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RESEARCH ARTICLE

THE EFFECT OF DIFFERENT OVARIAN STIMULATION PROTOCOLS ON MULTIFUNCTIONAL FETUIN_A CONCENTRATION IN THE SERUM AND FOLLICULAR FLUID OF WOMEN WITH PCOS UNDERGOING ICSI/IVF.

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Abstract

Objective: To measure the concentration of fetuin-A in serum and follicular fluid of patients with PCOS at day of ova pick up after using of different IVF/ICSI protocols and to assess the pregnancy outcome.

Study design: A total of 90 infertile women (55 women with Polycystic ovary syndrome and the other 35 women as a control (non PCOS) undergoing controlled ovarian hyperstimulation for intracytoplasmic sperm injection cycle were prospectively recruited for this study in High Institute of Infertility Diagnosis and Assisted Reproductive Technology / AL-Nahrian University and Kamal AL-Samarai Hospital, center of fertility and IVF (Baghdad/Iraq) during the period from December 2015 to the end of April 2017.The fifty five PCOS women were divided into three groups: twenty five (25) infertile women have had PCOS undergoing long Agonist protocol for ICSI cycle, fifteen (15) infertile women have had PCOS undergoing Antagonist protocol for ICSI and fifteen (15) infertile women have had PCOS undergoing short Agonist protocol for ICSI. The controlled group included 35 women were free of signs and symptoms of PCOS with regular cycles and no endocrine abnormalities undergoing ICSI cycle (15 infertile women underwent long agonist protocol, 10 women underwent short agonist and 10 women underwent antagonist protocol). In all patients, serum and follicular fluid fetuin-A levels were measured on the day of oocyte retrieval by using Enzyme linked immuno sorbent assay.

Results: There was significant difference ($p<0.05$) in the level of fetuin-A in follicular fluid between PCOS and control group. Follicular fluid fetuin-A level was high in non PCOS patients than PCOS patients but no significant difference ($p>0.05$) in the level of fetuin-A in serum between PCOS and control group. Also there was no significant difference in the effect of different IVF/ICSI protocols (Long agonist, Antagonist, Short agonist) in the level of follicular & serum fetuin-A of patients with PCOS ($P>0.05$) and no significant difference ($P>0.05$) in level of serum and follicular fluid fetuin-A between pregnant &non pregnant PCOS patients.

ž **Conclusion:** It was concluded from the present study that FF fetuin- A concentration of PCOS patients at the day of oocyte retrieval is significantly lower than that of non PCOS patient. But no significant

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- difference in S-Fetuin A concentration between PCOS and non PCOS patients, no significant difference in the effect of different IVF protocols on serum and follicular Fetuin A and no significant difference in S-FA and FF-FA between pregnant and non- pregnant PCOS patients underwent IVF protocols.

Introduction:-

Polycystic ovary syndrome (PCOS) is one of the most common endocrinopathies and metabolic abnormalities, affect 10–15% of women of reproductive age was initially described by Stein and Leventhal in 1935, characterized by ovulatory dysfunction, biochemical and/or clinical hyperandrogenism including hirsutism, alopecia, obesity and polycystic ovarian morphology including the presence of hyper vascularized androgen secreting stroma and enlarged ovaries with multiple small follicles 2-8 mm in diameter[1]. It may have complex effects on the ovulation, oocyte quality and the endometrium contributing to a lower fertility potential [2]. Fifty to seventy percent of women with PCOS are frequently associated with insulin resistance accompanied by compensatory hyperinsulinaemia and obesity. Insulin resistance is thought to play an important role in the aetiology of PCOS [3].

Fetuin-A (alpha2-Heremans-Schmid glycoprotein (AHSG)), is a carrier protein like albumin and belongs to the fetuin family and a negative acute phase reactant. It is more abundant in fetal than adult circulation[4] and synthesized in the liver secreted into serum processed from a single chain precursor to the mature circulating two-chain form by proteolytic processing in the Golgi apparatus to form a light and a heavy chain consisting of 27 and 321 amino acids, respectively. The mature form of fetuin-A has three domains so this complex structure of fetuin-A suggests multiple biological functions. Fetuin-A it is a natural inhibitor of insulin receptor autophosphorylation and tyrosine kinase activity in the skeletal muscles and liver [5]. It has been found to be one of the important factors involved in the pathogenesis of impaired insulin sensitivity, and thereby a blocker of insulin signal transduction and resulting in insulin resistance in these target tissues[6]. Its circulating levels show negative correlation to the insulin. AHSG is thought to play a significant role in weight gain, fat accumulation and the development of type 2 diabetes [7, 8].

