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MEASUREMENT OF ANTI-MÜLLERIAN HORMONE IN SERUM AND FOLLICULAR FLUID AS A CORRELATION WITH ICSI OUTCOME

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ABSTRACT

Background: Anti-Müllerian hormone is a dimeric glycoprotein. In the ovary, it is exclusively produced by the granulosa, it has an important role in ovarian function, especially in follicle development and selection. The objectives of this study is to determine the usefulness of Anti-Müllerian hormone level in serum and follicular fluid on the day of oocyte pick up with the pregnancy outcome of intracytoplasmic sperm injection treatment. Forty infertile women undergoing intracytoplasmic sperm injection cycles were enrolled prospectively in this study in Kamal Al-Samarrai hospital for infertility and *in-vitro*fertilization (Baghdad/Iraq). All patients received mid-luteal long gonadotropin releasing hormone agonistprotocol. Serum and follicular fluid AMH levels were measured on the day of oocyte retrieval by using Enzyme Linked Immuno Sorbent Assay. The diagnostic accuracy of AMH in serum and follicular fluid, was assessed by the area under the receiver operating characteristic curve. **Results:** Ten women (25%) achieved pregnancy and was defined as pregnant group, while 30 women (75%) failed in achieving pregnancy who defined as non-pregnant group. The concentration of Anti- Müllerian Hormone in both serum and follicular fluid was significantly higher ($P<0.05$) in the pregnant group than in the non-pregnant group.Significant positive correlation ($r=0.380$, $P<0.05$) between serum and follicular fluid Anti- Müllerian Hormone.A significant positive correlation between serum Anti-Müllerian hormone and total oocyte number, metaphase II oocyte number, and fertilization rate.The diagnostic accuracy of serum Anti- Müllerian Hormone between the pregnant and the non-pregnant women was good, while follicular fluid Anti- Müllerian Hormone was poor because the results of the area under the receiver operating characteristic curve for Anti- Müllerian

Hormone in serum and follicular fluid were (0.72and 0.340 respectively). We can conclude that serum AMH was significant and has more valid predictive value compared tofollicular fluid AMH in predicting pregnancy in women underwent intracytoplasmic sperm injection treatment.

KEYWORDS: AMH, follicular fluid, ICSI, AUC.

INTRODUCTION

Infertility is a complex disorder with important psychologic, economic, demographic and medical implications.[1] Since the birth of the first test tube baby, Louise Brown in July 1978 by Steptoe and Edwards, Assisted reproductive technology (ART) with various treatment protocols for ovarian stimulation developed to be used in infertility treatment.[2] After that, intracytoplasmic sperm injection (ICSI) discovered by Palermo and colleagues in 1991that has the ability achieve higher fertilization and pregnancy rates regardless of sperm characteristics that makes it the most useful procedure yet with which to treat male factor infertility.[3]

A patient's ovarian response to stimulation drug is mainly determined by her ovarian reserve, which comprises the quantity and functional capacity of follicles.[4] Traditional criteria used to predict ovarian response to ovarian stimulation drugs include the patient's age, baseline serum concentration of hormones such as Follicle stimulating hormone (FSH), Luteinizing hormone (LH), Estradiol (E2),and inhibin-b.[5] Anti-Mullerian hormone (AMH), a member of the transforminggrowth factor-β (TGF-β) superfamily, is known to bederived from the granulosa cells of growing follicles (fromthe primary to the small antral follicle stages).[6] AMH is emerging as an important regulator of ovarian function[7], by inhibiting follicle recruitmentand FSH-dependent follicle growth as well as selection, and prevents depletion of the primordial follicle pool.[8] Serum basal levels of AMH significantlydecrease over time in young normo-ovulatory women.[9] Serum basal AMH concentrations are useful for predictingovarian response in women undergoing IVF treatment[10], however, concerning the relationship between serum AMHlevels and the pregnancy rates after assisted reproductiontherapies, there are conflicting data in the literature. Some investigators[11] could not find a correlation betweenbasal AMH levels and pregnancy, whereas others[12] observed a better prognostic value for clinical pregnancy thanother markers.

