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Association of Ovarian Steroids in Follicular Fluid with Pregnancy Rates in Patients with or without Polycystic Ovary during *in vitro* Fertilization

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ABSTRACT

The point of this consideration is to examine the possible influence of progesterone (P4), estradiol (E2), and testosterone (T) concentrations in follicular fluid (FF) on fertilization, implantation, and biochemical pregnancy rates in patients with polycystic ovary (PCO) and normal women during *in vitro* fertilization (IVF) treatment. 30 normal-ovulatory women between 24-42 years old and 26 women with the PCO, between 21-37 years old under assisted reproductive therapy were recruited from two university hospital IVF centers during this study. The fluid from more than one preovulatory follicle was obtained on the day of ovulation for hormone measurement using the Elisa method. Association between the mentioned steroid levels and fertilization, implantation, and biochemical pregnancy rates were assessed by using spearman, Mann-Whitney, and regression statistical tests. Among the assessed outcomes, in the PCO group, the FF, E2, and T levels appeared a noteworthy reverse relationship with fertilization rate (P= 0.050). In both groups, intrafollicular levels of the E2/T ratio correlated positively with implantation rate (P=0.024 in control *vs*. P= 0.009 in PCO) and biochemical pregnancy (P= 0.020 in control *vs*. P= 0.012 in PCO). Furthermore, intrafollicular E2/P4 levels showed a positive relationship with both implantation (P= 0.047) and biochemical pregnancy (P= 0.050) rates in the control group. Our results for the first time suggest that high intrafollicular levels of E2 and T may influence reduced fertilization rate. The E2/T ratio would be a good predictor of successful implantation and pregnancy rates regardless of infertility causes. Moreover, the E2/P4 ratio could be a valuable prescient marker for implantation and biochemical pregnancy rates in normal-ovulatory women. Beyond the mentioned results, the underlying pathological processes, and pathways, including hyperandrogenism and interruptions in the hypothalamic-pituitary-ovarian (HPO) axis could be the potent concepts in polycystic ovary syndrome (PCOS) etiology and an alteration in genetic variants of SHBGs and catalytic enzymes responsible in steroidogenesis pathways. Furthermore, possible correlations between the mentioned steroid hormones and the functions of genes related to gonadotropins and steroid hormones in the pathophysiological pathways involved in PCOS were explored.

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Introduction

Polycystic ovary syndrome (PCOS) is a common disease with diverse genetic, metabolic, endocrine, and environmental aberrations that cause anovulation in women (Azziz, 2018). This syndrome has heterogeneous characteristics; thus, various mechanisms, such as elevated Estrogen levels, decreased serum progesterone and hyperandrogenism expanded Luteinizing

Hormone (LH)/ Follicle-Stimulating Hormone (FSH) ratio, menstrual irregularity, and other reproductive disorders may be implicated in its pathogenesis (De Leo *et al.*, 2016). PCOS is also related to changes in Follicular Fluid (FF) components (Niu *et al.*, 2017) which may lead to poor oocyte quality and pregnancy outcomes during Assisted Reproductive Techniques (ARTs) (Artini *et al.*, 2018).

The FF provides a significant microenvironment for oocyte growth. It is obtained from constituents of blood plasma that cross the blood follicular obstruction as well as granulosa and theca cell production (Blaschka *et al.*, 2020). Amid follicular development and advancement, the composition of FF changes particularly within the steroid substance (Marchiani *et al.*, 2020). It is sensible to consider that several components of the FF supporting the oocyte, including sex hormones may have a determinative impact on oocyte efficiency and the further prospects for fertilization and embryo development (Nagy et al., 2019; O'Brien *et al*., 2019). The characterization of the FF substances may also reflect metabolic alterations in blood serum (Forde *et al.*, 2016).

Similar to assisted reproduction, multiple studies evaluated the steroid content and content of the most viable oocyte with a greater chance of implantation success. In any case, the preovulatory FF as it were, reflects the follicle improvement near ovulation time, which could be an exceptionally energetic period in which all the changes are actuated by the gonadotropins midcycle waves for moving to the luteal stage (Andersen and Byskov, 2006). Some studies have indicated that a high level of FF progesterone (P4) (O'Brien *et al.*, 2019) (or low estradiol (E2)/P4 ratio) (Salehnia and Zavareh, 2013) is a great predictor of successive pregnancy and implantation (Enien *et al.*, 1995) and can be acknowledged as a sign of a reduction in aromatase activity linked to the final stages of follicle maturation (Revelli *et al.*, 2009). Androgens arouse follicular growth in the early stages and can increase the number of follicles in the rat ovary (Gervásio *et al.*, 2014). In addition, it seems to be a scale of reciprocal control between Follicle-Stimulating Hormone (FSH) and androgens in the course of follicle development (Fujibe *et al.*, 2019). Yet excessive FF androgen levels (testosterone (T)) are linked to poor oocyte performance, notably in the inclination to lower cleavage rates after fertilization (Sun *et al.*, 2018).

