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# Effects of acupuncture on the levels of serum estradiol and pituitary estrogen receptor beta in a rat model of induced super ovulation

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ABSTRACT

*Aims:* Acupuncture is frequently recommended as a complementary therapy for infertility. However, whether acupuncture can prevent early ovarian hyperstimulation syndrome has not been examined and its potential mechanisms are not well understood.

*Main methods:* Forty rats were randomized into four groups: Control, Ovarian Stimulation Model, Acupuncture, and Human Chorionic Gonadotropin (HCG). Serum estradiol, progesterone, testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) levels were measured by enzyme-linked immunosorbent assay. Pituitary ER mRNA and ERβ expression were detected by real-time PCR and western blotting respectively. The pathology of rat ovaries were observed by light microscopy.

*Key findings:* We observed significantly lower estradiol levels in the Acupuncture group than in the Model group and increased LH levels in the HCG group than in Model and Acupuncture groups. Testosterone and FSH levels were significantly lower in the Acupuncture group than in the HCG group. Western blotting showed significantly lower pituitary ERβ expression in the Model group than in the Control group and higher expression in the Acupuncture group than in the Model group. Real-time PCR showed lower pituitary ER mRNA expression in the Acupuncture group than in the Model group. Hematoxylin and eosin staining showed a lower proportion of atretic follicles in Acupuncture and HCG groups than in Model and Control groups. Instead, Acupuncture and HCG groups showed growing and mature follicles.

*Significance:* Our results demonstrate a relationship between acupuncture and the hypothalamic-pituitary-gonadal axis, and the potential mechanism underlying the preventative effects of acupuncture on the incidence of early ovarian hyperstimulation syndrome.

## 1. Introduction

The most feared complication of *in vitro* fertilization (IVF)-related ovarian stimulation is the development of ovarian hyper stimulation syndrome (OHSS) [1]; a syndrome, which in its severe form leads to hospitalization and can cause fatal complications. The incidence of clinically significant OHSS is 2–3%; however, milder forms of OHSS might develop in 20–30% of IVF patients [2,3]. Although the pathophysiology of OHSS is not fully understood, the involvement of increased vascular permeability due to the effect of human chorionic gonadotropin (HCG) has been proposed.

Traditionally, high or rapidly rising serum estradiol ($E_2$) levels on the day of the HCG trigger, denoting hypersensitivity to HCG, is used as a predictor of OHSS [4]. However, a high $E_2$ level alone is a poor predictor of OHSS [4,5]. However, the number of follicles in combination with serum $E_2$ levels predicts OHSS with high sensitivity and specificity [2,6]. Although the $E_2$ level alone is a poor predictor of OHSS, it is often closely monitored, and secondary OHSS prevention strategies are advised when high levels are present. As OHSS is associated with HCG, when several risk factors are present, terminating the ovulation cycle by cancelling the HCG trigger is the most effective technique for preventing OHSS [7].

Acupuncture is believed to improve autoregulation of the body to enhance the regulatory action of the hypothalamic-pituitary-gonadal

*Abbreviations:* HCG, human chorionic gonadotropin; E2, serum estradiol; P, progesterone; T, testosterone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; ELISA, enzyme-linked immunosorbent assay; ERβ, estrogen receptor beta; IVF, *in vitro* fertilization; PCOS, polycystic ovarian syndrome; OHSS, ovarian hyperstimulation syndrome; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SD, standard deviation; Model, model group; Acu, Acupuncture group; OVX, ovariectomized

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axis and normalize the secretion of follicle-stimulating hormone (FSH), luteinizing hormone (LH), $E_2$, and progesterone (P); regulated secretion of these hormones, in turn, restores ovulation and increases the conception rate [8]. Chen et al. reported that acupuncture can increase $E_2$ levels and the expression of estrogen receptor gene in a one-ovary rat model [9].

The present study aimed to investigate the effects of acupuncture on ovarian overstimulation and whether acupuncture could prevent early OHSS by downregulating $E_2$ levels and pituitary estrogen receptor (ER) expression in a rat superovulation model.

## 2. Materials and methods

### 2.1. Experimental animals

Forty 22-day-old female Wistar rats, weighing 40–50 g, were housed in groups of four in cages at 22 °C and under a 12-h light-dark cycle during the acclimation period (at least one week) and throughout the experimental period. The rats had free access to pelleted food and tap water.

The study protocol was approved by the Ethics Committee of the Affiliated Hospital of Nanjing Chinese Medicine University, Nanjing. Experimental procedures involving animals were performed in accordance with the Guidelines of the Institutional Animal Care and Use Committee of the China Academy of Chinese Medical Sciences (Beijing, China).

