The effect of serum and follicular fluid anti-Mullerian hormone level on the number of oocytes retrieved and rate of fertilization and clinical pregnancy

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ABSTRACT

OBJECTIVE: The objective of this study was to evaluate the relationship between oocyte yield, fertilization, and clinical pregnancy (CP), and anti-Mullerian hormone (AMH) level in serum and follicular fluid during in vitro fertilization treatment.

METHODS: Forty-four infertile women who underwent IVF treatment using multiagonist protocol were included in this study. Baseline level of AMH in serum and follicular fluid was measured on third day of menstrual cycle. AMH level in serum and follicular fluid was then measured again on day of oocyte pick-up. Pearson correlation and binary regression tests were used for statistical analysis. For Type 1 error, p=5% was selected as cut-off value for statistical significance.

RESULTS: Serum AMH level was positively correlated with total number of oocytes retrieved and rate of fertilization and CP (r=0.397, p=0.008; r=0.401, p=0.007; and r=0.382, p=0.011, respectively). There was significantly negative correlation between serum level of follicle-stimulating hormone (FSH) and fertilization rate (r=-0.320; p=0.034), as well as serum FSH level and CP rate (r=-0.308; p=0.042). There were no significant correlations between AMH level in follicular fluid and IVF treatment outcomes.

CONCLUSION: Serum AMH levels may be more reliable for prediction of total number of oocytes retrieved and rate of fertilization and CP than AMH levels in follicular fluid.

Keywords: Anti-Mullerian hormone (AMH); clinical pregnancy; fertilization; follicle-stimulating hormone (FSH); follicular fluid; infertility; in vitro fertilization.

Anti-mullerian hormon (AMH) is a member of the super family of "transforming growth factor-β" and it is synthesized by granulosa cells which develop from primary follicle up to the stage of a large antral folllicle [1]. AMH inhibits selection, and maturation of follicle- stimulating hormone – dependent follicle [2] with resultant prevention of rapid depletion of primordial follicle pool [3].

Up to now, despite the presence of publications indicating the presence of a positive association between serum AMH levels, and clinical pregnancy (CP) rates [4, 5], some research findings have noted lack of any significant correlation between them [6, 7]. In the literature a study asserted favourable contribution of higher AMH levels in follicular fluid on CP rates [7], however a separate publication reported negative effects of these levels on CP rates [8]. Nevertheless, follicular fluid (FF) AMH level is generally thought to be a reliable marker for successful CP in patients who underwent embryo transfer [9]. In some studies a positive correlation was found between serum baseline AMH level, and fertilization rates [5, 10], nevertheless, in another study such a correlation could not be demonstrated. [11]. As deduced from the results of the publications, direct, and indirect effects of serum, and follicular fluid AMH levels on in vitro fertilization (IVF) have not been clarified yet.

In our study, we planned to investigate the effectiveness of serum, and FF AMH levels on the number of oocytes retrieved, fertlization, and rates of CP. According to our research hypothesis, serum, and FF AMH levels may be useful in the prediction of CPs realized after IVF treatment.

MATERIALS AND METHODS

Our study was planned as a prospective cohort research, and consisted of 44 women with the diagnosis of infertilitywho were included in the IVF treatment program between January 2014, and June 2014. The study was approved by the Ethics Committee of A.I.B.U. Faculty of Medicine with the decision #2014–03. The study was perfromed in Infertilty Unit of A.I.B.U. Faculty of Medicine, and Ankelife Test Tube Baby Center. Written, and undersigned informed consent of all study participants were obtained.

Patients with only female-factor infertility were included in the study, while those with male-factor infertility were ruled out. Despite induction of ovulation using high dose FSH, women whose follicles did not mature or oocytes could not be retrieved were excluded from the study During pre-interview with the patients, their medical histories, and previous treatments for infertility were recorded. Following general physical, and gynecologic examinations, for baseline hormonal evaluation immunoenzymatic method was employed on the third day of the menstrual cycle to measure serum FSH, E2, and LH levels. On the same day number of antral follicles, dimensions of the ovaries, and uterus were evaluated ultrasongraphically using 5 mHz with vaginal ultrasound probe (General Electric Alfa Logic 200, USA). Hysterosalpingographic examinations (between 6, and 11. days of the menstrual period) serologic tests (HBsAg, anti-HBs, anti-HIV, anti-HCV), servicovaginal smear, antibiogram of the servical smear, blood typing, whole blood count, and spermiogram (following 3 days of sexual abstinence) were requested.

On the 2. day of the menstrual cycle, follitropin –alpha (Gonal-F) treatment was initiated, and multiple-dose antagonist protocol was administered taking baseline FSH value, body mass index, number of antral follicles into consideration. From the initiation of the treatment up to the day of HCG administration, the patients underwent transvaginal control US examinations daily or on alternate days, and beginning from the 7. day of the menstrual cycle up to the initiation of HCG , the treatment was maintained with the addition of daily doses of 0.25 mg of a GnRH antagonist (Cetrorelix).

