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# Electro-acupuncture attenuates the mice premature ovarian failure via mediating PI3K/AKT/mTOR pathway

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## Abstract

**Aims:** Electro-acupuncture(EA) is frequently recommended as a complementary therapy for premature ovarian failure(POF) in the clinical. However, little information exists about its potential treatment mechanisms. The study was designed to observe the effect of EA to ovarian function and fertility in POF mice model, and investigated its potential mechanisms on PI3K/AKT/mTOR signaling pathway.

**Materials and Methods:** Forty-five female C57/BL6 mice were divided into the Control, the Model and the EA group. The ovaries morphology of mice was observed by hematoxylin and eosin(HE) staining, and all follicles were counted under microscope. The protein expression of PI3K, phospho-PI3K, AKT, phospho-AKT, mTOR, phospho- mTOR, S6, phospho-S6, 4E-BP1 and phospho-4E-BP1 were detected by western blotting. The data was presented as the ratio of phosphorylation protein to total protein. Serum follicle-stimulating hormone(FSH), luteinizing hormone(LH), estradiol(E<sub>2</sub>) and anti-mullerian hormone(AMH) levels were

measured by enzyme-linked immunosorbent assay(ELISA). The fertility was observed by giving treated mice 8 weeks for breeding.

**Key findings:** We found that primordial follicle counts were increased in EA group compared to Model group. The phosphorylation of PI3K, AKT, mTOR, 4E-BP1 and S6K in EA group significantly reduced compared to Model group. Serum FSH and LH levels in EA group were decreased compared to Model group, while, serum E<sub>2</sub> and AMH levels in EA group were increased compared with Model group. The litter size in EA group was improved compared to Model group.

**Significance:** The effects of EA on the PI3K/AKT/mTOR signaling pathway may represent one of the mechanisms involved in attenuating the mice POF.

**Keywords:**

Electro-acupuncture; Premature ovarian failure(POF); Cell signaling; Ovarian reserve; Fertility

## 1. Introduction

At birth, the ovaries have a fixed pool of primordial follicles constituting the ovarian reserve, its activation or loss results in an irreversible decline in reproductive function, eventually led to the menopause[1]. To produce mature oocytes for fertilization, primordial follicles are recruited from the reservoir of dormant follicles into the growing follicle pool through a process termed follicular activation, and subsequently undergo a series of development steps. Ovarian follicles initiate from the primordial follicle stage, where oocytes arrested in prophase of meiosis I and surrounded by a single layer of squamous granulosa cells. They are activated to grow and transition to primary follicles, secondary follicles, and finally preovulatory antral follicles(Fig.5B). During the entire process, a massive proportion of follicles undergo apoptotic death(atresia) if not selected for further growth[2]. Anti-Mullerian hormone(AMH) is produced by the granulosa cells of preantral and small antral follicles, correlates with histological primordial follicle numbers, and is one of the most important measures of ovarian reserve used clinically[3, 4].

Premature ovarian failure(POF), also known as premature menopause, is a common disease referring to the ovulation obstacle and ovarian hormones disorder in women before the age of 40[5]. It is mainly characterized by secondary amenorrhea, menopause, infertility, and partly accompanied by peri-menopausal symptoms such as hot flashes, cold sweats, bone loss, irritability and osteoporosis. According to general examination, POF is shown by decreased estrogen levels and high gonadotropin secretion(follicle stimulating hormone, FSH), its pathogenesis is related to heredity, radiotherapy, chemotherapy, ovarian surgery, immune dysfunction[6, 7]. Luteinizing hormone(LH)is a glycoprotein gonadotropin secreted by

pituitary cells, it works together with FSH to promote follicular maturation, secrete estrogen, ovulation, and the production and maintenance of the corpus luteum[8].

Studies have shown that PI3K signaling in the oocyte controls the survival, loss and activation of primordial follicles[2, 9], and POF is closely related to the decline of estrogen level[10]. Estradiol(E2) binds to estrogen receptor  $\alpha$ (ER  $\alpha$ ) and estrogen receptor  $\beta$ (ER $\beta$ ) to participate in female sexual behavior. ER  $\alpha$  binding to the regulatory subunit p85a of PI3K in a ligand-dependent manner induces phosphatidylinositol 3-kinase(PI3K) activity, leading to the activation of protein kinase B(AKT). AKT is known to lead to the activation of mammalian target of rapamycin complex 1(mTORC1) through multiple mechanism[11]. mTORC1 positively regulates cell growth and proliferation by integrating various signals[12]. A series of genetics experiments have shown that increased activation of mTORC1 in mouse oocytes leads to the premature activation of primordial follicles[13]. For example, oocyte-specific deletion of either Tsc1[14]or Tsc2[15]in mice, which leads to increased levels of active mTORC1, causes the activation of all primordial follicles around the time of puberty and leads to follicular depletion in early adulthood and the associated development of POF. Activation of mTORC1 promotes the phosphorylation of two downstream targets, S6 kinase 1(S6K1) and eukaryotic initiation factor 4E binding protein-1(4E-BP1), stimulating ribosome biogenesis and protein synthesis[16, 17].

