple layers of granule cells. According to the different stages of the oocytes, there are primordial follicle, primary follicle, secondary follicle, antral follicle, and preovulatory follicle. Primordial follicles locate in the resting follicle pool of the ovary with a certain number. The number of human primordial follicles in the ovaries at birth is around 400,000, and decreases with age. When the number of primordial follicles decreases to less than 1000, a female enters the menopause at about 51 years old with disabled fecundity [1, 2]. The optimal age for childbearing is around 25 years old, and ovarian function begins to decline in 30 years old. After the age of 35, ovarian reserve decreases dramatically with the acceleration of primordial follicle activation, meanwhile oocyte quality is declining with the manifestation of high proportion of mature oocyte aneuploidy, low fertilization rate and embryo implantation rate, and high miscarriage rate. Ageing woman is suggested to consult in assisted reproductive center if she has prepared for pregnancy over 6 months but fails in conception. However, it is noteworthy that the success probability of assisted reproduction technology (ART) in aging woman is relative low, because traditional ovarian stimulation is not very effective. Oocyte donation is the effective treatment for aging woman with low fecundity or even infertility, but most women want to have their own babies, and hardly accept it in the mind. Personalized ovarian stimulation protocol and preimplantation

genetic testing are used as clinical intervention, but the treatments increase the costs, and low chance of pregnancy still exists. It is closely related to poor oocyte quality caused by ovarian aging. Therefore, how to protect ovarian reserve and improve oocyte quality is the key to improve female fertility [3-5].

Granulosa cells differentiate into mural granulosa cells and cumulus cells at the stage of the secondary follicle. The cumulus cells are connected to the oocyte by gap junctions, and gradually separate from the mural granulosa cells to form follicular cavity. The follicular cavity is filled with follicular fluid composed of plasma exudate and local secretions, which constitutes the internal environment of oocyte development and plays an important regulatory role in follicular development, maturation, ovulation and atresia. In the process of ovarian aging, the ovarian chronic inflammatory environment and angiogenesis abnormality around the follicle increase the oxidative stress level, decrease mitochondrial vitality, and increase cell apoptosis level, which lead to abnormal granulosa cell function and follicular microenvironment changes, and finally oocyte is damaged with poor quality. Human preovulatory follicular fluid contains a large number of antioxidant enzymes, such as CAT, GSSPx, GST, etc, which mainly prevent the damage of oxidative stress to oocytes. The decreased level of GST and CAT and increased SOD activity are observed in the follicular fluid of aging woman. The decrease in the CAT / SOD ratio indicates a diminished scavenging ability of activating oxide in the follicle [6, 7]. Due to the important role of oxidative stress in ovarian aging, a large number of studies have shown that the application of antioxidants such as Vitamin C, Vitamin E and CoQ 10 effectively improve oxidative stress levels in oocytes, restore mitochondrial function and improve oocyte quality [8-10]. In 2021, consensus on reproductive health and multiple micronutrient in China recommends that infertile woman takes multiple micronutrient containing folic acid of 0.8mg before ART or on the day of embryo transfer to reduce the risk of neural tube defects. However, the adjuvant drugs have not been fully confirmed in evidence-based medicine clinical research, and have not been routinely recommended to aging woman in the guideline.

Mitochondrial function is closely related to oocyte quality, and the effects may be more pronounced in infertile woman. The lack of multiple micronutrients is more common in infertile woman, and the supplementation of multiple micronutrients has an antioxidant effect, which reduce the damage of oxidative stress to fertility and help to improve the outcomes of ART. RECO-18 is a functional food containing a variety of plant extracts (sophora flower bud, clove, yam) and enzymatic soybean phospholipids, and its specific nutrients are list in Table 1. In previous in vivo and in vitro senescent models, RECO-18 was found to improve oocyte quality mainly by regulating the mitochondrial apoptosis pathway. Animal experiment has shown that RECO-18 significantly improved the female fertility in mice (Figure 1, Figure2, Figure 3), and the specific mechanism was related to reducing follicular atresia, promoting follicle development and improving oocyte quality. Therefore we aim to conduct a pilot study to explore whether RECO-18

plays a role in improving oocyte and embryo quality and pregnancy outcomes in infertile women undergoing IVF-ET.