Follicular-fluid (FF) is the fluid that occupy the antrum of the Graafian follicle and provide an important microenvironment for oocyte growth and development. It containshormones, proteins, sugars, growth factors of the transforming growth factor-beta (TGF-β) superfamily, reactive oxygen species and antioxidants, and other factors.[13] The aim of the present study was to investigate and compare the relationship of AMH, (E2), progesteron(P), levels in serum and FF on the day of oocyte pick up (OPU) with the basal FSH, cycle parameters, prognostic parameters and the outcome of IVF/ICSI treatment.

PATIENTS AND METHODS

Forty women aged between 19-40 years undergoing IVF/ICSI treatment were enrolled in this prospective studyin Kamal Al-Samarrai Hospital (Baghdad /Iraq) from the1st of November 2013 to the 1st of March 2014, and an informed consent of the participants for AMH-measurement and link to their treatment outcome was obtained.The Inclusion criteria were the presence of both ovaries, regular menstrual cycles, no evidence of endocrine disorders (normal TSH, prolactin, and testosterone), A BMI ranging from 18 to 35 Kg/m^2, not on hormone therapy for previous 3 months, and no history of ovarian surgery.While theexclusion criteria were acute or chronic infectious disease of the women or her partner, hypo gonadotrophic hypogonadism,polycystic ovary syndrome,history of one or both oophorectomy and sever psychiatric illness.

Main indications, were: male subfertility (60%), unexplained infertility (22.5%) and tubal pathology (17.5%). All the females involved in this study were examined generally and gynecologicaly after thorough history taking.

All patients were enrolled in long protocol IVF/ICSI cycle, pituitary down regulation was achieved by mid-luteal gonadotropin releasing hormone agonist, triptorelin (Decapeptyl 0.1 mg, Ferring Co, Kiel, Germany)® Ovarian stimulation was initiated onthe second day of the subsequent withdrawal bleeding by recombinant human follicle stimulating hormone (rhFSH) (Gonal F, Merck Serono)®. Transvaginal ultrasound was performed on cycle day 7 and subsequently every 2-3 days till at least 3-4 follicles reach >18 mm diameter. Then, ovulation was induced by administration of recombinant human chorionic gonadotropin (hCG), (Ovitrelle 6500 IU; Merck Serono)®. Transvaginaloocyte retrieval was performed 36 hours later.All patients received luteal phase support of progesterone for 2 weeks(Cyclogest, Barnstable)® starting fromthe day of the OPU. Intracytoplasmic sperm injection (ICSI) was performed for all patients. Embryos weretransferred on day 2 or 3 after OPU.

Collection of serum and follicular fluid

Blood samples were obtained on the day of OPU immediatelybefore the procedure. Sera were obtained after centrifugation for15 minutes at 3000 rpm and stored at -20°C in freezer. Follicular fluid was obtained from the firstretrieved follicle to avoid contamination of blood and flushmedium.Follicular fluid samples were centrifuged for 15 minutes at 3000 rpm and stored at -20°C before the analysis.

Determination of AMH, E2, and P in serum and follicular fluid

Serum and FF AMH levels were determined by using an ultrasensitive ELISA (human AMH ELISA kit; CSB-E12756h Diagnostic System Laboratories, China). Results were expressed as ng/ml. Serum and FF E2 and P concentrations were determined by Cobase411,an automated electro- chemiluminescence technique (ADVIA Centaur CP, Tarrytown, USA).Basal FSHin serum was determined by an automated miniVIDAS system (miniVIDAS made in France by bio Mericux Company).