The point of this study is to reveal the connection between intrafollicular E2, P4, and T levels and the IVF-Embryo Transfer (ET) outcomes, including fertilization rate, and implantation rate, as well as biochemical and clinical pregnancy rates in patients with and without PCOS. Also, we attempted to explain the possible relationships between the pathophysiological function of the PCOS candidate genes and pathways, and our target steroid hormones.

Materials and Methods

This study was approved by the Institutional Review Board of the Vali-e-Asr Reproductive Health Research Center and Shariati University Hospital of Tehran. This prospective study was performed on a total of 26 infertile PCO patients (21–37 years of age, BMI between 17-32 kg/m^2) as a PCO group and, a control group consisting of 30 infertile normal-ovulatory women without any history of diseases influencing on gonadotropin, ovaries or sex steroid release, which all fulfilled the mentioned eligibility requirements: 1) 24-42 years of age, 2) BMI ranging 17-32 kg/m2, and 3) normal menstrual cycle duration (21-40 days. In all cases, the cause of infertility was male abnormalities, endometriosis, or no clear reason.

The PCOS diagnosis was confirmed through the association of at least two of the three following benchmarks mentioned in Rotterdam criteria 2003 for patients in the PCO group: 1) Oligomenorrhea (irregular menstrual periods) or amenorrhea (absence of menstrual periods) or other ovulary disorders, 2) biochemical and/or clinical indication of hyperandrogenism 3) more than 12 follicles in the 2- to 9-mm rang in each ovary based on ultrasound imaging and/or ovarian volume higher than 10 ml. (Rotterdam ESHRE/ASRM, 2004). It should be noted that patients with male infertility count were excluded from this group.

The IVF/ET treatment was started by taking the oral contraceptive pill (OCP) on day 3 or 5 of the cycle. On day 21, 0.5 mg of Buserelin

(Suprefact®, 1mg/cc, Aventis, Germany) was used in a daily routine until the day of human chorionic gonadotropin (hCG) administration. On day 3 of the new cycle, human menopausal gonadotropin (hMG) (Menopur® 75 IU FSH: 75 IU LH, Ferring, Germany) or recombinant follicle-stimulating hormone (Gonal-F® 75 IU, Serono, Switzerland) was injected as an ovary stimulator. The follicular growth observation, via ultrasonography, was used as a determining factor for the gonadotropin dosage. Finally, patients took 10 000 IU of hCG (Pregnyl®, Organon, Iran) when at least three follicles became >17 mm. After 34 h, the oocytes were picked.

The fluids of follicles (16–20 mm) were tenderly aspirated and frozen at -20 °C on the day of oocyte collection. In this step, the ultrasensitive linked-Enzyme immune sorbent assay (ELISA) measured E2, P4, and T (DRG, Germany). The process began with making buffered-phosphate saline (PBS) (pH 7.4) by mixing Na_2CO_3 and $NaHCO_3$ and continued with pipetting immunoglobulin-specific capture antibody (1 µg/mL) in PBS. Next, the plate was covered and incubated for 1 h at room temperature, then washed three times with 300 µL of wash buffer. For blocking the remaining protein binding sites, 200 µL of blocking buffer was added and then incubated at room temperature for 1 h. In the next step, 100 µL of the antibody solutions were pipetted in each coated well and incubated for 2 h at room temperature. The antibody solution was aspirated and washed. Next, 100 µL of the antigen (1 µg/mL) in PBS was added to each well (except antigen blanks and assay blanks) and was incubated for 2 h at room temperature. The antigen solution was aspirated and washed. The 100 µL labeled antibody which is specific to antigen (1 µg/mL) in PBS was pipetted to all the wells and incubated as mentioned before. Then the reporter-labeled detection antibody solution was aspirated and washed. In the last final steps, 100 µL of the substrate solution (Rockland Immunochemicals, #TMBE-1000) was added and incubated for 1 h at room temperature in the dark. Finally, the reaction was stopped by adding a 100 µL stop solution to all wells. In the end, the results were detected on a reader set to 450 nm (Kohl *et al.*, 2017).

