

The Role of Ovarian Reserve Tests in predicting Intra-Cytoplasmic Sperm Injection Cycles Outcome

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ABSTRACT---- *Aims: 1. to assess the ovarian reserve (OR) for subpopulations of Iraqi infertile couples seeking Intra Cytoplasmic Sperm Injection (ICSI) treatment. 2. To correlate the OR markers with ICSI outcome. Study design: a prospective randomized controlled trial. Patients and methods: Eighty -seven participants were enrolled during their attendance to fertility center of Al-Sadar Medical Teaching city in Al- Najaf Province; twenty subjects as a control group (n=20), and 67 as a study group (n=67). Ovarian reserve parameters were assessed and ICSI were performed. We correlate the OR markers with: No. of follicles obtained, No. of mature oocytes, Fertilization rate, Cleavage rate, Embryo scoring, No. of embryo transferred and Pregnancy rate (biochemical pregnancy). Results: Age resulted in significant negative correlation with No. of follicles, No. of mature oocytes at metaphase II (MII), No. of two pronuclear phase (2PN),No. of best quality embryos grade one and two (G1+G2),and to the total No. of embryos transferred ;while basal serum level of anti mullerian hormone (AMH) follows age as it shows positive significant correlation with The No. of MII oocytes obtained , No. of best quality embryos ,it correlate positively and significantly to the level of serum AMH. Basal serum level of Follicle stimulating hormone (FSH) shows significant negative correlation with No. of follicles aspirated and to No. of MII oocytes. Basal serum levels of estradiol and inhibin B didn't show significant correlation with ICSI outcomes. None of the OR markers correlates with pregnancy rate.Conclusion: Age, Anti-Mullerian hormone and follicle stimulating hormone are better markers than inhibin B, estradiol or antral follicle count in predicting the outcome of ICSI cycles.*

Key words: Ovarian reserve, ovarian reserve markers, Antimullerian hormone, Intracytoplasmic sperm injection outcomes.

1. INTRODUCTION

The primary function of the female ovary is the production of a mature and viable oocyte capable of fertilization, subsequent embryo development and implantation. Even before birth, a woman's eggs begin to diminish in number. The number of eggs decline as the woman ages (1). In general, ovarian age parallels chronological age, but since that is not always the case; it is vitally important for reproductive endocrinologists routinely perform some screening tests to assess their patients' ovarian reserve. This is particularly true for women over the age of 35, where egg loss will dramatically increase (2, 3). Ovarian reserve: It is basically an estimate of how many oocytes are left in the ovaries, and that often translates into how many eggs we are going to be able to work with over the course of any given monthly treatment. By estimating the OR, a prediction of the remaining reproductive lifetime could be assessed as well as the likely success of assisted reproductive techniques (ART) such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) (4). Because more women are delaying childbearing to pursue higher education and career opportunities, chances to conceive are further threatened, when they are ready to start a family these women often turn to ART (5). Although ART offer a popular solution, they come with a high patient burden, expensive drug regimens, and a substantial chance of failure or developing complications. To minimize these effects it is important to be able to accurately predict treatment outcome prior to treatment initiation and to consequently tailor protocols to the individual response (6). The associated clinical factors for successful treatment of IVF/ICSI have been studied extensively (7, 8). The major clinical factors related to pregnancy outcome in ART cycles include the following: age of the patient (8), embryo morphology (9), cause

of infertility, and number of embryos transferred (7). Among these factors, the age of the patients and the cause of infertility, together with the OR markers are available prior to Controlled Ovarian Stimulation COS in the general practice of IVF treatment. It has been reported that the prevalence of unexplained infertility increases in female patients of advanced age (> 35 years) seeking infertility treatment (10, 11). A putative cause of such unexplained infertility was attributed to diminished OR. The OR markers would probably connect with the outcome of pregnancy for such patients. Herein we correlate in one of Iraqi IVF centers, the OR markers to the outcome of ICSI couples with exclusive female cause due to tubal factor or unexplained infertility.

The aim of this study was:

1-To assess the OR for subpopulations of Iraqi infertile couples seeking ICSI treatment, who were referred to the fertility center of Al-Sadder Medical Teaching City in Al -Najaf province and were selected for ICSI programme.