Results from clinical studies have indicated that electro-acupuncture(EA) is able to treat premature ovarian failure[18]. While the capacity for EA to preserve ovarian function has been demonstrated, the specific mechanisms underlying these effects remain unclear. Therefore, based on clinical evidence, we devoted our efforts to examining the effects of EA on the P13K/Akt/mTOR signaling pathway in POF.

## 2. Materials and methods

### 2.1. Experiment animals

Female C57BL/6 mice(n=45, 8 weeks old, SPF grade, weigh 18-20g) were purchased from Shanghai Xipuer-Bikai Lab Animal Co., Ltd. The license number for animal manufacture was SCXK (HU) 2013-0016. Mice were treated in accordance with guidelines of Nanjing University of Chinese Medicine Animal Research Committee. Nanjing University of Chinese Medicine Biosafety and Animal Research Committees approved all experimental protocols.

### 2.2. Animal groups and electro-acupuncture(EA)

The mice were divided into the Control group(n=15), the Model group(n=15) and the EA group(n=15). The mice in the Model and EA group were injected intraperitoneally with 75mg/kg cyclophosphamide(CY, Aladdin, 6055-19-2), and the mice in the control group received the same volume of saline. Electro-acupuncture was performed similar to that

described by Torres-Rosas et al[19]. All of the mice were restrained under light isoflurane anesthesia. The acupuncture points were selected on the basis of the previous clinical studies[20] (Fig.1A). EA stimulation was applied to the acupuncture points of Guanyuan(CV 4) and bilateral Zusanli(ST 36) using an EA stimulator instrument(Hans-200). Sterile acupuncture needles(diameter:0.18millimeter, length:10millimeter, made by Beijing Zhongyan Taihe Medical Instrument Co., Ltd) were inserted perpendicularly into the above-mentioned acupuncture points and connected with the output terminal. The stimulation with 2/15Hz frequencies was generated at an intensity of muscle twitch threshold and lasted 30min a day for 7 days(Fig.1B). The Control and Model group included the same procedure but using a non-electrical wood “toothpick” instead of the electrodes.

All the experimental animals were weighed and anesthetized with intraperitoneal injection of 10% chloral hydrate (350mg/kg)(Aladdin). After collecting blood samples for hormonal assays, the mice were euthanized. The ovaries were removed and weighed, then half of ovaries was snap-frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  for further analysis, another part was fixed in 4% PFA.

### **2.3. Hematoxylin and Eosin Staining and Follicle Classification.**

The ovaries were fixed in 4% PFA at  $4^{\circ}\text{C}$  overnight, dehydrated in a series of ethanol concentrations, cleared in xylene, and embedded in paraffin. Ovarian sectioning was performed on all ovaries in 6- $\mu\text{m}$  sections taken from five intervals 100- $\mu\text{m}$  apart throughout each ovary. At each consecutive interval, six sections were cut serially, resulting in five sets with nearly identical ovarian sections. The first slide of each 100- $\mu\text{m}$  interval was stained with HE, and five consecutive blank slides were cut at 6- $\mu\text{m}$  intervals. Performing follicle counts by sectioning at five 100- $\mu\text{m}$  intervals was deemed equivalent to whole-ovary sectioning in a pilot study. Blinded follicle counts were conducted by one investigator, and follicle counts were independently confirmed by a second blinded reviewer. Where discordant, sections were reviewed. Examination of primordial follicles was performed at 40 $\times$  magnification, primary follicles, secondary follicles and antral follicles at 20 $\times$ . All primary and secondary follicles were grouped together as “growing” follicles for the purposes of analysis. Follicle counts were reported as the average number of follicles per section as well as the average number of follicles per ovarian surface area( $\text{mm}^2$ ) to account for differences in the section area. OVARIAN SECTION MEASUREMENTS WERE TAKEN IN TWO PERPENDICULAR DIAMETERS, AND SECTION AREA WAS CALCULATED BY MULTIPLYING TWO PERPENDICULAR MEASUREMENTS OF EACH SECTION( $\text{MM}^2$ ).

