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**Basic Study**

# Effect of electroacupuncture on anti-Mullerian hormone expression in rats with polycystic ovarian syndrome

## 电针对多囊卵巢综合征大鼠抗苗勒氏管激素水平的影响

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**Abstract**

**Objective:** To observe whether the effect of electroacupuncture (EA) on improving sex hormone disorders and follicle development is by decreasing the expression of anti-Mullerian hormone (AMH) in rats with experimental polycystic ovarian syndrome (PCOS).

**Methods:** Forty rats were randomly divided into four groups, a normal group (NG), a model group (MG), an EA at acupoints group (EAAG), and an EA at non-acupoints group (EANAG), with 10 rats in each group. The rats in the EAAG and EANAG were intervened by EA treatment for consecutive 14 d. Zhongji (CV 3) and Guanyuan (CV 4) were selected as the acupoints in the EAAG, and the tip of the tail and 1 cm up from the tail tip were selected as the non-acupoints in the EANAG. After treatment, the histomorphological changes of the ovary, the levels of aromatase P450 (P450arom), testosterone and estradiol in the ovarian tissues, and the expressions of follicle stimulating hormone (FSH) and AMH were observed.

**Results:** After treatment, compared with the MG and EANAG, the expression of AMH decreased ($P$<0.05), the levels of P450arom and estradiol increased significantly, and the level of testosterone decreased significantly (all $P$<0.01) in the EAAG. Additionally, several normal follicles were present and the number of cystically dilated follicles decreased in the EAAG. Compared with the MG and EANAG, the EAAG obviously had more follicular granulosa cells.

**Conclusion:** EA can down-regulate the abnormally increased expression of AMH to improve sex hormone disorders and follicle development in PCOS rats.

**Keywords:** Acupuncture Therapy; Electroacupuncture; Point, Zhongji (CV 3); Point, Guanyuan (CV 4); Anti-Mullerian Hormone; Polycystic Ovary Syndrome; Follicle Stimulating Hormone; Rats

**【摘要】目的：**观察电针是否通过降低抗苗勒氏管激素(AMH)的表达实现对多囊卵巢综合征(PCOS)大鼠性激素紊乱和卵泡发育障碍的改善作用。**方法：**将 40 只大鼠随机分为正常组、模型组、电针经穴组和电针非经穴组，每组 10 只。电针经穴组和电针非经穴组大鼠连续治疗 14 d。电针经穴组大鼠取中极和关元；电针非经穴组取尾尖和尾尖上 1 cm 处。观察治疗后卵巢组织形态学变化，P450 芳香化酶、睾酮和雌二醇水平以及促卵泡生成素(FSH)和 AMH 的表达。**结果：**同模型组和电针非经穴组比较，电针经穴组 AMH 表达降低($P$＜0.05)，P450 芳香化酶和雌二醇水平显著升高，睾酮水平显著降低(均 $P$＜0.01)。此外，电针经穴组存在数个正常卵泡，囊性扩张卵泡减少，卵泡颗粒细胞数明显多于模型组和电针非经穴组。**结论：**电针可下调 PCOS 大鼠 AMH 的异常过度表达，从而改善性激素紊乱和卵泡发育障碍。

