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Original Article

Evaluation of serum anti-mullerian hormone as a biomarker of early ovarian aging in young women undergoing IVF/ICSI cycle

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Abstract: Objective: To determine whether or not the level of serum anti-Müllerian hormone (AMH) is related to early ovarian aging in young women (< 35 years of age) undergoing in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI) cycles. Design: Retrospective cohort study. Setting: An IVF laboratory in a university hospital in Taiwan. Patient (s): 70 young women (< 35 years of age) with low level of serum AMH (< 2 ng/ml) and 104 young women with level of serum AMH (≥ 2 ng/ml) who underwent IVF/ICSI cycles between January 2011 and November 2012 were enrolled. Intervention (s): None. Main outcome measure (s): Number of oocytes, fertilization rate, embryo quality, cycle cancellation rate, clinical pregnancy/abortion rate, and perinatal/infant outcomes. Results: The clinical pregnancy rate per transfer was favorable (low AMH group vs. normal AMH group [47.2% and 47.9%]) for women < 35 years of age, including women with a low serum AMH. Similarly, the live birth rate per transfer (low AMH group vs. normal AMH group [37.7% and 35.4%]) and perinatal outcomes were also comparable between the two groups. A significantly higher cycle cancellation was noted in the low AMH group than the normal AMH group (24.2% vs. 7.6%). Conclusion: Although early ovarian aging should be taken into consideration for young and infertile women with low AMH level than expected, our results suggest that low serum AMH level may suggest early ovarian aging in accelerated oocyte loss only, but may not fully represent "early ovarian aging" based on the favorable outcomes of pregnancy.

Keywords: Anti-müllerian hormone, early ovarian aging, pregnancy outcomes, perinatal outcomes, low ovarian reserve

Introduction

Ovarian aging is thought to be dominated by a gradual decrease in the number of follicles with oocytes of diminished quality, and menopause is the final destination. The onset of ovarian aging presents as an accelerated loss of ovarian follicles and normally occurs in the late thirties [1]. Faddy et al. offered a mathematical model that shows this accelerated decline of fertility takes nearly 13 years to result in menopause [2]. Therefore, women who become menopausal by 45 years of age will have begun an accelerated decline of fertility at 32 years of age. On the basis of a fixed interval between menopause and an accelerated decline in the ovarian reserve, it was hypothesized that up to 10% of women in the general population may undergo "early ovarian aging" [2, 3]. Such women will be asymptomatic and have regular menstrual cycles, thus making early ovarian aging difficult to diagnose. Early ovarian aging negatively affects female fertility, primarily through a sub-optimal quantity and quality of oocytes. The possible etiopathogenesis of early ovarian aging includes deficient initial follicle number, accelerated follicle atresia, and follicle dysfunction [4]. There are no reliable tools to screen for early ovarian aging until the symptoms of premature ovarian insufficiency are manifest.

Anti-Müllerian hormone (AMH) is good biomarker with which to predict ovarian response before utilizing assisted reproductive technology (ART) [5-7]. The serum AMH level offers an alternative method for evaluating the non-growing follicle pool and may play a role in assessing early ovar-

ian aging. Previous studies have shown that serum AMH appears to be a better parameter with which to assess the extent of ovarian follicle depletion and possibly be a better marker for diagnosing premature ovarian insufficiency (POI) in women with varying degrees of hypogonadism (ranging from imminent ovarian failure to full-blown premature ovarian failure) [5, 6]. Some studies have suggested that AMH will provide an index of age at menopause [7-10].

The serum AMH level declines with age and the peak serum AMH level occurs at 24.5 years of age [11]. Extensive use of the serum AMH level before ART causes controversy in pre-treatment counseling, especially in young infertile women with low serum AMH levels. For infertile young women with low ovarian reserve relative to what is expected, it is confusing that low ovarian reserve not only suggests a low number of oocytes, but also the tip of ice about early ovarian aging.

The aim of this study was to evaluate whether or not serum AMH levels are associated with early ovarian aging in young women undergoing IVF/ICSI cycles.

Materials and methods

Patients

Data were collected from infertile couples who utilized ART and underwent transcervical embryo transfer between January 2011 and November 2012. To evaluate the association between a low serum AMH level and early ovarian aging, we focused on women ≤ 35 years of age.