Subject, Material and methods:-

A prospective case control study conducted in High Institute of Infertility Diagnosis and Assisted Reproductive Technology / AL-Nahrian University and Kamal AL-Samarai Hospital, center of fertility and IVF (Baghdad/Iraq) during the period from December 2015 to the end of April 2017.Ninety infertile couples (PCOS and controlled) have been enrolled in this study and enter their ICSI cycle. Informed consent of all patients was taken before inclusion in the study. All infertile couples were subjected to a full history taking, complete general and gynecological examination and full infertility investigations including: husband's seminal fluid analysis, hormonal assay, trans-vaginal ultrasound and hystrosalpingography for evaluation of uterine cavity and tubal patency and/or laproscopy for evaluation of tubal patency and exclusion of pelvic pathology. Fifty five women (55) with PCOS were included selected from those who undergoing COH for ICSI cycle. These fifty five women were divided into three groups: I. twenty five (25) infertile women have had PCOS undergoing long Agonist protocol for ICSI cycle. II. Fifteen (15) infertile women have had PCOS undergoing Antagonist protocol for ICSI. III. Fifteen (15) infertile women have had PCOS undergoing short Agonist protocol for ICSI. The diagnosis of PCOS depend on fulfilling at least two of three criteria based on the Rotterdam ESHRE/ASRMS sponsored PCOS consensus workshop group [9] (Rotterdam 2004). Rotterdam's criteria of PCOS which was based on: 1- Typical picture of polycystic ovaries on ultrasonography (ten or more follicles in each ovary, each follicle measuring 2-9 mm in diameter).2- Anovulation or Oligo-ovulation.3- Hyperandrogenism; clinical or biochemical. Clinically hyperandrogenism manifested as a hirsutism, acne and biochemically as elevated serum testosterone level.

The controlled group included 35 women were free of signs and symptoms of PCOS with regular cycles and no endocrine abnormalities undergoing ICSI cycle (15 infertile women underwent long agonist protocol, 10 women underwent short agonist and 10 women underwent antagonist protocol) (13 with male factor infertility, 10 with unexplained infertility, and 12 with tubal infertility). The average age of women enrolled in this study ranged between 17 and 40 years had primary infertility (78 infertile couples) and secondary infertility (12 infertile couples) with duration between 2 and 15 years