### **2.4. Western blot analysis.**

Proteins from ovary tissues were separated in 4-20% gradient SDS-polyacrylamide gel electrophoresis(SDS-PAGE) and transferred onto polyvinylidene difluoride(PVDF) membranes(Millipore, USA). The PVDF membranes were blocked in a blocking solution containing TBST(10mM Tris-HCl, Ph7.4, 150mM NaCl, and 0.05% Tween 20), 4% nonfat dried milk, and 1% BSA at room temperature for 1h, and incubated with PI3K(CY5355,

1:1000 dilution; Abways), phosphor-PI3K(CY6427, 1:1000 dilution; Abways), AKT(9272, 1:1000 dilution; Cell Signaling Technology, USA), S473 phospho-AKT(9271, 1:1000 dilution; Cell Signaling Technology, USA), mTOR(YT2913, 1:1000 dilution; ImmunoWay), phospho-mTOR(YP1220, 1:1000 dilution; ImmunoWay), S6(2217, 1:1000 dilution; Cell Signaling Technology, USA), phospho-S6(2211, 1:1000 dilution; Cell Signaling Technology, USA), 4E-BP1(9452, 1:1000 dilution; Cell Signaling Technology, USA), S65 phospho-4E-BP1(9451, 1:1000 dilution; Cell Signaling Technology, USA) in the blocking solution at 4°C overnight. After washing with TBST buffer, the membrane was incubated with HRP-conjugated anti-rabbit antibody(7074, 1:3000 dilution; Cell Signaling Technology, USA) in the TBST buffer at a room temperature for 1h. After washing with TBST buffer, signals were detected by using a chemiluminescence reagent(Millipore, USA), imaged with a gel imaging system(Tanon, China), and quantified using Tanon image. Data are presented as the ratio of phosphorylation proteins to total proteins and are expressed as the fold-change compared to the Control group.

### **2.5. Enzyme-linked immunosorbent assay(ELISA)**

The levels of serum FSH, LH, E2 and AMH were measured by mouse-specific ELISA kits(Nanjing Jin Yibai Biological Technology Co., Ltd., Shanghai, China) according to the manufacturer's instructions(Catalog Nos.JEB-12673, JEB-12597, JEB-10719, JEB-12991). The concentration was determined by the absorbance at 450nm. Every experiment was done three repeats.

### **2.6. Mating Protocol**

Eight weeks following treatment, mice were housed with proven male breeders(C57BL/6, 3- to 5-month-old). Harem mating of two females per one male was used where applicable. Female mice were removed from the breeding cage at confirmed signs of pregnancy, including a weight >31g. Separated females were monitored every 24 to 36 h for pups. Following birth, pups were counted and weighed. Data recorded following birth included female weight post-birth, number of pups, percent of living pups, weight of pups, pup anomalies, and days from male interaction to birth. Mice were given up to 8 weeks for breeding and successful birth of a first litter.

### **2.7. Statistical Analysis.**

One-way analysis of variance, Student's t test, chi-square test, and Fisher's exact test were used where appropriate. Data are presented as mean±SEM with significance set at P<0.05.

## **3.Results**

### **3.1. Electro-acupuncture protects the primordial follicle pool in POF mice.**

Mean change in mouse weight from baseline, mean ovarian weight and ovarian surface area were used as proxies for systemic and ovarian toxicity. Mouse weight was similar at baseline

between three groups. Mice in the Control group weighed more at sacrifice than they did at baseline ( $P < 0.05$ ). Mice in the Model and EA group gained weight from baseline to sacrifice (Fig.1C). There were no differences between three groups when comparing ovarian weight and ovarian surface area ( $\text{mm}^2$ ) at sacrifice (Fig.1D and E).

Following the schema, the effects were assessed of EA on follicle counts. Primordial follicles per  $\text{mm}^2$  were reduced in the Model group compared with the Control group ( $P < 0.005$ ). Mice in the EA group trended toward more primordial follicles compared with mice in the Model group ( $P < 0.005$ , Fig.2B). Primary follicle counts and secondary follicle counts were not statistically different in the Control group and the EA group, despite a trend toward fewer primary follicles and secondary follicles in the Model group (Fig.2C and D). There were fewer antral follicles per  $\text{mm}^2$  in the Model group compared with the Control group ( $P < 0.05$ , Fig.2E). Ovaries of the EA group demonstrated a ratio of growing to primordial follicles less than that in the Model group ( $P < 0.05$ , Fig.2F), all of these supporting our finding that EA maintains ovarian quiescence and preserves the primordial follicle pool.

