Serum and follicular fluid Anti-Mullerian hormone concentrations at the time of follicle puncture and reproductive outcome

Follikül aspirasyon zamanı serum ve follikül sıvısında Anti-Mullerian hormon konsantrasyonu ve reprodüktif sonuçlar

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Abstract

Objective: The objective of the study is to determine and compare the levels of Anti-Mullerian hormone (AMH) and estradiol (E2) in serum and follicular fluid (FF) on the day of oocyte pick up (OPU) with the cycle parameters and the outcome of in vitro fertilization (IVF) treatment.

Material and Methods: The long stimulation protocol was used in 37 (86%) women; the microdose flare-up protocol was used in 6 (14%) women. Concentrations of AMH and E2 were measured in serum and FF of 43 women undergoing IVF treatment on the day of OPU.

Results: Significant positive associations were observed between serum AMH concentrations and the total number of oocytes retrieved (r=0.343, p=0.024). Serum AMH and FF AMH levels on the day of OPU were significantly increased in the group of women who achieved clinical pregnancy (p=0.017, p=0.028). For serum AMH, a cut-off level of 1.64 ng/ml was used for the prediction of clinical pregnancy; for FF AMH, a cut-off level of 3.8 ng/ml was used for the prediction of clinical pregnancy. Serum AMH and FF AMH levels were significantly and positively correlated with implantation rate (r=0.401, p=0.008; r=0.317, p=0.039). No significant correlation was found between serum and FF AMH concentrations and fertilization rate.

Conclusion: Serum AMH and FF AMH concentrations are positively correlated with implantation and clinical pregnancy rates.

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Key words: AMH, E2, follicular fluid, IVF

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Özet

Amaç: Çalışmanın amacı oosit aspirasyon günü serum ve follikül sıvısında Anti-Mullerian hormon (AMH) ve estradiol seviyelerini saptamak ve siklus parametreleri ve in vitro fertilizasyon tedavisi sonuçları ile karşılaştırmaktır.

Gereç ve Yöntemler: Otuz yedi (%86) hastada long stimulasyon, 6 (%14) hastada mikrodoz flare-up protokol uygulanmıştır. Oosit aspirasyon günü in vitro fertilizasyon tedavisi alan 43 hastanın serum ve follikül sıvısında AMH ve estradiol konsantrasyonları saptanmıştır.

Bulgular: Serum AMH konsantrasyonları ve toplanan oosit sayıları arasında anlamlı pozitif ilişki saptanmıştır (r=0.343, p=0.024). Oosit aspirasyon günü serum AMH ve follikül sıvısı AMH seviyeleri klinik gebelik saptanan hastalarda anlamlı olarak yüksek bulunmuştur (p=0.017, p=0.028). Serum AMH için 1.64 ng/ml, follikül sıvısı için 3.8 ng/ml klinik gebeliğin öngörülmesinde cut-off değeri olarak saptanmıştır. Serum AMH ve follikül sıvısı AMH seviyeleri ile implantasyon oranı arasında anlamlı pozitif korelasyon saptanmıştır (r=0.401, p=0.008; r=0.317, p=0.039). Serum ve follikül sıvısı AMH konsantrasyonları ve fertilizasyon oranları arasında anlamlı ilişki bulunmamıştır.

Sonuç: Serum ve follikül sıvısı AMH konsantrasyonları implantasyon oranları ve klinik gebelik oranları ile ilişkilidir.

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Anahtar kelimeler: AMH, E2, follikül sıvısı, IVF

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Introduction

Anti-Mullerian hormone (AMH), a member of the transforming growth factor-β (TGF-β) superfamily, is known to be derived from the granulosa cells of growing follicles (from the primary to the large antral follicle stage) (1). The TGF-β superfamily members inhibin A, inhibin B, and activin are related to ovarian follicle development as well as AMH, which is emerging as an important regulator of ovarian function, especially follicle selection and development (1, 2). Serum basal AMH levels correlate with the number of antral follicles and age (3). Serum basal levels of AMH significantly decrease over time in young normoovulatory women, whereas other markers associated with ovarian aging do not change (4). AMH has been shown to inhibit FSH-induced follicle growth in female mice (5). The period of AMH production coincides with the period of oocyte meiotic arrest (6). Within the human ovary, AMH inhibits follicle recruitment and FSH-dependent follicle growth as well as selection (2, 7), and prevents depletion of the primordial follicle pool (8).