After formation of two follicles greater than 18mm in diameter was achieved, 500 mcg chorionic gonadotropin–alpha (Ovitrelle) was injected subcutaneously. Nearly 36 hours following administration of chorionic gonadotropin –alpha, follicular aspiration was performed through vaginal route with the patient in the lithotomy position using oocyte aspiration needle mounted in the transvaginal 17 mm-caliber, and 30 cm- long, single -or dual -lumen US

probe under sterile conditions, and general anesthesia. Follicular aspiration was performed under 125 mmHg negative pressure. Mature oocytes retrieved from the follicular fluid were transferred on IVF-GI, and G2 culture media. On oocyte aspiration day, following 12 hours of overnight fasting, venous blood samples (8 ml) were drawn while the patients were sitting erect. Serum portions of the blood samples were obtained using dry serum separator tubes containing clot activator, and serum separator gel (Vacuette, Greiner Bio-one GmbH, Kremsmünster, Austria). Blood samples were left under room temperature for 30 minutes. Samples of follicular fluid were transferred into absolutely empty tubes. Both follicular fluid, and blood samples were centrifufed under +4°C, and at 1250 g for 15 minutes to separate their serum portions. Samples of serum, and follicular fluid were kept under -80°C till analysis of the supernatant portion of the follicular fluid. Immediately before the analysis, frozen samples were thawed in a stepwise fashion. Recurrent freezing, and thawing procedures were not performed. In pregnant women who may develop severe ovarian hyperstimulation syndrome "agonist triggering" protocol was used to invoke HCG production. Lucrin Daily was injected subcutaneously at a dose of 0.2 mg using a PPD syringe. Thirty-six hours later oocytes were retrieved. The patients who received "agonist triggering" were also subjected to "total freezing".

Serum AMH levels were measured in blood samples drawn just before OPU, and on the day of OPU. AMH levels were measured using, AMH Gen II ELISA commercial kit (A79765, Beckman Coulter, Inc, California, USA). Principle of the test was evaluated based on enzymatically induced two-way immunoassay method. Calibration curve was drawn using a calibrator at 0.16–0,4–1.2–4–10–22.5 ng/ ml concentrations. Detection limit was determined as the lowest value, ie. 0.08 ng/ml. İntra-, and inter-experimental CV (coefficient variation) were 5.4, and 5.6% at a concentration of 4.42 ng/ml, while the corresponding CVs at a concentration of 14.03 ng/ml were reported as 3.6 and 4.5%, respectively. The measurements were performed at 450 nm using Biokit (ELX800, Barselona, Spain) device, and the results were expressed in ng/ml.

Observation of two pronuclei or two bipolar bodies under microscope 18–20 hours after intracytoplasmic sperm injection was accepted as the presence of fertilization. Embryo transfer was performed between 3–5 days after OPU. Embryos at 16-cell blastocyst stage were transferred. The advancement of embryo transfer catheter within the uterine cavity was monitored using an abdominal ultrasound probe so as to place the embryos in the uterine cavity. Following the procedure, catheters were flushed with physiologic saline to determine if any embryos were retained within the catheter, and the presence of embryos was checked under microscope. Ultrasonographic detection of fetal cardiac activity at sixth gestational week was considered as an evidence of clinical pregnancy.

Descriptive statistics were used to define demographic data. Intergroup statistical analysis of CP, and fertilization rates was realized using a parametric test, namely Student t-test. Variable predictive values of data which revealed statistically significant differences between groups, and related clinical outcomes were analyzed with binary logistic regression models. Correlation coefficients were graded as weak (0–0.30), moderate (0.31–0.50), strong (0.51–0.70), and complete (0.71–1.0). P=0.05 was accepted as the cut-off value for statistical significance.

RESULTS

A total of 48 patients were included in the study. One patient with FSH value of 13 IU/ml, and three patients refractory to stimulation of ovulation were excluded rom the study. Embryos were retrieved from 44 infertile patients aged between 24–40 years who were eligible for the study. Embryos were harveste from. 37 (84.9%) patients. In 36.4% (16/44) patients clinical pregnancy could be achieved. A statistically significant difference was detected between mean (±SD) ages of the patients in whom CP was achieved or failed. (30.8±4.8 vs 34.4±4.2 years, p=0.014). Period of infertility among patients who became pregnant was statistically significantly different from that of those who couldn't achieve pregnancy. (7.64±3.0 vs 10.4±2.9 years, p=0.004) (Table 1). Serum AMH levels

TABLE 1. Demographic characteristics of the pregnant, and non-pregnant patients after application of IVF

	CP (+) n=16	CP (-) n=28	p
Age (year)[a]	30.8±4.8	34.4±4.2	0.014*
Body mass index (kg/m²)[a]	29.2±5.4	29.9±3.5	0.844
Duration of infertility (year)[a]	7.6±3.0	10.4±2.9	0.004*

a: Student t-test was used; *: Statistically significant; IVF: In vitro fertilization; CP: Clinical pregnancy.