### **3.2. Electro-acupuncture down-regulates the phosphorylation of PI3K/AKT/mTOR pathway proteins in ovaries of POF mice.**

POF mice were treated with one-week EA treatment and whole ovaries were harvested. CY added PI3K/AKT/mTOR pathway activation moderately, mainly shown by phosphorylation of PI3K, S473 AKT, mTOR, Thr389 S6 kinase and S65 4E-BP1 to P-PI3K, P-AKT, P-mTOR, P-S6K and P-4E-BP1. EA reduced phosphorylation of PI3K, AKT, mTOR, S6K and 4E-BP1 (Fig.3A). Downstream targets of the pathway were assessed by immunoblot analysis of the ratio of phosphorylated to total protein. The ratios of phosphorylation of PI3K, AKT, mTOR, S6K and 4E-BP1 were increased in Model compared with Control, while the ratios were decreased after EA treatment (Fig.3B-F,  $P < 0.05$ ).

### **3.3. Electro-acupuncture can up-regulate serum AMH, E2 levels, and down-regulates serum FSH, LH levels in POF mice.**

To investigate the impact of EA, serum FSH, LH, E2, AMH levels were measured by ELISA in all groups. The Model mice had significantly higher levels of serum FSH compared with the Control mice ( $P < 0.005$ ), which decreased after EA treatment ( $P < 0.05$ , Fig.4A). Serum LH was significantly increased in the Model group than in the Control, and the level was reduced after EA treatment ( $P < 0.05$ , Fig.4B). Serum E2, AMH in the Model group were significantly lower than those in the Control group ( $P < 0.005$ ), and fewer than those in the EA group ( $P < 0.05$ , Fig.4C and D). The results above indicate that EA up-regulates serum AMH to preserve the ovarian reserve function.

### **3.4. Electro-acupuncture preserves fertility in POF mice.**

We investigated whether EA also preserves fertility in POF mice. Studies have shown that rodents are able to start normal mating behavior at the time of 14 days following CY treatment. To ensure more rigorous, mice were harem-bred with proven male breeders and

given 8 weeks to breed after 4 weeks at the end of the final treatment. Compared with the Control group, the Model group had a reduction in litter size ( $P < 0.005$ ), and the EA group had more litter sizes than the Model group ( $P < 0.05$ , Fig.5A). There were no differences in the percentage of pups live-born, pup weight (Fig.S1A and B). The time from beginning breeding to first birth was similar between groups, while there was a trend toward increased time from male introduction to birth in the Model group, which did not reach significance (Fig.S1C).

## 4. Discussion

The etiology and pathogenesis of POF is not yet known, and it is believed that it is mainly related to the permanent damage of ovaries. Traumatic factors include trauma, radiotherapy and chemotherapy, immunity and inheritance [21]. Although it is not lethal, POF has brought catastrophic injury to patients. The difficulty of treatment is a major challenge for in clinic. Chemotherapy is considered as one of the leading causes of POF, especially the widely used alkylating agents, such as CY, the core of breast cancer chemotherapy, with high clinical toxicity, and induces ovarian damage by activating PI3K/AKT/mTOR Pathway, causing primordial follicle activation and follicular burnout, leading to loss of ovarian reproductive and endocrine function [22, 23]. The follicular burnout refers to the repeated ovarian exposure to chemotherapy and damage to growing follicles, mobilizing dormant primordial follicles to activate and grow to replace damaged antral follicles [24, 25].

Considerable clinical research effort has been directed toward treatment of EA in POF patients in recent years, and results indicate that EA can regulate ovarian secretion to promote ovarian function recovery in POF patients [26, 27]. Findings from previous studies have demonstrated that PI3K/AKT signaling pathway exists widely in cells and participates in cell growth, proliferation and differentiation, while EA can regulate the PI3K/AKT signaling pathway [18, 28]. However, whether it is involved in EA treatment of POF remains to be determined.

At present, studies have shown that mice were injected in intraperitoneal with 75mg/kg of CY, all follicles in the ovary were reduced, and primordial follicles decreased to half [29]. Therefore, a single intraperitoneal injection of 75mg/kg CY was used to establish a mouse model for ovarian dysfunction. In this study, we examined the effects EA on PI3K/AKT/mTOR pathway in POF mice. Parameters assessed in our study included all follicles counts, ovarian morphology, serum hormone, protein expression, fertility. In the Model group, we observed some changes in follicles counts, hormone levels, proteins expressions and fertility. Such findings indicate that CY can increase the phosphorylation of PI3K, AKT, mTOR, S6K and 4E-BP1, regulate serum AMH, E2, FSH, LH levels, reduce the primordial follicles, and have an impact on fertility. Following EA treatment, the PI3K/AKT/mTOR signaling pathway was activated (Fig.5B), included decreased phosphorylation of the pathway proteins, recovery of serum AMH, E2, FSH, LH levels, more litter sizes. The ovarian function was protected and the fertility was improved.