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These inhibitory actions of AMH on early follicle development are overcome by the FSH treatment during *in vitro* fertilization (IVF). After gonadotropin treatment during IVF, the AMH concentrations in small and large antral follicles positively correlate with granulosa cell responsiveness to FSH (9).

Serum basal AMH concentrations are useful for predicting ovarian response in women undergoing IVF treatment (10); however, concerning the relationship between serum AMH levels and the pregnancy rates after assisted reproduction therapies, there are conflicting data in the literature. Some investigators (11-14) could not find a correlation between basal AMH levels and pregnancy, whereas others (15-18) observed a better prognostic value for clinical pregnancy than other available markers. The putative correlation of AMH in the follicular fluid (FF) and reproductive outcome has been investigated in several studies.

The aim of the present study was to investigate and compare the relationship of AMH and estradiol (E2) levels in serum and FF on the day of oocyte pick up (OPU) to the cycle parameters, prognostic parameters and the outcome of IVF treatment.

Materials and Methods

Patient characteristics and IVF procedure

Forty-three women aged between 18-40 years and undergoing IVF treatment were studied prospectively. Inclusion criteria included the presence of both ovaries, normal pretreatment hormonal status, and the absence of other endocrine pathologies, such as thyroid and adrenal disorders or hyperprolactinemia. Women with polycystic ovary syndrome were not included in the study. Informed consent was obtained from all women. The study was approved by the institutional review board of the hospital.

The long stimulation protocol was used in 37 (86%) women; the microdose flare-up protocol was used in 6 (14%) women. For the patients treated using the long protocol, pituitary down-regulation was achieved by gonadotropin-releasing hormone (GnRH) agonist, leuprolide acetate (Lucrin daily injection, Abbott, Turkey). Gonadotropin stimulation was initiated on the third day of the subsequent withdrawal bleeding. For the patients treated using the microdose flare-up protocol, a GnRH agonist was administered starting on the 24th day of the menstrual cycle, with the addition of gonadotropins on the 26th day. Dose adjustments were performed on the 4th day of stimulation and thereafter according to the sonographic findings and serum E2 levels. The women were administered human chorionic gonadotropin (HCG, Ovitrelle 250 mcg, Serono, Turkey) when at least 3 follicles had reached a diameter of >18 mm. Transvaginal oocyte retrieval was performed 36 hours later. All patients received luteal phase support of progesterone (Crinone 8% vaginal gel, Serono, Turkey) daily starting from the day of the OPU. Intracytoplasmic sperm injection (ICSI) was performed for all patients. Fertilization was assessed using an established pronuclei (PN) scoring system. Embryos were transferred on day 3 or 5 after OPU. One or 2 embryos were transferred depending on the woman's age and embryo quality. Embryo transfers were performed under transabdominal ultrasonographic guidance. Biochemical pregnancy was established when serum BHCG level >20 IU/l was found on day 12 after embryo transfer. Clinical pregnancy was defined as a positive serum BHCG result with ultrasound evidence of a gestational sac and fetal heart beat at 6 weeks from the date of embryo transfer.

Collection of serum and follicular fluid

Blood samples were obtained on the day of OPU immediately before the procedure. Sera were obtained after centrifugation and stored at -80°C. Follicular fluid was obtained from the first retrieved follicle to avoid contamination of blood and flush medium. Care was taken to avoid contaminated samples. Follicular fluid samples were centrifuged for 15 minutes at 3000 rpm and stored at -80°C before the analysis.

