Antiovarian Antibody and Free Androgen Index in Subfertile Women and Their Relation to Intracytoplasmic Sperm Injection Outcome

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Abstract

A successful pregnancy was the consequence of numerous complex interactions between receptive uterus and the mature blastocyst under immune-hormonal control. The objectives of this study were to determine the relation between hyper androgenemia and autoimmunity, and their effect on intracytoplasmic sperm injection (ICSI) outcome. This study was carried out on 110 subfertile women aged between 18-45 year (30.01±6.02), referred to fertility clinic in Al-Sadder teaching hospital at AL-Najaf city who undergone intracytoplasmic sperm injection between the period from March to October 2014. The study participants were then subclassified into four subgroups according to the causes of subfertility were 33 ovulatory, 47 male cause, 8 unexplained, 27 tubal). Antiovarian antibody (AOA) and free androgen index (FAI) were measured in serum collected on day of pick up using ELISA kit for AOA, and special formula for FAI, then evaluated the correlate to ICSI outcome. The results of confirmed that the pregnancy number was 38 women out of 110 embryo transferred after 48-72 hours, there was no significant difference between pregnant and non pregnant women regarding AOA and FAI at $P>0.05$, furthermore there was positive but not significant correlation between FAI and ICSI, GI, FR, pregnancy rate. While regarding AOA there was significant difference between causes of subfertility groups and with unexplained subfertility at $P<0.05$. Furthermore, 88% of women with ovulatory factor (mostly PCOS) and negative AOA got pregnant. 100% of unexplained subfertility with negative AOA got pregnant, women with positive AOA had less median retrieved oocyte number, MII, injected, but more fertilization rate, cleavage rate at $p=0.01$, percent of embryo at $p= 0.02$, and less pregnancy rate. Also, there was positive non-significant correlation between FAI, total testosterone and AOA in women with positive AOA at $P>0.05$. There was negative non-significant correlation between TT/FT ratio, SHBG and AOA. The best cutoff value of FAI associated with pregnancy was 0.39 and for AOA was 7.22 IU/L. Conclusion: There was an autoantibody complex possibility, that having a role in controlling production of androgen from zona reticularis of adrenal gland or near the ovarian theca cell, that was named as APF. Mild degree of autoimmunity will enhance androgen level, while high degree of positive AOA will decrease androgen level and AOA affect the pregnancy out come and is mainly distributed in unexplained infertility.

Keywords: AOA, FAI, autoimmunity, TT/FT ratio, SHBG

Abbreviations: PCOS: polycystic ovarian syndrome, PN: pronuclear, NO: number, TT: total testosterone, OR: odds ratio

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Introduction

As a routine part of every patient's initial evaluation of androgen testing using commercial laboratories (Gleicher, *et al*., 2013). Androgen in human plays an important role at stages of development of the follicle, this was suggested by its association with IVF/ICSI outcome (Frattarelli and Gerber, 2006; Hossein *et al*., 2009) and by its role in improvement of subfertility treatment outcomes after providing women at all ages with low functional ovarian reserve with androgen supplementation (Gleicher and Barad, 2011).

About 60 percent of testosterone is bounded to sex hormone-binding globulin (SHBG) which is a specific high-affinity protein, the remaining of hormone about 38% was binding to albumin. While the last small portion about 1% in females and 2% in male represents the physiologically active free form of hormone mediating its biological action at the target tissues in both sexes (Murray *et al.*, 2009). Free androgen index was defined as the ratio that measuring abnormal androgen status in humans. It is equal to the total testosterone value over sex hormone binding globulin (SHBG), totally multiplying by 100. The unit used for measurement of testosterone concentration and SHBG is nanomols per liter. So the index has no units.

$$FAI = 100 \times (\frac{Total\ Testosterone}{SHBG})$$ (Marianne and Sohrabi, 2014)

Androgens commonly considered to have an immuno-modulatory effect, either immuno-enhancing or -suppressive (Roberts and Peters, 2009). The supposing that immune system-induced hyperandrogenemia and the subsequently such androgen immuno-modulatory effects could control androgen production by adrenal glands or ovaries that able to feed back. Sen, *et al.* (2014) found that there is a possibly immune system-associated androgen production process and report a supportive evidence for their theory that located primarily in adrenal glands and/or ovaries. They also report that this process maybe has an autoantibody-driven analogues to other endocrine organs immune processes.