Bradford assay was used to adjust the steroid ratios in the FF to its protein content to prevent any bias brought on by the FF volume fluctuation. In brief, a Bradford reagent was made in the following approach, for 2-3 hours at room temperature, 0.05% (w/v) Bradford reagent + 25% ethanol (v/v) + 42.5% phosphoric acid were combined with continual shaking. The mixture was then (mentioned reagents were all provided by Merck and Co. Inc, USA Company), and enumerating the standard curve, multiple dilutions of BSA (µg /mL) were made from a stock solution of BSA 1 mg/mL (Sigma-Aldrich Co, UK). To measure the absorbance of standard and unknown solutions by spectrophotometer, diluted FF samples (1/2000 with BSA) were used (Kielkopf *et al.*, 2020). Microsoft Excel was used to measure the protein concentration (linear regression).

To evaluate the IVF outcomes, such as biochemical pregnancy and implantation rate, the patient's medical records were used with the help of a physician. Biochemical pregnancy refers to the HCG hormone produced by the embryo. If the embryo produces enough hCG, it will be shown on an hCG blood test. This is recommended that the women get tested for pregnancy 12 days following the oocyte retrieval, 9 days after a day-3 embryo transfer, and 7 days after a blastocyst transfer to ascertain if there is a clinical pregnancy. A clinical pregnancy could be a pregnancy that's affirmed by both high levels of β-hCG and ultrasound affirmation of a gestational sac or pulse. The Mann-Whitney U test was applied to reveal group differences, and associations were analyzed using Spearman correlation coefficients. The predictive effect of the independent variables, *i.e.*, P4, E2 ,and T concentrations, and the steroid ratios in the FF on the dependent variables fertilization and implantation rates were evaluated using backward multiple linear regression analysis. The presence or absence of the PCO was taken into account as an independent variable in the regression model. The independent connection between a group of variables was investigated using multiple logistic regression analysis. The threshold for statistical significance was P.05. SPSS was used to conduct all statistical analyses (SPSS Inc., Chicago, IL).

Results

There were significantly higher amounts of Intrafollicular E2, P4, and T in the control group (P= 0.009; P< 0.001 and P= 0.013, respectively), (Table 1). In the control and PCO groups, there was a positive relationship between the FF steroids (E2, P4, and T) levels (Fig. 1). No meaningful differences in fertilization and implantation rates between the control and PCO groups were observed (data are not shown).

Table 1. The median range of Follicular Fluid (FF) levels of E2, P 4, and T in control and PCO groups.

FF (ng/g protein)	Control(n=30)	PCO (n=26)	P
E2	4.67 (0.75-72.5)	1.93 (0.68-45.77)	0.009
P4	137.68 (23.45-2206.1)	33.77 (0.29-118.49)	<0.001
T	0.01 (0.002-0.054)	0.0045 (0.001- 0.024)	0.013

P-value < 0.05 was considered significant.