2-To correlate the OR markers with ICSI outcome.

2. PATEINTS AND METHODS

2-1: Work Design:

This study was designed as a prospective randomized controlled trial. Eighty -seven participants were enrolled; twenty subjects as a control group (n=20), and 67 as a study group (n=67). These subjects were referred from many Iraqi governorates to the fertility center of Al-Sadar Medical Teaching City in Al-Najaf province e from December 2010 - September 2011. These participants were seeking treatment for their infertility, and we design to treat them by ICSI.

2.2:Participants Selection:

The sixty-seven study group was selected from 1400 attender to the fertility center during the period of the study. All couples were surveyed for their etiology of infertility by semen analysis for the males, and the female were asked about their gynecological and medical history; they underwent complete medical examination; height and weight measurements. Gynecological examination was performed; cycle day two (CD2) vaginal ultrasound (U\S) and blood tests for follicle stimulating hormone(FSH), luetinising hormone(LH), estradiol (E2), serum prolactin, testosterone and thyroid function test. Hysterosalpingiography was arranged and some have been prepared for laparoscopy. Once the couple have been screened and found to be eligible according to our selection criteria, they were randomized to go on with the designed programme.

Selection Criteria:

1. Male partner with normal semen analysis according to World Health Organization 2010 guideline, (concentration> 15 million /ml, motility > 32% and strict morphology > 4%), (12).

2. Regular menstrual cycle of 21-35 days.

3. Female partner have normal both ovaries; visible on U\S not having cystic ovaries (PCO) defined by The Rotterdam ESHRE / ASRM criteria (2003) (13).

4. The cause of infertility is either unexplained (n=43) and the couple underwent at least three trials of intrauterine insemination; or tubal factor infertility (n= 24) but not hydrosalpinx.

5. Vaginal U\S show no uterine fibroid; anomaly or ovarian cyst measuring 20 mm or more in diameter.

6. No endocrine cause for their infertility such as hyperprolactinaemia or hyperandrognesim.

7. Screening tests for hepatitis B and C as well as for human immune deficiency viruses (HIV) prove to be negative.

8. The participants have their first or previous trials of ART.

9. Informed written consent was obtained from the patients.

Hormonal Assay: Five mL of blood was drawn on CD 2. The blood was centrifuged and sera were stored at -20 °C. AMH sera levels were measured with AMH\Gen II analysis kit (Beckman coulter, USA) using Enzyme Linked ImmunoSorbant Analysis (ELISA). Kits for measurement of FSH was (bioMérieux® France) using Mini VIDAS

analysis. The kit for E2 measurement was (bioMérieux® France) using Mini VIDAS analysis. Inhibin was measured by ELISA using (Diagnostic system Laboratory, USA) kit.

Ultrasound was performed CD 2 by the gynecologist of the center using real time ultrasound device (Philips 11*E), using vaginal probe (7 MHZ) , Follicles measuring 2-8 mm were counted from the lateral to medial margin of each ovary to determine the antral follicle cohort. The total number of the follicles per patient counted in both ovaries was used for calculation.

In all patients treated for ICSI, the protocol for pituitary down-regulation was by short Protocol; at cycle day 2 the patient receives 0.1 mg/day of GnRH analogue as a morning dose and FSH as an evening dose. These are given subcutaneously. Decapeptyl in the form of Diphereline 0.1 mg (triptoreline Beaufour pharma, France) and FSH in the form of (Gonal-f, Follitrop alfa 75 IU /ampul ,serono .Switzerland). The dose can be administered either via step up or step down protocol where the dosage of FSH is maintained or gradually increased or initial high dose are tailored down.

