COMPARATIVE ANALYSIS OF SERUM ANTI-MULLERIAN HORMONE, SERUM FOLLICLE STIMULATING HORMONE AND SERUM ESTRADIOL IN THE PREDICTION OF OVARIAN RESERVE.

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ABSTRACT: A number of ovarian reserve tests are being used to determine oocyte reserve to help predict in vitro fertilization outcome. This study was undertaken to find if any correlation exists between levels of Anti-Mullerian (AMH), follicle stimulating hormone (FSH) and estradiol (Et), with the ovarian reserve. Purposive sampling was undertaken. Infertility patients seeking treatment at Jaipur Fertility and Medical Research Centre, a tertiary care unit of Mahatma Gandhi Medical College and Hospital from May-Dec2011 were included in the study. In all 105 patients were studied. Serum levels of hormones were determined at day 3 and ovarian follicle response was assessed on day of administration of HCG. Results revealed that Serum AMH was significantly correlated (r 0.302 $p<0.001$) with higher follicular response while FSH (r 0.0283 $p>0.1$) and Et (r 0.999 $p>0.1$) were not found to have any such association with ovarian reserve in the study.
KEY WORDS: ovarian reserve, AMH, FSH, estradiol, IVF.

INTRODUCTION: Infertility, whether male or female, is defined as the inability of a couple to achieve conception after a year or more of regular, unprotected intercourse. The World Health Organization (WHO) estimates that approximately 8-10% of couples experience some form of infertility problems. Based on these estimates and on the current world population, 72.4 million women are currently infertile; of these, 40.5 million are currently seeking infertility medical care [1]. Good ovary reserve is necessary for successful conception. Assessment of ovarian reserve is routinely undertaken in all cases seeking assisted reproductive techniques (ART) with the aim to identify women with a high risk of producing a poor response to ovarian stimulation and/or a very low probability of becoming pregnant through in vitro fertilisation (IVF), as well as those who still produce enough oocytes to have a good chance of becoming pregnant even if female age is advanced.

The biochemical markers indicative of ovarian reserve (OR) are serum levels of Anti-Müllerian Hormone (AMH), Follicle Stimulating Hormone (FSH) and estradiol (Es). Their levels are determined on the third day of the menstrual cycle. The determination of the quantity of the follicular pool would allow the prediction of women who may under-respond or over-respond to controlled ovarian hyper stimulation protocols in ART programs [3]. In clinical terms, all IVF programs have criteria for cancellation of IVF cycles that do not produce an adequate number of follicles to go through with egg retrieval procedure followed by IVF. Such patients, whose prevalence is estimated to be 5–20% of the artificial reproductive treatment population, have been referred to as low responders and having diminished ovarian reserve.

Day 3 post menstruation level of FSH and serum Es biomarkers are considered indirect measures of ovarian reserve, as they require stimulation from either a feedback inhibition or stimulation loop. As women and their follicles age, the amount of FSH secreted increases due to the lack of responsiveness of the ovary [4]. As day 3 FSH levels climb, it is indicative of a diminished ovarian reserve. Estradiol is a product of the granulosa cells and is indicative of follicular activity. An increased estradiol level early in the menstrual cycle suggests that follicular development is in advanced stage which is inappropriate for day 3 [4].

AMH is an example of direct measures of ovarian reserve as these hormones are produced during specific stages of follicular development, rather than by follicular stimulation. AMH is produced by the granulosa cells of pre-antral and small antral follicles. Follicular growth is modulated by AMH, which inhibits recruitment of follicles from the primordial pool by modifying the FSH sensitivity of those follicles [6]. AMH is considered to be reflective of the non-FSH dependant follicular growth. AMH is the only marker of ovarian reserve that can be tested in follicular as well as luteal phase [7].

From the chemical point of view AMH is a peptide homodimer of molecular weight 140 kDa, consisting of two identical glycoprotein subunits, connected by disulfide bridges. These receptors for AMH are transmembrane heteromeric proteins, composed from two subunits, denoted Type I and II. As all the receptors for growth factors of the TGF beta family, they do not use G-proteins and possess intrinsic kinase activity. Type II (better subunit) binds specifically the ligand leading thus to activation of the Type I, the intracellular part of which acts as threonine kinase. Activation of the latter starts a signal cascade resulting in a respective biological response.

