Original Article

Ovarian reserve tests and ovarian response to increasing doses of clomiphene citrate in infertile women

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Abstract

Background: Ovarian reserve is affected by many factors and repeated ovulation is one of the factors thought to be responsible for the same. Clomiphene citrate (CC) is the most common drug used for ovulation induction in anovulation and also empirically for unexplained infertility. The study aimed to determine the ovarian reserve by day 3 follicle-stimulating hormone (FSH), anti-Mullerian hormone (AMH), and antral follicle count (AFC) in infertile women and also tried to determine ovarian response and any effect in ovarian reserve to increasing doses of CC. **Materials and Methods:** This prospective interventional study included 50 infertile women, who did not undergo ovulation induction for six or more cycles. Day 3 AFC, FSH, and AMH were determined in each cycle and treated with increasing doses of CC 50, 100, and 150 mg and monitored for follicular growth and ovulation by transvaginal scan (TVS) to know the ovarian response. Pregnancy rate and side-effects were monitored. **Statistical Analysis:** Correlation between the ovarian reserve tests was conducted with Pearson and Spearman correlation. Differences in parameters in three cycles were analyzed by repeated measures analysis of variance, statistical test (ANOVA). Ovarian reserve and response with increasing doses of CC were analyzed by logistic regression. **Results:** AFC and FSH emerged as better tests to predict ovarian reserve. A significant negative correlation was noted between AFC and FSH ($r = -0.366$; $P = 0.0001$). Positive correlations were obtained between AFC vs AMH and FSH vs AMH. Ovarian response significantly increased with increasing doses of CC. Ovarian reserve suffered a decrease with increasing doses of CC, but this did not reach significant levels. **Conclusion:** Although ovarian response increased with increasing doses of CC, there is a risk of decreased ovarian reserve with such therapy, and this finding should be confirmed by large sample size.

Keywords: Anti-mullerian hormone, antral follicle count, clomiphene citrate, follicle-stimulating hormone, infertility, ovarian reserve, ovarian response

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INTRODUCTION

Ovarian reserve refers to the resting primordial follicles, of which few grow into graafian follicles and result in ovulation. Ovarian response represents the number of follicles that develop in a cycle when ovulogens are employed. Ovarian reserve is affected by many factors, and repeated ovulation is thought to be one of them. The available literature shows that the ovarian response

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appears to be maintained with repeated treatment, and the only significant decline in ovarian reserve is because of an increase in age of the woman.[1,2] Ovulation induction alters the physiologic selection of a single dominant follicle but do not accelerate the recruitment of follicles from further cycles, and it was reported that there is no detrimental effect on ovarian function after repetitive ovarian stimulation.[3]

Clomiphene citrate (CC) is the most common drug used for ovulation induction in anovulation and also empirically for unexplained infertility. It is essential to find out the ovarian reserve before contemplating ovulation induction in women who have had multiple cycles of ovulation induction. There are many static and dynamic tests available to determine ovarian reserve, of which antral follicle count (AFC), serum anti-Mullerian hormone (AMH), and day 3 follicle-stimulating hormone (FSH) are the most commonly employed. Although ovarian reserve is within normal limits, ovarian response is not optimum sometimes.

This study aimed to (1) determine ovarian reserve in infertile women, (2) assess ovarian response to increasing doses of CC in the same woman, and (3) differentiate good responders from poor ones.

MATERIALS AND METHODS

This was a prospective cohort study conducted with the Department of Obstetrics and Gynaecology, JIPMER, Puducherry, India, between 2012 and 2013. The inclusion criteria were women with infertility, willing to come for follow-up of a minimum period of 3 months, and were in the age group between 20 and 40 years. The exclusion criteria were women who underwent ovulation induction for more than six cycles, male factor infertility, infertility due to tubal factors, presence of ovarian cysts, documented ovarian failure, and past history of ovarian neoplasm. Fifty women who fulfilled the criteria were recruited after taking informed consent as per ethical approval (SEC/2011/4/70).

On the third day of menstrual cycle, 5 ml of venous blood was drawn from all participants for the measurement of FSH and AMH, for three consecutive menstrual cycles. The blood samples were centrifuged, and serum was separated and stored under −80°C until further assay.