The inclusion criteria were as follows: (1) female patients ≤ 35 years of age; (2) controlled ovarian stimulation with long or short protocols of pituitary down-regulation until oocyte retrieval; (3) female patients who had detailed medical records after pregnancy and delivered at Chang Gung Memorial Hospital Kaohsiung Medical Center; and (4) complete perinatal medical records for the newborns of these patients in our center until 1 year of age. The exclusion criteria were as follows: (1) history of ovarian surgery; (2) history of chemotherapy or radiation therapy; and (3) incomplete medical records or lost to follow-up. All of the charts were retrospectively reviewed by one physician.

The serum AMH levels were measured at the time of initial presentation and prior to all treatment cycles. Previous reports have shown that the median serum AMH level at 35 years of age in healthy women is 3.127 ng/ml [12] or 2.58 ng/ml [13]. An AMH cut-off level of 2 ng/ml was chosen because it represents a group of women with an AMH level below 25th~50th percentile [12, 13] and the cut-off level is also easy to use clinically.

One hundred seventy-four young women (≤ 35 years) undergoing IVF/ICSI treatment with a down-regulation protocol were enrolled in the current study. There were 70 young women in the low AMH group (AMH < 2 ng/ml) and 104 young women in the normal group (AMH ≥ 2 ng/ml). The cycle outcomes were then analyzed and compared between the two groups. The follow-up for pregnancy, and perinatal/infant outcomes 1 year after delivery were completed in December 2013.

The serum AMH levels were measured using a diagnostics system laboratories

(DSL) assay (Beckman Coulter, Inc., Brea, CA, USA). The assay for serum AMH involved an enzymatically-amplified two-site immunoassay. According to the manufacturer's manual, the sensitivity of the assay is 0.14 ng/ml and the intra- and inter-assay coefficients of variation were 12.3% and 14.2%, respectively.

The Institutional Review Board of the Ethics Committee of Chang Gung Memorial Hospital approved this study.

Controlled ovarian hyperstimulation and oocyte retrieval

The protocol for controlled ovarian hyperstimulation followed a standard down-regulation regimen, as we have reported in previous studies [14, 15]. Briefly, all women received the long or short protocol of pituitary down-regulation with leuprolide acetate (Lupron®; Takeda, Tokyo, Japan), depending on ovarian reserve, patient age, baseline serum FSH concentration, and previous response to ovarian stimulation. Exogenous FSH was administered at an initial dose of 150-300 IU, with further doses given according to the individual's ovarian response, as analyzed by the serum estradiol (E2) level and ultrasonographic follicular growth monitoring.

When the lead follicle reached 16-18 mm in diameter, leuprolide acetate and FSH were discontinued, and hCG was administered. Oocyte retrieval was performed by transvaginal ultrasound-guided follicle aspiration 36-38 hours after hCG administration. A single team of embryologists coordinated all procedures, thereby ensuring that both the culture protocols and the embryo assessment were standardized.

Oocyte preparation, assessment of fertilization, and embryo culture

Standard IVF or ICSI procedures were used to achieve oocyte fertilization. Gametes were fertilized in universal IVF medium (MediCult, Jyllinge, Denmark), and fertilization was evaluated 16-18 hours after IVF or ICSI.