**【关键词】**针刺疗法；电针；穴，中极；穴，关元；抗苗勒氏管激素；多囊卵巢综合征；促卵泡激素；大鼠

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Polycystic ovary syndrome (PCOS) is an endocrine disorder caused by sex hormone dysfunctions that disturb follicle development and lead to ovulation failure[1]. Increased androgen level or hyperandrogenism is the main factor leading to a series of reproductive, endocrine and metabolic disorders[2]. Aromatase P450 (P450arom) has been demonstrated to be the last rate-limiting enzyme in the conversion of androgens to estrogen, and insufficient estrogen expression is considered to be a key mechanism resulting in hyperandrogenism and abnormal follicle development in PCOS[3]. In the late stage of preantral follicle development period (secondary follicles and antral follicles), follicle stimulating hormone (FSH) induces the synthesis of P450arom, which promotes the conversion of testosterone to estradiol and improves the formation of a dominant follicle[4]. Recent studies have indicated that normal expression of P450arom is suppressed by excessive expression of anti-Mullerian hormone (AMH) in PCOS[5]. AMH is an important regulator of folliculogenesis in the ovaries and is exclusively secreted by granulosa cells of the ovarian follicles. Physically, the highest expression of AMH is observed in preantral and small antral follicles, and then it decreases with the selection of dominant follicles[6]. However, serum level of AMH is 2-3 times in women with PCOS compared with that in healthy women[7-8]. It is demonstrated that AMH reduces the aromatase mRNA expression in human granulosa cells[9]. Therefore, the inhibitory effect of AMH on P450arom is considered the main cause of excessive androgen level in PCOS, which contributes to sex hormone disorders and ovulation failure[10].

In recent years, electroacupuncture (EA) has been used to treat PCOS patients because of its fewer side effects than drug therapies[11-12]. Experimental and clinical evidences have indicated the effect of EA in improving follicle development by affecting the functions of hypothalamus, pituitary gland and ovaries[13-16]. Our previous study has demonstrated that EA increases the expression of P450arom and improves follicle development[17-18]. In addition, acupuncture was proved to reduce the serum level of AMH and ovarian volume in a randomized controlled trial[19]. Therefore, based on the previous study results, this study was to find out whether the effect of EA in increasing the expression of P450arom to improve sex disorders and follicle development results from down-regulation of the excessive expression of AMH in the granulosa cells in PCOS rats established by letrozole[20].

## 1 Materials and Methods

### 1.1 Experimental animals

The 42-day-old specific-pathogen-free inbred female Sprague-Dawley (SD) rats were purchased from the Experimental Animal Science Department of Shanghai University of Traditional Chinese Medicine. The experiments were performed in strict accordance with the *Opinion on the Treatment of the Experimental Animals* issued by the Ministry of Science and Technology of the People's Republic of China. All rats received humane care in a temperature-controlled room with a 12-hour light-dark cycle, and free accessed to water and food. The treatments to animal in this study were approved by the Ethics Committee on Use of Experimental Animals in Teaching and Research, Shanghai University of Traditional Chinese Medicine.

### 1.2 Study procedure

Forty 42-day-old female SD rats were randomly divided into four groups, a normal group (NG), a model group (MG), an EA at acupoints group (EAAG) and an EA at non-acupoints group (EANAG) by the random number table method, with 10 rats in each group. The rats in the MG, EAAG and EANAG were administered with letrozole solution at 1.0 mg/(kg·bw) by gavage, once a day for consecutive 21 d. The rats in the NG were administered with normal saline via gavage over the same period of time. Since the second day after modeling (i.e., the 64th day after birth), the rats in the EAAG and EANAG were intervened by EA treatment for consecutive 14 d. Zhongji (CV 3) and Guanyuan (CV 4) were selected as the acupoints in the EAAG, and the tip of the tail and 1 cm up from the tail tip were selected as the non-acupoints in the EANAG. During the procedure, acupuncture needles of 0.22 mm in diameter and 13 mm in length were quickly inserted by 2-3 mm into the acupoints. The needles were then connected to a HANS-100 pain treatment device (Nanjing Jisheng Medical Technology Co., Ltd., China) with a continuous wave (frequency: 2 Hz, current: 2 mA) until the stimulated areas showed visible quivering. The needles were retained for 20 min, once a day for 14 d. During the course of treatment, the rats in the NG and MG did not receive any treatments.

### 1.3 Ovarian tissues collection

Following the completion of EA treatments on the 78th day after birth, 2% pentobarbital sodium solution was injected into the abdominal cavity of each rat at a dose of 100 μg/(g·bw), and then the bilateral ovaries were extracted from the opened cavity. The adipose tissues of ovaries were immediately removed and weighed, and then the ovaries were submerged in 4% polyformaldehyde solution and stored at −80 ℃ separately.