In different situations of human ovary might be targeted of an autoimmune attack, including systemic autoimmune or several organ-specific diseases. The consequential clinically of ovarian dysfunction results usually in premature ovarian failure (POF), ovarian disturbances as Polycystic Ovarian Syndrome (PCOS), endometriosis, and unexplained subfertility were related to anti-ovarian autoimmunity. The presence of AOA had been detected in patients with unexplained subfertility (Luborsky *et al.*, 1999, 2000, 2002), Antiovarian antibodies are found in the serum of about 50% of

PCOS women (VanGelderen and Gomes, 1993; Fenichel *et al*., 1999). The outcomes collectively suggested that the AOA are independent markers for the future onset of ovarian failure. So screen for the presence of AOA before the beginning of the IVF/ICSI cycle might be recommended, since AOA are thought to be associated with poor results in subfertility patients involved in the programs for assisted reproduction (Pires *et al.,* 2007).

Even healthy individuals frequently demonstrated the laboratory evidence suggestive of immune system hyperactivity, normal latency versus activation is not well defined. However, the autoimmune abnormalities are frequently found in association with allergies (Higashi, *et al*., 2009) or even in normal patient populations of normal ovulation, implantation, pregnancy are an immune mediated inflammatory process (Grümmer and Winterhager, 2011; Mariz, *et al*., 2011; Bruner *et al*., 2012). The sub-clinicals have been associated with reproductive problems, including subfertility, miscarriage risk as antiphospholipid antibody (Turi *et al*., 2010 and Gleicher *et al*., 2012) and complications of pregnancy, such a premature labor and preeclampsia or eclampsia (Gleicher, 2010 and Gleicher, 2014). These regulations might suppose the process for androgen in adrenals and ovaries, so the possibility of an immune system derived androgen production factor overproduction must be supposed. Responsible for maintaining appropriate androgen levels for normal female fertility, which may be potentially increased in association with polycystic ovarian syndrome (PCOS). APF overproduction may result hypothetically, in hyperandrogenemia, an ovarian PCOS- like phenotype that may be either hyper- or normo-androgenic (Zhang, *et al*., 2009). While in women with prematurely diminished functional ovarian reserve (premature ovarian aging (POA)) it decreased. Leading to lack immune system's ability for production of APF. Consequently, decreasing testosterone levels. Thus its etiology was from adrenal and ovary, that mimic in opposing ways the androgen excess and excessive production of follicle in some women with polycystic ovary syndrome (PCOS) (Gleicher *et al*., 2013). Therefore, PCOS and DOR (diminished ovarian reserve) considered as an opposing extremes of immune-mediated effects on adrenals and/or ovaries (Gleicher, *et al*., 2007). The objectives of this study were to determine the relation between hyper androgenemia and autoimmunity, and their effect on intracytoplasmic sperm injection (ICSI) outcome

Materials and Methods

The protocol of this study was planned according to the declaration of ethical philosophies of medical research, as a prospective cohort study with acceptation from the committee of ethical research in the Physiology and Obstetrics and Gynecology Departments, Faculty of Medicine, University of Kufa. A total of 120 cases included subfertile couples referred to the fertility center of Al-Sadder teaching hospital in Al-Najaf city between the period from March to October 2014. All women considered underwent of 120 IVF-ICSI cycles after a certain ovarian stimulation protocol, 110 reached the ovum pick up stage, and the remained either cancelled cycle or had no