Participants were monitored for follicular recruitment and growth by serial transvaginal ultrasound and serum E2 from the 6th day of stimulation with gonadotropins. Titration of FSH upward or downward is based on response of folliculogenesis. Participant that did develop less than 3 follicles measuring 18 mm after 14 days of FSH treatment ,or E2 level < 200 Pg/ml, had their treatment cycle cancelled or was converted to have intra uterine insemination depending on their clinical factor of infertility (poor responder, n= 18); those considered to have an oocyte retrieval = zero. The remaining 49 women having a completed ICSI cycle When at least 2 dominant follicles of 17 mm in diameter in each ovary is ready, ovulation is triggered by human chorionic gonadotropin (HCG), pregnyl ,5000- 10000 IU (HCG, Prygnyl, Organon, Holland) intramuscular. Thirty six hours after HCG trigger, follicular aspiration is done by transvaginal ultrasound guidance. Participants who developed more than 20 follicles measuring more than 10 mm in diameter or E2 level more than 3000pg/ml (ovarian hyper stimulation) didn't proceed to embryo transfer(n=2). The oocytes were incubated and evaluated for maturity after their denudation ICSI was done under inverted microscope with manipulators (Bickland industrial, UK). On day 1 pronuclear stage was assessed and fertilization rate calculated. Day2, subsequent division was assessed and cleavage rate was calculated. Embryo transfers were done 48-72 hours after ICSI. Embryos were graded and scored under the microscope according to Steer et al., (14); and Bączkowski et al., (15), and then transferred to the uterus with Labotec catheter. Luteal phase was supported by Progesterone vaginal cream (Crinon 8%, Serono,U.K), Duphastone tablets(10mg,Solvay phasmaceutical, Holland),Aspirin tablet(Aspin-100mg/ enteric coated tab. SDI. Iraq), and Folic acid tablet (5 mg/tab. SDI- Iraq). ET was done only in 44 cycles because of fertilization failure in one case and cleavage failure in two case added to the two cases of ovarian hyper stimulation and 18 poor responders, so total of 23 cases with no ET). On the fourteenth day of embryo transfer serum B- HCG was performed to confirm pregnancy, by that time pregnancy rate is stated.

Table (1): Demographic data regarding the study group (n= 67) participating in ICSI treatment of different age group. Values are mean ± standard deviation or %.

Base line characteristic	
Age(years) female	31.76 ± 6.85
male	38.65 ± 54
female BMI(Kg/m2)	28.23 ± 5.08
Duration of infertility(years)	8.09 ± 4.79
Type of infertility	
Primary (no)%	(54) 80.59
Secondary(no) %	(13) 19.41
Etiology	
Unexplained (no)%	(43)64.2
Blocked tube (no)%	(24) 35.8

Cycle day 2	
Basal FSH(mIU/ml)	5.75 ± 2.61
Basal E2(pg/ml)	39.64 ± 19.67
Basal AMH(ng/ml)	2.90 ± 3.44
Basal inhibin B(pg/ml)	74.25 ± 1.55
Total AFC	7.42 ± 2.87

The control group was volunteers either relatives or staff of the fertility center, their age range from 22-45 years who had delivery at least within the last 4 years; and now using either rhythm method or intra uterine contraceptive device, as methods for birth control. They underwent height and weight measurement, cycle day 2 blood sampling for FSH, LH, E2, AMH and Inhibin B. Vaginal U/S was done for AFC (Table 2).

Table 2: Demographic character of the control group. Values are mean ± SD. (n= 20).

Age (years)	32.5 ± 7.41
Duration of child free(years)	2.25 ± 1.06
Basal FSH(mIU/ml)	4.71 ± 1.35
Basal E2(Pg/ml)	41.83 ± 15.79
Basal AMH(ng/ml)	2.54 ± 1.48
Basal Inhibin B(Pg/ml)	118.91 ± 1.00
AFC	7.60 ± 1.87
BMI(Kg/m ²)	28.02 ± 3.46

Statistical analysis of the data was performed with SPSS version 17(Statistical package for Social Sciences; SPSS, Inc., Chicago, IL) for Windows. Continuous variables were expressed as mean ± SD and range, categorical variables as percentages. Between-group differences were tested with (unpaired) independent t- test for continuous parameters .A p value of <0.05 was considered statistically significant for all analysis.

Pearson's correlation was used to examine the relation between continuous variables, (age, basal FSH, E2, Inhibin B, AMH and AFC) and as determinant of ovarian response to COS expressed as no. of oocyte, and ICSI outcome (expressed as no. of PN, embryo parameters and no. of embryos transferred and pregnancy rate ..

3. RESULTS

Table (3): comparison between study group and control group regarding base line evaluation. Values are mean ± SD.

