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STUDY PROTOCOL

Acupuncture for Premature Ovarian Insufficiency: A Randomized, Non-Inferiority Protocol with Exploratory Epigenetics Analysis

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Background and Objectives: Premature ovarian insufficiency (POI), characterized by the loss of ovarian function before age 40, significantly impairs reproductive health. While Hormone Replacement Therapy (HRT) remains the standard of care, its efficacy is often limited by incomplete functional restoration and potential adverse effects. Acupuncture has emerged as a promising adjunct; however, robust clinical evidence and a clear mechanistic understanding are currently lacking. This study aims to evaluate the non-inferiority of acupuncture compared to HRT in improving clinical pregnancy rates among women with POI, while concurrently exploring potential epigenetic mechanisms, specifically DNA methylation.

Methods: This is a randomized, controlled, non-inferiority trial. A total of 572 participants with POI undergoing assisted reproductive technology (ART) will be recruited and randomized to receive either a standardized acupuncture protocol or HRT (following 2024 ESHRE guidelines). Additionally, 286 women with normal ovarian function undergoing in vitro fertilization and embryo transfer (IVF-ET) for male-factor or tubal infertility will be enrolled as a baseline control group for epigenetic analysis. The primary outcome is the clinical pregnancy rate. Secondary outcomes include serum sex hormones, ovarian reserve markers, ovulation induction parameters, pregnancy outcomes, the Modified Kupperman Index, and DNA methylation profiles in ovarian granulosa cells, characterized via whole-genome bisulfite sequencing (WGBS).

Discussion: This protocol is designed to establish clinical evidence regarding the non-inferiority of acupuncture to HRT for POI management. Furthermore, the exploratory epigenetic analysis seeks to elucidate the underlying molecular mechanisms, potentially identifying DNA methylation as a key regulatory pathway.

Keywords: acupuncture, premature ovarian insufficiency, epigenetics, non-inferiority trial, hormone replacement therapy

Introduction

Premature ovarian insufficiency (POI) is defined as the cessation of ovarian function before the age of 40, characterized by menstrual irregularities and pathologically elevated follicle-stimulating hormone (FSH) levels.[1] Current diagnostic criteria identify POI by the presence of oligomenorrhea or amenorrhea for at least four months, alongside FSH levels exceeding 25 IU/L on two separate occasions at least 4–6 weeks apart. POI is a prevalent condition that poses a significant clinical challenge due to its profound impact on fertility and long-term systemic health.[2,3] Beyond infertility, POI is associated with an increased risk of chronic morbidities, including cardiovascular disease, osteoporosis, and cognitive impairment.[4]

The etiology of POI is multifactorial and frequently idiopathic, though genetic and autoimmune factors are recognized as primary contributors.[5,6] Recently, epigenetic mechanisms have emerged as important regulators in the

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pathogenesis of POI. Epigenetic modifications—including DNA methylation, histone modification, and non-coding RNA regulation—modulate gene expression without altering the underlying DNA sequence and are indispensable for normal folliculogenesis and oocyte development.[7–9] Mounting evidence suggests that aberrant epigenetic alterations, particularly DNA methylation, are closely linked to ovarian dysfunction.[10–12] Abnormal DNA methylation patterns have been identified in the ovarian tissues of POI patients, specifically within genes central to follicular development and endocrine signaling, such as GDF9, BMP15, FSHR, and AMH.[10,13,14] These findings indicate that epigenetic dysregulation may drive POI progression. Importantly, the potential reversibility of epigenetic modifications makes them compelling therapeutic targets for novel interventions.

Hormone replacement therapy (HRT) remains the cornerstone of POI management, effectively alleviating symptoms of hypoestrogenism. However, HRT does not restore natural fertility and is associated with potential long-term safety concerns, necessitating the exploration of alternative or complementary therapeutic strategies.[15–18]

Acupuncture, a non-pharmacological intervention, is increasingly utilized in POI management. Clinical studies suggest it may improve menstrual regularity, normalize hormone profiles, and enhance ovarian reserve.[19–21] Emerging research indicates that acupuncture's effects may extend beyond systemic regulation to molecular modulation. Experimental and omics-based studies have shown that acupuncture can influence gene expression, inflammatory pathways, and cellular signaling. Furthermore, recent studies indicate that acupuncture may modulate epigenetic processes, including DNA methylation and microRNA expression, suggesting a potential mechanistic link between acupuncture and epigenetic regulation.[22–25] However, robust clinical evidence directly comparing acupuncture with HRT is lacking, and the role of epigenetic modulation in mediating these clinical effects remains poorly understood.

Given the favorable safety profile of acupuncture and preliminary evidence suggesting its clinical efficacy may be comparable to HRT, a non-inferiority trial design is employed here.[26,27] This study aims to evaluate whether acupuncture can achieve similar clinical outcomes to HRT while offering advantages in tolerability. Additionally, an exploratory analysis of genome-wide DNA methylation in ovarian granulosa cells will be conducted via whole-genome bisulfite sequencing to elucidate the potential molecular mechanisms underlying the intervention.