stimulated oocyte by ultrasound. All 110 positive cases were distributed into four subgroups according to the cause of subfertility: female factor (Ovulatory factor, endometriosis), male factor, Tubal and Unexplained subfertility. All cases were subjected to the ICSI according to the indication. They were of reproductive age ranging from 18-45 years old. Their body mass indices (BMI) range from (20.8-38.8). Free from hepatitis and HIV confirm by screening tests. They did not show any history of rheumatological or immunological diseases related to antiovarian antibody (Pires *et al*., 2007). Female with primary ovarian failure (POF) as result of surgery, chemotherapy or radiotherapy, infection like oophoritis as a result of viral infection or primary ovarian failure induced by galactosemia, large ovarian cyst, and hyperprolactinemia. Also, the cases had pelvic surgery such as appendicitis or uterine or ovarian operation through the previous 2 month or women with pelvic inflammatory diseases at last 3 month of infection, sever endometriosis (stage IV), significant abnormalities in the uterine cavity, submucous, intramural fibroid and hydrosalpinx were excluded in this study.

All women underwent ovulation induction were divided into different protocol groups, the long protocol group was involved 14 cases, short protocol group was involved 66 cases and antagonist group that involved 30 cases depending on hormonal conditions, timing, and ovarian reserve status of the women on the discretion of the clinician. After the menstrual cycle end, the vaginal ultrasound guidance of HCG injection was given to trigger the final stage of oocyte maturation with oocyte pick up guided was performed 34-36 hours later end of menstrual cycle end. Then good quality embryos were returned to mother after a period ranging from 3-5 days, and embryos classified according to their morphology and percentage of fragmentation. Five ml blood sample was taken from patient on day of pickup and serum obtained and freeze till the time of measurement using ELISA kits for AOA, total testosterone (TT), SHBG (sex hormone binding globulin). Total testosterone LOT, 14001 (Human, Germany) (Kicklighter and Norman, 1989), SHBG DE2996, Demeditec Diagnostics, GmbH LOT 302k054 (Selby, 1990), FAI (Total Testosterone/SHBG) X100, AOA (Demeditec Diagnostics GmbH DE2937, LOT ELC09141009).

Statistical analysis in this study was performed using SPSS (Statistical Package for Social Science; Version 20) program. Expression of values as mean ± SD, independent t-test was used. Also, one-way ANOVA was used to evaluate differences of means among multiple groups. Pearson's correlation analysis had been used to correlate among the measured variables and other clinical variables or data. The odds ratio was determined by binary logistic regression and regarded as a test of a correlation among pregnancy parameters as categorical data as dependent variable and AOA. Whereas the odds ratio for continuous data as dependent variable was determined by the general linear model. Using receiver operating characteristic (ROC) curves to investigate the expectedness of ICSI variables and AOA, FAI, TT, SHBG levels for pregnancy and to determine the sensitivity and specificity, the results were described as mean ±SD, with P value less than 0.05 that was reflect a statistical significant (Daniel, 1999).

Results

Free Androgen Index (FAI)

The results of this study showed the 38 women were pregnant while non pregnant women were 72 from total 110. SHBG there is increased in the pregnant was 130.89±48.36 more that non-pregnant but the mean±SD was 118.4±51.25 within normal. The FAI in non-pregnant was 0.97±0.81 more than pregnant with mean±SD was 0.89±0.66 compared with the reference range of adult female premenopausal SHBG was 40-120 nmol/L and Free Androgen index <5. The result of TT/FT ratio was 1.30±0.48 in the pregnant and it was higher than non-pregnant. There were no significant difference regarding the previous mentioned parameters table (1).

Interactivity of Free androgen index to ICSI outcome

There is positive correlation between FAI to some ICSI outcome but not significant correlation with most variable apart from GI, FR, and pregnancy rate table (2).