Parameter	Study group (n=67)	Control group (n=20)	P-value
Age(years)	31.76 ± 6.83	32.5 ± 7.41	NS*
Duration (years)	8.09 ± 4.79	2.25 ± 1.06	P < 0.05**
FSH (mIU/ml)	5.75 ± 2.61	4.71 ± 1.35	P < 0.05**
E2 (Pg/ml)	39.64 ± 19.67	41.83 ± 15.79	NS*
AMH (ng/ml)	2.90 ± 3.44	2.54 ± 1.48	NS*
Inhibin B (Pg/ml)	74.25 ± 1.55	118.91 ± 1.00	NS*
AFC	7.41 ± 2.87	7.60 ± 1.87	NS*
BMI (Kg/m ²)	28.24 ± 5.07	28.02 ± 3.46	NS*

* NS: non-significant difference.

** significantly different from the corresponding group.

Table (3) compares the base line characteristic of the females of the studied group and the control. No statistically significant differences existed in age of the studied and control group ($p > 0.05$) (31.76 ± 6.83 ; 32.5 ± 7.41 years) respectively. The duration of voluntary infertility which correspond to the period of last child birth in which the non-hormonal contraception was used in the control group was (2.25 ± 1.06 years), and it differs significantly from the involuntary period of infertility of the studied group (8.09 ± 4.79 years), ($p < 0.05$). Only Significant differences ($P < 0.05$) were identified in CD2 serum levels of FSH, the control group demonstrate 5.75 ± 2.61 mIU/ml value while 4.71 ± 1.35 mIU/ml in the study group, while other markers demonstrates no significant difference ($P > 0.05$).

For E2, CD2 serum level: 39.64 ± 19.67 Pg/ml in the study group while it was 41.83 ± 15.79 Pg/ml in the control, for AMH: 2.90 ± 3.44 vs. 2.54 ± 1.48 (ng/ml), in the control. Inhibin B at CD2 (74.25 ± 1.55 for study group vs. 118.91 ± 1.00 (Pg/ml) in the control; ultrasound scan for AFC (7.41 ± 2.87 in the study group vs. 7.60 ± 1.87 in the control). BMI measurement for both groups (28.24 ± 5.07 vs. 28.02 ± 3.46) (Kg/m²).

Table (4): Correlation of age, FSH, E2, AMH, inhibin B and AFC, to the outcome of ICSI cycles, for the completed ICSI cycles (n=44).

OR Marker	No. of follicle	No. of MII oocyte	No. of 2PN	G1+G2	G3+G4	No. of ET	B-HCG-
Age	-0.47*	-0.53*	-0.49*	-0.34*	-0.01	-0.30*	-0.19
FSH	-0.31*	-0.27*	-0.26	-0.02	0.09	-0.08	-0.25
E2	0.08	-0.02	0.07	0.17	-0.45	0.00	-0.18
AMH	0.27	0.35*	0.24	0.34*	0.07	0.60	0.21
Inhibin B	-0.02	0.12	0.08	0.08	-0.28	0.94	0.10
AFC	0.12	0.14	0.06	-0.23	0.57*	0.42	0.47

* Significant correlation

Table (4) shows the correlation of various ovarian reserve tests to the outcome of ICSI. Age reflect negative significant association with no. of follicles, no. of MII, no. of 2PN, no. of best quality embryos (G1+G2), and to the total no. of embryos transferred ($r = -0.47, -0.53, -0.49, -0.34, -0.30$) respectively. Age didn't show significant association to no. of least quality embryos (G3+G4) ($p > 0.05$).

Serum levels of FSH at cycle day 2 demonstrate significant negative correlation to total no. of follicles aspirated ($r = -0.31$) and to no. of MII oocytes ($r = -0.27$) ($p < 0.05$). It has negative correlation with no. of 2PN, no. of best quality embryos, and to the no. of transferred embryos. ($r = -0.26, -0.02, -0.12, -0.08$) respectively. None of these correlation reached significant level ($p > 0.05$). The positive correlation with the no. of least quality embryos ($r = 0.09$) also was insignificant.