STATISTICAL ANALYSIS

Data were summarized, presented and analyzed using SPSS version 16 and Microsoft office Excel 2007. Numeric variables were expressed as mean $\pm$ standard error (SE), while nominal variables were expressed as number and percent. Independent sample student t-test was used to compare mean of numeric variables between any two groups. Chi-square test was used to study association between any two nominal variables. Pearson's correlation coefficient was used to evaluate correlation between numeric variables. ROC (receiver operator characteristic) curve analysis was used to calculate cutoff values of variables that predict positive pregnancy outcome, While the diagnostic accuracy of variables, were assessed by the area under the receiver operating characteristic curve (AUC). P-value was considered significant when it was equal or less than 0.05.

RESULTS

The total 40 infertile women were divided into two groups according to ICSI- outcome, the pregnant group 25% (10/40) and the non-pregnant group 75% (30/40). The hormonal parameters (E_2, P and AMH) (Figure 1) and (Figure 2) showed a significant increase($p<0.05$) in the AMH levels in both compartments (serum and FF) and a significant increased ($p<0.05$) in FF $E_2$and P in the pregnant group more than in the non-pregnant group.

Pearson's correlation analysis of associations between the serum AMH levels and the FF (E_2, P, and AMH), clinical (age, BMI, basal FSH, basal E_2), treatment (oocytes (total, MII), fertilization rates and FSH ampoules,) parameters (Table 1), showed significant positive correlation betweenserum AMH andthe FF AMH, oocytesno. (total, MII), and fertilization rates (r=0.380, p<0.022r=0.484, p=0.010; r=0.315, p=0.015; and r=0.361, p=0.019) respectively. On the other hand, there were significant negative association between serum AMH level and the patient age, and the number of the FSH ampoules that are used (r=-0.491, p=0.001; r=-0.365, p=0.013). Table (2) showed the correlation of FF AMH with the serum (E_2, P, and AMH), clinical (age, BMI, basal FSH, basal E_2), treatment (oocytes (total, MII), fertilization rates, and FSH ampoules,) parameters, there was only a significant negative correlation with basal FSH(r=-0.312, p=0.015).

To investigate the diagnostic accuracy of AMH to discriminate between the pregnant and the non-pregnant patients, area underthe receiver operating characteristic curve (AUC) values were determined by ROC analysis, as shown in (Figure 3andTable 3). Using the value of 3.42 ng/ml for serum AMH at the day of oocyte retrieval as the cut-off point had (sensitivity 60%, specificity 86.7%, positive predictive value 60%, negative predictive value 86.67%). While, the value of 5.52 ng/ml for FF AMH as the cut-off point had (sensitivity 60%, specificity 73.3%, positive predictive value 42.85%, negative predictive value 84.61%). Area Under Curve (AUC) for serum and FF AMH was 0.723 and 0.340 with P-value (0.036 and 0.134) respectively, this mean serum AMH was significant P<0.05 and has more valid predictive value compared to FF AMH.

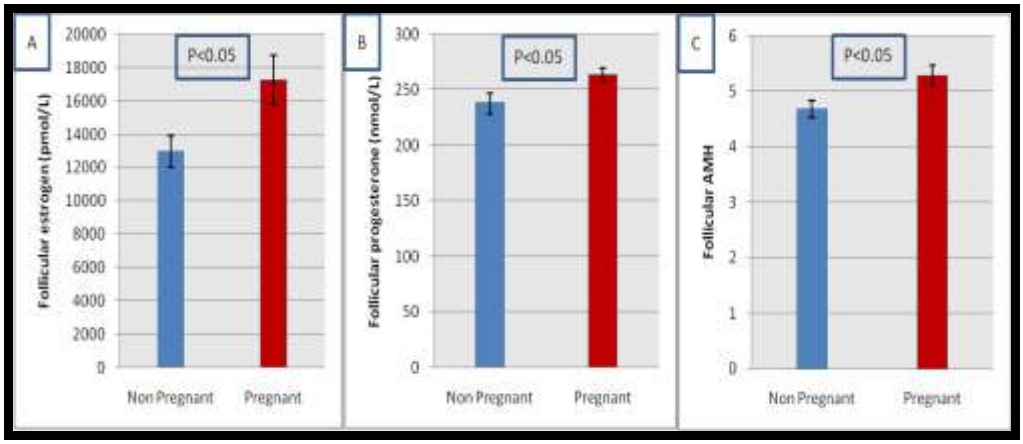

Figure: 1 The Comparison of Mean FF Hormones Levels (A) Estrogen, (B) Progesterone, and (C) AMH Between Pregnant and Non Pregnant Groups. (p>0.05:not significant, p<0.05: significant).