Table (1) Free Androgen Index mean and standard deviation in the studied group and between pregnant and non-pregnant women

Test	mean±SD	pregnant	Non-pregnant	P value	Odd's ratio
SHBG	118.4±51.25	130.89±48.36	111.2±51.9	0.094	1.008
FAI (bioavailable test)	0.89±0.66	0.85±0.62	0.97±0.81	0.480	0.850
TT/FT ratio	1.2±0.5	1.30±0.48	1.13±0.49	0.127	0.565

Table (2) correlation between FAI and ICSI outcome

ICSI outcome variable	FAI	
	r	p
Oocyte number	0.086	0.443
MII	0.126	0.261
pronuclear	0.019	0.867
Embryo NO.	0.054	0.632
G1	-0.070	0.548
G2	0.033	0.780
Embryo transferred	0.015	0.894
CR%	0.161	0.163
FR%	-0.077	0.508
Pregnancy rate	-0.080	0.480

MII: metaphase IIGI: grade 1 embryo, GII grade 2 embryo, CR: cleavage rate, FR: fertilization rate

Antiovarian Antibody (AOA)

The measurement of AOA according to the causes of subfertility showed a significant difference between the groups in the mean plot in women with unexplained infertility (10.3 IU/L), followed by ovulatory cause (7.72 IU/L), male factor (7.2 IU/L) and then women with tubal cause (6.65 IU/L) figure (1).

The cutoff point between the AOA positive and negative was 10 IU/L, where AOA was >10 IU/L positive women, while negative women had AOA <10 IU/L. About 88% of women with ovulatory factor and negative AOA got pregnant, while only 12% of them got pregnant with AOA positive. Also, 100% of unexplained infertility women with negative AOA got pregnant figure (2). The serum of AOA test between pregnant and non-pregnant women on day of pick up did not show a significant differences.

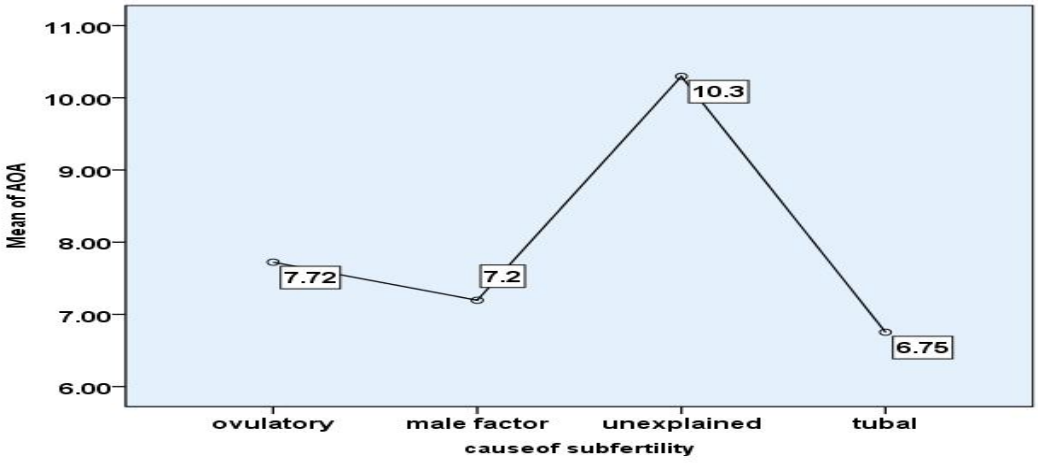
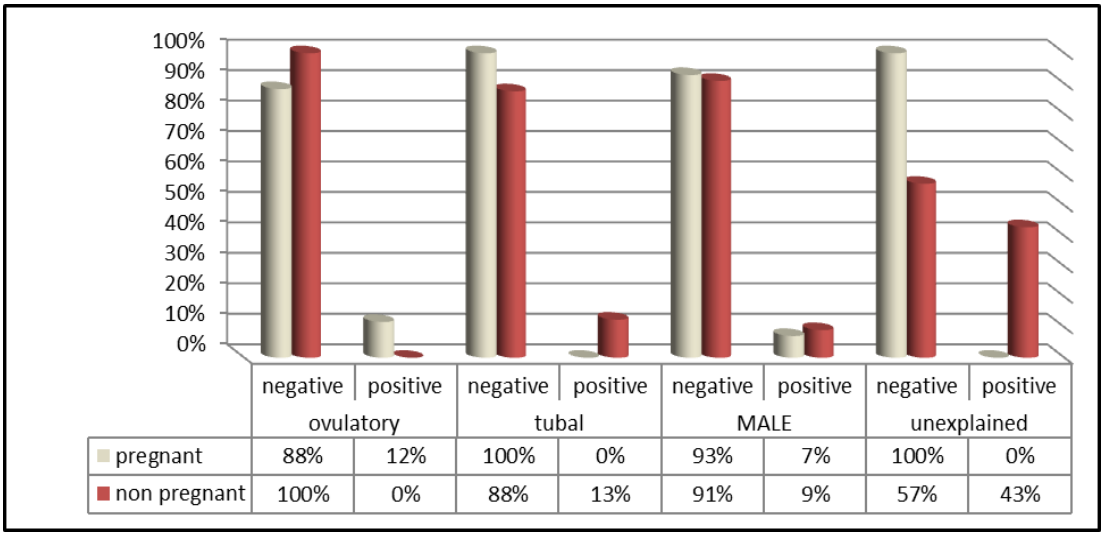