Basal serum levels of E2 have positive correlation with the no. of follicles, no. of 2pn, no. of best quality embryo ($r = 0.08, 0.07, 0.17,$) respectively. Basal serum levels of E2 have negative correlation with no of MII oocytes, no. of least quality embryo, ($-0.02, -0.45,$) respectively, both correlations are insignificant.

AMH measurement at CD2 got positive correlation with no. of follicle, No. of 2PN, No. of least quality embryos and to the no. of ET ($r = 0.27, 0.24, 0.07, 0.60$) respectively all have p value (> 0.05). The no. of MII oocytes obtained, no. of best quality embryos correlate positively and significantly to the level of serum AMH ($r = 0.35, 0.34, 0.34$) ($p < 0.05$).

Basal serum inhibin B measurement showed no significant correlation with any of the outcome measured (no. of follicles, no. of MII oocytes, no. of 2PN, no. of best quality embryos, no. of bad quality embryos, to the no. of ET. All are statically insignificant ($r = -0.02, 0.11, 0.08, 0.08, -0.28, 0.94$). Sonographer assessment of AFC CD didn't show any significant correlation to no. of follicle obtained after COS, no. of MII oocytes, no. of 2pn, no. of best quality embryos, no. of embryos no. of ET) ($r = 0.12, 0.14, 0.06, -0.23, 0.10, 0.42$) respectively. Except for the no. of bad quality embryos. ($r = 0.57$) which is of significant level ($p < 0.05$).

The study further separately analyzed the significance of those ovarian reserve markers in predicting pregnancy outcome demonstrated by the B-HCG. None of them showed predictive value in this respect (Age: $r = -0.19$; FSH: $r = -0.25$; E2: $r = -0.18$; AMH: $r = -0.18$; Inhibin B: $r = 0.10$; AFC: $r = 0.47$) ($p > 0.05$).

4. DISSCUSSION

Traditional methodology used to assess OR has consisted of baseline serum levels of hormones such as FSH, estradiol and inhibins, and chronological age (16, 17). Also, a number of provocative tests have been devised to indirectly assess OR and identify patients who might not be detected by basal hormone screening alone (16, 18, 19). However, neither basal hormone measurements nor such dynamic tests provide direct information concerning the responsiveness of the ovaries to exogenous gonadotropins used in ovarian stimulation for assisted reproductive treatment.

AMH is formed in females' ovaries from the 36th week of gestation, during the female life until menopause it is expressed in granulosa cells of small growing follicles (primary and preantral). The biological activity of AMH in women is not completely understood, but data along the last years suggest that AMH modulates follicular growth in a way that it inhibits the recruitment of follicles from the primordial pool by modifying the FSH sensitivity of those follicles and regulating ovarian steroidogenesis and intrafollicular androgen to estrogen ratio (19, 20, 21). There is a linear decline of AMH levels over time (22,20). The fact that AMH acts first of all paracrine and is not involved in feed-back mechanisms of hypothalamo-pituitary-gonadal axis, and that AMH is expressed at a constant level and demonstrates less individual intra- and inter-cycle variation, makes AMH very attractive, promising and reliable economic marker as a direct measurement of OR (20, 23, 24).

The basic demographic characters of the control were not different significantly ($p > 0.05$) from the studied group regarding age, basal E2, AMH inhibin B, AFC nor BMI as demonstrated in (table 3). These findings are expected since our study group have extra ovarian cause for their infertility. The only difference was in duration of the involuntary infertility of our study group which is significantly higher than the voluntary infertility of the control group. This difference cannot be explained on scientific bases since the involuntary one is out of the wish of the couple and the other is according to the need of the couple's. Basal serum FSH level is significantly higher in the study group; this may be a contributing cause to their infertility since high FSH serum levels are associated with decrease fecundity (25). (Table 3).

The correlation of ovarian reserve markers to the outcome of ICSI programme regarding no. of follicle, no. of MII oocyte, no. of 2PN, no. of best quality embryos (G1+G2), bad quality embryo (G3+G4), no. of ET and B-HCG is demonstrated in table 4.

Our results demonstrate that age has a significant negative association with these outcomes of ICSI. This is widely concluded by other researchers since age is considered to be the single most important factor in determining quality and quantity of ovarian reserve. Not surprisingly, in IVF cycles, older women tend to produce lesser number of oocytes and embryos derived from them consequently have lower implantation potential (26; 27).