All of them were subjected to transvaginal ultrasound using diagnostic ultrasound system Toshiba Model SSA-340A, Toshiba, Chiba, Japan. The size of the ovaries and the number and size of antral follicles of each ovary were noted, and the total AFC was calculated. Subsequently, patients were given CC in increasing doses 50, 100, and 150 mg once a day for 5 days from days 3 to 7 of each cycle on their cycles 1, 2, and 3, respectively. Transvaginal sonography was carried out from days 10 to 22 every 48 h to monitor the number of follicles and their sizes.

Based on transvaginal scan (TVS), a follicular size of 16 to 18 mm in any one of the three cycles was taken as a good ovarian response to CC. If there was no development of dominant follicle in all cycles, these women were classified as poor responders. The woman was labeled as having good ovarian reserve if she had met any two of the criteria, that is, day 3 FSH $<$ 10 IU/L, day 3 AMH $>$1 ng/mL, and day 3 AFC $>$ 12.

FSH levels were estimated by using immunoenzymometric assay based on the principle of sandwich method. The data were analyzed by Gen 5 software (Biotek Powerwave XS). The range of the assay was between 0 and 100 mIU/mL. Intraassay coefficient of variation was 3.4 to 7.9%. Interassay coefficient of variation was 4.75 to 2.58%. AMH levels were estimated by competitive enzyme immunoassay technique. Data were analyzed by five-parameter logistics using Masterplexl Reader fit software (Hitachi Solutions America Ltd, Irvine, California, USA). The range of detection was between 0.375 and 150 ng/mL. Both intraassay and interassay coefficients of variation were $<$15%.

Statistical analysis

Data were entered in Excel 2007 and analyzed in STATA 11.0 (Stata Corp, Texas, USA). Continuous variables were reported as mean ± SD, and median with interquartile range was reported wherever necessary. Difference between means was analyzed by either one-way analysis of variance, statistical test (ANOVA) or Wilcoxon rank-sum test, depending on the distribution. *P* value of less than 0.05 was considered significant. Categorical variables were analyzed either by χ^2-square or Fisher's exact test. Averages of three cycles of AFC, FSH, and AMH were taken for calculating their diagnostic utility in prediction of ovarian reserve. Receiver operating characteristic (ROC) curve for each parameter was determined. Correlation between AFC, FSH, and AMH, and between age and these parameters were shown in scatter plot. Differences in parameters of these three cycles were analyzed by repeated measures ANOVA. Ovarian reserve and response with increasing doses of CC were analyzed by logistic regression.

RESULTS

The clinical characteristics of the study population are shown in Table 1. More than 50% were between 26 and 30 years of age, and the mean age was 27.48 ± 3.45 years. Ten percent were between 31 and 35 years, and only 6% belonged to 36 to 40-year age group. More than 70% had short duration of infertility (1–5 years), and polycystic ovarian syndrome (PCOS) morphology was present in 72% of women. More than 80% underwent three or less cycles of ovulation induction.

AFC, FSH, and serum AMH were measured for each patient on day 3 of three consecutive menstrual cycles. AFC values followed normal distribution, and thus, it is represented as mean with standard deviation. FSH and AMH lack normality, and they are represented as median with interquartile range in Table 2. ROC curves were estimated to evaluate the performance of AFC, FSH, and AMH in predicting the ovarian reserve. Ovarian reserve was classified as good when the patient had any two of the following: mean AFC > 12, mean FSH < 10 mIU/mL, and mean AMH > 1 ng/mL. ROC curve analysis for good ovarian reserve demonstrated that AFC had the largest area under the curve (AUC) (0.6664; $P = 0.0001$) relative to FSH (0.0960; $P = 0.00001$) and AMH (0.5794; $P = 0.00001$), which is shown in Table 3 and Figure 1. Correlation between the ovarian reserve tests was conducted with Pearson and Spearman correlation, and the results are shown in Table 4. A significant negative correlation was noted between AFC and FSH ($r = -0.366$; $P = 0.0001$). Positive correlations were obtained between AFC vs AMH and FSH vs AMH.