Determination of AMH and E2 in serum and follicular fluid

Serum and FF AMH levels were determined by using an ultrasensitive ELISA (AMH ELISA kit; Diagnostic System Laboratories, Texas, USA). Results were expressed as ng/ml. Serum and FF E2 concentrations were determined by an automated chemiluminescence technique (ADVIA Centaur CP, Tarrytown, USA).

Statistical analysis

Data analysis was performed using SPSS for Windows, version 11.5 (SPSS Inc., Chicago, IL, United States). Whether the distributions of continuous variables were normal or not was determined using the Shapiro-Wilk test. Continuous variables are expressed as mean±standard deviation or median (minimum-maximum), where applicable. The mean differences between groups were compared using Student's t-test. Otherwise, the Mann-Whitney U-test was applied for the evaluation of the median values. Degrees of associations between continuous variables were tested using Spearman's correlation analysis. Whether the effects of both serum and FF AMH on clinical pregnancy were statistically significant was evaluated by multiple logistic regression analysis adjustment for age and AFC. A p-value less than 0.05 was considered statistically significant.

Results

The study group consisted of 43 women with a mean age of 30.1±4.2 years. Thirty-one patients (72%) had unexplained infertility, and 12 patients (28%) had male factor infertility. Clinical and cycle parameters and FF and serum concentrations of AMH and E2 of the patients with respect to the pregnancy outcome are shown in Table 1. No difference was observed between the groups that did or did not achieve clinical pregnancy with respect to age, BMI, baseline E2, FSH, AFC, amount of gonadotropin used, number of MII oocytes or fertilization rate. Serum AMH and FF AMH levels on the day of OPU were significantly increased in the group of women who achieved clinical pregnancy (p=0.017, p=0.028). To investigate the correlation of

Table 1. Clinical and cycle parameters, FF and serum concentrations

Variables	Clinical Pregnancy (n=14)	No pregnancy (n=29)	p value
Clinical and cycle parameters			
Age (years, mean±SD)	29.3±4.4	30.5±4.2	0.249
BMI (kg/m^2, mean±SD)	24.4±3.7	26.0±4.9	0.299
Basal E2 (pg/ml,range)	52.5 (4-119)	52 (8-79)	0.816
Basal FSH (ıu/l, mean±SD)	6.2±1.59	6.6±2.65	0.657
AFC (n, range)	13 (6-24)	11 (0-24)	0.126
Dose of gonadotropins (IU, range)	1225 (900-4113)	1872 (600-7500)	**0.020**
MII oocytes (n, range)	7 (2-15)	7 (1-16)	0.516
Fertilization rate (%, range)	63.3 (9.1-100.0)	50.0 (12.5-100.0)	0.567
FF hormone levels			
AMH (ng/ml, range)	4.1 (0.35-13)	1.87 (0.32-16)	**0.028**
E2 (pg/ml, range)	544.6 (171.7-849.8)	422.2 (16,6-1385.0)	0.162
Serum hormone levels on the day of OPU			
AMH (ng/ml, range)	1.87 (0.04-4.15)	0.35 (0.03-14)	**0.017**
E2 (pg/ml, range)	1118.5 (59.1-2015.2)	712.4 (66.1-1984.1)	0.058

BMI: body mass index, AFC: antral follicle count, AMH: Anti-Mullerian hormone, FF: follicular fluid. Statistically significant p values are shown in bold

serum and FF AMH and clinical pregnancy, cut-off values were calculated using ROC analysis. For serum AMH, a cut-off level of 1.64 ng/ml was used to predict clinical pregnancy (sensitivity 71.4%, specificity 69%, positive predictive value 52.6%, negative predictive value 83.3%). For FF AMH, a cut-off level of 3.8 ng/ml (sensitivity 64.3%, specificity 79.3%, positive predictive value 60%, negative predictive value 82.1%) was used.

When multivariant logistic regression analysis was used for the prediction of clinical pregnancy, serum and FF AMH concentrations lost their statistical significance when adjusted for age and AFC. Serum and FF AMH were not the only independent factors for the prediction of clinical pregnancy.