### 1.4 Morphological observation

Pieces of ovarian tissue (0.5 $cm^3$ for each) were fixed in 4% polyformaldehyde solution; embedded in paraffin; sectioned at 4 μm; stained with hematoxylin-eosin (HE); dehydrated in 95%, 90% and 70% ethanol; and then cleared in xylene. The sections were viewed using an Olympus DP73 microscope (Olympus, Japan).

**1.5 Enzyme-linked immunosorbent assay (ELISA)**

The ovarian tissue supernatant was extracted and purified, and the concentrations of testosterone, estradiol, and P450arom were analyzed using ELISA kits (Beinglay Biotechnology Co., Ltd., China).

**1.6 Immunohistochemical assay**

The ovarian tissue sections were deparaffinized, hydrated, and pretreated in a microwave (antigen retrieval). Endogenous peroxidase activity was inhibited with 0.3% $H_2O_2$, and nonspecific binding was blocked with 20% normal goat serum. All sections were incubated with FSH (rabbit polyclonal anti-rat FSH 1:500, LifeSpan, USA) and AMH (rabbit polyclonal anti-rat AMH 1:10, Novus, USA) antibodies for 2 h at 37 ℃. The samples were washed and then incubated for 30 min at room temperature with the appropriate pre-absorbed biotinylated secondary antibodies. Visualization of the antigens was achieved using the streptavidin-peroxidase method (JRDUN Biotechnology Co., Ltd., China), and 3, 3-diaminobenzidine (DAB) (liquid DAB-plus substrate kit, JRDUN Biotechnology Co., Ltd., China) was used as a chromogen. The slides were washed in distilled water and counterstained with Mayer's hematoxylin, and then they were dehydrated and mounted. The antibodies were replaced with phosphate buffer saline (PBS) for the negative control. For evaluation, a semi-quantitative analysis of the staining results was achieved using the IMS medical image quantitative analysis system (JRDUN Biotechnology Co., Ltd., China). Positive expressions of FSH and AMH were brown or yellow particles stained among the granulosa cells. The positive area and the optical density (OD) values in three different high-power optical fields (×200) of every slice were measured. The immune positive area index (Positive area ÷ Total area × OD) of FSH and AMH was calculated for each high-power optical field.

**1.7 Statistical analysis**

The experimental data were shown as mean ± standard deviation ($\bar{x} \pm s$), and the SPSS version 19.0 statistical software package was used for the statistical analysis. Multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by the correction of *P* values with Dunnett's post hoc test. $P<0.05$ was considered as statistical significance.

## 2 Results

**2.1 Comparison of ovarian weight between different groups**

Compared with the NG, the bilateral ovarian weights of rats in the MG and EANAG increased significantly (both $P<0.01$), and there was no significant difference between the NG and EAAG ($P>0.05$). Compared with the MG and EANAG, the bilateral ovarian weights of rats in the EAAG decreased significantly (both $P<0.01$), but there was no significant difference between the EANAG and MG ($P>0.05$), (Figure 1).