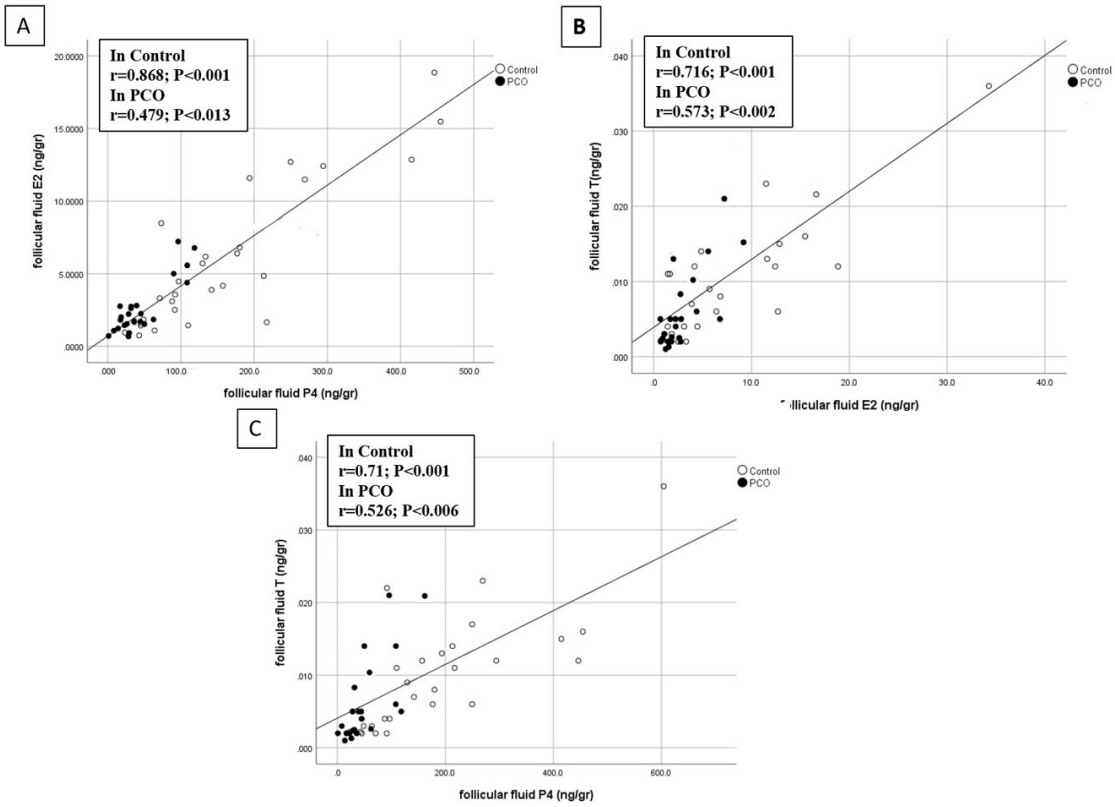

Fig. 1. Relationship between intrafollicular E2, P4, and T in control and PCO groups. A. Correlation of E2 and P4, B. Correlation of T and E2, C. Correlation of T and P4 in control and PCO groups. P-value ≤ 0.05 was considered significant. Spearman rank correlation coefficient test (SRCC).

To better understand the relationship between these hormones, the ratio of the steroids was assessed by statistical analysis. The ratio of FF E2/T was positively correlated with implantation and biochemical pregnancy rates (as discussed in the materials and method section) in the control group (r= 0.411, P= 0.024; P= 0.020, respectively) and in the PCO group (r= 0.05, P= 0.009; P= 0.012, respectively). Similarly, in the control group, E2/P4 ratios were related to implantation and biochemical pregnancy rates (r= 0.366, P= 0.047; P= 0.050, respectively). As can be seen in Fig. 2, intrafollicular E2 and T levels were negatively connected with fertilization rate (P= 0.05, P= 0.050, respectively) only in the PCO group (Fig. 2).

Backward multiple linear regression was performed, including fertilization and implantation rates as dependent variables, and hormones value comprising the presence of the

PCO as independent variables. Intrafollicular E2 and E2/T concentrations contributed positively to the implantation rate (P= 0.004, P= 0.012, respectively) (Table 2). However, intrafollicular P4 and E2/P4 levels negatively influenced the implantation rate (P= 0.003, P= 0.004, respectively) (Table 2). None of these hormones significantly affected the fertilization rate. Further to that, none of the individual variables severely altered the biochemical/clinical pregnancy rates as measured by multiple logistic regression analysis.

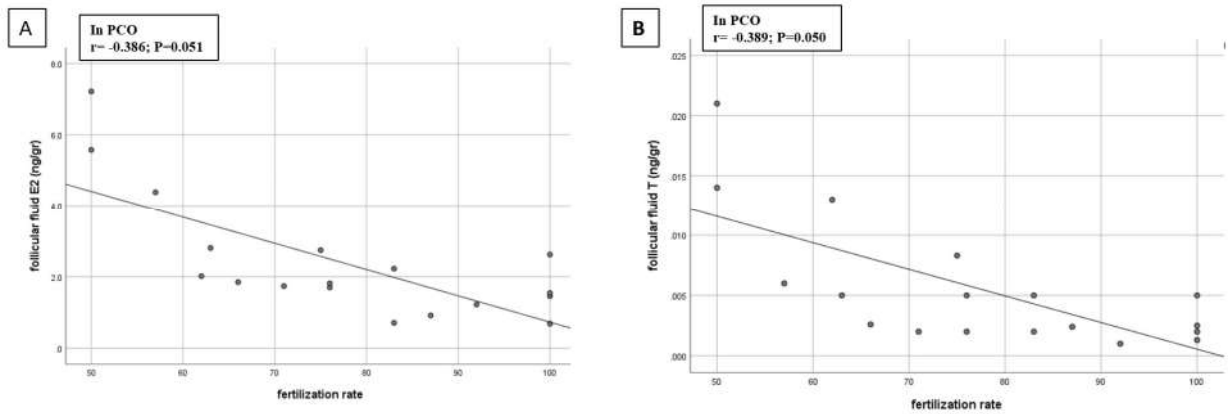

Fig. 2. Correlation of E2 (A) and T (B) with Fertilization in PCO group. P-value ≤ 0.05 was considered significant. SRCC test. *: Mann-Whitney U test.

