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Follicular fluid cell free DNA in poly cystic ovary syndrome patients undergoing intracytoplasmic sperm injection program: relation to ovarian reserve and embryo quality

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Abstract: *Poly cystic ovary syndrome [PCOS] represents the most common disorder in women during reproductive age. Because of the expensiveness and invasiveness of in vitro fertilization procedures [IVF], it has been tried to find a trustful, non invasive way for choosing embryos. Cell free DNA [cfDNA] extracted from follicular fluid can provide an indicator for embryo quality. Ovarian reserve has an important role in the occurrence of pregnancy in IVF programs, the aim of the study is measure the amount of cfDNA in the follicular fluid in females with PCOS undergoing intracytoplasmic sperm injection program and relating it to the ovarian reserve and embryo quality. This prospective study was accomplished in AL-Samaray Teaching Hospital in Baghdad from December 2017 to March 2017. Sixty females were involved in this study. Thirty one did not have PCOS [control] and 29 had PCOS. Blood samples were withdrawn on day 3 of the cycle for hormonal analysis. All of the patients had entered through the ovarian stimulation protocol [antagonist protocol]. Follicular fluid was collected at time of oocyte retrieval; cfDNA was extracted, and calculated. Embryo quality was assessed too. In age matched POCS and control groups, the correlation between body mass index and cell free DNA in all cases was a non significant positive correlation [p>0.05].There was also a non significant positive correlation between cfDNA and FSH level in both group [r=-0.080 and 0.228 respectively]. The correlation between AMH and cfDNA was a significant negative one in PCOS group while it was not significant in control group. there is a significant difference in cfDNA levels according to the embryo quality being less in grade1 and 2 embryos and more in bad grade 3 and 4 embryos in both groups.Cell free DNA in follicular fluid correlated negatively and significantly with the level of AMH in polycystic ovary syndrome patients, so the low levels of cfDNA indicates high ovarian reserve. The quality of embryos after intracytoplasmic sperm injection increased in embryos yielded from follicles with low levels of cfDNA.*

Keywords: *AMH , cfDNA, embryo quality, ICSI, ovarian reserve , PCOS.*

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I. Introduction

Infertility is considered as the most important, as it`s percentage increased worldwide and about 72.3 million female are now infertile [1]. Infertility can be defined as the inability of a female to become a pregnant or the inability to bear a fetus till the end of pregnancy [2]. It can be divided to primary or secondary infertility. One of the causes of infertility is poly cystic ovary syndrome [PCOS], as it is responsible for about 75% of infertility causes [3]. It is a common endocrine disorder that affects females in their reproductive age and it has much cardiovascular, endocrine and reproductive consequence [4].

Artificial reproductive technology [ART] is considered to be costy and invasive procedure, that is why is it used as a last method, so researchers had tried to find an accurate way in order to increase the success rate by improving embryo selection [5]. The selection of embryo depends only on morphological criteria to predict the successful ART procedure, and it is found by observation that this procedure has limited expectations [6]. The work was hard to find out a biomarker to improve ability to expect embryo quality and later implantation possibility and as a consequence improvement of IVF outcomes, but most of these methods are under investigation till now [7].

Follicular fluid nowadays is thought to be useful as a biomarker for embryo quality as it is derived mainly from plasma and granulose cell secretion [8] it a mixture of many proteins ,metabolites and ionic component , also it is found that it reflects the degree of maturation of the ovarian follicles [9].

Cell free DNA fragments are considered as important biomarker for many diseases. They are found normally in plasma and result mainly from apoptosis and\or necrosis; also they are present in follicular fluid of ovarian follicles [10]. Cf DNA fragments are normally found in small amount in blood but their levels are increased in certain conditions [11].These fragment also had been found in maternal bloods which provide great approach in obstetrics and gynecology [12].

To achieve pregnancy, it is important to consider ovarian reserve especially during IVF cycles in order to make prediction of the IVF outcome. The most common used test is antimullarian hormone [AMH] [13].