Table 1. Nutrients of RECO-18

Project	Per 100g
Energy	1860k.J
Protein	20.2g
Fat	14.9g
Carbohydrate	56.8g
Sodium	334mg
Vitamin A	14072μgRE
Vitamin D	245.0μg
Vitamin B_1	57.00mg
Vitamin B_2	35.70mg
Vitamin B_6	47.00mg
Vitamin B_{12}	64.20μg
Vitamin C	2801.0mg
Niacin	347.00mg
Folic acid	9527μg
Iron	447.0mg
Zinc	202.00mg

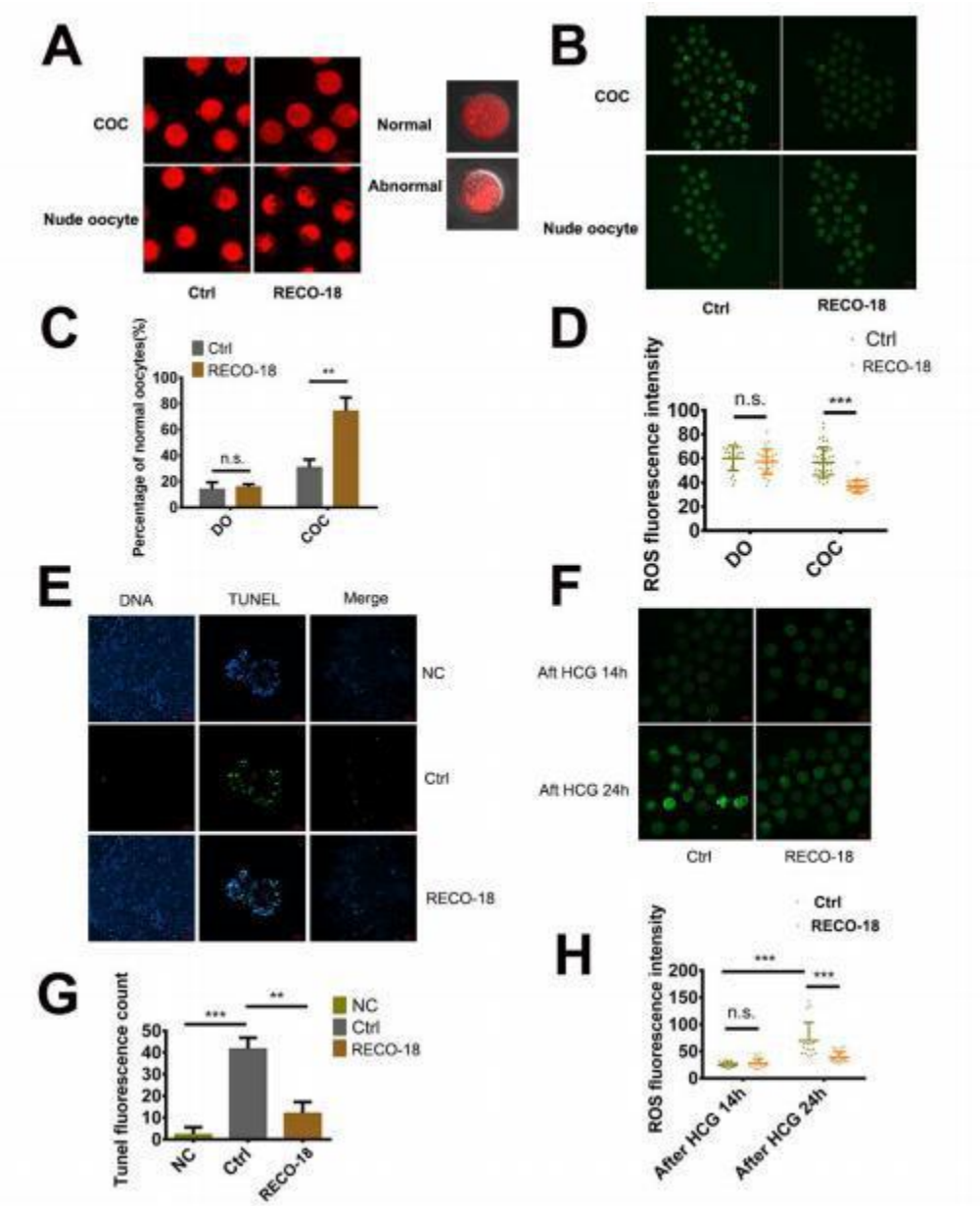

Figure 1: RECO-18 improved oocytes quality in in vitro and in vivo senescent models. A and C, RECO-18 improved mitochondrial distribution in in vitro aging COC; B and D, RECO-18 reduced ROS level in in vitro aging COC; E and G, RECO-18 reduced TUNEL fluorescence signal in in vivo aging COC ; F and H, RECO-18 reduced ROS level of oocytes in in vivo aging COC.