First group of PCOS patients(25) were enrolled in long protocol type of IVF/ICSI cycle, an ultrasound examination was performed in order to exclude those women with ovarian cyst and assess the endometrial thickness and started on day 21 of the previous menstrual cycle (mid-luteal) with a daily administration of subcutaneous injection of GnRH-a, triptorelin (Decapeptyl®; 0.1 mg Ferring Co, Kiel, Germany)® for pituitary down-regulation and desensitization and continue till the day of HCG administration. When pituitary down-regulation was achieved (menstruation occur, E2 level reaching < 50 pg/ml and endometrial thickness was ≤ 2-3 mm on ultrasound examination [10] ovarain stimulation started with recombinant human follicle stimulating hormone (rhFSH) (Gonal F, Merck Serono® 75 IU of FSH activity per ampoules) by daily subcutaneous injection in a dose of 150-225 IU depending on the women's age and previous response of ovulation induction. The follicle growth and the doses of Gonal-F® were monitored by trans-vaginal ultrasound (cycle day 5 and subsequent scan were done every 2-3 days as required) and by serum E2 level (day 6-8 of (Gonal-F®) injection and till the day of hCG administration) [11]. When either two or three lead follicles have reached 17-18 mm ovulation induction was induced by the administration of recombinant hCG (rhCG 6500 IU, Ovitrelle®; Merck Serono, Italy) subcutaneously [12]. Second group (15) infertile couples in whom PCOS women were undergoing antagonist protocol, started daily administration of 150-225 IU of (Gonal-F®) injection subcutaneously from day two of menstrual cycle. GnRH antagonist (Cetrorelix) is usually given in a dose of 0.25 mg daily when the leading follicle reaches a certain size by ultrasound monitoring (12-14mm). The antagonist is continued together with the (Gonal-F®) stimulation until an adequate response is obtained and hCG injection required for ovulation induction [13]104 (Al-Inany H 2002). Third group (15) infertile couples in whom PCOS women were undergoing short agonist protocol, which started on day two of menstrual cycle with a daily administration of subcutaneous injection of GnRH-a, triptorelin (Decapeptyl®; 0.1 mg Ferring Co, Kiel, Germany) ® .ovarain stimulation started on day three of menstrual cycle with daily administration of 150-225 IU of (Gonal-F®) injection subcutaneously. Decapeptyl® is continued together with the (Gonal-F®) stimulation until an adequate response is obtained and hCG injection required for ovulation induction [14]. The fourth controlled group contain thirty five infertile couples (35) in whom(15 undergoing long agonist protocol,10 undergoing short protocol,10 undergoing antagonist protocol). The oocytes were retrieved by transvaginal ultrasound-guided follicle aspiration 34-36 h after hCG administration. Sperm preparation and IVF/ICSI were performed and embryo transfers were carried out 2- 3 days after oocyte retrieval. Luteal phase support was done by giving 400 mg/bid of vaginal progesterone (Cyclogest®, Actavis). A 5-ml blood sample was collected from all patients on the day of oocyte retrieval. After collection each sample was immediately centrifuged at 3000 rpm for 10 min at room temperature and the supernatant was separated and stored at −20 °C until assayed. A 2 ml of clear Follicular fluid was collected from aspirated follicles, and then it was centrifuged at 3000 rpm for 10 min at room temperature and stored at −20 °C until assayed. Fetuin -A levels were detected in both serum and follicular fluid samples by enzyme-linked immunosorbent assay technique using diagnostic kit (SHANGHAI, YHB1184HU).

Statistical analysis:-

The Statistical Analysis System- SAS (2012), version 9 was used to evaluate effect of different factors in study parameters. Numeric variables were expressed as mean ± standard error (SE). Least significant difference –LSD test (ANOVA) and T test were used to significant compare between means [15].

Results:-

Clinical characteristics of PCOS and control (according to certain parameters):

Female age: The mean age± standard error (SE) of all females with PCOS participate in this study was (29.01±0.63) years, which is ranging between 17 and 40 years, and for control (the mean age± SE was (28.57±0.81) years which ranging between 19 and 36 years. The statistical analysis showed no significant difference ($p>0.05$) in the age between two groups (Table 1).

Body Mass Index (BMI): The mean ± SE of BMI of PCOS patients in the present study was (28.68±1.06)Kg/m2, and in control group the mean ± SE of BMI was (28.29±0.99)Kg/m2, (Table 1). The statistical analysis showed no significant difference ($p>0.05$) in the BMI between PCOS and control groups.

Duration of Infertility: The mean ± SE regarding duration of infertility for all PCOS patients in the present study was (6.67 ±0.37) years, and in control group was (6.80±0.44) years. The statistical analysis showed no significant difference ($p>0.05$) in the duration of infertility between two groups (Table 1).

Table 1:- Clinical Characteristics of PCOS patients and non-PCOS groups

Characters	PCOS		Non-PCOS		LSD
	Mean	SE	Mean	SE	
Age(years)	29.10	0.63	28.57	0.81	0.492 NS
BMI (kg/m2)	28.68	1.06	28.29	0.99	0.562 NS
Duration of infertility	6.67	0.37	6.80	0.44	0.465 NS

All values are expressed as mean ± SE (Standard Error).PCOS= poly cystic ovary syndrome; BMI=Body Mass Index; NS: Non-significant.