Correlations between serum and FF AMH and E2 concentrations and clinical and hormonal parameters are shown in Table 2. Serum AMH concentrations were positively and significantly correlated with FF AMH concentrations (r=0.638, p<0.001). There was a significant positive correlation between serum AMH and E2 concentrations (r=0.429, p=0.004). Significant positive associations were observed between serum AMH concentrations and AFC, total number of oocytes retrieved, MII oocytes and implantation rate (r=0.476, p<0.001; r=0.343, p=0.024; r=0.389, p=0.01; and r=0.401, p=0.008, respectively). However, negative correlations were observed between serum AMH levels, age and the amount of gonadotropins used (r=-0.311, p=0.043; r=-0.596, p<0.001). FF AMH levels were significantly and positively correlated with AFC and implantation rate (r=0.343, p=0.025; r=0.317, p=0.039) and negatively correlated with baseline FSH levels (r=-0.333, p=0.029). No significant correlation was found between serum and FF AMH concentrations and fertilization rate.

Discussion

The assessment of the ovarian reserve before an IVF treatment is important to identify both poor- and high-responder patients. Poor ovarian reserve also predicts decreased fertility rates, although the relationship between declining ovarian reserve and decreased fertility rates remains incompletely understood (19). Several parameters have been proposed for the estimation of ovarian reserve including basal or clomiphene citrate-stimulated FSH levels, follicular phase inhibin B levels, ovarian volume, AFC and ovarian stromal blood flow. However, the associated predictive values remain controversial. In addition, it remains a challenge to identify young women with normo-ovulatory cycles but with a low ovarian reserve. Gnoth et al. 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 (20). Consistent with the study by Wunder et al. a significant correlation of serum AMH levels with ovarian response as expressed by the number of oocytes retrieved is observed in the present study (21). In a study by Elgindy et al. that investigated longitudinal changes in AMH levels during different phases of the menstrual cycle in patients undergoing IVF treatment, no significant differences were found between AMH levels taken on day 3, on the day of ovulation and 7-8 days later (midluteal phase). The number of oocytes retrieved was found to be significantly correlated with midluteal AMH, day 3 AMH and ovulatory AMH (22). In addition, Fanchin et al. observed

Table 2. Correlations between serum and FF AMH and E2 levels and clinical and hormonal parameters

	FF AMH		Serum AMH		FF E2		Serum E2	
	r	p	r	p	r	p	r	p
FF AMH	1.000	.	0.638	<0.001	0.218	0.161	0.062	0.694
Serum AMH on day of OPU	0.638	<0.001	1.000	.	0.170	0.275	0.429	**0.004**
FF E2	0.218	0.161	0.170	0.275	1.000	.	0.221	0.154
Serum E2 on day of OPU	0.062	0.694	0.429	0.004	0.221	0.154	1.000	.
Age	-0.174	0.264	-0.311	0.043	0.031	0.845	-0.277	0.073
AFC	0.343	0.025	0.476	<0.001	-0.171	0.273	0.165	0.291
Basal FSH	-0.333	0.029	-0.281	0.068	0.184	0.236	-0.065	0.679
Basal E2	-0.062	0.695	0.056	0.723	0.142	0.363	0.214	0.168
Dose of gonadotropins	-0.264	0.087	-0.596	<0.001	-0.087	0.579	-0.376	**0.013**
Total oocytes retrieved	0.072	0.647	0.343	0.024	0.072	0.648	0.568	**<0.001**
MII oocytes	0.143	0.361	0.389	0.010	0.055	0.725	0.586	**<0.001**
Fertilization rate	-0.148	0.344	0.029	0.854	0.047	0.767	0.125	0.424
Implantation rate	0.317	0.039	0.401	0.008	0.136	0.386	0.239	0.123

Statistically significant p values are shown in bold. Positive correlations (r>0), negative correlations (r<0), FF: follicular fluid, AMH: Anti-Mullarian hormone, OPU: oocyte pick-up, E2: Estradiol, FSH: Follicule stimulating hormone

a remarkable lack of variation of serum AMH levels between two distinct points in the menstrual cycle (day 3 and the day of OPU), showing that AMH levels remain stable throughout the cycle independent of FSH (9, 23). Our results, together with those of other studies in the literature, support the assumption that serum AMH levels can predict the number of oocytes that will be yielded by an IVF cycle. We also found a positive correlation between serum AMH and serum E2: the expected serum E2 concentrations are associated with the number of oocytes retrieved.