Methods

Study Design

This study is a prospective, randomized, non-inferiority, parallel-controlled clinical trial conducted at Shenzhen Maternity and Child Healthcare Hospital. A total of 572 women diagnosed with POI and planning to undergo in vitro fertilization and embryo transfer (IVF-ET) will be prospectively enrolled and randomly assigned in a 1:1 ratio to either the acupuncture group or the HRT group (n = 286 per group).

This study comprises an 18-month recruitment period, followed by a 12-week intervention phase and a subsequent follow-up period. Participants will be assigned to either the acupuncture group, receiving standardized acupuncture treatment, or the Hormone Replacement Therapy (HRT) group, receiving estradiol/estradiol-dydrogesterone. The 12-week intervention duration corresponds to approximately three menstrual cycles in women with regular cycles. In participants with irregular or absent menstruation, the intervention duration will be strictly defined by time (12 weeks) rather than by the number of menstrual cycles. During this time, the acupuncture group will be restricted from using HRT or any other hormonal therapies. Adherence will be monitored through clinical visits and standardized treatment logs. Following the intervention, all participants will undergo IVF-ET via a mild stimulation protocol. Participants will be followed until the confirmation of clinical pregnancy (approximately 4–6 weeks post-embryo transfer) and through delivery to record live birth outcomes.

In addition, a control group of women with normal ovarian reserve undergoing IVF-ET due to male or tubal factors will be prospectively recruited from the same clinical population. These participants will be matched to the POI group based on age and BMI and will be included for DNA methylation analysis only.

The study flow is presented in Figure 1, and the schedule of enrolment, interventions, and assessments is summarized in Table 1. Written informed consent will be obtained from all participants prior to enrollment.