TABLE 2. Laboratory values, and number of oocytes retrieved of the pregnant, and non-pregnant patients after application of IVF

	CP (+) n=16	CP (-) n=28	p
Serum AMH (ng/ml)[a]	1.52±1.04	0.86±0.58	0.011*
Follicular AMH (ng/ml)[a]	2.1±0.73	1.66±0.82	0.057
Serum FSH (mIU/ml)[a]	7.66±2.81	6.20±1.38	0.042*
Number of oocytes retrieved	11.5±5.3	4.29±2.24	0.001*

a: Student t-test was used; *: Statistically significant; IVF: In vitro fertilization; CP: Clinical pregnancy.

were statistically significantly higher in the group who achieved CP. (mean±SD: 1.52±1.04 ng/ml vs 0.86±0.58 ng/ml; p=0.011). Serum FSH levels in women who became pregnant were statistically significantly higher (ortalama±mean±SD: 7.66±2.81 mIU/ml vs 6.20±1.38 mIU/ml p=0.042). The number of embryos retrieved was statistically significantly different between groups with successful, and failed IVF (mean±SD: 11.45±5.31 vs 4.29±2.24; p<0.001) (Table 2).

Moderately positive correlations were found between serum AMH levels, and both number of oocytes retrieved (r=0.387, p=0.008),and also fertilization rates (r=0.401, p=0.007). However a moderately positive but statistically insignificant correlation was detected between serum AHM levels, and CP rates (r=0.382, p=0.11). A statistically but moderately significant negative correlation was found between serum FSH level, fertilization and CP. A statistically significant but negative correla-

tion was detected between serum FSH level, and fertilization, while serum FSH level was statistically significantly but moderately correlated with CP rates (r=-0.320 p=0.034 vs r=-0.308, p=0.042, respectively) (Table 3). Binary logistic regression analysis revealed that the number of oocytes retrieved was the only parametre which increased CP rates (Table 4).

DISCUSSION

Since reduced ovarian reserve is the only significant cause of infertility, serum AMF level, and the number of antral follicles are the most important parametres during the evaluation process of the ovarian reserve before treatment of patients scheduled for IVF [6, 9]. Because of the presence of a relationship between serum AMH concentration,

TABLE 3. Correlations between serum, and follicular fluid hormone levels, and the number of oocytes retrieved, fertilization, and CP rates

	Number of oocytes retrieved		Fertilization rates		CP rates	
	r	p	r	p	r	p
Serum AMH (ng/ml)	0.397	0.008*	0.401	0.007*	0.382	0.011*
Follicular AMH (ng/ml)	0.144	0.351	0.250	0.102	0.128	0.408
Serum FSH (mIU/ml)	-0.280	0.065	-0.320	0.034*	-0.308	0.042*

r: Pearson correlation coefficient; *: Statistically significant; CP: Clinical pregnancy; AMH: Anti-mullerian hormone; FSH: Follicle-stimulating hormone.

Table 4. Binary logistic regression analysis of factors effecting clinical pregnancy rates

	Odds ratio	Odds ratio incl. 95% Confidence Interval		p
		Min.	Max.	
Age (year)	0.984	0.786	1.232	0.886
Serum AMH	1.358	0.190	9.729	0.761
Serum FSH	0.432	0.092	2.024	0.287
NOOR	9.993	1.295	77.088	0.027*
Constant	0.000	–	–	0.146

NOOR: Number of oocytes retrieved; *: Statistically significant (p<0.05); FSH: Follicle-stimulating hormone; AMH: Anti-müllerian hormone.

and the number of of antral follicles [12], measurements of AMH levels are being used in clinical practice to determine the number of antral, and pre-antral follicles [13]. FSH eliminates, inhibitory effects of AMH on follicular development during IVF treatment [14].

In our study, we made a correlation analysis serum AMH levels, the number of oocytes retrieved, fertilization, and CP rates in patients who received IVF treatment, and found a moderately positive correlation among these parametres. However since according to correlation analysis more than one parametre positively induced increases in CP rates, we applied binary logistic regression analysis, and disclosed that only the number of oocytes retrieved was effective on increased CP rates.