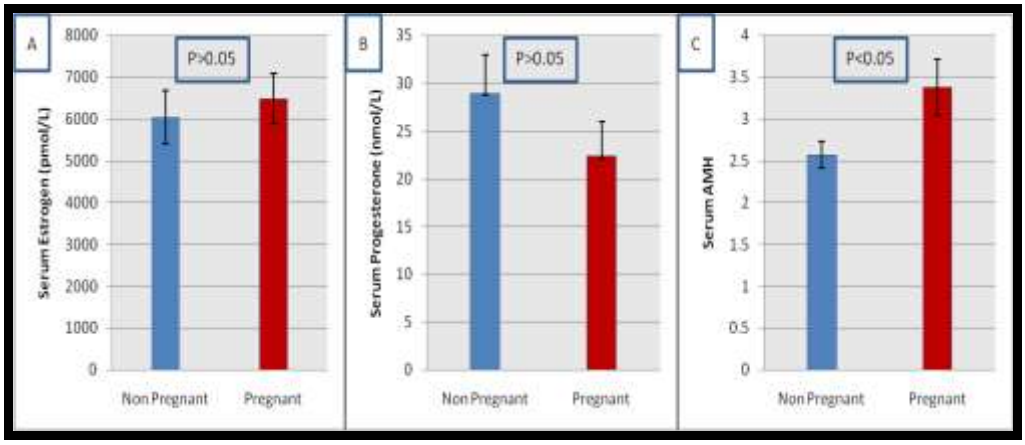

Figure: 2 The Comparison of Mean Serum Hormones Levels (A) Estrogen, (B) Progesterone and (C) AMH Between Pregnant and Non- Pregnant groups. (p>0.05: not significant, p<0.05: significant).

Table 1 Correlation between serum hormones and other parameters in all infertile females.

	Serum AMH	Serum Progesterone	Serum Estrogen
FF estrogen	0.185	-0.097	-0.044
	0.254	0.552	0.789
FF progesterone	0.104	-0.254	-0.107
	0.525	0.113	0.512
FF AMH	0.380	0.180	-0.104
	0.022	0.265	0.525
Age	-0.491	-0.031	0.205
	0.001	0.850	0.204
BMI	-0.007	0.114	0.026
	0.966	0.486	0.872
Pregnancy	0.104	-0.144	0.063
	0.525	0.375	0.699
Total o'ocytes	0.484	0.055	0.078
	0.010	0.738	0.633
MII	0.315	0.146	0.078
	0.015	0.368	0.633
Fertilization rate	0.361	-0.162	-0.103
	0.019	0.319	0.529
Basal Estrogen	0.299	-0.222	0.055
	0.061	0.169	0.735
Basal FSH	0.173	0.083	0.084
	0.285	0.612	0.604
FSH ampoules	-0.365	0.132	0.092
	0.013	0.418	0.574

(FF=Follicular Fluid; BMI=Body Mass Index; FSH=Follicle Stimulating Hormone. MII=Metaphase II). P>0.05: Not significant, *P<0.05:significant.

Table: 2 Correlation between Follicular hormones and other parameters in all infertile females.