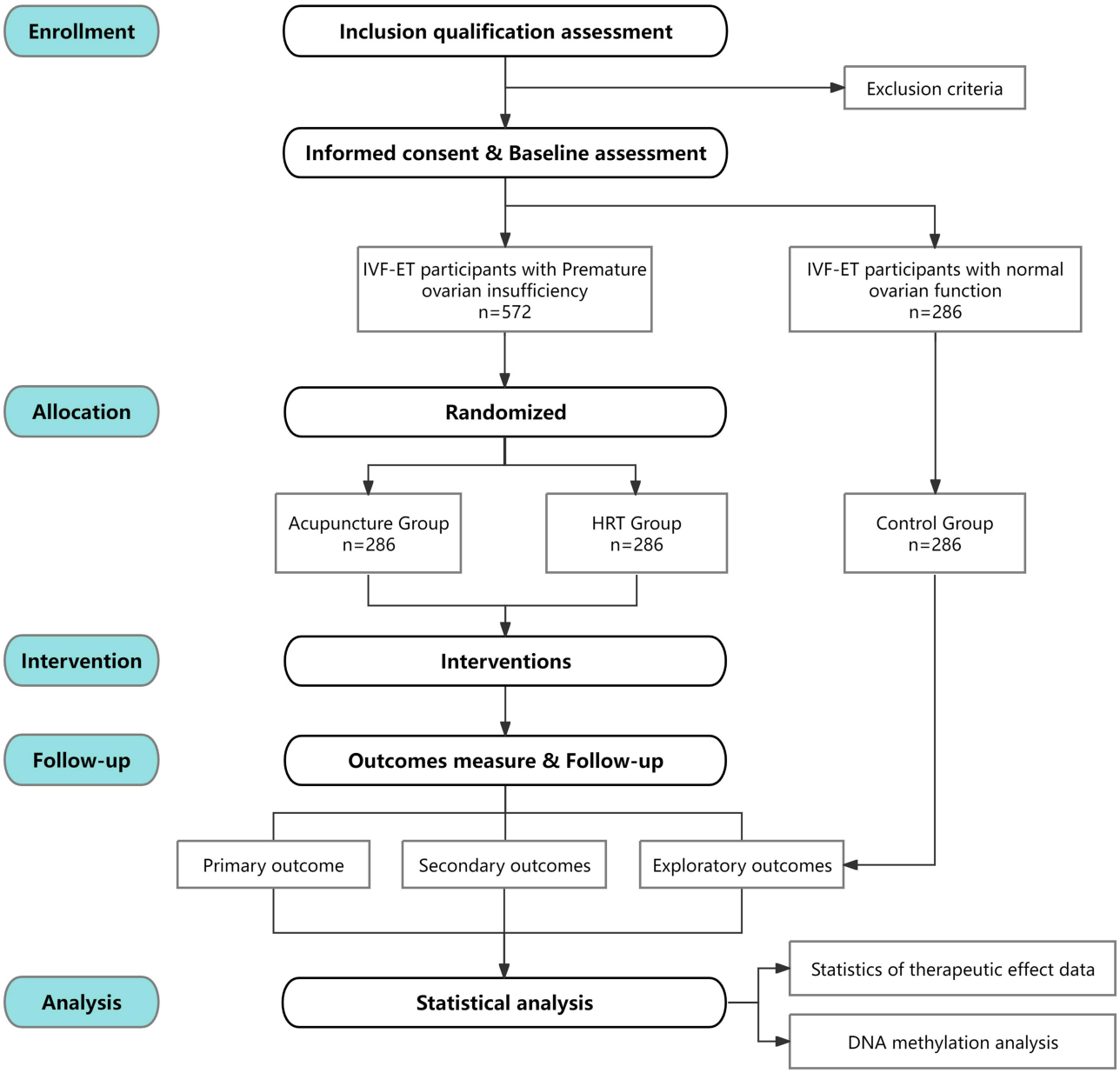

Figure 1 Study flow chart. A total of 286 participants will be enrolled in both the acupuncture group and the HRT group. In addition, women with normal ovarian function undergoing IVF-ET due to male or tubal factors will be included as the control group at a 1:1 ratio.

Study Population

Participant Recruitment

The target population consists of women diagnosed with POI according to the European Society of Human Reproduction and Embryology (ESHRE) guidelines. The reference population, from which control samples will be collected, comprises women with normal ovarian function who undergo IVF-ET due to male or tubal factors. These control participants will be matched to the POI participants as described below.

Inclusion and Exclusion Criteria of POI Participants

The participation in this study is subject to a series of rigorous inclusion and exclusion criteria, which have been meticulously devised to ensure a homogeneous study population and to minimise the influence of confounding variables that could otherwise compromise the study's findings. According to the ESHRE guidelines,[1,28] the

Table 1 Schedule of Enrolment, Interventions, and Assessments

Item	Screening	Baseline (Week 0)	Intervention Period (Week 1–12)	End of Treatment (Week 12)
Informed consent	●			
Medical history	●			
BMI	●	●		●
Transvaginal ultrasound	●	●		●
Routine tests	●	●		●
Hormone biomarkers	●	●		●
Acupuncture (Acupuncture group)			▲	
HRT (HRT group)			▲	
Adverse events		●	●	●
DNA methylation analysis		●		●

Notes: ● indicates that the assessment is performed at the specified time point; ▲ indicates intervention administration during the treatment period. Routine tests include blood routine examination, liver and kidney function tests, as well as urine and stool analysis. Transvaginal ultrasound assessments include ovarian volume, AFC, endometrial thickness, blood flow resistance index, and follicular development parameters.

Abbreviations: BMI, body mass index; AFC, antral follicle count; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E2, estradiol; AMH, anti-Müllerian hormone.

inclusion criteria are as follows: (a) female patients aged 18 to 40 years with a BMI between 17 kg/m^2 and 30 kg/m^2; (b) spontaneous amenorrhea or irregular menstruation lasting for ≥ 4 months without the use of oral/injectable/intrauterine hormonal contraceptives (including combined oral contraceptives, injectable or long-acting contraceptives);[1] (c) in two tests conducted with an interval exceeding 4 weeks, FSH level exceeded 25 U/L in both instances.[28] (d) to further ensure homogeneity of ovarian reserve status, additional inclusion criteria of AMH <1.2 ng/mL and antral follicle count (AFC) <5 will be applied (these thresholds are consistent with clinically recognized cut-offs for ovarian reserve markers in POI reported in previous studies);[29–31] (e) intention to undergo a mild stimulation protocol for IVF-ET; (f) voluntary provision of written informed consent and agreement to follow-up procedures.

Exclusion criteria include: (a) known chromosomal abnormalities, gene mutations (eg., X chromosome monosomy), or clearly identified etiologies of POI; (b) uterine malformations, significant uterine myomas, and endometrial lesions that affect embryo implantation; (c) use of hormone replacement therapy, oral contraceptives, or other medications affecting ovarian function, or acupuncture treatment targeting ovarian function, within the past 3 months; (d) severe systemic diseases, including thyroid dysfunction, diabetes, uncontrolled systemic lupus erythematosus, or significant impairment of cardiac, hepatic, renal, or pulmonary function; (e) history of ovarian surgery (unilateral or bilateral oophorectomy) or damage to ovarian blood supply; (f) current pregnancy or plans for natural conception during the study, or confirmed menopause; (g) mental illness or cognitive impairment that limits compliance with the study protocol; (h) participation in other clinical trials or experimental treatments during the study period; (i) contraindications to acupuncture or hormone therapy, such as skin infection at acupuncture sites, bleeding disorders, or known hormone hypersensitivity.

Inclusion and Exclusion Criteria of the Control Group

The control group consists of women with normal ovarian reserve undergoing IVF-ET due to male or tubal factors. The inclusion criteria are as follows: (a) female patients aged 18 to 40 years with a BMI between 17 kg/m^2 and 30 kg/m^2; (b) normal ovarian reserve, defined as baseline serum FSH <10 IU/L, AMH within the normal reference range based on age-adjusted and laboratory-specific standards, and AFC between 9 and 20;[31,32] (c) intention to undergo IVF-ET; (d) voluntary provision of written informed consent and agreement to follow-up procedures. The exclusion criteria are consistent with those applied to the POI group (criteria a–h), in order to ensure comparability between groups. In addition, individuals with polycystic ovary syndrome (PCOS) or abnormally elevated ovarian reserve will be excluded to avoid potential bias.