With respect to fertilization, we did not find a significant association between fertilization rate and serum or FF AMH levels. This finding is consistent with some, but not all, studies in the literature. Takahashi et al. (24) reported that the FF AMH levels of fertilized patients were 3.42 times higher than those of non-fertilized patients. However, they found no correlation between serum AMH and high-quality embryos. These results indicate that serum AMH levels did not reflect high-quality fertilization. In a study by Silberstein et al. (25), which included 257 patients, the authors found that AMH levels at the time of HCG administration reflect both ovarian reserve and better embryo morphology. However, Fanchin et al. and Wunder et al. (9, 21) found similar fertilization rates regardless of the AMH concentrations in serum or FF. Due to contradictory reports regarding fertilization, oocyte quality and embryo morphology in the literature, these issues deserve further investigation.

The results of this study indicate that serum and FF AMH levels are good predictors of implantation and clinical pregnancy. Cut-off values for both serum and FF AMH were calculated for the prediction of clinical pregnancy. Cut-off concentrations for serum AMH and FF AMH were found to be 1.64 ng/ml (71.4% sensitivity, specificity 69%) and 3.8 ng/ml (64.3% sensitivity, 79.3% specificity), respectively. High FF AMH levels constitute a useful marker for implantation. FF AMH concentrations reflect granulosa cell functioning. It is postulated that granulosa cell metabolism and embryogenic competence of the oocyte are interrelated (9). 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. Our results are consistent with the results of a study by Fanchin et al. (9) They found that high clinical pregnancy and implantation rates correlated with FF AMH levels and concluded that FF AMH measurements could help to identify the embryos that are most likely to achieve implantation in IVF cycles. Silberstein et al. (25) found that AMH levels at the time of HCG administration (≥2.7 ng/ml) portended improved oocyte quality as reflected by higher implantation rates and a trend toward improved clinical pregnancy rate. In the study by Nelson et al. (26), which investigated the value of serum AMH in the prediction of live birth and ovarian response to stimulation, it was found that plasma AMH is an accurate predictor of live birth and strongly correlated to the risk of excessive response to ovarian stimulation.

There are several clinical implications of these findings. Serum AMH seems to be a predictor of ovarian reserve as represented by oocyte yield in IVF cycles. This may allow development of individualized and optimized treatment strategies prior to the first cycle of ovarian stimulation. It is also important for the counseling of couples for making decisions after a failed IVF cycle, especially in patients with low ovarian reserves.

Recent studies have shown that a low response to ovulation induction is associated with early menopause (20, 27, 28). AMH seems to be a promising parameter for the occurrence of menopausal transition (20, 29). Thus, AMH may serve as a screening test for diminished ovarian reserve, especially in patients with regular cycles (3).

The results of our study, which were confirmed by the study of Wunder et al. (21), demonstrated a positive correlation between serum and FF AMH concentrations. A finer determination of AMH in FF is not necessary because serum concentrations of AMH yield similar data.

Because FF AMH concentrations are positively correlated with implantation and clinical pregnancy rates, FF AMH measurement during embryo transfer may be an additional parameter aside from embryo morphology with which to distinguish the embryos that are most likely to achieve implantation and thus clinical pregnancy.

In conclusion, the results of the study indicate that serum AMH and FF AMH concentrations are positively correlated with implantation and clinical pregnancy rates. In addition, serum AMH concentrations are associated with the number of oocytes and the number of mature oocytes retrieved.

Conflict of interest

No conflict of interest was declared by the authors.

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