Normal fertilization was defined as zygotes with two pronuclei (2PN) after IVF or ICSI. Fertilization failure was defined as zero oocytes achieving zygote stage with 2PN. The normal fertilization rate was reported as a percentage of the total number of oocytes undergoing IVF or ICSI. The zygotes with 2PN were cultured for another 48 hours in 100 μL microdrops of G1 medium (Vitrolife, Göteborg, Sweden) under oil. Any zygotes with 2PN were cultured until the day of embryo transfer. G1™ medium (Scandinavian IVF Science) was used for culture of embryos on days 1-3. G2™ medium (Scandinavian IVF Science) was used for culture of embryos from days 3-5 or 6. The zygotes were scored according to the Z-scoring system [15]. Zygotes with an equal number of nucleolar precursor bodies aligned on the PN junction were designated Z-1. Zygotes with an equal number of nucleolar precursor bodies that were scattered were designated Z-2. Zygotes with an inequality of number or alignment were designated Z-3, and zygotes with PN of unequal size or PN that were not aligned in a central position within the oocyte were designated Z-4. The Veeck's morphologic grading system was adopted for day 3 embryo scoring. Pre-embryos with eight cells and blastomeres of equal size and no cytoplasmic fragments were scored as grade I. Pre-embryos with eight cells and blastomeres of equal size and minor cytoplasmic fragments were scored as grade II. Pre-embryos with eight cells and blastomeres of distinctly unequal size and no cytoplasmic fragments were scored as grade III. Pre-embryos with four-to-eight cells and moderate-to-heavy fragmentation were scored as grade IV. Pre-embryos with few blastomeres of any size and major and complete fragmentation were scored as grade V. We defined "good embryos" as zygotes that had a Zscore of grade I and the Veeck's scoring system on day 3 embryos of grade I.

Luteal support and confirmation of pregnancy

Luteal phase supplementation of micronized progesterone was started on the day of oocyte retrieval, and 5000 IU of hCG was administered on day 6 after oocyte recovery in all patients. Luteal phase support continued until the day pregnancy was confirmed by detecting hCG in the urine. The study population received Crinone 8% gel (90 mg daily; Fleet Laboratories Ltd., Watford, UK) or Utrogestan vaginal capsules (200 mg 4 times daily; Piette International Laboratories, Belgium). Clinical pregnancy was determined by identifying a gestational sac at 7 weeks gestation with transvaginal ultrasonography. If conception had occurred, micronized progesterone supplementation was provided for an additional 4 weeks.

Maternal, infant, and perinatal outcome survey

The medical records of the live births from IVF/ICSI cycles underwent a detailed review.

Adverse infant outcomes in this study included major birth defects, fetal death, preterm birth, fetal growth restriction, an Apgar score < 7 at 5 minutes, intracranial hemorrhage, seizures, sepsis, and the need for mechanical ventilation. Birth defects were grouped into the following categories: congenital heart defects, gastrointestinal anomalies, musculoskeletal anomalies, and chromosomal anomalies. Only birth defects with major morphologic or functional importance were considered as anomalies. Preterm birth was defined as birth at a gestational age < 37 weeks completed weeks and fetal growth restriction (small-for-gestational age) was defined as a birth weight less than the third percentile in the corresponding standard population stratum. Fetal death was defined as an intrauterine death at a gestational age> 20 weeks or a birth weight > 500 g.

Statistical analysis

Continuous data were summarized as the mean ± standard deviation. The Mann-Whitney

Table 1. Comparison of patient characteristics, IVF/ICSI cycle parameters, and pregnancy outcomes in 174 young women (< 35 years of age) with low serum anti-Müllerian hormone level (< 2 ng/ml) and normal level (≥ 2 ng/ml)