Although POF was not nominally recorded in TCM, its clinical manifestations can be classified into the category of amnesic amenorrhea, blood dryness and infertility[30]. The TCM etiology includes insufficient transformation of blood of the spleen and stomach, severe consumption of yin blood and exhaustion of blood source. Its pathogenesis is mainly kidney deficiency, and dysfunction, while kidney deficiency is the most fundamental[31]. Guan Yuan is the intersection of Renmai and Zusanyin. It is the place where the yin and yang meet, and the place where the gas is condensed. Zusanli is a point of the foot-yangming stomach meridian, which has the effect of warming the spleen and stomach, nourishing qi and nourishing blood, and supporting the righteousness. The combination of the two points can reinforce liver and kidney, regulating Qi and blood, and adjusting mental activity[32].

Although our study is limited by the mouse model, ovaries of mouse and human maintain many functional similarities in that the ovarian reserve is maintained in primordial follicles that localize to the ovarian cortex, and follicular development occurs through the same stages. EA treatment regimens in our study were extrapolated from human to mice, and may thus exist differences. In the Future, clinical studies will be conducted, and the EA frequency, duration, intensity, acupoints will be determined simultaneously.

## 5. Conclusion

Our data provide evidence for the improvement of EA on ovarian function, and EA may be used for complementary therapy to POF. However, in our study, we only focused on the PI3K/AKT/mTOR signaling pathway to investigate its potential mechanisms. Therefore, further research need to be carried out to clarify the mechanism of EA to anti-POF.

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### Conflict of interest statement

THE AUTHORS DECLARE THAT THERE ARE NO CONFLICTS OF INTEREST.

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## Legends

**Fig.1.**(A)Acupoint site.(B)An experimental schema.(C)Mouse weight at baseline and sacrifice(g). All mice gained weight from baseline to sacrifice: the Control group(\*P<0.05), the Model group (n.s., P>0.05), the EA group(n.s., P>0.05). (D)Ovarian weight at time of sacrifice(mg)(n.s., P>0.05). (E)Ovarian surface area(mm<sup>2</sup>)(n.s., P>0.05). Data are presented as SEM.

**Fig.2.**EA protects the primordial follicle pool in ovaries of the Model group mice.(A)Ovarian sections stained with H&E and representative images are shown (20×, 40× magnification). Representative growing follicles are marked with arrows.(B)Primordial follicles were reduced in the Model group mice compared with the Control group mice (\*\*P<0.005). The EA group mice trended toward more primordial follicles compared with the Model group mice(##P<0.005).(C and D)Primary follicle and secondary follicle counts were not statistically different in the Control group and EA group despite trending toward fewer primary follicles and secondary follicles in the Model group(n.s., P>0.05).(E)There were fewer antral follicles in the Model group than in the Control group(\*P<0.05), and mice in the EA group trended toward more antral follicles compared with the Model group(n.s., P>0.05).(F) Ovaries of the Model group demonstrated a ratio of growing to primordial follicles more than that in the Control group(\*P<0.05) and EA group(#P<0.05). Results are derived from five mice per treatment group with SEM shown.

**Fig.3.**EA down-regulates downstream targets of the PI3K/AKT/mTOR pathway in the ovaries. Immunoblot analysis of whole-ovary lysates assessing downstream targets of EA.(A)The representative immunoblots.(B)The ratio of p-PI3K to PI3K was increased in the Model group compared with the Control group(\*P<0.05), then decreased after EA treatment(#P<0.05).(C)The ratio of p-AKT to AKT was increased in the Model group compared with the Control group(\*P<0.05), then decreased after EA treatment(#P<0.05).(D)The ratio of p-mTOR to mTOR was increased in the Model group compared with the Control group(\*\*P<0.05), then decreased after EA treatment(#P<0.05).(E)Phosphorylation of S6K was increased in the Model group compared with the Control group(\*\*\*P<0.001), while EA treatment decreased phosphorylated S6K levels without affecting total protein content(#P<0.05).(F)Phosphorylation of 4E-BP1 in the Model group was increased compared with the Control group(\*P<0.05), and reduced after EA treatment(#P<0.05). All ovaries from five mice per group of 8-week-old mice. Results are quantified from three series of representative immunoblots. Data are presented as mean ± SEM.

