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PREDICTIVE VALUES OF DHEAS, TT AND IGF1 IN SUCCESSFUL PREGNANCY OUTCOME OF PATIENTS UNDERGOING IVF/ICSI-ET

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ABSTRACT : Clinical index is needed to predict the successful pregnancy after *in vitro* fertilization/ intracytoplasmic sperm injection-embryo transfer (IVF/ICSI-ET) for infertile patients. Insulin like growth factor 1 (IGF1), Dehydroepiandrosterone sulfate (DHEAS) and Total Testosterone (TT) may have a role on follicle growth, oocyte quality, embryo quality and subsequent pregnancy in patients undergoing IVF cycles. To explore the possibility of using DHEAS, TT and IGF1 as predictive indicator for successful pregnancy in patients undergoing (IVF/ICSI-ET). A prospective study was performed enrolling (12) non pregnant control group, (7) pregnant control group, (18) non-pregnant PCOS group and (12) pregnant PCOS group. The collection of blood and follicular fluid (FF) samples was done at the day of oocyte aspiration. Electrochemiluminescence immunoassay (ECLIA) method was used to measure DHEAS levels, Chemiluminescence immunoassay (CLIA) method was used to measure TT levels and competitive enzyme-linked immunosorbent assay (ELISA) was used to measure IGF1 levels. Mean of FF DHEAS, serum DHEAS, FF TT and FF IGF1 were higher in pregnant control group than those of non-pregnant control group. The differences were statistically significant (except for serum DHEAS). Additionally, mean of FF DHEAS, serum DHEAS, FF TT, and FF IGF1 were statistically significant lower in pregnant PCOS group than those of non-pregnant PCOS group. Correlation analysis of control group revealed positive association between FF DHEAS and serum DHEAS levels with fertilization rate and cleavage rate. Correlation analysis of PCOS group found negative association between FF TT and M II oocyte, positive association between FF IGF1 and serum DHEAS and positive association between serum DHEAS and FF TT. In control group and PCOS group, ROC analysis indicated that FF DHEAS is a good marker for predicting pregnancy, followed by FF TT. Intrafollicle DHEAS and TT has a predictive use for the successful pregnancy in both of control and PCOS women.

Key words : Dehydroepiandrosterone sulfate, *in vitro* fertilization, oocyte, follicular fluid, intra-cytoplasmic sperm injection.

INTRODUCTION

Assisted reproductive techniques (ART) seek to achieve largest possible number of oocytes of good quality (Macklon *et al*, 2006). *In vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are widely used techniques to solve human infertility. These techniques provided great benefits for couples, who have struggled with infertility disorders (Lu *et al*, 2013).

Follicular fluid (FF) is biological microenvironment that promotes the growth of the oocyte and the following embryo. FF is the product of theca cells and granulosa cells secretion that encircle the follicular wall. The content of FF includes different substances such as hormones, cytokines, growth factors, and antioxidants. These mediators may a have a direct effect on oocytes quality and maturation ability (Revelli *et al*, 2009).

The insulin like growth factor (IGFs) family have important role in initiation of follicle growth (Franks *et al*, 2008) and affecting mammalian embryo development (Miese-Looy *et al*, 2011).

In the ovaries, growth hormone binds to growth hormone receptors found on granulosa, luteal, theca cells. This supports the process of steroidogenesis and gametogenesis. The production of growth hormone induces the liver to synthesize IGF1. The IGF1 and growth hormone releasing hormone increase ovarian sensitivity to gonadotropin stimulation, which in turn affects the follicle marturation and the gamete (Kingsberg Medical, 2018).

The stimulatory effect of gonadotropins depends on the expression of IGF1 receptor in granulosa cells of human, rat and mouse (Zhou *et al*, 2013).

Theca cells control granulosa cells proliferation by enhancing IGF1 expression (Shiomi-Sugaya *et al*, 2015). Moreover, locally produced IGF1 may affects the mechanisms of folliculogenesis, inducing a larger number

of follicles to grow (Br¹ zert *et al*, 2015).

Dehydroepiandrosterone (DHEA) is the most considerable circulating steroid hormone in human (Barrett *et al*, 2012). The DHEA is converted to T in connective tissue (stroma/theca) of the ovary, and processed by the granulosa cells to give E_2 (Mo *et al*, 2006). However, DHEA may considered a key molecule at crossroads, directing a critical balance between estrogen and androgen production; as well as, affecting oocyte maturation (Chimote *et al*, 2015).