Figure (1): Mean plot of AOA according to the cause of subfertility.



	negative	positive	negative	positive	negative	positive	negative	positive
	ovulatory		tubal		MALE		unexplained	
pregnant	88%	12%	100%	0%	93%	7%	100%	0%
non pregnant	100%	0%	88%	13%	91%	9%	57%	43%

Figure (2) Distribution of AOA positive and negative group in pregnant and non pregnant women according to their cause of infertility. Reference range of AOA: 10IU/L.

Antiovarian antibody and ICSI Outcome

Women with positive AOA on female infertility and their response to intracytoplasmic sperm injection (ICSI) had less median retrieved oocytes number, MII, injected, percentage of fertilization rate and cleavage rate at p= 0.01. Less

percentage of pregnancy rate and embryo at p= 0.02. Binary logistic regression showed that increase the odd's ratio for the negative AOA regarding retrieved oocytes OR: 0.993, MII OR: 1.05, NO. of injected oocytes OR:1.04, PN. OR: 1.007, non-fertilized OR: 1.644, embryo NO. OR: 0.892, embryo transferred OR: 0.937, GI, GII, GIII OR (0.867, 0.885, 1.074 table (3).

Table (3): Differences in ICSI characters between AOA positive and negative group

ICSI characters		AOA negative Median(min-max) sum	AOA positive Median(min-max) sum	P value	Odds ratio
Oocyte NO		9(0-28)664	7(4-30)114	0.944	1.021
MII		7(0-23)831	6(4-14)68	0.438	1.05
Injected		6(0-23)801	5(4-14)68	0.523	1.04
pronuclear		4(0-15)490	4(1-12)48	0.939	1.007
Non fertilized		2(0-11)157	2(1-2)10	0.148	1.644
Embryo number		4(0-13)373	4(2-10)47	0.297	0.892
Embryo transferred		4(0-7)265	4(2-4)31	0.797	0.937
GI		1(0-7)136	2(0-3)18	0.408	0.812
GII		2(0-10)177	3(1-8)25	0.683	0.934
GIII		0(0-5)43	0(0-5)47	0.859	1.074
Fertilization rate		0.64±0.25	0.73±0.24	0.301	0.209
Embryo percentage		0.54±0.21	0.74±0.26	0.000[1**]	1.021
Cleavage rate		0.75±0.36	0.89±0.18	0.016[*]	1.011
Pregnancy out come	pregnant	35(38.6%)	3(25%)	0.367	1. 88
	Non pregnant	63(61.4%)	9(75%)		

*P value< 0.05, **P value <0.001, MII metaphase II, GI, GII, GIII: Grade1, II, III embryos.

Relation of Free androgen index (FAI) and Antiovarian antibody (AOA) to intracytoplasmic sperm injection outcome

Mean value of FAI was higher among women with positive AOA at p=0.601, it was positive non-significant correlation to the androgenic etiology of ovarian autoantibody figure (3). Also, there was positive non-significant correlation at p=0.078 between total testosterone in women with positive AOA. A positive non-significant correlation between TT/FT ratio and AOA figure (4), with positive non-significant correlation between SHBG and AOA in figure (5) with P value >0.05.