	Serum AMH (< 2 ng/ml)	Serum AMH (≥ 2 ng/ml)	*P* value
No. of cycles	70	104	
Age of female partners (y)	32.3±2.46	31.4±3.27	0.063
Age of male partner (y)	35.2±4.52	34.8±4.72	0.619
Serum AMH, ng/mL	1.14±0.57	5.38±3.42	< 0.001[a]
Baseline FSH (mIU/ml)	7.84±4.4	6.67±3.87	0.249
Body mass index (kg/m^2)	22.3±3.86	21.6±2.86	0.216
Duration of infertility (y)	3.83±1.9	3.61±2.1	0.668
Infertility, % (n)			
Primary	77.1% (54/70)	62.5% (65/104)	0.047[a]
secondary	22.8% (16/70)	37.5% (39/104)	
Days of FSH treatment	9.25±1.97	8.90±1.33	0.161
Endometrial thickness on hCG day	1.27±0.33	1.35±0.25	0.100
Ampules of 75 IU FSH	31.36±10.47	28.2±6.70	0.025[a]
E2 on hCG day (pg/mL)	1967±1599	3079±1530	< 0.001[a]
P (ng/mL) on hCG day	1.03±0.9	1.04±0.45	0.961
Mean no. of oocytes retrieved	4.55±3.58	8.54±4.11	< 0.001[a]
Mean no. of mature oocytes retrieved	2.43±1.86	4.15±2.24	< 0.001[a]
2PN formation rate, % (n)	80.5% (247/307)	83.8% (681/813)	0.213
Abnormal fertilization and fertilization failure rate, % (n)	19.5% (60/307)	16.2% (132/813)	0.213
Cycle cancellation rate, % (n)	24.2% (17/70)	7.6% (8/104)	0.004[a]
Causes of cancellation:			
No oocytes % (n)	29% (5/17)	12.5% (1/8)	0.624
Fertilization failure % (n)	41.1% (7/17)	50% (4/8)	1.000
Embryo arrest and others % (n)	29.5% (5/17)	37.5% (3/8)	1.000
Zygote score % (n)			
Z1	40.4% (100/247)	40.5% (276/681)	1.000
Z2	41.7% (103/247)	40.7% (277/681)	0.821
Z3	16.2% (40/247)	15.3% (104/681)	0.758
Z4	1.6% (4/247)	3.5% (24/681))	0.191
Mean no. of embryos transferred	1.84±1.23	2.32±0.84	0.001[a]
Cleavage embryo formation rate, % (n)	92.5% (248/268)	98.8% (711/720)	< 0.001[a]
Mean embryo score per transferred embryo	2.35±1.68	3.19±1.57	0.001[a]
Mean no. of D3 good embryo	1.11±1.17	1.89±1.34	< 0.001[a]
D3 good quality embryo in transferred embryos % (n)	55.8 % (76/129)	65.7% (159/242)	0.072
Implantation rate, % (n)	25.6% (33/129)	24.4% (59/242)	0.802
Clinical pregnancy rate per transfer, % (n) transfer, % (n)	47.2% (25/53)	47.9% (46/96)	1.000
Abortion rate per transfer, % (n)	7.5% (4/53)	7.2% (7/96)	1.000
Live birth rate per transfer, % (n)	37.7% (20/53)	35.4% (34/96)	0.859

Note: Values are mean ± SD; % (*n*) or median (interquartile range) [a]*P* < 0.05.

rank sum test was used for comparisons of means, and the Fisher's exact test was used for comparisons of proportions. The significance of group differences was evaluated using the Student's t-test. All statistical analyses were performed with Statistics Package for Social Sciences software (SPSS, version 17.0; SPSS, Inc., Chicago, IL, USA). All *P* values were two-sided, and a *P* value < 0.05 was considered to be statistically significant.

Table 2. Demographic characteristics of live birth children from low serum anti-Müllerian hormone level (< 2 ng/ml) group and normal level (≥ 2 ng/ml) group

	Serum AMH (< 2 ng/ml)	Serum AMH (≥ 2 ng/ml)	*P* value
Birth parameters			
All deliveries (n)	25	42	
CS delivery % (n)	70% (14/20)	41.1% (14/34)	0.052
Multiple gestational rate % (n)	25% (5/20)	23.5% (8/34)	1.000
Female/male, n (%)	11 (44%)/14 (56%)	24 (57%)/18 (43%)	0.324
Gestational age (range), wk	37.3±2.0 (32~40)	38.1±1.5 (35~40)	0.382
Prematurity, % (n) (gestationalage < 37 wks)	28% (7/25)	21.4% (9/42)	0.496
Birth weight (range), gm	2765 ± 511 (1810~3780)	2760±479 (1850~353)	0.854
Apgar 1 min < 5 or 5 min < 7, n (%)	0% (0)	0% (0)	0.566
Birth weight < 2500 gm, % (n)	28% (7/25)	33.3% (12/42)	1.000
Neonatal respiratory distress syndrome, n (%)	8% (2/25)	0% (0/42)	1.000
Minor congenital anomalies % (n)	8% (2/25)	9.5% (4/42)	1.000
Congenital heart defect	0	1	
Musculoskeletal system	0	1	
Brain	2	2	
Major congenital anomalies % (n)	0% (0/25)	2.3% (1/42)	
Gastrointestinal tract anomalies (esophageal fistula)	0	1	

Note: Values are mean ± SD; % (*n*) or median (interquartile range) $^{a}P < 0.05$.