The DHEA is converted reversibly to the sulfate ester DHEA sulfate (DHEAS) by sulfotransferase (NCBI, 2018). The DHEAS is also generated by the adrenal cortex (Miller *et al*, 2011).

Chimote *et al* (2015) improved that FF DHEAS level correlated positively with oocyte maturation process and is predictive of fertilization, embryo growth to blastocyst stage and live birth rates.

Similarly, other researchers improved that DHEAS could be predictor for pregnancy in younger women after the first IVF cycle (Alebiæ*et al*, 2013).

The T is synthesized in smaller amounts in women by the adrenal glands, theca cells of the ovary and by the placenta during pregnancy (Neal, 2016). Higher FF T levels may negatively affects IVF outcomes, by reducing the expression of aromatase in luteinized granulosa cell from PCOS women with Yang *et al* (2015).

By contrast, the association between increased live birth and pregnancy rates and use of transdermal T has been described in patients with low ovarian response (Jeve *et al*, 2016).

This study aimed to explore the possibility of using DHEAS, TT and IGF1 as predictive indicator for successful pregnancy in patients undergoing IVF/ICSI-ET.

MATERIALS AND METHODS

Subjects

A prospective case control study conductedat Kamal Al-Samarai IVF Hospital in Baghdad, Iraq, from December 2017 to June 2018. The medical ethics committee in University of Baghdad approved this study protocol. All patients signed a written informed consent.

The study included the following groups:

Non pregnant control group (n = 33) and pregnant control group (n = 7), non-pregnant PCOS group (n = 28) and pregnant PCOS group (n = 12).

Therefore, the number of non-pregnant women were selected as close to the number of pregnant women to be statistically acceptable for comparison; hence, non-pregnant control group was selected to be (n = 12) and non-pregnant PCOS group was selected to be (n = 18).

Control group included women with male cause infertility group.The diagnosis of PCOS was according to the revised Rotterdam European Society of Human Reproduction and Embryology/American Society for Reproductive Medicine Criteria (Rotterdam, 2004).

Exclusion criteria : women laparoscopically diagnosed with endometriosis, poor ovarian reserve, endocrine disorders (such as hyperprolactinemia and thyroid dysfunction), pathologies of ovarian or fallopian tubes (*e.g.* Adenomyosis, Hydrosalpinx) and women with severe pelvic adhesions.

Controlled ovarian hyperstimulation, oocyte collection and sampling

All subjects were hyperstimulated by gonadotropin releasing hormone (GnRH) antagonist protocol. Administration of 150-225 IU of recombinant FSH (Gonal-F®) injection from day two of menstrual cycle. GnRH antagonist (Cetrorelix) is given (0.25 mg) daily when the follicle reach (12-14mm), as detected by ultrasound. Cetrorelix and Gonal-F® are continued together until either two or three follicles reach 17-18 mm in the ovary. Then, ovulation induction using recombinant human chorionic gonadotropin administration (rhCG 6500 IU, Ovitrelle®; Merck Serono, Italy) was done.

Oocytes were picked up after 34-36 hours from hCG injection using needle aspiration with a transvaginal ultrasound transducer guidance. Uncontaminated FF samples were centrifuged at 3000xg for 10 min at room temperature. Venous blood were withdrawn at the day of oocyte aspiration and allowed to clot in a gel tube and then centrifuged. Serum and FFsamples were stored in sterile eppendorftubes at -20^{0}C until use.

Assessment of oocyte morphology and oocyte maturation

Maturation of oocytes was dictated by the identification of the first polar body. Oocytes morphology was assessed by counting the oocyte maturation status (metaphase II (MII) oocyte) (Xia, 1997; Rienzi *et al*, 2008).

ICSI procedure, assessment of fertilization and embryonic development

After visualization of the oocyte-cumulus complex in the FF, each oocyte was maintained at 37ºC in culture medium with proper pH using 6% CO_2 in air through all steps. After 1-2 hour of oocyte retrieval, the denudation

was performed by exposure to buffered medium containing 80 IU/ml hyaluronidase to enhance the enzymatic removal of corona cells and cumulus. The oocytes were aspirated in and out of a Pasteur pipette, then they were rinsed several times and incubated for ICSI (Palermo *et al*, 1995). The denuded oocytes were examined for oocyte maturation.