Serum AMH levels are in the literature usually given in mass units (ng/ml or pg/l), which are used also in this article, though according to a good laboratory practice, more accurate is usage of S.I. units (pmol/l). The respective converting factor is [pmol/l] = 7.14 x [ng/ml. As mentioned already, AMH levels in healthy women decline continually with age reaching non-measurable values after menopause. Since the end of nineties the immunological kits are available for AMH determination in body fluids (serum, plasma and also a follicular fluid). They usually apply sandwich systems with two specific antibodies to AMH: the first bound to a solid phase (in most instances the tube walls or titration wells), and the second labeled with biotin, to which the streptavidin-labeled enzyme is bound (usually horse-radish peroxidase). After addition of the substrate (a chromogenic conjugate which affords a coloured product after cleavage by the enzyme) its absorbance is measured. The inserted streptavidin-biotin system increases the specificity and sensitivity of the method, which amounts approximately 1 ng/ml.

MATERIAL AND METHODS: This study was carried out at Jaipur Fertility and Medical Research Centre, which is the tertiary care unit of Mahatma Gandhi Medical College and Hospital Jaipur. One hundred and five patients of infertility entering their first IVF cycle from May to Dec2011 were the studied by purposive sampling. All patients were subjected to a detailed history taking which included duration of infertility, prior infertility workup and treatment taken and significant past, medical and surgical history.

In order to be included, the candidate had to meet the following criteria: regular ovulatory menstrual cycles, normal BMI (18–25 kg/m2), no current hormone therapy, and no current or past diseases affecting ovaries or gonadotrophin or sex steroid secretion, clearance or excretion and adequate visualization of both ovaries at transvaginal ultrasound scanning. Women who had polycystic ovarian syndrome were excluded.

On day-2 of the menstrual cycle, morning blood sample around 10 AM were taken for measurement of serum levels of AMH, FSH and E2.Serum AMH levels were determined using an ultrasensitive enzyme-linked immunosorbent assay (ELISA). Serum levels of FSH and E2 were determined using an automated multi-analysis system with chemiluminescence detection. Trans-vaginal ultrasonography was used to estimate the number of follicles in the ovary. Human chorionic gonadotrophin (HCGH) was used to bring about follicle rupture prior to performing oocyte retrieval. The resultant ovarian response was monitored by transvaginal ultrasound. Follicular response on day of human chorionic gonadotrophin administration was recoded. On the basis of number of follicles, the patients were divided into two groups i.e. poor responders i.e. those patients having less than 4 follicles on ovulation study by ultrasonography and good responders i.e. those patients having more than 4 follicles on ovulation study. Scope of the study objects was limited to only correlating hormone levels with ovarian reserve and not to correlate with age as the difference in age was negligible among subjects.

The results were tabulated and statistically analyzed. The sample mean (X), standard deviation (SD), and standard error of the mean as well as the range were obtained for numerical variables. For non-numerical variables, the frequency, distribution and percentage were calculated. The student's (t) test was used to test the significance of the difference between the two independent means as per normal distribution. The Chi square test ($\chi 2$) was used to test whether the distribution of a certain phenomenon among two or more groups was equal or not.

Correlation coefficient (r) was used to find out a correlation between two parameters where (r) value will be either +1 (positive correlation), 0 (no correlation) or −1 (negative correlation). To evaluate significance of a test, we determined its sensitivity, specificity, +ve predictive value (PPV) and −ve predictive value (NPV). The probability (P) value was calculated and a P-value <0.05 was considered statistically significant.

RESULTS: A total of 105 oocyte retrievals were performed. The mean age of patients was 31.61 years. On ultrasonography, the number of patients showing good response (>4 follicles) and poor response (<4 follicles) in relation with serum levels of the hormones is shown in Table 1.

TABLE 1: Showing Serum levels of Hormones in relation with Ovarian reserve response

Hormone levels	Good responders	Poor responders
Mean AMH (ng/l) and SD	3.923 .+ 3.007	1.274_ ⁺1.098

Mean FSH (IU/l) and SD	5.015 _ + 2.16	3.500 + 1.612
Mean Et (IU/l) and SD	40.927 +24.466	28.000 +10.58

The mean serum basal levels of AMH, FSH and Es were higher in good responders and low in poor responders (Table 1 and Table 2).

TABLE 2: Correlation and significance levels of Hormones with ovarian reserve

Hormone	Correlation factor (r value)	Significance value*
AMH	0.302	P<0.001
FSH	0.028	p>0.1
Es	0.084	p>0.1

*t value at df103 and also normal curve analysis values

However the level of AMH was significantly high among good responders as compared to low responders (p<0.001) while no such difference was noticeable between levels of FSH and Et vis a vis ovarian reserve. The validity of the various tests of hormone assay also confirms the similar findings (Table 3).However uniformly low NPV suggest bigger sample need to confirm the findings.