**Fig.4.** Serum AMH, E2 decrease and serum FSH, LH increase in the Model group mice whereas EA maintain their concentration. (A)The Model group mice had significantly higher levels of serum FSH compared with the Control group(\*\*P<0.005), while the level declined after EA treatment (#P<0.05). (B)Compared with the Control group, CY slightly up-regulates the serum LH(\*P<0.05), then decreased after EA treatment(#P<0.05). (C, D)Serum AMH, E2 in the Model group had lower levels compared with the Control group(\*\*P<0.005), however, the levels increased after EA treatment (#P<0.05). Results are derived from five mice per treatment group with SEM shown.

**Fig.5.** (A)Quantification of the number of pups born in first litters. CY reduces litter size compared with the Control group(\*\*P<0.05), and the EA group had more litter sizes than the Model group(#

P>0.05). Results are derived from three mice per treatment group with SEM shown. (B) A diagram of the sequence of development from primordial follicles to antral follicles is shown. Also shown is the PI3K/AKT/mTOR pathway. Physiologic ovarian folliculogenesis is shown proceeding from the primordial follicle stage, where an oocyte arrested in prophase of meiosis I and surrounded by a single layer of squamous granulosa cells is activated, grows and transitions to a primary follicle, a secondary follicle, and then a preovulatory antral follicle.