Table 2. Multiple Linear Regression analysis of predictive value on implantation rate.

Variables	Unstandardized coefficient B	Standardized coefficient Beta	P-value
Constant	- 4.942		0.281
FF E2	3.311	1.751	0.004
FF P4	- 0.115	- 1.564	0.003
FF E2/P4	- 10.014	- 0.927	0.004
FF E2/T	0.016	0.332	0.012

P-value < 0.05 was considered significant. Backward multiple linear regression.

Discussion

In our research, we found that intrafollicular E2 concentration contributed positively to implantation rate (P= 0.004) also, E2/P4 levels negatively influenced implantation rate (P= 0.004). In this regard, a study has shown that the high levels of FF, E2, and E2/P4, reflect the later point in time of oocyte maturation and have been linked to a higher pregnancy rate (Carpintero *et al.*, 2014). However, other trials did not endorse this observation (Lv *et al.*, 2020; Wen *et al.*, 2010). Furthermore, a study in 2006 has shown that lower baseline and peak E2 levels were associated with the reduced number of retrieved and fertilized oocytes (Velasco *et al.*, 2006).

The elevated FF P4 concentration mostly resulted in the abnormal fertilization of the oocytes that usually induce multi-nuclear embryos (Ben-Rafael *et al.*, 1987). Moreover, while appropriate input to P4 had a good impact on the oocyte quality, excessive access significantly reduced the quality of the cells. It should be noted that enough knowledge of the P4 threshold that damage the oocyte is lacking (Revelli *et al.*, 2009; Sun *et al.*, 2018). Among these results, we observed that intrafollicular P4 negatively influenced implantation rate (P= 0.003). Also, we reported that intrafollicular E2/T concentration contributed positively to the implantation rate (P= 0.012). The E2/T ratio was shown to be greater in the follicles connected to pregnancy (Xia and Younglai, 2000).

Overall, it could be considered that the FF's low estrogen/androgen ratio may be related to early follicular atresia, which negatively influenced the viability of the embedded oocyte, the chance of successful pregnancy, and fertilization. Arguably, it was well accepted that follicular atresia was the result of a principally androgenic intrafollicular environment, and also the optimal amount of intrafollicular androgens was

necessary for follicular growth. Adding LH (and thus stimulating testosterone synthesis by theca cells), during IVF cycles with poor FSH response, has been shown to increase oocyte maturation and follicle growth. (Fujibe *et al.*, 2019). However, blastocyte quality and pregnancy outcomes depended on several factors such as maternal age. It has been reported that there was a significant reverse relationship between the female age and blastocyst rate (Aghajani *et al.*, 2021). Furthermore, single embryo blastomere quality could be a predictive factor in embryo transfer outcomes (Salehzadeh *et al.*, 2022).

So far, several studies from China, the USA, and Europa have identified ten locus and genetic variants associated with PCOS etiology, including 2p16.3, 2p21, 9q33.3 (Chen *et al.*, 2011), 9q22.32, 11q22.1, 12q13.2, 12q14.3, 16q12.1, 19p13.3, and 20q13.2 (Shi *et al.*, 2012; Day *et al.*, 2015; Welt *et al.*, 2012; Brower *et al.*, 2015). These genes are in general involved in the synthesis of proteins that have potential roles in the pathophysiological features of PCOS, especially in the secretion and actions of gonadotropin, and the steroid hormones. Despite the name, PCOS is thought to be caused by disrupted neuronal pathways within the brain that administer the Hypothalamic-Pituitary-Ovarian (HPO) axis (Moore and Campbell, 2016). The HPO axis is the primary regulator of female reproduction; therefore, it is rational to assume that some coded genes in this axis play a crucial role in PCOS pathogenesis, including FSHR, LHCGR (LH Choriogonadotropin Receptor) (Schüring *et al.*, 2012), GnRHR (GnRH receptor) (El-Shal *et al.*, 2016), FSHβ (Tian *et al.*, 2016) and LHβ. The main neuroendocrine abnormalities associated with PCOS and the elevation in amplitude and frequency of GnRH secretion are mirrored in the high and low concentrations of LH and FSH, respectively, in the 2/1 to 3/1 amounts (Tian *et al.*, 2016; Sheikhha *et al.*, 2007). FSH stimulates the formation of follicles in the ovary through binding to FSH receptors on granulosa cells. This is while the follicular development and ovulation do not occur when the FSH levels are low for an extended period, resulting in infertility. The ovary eventually develops tiny cysts from these immature follicles which are recognized as a sonographic hallmark of PCOS (Barzegar *et al.*, 2018). Furthermore, follicular development, steroidogenesis, and corpus luteum formation are all aided by LH (lee *et al.*, 2021) and ovulation happens as the result of an increase in LH (Carrasco *et al.*, 2020). Hence. abnormalities in GnRH secretion and hormonal imbalances can be known as the main underlying pathophysiological processes in PCOS.