Comparison between PCO and Non-PCOS in ICSI Parameters:

The Total Number of Oocytes Retrieved: The mean total number of oocytes ± SE in both PCOS and non-PCOS group were (11.02 ± 0.61 and 9.08 ± 0.83) respectively. Total number of oocytes retrieved was significantly higher ($P<0.05$) in PCOS than that in non-PCOS (table 2).

Number of mature Oocytes (Metaphase II): In this study, the mean number of mature oocytes [metaphase II (MII)] ± SE in both PCOS and non-PCOS group were (6.89 ± 0.37 and 5.05 ± 0.46) respectively. The statistical analysis showed a highly significant difference ($P<0.001$) among the two groups (Table 2).

Fertilization Rate (FR %): Our study found that the mean number of fertilization rate ± SE in both PCOS and non-PCOS group (57.76 ± 3.56 and 55.16 ± 6.08) respectively. There was no significant difference ($P>0.05$) between the two groups (Table 2).

Table 2:- Comparison between PCO and Non-PCOS in ICSI Parameters

Group	No.	Mean ± SE		
		No. of Oocyte	No. of metaphase II(MII)	Fertilization Rate FR%
Non-PCO	35	9.08 ± 0.83	5.05 ± 0.46	55.16 ± 6.08
PCO	55	11.02 ± 0.61	6.89 ± 0.37	57.76 ± 3.56
P-value	---	0.051*	0.0029**	0.695 NS
* ($P<0.05$), ** ($P<0.001$).				

Comparison between PCOS patients and non PCOS in the level of follicular fluid & serum Fetuin-A who underwent IVF/ICSI protocols:

In the current study, the mean ± SE of the level of fetuin-A in follicular fluid (F) in PCOS group was (1569.06 ± 143.16) pg/ml and in control group was (2028.62 ± 149.65) pg/ml. There was significant difference ($p<0.05$) in the level of fetuin-A in follicular fluid between PCOS and control group. On the other hand the mean ± SE for the level of fetuin-A in serum(S) at the day of oocyte retrieval in PCOS group was (1526.86 ± 159.34)pg/ml and in control group was(1504.88 ± 125.49)pg/ml. The statistical analysis shows no significant difference ($p>0.05$) in the level of fetuin-A in serum between PCOS and control group (Table 3).

Table 3:- Comparison between PCOS and Non-PCOS in FF-F.A&S-F.A.

Group	No.	Mean ± SE	
		Fetiun follicular fluid (F.A-FF)	Fetiun serum(F.A-S)
Non-PCO	35	2028.62 ± 149.65	1504.88 ± 125.49
PCO	55	1569.06 ± 143.16	1526.86 ± 159.34
T-Test	---	428.54 *	444.45 NS
P-value	---	0.0359	0.921
* ($P<0.05$).			

Comparison between the effect of different IVF/ICSI protocols (Long agonist, Antagonist, Short agonist) on the level of follicular & serum Fetuin-A of patients with PCOS:

In the current study, the mean ± SE of the level of fetuin-A in follicular fluid (F.F) in PCOS groups who underwent long agonist, Antagonist, short agonist protocols were (1656.25 ± 313.90, 1430.55 ± 59.40 and .1562.26 ± 50.44)pg/ml respectively. There was no significant difference ($p>0.05$) in the level of fetuin-A in follicular fluid between the three PCOS groups. On the other hand the mean ± SE for the level of fetuin-A in serum(S) at the day of oocyte retrieval in PCOS groups who underwent long agonist, Antagonist, short agonist protocols were (1611.69 ± 269.92, 1634.35 ± 373.40 and 1278.01 ± 73.23)pg/ml respectively. The statistical analysis shows no significant difference ($p>0.05$) in the level of fetuin-A in serum between three groups of PCOS (Figure 3, 4).