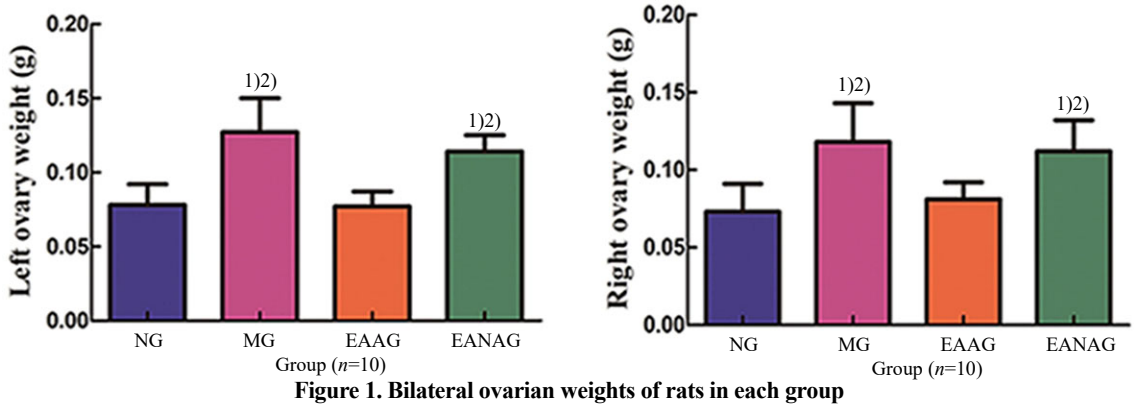


**Figure 1. Bilateral ovarian weights of rats in each group**

Note: Compared with the NG, 1) $P<0.01$; compared with the EAAG, 2) $P<0.01$

**2.2 Observation of ovarian morphology of rats**

In the NG, well-developed ovarian follicles and some luteum were seen, and the oocytes and corona radiata were visible in the follicles. There was morphological integrity in the granulosa cells, which were neatly and closely arranged in the follicles in multiple layers. In the MG, the thickening albuginea was seen, under which there were many cystic follicles and less luteum, as well as thinning layers of reduced and loosely arranged follicular granulosa cells, and oocytes and corona radiata were missing within the follicles. It was rare to see normal follicles in different stages. In the EAAG, the number of well-developed follicles increased, in which the oocytes and corona radiata were visible, and there were more closely-arranged granulosa cells. However, there were only few luteum and cystic follicles. In the EANAG, the thickening albuginea was observed, under which there were many cystic follicles, and the follicular granulosa cell layers were thinning with fewer loosely-arranged granulosa cells. However, there were no normal follicles. The detail is shown in Figure 2.

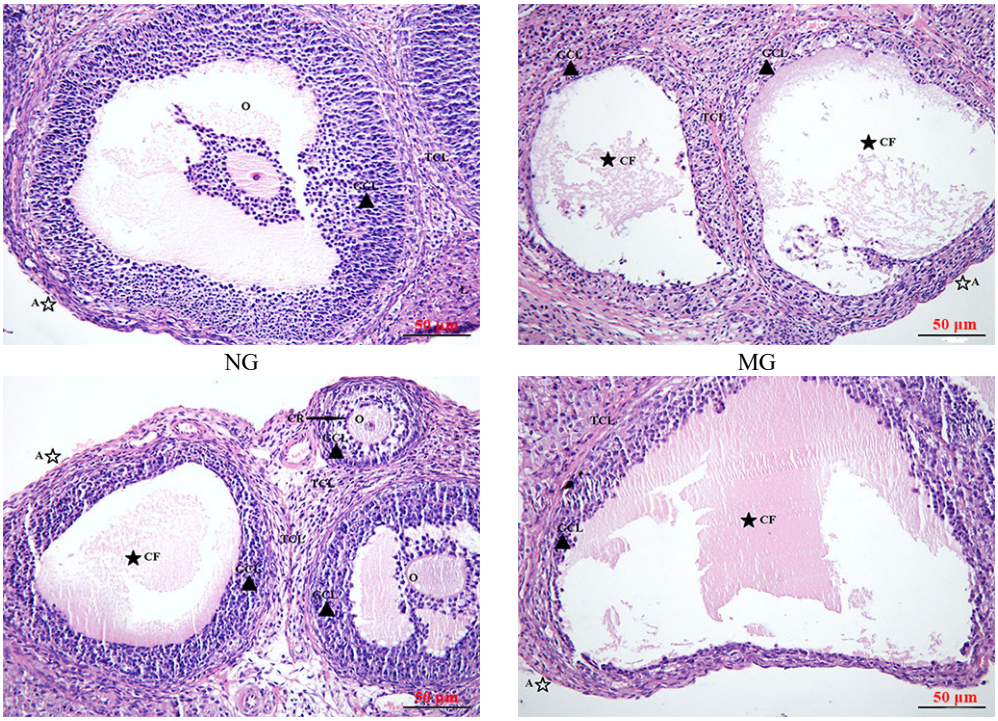


**Figure 2. The morphology of the rats' ovarian tissues (HE, ×200)**

Note: A=Albuginea, CF=Cystic follicle, CR=Corona radiata, GCL=Granular cell layer, L=Luteum, O=Oocyte, TCL=Theca cell layer; ☆indicates changes in albuginea; ★indicates the appearance of a cystic follicle; ▲indicates changes in the granular cell layer