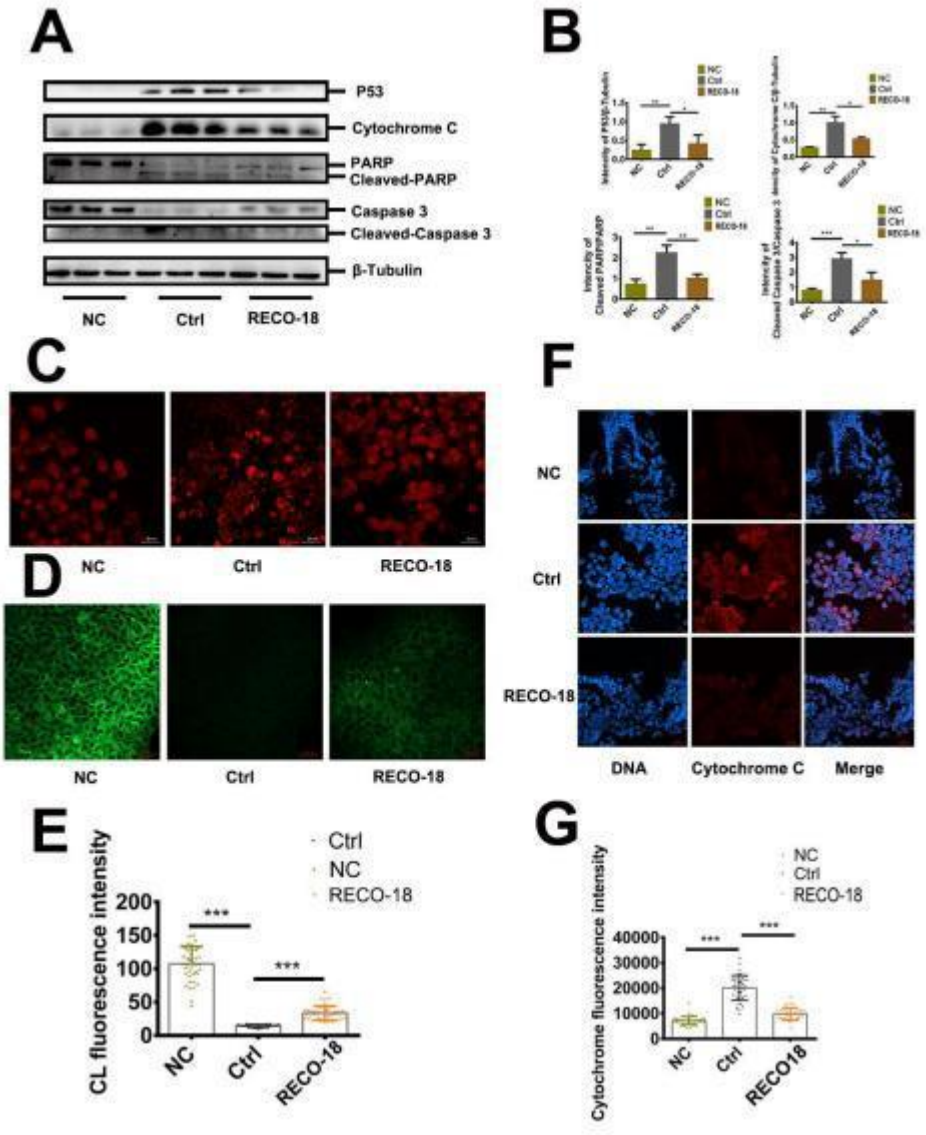

Figure 2: RECO-18 improved the aging oocyte quality mainly by improve the mitochondrial apoptosis pathway of cumulus cells. A and B, Western blot showed that RECO-18 improved the apoptosis pathway in aging COC; C, RECO-18 improved the mitochondrial distribution in aging COC; and D and E, RECO-18 increased the cardiolipin level in aging COC; F and G, RECO-18 reduced cytochrome C level in aging COC.

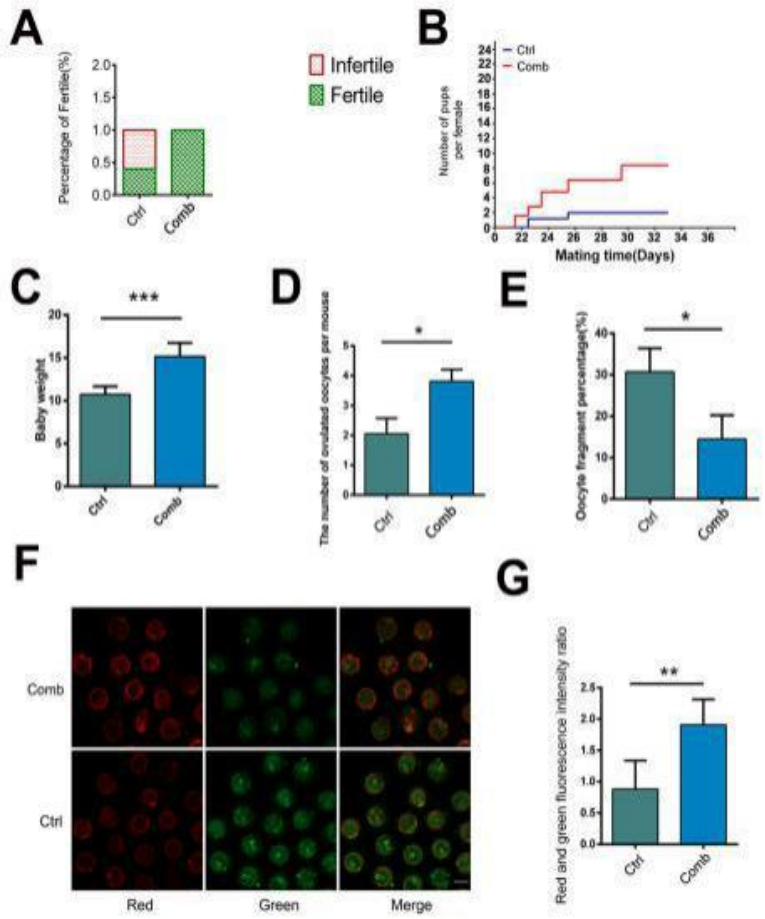

Figure 3: RECO-18 improved oocyte quality and fertility in aging mice. A, RECO-18 increased pregnancy rate of aging mice; B, RECO-18 increased litter size of ageing mice; C, RECO-18 increased the weaning weight of offspring in mice; D, RECO18 increased the ovulation of aging mice; E, RECO-18 reduced the fragmentation rate of oocyte in aging mice during ovarian stimulation; F and G, RECO-18 improved the mitochondrial membrane potential of oocyte in aging mice.

3. Research purpose

To investigate the effect of RECO-18 pretreatment on pregnancy outcomes of IVF-ET in infertile women when compared with oral administration of multi-vitamins.

4. Research design

This study is a prospective, randomized, controlled and superiority clinical trial. Subjects will be recruited from the infertile patients undergoing IVF/ICSI at the Reproductive Medicine Center of Sun Yat-sen Memorial Hospital, Sun Yat-sen University.