### 2.2. Animal groups and acupuncture

The rats were divided into four experimental groups, the Control ($n = 10$), ovarian stimulation (Model; $n = 10$), acupuncture (Acu; $n = 10$), and HCG (HCG; $n = 10$) groups. The Control group received no treatment. The Model group was injected subcutaneously with pregnant mare's serum gonadotropin (PMSG) at a dose of 10 IU (Folligon, $5 \times 10^3$ IU Diluent, MSD; Animal Health, Intervet International, Netherlands) at 39 days of age. The Acu group included Model rats treated with acupuncture on bilateral EX-CA1, bilateral SP6, CV4, and CV6 for 30 min once a day for two consecutive days during the ovulatory period beginning at 56 h after PMSG injection. The locations of the EX-CA1, SP6, CV4, and CV6 points were selected as described previously [10–12]. The rats were fixed in a special frame, and then their skin was disinfected with 75% alcohol. Acupuncture was performed on rats in the Acupuncture group using a Huatuo filiform needle measuring $0.25 \times 15$ mm$^2$. The HCG group included Model rats that were injected with 10 IU of HCG, beginning 56 h after PMSG injection, once a day for two consecutive days during ovulation. Rats in the HCG group were not treated with acupuncture.

### 2.3. Tissue collection

All the experimental animals were weighed and anesthetized with a subcutaneous injection of 5 mg/kg xylazine and 35 mg/kg ketamine (Alfamine 10% and Alfazyne 2%; Alfasan International B.V., Netherlands). After obtaining cardiac blood samples for hormonal (estradiol) assays, the animals were euthanized. The ovaries and hypophysis cerebri were excised and quickly dissected on dry ice. Both the ovaries and hypophysis cerebri were weighed, snap-frozen in liquid nitrogen, and stored at −80 °C until use. At the end of the experiment, all rats were euthanized at 43 days of age.

### 2.4. Ovarian morphology

The morphological changes in the ovary were observed in the Department of Pharmacology of the Affiliated Hospital of Nanjing University of Chinese Medicine. At the end of the experiment, the rats were sacrificed, and their ovaries were removed to observe any pathological changes. The ovarian tissues were fixed with 10% formalin and subjected to conventional dehydration. The processed tissues were embedded in paraffin and sliced into 4-μm-thick sections. Hematoxylin and eosin staining was performed [13], and the structures of the rat ovaries were observed under an optical microscope (Olympus IX71; Japan).

### 2.5. Quantitative real-time PCR

Total RNA was isolated from the hypophysis cerebri using TRIzol reagent (Invitrogen, USA) and stored at −80 °C. Then, the isolated RNA (2 μg) was reverse transcribed to cDNA using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher, USA), and 20 μL of the reverse transcription product was used as template in the subsequent amplification. The conditions for reverse transcription were as follows: 5 min at 25 °C, 60 min at 42 °C, and 10 min at 70 °C. The synthesized cDNA was diluted with sterile deionized water to a final volume of 100 μL, and 2 μL of the cDNA was used for real-time polymerase chain reaction (PCR). The real-time PCR was performed using the Applied Biosystems Step One Plus Real-Time PCR System (ABI) with SYBR Green I real-time PCR Master Mix (TOYOBO, Japan), which was used to detect the PCR products. Reactions were performed in triplicate. The relative expression of the target gene *esr1* was analyzed, and its expression was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (*gapdh*).

The sequences of the primers used for *esr1* were 5′-TGC CTC AAA TCA ATC ATT TTG C-3′ (forward) and 5′-GTG GAT GTG GTC CTT CTC TTC C-3′ (reverse), and the size of the synthesized cDNA was 90 bp. The primers used for *gapdh* were 5′-GGC CTT CCG TGT TCC TAC C-3′ (forward) and 5′-CGC CTG CTT CAC CAC CTT C-3′ (reverse), and the size of the synthesized cDNA was 103 bp. The PCR conditions were as follows: 95 °C for 5 min (DNA denaturation), followed by 40 cycles of 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 40 s. Melting curve analysis was performed at the end of the amplification cycles.

### 2.6. Western blot analysis

Total protein was measured by the bicinchoninic acid disodium (BCA) assay (Bio-Rad, USA) using bovine serum albumin as the standard. A fixed amount of protein (20 μg) from each sample was separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Pall, USA). These membranes were incubated with 5% skimmed milk in TBS buffer for 1 h at room temperature. To detect estrogen receptor beta (ERβ), membranes were exposed to a primary anti-ERβ antibody (ab3576, 1:250 dilution; Abcam, USA). After washing, the membranes were incubated with the secondary antibody (HRP-conjugated anti-rabbit antibody, 1:5000 dilution; KBL, USA). The bound antibodies were detected using enhanced chemiluminescence reagent (GE Health Care, USA). Data are presented as the ratio of ERβ to actin and are expressed as the fold-change compared to the Control group. The actin-specific antibodies and peroxidase-conjugated goat anti-rabbit IgG secondary antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

### 2.7. Elisa

The serum sex hormones ($E_2$, P, T, LH, and FSH) were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instruction (Catalog Nos. E30608, E50005, E30610, E30623, and E30597 respectively; Shanghai Boyun Biotech, Co., Ltd., Shanghai, China). The absorbance in each well was measured at 450 nm using a microplate reader (Thermo Fisher, USA).

### 2.8. Statistical analyses

GraphPad Prism software (v6.01) was employed for all statistical

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