ACCEPTED MANUSCRIPT

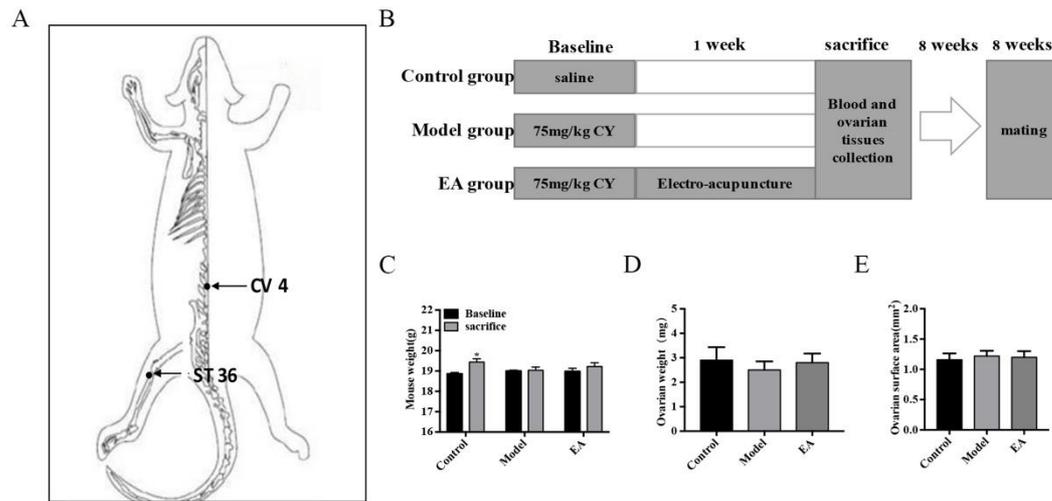


Figure 1

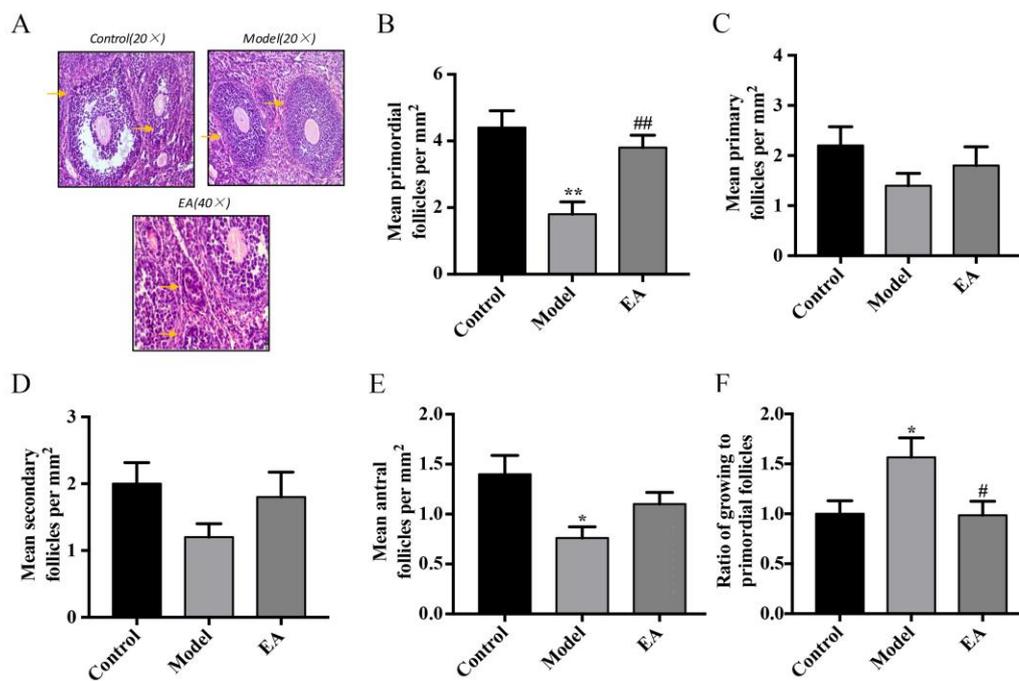


Figure 2