Fresh or frozen sperms were collected at time of oocytes picked up by masturbation, sperm aspiration from the testes by fine needle aspiration or testicular biopsy.

ICSI was performed 3-5h after oocyte aspiration by choosing mature MII oocytes, which had a polar body (Xia, 1997; Rienzi *et al*, 2008).

Fertilization results were evaluated by appearance of two pronuclei and two polar bodies. Cleavage was done (24 hours after fertilization). Embryo transfer was done on day 2 of embryonic development with assisted hatching or without, mostly two or three embryo were transferred depending on the recommendation of the couple and the quality of the embryos. Embryos were graded morphologically as recommended by Palermo *et al* (1995). All these steps were done by a clinical embryologist in charge at test tubes babies'laboratory in Kamal Al-Samarai Hospital.

Support of the luteal phase was performed by injecting 1500 IU hCG immediately after oocyte retrieval. Additionally, vaginal administration of 200mg of micronized progesterone, three times a day (was started in the evening after oocyte pickup) (Fatemi *et al*, 2013) until pregnancy test day. Pregnancy test using serum hCG assay was performed on day 12 after embryo transfer.

IVF Outcomes

The percentage ratio of oocyte maturity rate, cleavage rate and fertilization rate were calculated.

Oocyte maturation rate: Total no. of mature oocytes/ total no. of all oocytes (Trounson *et al*, 1998).

Cleavage rate : Total no. of informed embryos/Total no. of fertilized oocytes (Greenblatt *et al*, 1995).

Fertilization rate : Total no. of zygotes (2 pro nucleus)/ Total no. of mature oocytes MII (Jiaen *et al*, 1995).

Measurement of DHEAS

DHEAS levels were measured by Electrochemiluminescence immunoassay (ECLIA), using Competitive immunoassay method according to the manufacture's protocol from (Roche, Switzerland). The intermediate precision is 2.4–4.7%.

Measurement of TT

TT levels were measured by Chemiluminescence Immunoassay (CLIA), the precision is within run 3.2% and total 7.9%.

Measurement of IGF1

Human IGF1 levels were measured by human IGF1 ELISA Kit, using competitive ELISA method according to the manufacture's protocol from (Demeditec, Germany). The intra-assay and the inter-assay is CV 6.62% and CV 7.79%, respectively.

Statistical analysis

Data analysis was done by utilizing SPSS for Windows, version 17(SPSS Inc. Chicago, IL, United States). Data were appeared as mean ± standard deviation. Statistical analysis performed by Independent sample's T-test. The association degrees between variables were analyzed by Pearson correlation analysis. In addition, ROC used to evaluate the AUC. The best cut off point of the studied parameters, sensitivity, specificity, PPV and NPV were also calculated. A p value less than 0.05 was considered statistically significant (Glover *et al*, 2008).

RESULTS

Comparisons of the studied parameters between pregnant and non pregnant groups

In control pregnant group, the mean of FF & serum DHEAS, FF TT and FF IGF1 were higher than those of non-pregnant group. The differences were statistically significant ($P< 0.05$) (except for serum DHEAS $p > 0.05$) (Table 1).

In PCOS pregnant group, the mean of FF & serum DHEAS, FF TT and FF IGF1 were statistically significant ($P< 0.05$) lower than those of non-pregnant group (Table 2).

Pearson correlation analysis of the studied parameters with IVF outcomes of control group and PCOS group

In the present investigations, it was found that FF DHEAS and serum DHEAS levels associate directly with fertilization rate (Fig. 1A) and cleavage rate (Fig. 1B).

The present study displayed a negative relationships between FF TT and M II oocyte (Fig. 2A) and a positive association between IGF1 level in FF and DHEAS level in serum of PCOS women (Fig. 2B) and a positive association between serum DHEAS and FF TT level (Fig. 2C).

Receiver operating characteristics (ROC)

The above data showed that the parameters (except serum DHEAS in control group) have significant P value in relation to pregnancy statue in control group and PCOS