Logistic regression was carried out with first cycle (50 mg CC) and taken as reference. Odds ratio for the second cycle (100 mg CC) was 3.187, which was statistically significant ($P = 0.006$). Odds ratio for the third cycle (150 mg CC) was 3.5 with statistically significant *P* value of 0.003. These results are shown in Tables 5 and 6. Hence, a significant increase in response with increasing doses of CC was observed more from 50 to 100 mg CC. ROC curves were estimated to evaluate the performance of AFC, FSH, and AMH in predicting the good ovarian response. Good ovarian response was defined as development of dominant follicle (16–18 mm) in any one of the three observed cycles. ROC curve analysis [Figure 2] for good ovarian response demonstrated that AFC had the largest AUC (0.5894; $P = 0.00001$) relative to FSH (0.5437; $P = 0.00001$) and AMH (0.5529; $P = 0.00001$). All the parameters had almost equal AUC,

Table 1: Clinical profile

S. No.	Characteristic	Cases ($n = 50$)
I	Age in years	
	21–25	16 (32%)
	26–30	26 (52%)
	31–35	5 (10%)
	36–40	3 (6%)
	Mean age (mean ± SD)	27.48 ± 3.45
II	Body mass index in kg/m^2 (mean ± SD)	23.19 ± 3.75
III	Duration of infertility in years	
	1–5	36 (72%)
	6–10	9 (18%)
	11–15	4 (8%)
	16–20	1 (2%)
IV	Number of cycles of prior ovulation induction	
	0–3	44 (88%)
	4–6	6 (12%)
V	Menstrual cycles	
	Regular cycles	28 (56%)
	Irregular cycles	22 (44%)
VI	Polycystic ovarian morphology by USG	36 (72%)
VII	Hypothyroidism	2 (4%)
VIII	Type II diabetes mellitus	2 (4%)

Table 2: Ovarian reserve tests (of all cycles)

S. No.	Ovarian reserve tests	Value
1	Antral follicle count (mean ± SD)	10.59 ± 4.41
2	Serum follicle-stimulating hormone (mIU/mL)(median with range)	1.39 (1.01–1.98)
3	Serum anti-Mullerian hormone (ng/dl) (median with range)	3.12 (2.58–5.94)

Table 3: Best marker of ovarian reserve [Figure 1]

S. No.	Tests	ROC area under the curve	*P* value
1	AFC	0.6664	<0.001
2	FSH	0.0960	<0.001
3	AMH	0.5794	<0.001

Table 4: Correlation between the ovarian reserve tests

S. No.	Correlation	*r* value	*P* value
1	AFC with FSH	−0.366	0.0001*
2	AFC with AMH	0.025	0.757
3	FSH with AMH	−0.006	0.938

*Significant.

Table 5: Ovarian response with increasing doses of clomiphene citrate

Cycle	Dose of clomiphene citrate (mg)	Odds ratio	95% confidence interval	*P* value
1	50	Reference	–	–
2	100	3.187	1.40–7.24	0.006*
3	150	3.5	1.53–8.01	0.003*

*Significant.

Table 6: Best marker of ovarian response [Figure 2]

S. No.	Tests	ROC area under the curve	*P* value
1	AFC	0.5894	<0.001
2	FSH	0.5437	<0.001
3	AMH	0.5529	<0.001

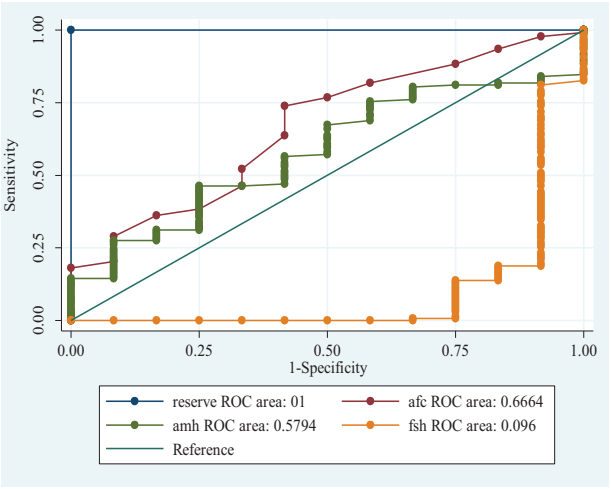

Figure 1: AFC, FSH, and AMH ROC curves for good ovarian reserve. ROC curves ovarian reserve is classified as good when the patient had any two of the following: mean AFC > 12, mean FSH < 10 mIU/mL, and mean AMH > 1 ng/mL; area under curve (AUC) was significant for all the parameters. AFC had the largest AUC (0.6664; $P < 0.001$) relative to FSH (0.0960; $P < 0.001$) and AMH (0.5794; $P < 0.001$).