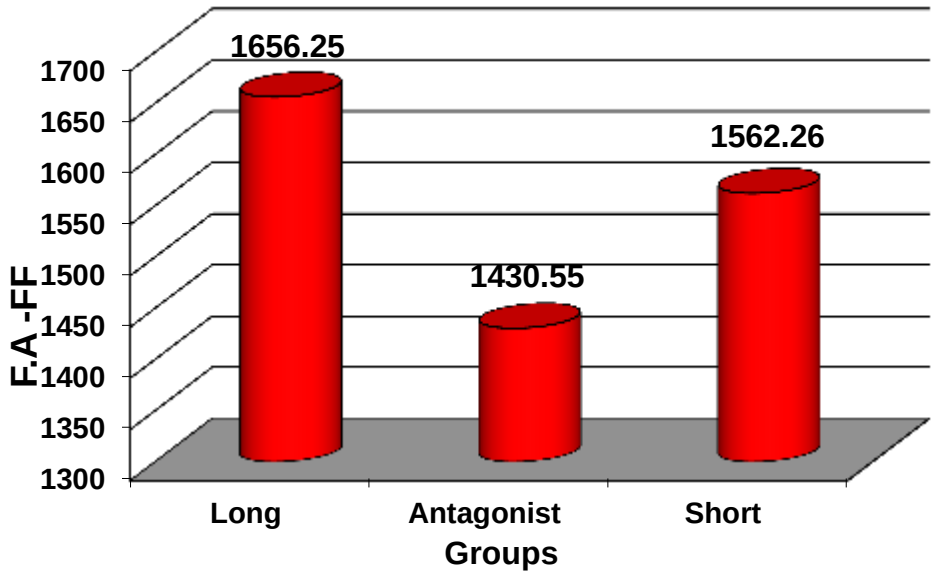

Figure 3: Comparison between different PCOS groups in FF- F.A

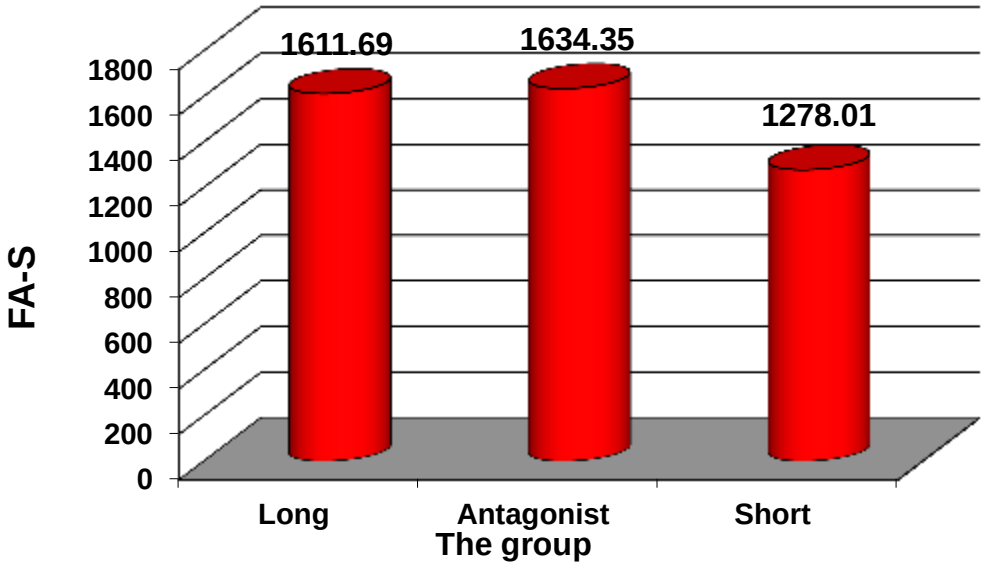

Figure 4: Comparison between difference PCOS groups in S-FA

Comparison of serum & follicular fluid Fetuin-A level between pregnant &non pregnant PCOS patients:
The mean of serum and follicular F.A levels at the day of oocyte retrieval ±SE of pregnant PCOS patients shared in this study were (1554.20±300.54 and 1561.73±186.88) respectively. The mean of serum and follicular F.A levels at the day of oocyte retrieval ±SE of non-pregnant PCOS group shared in this study were (1518.39±188.67 and 1571.33±186.88) respectively. The statistical analysis showed no significant difference (P>0.05) in level of serum or follicular F.A between the two groups (Table 4).