The treatment group begins to take Reco-18 on the 1st to 5th day of menstruation with a dosage of 4 pills per day for the whole menstrual cycle, then perform ovulation induction on the second menstrual cycle, and continue to take Reco-18 until the day of oocyte retrieval. The control group

takes the multi-vitamins (Elevit, Bayer S.A.) with the dosage of one tablet per day as the same period in the treatment group.

5. Research protocol

5.1 Sample size

At first it is designed to include 80 infertile patients (40 cases in each group), then the sample size is calculated based on the preliminary study results.

5.2 Inclusion criteria

- female, 20 to 40 years old;
- the 1st or 2nd cycle of IVF/ICSI treatment;
- BMI≤30Kg/m^2, 4) with bilateral ovaries;
- be eligible for IVF/ICSI treatment.

5.3 Exclusion criteria

- repeated implantation failures (with previous 3 or more IVF/ICSI failures);
- moderate to severe endometriosis;
- untreated hydrosalpinx;
- untreated endometrial disease;
- contraindications for assisted reproductive techniques or gestation;
- a history of ovarian surgery;
- expected poor ovarian response (POR) or previous POR;
- polycystic ovarian syndrome;
- participants in clinical trials of other drugs within one month prior to enrollment;
- hypersensitivity to follicle-stimulating hormone-α, FSH, human menopausal gonadotropin, LH or excipients;
- uncontrolled endocrine diseases (such as hyperthyroidism, hypothyroidism, adrenal gland disease, obesity, etc.);
- percutaneous epididymal sperm aspiration or testicular sperm aspiration.

5.4 Exit criteria

1) Patients request to drop out; 2) the cycle is cancelled due to low ovarian response or premature ovulation; 3) actual treatment deviates from the protocol; 4) patients lost follow-up.

5.5 The measurement of research results

Primary indicator: the ongoing pregnancy rate at 12 weeks' gestation.

Secondary indicators: the number of oocytes retrieved, the normal fertilization rate and the rate of high quality embryos, implantation rate, clinical pregnancy rate, and early miscarriage rate.

Randomization: randomized envelope method is used in the study. Random sequence is firstly generated by an independent statistician, secondly the grouping is randomized according to

random number and put into an opaque sealed envelope by the independent personnel, at last the investigator divides the subjects in line with the sealed envelope.

Random ratio: 1:1

Grouping: the treatment group takes RECO-18 while the control group takes the multi-vitamins.

5.6 Informed consent

Before the initiation of study, the informed consent form (ICF) should be used to inform the potential subjects of benefits and risks in simple language. ICF should specify that the informed consent is signed voluntarily, and the potential benefits and risks associated with study are specified, and subjects may drop out from the study at any time.

5.7 Ethics

The study process and informed consent acquisition should comply with Helsinki Declaration. This study has been approved by the Medicine Ethics Committee of Sun Yat-sen Memorial Hospital of Sun Yat-sen University. Every subject should sign the informed consent. The informed consent copy, and contact information of the investigator and Medicine Ethics Committee must be provided to the subjects.

5.8 Research method

5.8.1 Collection of basal data: age, infertility legnth, weight, and body mass index (BMI).

5.8.2 Collection of basal endocrine indexes: follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol, testosterone, dehydroepiandrosterone sulfate (DHEAS), sex hormone binding globulin (SHBG), and anti-müllerian hormone (AMH).

5.8.3 Collection of biochemical indexes: alanine aminotransferase (ALT), aspertate aminotransferase (AST), creatinine, total cholesterol (TC), triglycerides (TG) , high-density lipoprotein (HDL), low-density lipoprotein (LDL), apolipoproteins B (ApoB), high-sensitivity C-reactive protein (Hs-CRP), fasting glucose, and fasting insulin.