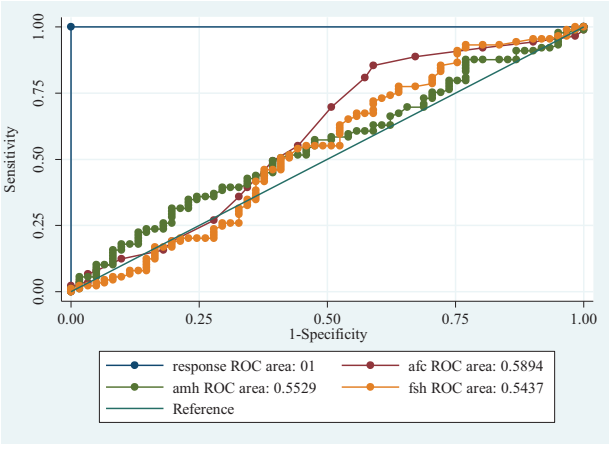

Figure 2: AFC, FSH, and AMH ROC curves for good ovarian reserve. ROC curves of AFC, FSH, and AMH in predicting the good ovarian response. Good ovarian response was defined as development of dominant follicle (16–18 mm) in any one of the three observed cycles. ROC curve analysis for good ovarian response demonstrated that AFC had the largest area under the curve (AUC) (0.5894; $P = 0.00001$) relative to FSH (0.5437; $P = 0.00001$) and AMH (0.5529; $P = 0.00001$).

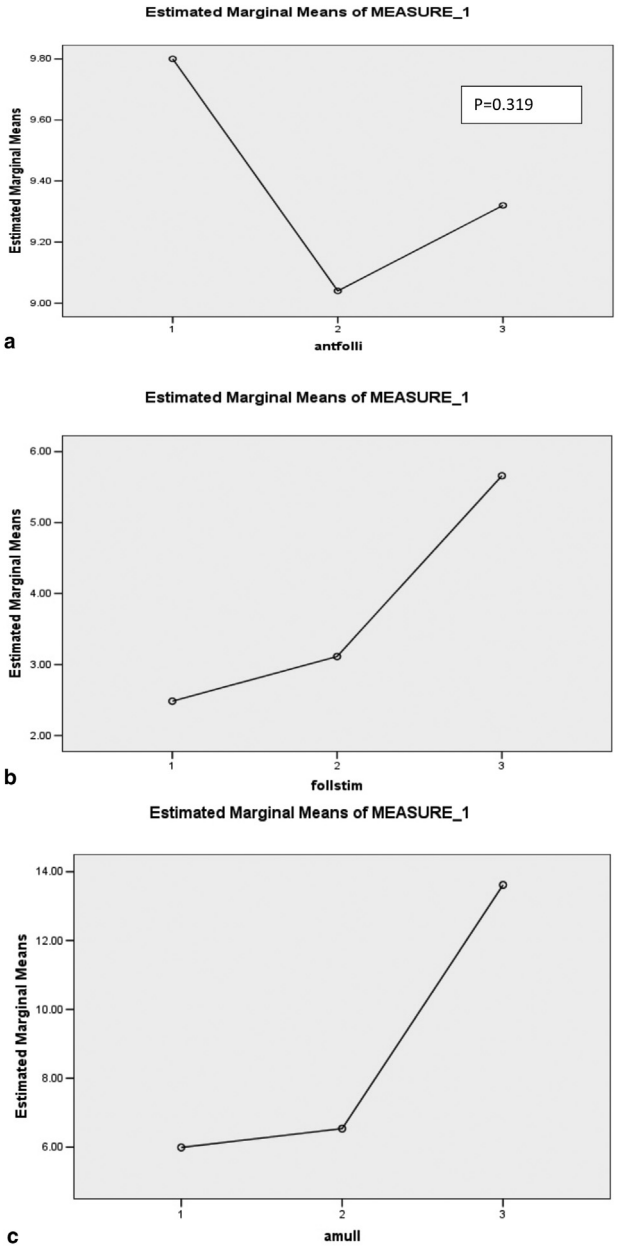

Figure 3: (a) AFC with increasing doses of clomiphene citrate. (b) FSH with increasing doses of clomiphene citrate. (c) AMH with increasing doses of clomiphene citrate

and thus, they are almost equal in predicting good ovarian response. The sensitivity, specificity, positive predictive value, and negative predictive value were less because of small sample size.