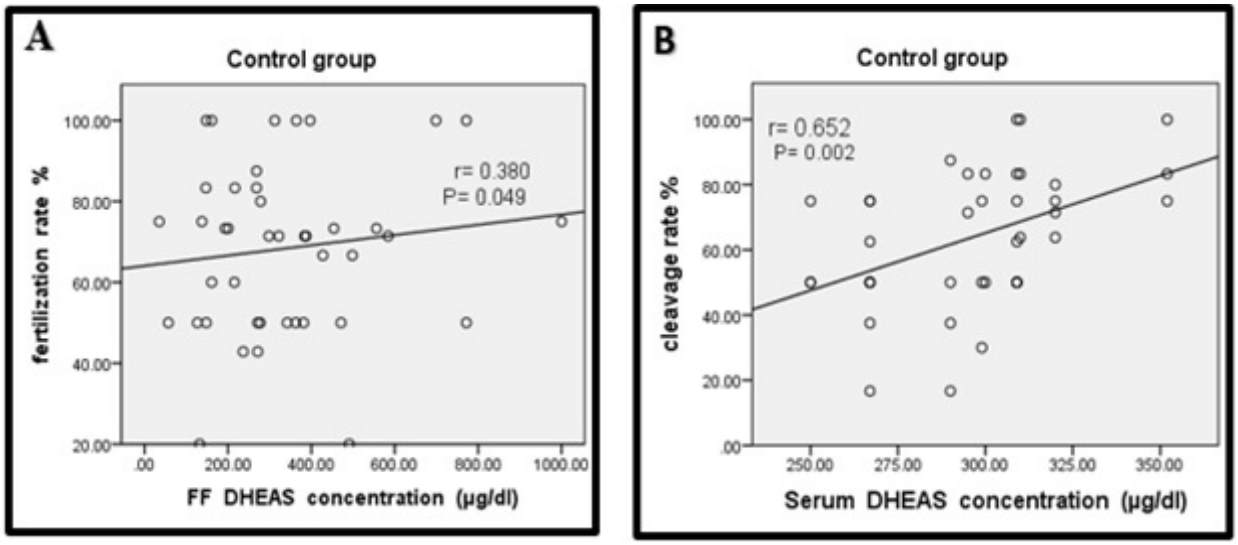

Fig. 1 : The relationship between: A. FF DHEAS and fertilization rate, B. serum DHEAS and cleavage rate in control group.

Table 1 : Comparisons of the studied parameters between pregnant and non pregnant control groups.

Parameters	Non pregnant group (n=12)	Pregnant group (n=7)	*P* value
FF.DHEAS (µg/dl)	224.05±70	328.56±112.0	0.015
S.DHEAS (µg/dl)	285.1±42.5	316.8±50.2	0.152
FF. TT (ng/ml)	3.17±0.78	4.30±1.38	0.018
FF. IGF1 (ng/ml)	147.76±40.88	185.84±34.75	0.049

Table 2 : Comparisons of the studied parameters between pregnant and non-pregnant PCOS groups.

Parameters	Non pregnant group (n=18)	Pregnant group (n=12)	*P* value
FF.DHEAS (µg/dl)	379.8±90.36	300.0±109.0	0.038
S.DHEAS (µg/dl)	388.5±60.5	330.7±55.8	0.037
FF. TT (ng/ml)	9.21±2.2	6.52±2.8	0.031
FF. IGF1 (ng/ml)	201.5±40.3	163.3±33.4	0.020

Table 3 : The ROC curve data of the studied parameters in control group.

Parameters	AUC	*P* value	Cut off	Specificity%	Sensitivity %	PPV%	NPV%
FF.DHEAS	0.79	0.039	268.5 (µg/dl)	71.4	76.5	28.6	23.5
FF. TT	0.77	0.05	3.8 (ng/ml)	60	63	40	37
FF. IGF1	0.75	0.06	170.5 (ng/ml)	66.7	66.7	33.3	33.3

AUC = area under the curve, PPV = positive predictive value, NPV = negative predictive value.

Table 4 : ROC curve data of the studied parameters in PCOS group.

Parameters	AUC	*P* value	Cut off	Specificity%	Sensitivity %	PPV%	NPV%
FF.DHEAS	0.83	0.003	301.0 (µg/dl)	90	91	10	9
S. DHEAS	0.73	0.08	362.5 (µg/dl)	57.1	57.9	42.9	42.1
FF. TT	0.80	0.045	7.4 (ng/ml)	80	75	20	25
FF. IGF1	0.78	0.016	167.6 (ng/ml)	66.7	70	33.3	30

AUC = area under the curve, PPV = positive predictive value, NPV = negative predictive value.

group, so the area under curve (AUC) and predictive cut off value for pregnancy of these parameters was next analyzed. Furthermore, the sensitivity, specificity, PPV, and NPV for these parameters was also analyzed (Tables 3 and 4).