Gnoth et al. have demonstrated that AMH is an important parametre to be used as a screening test for the determination of reduced ovarian reserve [15]. The same team found that AMH levels below 1.26 ng/ml had identified women with lower ovarian reserve (number of oocytes ≤4) with 97% sensitivity. While; AMH levels below 0.5 ng/ml could detect women with very low ovarian reserve (number of oocytes, ≤2) with 88% sensitivity [15]. With aging, AMH levels in circulation, number, and quality of oocytes decrease [16, 17]. Number of oocytes retrieved was found to be higher in wom-

en with higher AMH levels when compared with age-matched women with relatively lower AMH levels [18]. As an outcome of our study, we found a positive correlation between serum AMH levels, and the number of of oocytes retrieved. Although publications supporting the outcome of our study which reportedly indicated benefit of using serum AMH values in the prediction of the number of oocytes retrived during IVF cycles [2, 4, 5, 9, 19, 20, 21] in a study by Takahashi et al. could find absence of a correlation between AMH, and the number of antral follicles [11]. The reason of inability to detect a correlation between serum AMH levels, and the number of oocytes retrieved might be related to failure to collect oocytes from follicles smaller than 17 mm in diameter. Besides measurement of serum AMH levels during oocyte retrieval process might be the reason for these totally different outcomes. [11]. Hypothetically, elimination of inhibitory effect of AMH on follicular development by the action of FSH [14] might play a role in the reduction of AMH levels after treatment with FSH.

Some publications have demonstrated the presence of a positive correlation between serum AMH concentration, and CP rates [4, 5, 9] while others have reported lack of any correlation between CP rates, and serum AMH levels [6, 7, 8]. Wunder DM et al. (2008) detected significantly higher AMH concentrations both in serum, and follicular fluid of the patients who achieved clinical pregnancy [9]. A positive correlation was reported between AMH levels in follicular fluid, implantation, and CP. Besides determination of follicular fluid AMH levels has been reported to be of use in the identification of potentially implantable embryos. However in a study by Metha et al. the authors detected higher CP rates in patients with lower follicular fluid AMH levels [8]. Any difference between post-IVF CP levels could not be found between women aged 40 years with serum AMH levels lower, and higher than 1 ng/ml [18]. Detection of live births among women with very low (<0.4 ng/ml) serum AMH levels [19] has suggested potentially effective roles of factors other than serum AMH levels (especially quality of oocytes, and embryos) on CP rates. In two literature publications, it has been reported

that serum AMH levels did not contribute to the determination of quality of oocytes [22], and also they were not effective in the prediction of quality of embryos [11, 22]. In another study, the authors claimed that serum, and follicular fluid AMH levels were correlated with the quality of oocytes, and embryos [7]. Follicular fluid AMH levels can increase quality of oocytes, and embryos due to their roles in the metabolism of granulosa cells. Also in our study, although a statistically significantly positive correlation existed between serum AMH levels, and CP rates, we could not detect any relationship between follicular fluid AMH levels, and clinical pregnancy. However in another study a correlation was found between follicular fluid AMH levels, and CP rates. [7]. In various studies, diverse effects of AMH levels on CP may be related to quality, and number of oocytes, and embryos, endometrial or uterine factors, and problems arising during embryo transfer. We think that serum AMH levels are not singly, and directly effective on increased CP rates.

In our study, we found a positive correlation between serum AMH level, and fertilization rates. In some studies a positive [5, 7, 10] and a strong [23] correlation has been detected between fertilization, and baseline AMH levels. Lower fertilization rates were detected in patients with lower serum AMH levels relative to those with higher serum AMH levels [6]. However, Takahashi et al. could not find a significant difference between serum AMH levels in women with and without fertilization potential [11].

In our study any correlation could not be found between follicular fluid AMH levels, and fertilization. Also in a study by Capkin any correlation between follicular fluid AMH levels, and fertilization was not reported [4]. In another study, 3.42 fold higher AMH levels in follicular fluid samples of the fertilized group were reported [11]. However in a study by Mehta [8], higher fertilization rates, and improved embryo quality were indicated in the infertile group with lower follicular fluid AMH levels In a study by Majumder (2010) baseline serum AMH levels were reported to be more important in the determination of the number of embryos when compared with other parametres [20].

Decreased ovarian reserve may be associated with lower CP rates, decreased number of oocytes, and embryos retrieved, and consequently reduced number of embryos of higher quality. Relationship between ovarian reserve, and lower CP rates constituted a significant problem in the context of our study design, however herein the most important issue of the debate, should be the ways of predicting the presence of an embryo of higher quality

In conclusion, despite existence of a strong correlation between CG, and serum AMH levels, among parametres we investigated, only number of oocytes retrieved increased CP rates. Positive correlation between serum AMH levels, and number of oocytes retrieved suggested a possibly indirect role of serum AMH levels in the prediction of CP rates. At the same time for the clarification of the controversial literature information on serum, and follicular fluid AMH levels concerning stages of IVF treatment (oocyte retrieval, fertilization, and embryo) due to diverse outcomes of the studies performed, we suggest that further multicentered randomized controlled studies involving greater number of participants should be performed.

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