Withdrawal Criteria

Participants may be withdrawn from the study under the following circumstances:

(a) voluntary withdrawal of consent at any time;

(b) investigator-initiated discontinuation due to serious adverse events related to the intervention, emergence of protocol-specified contraindications (eg., severe infection, thromboembolic events, or uncontrolled bleeding), or use of prohibited concomitant medications affecting ovarian function or hormone levels;

(c) loss to follow-up, defined as failure to attend scheduled key visits;

(d) non-adherence to the assigned treatment protocol, including missed acupuncture sessions or deviation from the hormone therapy regimen.

Randomization and Blinding

The randomization and allocation process is fundamental to the rigor of this study. Eligible participants with POI will be randomly assigned to either the acupuncture group or the HRT group in a 1:1 ratio. The randomization sequence will be generated using computer software, with allocation concealment maintained through the use of sequentially numbered, opaque, sealed envelopes. These envelopes will be managed by an independent researcher not involved in participant recruitment or treatment. Due to the inherent nature of the interventions, blinding of participants and practitioners is not feasible. To mitigate potential bias and ensure therapeutic consistency, all acupuncture procedures will be performed by licensed acupuncturists with over five years of clinical experience. These acupuncturists will undergo standardized training to unify acupoint localization, insertion depth, and manual manipulation techniques. Furthermore, outcome assessors and statistical analysts will remain blinded to the group assignments to minimize detection and reporting bias.

Interventions

Acupuncture Intervention

Adhering to the Standards for Reporting Interventions in Clinical Trials of Acupuncture (STRICTA) guidelines,[33] our acupuncture intervention is designed for participants with POI and spans a 12-week intervention period. Treatment is administered on alternate days, three times per week, for a total of 12 consecutive weeks. The selection of acupoints is grounded in Yin-Yang theory and Traditional Chinese Medicine (TCM) principles, utilizing two distinct sets of points corresponding to the supine and prone positions. As illustrated in Figure 2, the supine acupoint set includes: Baihui (DU20), Shenting (DU24), Benshen (GB13), Zhongwan (RN12), Xiawan (RN10), Tianshu (ST25), Qihai (RN6), Guanyuan (RN4), Zigong (EX-CA1), Zusanli (ST36), Sanyinjiao (SP6), and Taichong (LR3). The prone position acupoint group includes: Baihui (DU20), Shenting (DU24), Benshen (GB13), Pishu (BL20), Shenshu (BL23), Dachangshu (BL25), Ciliao (BL32), Zhongliao (BL33), Zhishi (BL52), and Sanyinjiao (SP6). All points conform to the WHO Standard Acupuncture Point Locations for the Western Pacific Region.[34,35]

Prior to each session, the acupoints and the acupuncturists' hands will be disinfected with 75% alcohol. Sterile, disposable needles (specifications: 0.25 mm × 25 mm, 0.25 mm × 40 mm, and 0.35 mm × 75 mm) will be employed. To ensure standardized delivery, unilateral acupoints are selected per session, alternating between the supine and prone prescriptions for every other visit. Participants in the supine position are instructed to empty their bladder prior to needling. Insertion depth and standardized manipulation techniques—including twirling, lifting, and thrusting—are applied to elicit the De Qi sensation, a clinical indicator of effective stimulation. The needle retention time is 30 minutes. Throughout the 12-week period, menstrual cycles will be recorded, and adverse events will be monitored from the initiation to the conclusion of therapy.

HRT Administration Intervention

In the HRT arm, participants will receive a standardized oral sequential estrogen-progestin regimen using estradiol and estradiol/dydrogesterone composite tablets. This protocol aligns with the 2024 ESHRE guidelines and the concurrent consensus from the Chinese Obstetrics and Gynecology Association regarding the management of premature ovarian insufficiency.[36]