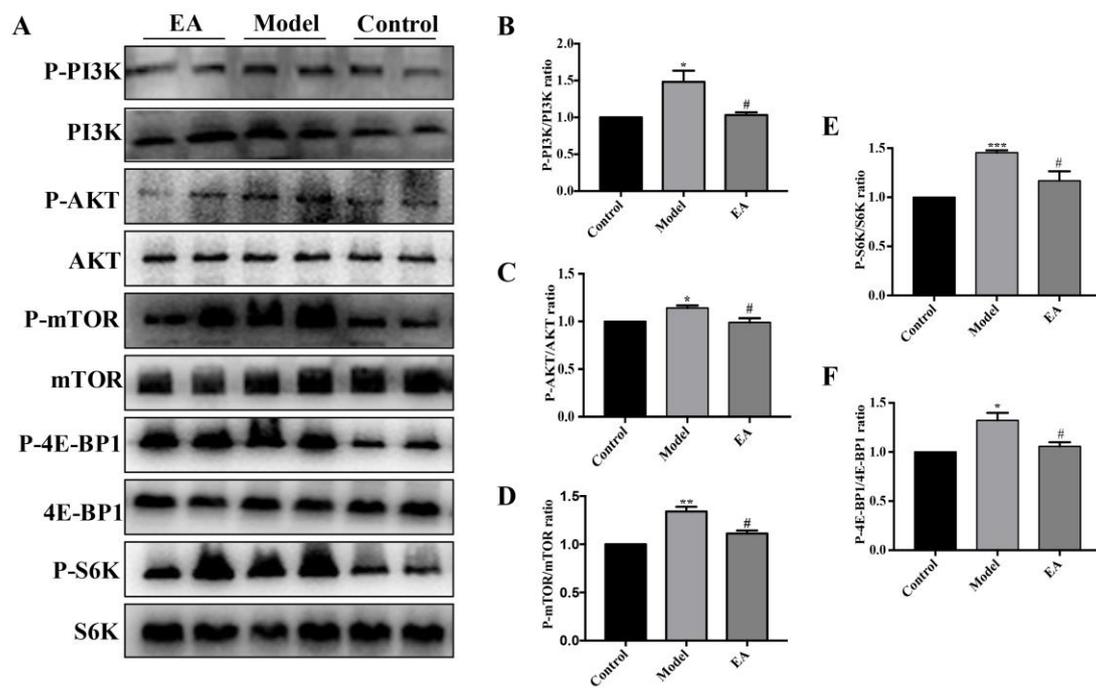


Figure 3

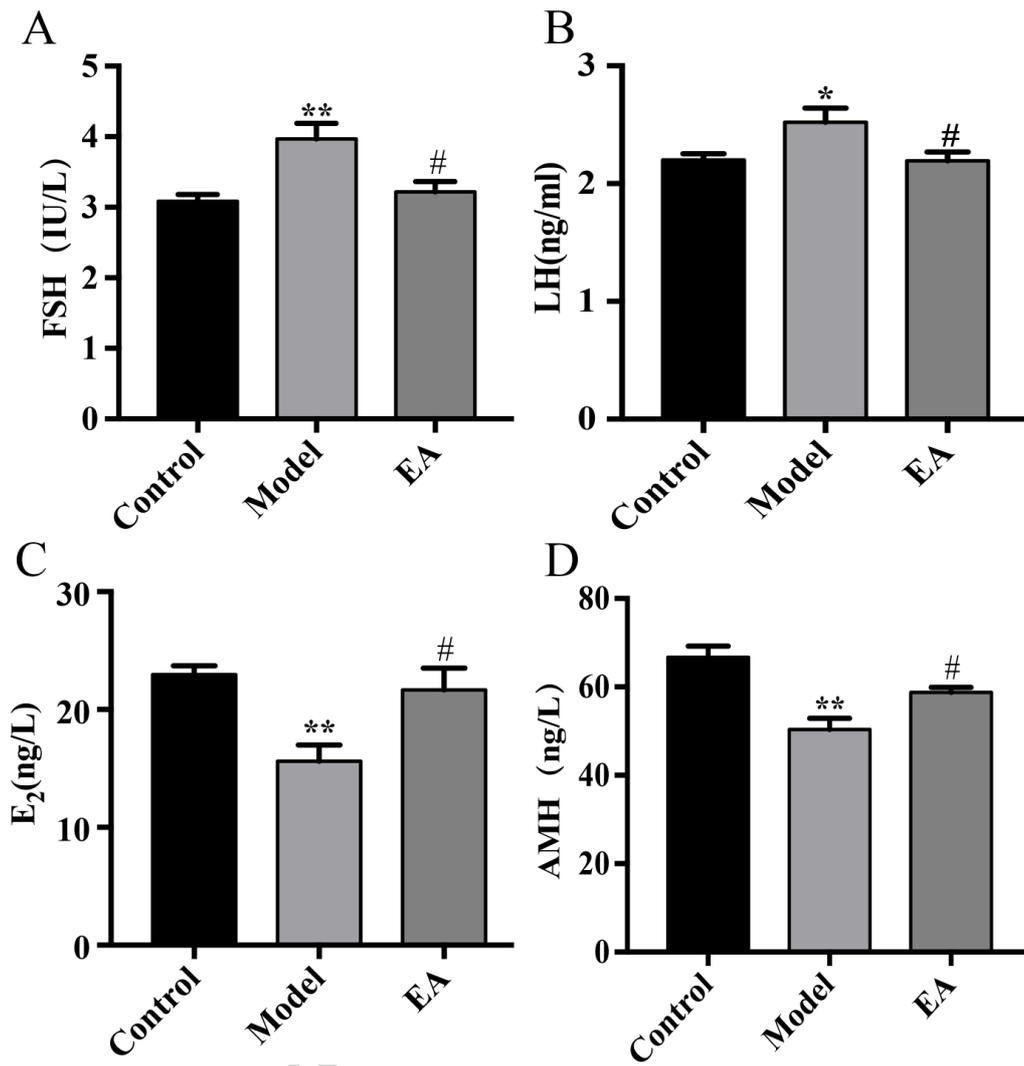


Figure 4

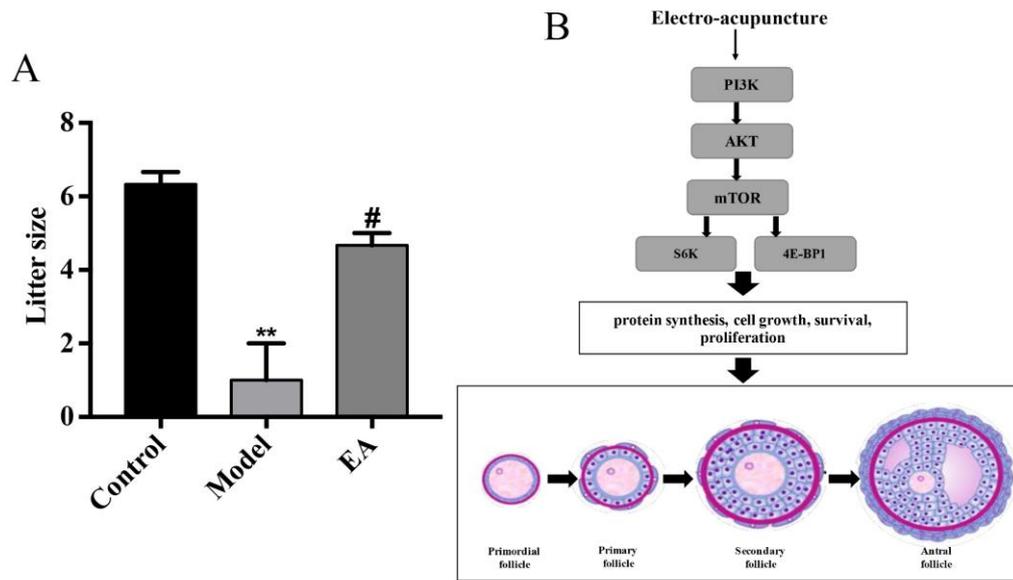


Figure 5