	Follicular estrogen	Follicular progesterone	Follicular AMH
Serum AMH	0.185	0.104	0.080
	0.254	0.525	0.622
Serum Progesterone	-0.097	-0.254	0.180
	0.552	0.113	0.265
Serum Estrogen	-0.044	-0.107	-0.104
	0.789	0.512	0.525
Age	0.358	0.071	-0.058
	0.023	0.639	0.724
BMI	-0.062	-0.192	-0.057
	0.705	0.234	0.727
Pregnancy	0.353	0.247	-0.228
	0.025	0.124	0.157
Total o'ocytes	-0.070	-0.117	0.018
	0.667	0.471	0.914
MII	-0.064	-0.131	0.042
	0.693	0.422	0.795
Fertilization rate	-0.001	-0.097	0.078
	0.993	0.551	0.633
Basal Estrogen	-0.078	0.178	0.076
	0.633	0.272	0.641
Basal FSH	0.028	-0.018	-0.312
	0.862	0.911	0.015
FSH ampoules	0.067	-0.007	0.236
	0.682	0.965	0.143

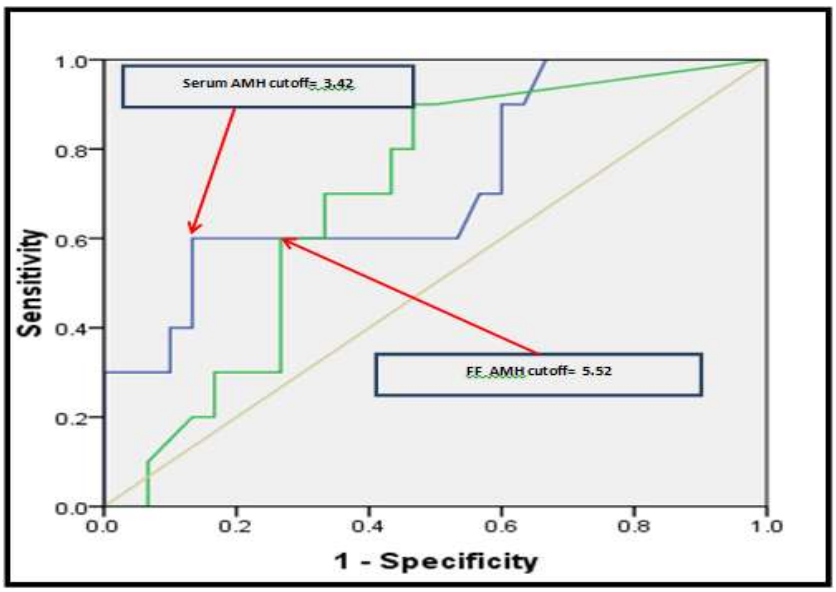

Figure: 3 ROC (receiver operator curve) analysis for calculation of follicular fluid and serum AMH(ng/ml) pregnancy predictive cut-off value.

Table: 3 ROC curve parameters

Parameter	Serum AMH	FFAMH	Interpretation
Cut off value	3.42	5.52	Follicular is higher
Sensitivity	60 %	60 %	Good sensitivity (Both)
Specificity	86.7 %	73.3 %	Good specificity (serum AMH)
PPV	60%	42.85%	Serum is better
NPV	86.67%	84.61%	Serum is better
AUC	0.723	0.340	Acceptable (serum AMH)
P-value	0.036	0.134	Significant for (serum AMH)

FF= Follicular Fluid, PPV=Positive Predictive Values; NPV= Negative Predictive Values; AUC= Area Under ROC Curve. P>0.05:not significant,*****P<0.05:significant.

DISCUSSION

One of the most serious aspects before starting an ART procedure is the initial evaluation of the female's ability to produce healthy and developmental competent oocytes. Serum AMH has become a standard determination to evaluate the ovarian reserve.[14]