In control group, ROC analysis indicated that the FF DHEAS is a good marker for predicting pregnancy. The rest parameters showed less sensitivity in predicting pregnancy.

In PCOS group, ROC analysis indicated that the FF DHEAS is a good marker for predicting pregnancy, followed by FF TT. The rest parameters showed less sensitivity in predicting pregnancy.

DISCUSSION

Comparisons of the studied parameters between pregnant and non pregnant groups

The present study findings about DHEAS level in FF and serum of control pregnant group *vs*. control non-pregnant group confirms the previous observation of Chimote *et al* (2015) whom they showed that patients with higher FF DHEAS level have higher percentage of

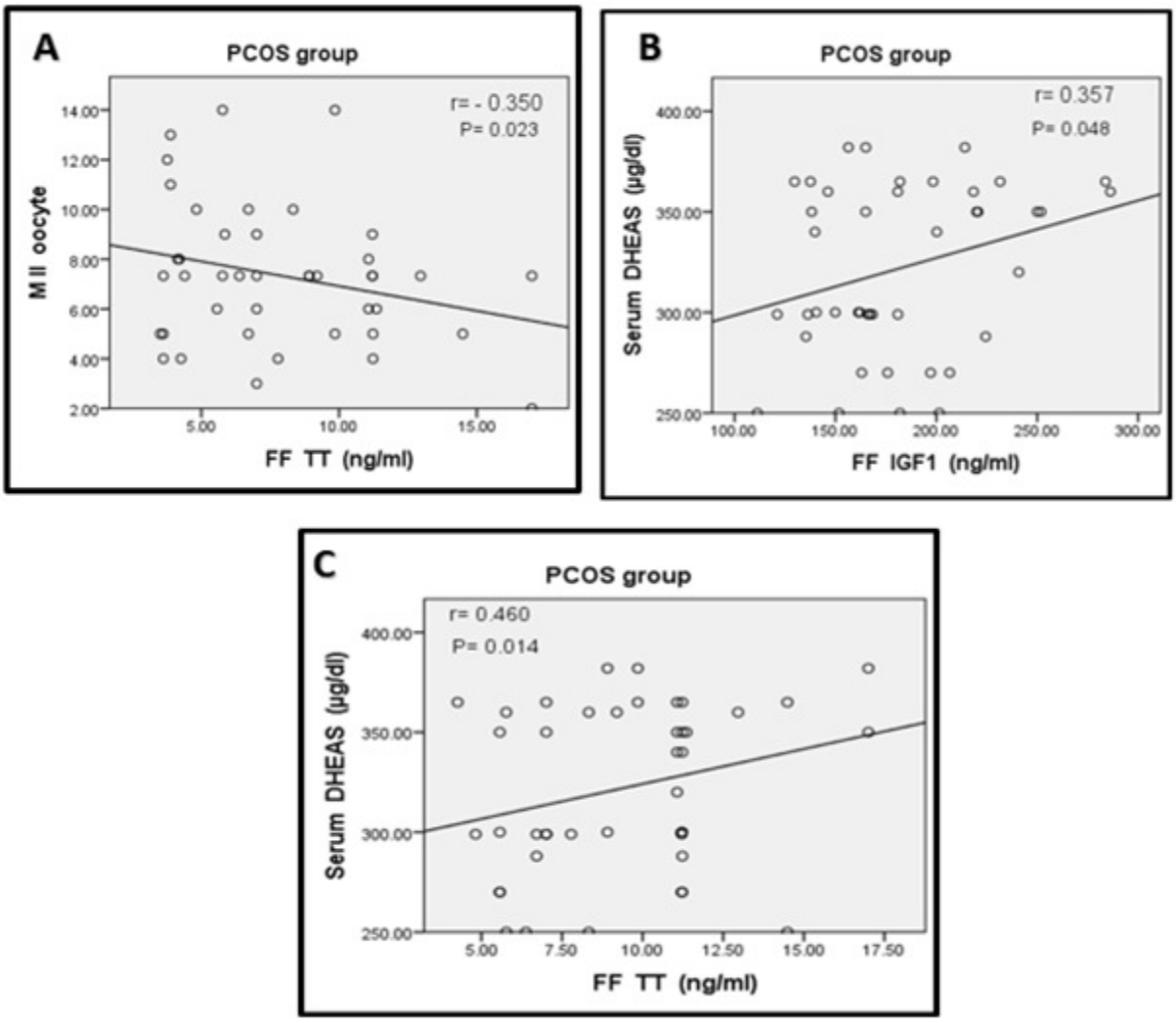

Fig. 2 : The relationship between: A. FF TT and M II oocyte, B. serum DHEAS and FF IGF1, C. serum DHEAS level and FF TT level in PCOS group.