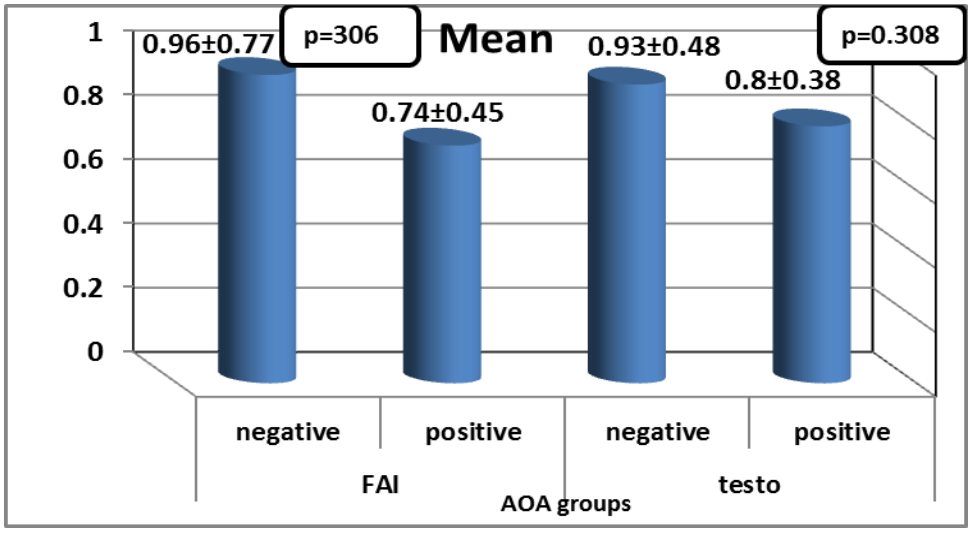


Figure (3) Free androgen index & testosterone mean and standard deviation among AOA groups.

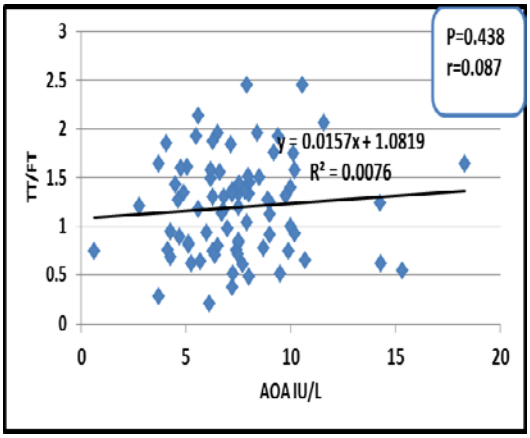


Figure (4): Correlation between antiovaria group and TT/FT rat

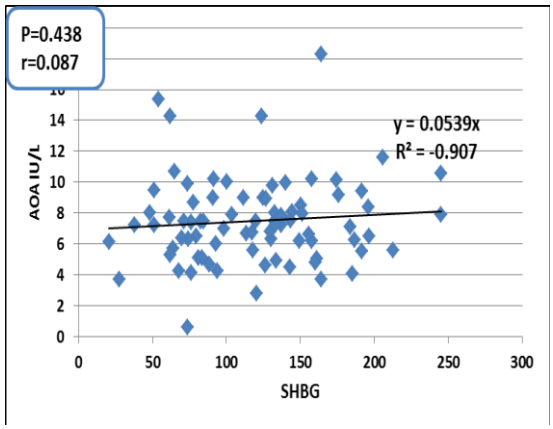


Figure (5) Correlation between antiovarian group and SHBG

An Estimated area under curve (AUC) and predictive cut-off point values for the individual some seroimmunological and hormonal parameters with the receiver operating characteristics (ROC) curve analysis between pregnant and non-pregnant women revealed that the SHBG showed a highest AUC 0.603, a threshold of 111.04 gave sensitivity of 74% and specificity 44%, followed by total testosterone (AUC 0.544, threshold of 0.51 sensitivity of 94% and specificity 20%), then AOA (AUC: 0.529, threshold of 7.22 sensitivity of 63% and specificity 56%).