Repeated measures ANOVA was used to determine changes in AFC, FSH, and AMH over three cycles. There was no statistically significant change in AFC, whereas significant changes occurred in FSH and AMH values over three cycles with increasing doses of CC. AFC remained almost the same in three cycles, whereas FSH and AMH values increased with increasing doses of CC, which is shown in Table 7 [Figure 3a–c].

Logistic regression was applied to determine the change in ovarian reserve over three cycles with increasing doses of CC, taking first cycle as reference. Odds ratio for the second cycle was 0.234 ($P = 0.202$) and third cycle was 0.125 ($P = 0.057$) [Table 8]. Ovarian reserve suffered a decrease with increasing doses of CC, but that was not statistically significant, which may be because of small sample size. Agreement

Table 7: Ovarian reserve tests with increasing doses of clomiphene citrate [Figure 3a–c]

S. No.	Ovarian reserve tests	Cycle 1	Cycle 2	Cycle 3	*P* value
1	AFC	9.8 ± 5.64	9.8 ± 5.64	9.32 ± 5.27	0.319
2	FSH (mIU/mL)	2.48 ± 2.75	3.11 ± 3.46	5.65 ± 4.3	0.0001*
3	AMH (ng/mL)	5.99 ± 12.59	6.53 ± 12.46	13.61 ± 28.45	0.005*

*Significant.

Table 8: Ovarian reserve with increasing doses of clomiphene citrate in three cycles

Cycle	Dose of clomiphene citrate (mg)	Odds ratio	95% confidence interval	*P* value
1	50	Reference	–	–
2	100	0.234	0.25–2.17	0.202
3	150	0.125	0.15–1.06	0.057

Table 9: Association between ovarian reserve and ovarian response

Agreement	κ value	*P* value
70%	0.1007	0.2345

between ovarian reserve and response was 70%. Although it looked like a fair agreement, κ value was 0.1007, which is less than the recommended value of 0.5. Hence, women with good ovarian reserve do not always mean that they are good responders to CC [Table 9].

Differentiation between good and poor responders is shown in Table 10. The differences of age, body mass index (BMI), and AFC were found using two sample *t* test. The differences of duration of infertility and number of cycles of prior ovulation induction were calculated using Wilcoxon rank-sum test. There is no statistical difference in age between a good and a poor responder. Only those women with more than 5 years of infertility were poor responders as per the criteria used in this study. AFC was less among poor responders, though it was not statistically significant. The pregnancy rate was 8% and the percentage of women with hyperstimulation was 12%.

DISCUSSION

Demographic and epidemiological studies have consistently demonstrated that fertility declines with age. There are various tests used to determine the ovarian reserve at any age, out of which, AMH and AFC are being employed most often, and AMH being an expensive test and not readily available for all the strata of women. In practice, we encounter women who have already taken several cycles of CC empirically, and it is good to know the ovarian reserve among them and their

Table 10: Differentiation of good and poor responders

S. No.	Parameter	Good responder	Poor responder	*P* value
1	Age (years)	27.05 ± 0.52	28.83 ± 1.13	0.119
2	Duration of infertility (years)	4 (3–6)	5 (4–10)	0.04*
3	No. of cycles of prior ovulation induction	1 (0–2)	1 (0–2)	0.24
4	Body mass index (kg/m^2)	23.14 ± 3.83	23.33 ± 3.64	0.88
6	Antral follicle count	10 ± 5.02	8.49 ± 5.26	0.078
7	Follicle-stimulating hormone (mIU/mL)	2.71 (1.55–4.15)	2.37 (1.16–4.93)	0.36
8	Anti-Mullerian hormone (ng/mL)	3.67 (2.36–5.66)	3.16 (2.1–4.71)	0.27

*Significant.

response to CC to counsel them regarding the need for asisited reproductive techniques (ART).