'clinical pregnancy rate' and 'live birth rate' compared to that with lower DHEAS level. They demonstrated that DHEAS level in FF may influence oocyte maturation, as well as, predictive of fertilization rate, embryonic development to blastocyst stage and live birth rates in non PCOS patients undergoing IVF. The possible interpretation of this result is that DHEAS may act as the primary regulator of estrogen formation; as well as, the utilization of DHEAS as precursor for estrogen production (McEvoy, 2012).

A previous study by Wiser *et al* (2010) showed improvement in embryo quality and higher live birth rate in diminished ovarian reserve patients treated with DHEA supplementation, recombinant FSH and LH administration during IVF cycles.

The DHEA is precursor to androgens and a prohormone for FF T (Haning *et al*, 1991). They speculated that the better IVF outcomes of DHEA supplementation group were derived from the synergistic effect of both DHEA and recombinant LH that increase intrafollicular androgen production (Wiser *et al*, 2010).

The present study showed that FF TT level were higher in control pregnant group compared to control non-pregnant group.

This result confirms other study (Carpintero *et al*, 2014), whom they stated that higher FF TT level in 'normal fertilized patient' group compared to 'failed fertilization patients' group with unknown infertility or male infertility.

The conversion of androgens to estrogens is favored by FSH. FF with higher androgen levels would also have increased levels of estrogen. The predominant follicular androgenic environment can lead to follicular atresia although a certain amount of follicular androgens are necessary for optimal follicle growth (Nielsen *et al*, 2010; Carpintero *et al*, 2014).

Other study displayed that serum T level of pregnant women at both of third day of IVF and fourteenth day

after ET were higher than that of non-pregnant women. They demonstrated that serum T level on fourteenth day of ET can predict successful IVF cycles. They expected regulating androgen levels may improve pregnancy rates (Hossein Rashidi *et al*, 2009).

The present study and others (Mehta *et al*, 2013; Faraj *et al*, 2017) revealed that FF IGF1 level were higher in control pregnant group compared to control non-pregnant group.

The increased IGF1 concentrations in FF can modify the regulatory system of ovarian folliculogenesis to enable development of more preantral follicles, maintenance of larger pools of small antral follicles, recruitment of more follicles within the cohort of developing follicles, and selection of two or more dominant follicles within a follicular wave. This interpretation could explain how FF IGF1 could influence on the folliculogenesis, hence on the pregnancy rate in the future (Genc *et al*, 2011).

By contrary to the DHEAS levels of control group, the present study showed that DHEAS level in FF and serum were lower in PCOS pregnant group compared to PCOS non-pregnant group.

The depletion in FF and serum DHEAS level in pregnant PCOS women compared to that of non-pregnant PCOS women, might be explained depending on steroid synthesis pathway in FF from PCOS (Naessen *et al*, 2010), may be due to increase both of 3βHSD activity and CYP19 aromatase activity in pregnant PCOS women.

The present study showed that FF TT level were lower in PCOS pregnant group compared to PCOS non-pregnant group (this finding may be strengthen by the negative association between FF TT with MII oocyte in PCOS group, which mean that increase FF TT in non-pregnant PCOS may decrease MII oocyte and may in turn, decease pregnancy rate).

The depletion in FF TT level in PCOS pregnant women compared to that of PCOSnon-pregnant women, might be may be interpreted as increase CYP19 aromatase activity and reduce 17HSD3 activity in pregnant PCOS women (Naessen *et al*, 2010).

The present study displayed that FF IGF1 level were lower in PCOS pregnant group compared to PCOS non-pregnant group. The IGF1 and its binding protein have been linked to androgen production in PCOS patients (Carmina *et al*, 1999).

In the current study, higher FF IGF1 in PCOS non-pregnant group, may increase hyperandrogenisim. Indeed, this was proven by significant increase in FF DHEAS, serum DHEAS, FF TT in nonpregnat group; as well as, the positive association between FF IGF1 and serum DHEAS in PCOS group.