TABLE 3: Validity of the serum Hormones levels at threshold values

Hormone levels	Sensitivity	Specificity	Positive predictive value	Negative Predictive value	Significance
AMH TV 1.8ng/L	73.91	66.67	94.45	24.24	P<0.001
FSH TV 10 iu/L	0.00	100.00	7.67	7.61	P<0.01
Es TV 50 iu/L	25.77	75.00	92.59	7.69	p>0.1

DISCUSSION: The ability of the ovaries to respond to gonadotropin with adequate follicular development has been referred to as ovarian reserve. Ovulation stimulation allows the retrieval of more than one mature oocyte, thus increasing the chance of embryonic implantation during an IVF (in vitro fertilization) cycle. The assessment of ovarian reserve can be beneficial to patients undergoing assisted conception treatment. As suggested above it helps in determining the dose of the medication to induce multiple follicular developments [8]. Patients with reduced reserve will require higher doses of medication and those with sensitive ovaries require a more measured approach.

Anti-Mullerian hormone (AMH) has been suggested as an indicator of ovarian response and been found to decline with advanced female age [9]. Our study showed a significant correlation of AMH and ovarian response to follicular stimulation. Earlier studies have even suggested that AMH is the single best predictor of poor response for ART [10]. As AMH may permit the identification of both the extremes of ovarian stimulation, a possible role for its measurement may be in the individualization of treatment strategies in order to reduce the clinical risk of ART along with optimized treatment burden [11,12].

The fact that AMH is secreted without dependence on other hormones, particularly the gonadotropins, and that AMH is expressed at a constant level, independent of cycle day make AMH very attractive as a direct measurement of OR [13]. The freedom that AMH testing offers both clinicians and patients by allowing collections to be performed on any day during the menstrual cycle is a vast logistical advantage over other biomarkers. It also helps predict excessive response to ovarian hyperstimulation [14]. A recent study has also concluded that AMH is able to specify a woman's reproductive age more realistically than chronological age alone [15]. However some studies have showed that AMH cannot predict pregnancy [16]. Even though the true place of AMH in reproductive medicine remains to be fully elucidated, undoubtedly AMH measurement allows an assessment of ovarian reserve with several advantages over other biochemical and biophysical markers [17]. In fact, even reports by oncologists indicate that it may be feasible to assess ovarian reserve in cancer patients by emerging markers such AMH which may be more sensitive than FSH measurements [18].

In our study, FSH did not show a significant correlation with ovarian reserve. Studies by Brodin and colleagues showed that low FSH level probably reflects a well-preserved ovarian reserve and is associated with good success rates at IVF/ICSI although they suggested it to be combined with high LH to increase the predictive value [19]. However, patients with mildly elevated levels (10-15IU/L) seem to have a good probability of getting pregnant. Also, between cycle fluctuation in day 3 FSH levels make OR estimation difficult [4]. Additionally, an increased day 3 FSH level is considered a late indicator of marked decreased fertility potential. Our findings suggest that it is not to essential to evaluate a sub fertile patient routinely by FSH values nor is it justified to exclude patients with regular cycles from treatment on the basis of FSH value alone. Increased estradiol level early in the menstrual cycle suggests that follicular development is in an advanced stage that is inappropriate for day 3 [4]. However, estradiol levels can be increased for two very different reasons. Estradiol levels can become elevated due to the occurrence of rapid folliculogenesis. Alternatively, an increased estradiol level can be due to an enhanced OR, such as in women afflicted with polycystic ovarian syndrome (PCOS), where a small amount of estradiol is being produced by a large number of antral follicles [20].

Regarding day 3 estradiol, no significant correlation was detected by us between elevated levels ≥ 50 pg/ml and follicular response. In contrast, this level was associated with higher cancellation and lower pregnancy rates independent of FSH levels in an earlier study [21]. Thus, the role of estradiol remains at best debatable. Analyses of our results show that among the biochemical ORTs known to date, AMH is most suitable for relevant clinical use. Serum AMH levels may reflect ovarian response better than the usual hormone markers [22]. Accuracy of testing on the basis of biochemical markers for the occurrence of poor ovarian response appears to be modest. Simultaneous evaluation of a combination of tests could be used as a marker of diminished ovarian reserve and a sensitive predictor of response to ovarian stimulation in patients undergoing in vitro fertilization treatment.

CONCLUSION: Amongst the biochemical markers AMH, FSH and estradiol; AMH level is a reliable and a good predictor of ovarian reserve while level of FSH has a poor association and levels of estradiol have no association with the ovarian reserve in our study.

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