The present study demonstrates that increasing basal FSH level was associated negatively and significantly with reduced number of follicles, number of MII, and negatively but not significantly with fertilized oocytes and the number of embryos obtained as shown in table 4, a finding concluded by Karimzadeh and Ghandi, (28). In our study basal E2 level as an OR marker have been incapable to correlate with follicular development or demonstrate its ability to predict oocyte quality, embryo parameters or the occurrence of pregnancy (Table 4), a finding which is highlighted by others (29; 30). E2 may be used to guide the clinician as to whether the stimulation with gonadotropins can be started, but it does not have value as IVF prognostic tool.

Basal inhibin B level in our study group couldn't demonstrate a significant association with ICSI-ET outcome as demonstrated in table 4, a conclusion reached by Cerus and Lin with their co-workers (31; 32), while other studies showed different results (33,34) this may be due to the smaller highly selected study group compared to them.

Our results demonstrate the significant association between basal AMH serum concentration with the total number of oocyte quality and embryo development in ICSI cycles (Table 4), an observation seen by others (35; 34). Arabzadeh and working team observed that early embryo cleavage rate was significantly higher among patients with AMH serum levels between 1.40 ng/mL and 4.83 ng/mL which was comparable to our mean AMH (2.90 ng/ml) (36), and at the same time reflects the significant association of AMH with embryo quality, this consolidates our results. On account of the fact that AMH is produced by the granulosa cells at earlier follicular stages, lower levels of the hormone may be associated with a failure in granulosa cell expression, which could irreversibly harm the gamete.

This study suggests that AFC cannot be used to predict oocyte/embryo quality or ICSI outcome (table 4). A conclusion demonstrated by Melo et al., 2009 (37), while Majumder and team demonstrate that AFC are predictors of oocytes retrieved and of the number of good quality embryos available for transfer and freezing (38), the difference may be due to the sample size, to the stimulation protocol since we haven't introduced the antagonist analogue yet, and limited types and options of gonadotropins available in our country. Our study group may have responded differently to gonadotropins as they may have certain specific FSH receptors genotype present in them which demonstrate ovarian

resistance to FSH. However, different genetic characteristics with respect to the FSH receptor may mean that using AFC as a sole assessment of IVF prognosis may be confounded in some women (39).

Our study highlighted that any OR test to be inaccurate for the direct prediction of pregnancy, (Table 4), although age, basal FSH and E2 have negative correlation to pregnancy occurrence demonstrated by positive B-HCG. On the other hand basal AMH, Inhibin B and AFC have positive correlation to it, non-have reached significant level ($p > 0.05$). Our findings have been shown by many other works, (33, 34, and 40).

The natural monthly fecundity rate which is about 25% between 20 and 30 years of age decreases to below 10% above the age of 35 (41). As a woman become older, the ovarian reserve and her ability to have pregnancy decrease both in natural and stimulated cycles, and the development of diminished OR generally reflects the process of follicular depletion and decline in oocyte quality (42). With age a numerical abnormality in the number of chromosomes (aneuploidy) is very common in the human embryo. Indeed, chromosomal abnormalities seem much more common in oocytes compared with sperm (43). Some mechanisms that have been implicated in the age-related increase in oocyte aneuploidy involve the different stages of fetal ovarian development, the depletion of the follicle pool throughout life, the number of years of primordial follicle arrest, direct effects of increasing FSH concentrations with age, and many others (44).

The main purpose of OR evaluation, especially before ART, is to identify women with poor ovarian reserve for their chronological age. This is why age must always be the first marker to be considered in ovarian reserve assessment. Older women may benefit from OR tests since they can help clinicians to find out acceptable chances of pregnancy through IVF. Younger women with altered tests should be first classified as potentially poor responders and must gain benefits from individualized stimulation protocols by quantitative estimation of the pool of FSH-sensitive follicles (45).

5. CONCLUSION

Age, Anti-Mullerian hormone and follicle stimulating hormone are better markers than inhibin B, estradiol or antral follicle count in predicting the outcome of Intracytoplasmic sperm injection cycles.

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