It has been suggested that insulin may induce ovarian hyperandrogensim in the face of insulin resistance by binding to the IGF1 receptor (Medscape, 2009). Although, the present study did not analyze serum Insulin levels, but it can speculate that non-pregnant group of PCOS women where under insulin resistance, which may in turn decrease pregnancy rate (Refaie *et al*, 2005).

Pearson correlation analysis of the studied parameters with IVF outcomes of control group and PCOS group

The present study findings regard DHEAS level in FF and serum of pregnant group *vs.*non-pregnant group of control group, may be strengthen by the positive association between control group serum DHEAS and FF DHEAS with cleavage rate and fertilization rate, respectively.

The possible interpretation is that DHEAS convert to DHEA in ovary by the action of granulosa cell sulphatase (Burger, 2002); indeed, the converted DHEA may improve IVF outcomes. DHEA affects functional ovarian reserve, oocyte quality, embryo quality and improve pregnancy outcomes in women with diminished ovarian reserve (Gleicher *et al*, 2011; Gleicher *et al*, 2013). Moreover, DHEA converts to T and E_2, therefore, can affects the process through androgen receptor or estrogen receptors (Engdahl *et al*, 2014). It was stated that T levels after DHEA supplementation are associated with IVF pregnancy chances (Weghofer *et al*, 2012).

Cappel *et al* (2005) revealed direct relationship between serum IGF1 and DHEAS in adult women with clinical acne. They stated that higher IGF1 levels and the androgen may affect acne in adult women; as well as, IGF1 appears to have a stronger effect than DHEAS on acne in women.

Similarly, IGF1 level was increased as a result of DHEAS administration to postmenopausal women (Genazzani *et al*, 2001).

By contrast, the publication of Szczuko *et al* (2016) which studied concentration of tumor necrosis factor alpha and IGF1 in woman with PCOS phenotypes based on androgen levels. They found no relashionship between IGF1 and DHEAS levels in both of hyperandrogenic PCOS women and normoandrogenic PCOS women; while, they found a positive relationship between IGF1 with LH level and a negative relationship between tumor necrosis factor alpha with DHEAS level in hyperandrogenic PCOS women group. They demonstrated that inflammatory state involving tumor

necrosis factor alpha in women with hyperandrogenic PCOS is caused by massive progesterone pathway and restricted DHEA pathway of T biosynthesis.

Vendola *et al* (1999) reported that androgens treated rhesus monkeys resulted in increased ovarian IGF1 and IGF1 receptor mRNA in granulosa and theca cells. They suggested that androgen may induce ovarian follicular growth and theca interstitial growth throughIGF1 and its receptor.

DHEAS is derived from the adrenal gland (exclusively) with essentially none coming from the ovary (Haning *et al*, 1991); whereas, circulating DHEA originates from the adrenal zona reticularis, ovarian theca, and peripheral conversion of DHEAS (Burger, 2002).

In the present investigations, although there was no significant relationship between serum and FF DHEAS concentration, but it was shown that DHEAS concentration in serum is higher than that in FF of control group and PCOS group. This observation confirmed by Haning *et al* (1991) in that intrafollicular DHEAS arises (exclusively) by transport into the follicles from the peripheral circulation.

Moreover, plasma DHEAS accounts for intrafollicular T and DHEA, which is also a precursor of androstenedione and T. Therefore, both serum DHEA and DHEAS appear to be important for follicular androgen. To our knowledge, utilization of DHEAS by granulosa cellsoccur via sulfatase activity (Burger, 2002). This relationship support the hypothesis that DHEAS function as a precursor of steroid production, especially T ovarian production (Burger, 2002).

The present study displayed negative relationships between FF TT and both of serum FSH level (data not shown) and M II oocyte.

These relationships can be interpreted by the excessive ovarian androgen production, which is a major pathophysiological feature of PCOS. The basis for androgen overproduction has been attributed to altered theca cell responsiveness to gonadotropin stimulation in association with increased pituitary LH secretion in women with PCOS and this along with low levels of FSH contributes to poor oocyte development and the inability to ovulate (Ehrmann, 2005).

It is concluded from the results of current study that intrafollicle DHEAS and TT has a predictive use for the successful pregnancy in both of control and PCOS groups.

It's worth mentioning that, DHEAS, TT and IGF1 may depend on the etiology of infertility, since the (elevation/depletion) in their levels (in pregnant *vs*. non-pregnant) clearly differentin control group than PCOS group.

CONCLUSION

Intrafollicle DHEAS and TT has a predictive use for the successful pregnancy in both of control and PCOS women.

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