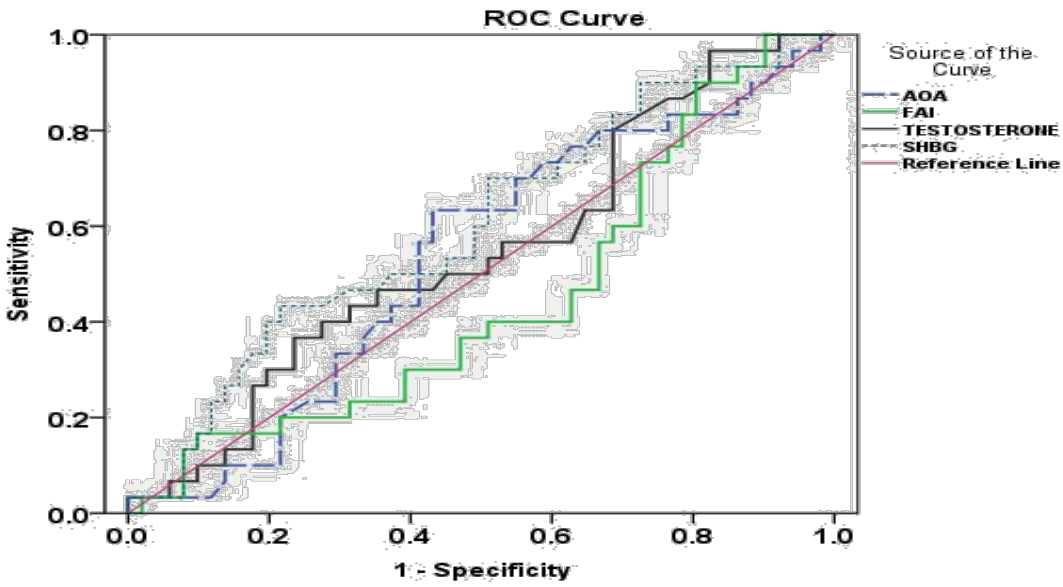


Figure (6) Estimated area under curve (AUC) sensitivity and specificity.

Table (4) Estimated area under curve (AUC) and predictive cut-off point values of seroimmunological and hormonal parameters with the receiver operating characteristics (ROC) curve analysis between pregnant and non-pregnant women

variables	AUC	Cut off point	sensitivity	specificity	CI 95%	
AOA	0.529	7.22	63%	56%	0.416	0.642
Testosterone	0.544	0.51	94%	20%	0.430	0.659
SHBG	0.603	111.04	74%	44%	0.493	0.714
FAI	0.465	0.39	89%	19%	0.352	0.579

CI: confidence interval

Discussion

The ova and embryo development might be affected by AOA which might be responsible for failures of implantation. This result agree with previous reported that AOA was identified in 33-61% of women complaining of unexplained subfertility, meaning that unexplained subfertility may be the early step of autoimmune ovarian failure (AOF) Other reports that inflammatory reaction may occurred in many cases of unexplained subfertility, or antibodies could attack or hit against hormones,

reproductive tissues (ovaries or testes), or clotting factors (Luborsky *et al*., 1999a, 2000, 2002).

The incompatibility in the result may be due to the heterogeneity of PCOS with antibody tests. AOAs were discovered in about 50% of PCOS women (VanGelderen and Gomes, 1993; Fenichel, *et al*., 1999) while these results were disagreed with others (Rojanski *et al*., 1997 and Luborsky, *et al*., 1999b). The difference between the results of the above researchers could be due to using multiple antigenic substrates for these studies like: VanGelderen and Gomes (1993) used granulose cell in their study and Fenichel *et al*. (1999) used extract of human ovary, while Rojanski *et al*. (1997) used ovarian follicle theca interna, Luborsky *et al*. (2002) used 'microsomal' antigens in their study to measure antiovarian antibody.