5.8.4 Ultrasound examination: bilateral ovarian size, follicle number, and antral follicle count (AFC);

5.8.5 Controlled ovarian stimulation and IVF/ICSI:

a. Controlled ovarian stimulation

Gonadotropin-releasing hormone (GnRH) antagonist protocol: gonadotropin (Gn) 100 IU to 250 IU/day is individually administered to stimulate follicular growth on the 2nd to 4th day of menstruation. GnRH antagonist is added when dominant follicle reaches ≥14mm in diameter and LH level is ≥10IU/L, and is stopped until the day of human chorionic gonadotropin (HCG) administration.

GnRH agonist long protocol: in the midluteal phase of the menstrual cycle confirmed by ultrasound or hormone test or after 2 weeks of oral contraceptive use, pituitary downregulation is achieved by injection of 0.93-1.87mg long-acting GnRH agonist. 14 to 21 days later, vaginal ultrasound and blood hormone tests are performed to evaluate the complete downregulation of the

pituitary gland. Ovarian stimulation is initiated with Gn 100U-250U/day, and adjustment of Gn dosage is performed according to serum hormone level and follicle growth.

b. Trigger: recombinant HCG of 250ug is administered to induce oocyte maturation when at least two follicle ≥18mm or three follicles ≥17mm or four follicles ≥16mm in diameter.

c. Oocyte retrieval, luteal support and embryo transfer: oocyte recovery is performed 34 to 38 hour after the trigger. Luteal support is provided on the day of oocyte retrieval, with vaginal micronized progesterone capsules (600mg/day) combined with oral dydrogesterone (30mg/day) until the pregnancy test. With the use of conventional IVF or ICSI fertilization, 1 to 2 cleavage stage embryos or day 5 blastocysts are transferred to the uterus, and the rest available embryos are cryopreserved.

d. If fresh embryo transfer is cancelled due to medical reasons, pregnancy outcome of the first thawed embryo transfer is collected. The endometrial preparation, thawed embryo transfer and luteal support are based on the routine operation.

5.8.6 Collection of discarded sample:

a. Follicular fluid is collected during oocyte retrieval.

b .The cumulus oocyte complex (COCs) is put into hyaluronidase by the digestion for 10 to 30 seconds to remove the cumulus cells, and the oocytes are picked up and cumulus cells are collected.

5.8.7 Follow-up:

A total of three visits are followed up during the treatment procedure.

a. At the 14th day after embryo transfer: to record urinary pregnancy test, serum HCG and progesterone level.

If pregnancy test is positive, the following follow-up continues, otherwise it discontinues.

b. Four to five weeks after embryo transfer: to perform transvaginal ultrasound. Clinical pregnancy is defined as a gestational sac and fetal heart beat detected on ultrasound.

c. At 12 weeks' gestation: to perform ultrasound. Ongoing pregnancy is defined as live fetuses detected on ultrasound.

6. Safety evaluation

6.1 Adverse events: Any adverse medical events occurring during the study period are defined as adverse events, such as drug reactions, adverse reactions associated with ovarian stimulation, complications associated with pregnancy, and other discomfort complaints

6.2 Adverse events report:

Mild adverse events are recorded in the progress note.

Serious adverse events are submitted to Medicine Ethics Committee within 48 hours of occurrence.

6.3 Safety indexes are adverse events occurring in the treatment group and the control group, such as gastrointestinal reactions and allergies.

7. Data collection and management

The data are collected and managed by the study team members, and personal information involving subject privacy will not appear in the results except for necessary. It should be applied to view subject information in accordance with the rules and regulations of department. The information is only open to the team members, and strictly forbidden to share with irrelevant personnel.

Data are analyzed using SPSS 19.0 statistical software. Measurement data are expressed as mean ± standard deviation, and categorical variables are described as percentages. Data are compared using the independent sample t-test or chi-squared test. $P<0.05$ is considered statistical significance.

7.1 Data Analysis Set:

Full Analysis Set (FAS): the FAS include all randomized subjects receiving treatment at least once. According to the intent-to-treat population (ITT) principle, all randomized cases accepting intervention at least once should be analyzed.

Safety Set (SS): all randomized subjects accepting intervention at least once and receiving post-treatment safety evaluation at least once should be included in the SS. SS is the safety evaluation population for this trial.