### 2.3 P450arom, testosterone, and estradiol levels in the ovarian tissues of rats

#### 2.3.1 Comparison of the P450arom level in the rats' ovarian tissues between different groups

Compared with the NG, the levels of P450arom in the MG and EANAG decreased significantly (both $P<0.01$), and there was no significant difference between the NG and EAAG ($P>0.05$). Compared with the MG and EANAG, the level of P450arom in the EAAG increased significantly (both $P<0.01$), but there was no significant difference between the EANAG and MG ($P>0.05$). The details are shown in Figure 3.

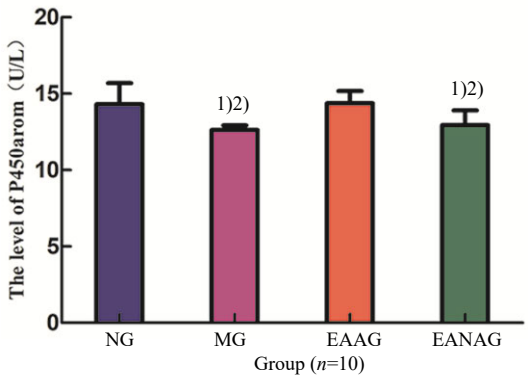


**Figure 3. Comparison of the P450arom level in the rats' ovarian tissues**

Note: Compared with the NG, 1) $P<0.01$; compared with the EAAG, 2) $P<0.01$

#### 2.3.2 Comparison of the testosterone level in the rats' ovarian tissues between different groups

Compared with the NG, the levels of testosterone in the MG and EANAG increased significantly (both $P<0.01$), and there was no significant difference between the NG and EAAG ($P>0.05$). Compared with the MG and EANAG, the level of testosterone in the EAAG decreased significantly (both $P<0.01$), but there was no significant difference between the EANAG and MG ($P>0.05$). The details are shown in Figure 4.

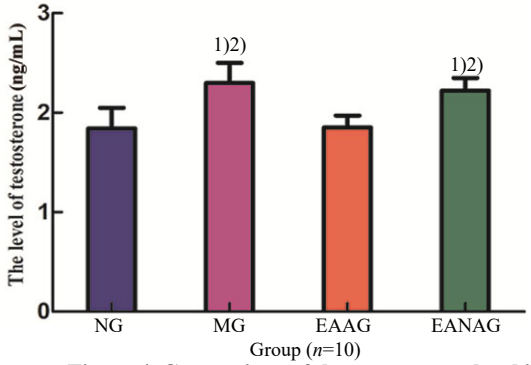


**Figure 4. Comparison of the testosterone level in the rats' ovarian tissues**

Note: Compared with the NG, 1) $P<0.01$; compared with the EAAG, 2) $P<0.01$

2.3.3 Comparison of the estradiol level in the rats' ovarian tissues between different groups

Compared with the NG, the levels of estradiol in the MG and EANAG decreased significantly (both $P<0.01$), and there was no significant difference between the NG and EAAG ($P>0.05$). Compared with the MG and EANAG, the level of estradiol in the EAAG increased significantly (both $P<0.01$), but there was no significant difference between the EANAG and MG ($P>0.05$). The details are shown in Figure 5.

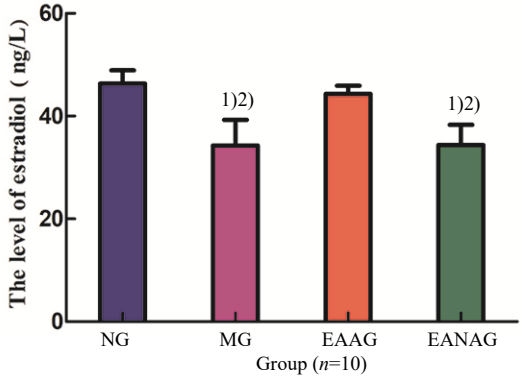


**Figure 5. Comparison of the estradiol level in the rats' ovarian tissues**

Note: Compared with the NG, 1) $P<0.01$; compared with the EAAG, 2) $P<0.01$

**2.4 FSH and AMH expressions in the follicular granulosa cells of rats**

2.4.1 Comparison of the FSH expression in the rats' follicular granulosa cells between different groups

The expression of FSH in each group did not differ significantly (all $P>0.05$). The details are shown in Figure 6 and Figure 7.