Although the exact mechanisms of the abnormal increase of LH in PCOS are still unknown, several theories have been offered to elucidate the effect of peripheral hormones on the pathophysiology of PCOS. One of the most relevant hypotheses is "Hyperandrogenism" (Rosenfield and Ehrmann, 2016).

Hyperandrogenism can occur for a variety of causes, and it can interfere with the ovary's normal function. Hyperandrogenism disrupts the negative feedback of the steroid hormones by changing its crucial neuronal circuits (Moore and Campbell, 2016) and subsequently disrupting the function of the GnRH generator which reduces the function of GnRH inhibitors such as Gonadotrophin Inhibitory Hormone (GnIH) or its mammalian analog, RFRP-3 (Ubuka *et al.*, 2009). A study from Iran has shown that mRNA expression of hypothalamic RFRP-3 neuropeptide is lowered in rats after induction of PCOS through continuous light exposure (Shaaban *et al.*, 2018). However, more studies are required in this area.

Although the incidence of hyperandrogenism is still unclear, it can be presumed that there are some vicious cycles behind its emergence: a) Hypothalamic-pituitary axis disruption: The destabilization of the HPA axis can lead to an increase in LH production, which affects theca interna cells and initiates the synthesis of androgens (Roland and Moenter, 2014). b) Exposure to androgens during fetal development is another cause of hyperandrogenism, which may also lead to PCOS phenotypes in adulthood (Fenichel *et al.*, 2017). c) Alteration in genetic variants of SHBGs and catalytic enzymes responsible in steroidogenesis pathway: altering gene expression genetic variants of genes-mediated steroidogenesis pathways, including HSD17B (Jones *et al.*, 2009; Ju *et al.*, 2015), AR (Hickey *et al.*, 2002; Baculescu, 2013), CYP19A1and SHBG (Moran *et al.*, 2013), lead

to hyperandrogenism (Petry *et al.*, 2005). d) Genetic disruption in CYP19A1 gene: this gene encoded aromatase enzyme, one that transforms androgens to estrogens. As demonstrated in the PCO, reduced aromatase activity in the ovarian follicles causes androgen cumulation. A study from Greece has found links between this gene and the occurrence of PCOS (Deligeoroglou *et al.*, 2009) According to Xu *et al*. (2013), polymorphism (TTTA)n in the CYP19 gene indicated that the PCO women had shorter alleles in this tandem repeat. This might have had a huge role in inhibiting aromatase activity in patients with the TC genotype of heterozygous CYP19 gene (Xita *et al.*, 2010). Some other studies have also claimed that the SNP rs2414096 in the CYP19 gene may be related to hyperandrogenism by reducing aromatase activity (Jin *et al.*, 2009; Mehdizadeh *et al.*, 2017; Mostafa *et al.*, 2016). Hence this gene seems to be valuable for further studies in PCOS.

Conclusion

In brief, our results suggest that high intrafollicular levels of E2 and T may influence the reduced fertilization rate. They can be negative predictors of fertilization rate, as an IVF outcome. The E2/T ratio would be a good predictor of successful implantation and pregnancy rates regardless of infertility causes. Moreover, in normal-ovulatory women, the E2/P4 ratio could be a valuable prescient marker for implantation and biochemical pregnancy rates. These hormonal changes in the FF can reflect disorders in the genetic pathways of the HPO axis. However, the underlying pathological processes and pathways before these emergencies in PCOS patients are essential to construct new outlooks for future investigation and studies. Therefore, we have attempted to collect new data in correlation with our results.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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