In the present study, AFC had the larger AUC than AMH and FSH, but all three are nearly equal. Our findings are in line with Nardo *et al.*[4] and Jayaprakasan *et al.*[5] regarding AMH and AFC, where they showed similar predictive value. In the present study, AFC had significant negative correlation with basal FSH ($r = -0.366$; $P = 0.0001$), which is in agreement with Haadsma *et al.* ($r = -0.288$; $P < 0.01$).[6] ROC curve analysis of the present study revealed that AFC and AMH were better than basal FSH in prediction of good ovarian reserve. In the prediction of ovarian response, all the three (AFC, FSH, and AMH) had nearly equal AUCs (AFC $ROC_{AUC} = 0.5894$, FSH $ROC_{AUC} = 0.5437$, and AMH $ROC_{AUC} = 0.5529$), which is in accordance with the findings of Nardo *et al.*[4] and Jayaprakasan *et al.*,[5] in which they demonstrated almost equal predictive accuracy between AFC and AMH. The findings of the present study did not corroborate with them regarding FSH and AMH, where they had established AMH as a significantly better predictor than basal FSH. The present study showed that patients with poor response were slightly older, but there is no statistical significance (poor vs good responders were 28.66 ± 4.06 vs 27.21 ± 3.29; $P = 0.25$) The results of the present study were in agreement with Van Rooij *et al.*, Hazout *et al.*, Eldar-Geva *et al.*, Kwee *et al.*, and Moawad *et al.* that there was no significant difference in age between good and poor responders.[7-11] This is because of the fact that only 10% of the study population belonged to age more than 31 years. Majority of them were young, and the duration of infertility was also less number of years. In the study of Jayaprakasan *et al.*, the mean age was 33.3 ± 3.6 in good and 35.7 ± 1.9 in poor responders.

Most of the studies in literature showed significant difference in AMH levels between good and poor responders. In the present study, none of the women

had AMH <1 ng/ml. This is because 72% were PCOS. In the present study, AMH values significantly increased in the three cycles with increasing doses of CC ($P = 0.005$), which is against the conclusion of Fanchin *et al.* that AMH is not influenced by gonadotropic status and reflects only the follicle population.[12] AMH had a negative correlation with basal FSH ($r = -0.006$; $P = 0.938$), which is in agreement with Milewicz *et al.* where $r = -0.40$; $P < 0.05$.[13] AMH stands next to AFC in ROC curve analysis for the good ovarian reserve and response, which is in concordance to Eldar-Geva *et al.*[9] No significant correlation was observed between AFC and AMH. This finding is against the results of Van Rooij *et al.*,[7] Visser *et al.*,[14] Majumder *et al.*,[15] Nardo *et al.*,[4] and Jayaprakasan *et al.*,[5] where they found that both AMH and AFC had highly significant correlation with ovarian response and similar predictive value.

By convention and in agreement with various studies (Gysler *et al.*[16] and Imani *et al.*[17]), the present study also showed a significant increase in ovarian response with increasing doses of CC. Ovarian reserve showed a decline but not to a significant level, which is in concordance with the results of Kelly *et al.*[18] and Luk and Arici.[2]

Good ovarian reserve does not always mean they respond well to ovarian stimulation. There are many predictive factors for ovarian response like free-androgen index, BMI, age, cycle history, duration of infertility, ovarian volume, fasting insulin levels, insulin-to-glucose ratio, serum leptin levels, and insulin-like growth factor levels as explained by Imani *et al.*[19,20]

Likelihood of pregnancy in a woman undergoing ovulation induction is subjected to a large number of factors other than ovarian reserve and response. Pregnancy rate in this series was 8%, which is very low compared to 30 to 40% rates of previous studies.[21-24]

Rate of hyperstimulation in the present study was 12%. Homburg[25] in 2005 stated that although mild ovarian enlargement was relatively common, he had never seen a full-blown ovarian hyperstimulation syndrome (OHSS) as a result of CC treatment in his 40 years of experience. The present study showed high rates because the study population were mostly women with PCOS. Predicting poor response to avoid treatment side-effects, expense, and psychological stress is very significant in the field of infertility, and identifying poor responders before undergoing expensive and time-consuming treatment is of paramount importance.

CONCLUSION

The present cohort of study population, which constituted mostly young infertile women with PCOS, AFC, and FSH, emerged as better tests to predict ovarian reserve. AMH levels did not differ significantly between women with good and poor ovarian reserve. This could be probably because the median value for poor responders was more than 3 ng/mL, as majority of study population belonged to polycystic ovarian morphology. Ovarian response significantly increased with increasing doses of CC. All the three ovarian reserve tests were not helpful to differentiate poor responders from good ones with the definition used. Ovarian reserve suffered a decrease with increasing doses of CC, but this did not reach significant levels. To confirm this, a large sample size would be necessary.

Limitations

(1) Nonhomogenous study population
(2) Small sample size
(3) Criteria used to differentiate good responders from poor ones were based on only minimal response to CC

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Conflicts of interest

There are no conflicts of interest.

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