Per-protocol population set (PPS): all subjects in the FAS without major protocol violation will be included. Major protocol violation should be defined in the statistical analysis plan before database locking and study unblinding. Subjects will be analyzed based on the treatment.

7.2 Analysis of primary indicator and secondary indicators:

Analysis of primary indicator: the two-sample rate test is used to compare the ongoing pregnancy rate at 12 weeks' gestation in the treatment group and the control group. Categorical variable is compared between groups using Pearson chi-squared test or Fisher's exact probabilistic method. Based on the FAS, the primary endpoint calculates the ongoing pregnancy rate at 12 weeks' gestation and its 95% confidence interval. Logistic regression is used to analyze the baseline effects, and the adjusted factors included age, infertility length, BMI, AMH, AFC, ovulation stimulation protocol, etc.

Analysis of secondary indicators: measurement data are compared using independent sample test or nonparametric analysis, and categorical variables are compared using Pearson chi-squared test or Fisher's exact probabilistic method between groups. Measurement data are analyzed by covariance analysis, and qualitative indicators are analyzed by Logistic regression to evaluate the baseline effects. Adjusted factors refer to the analysis of primary indicators.

7.3 Safety indicators

Use descriptive statistics to summarize laboratory examination and vital signs as safety evaluation indicators, and summarize the changes of each index relative to the baseline. The indexes are classified as normal or abnormal (without clinical significance), and calculated the case number and percentage in each classification. Other safety indicators are summarized or listed as appropriate.

7.4 Data management and quality control

7.4.1 Database establishment

The Epidata electronic database is established based on the case report form before the initiation of study.

7.4.2 Data filling and review

Case report forms should be filled by designated, trained researchers with uniform regulation. Project principal assigns the researcher to review the completeness and accuracy of the case report form, and guide the researcher to make any necessary corrections or additions. When the data are questionable, fill in the data query form, and the researcher should respond the question and confirm the correction. Keep the signed data query form.

7.4.3 Data entry and verification

In order to ensure the accuracy of the data, two data administrators check the data independently. For any questions in the case report form, the data administrators generate the question answer form (DRQ) and send it to the researcher through clinical research associate. The researchers recheck the data and reply in time, and the data administrators revise and input the data accordingly.

7.4.4 Data audit and database locking

After all questions of the data in the system are resolved, the principal investigator, statistician and data administrator first review the data and determine the association of adverse events and study, then determine the analyzed data set, last lock the audited data without any change. If there is any question after the database lock, it should be corrected in the statistical analysis process, and be recorded in detail.

8. Quality Management

8.1 The research team has rich clinical experience and has received GCP training in order to conform to the protocol and ethical requirements

8.2 Animal experiments have shown that RECO18 significantly improved the fertility of mice, which sets the foundation for clinical research.

8.3 Reco-18 is a food for special dietary use based upon "National Food Safety Standard and Nutritional Supplementary Food for Pregnant and Lactating Women" (GB31601-2015). It contains multiple micronutrients (vitamins and minerals) and active constituent of Reco-18, and has passed through food inspection by a third party. Production license number is SC11337082906741.

9. Pre-assessment of risks and benefits and risk control preplan

9.1 Potential benefits

9.1.1 Subjects may get more opportunities for pregnancy with the participation in the study. Meanwhile the study may provide indirect benefit for other patients and generate social benefits

9.1.2 Researchers may develop an adjuvant therapy to improve the fertility of infertility patients, so as to benefit the majority of patients.

9.1.3 Medical institution may provide efficient service for patients, and raise the visibility.

9.2 Potential risks

9.2.1 For researchers and medical institution, the study may not meet the expectation and achieve the research purpose, resulting in the waste of medical resources.

9.2.2 For the patients, the fertility of the subjects may not be ultimately improved.

9.3 Risk control measures

9.3.1 Subjects are fully informed and sign informed consent before participation.

9.3.2 If serious adverse events occur, stop the study immediately and report to the department and Medicine Ethics Committee in the first time, and control the impact of adverse events to a minimum.

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