Results

Patient characteristics

One hundred seventy-four patients (< 35 years of age) and with available serum AMH data were collected. The pregnancy and perinatal outcomes of patients < 35 years of age are presented in **Table 1**. After dividing the patients according to the AMH reference (AMH < 2 ng/ml and AMH ≥ 2 ng/ml), a negative trend of cycle parameters was observed toward the low AMH group. The mean AMH level in the low AMH group was 1.14±0.57 ng/dl and the level at 35 years of age represented approximately the 10th percentile from the reference of serum AMH in the general female population [12, 13, 16]. As shown in **Table 1**, a higher percentage of primary infertility was associated with higher doses of FSH, lower E2 level on the hCG day, a decreased number of oocytes, and fewer mature oocytes, which were all statistically significant in the low AMH group. The cycle cancellation rate was significantly higher in the low AMH group (24.2% vs. 7.6%).

In addition, a lower cleavage embryo formation rate (92.5% vs. 98.8%), lower mean embryo score (2.35 ± 1.6 vs. 3.19 ± 1.5), fewer embryos to transfer (1.84 ± 1.2 vs. 2.32 ± 0.8), and fewer day 3 embryos of good quality (1.11 ± 1.2 vs. 1.89 ± 1.3) were also statistically significant. The proportion of day 3 good embryos among the transferred embryos was similar between the two groups.

Comparison of pregnancy and perinatal outcomes

The clinical pregnancy rate per transfer (47.2% vs. 47.9%), implantation rate (25.6% vs. 24.4%), abortion rate (7.5% vs. 7.2%), and live birth rate per transfer (37.7% vs. 35.4%) had comparable results between the two groups.

We further analyzed the perinatal outcomes (**Table 2**). There were 25 live births from the low AMH group and 42 live births from the normal AMH group. There was a trend for cesarean delivery (CS) in the low AMH group, but there was not a significance difference in the CS rates between the two groups. The obstetric and perinatal outcomes between the two groups were comparable until one year after delivery. Two neonates in the low AMH group (choroid plexus and subependymal cysts) and four neonates in the normal group (pulmonary artery stenosis, torticollis, intraventricular hemorrhage and quadrigerminal cysts) had minor congenital anomalies. There were no major anomalies in the low AMH group and one neonate in the normal group with a major anomaly (tracheal-esophageal fistula).

Discussion

Currently, age, antral follicle count (AFC), and AMH level are generally acknowledged as the best predictors for ovarian reserve [17]. The value of the AMH level in the prediction of pregnancy has been investigated in various studies, but the results have been inconsistent. A number of studies have demonstrated associations between the AMH level and oocyte quality, fertilization rate, blastocyst development, embryo quality, pregnancy outcome, and live birth rate [18-21], but were not confirmed in other studies [22-24]. The latest systematic review and meta-analysis of the literature showed that the AMH level, independent of age, has an association with predicting live birth after ART [25, 26]; however, prediction of the qualitative aspects of assisted reproduction by measurement of the AMH level has not been fully reported [27].

With the widespread use of the serum AMH level in clinical practice, counseling dilemmas have occurred. In fact, female age has been shown to be the only independent predictor of ovarian reserve. For these young infertile women with low ovarian reserve relative to what is expected, it is uncertain whether or not a low ovarian reserve is an indicator of a low number of oocytes or early ovarian aging as well. Although a previous report [28] demonstrated that low AMH levels in healthy, young women in their mid-20 s does not predict reduced fecundability, the significance of a low AMH level, if any, is unknown in infertile women.