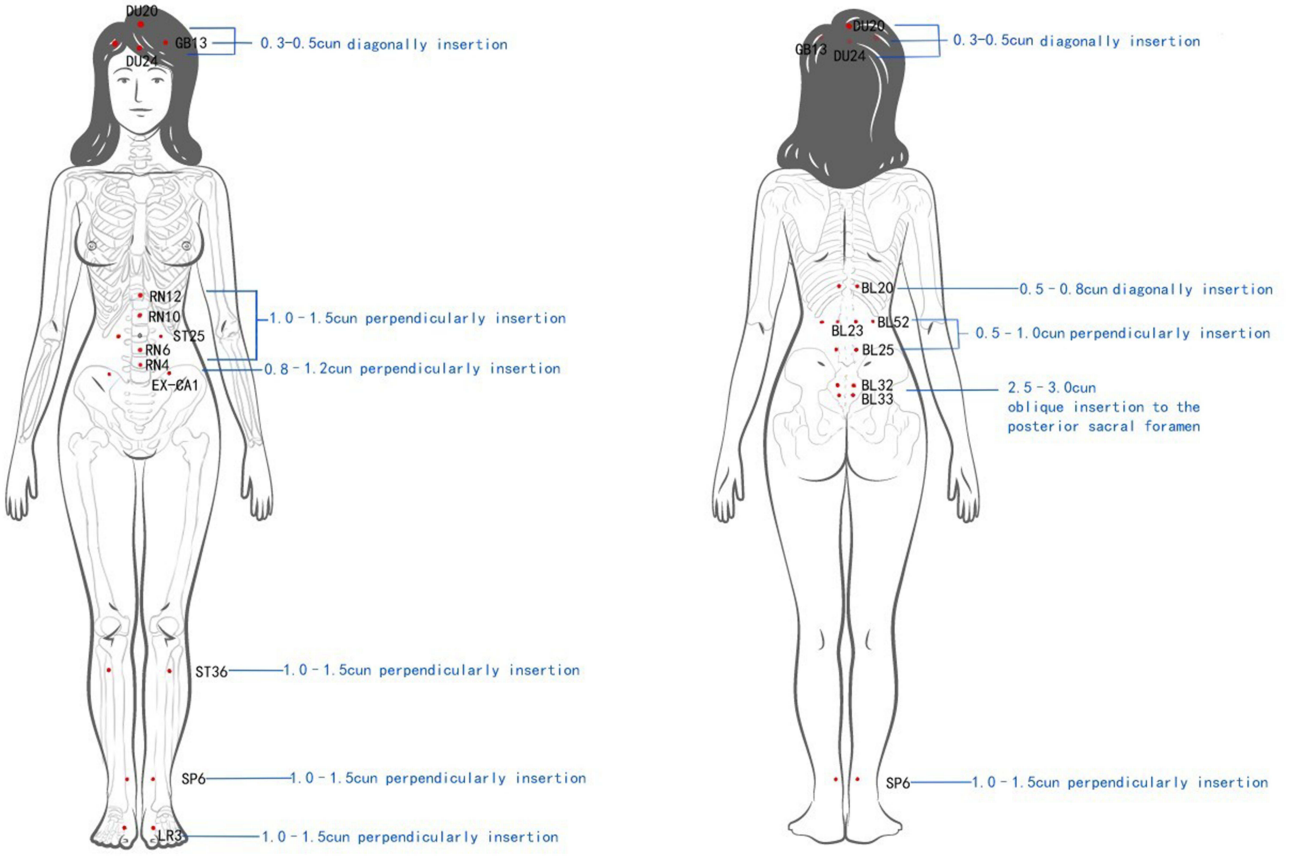

Figure 2 Acupuncture points and needling parameters. Two standardized protocols are shown, including 18 supine and 18 prone acupoints. Locations are shown according to standard anatomical references, with needling method and insertion depth indicated for each acupoint.

Each 28-day cycle consists of 14 days of estradiol-only administration followed by 14 days of combined estradiol and dydrogesterone. The intervention will be maintained for a total of 12 consecutive weeks. Participants with regular cycles or oligomenorrhea are instructed to initiate the regimen on the first and fifth day of their menstrual cycle, respectively. Amenorrheic participants may commence treatment immediately upon enrollment. If spontaneous menstruation occurs during the treatment period, the medication cycle should be restarted on the first day of the new menstrual flow to ensure synchronization. Treatment adherence will be monitored over the 12-week intervention period to ensure consistency across both study arms.

DNA Methylation Analysis

Sample Collection

Granulosa cells are obtained from follicular fluid collected during oocyte retrieval as part of the IVF-ET procedure. Following controlled ovarian stimulation, ovulation is triggered using human chorionic gonadotropin (hCG), and transvaginal ultrasound-guided follicular aspiration is performed 35–36 hours later.[37,38] The collected follicular fluid is immediately processed to isolate granulosa cells, which are used for subsequent DNA methylation analysis. All samples are handled according to standardized laboratory protocols to ensure consistency and integrity.

DNA Extraction and WGBS Library Preparation

Genomic DNA is extracted from granulosa cells using a commercially available genomic DNA extraction kit according to the manufacturer's instructions. DNA quality and concentration are assessed using a NanoDrop spectrophotometer and Agilent 2100 Bioanalyzer.

For whole-genome bisulfite sequencing (WGBS), genomic DNA undergoes bisulfite conversion using a commercially available bisulfite conversion kit, followed by library preparation using an Illumina-compatible protocol. Library construction includes end repair, A-tailing, adapter ligation, and PCR amplification. Library quality and fragment size distribution are evaluated prior to sequencing.[39]

Sequencing and Data Preprocessing

Sequencing is performed on the Illumina NovaSeq 6000 platform with 150 bp paired-end reads. Raw sequencing data are generated in FASTQ format and subjected to quality control using FastQC. Low-quality reads and adapter sequences are removed to obtain clean reads for downstream analysis.

Alignment and Methylation Calling

Clean reads are aligned to the human reference genome using a bisulfite-aware aligner (eg., Bismark). Methylation levels at CpG sites are extracted using the Bismark methylation extractor, generating genome-wide DNA methylation profiles at single-base resolution.