It is well known that FSH levels increase and AMH level as well as AFC decrease as women age.[15] Median and mean AMH levels decreased steadily with increasing age from 24 to 50 years of age.[16] The ages of the all study group range between (19-40) years and. This study showed a significant negative correlation between the age and serum AMH. Which mean that with increase in age, there is a decrease in AMH level. This result was in agreement with that reported by J. van Disseldorp, *et al*[17], who found that AMH is able to specify a woman's reproductive age more realistically than chronological age alone. This observation is very important, because it was found by Batista, *et al*[18] that female whose age is more than 34 years old, shows a decline of fertility. Such data may be of value to physicians and their patients who are considering reproductive options. Although it is known that AMH is produced from the ovarian granulosa cells in human, few studies have examined the relationship between FF AMH levels and the clinical outcome.FF AMH concentrations reflect granulosa cell functioning. It is postulated that granulosa cell metabolism and embryogenic competence of the oocyte are interrelated.[19] FF AMH concentrations on the day of OPU may contribute to embryo quality, which in turn yields competent embryos with high implantation potential, and thus results in a high probability of pregnancy.[20] These observations explains the results of the present study that showed a significant increase in AMH levels in both compartments (serum and FF) in the pregnant group than in the non-pregnant. Similarly a study done by Wunder *et al*[21] demonstrated a correlation between FF AMH and pregnancy outcome. In that study, not only the serum, but also the FF AMH levels

were found to be reliable predictors of pregnancy in response to IVF and ICSI. This study showed no significant correlation between FF AMH and no. of oocyte retrieved, M II oocytes, and fertilization rate. These results agree with a study done by Somaya Arabzada *et al*[22] showed a non-significant inverse relationship between intrafollicular AMH levels and oocyte quantity in both PCOS and control groups. Until now, the relationship between FF AMH levels and oocyte quality was uncertain. Also Cupisti *et al*[23] found that AMH levels in individual follicles were inversely correlated with the maturation and developmental potential of oocytes. On the contrary, Takahashi *et al*[24] observed that oocytes were more likely to be fertilized when their follicle was able to produce high levels of AMH, as FF AMH levels from follicles with fertilized oocytes were three times higher than from follicles with non-fertilized eggs in normo-ovulatory women. It is well established that AMH is correlated with the number of oocytes retrieved at the time of aspiration.[25] The results of this study are consistent with this observation. These results showed a significant positive correlation between serum AMH and total number of oocytes retrieved. Furthermore, from a practical standpoint, it shows that AMH is particularly useful to predict ovarian response to stimulation as evidenced by the number of oocytes retrieved. Also Gnoth *et al*[26] demonstrated that AMH was an important screening test for reduced ovarian reserve in women. They proposed that, by using AMH levels ≤1.26 ng/ml, it was possible to identify 97% of women with reduced ovarian reserve and predict low response to gonadotropin stimulation in 88% of cases in groups of comparable age.

With respect to fertilization, this study showed a significant positive correlation betweenserum AMH levelsand fertilization rate. This finding is consistent with some, but not all, studies in the literature. Chapkin et al[20] did not find a significant association between fertilization rate and serum AMH. In a study by Silberstein et al.[27], which included 257 patients, the authors found that AMH levels at the time of HCG administration reflect both ovarian reserve and better embryo morphology.

As well as AMH being a marker of the quantitative ovarian response, this study demonstrated a significant positive correlation between serum AMH and metaphase II oocytes number.consistently, one study demonstrated a positive and significant correlation between serum AMH levels and proportion of M II oocytes.[28] On the other hand, another study showed no correlation between serum day 3 AMH levels and oocyte maturation.[29] In this study, together with the results of other studies in the literature, support the assumption that

serum AMH seems to be a predictor of ovarian reserve as represented by oocyte yield in IVF/ICSI cycles.

Depending on a rough guide for classifying the accuracy of a diagnostic test, ROC curve analysis and AUC results of this study (Table 3 and Figure 3), showed that serum AMH was significant $P<0.05$ and has more valid predictive value compared to FF AMH.

In conclusion, although the concentration of AMH in both serum and FF on the day of oocyte retrieval was significantly higher in the group of patients who became pregnant than in those who did not conceive, but considering the sensitivity, specificity, PPV, NPV and AUC, serum AMH was significant and has more valid predictive value compared to FF AMH.

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