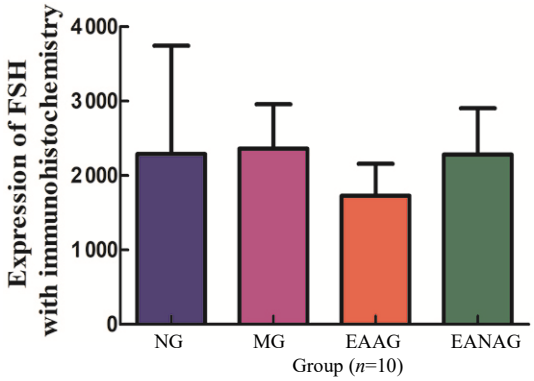


**Figure 6. The expression of FSH in the rats' follicular granulosa cells**

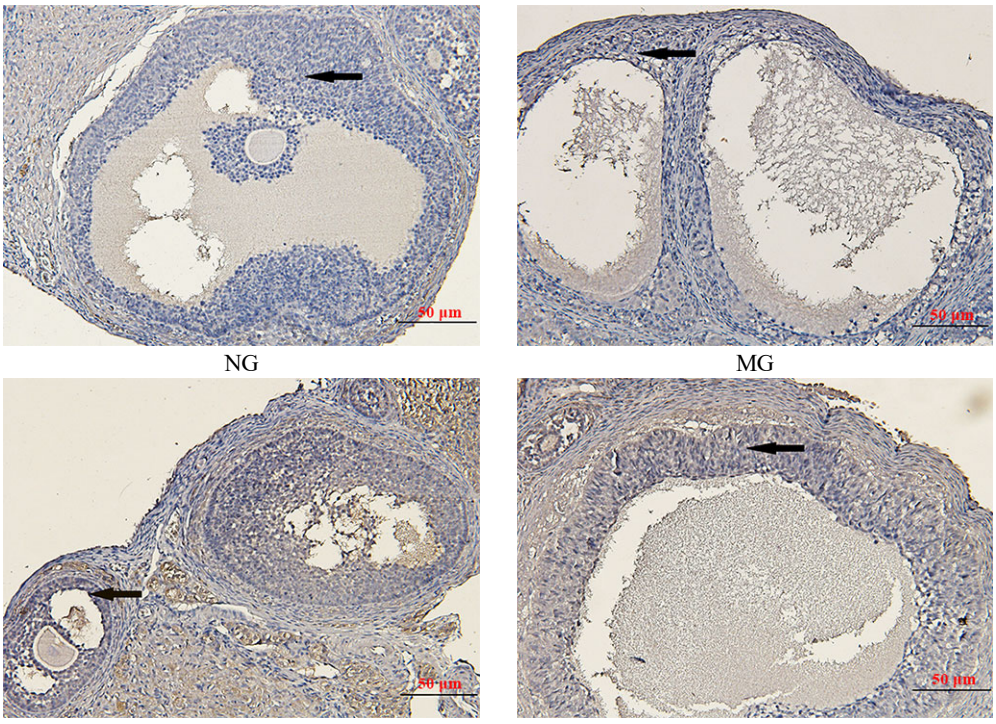


**Figure 7. The expression of FSH in the rats' follicular granulosa cells (immunohistochemistry, ×200)**

Note: Arrows indicate the positive expression of FSH

2.4.2 Comparison of the AMH expression in the rats' follicular granulosa cells between different groups

Compared with the NG, the expressions of AMH in the MG and EANAG increased significantly (both $P<0.01$), and there was no significant difference between the NG and EAAG ($P>0.05$). Compared with the MG and EANAG, the expression of AMH in the EAAG decreased significantly ($P<0.01$, $P<0.05$), but there was no significant difference between the EANAG and MG ($P>0.05$). The details are shown in Figure 8 and Figure 9.