Identification of Differentially Methylated Regions

Differentially methylated regions (DMRs) between groups are identified using R packages designed for WGBS data, such as DSS and methylKit, which account for biological variability and sequencing depth. To control for multiple testing, the Benjamini–Hochberg false discovery rate (FDR) correction is applied. DMRs are defined based on an adjusted P-value < 0.05 and an absolute methylation difference ($\Delta\beta \geq 0.10$).

Functional Enrichment Analysis

Genes associated with DMRs are subjected to functional enrichment analysis using the clusterProfiler package, including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses, to explore the biological relevance of methylation changes.

Sample Size Calculation

The determination of the sample size required for this experiment is based on the comparison of the clinical pregnancy rates of the two groups of samples, using the following formula:

$$N = \frac{\left(Z_{1-\alpha} + Z_{1-\beta}\right)^2}{\left(\pi_T - \pi_C + \Delta\right)^2} \times \left[\pi_T(1-\pi_T) + \pi_C(1-\pi_C)\right]$$

where π is the assumed clinical pregnancy rate in each group, Δ is the non-inferiority margin, α is the one-sided significance level, and $1-\beta$ is the power.

Based on prior experimental findings and literature,[40] the success rate of ART after hormone replacement therapy stands at 31.7%. Additionally, research indicates that the clinical pregnancy rate for IVF-ET in patients with POI who received acupuncture treatment is 26.1%. Given the scarcity of direct POI-specific data for HRT and the clinical similarity between diminished ovarian reserve (DOR) and POI populations, a common clinical pregnancy rate of 30% is assumed for both groups. A non-inferiority margin of 10% is predefined based on clinical judgment and previous reproductive medicine trials.[41] With a one-sided α of 0.025 and 80% power, the required sample size is calculated as 260 participants per group. Allowing for a 10% dropout rate, the final sample size is set at 286 participants per group, resulting in a total of 572 participants.

Because the actual clinical pregnancy rate in patients with POI may be lower than the assumed 30%, the robustness of the planned sample size is assessed under lower pregnancy rates of 20% and 15%. With the sample size fixed at 260 participants per group before dropout (286 after dropout), the non-inferiority margin ($\Delta = 0.10$) and the one-sided significance level ($\alpha = 0.025$) unchanged, the statistical power is calculated for each scenario. Under a true pregnancy rate of 20%, the power is approximately 89%; under 15%, the power is approximately 94%. Both exceed the pre-specified power of 80%, indicating that the planned sample size provides adequate power even if POI patients have

substantially lower pregnancy rates than initially assumed. Therefore, the non-inferiority test remains well-powered under plausible clinical scenarios.

To ensure comparability, we will rigorously select women whose age, BMI, and other demographic characteristics closely match those of POI patients. These women will have normal ovarian function and will have undergone IVF-ET treatment for non-ovarian reasons (eg., male factors or fallopian tube issues). They will be compared with either the acupuncture group or the HRT group in a 1:1 ratio. All participants will undergo DNA methylation analysis to enhance the depth of epigenetic research.

Outcomes Measures

Primary Outcome

Clinical Pregnancy Rate

Fourteen days after embryo transfer, a pregnancy test is conducted to determine whether pregnancy has occurred. At 28 days post-transplantation, ultrasound examination confirms the presence of one or more gestational sacs in the uterine cavity, along with visible fetal heartbeats or other evident clinical signs of pregnancy, excluding instances of positive bloodline β-hcg (biochemical pregnancy) or ectopic pregnancy.[40] Clinical pregnancy rate = (number of pregnancy cases ÷ number of initiated transplantation cycles) × 100%.

Secondary Outcomes

Sex Hormone Indicators and Ovarian Reserve Conditions

Before enrollment and on the 2nd to 4th days of the menstrual cycle following the intervention, venous blood is collected from the empty abdomen of all participants, and an ultrasound examination of ovarian follicle storage is conducted. At the heart of our study lies the meticulous quantification of changes in serum FSH, LH, E2, and AMH levels, all of which are crucial biomarkers for gauging the therapeutic efficacy of acupuncture in the realm of POI treatment. FSH is a pivotal cornerstone biomarker for gauging the therapeutic efficacy of acupuncture in the realm of POI treatment.[42] This particular parameter is chosen for its well-documented correlation with ovarian reserve and the broader implications for reproductive potential.[43] LH is another key hormone that plays a crucial role in regulating the menstrual cycle and ovarian function. It works in conjunction with FSH to stimulate the production of androgens in theca cells, which are then converted to E2 in granulosa cells, promoting follicle development. E2 is essential for maintaining the health of reproductive tissues and reflects ovarian activity. It exerts positive feedback on follicle development and supports endometrial growth, which is vital for a successful pregnancy. AMH is a reliable marker of ovarian reserve, reflecting the number of remaining ovarian follicles. It is secreted by granulosa cells in small follicles and is relatively stable throughout the menstrual cycle, making it a more accurate predictor of ovarian reserve compared to other hormones.