When ovarian aging occurring, it has also not been determined if loss in oocyte number and decrease in the quality of oocytes coincide. Clinically, no reliable tools are available to screen and diagnose early ovarian aging until the symptoms are manifest. In addition to the difficulty in early diagnosis, we also could not distinguish the causes of diminished ovarian reserve from a normal distribution of the entire population or early ovarian aging in these young, infertile women with low ovarian reserve. Therefore, ART is another means by which the existing issues about reproductive aging can be clarified. Due to lack of uniform criteria, a poor response to ovarian stimulation is considered to be a sign of early ovarian aging, making this entity a retrospective diagnosis [29]. Our study showed that the poor ovarian response in the low AMH group included a higher dose of exogenous FSH, fewer oocytes, less mature oocytes, a lower embryo score, and a lower cleavage embryo formation rate. The process of ovarian stimulation appeared to be more “resistant” in the low AMH group. In addition, poorer embryo scores suggest that low serum AMH levels in these young women may be correlated with a reduced quality of oocytes. Our results do not fully support previous reports that AMH achieving this just through its primary relationship with oocyte yield [6]. As expected, the prevalence of poor ovarian responses increases with age, and in women > 40 years of age the prevalence is > 50% [30]; however, young age does not completely protect against poor ovarian responses [31]. Based on a prior study [30], the poor ovarian response rate (cycle cancellation or ≤ 3 oocytes) is approximately 10% between 30 and 35 years of age; however, the poor ovarian response rate in our low AMH group was paradoxically higher, even at the same age (24.2%). The possible explanation for the higher percentage of poor ovarian response rate in our study is that these young patients with low ovarian reserve may constitute those with early ovarian aging, and therefore the clinical presentation in the low AMH group was similar to poor responders.

Although a poor ovarian response was noted in the low AMH group, the pregnancy outcome remained relatively good if the cycle was not cancelled and continued to embryo transfer. Based on our transfer policy, approximately 2 embryos were transferred to young women < 35 years of age. Therefore, the proportion of good day 3 embryos for transfer was also comparable in each group and resulted in comparable pregnancy outcomes. Prior studies focusing on the same issues also concluded that young, but low responders represent a unique subset because age protects low responders from the deleterious effects of a poor ovarian response [32, 33]. Furthermore, the embryos from the low serum AMH groups were also competent and achieved favorable live birth rates with good perinatal outcomes. No higher abortion or preterm delivery rates were noted in the low AMH group, hence we agree with a prior report that concluded premature ovarian aging is not a significant contributory factor for recurrent miscarriage [34]. These findings are also consistent with reports from Reichman et al. [35]; specifically, patients with extremely low levels of AMH can achieve reasonable treat-

ment outcomes and should not be precluded from attempting IVF solely on the basis of the serum AMH level.

Another important finding from our study is that a loss in oocyte number and a decrease in oocyte quality may not occur simultaneous with early ovarian aging. The quality of oocytes may decrease more slowly and manifest as a poor ovarian response and lower embryo score than expected. Nevertheless, the overall quality of oocytes in these young women still remained competent due to favorable pregnancy and perinatal outcomes, suggesting that ovarian aging may be not simultaneous with respect to quantity and quality. A decrease in the quantity of oocytes may be an earlier sign suggestive of ovarian aging.

Our study offers more reliable information with which to counsel young women with low ovarian reserve before treatment. A low serum AMH level does not appear to represent an appropriate marker for withholding fertility treatment. Detailed counseling about the potential for a poor ovarian response and a high cycle cancellation is necessary for these young patients to reduce the psychological burden of treatment failure or cancellation. If IVF/ICSI treatment proceeds to embryo transfer, a favorable pregnancy outcome with autologous oocytes occurs.

One of the limitations of the present study was the relative small sample size; a larger sample size is needed to confirm the results of this study. Additionally, long-term observations of the reproductive outcomes in women with low serum AMH levels are also needed to elucidate the relationship between ovarian aging and diminished ovarian reserve.

In conclusion, the use of the serum AMH level as a biomarker of ovarian reserve has major implications for the preventive management of age-related decreased fertility and general health risks associated with early-onset menopause in conditions, such as premature ovarian insufficiency. For these young females with a lower than expected ovarian reserve, a low ovarian reserve test may suggest early ovarian aging, but may not fully represent “early ovarian aging”. Clinically, it is necessary to remind women with low AMH levels that they may have a shorter reproductive span and family planning is thus recommended.

As demonstrated in this study, the live birth rate is favorable (low AMH vs. normal group [37.7% vs. 35.4%]) for women < 35 years of age with low serum AMH. Rather than withhold treatment, aggressive management of infertile issues is needed to counsel patients while they are still young because a low serum AMH level possibly results from early ovarian aging.

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Disclosure of conflict of interest

None.

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