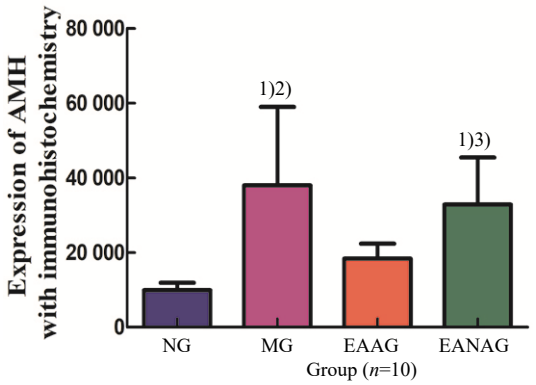


**Figure 8. The expression of AMH in the rats' follicular granulosa cells**

Note: Compared with the NG, 1) $P<0.01$; compared with the EAAG, 2) $P<0.01$, 3) $P<0.05$

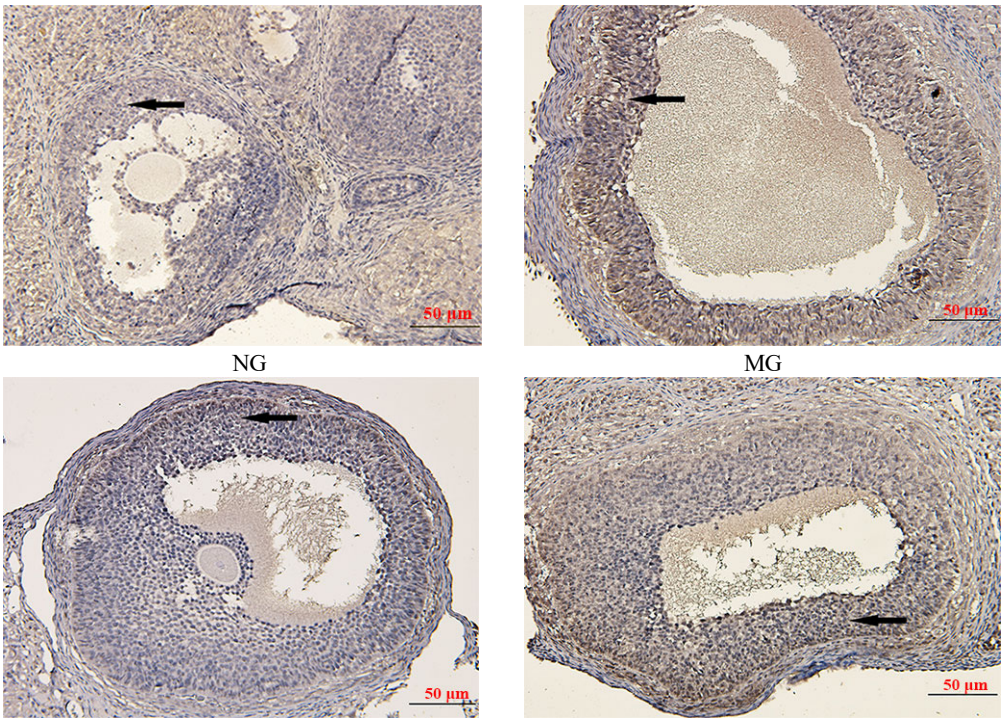


**Figure 9. The expression of AMH in the rats' follicular granulosa cells (immunohistochemistry, ×200)**

Note: Arrows indicate the positive expression of AMH

## 3 Discussion

In this study, letrozole, a nonsteroidal aromatase inhibitor, was used to establish the PCOS model[20]. The level of P450arom in the ovaries decreased due to the inhibition of steroid hormone production by letrozole, which affected the transition of androgens to estrogen, resulting in excessively increased testosterone and relatively deficient estradiol. Bilateral ovarian weights of rats in the MG increased significantly, and the albuginea ovarii were noted to become thickened under light microscope. According to morphological observation, follicular atresia, cystically dilated follicles and decreased layers of follicle granulosa cells were observed in the MG. In addition, the expression of testosterone increased significantly and the estradiol level decreased significantly in the ovarian tissues of rats in the MG compared with those in the NG. These results were consistent with the characteristics of the reproductive endocrine disorder of PCOS in humans[21-22].