Ovulation Induction Status and Pregnancy Outcomes

The quantity and rate of retrieved oocytes will be assessed on the day of oocyte retrieval. On the day of embryo transfer, the proportion of high-quality embryos, endometrial thickness, and blood flow resistance index will be evaluated. Follow-up will continue until approximately 40 weeks post-transfer to determine the live birth rate. The number of M II oocytes observed under the microscope corresponds to the retrieved oocytes. Fertilization is conducted using the droplet method, and after 16 to 19 hours, prokaryote formation is detected microscopically, with two prokaryotes indicating successful fertilization. Evaluation of embryo development is performed 72 hours post-fertilization, with embryos containing 7 to 9 cells and less than 10% fragments classified as high-quality embryos. Live birth rate = number of live deliveries ÷ number of embryo transfer cycles × 100%.

Modified Kupperman Index

Perimenopausal symptoms are assessed using the modified Kupperman Index (KI) scale both before and after treatment.[44] These symptoms are categorised into 13 items, including hot flushes, sweating, paresthesia, insomnia, irritability, depression, and urinary tract irritation. Symptom severity is rated on a scale of 0–3, with hot flushes and

sweating carrying a weighting of 40%, abnormal sensations, insomnia, irritability, dyspareunia, and urinary symptoms carrying a weighting of 20%, and other symptoms carrying a weighting of 10%. The total score ranges from 0 to 63 points, with higher scores indicating more severe symptoms.

Exploratory Outcomes

DNA Methylation Analysis

In this study, DNA methylation will be investigated as a key epigenetic mechanism potentially involved in the therapeutic effects of acupuncture in patients with POI. Whole Genome Bisulfite Sequencing (WGBS) will be employed due to its high resolution and comprehensive genome-wide coverage, enabling the detection of methylation changes at single-base resolution.[39] Genomic DNA will be extracted from ovarian granulosa cells using a standard DNA extraction kit according to the manufacturer's instructions. DNA quality and concentration will be assessed using a NanoDrop spectrophotometer and the Agilent 2100 Bioanalyzer to ensure suitability for downstream analysis. For library preparation, genomic DNA will undergo bisulfite conversion using a commercially available bisulfite conversion kit, followed by library construction using the Illumina-compatible DNA library preparation kit. The libraries will undergo end repair, A-tailing, adapter ligation, and PCR amplification. Library quality and fragment size distribution will be evaluated prior to sequencing.

Sequencing will be performed on the Illumina NovaSeq 6000 platform using 150 bp paired-end reads. Raw sequencing data will be generated in FASTQ format and subjected to quality control prior to downstream analysis.

Statistic Analysis

All statistical analyses will be performed using R software. A two-sided P value < 0.05 will be considered statistically significant unless otherwise specified.

Primary Outcome Analysis

The primary outcome is the clinical pregnancy rate. A non-inferiority framework will be applied to compare the acupuncture group with the HRT group. The difference in clinical pregnancy rates between the two groups and its corresponding two-sided 95% confidence interval (CI) will be estimated. Non-inferiority will be concluded if the lower bound of the 95% CI for the difference (acupuncture minus HRT) is above the predefined non-inferiority margin of −10%. For univariate analysis, comparisons between groups will be performed using the chi-square test or Fisher's exact test, as appropriate. In addition, multivariate logistic regression models will be applied to adjust for potential confounding factors, including age, BMI, and baseline ovarian reserve indicators (eg., AMH and AFC), to further evaluate the robustness of the treatment effect.

Secondary Outcome Analysis

Secondary outcomes include hormone levels, ovarian reserve indicators, pregnancy-related outcomes, and symptom scores. Continuous variables will be assessed for normality using the Shapiro–Wilk test. Normally distributed data: presented as mean ± standard deviation (SD) and analyzed using Student's *t*-test. Non-normally distributed data: presented as median (interquartile range, IQR) and analyzed using the Wilcoxon rank-sum test. Categorical variables will be expressed as frequencies and percentages and compared using the chi-square test or Fisher's exact test, as appropriate. For selected outcomes, multivariate linear or logistic regression models will be further applied to adjust for potential confounders.

Handling of Missing Data

Missing data will be handled using multiple imputation methods under the assumption of missing at random. Sensitivity analyses will be conducted to evaluate the impact of missing data on the primary outcome.

DNA Methylation Analysis

Genome-wide DNA methylation data will be analyzed using bioinformatics pipelines as described in the Method section.

DMRs will be identified using appropriate statistical models, with FDR correction applied to control for multiple testing. To explore the potential biological relevance of methylation changes, functional enrichment analyses will be performed using GO and KEGG databases. Correlation analyses between DNA methylation levels and clinical parameters (eg., hormone levels and ovarian reserve indicators) will be conducted using Spearman correlation coefficients. Receiver operating characteristic (ROC) curve analysis will be used to evaluate the potential diagnostic value of candidate methylation markers. In addition, multivariate models will be applied where appropriate to assess the association between methylation patterns and clinical outcomes while adjusting for potential confounding factors. The epigenetic component of this study is exploratory in nature, and the findings will be interpreted as hypothesis-generating, providing a basis for future mechanistic validation studies.