In addition, there was a large percentage of false positive AOAs that detected in the serum and comprises a big problem. Another factor responsible for the conflicting result regarding AOAs was poor specificity of the available commercial kit as about 33% of healthy women with regular cycle were tested positive (Dehaghani *et al*., 2013), this problem could occur probably as a result of the presence of naturally occurring antibodies that affect different cellular self-constituents. A possible association between immune system activation and embryo quality had never before been reported. It was not only statistically very forceful but even held up after adjustment for age and number of earlier IVF cycles, suggesting that it was independent of female age and, at least to a degree, independent of severity of subfertility. Combined, these observations, therefore, suggest that a mild form of immune system activation may represent a highly important theme throughout all stages of successful reproduction, starting with ova/embryo quality.

Ovaries as immunological "hot-spots" was reported based on the recognition that increasing numbers of fertility-associated genes in the ovary also have immunological functions (Albertini *et al*., 2012). Mean value of FAI was lower among women with positive AOA which was negative non-significant correlation this may give us clue about androgenic etiology of ovarian autoantibody. Also there was positive non-significant correlation between total testosterone in women with positive AOA. Mean mild degree of autoimmunity will enhance androgen level of negative non-significant with TT, FAI, while high degree of positive AOA will decrease androgen level.

There were positive non-significant correlation between TT/FT ratio and AOA, with positive non-significant correlation between SHBG and AOA. The association of TT/FT ratios and immunoglobulin abnormalities supports the previously reported association between mild immune system activation (Gleicher, *et al*., 2013), normal ovarian function and normal androgen levels; while women with abnormally low ovarian function not only lack evidence of immune system activation but also demonstrate comparatively low androgen levels. The explanations of that may be: either due to blocking of the effects of APF or interruption of APF production. Also the results suggested a decreased in APF production more likely than blockage of APF, because the later will involve evidence of immune system activation, the opposite of what they observed Gleicher, *et al*. (2013).

Based on these unexpected findings, the above result was consolidated by many authors as Sen, *et al*, 2014, who further hypothesized that normal androgen levels, in

females nearly equally adrenal and ovarian in origin, depend on mild immune system activation. In absence of activation by androgen production factor driven from immune system (APF), levels of androgen decrease and androgen-dependent early stages of follicle maturation wither. Under this hypothesis, the establishment of pregnancy, thus, would be dependent on mild immune system activation.

This is a potentially interesting new observation since TT/FT ratios were to a large degree dependent on sex hormone binding globulin (SHBG) (Ly, and Handelsman, 2005). These observations potentially link immunoglobulin concentrations via androgen levels to SHBG levels, as androgens are known to decrease SHBG, activated immune system seems to be related to normal or slightly increased androgen, on other hand androgen decreased in lack of immune system stimulation (Selby, 1990 and Nitsche, *et al*., 2014)

The best explanation to the previous results in that stimulated immune system accompanied by existence of androgen producing factor APF, which is an important fact to illustrate the tolerance production in paternal semi allograft implantation needing activated maternal immune system. The production of APF was broken up or its effect was blocked (Kim, *et al*., 2013). Our results are in agreement with previous studies (Gleicher *et al*., 2007 and Gleicher *et al*., 2009).

Furthermore there may be a regulation process for androgen production in both adrenals and ovaries, also there was a probability of over production of APF which in turn leading to increased androgen concentration resulting in ovarian PCOS- like phenotype Such phenotypes, which might be hyper or normo androgenic (Zhang, *et al*., 2009). There for DOR and PCOS functionally may considered as conflicting ends of effects that probably mediated by immune system on ovaries and/or adrenals.

The etiological factors for PCOS might an autoimmune factor (VanGelderen and Gomes, 1993 and Hefler-Frischmuth *et al*., 2010). Interestingly, increased androgen level seems to exert anti-inflammatory effects on PCOS women. From the above study we concluded that there was an autoantibody complex possibility, that having a role in controlling production of androgen from zona reticularis of adrenal gland or near the ovarian theca cell, that was named as APF. Mild degree of autoimmunity will enhance androgen level, while high degree of positive AOA will decrease androgen level and AOA affect the pregnancy out come and is mainly distributed in unexplained infertility.

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