According to recent studies, follicular arrest, increased number of preantral follicles and the appearance of non-dominant follicles suggest that growth retardation of primordial follicles in the ovaries may possibly be the leading cause of anovulation in

PCOS[23-24]. FSH secreted by the pituitary gland regulates the proliferation and differentiation of follicular granulosa cells and the maturation of follicles[25]. Studies have indicated that abnormal development of follicles should be due to inhibitory effect of FSH resulting from the secretion of AMH in the granulosa cells[26-27]. Over-expression of AMH in the follicular granulosa cells have been demonstrated to inhibit the expression of P450arom, resulting in follicular development retardation[28-30]. In this study, the expression of FSH observed by immunohistochemistry did not differ significantly, while the expression of AMH increased to different degrees in each group, and the expressions of AMH in the MG and EANAG were found increased significantly. Follicles, follicular atresia, and cystically dilated follicles in the albuginea ovarii increased significantly, while the corpus luteum and the layers of follicular granulosa cells decreased significantly. Furthermore, P450arom level declined, the testosterone level increased significantly, and the estradiol level dropped in both groups. These results revealed that the over-expression of AMH may be an important factor in affecting P450arom, leading to insufficient substrates for estrogen generation[31], and causing hyperandrogenism which is a key factor of follicular arrest and abnormal follicle development[32-33].

One study used Zhongji (CV 3) and Guanyuan (CV 4) to regulate ovarian and uterine dysfunction in patients with PCOS[34]. The innervation of ovary and uterus is at the level of $T_{10}$-$L_2$ segment. The innervation of Zhongji (CV 3) and Guanyuan (CV 4) is at the level of $T_6$-$L_2$ segment[19]. The overlapping segments of ovary and uterus with these two acupoints suggest that EA can regulate the function of ovary and uterus through stimulating Zhongji (CV 3) and Guanyuan (CV 4). In our previous study, we observed that low-frequency EA, stimulating at Zhongji (CV 3) and Guanyuan (CV 4), could increase the expression of P450arom and improve the granular layer, chondriosome and mitochondrial cristae of follicle in PCOS rats[17-18]. In the present study, low-frequency EA was applied to the same acupoints Zhongji (CV 3) and Guanyuan (CV 4) as in our previous study[17]. In the EAAG, bilateral ovarian weights of rats declined significantly and a few follicles similar to those in the NG were detected in the ovaries; additionally, the number of cystic follicles decreased, and the amount of corpus luteum and the granular layer increased significantly. Meanwhile, the over-expression of AMH dropped significantly and the P450arom level increased significantly. With the increased P450arom, the testosterone level declined significantly and the estradiol level increased significantly. However, these changes were not observed in the EANAG, in which the findings were similar to those in the MG. The different results between the acupoints group and non-acupoints group should be derived from the effect of the acupoints Zhongji (CV 3) and Guanyuan (CV 4) that affects the spinal innervation of the ovaries and uterus. Ovarian functional regulation can be mediated by the sympathetic fibers in the ovaries and controlled by a spinal pathway. But the non-acupoints, the tip of the tail and 1 cm from the tip, did not affect the same spinal pathway[35-36]. Compared with the EANAG, the EAAG had a better effect, which may result from the decrease in the over-expressed AMH in the follicular granulosa cells and the subsequent improvement in the expression of P450arom and abnormal follicle development.

In conclusion, our work has indicated that the efficacy of EA in improving sex hormone disorders and follicular development in PCOS may be related to the down-regulation of the excessively expressed AMH and the improved P450arom expression. This study has offered a better understanding of the effect of low-frequency EA on PCOS.

**Conflict of Interest**

The authors declared that there was no potential conflict of interest in this article.

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**Statement of Human and Animal Rights**

The treatment of animals conformed to the ethical criteria.

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