Adverse Events Management

Patient safety is paramount in this clinical trial, particularly concerning acupuncture interventions. Rigorous training will be provided to the acupuncturists involved in the study, who are required to have five years or more of experience, to ensure that they are proficient in uniform acupuncture practices. Regular assessments and supervision will also be conducted to ensure the consistency of the intervention and to reduce the likelihood of adverse events, which may include, but are not limited to, bruising, syncope, discomfort, and the risk of infection.[45] Adverse events are diligently monitored and documented throughout the trial. The clinical team stands at the ready to address any issues with promptness and efficiency; in the event of serious adverse events, the trial will be suspended without delay, and immediate medical interventions will be initiated, followed by a report to the institutional review board at the Shenzhen Maternity and Child Healthcare Hospital within a strict 24-hour timeframe.[46] A thorough and exhaustive documentation of every adverse event and the corresponding management strategies is imperative for safeguarding the safety and maintaining the quality of the trial.[47]

Discussion

This randomized non-inferiority trial is proposed to evaluate whether acupuncture demonstrates non-inferior to HRT in improving clinical pregnancy outcomes among women with POI undergoing IVF-ET. The primary endpoint centers on comparing clinical pregnancy rates between the two intervention groups. The study seeks to determine if acupuncture could represent a clinically relevant alternative for POI patients, particularly those for whom long-term hormonal therapy is contraindicated or undesirable. By exploring the potential of acupuncture in reproductive management, this trial aims to address the known limitations of HRT, such as its limited capacity to restore natural fertility.

In addition to clinical outcomes, the protocol outlines an exploratory epigenetic component to investigate potential molecular mechanisms. Genome-wide DNA methylation profiling of ovarian granulosa cells is planned to identify DMRs that may be associated with treatment response. Given the exploratory nature of these analyses, the intended findings are meant to provide preliminary insights into potential regulatory pathways.

Notably, differential DNA methylation does not inherently confirm functional biological impact. To address this limitation, the study proposes correlation analyses between methylation patterns and clinical parameters—including hormone levels and ovarian reserve markers—to identify candidate loci potentially linked to treatment efficacy. Nonetheless, subsequent studies incorporating transcriptomic profiling, proteomic validation, and functional assays (eg., quantitative PCR) would be essential to confirm the biological relevance of any observed epigenetic modifications.

The strengths of this protocol lies in its randomized non-inferiority design, which provides a framework for comparing acupuncture and HRT within a clinically relevant population. Furthermore, the implementation of standardized intervention protocols and stringent eligibility criteria is intended to enhance internal validity and mitigate heterogeneity. The integration of clinical outcomes with molecular data is designed to establish a foundation for exploring the biological underpinnings of the intervention.

However, several limitations of the study design warrant acknowledgment. First, the nature of acupuncture precludes the blinding of participants and practitioners, which could introduce expectancy bias. To mitigate this, the protocol specifies that all practitioners undergo standardized training to ensure consistency in treatment delivery. Second, the study does not stratify participants by traditional Chinese medicine syndrome differentiation; future research may be

needed to explore how this heterogeneity influences treatment response. Third, the epigenetic analysis is exploratory and lacks concurrent transcriptomic or proteomic validation; thus, any results should be interpreted as hypothesis-generating.

In conclusion, this study protocol aims to provide comparative clinical evidence regarding the non-inferiority of acupuncture to HRT for women with POI undergoing IVF-ET. Concurrently, the exploratory epigenetic analysis is intended to generate hypotheses regarding the potential molecular mechanisms of acupuncture, providing a basis for future mechanistic validation.

Abbreviations

POI, Premature Ovarian Insufficiency; DOR, Diminished Ovarian Reserve; FSH, Follicle-Stimulating Hormone; LH, Luteinizing Hormone; E2, Estrogen; AMH, Anti-Müllerian Hormone; HRT, Hormone Replacement Therapy; ESHRE, European Society of Human Reproduction and Embryology; AFC, Antral Follicle Counts; IVF-ET, In Vitro Fertilization and Embryo Transfer; BMI, Body Mass Index; STRICTA, Standards for Reporting Interventions in Clinical Trials of Acupuncture; hCG, Human Chorionic Gonadotrophin; WGBS, Whole Genome Bisulfite Sequencing; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DMRs, Differentially methylated regions; FDR, False Discovery Rate.

Ethic Statement

This study has been approved by the Ethics Committee of Shenzhen Maternity and Child Healthcare Hospital (Ethics approval numbers: SFYLS[2024]104) and will be in accordance with the Declaration of Helsinki (2013 version). Participants will provide written informed consent to participate in this research. Confidentiality of participants' data will be rigorously maintained throughout the study, in accordance with the standards of data protection and security in medical research. Trial registration: International Traditional Medicine Clinical Trial Registry (No. ITMCTR2024000470).

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Disclosure

The authors report no conflicts of interest in this work.

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