Table 4:- Comparison of S & FF-Fetuin-A level between pregnant &non pregnant PCOS patients:

PCOS Group	No.	Level of Fetuin A	
		F.A-S Pg/ml	F.A-F.F Pg/ml
Pregnant	13	1554.20±300.54	1561.73±186.88
Non pregnant	42	1518.39±188.67	1571.33±186.88
T-Test		102.693 NS	94.052 NS
NS: Non-significant.			

Discussion:-

The reason we studied liver-derived fetuin-A in women with PCOS and compare with non PCOS women was the previous observation that this protein may play a significant role in mechanisms at insulin sensitivity, weight gain and fat accumulation [8, 16]. Especially because, as in animal and human studies the inhibitory function of fetuin- A on insulin receptor (tyrosine kinase) in the muscle and in the liver has been proved [17,6]. AHSG may be considered as an important link between obesity and insulin resistance. On the other hand, decreased insulin sensitivity and subsequent hyperinsulinaemia, in PCOS more severe than expected on the basis of body weight, represent crucial metabolic disturbances in the majority of obese as well as of lean women with PCOS [18, 19]. Moreover, it is postulated that obesity in women with PCOS is associated not only with more severe insulin resistance but also with hyperandrogenemia and fertility disorders [20].A few other human studies, that did not involve women with PCOS, found higher levels of AHSG in obese subjects with metabolic disturbances compared to lean individuals. In obese children, initially elevated fetuin-A levels decreased during exercise- and diet-induced weight loss. In patients with morbid obesity, fetuin-A was markedly increased and significantly declined after weight loss resulting from bariatric surgery. This fall was related to changes in insulin resistance but not directly to BMI [21].

In our study, we found that PCOS women on ICSI cycle showed no significant difference in serum fetuin- A levels compared to ICSI-treated non-PCOS subjects (Table 3) (Figure 2), however, fetuin- A level in follicular fluid of non PCOS women was significantly higher than that in follicular fluid in PCOS women (Table 3) (Figure 1). Furthermore, there was no significant difference in serum or follicular fluid fetuin-A levels between the women who succeeded in becoming pregnant and those who did not in PCOS patients (Table 5), (Figure 5 & 6). To the best of our knowledge, this is the first study to compare fetuin-A levels in follicular fluid between PCOS and non PCOS women and between pregnant and non-pregnant PCOS patients undergoing ICSI protocols. In two published independent trials, there was divergent data on serum fetuin-A levels in women with PCOS. In the paper by Abali et al. (2013) [22], mean serum fetuin-A concentrations were considerably elevated compared to healthy controls, whereas in the study by Gulhan et al. (2012) [23], there was no difference between women with PCOS and healthy subjects with regard to fetuin-A levels. Probably, all these discrepancies may be related to differences in age, BMI, liver fatness, level of insulin resistance and other yet undefined metabolic factors. Yasar E. et al. (2013) Demonstrated that serum fetuin-A levels are similar in lean and obese women with PCOS. They found an association between fetuin-A levels and ALS activity in lean patients and between fetuin-A levels and DHEA-S in all women [24]. Antoaneta G. et al. (2013) confirmed for the first time in patients with PCOS there was no difference in fetuin-A levels between patients with obesity with and without PCOS, nor between patients with PCOS with and without obesity. These results suggest that fetuin-A is not independently linked to obesity and to PCOS status but rather depends on the presence of metabolic syndrome [25].

IVF Protocols: Before 20 years, GnRH agonists have been the "gold standard" protocol in COS. In recent years it has become more complex, and stressful for the couples due to overlong and complex ovarian stimulation protocols, GnRH antagonists, in contrast, are more often used as second-line agents in patients who are poor responders, in the elderly and in the ones with previous IVF failures. Also, the addition of antagonist protocol, to the semi natural cycle IVF protocol reduced the premature LH surge without affecting overall outcome. The short protocol seemed to

be an efficient and cost-effective protocol for the poor responders and old age. In present study, there is no significant difference in follicular and serum fetuin A levels among different PCOS groups (Figure3, 4). To the best of our knowledge, this is the first study to compare serum and follicular fetuin-A levels among PCOS patients underwent different IVF protocols, so further researches required to agree or disagree with our results.

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