II. Patients And Methods

This prospective study was performed in AL-Samaray Teaching Hospital for infertility and artificial reproductive technology [ART] in Baghdad from December 2017 to March 2017.

Sixty samples had been collected from Kamal AL-Samarrai hospital. Thirty one of them were free from PCOS [control group] and, 29 females had PCOS diagnosed according to Rotterdam criteria [14].The mean age of the women is between [23-40] years, with different infertility duration of both groups. Body weight and length were measured and BMI was calculated [BMI=weight/[length]^2]. Several hormone level estimations were done [prolactin, follicle stimulating hormone [FSH], luteinizing hormone [LH], estradiol [E2], and testosterone] at day 3 of cycle. Antimullarian hormone [AMH] was also measured using. All hormones were estimated using ELIZA technique. Both groups went through the intra cytoplasmic sperm injection [ICSI] program.

In vitro fertilization and follicular fluid collection :

All 60 patients had received antagonist treatment [Cetrorelix Merek Serono, Switzerland] and ovarian stimulation with recombinant FSH [Puregon, MSD, Courbevoie, France] or Gonal F [Merck-Serono, Switzerland]. Ovarian stimulation response was monitored by measuring 17ß estradiol [E2] concentration and by doing transvaginal ultrasound evaluation of endometrium thickness and follicular growth.

A single injection of human chorionic gonadotrophin had been given when at least 3 follicles reaches to 17 mm in diameter [Ovitrelle Merck Srono, Lyon ,France] in dose of 250 µg and is given at day 12 of cycle . After 34-35 hours, oocyte retrieval had been done by transvaginl ultrasound guided aspiration of the follicles.

Follicular fluid samples had been collected, fluids which contain blood [gross appearance] were neglected, and they were stored immediately except for about 1 ml of fluid which was centrifuged and stored for E2 estimation later on by ELIZA technique [human ELISA kit ,Germany], and the rest 1.5 ml was put in freezer immediately for cell free DNA measurement and PCR. The corresponding cumulus- oocyte complex were isolated for ICSI procedure and cultured in Gain 1 culture media [bicarbonate-buffer balanced salt solution with 10 mg\L Gentamicin and 3.5 g\L human serum albumin], after 4 hours ICSI procedure was done. Eighteen to 20 hours later, normal fertilization was confirmed by the presence of 2 polar bodies and after 25-27 hours ,early cleavage had been observed and embryo quality was reported using the grading from 1-4 according to morphological and fragmentation rate.

On day 3, embryo transfer was done for grade 1 and 2 while grade 3 or 4 [bad embryo quality] were discarded.

Table [1]: Morphological criteria for grading of embryos at day 3 [day of transfer according to the guidelines of Al-Samarai Hospital.

	Grade 1	Grade 2	Grade 3	Grade 4
Number of blastomere	6-8	6-8	6-8	>6
Blastomere regularity	regular	regular	irregular	irregular
Rate of fragmentation	<10	10-25	26-40	>40

cfDNA extraction from follicular fluid :

This was done by using Genomic kit [Geneaid, USA]. DNA extraction had been done by agarose gel electrophoresis.

Quantification of cf DNA concentration:

Purity and concentration of DNA solution extracted from fluid sample were determined by measuring the absorbance of DNA solution at 260nm [A260] and 280nm [A280] by using UV spectrophotometer.

DNA purity =A260\A280, the ratio for pure DNA range between 1.8 and 2.0 [14].

Cell- free DNA calculation:

Preparation of buffer containing [25 ml\L Tween 20 , 50 mmol\l Tris and 1mmol\l EDTA] [14] about[10 µl] of buffer with[10µl] of F.F. DNA digested in[8 µg] of proteinase K [Qiagen] mix with pipette then the mixture was put at [50o C] for [20 min] followed by heat inactivation and in solubilisation at [95oC] for [5 min.].

Statistical analysis: All data were analyzed using SPSS 20.0.All values were in mean ± SD .Comparison between means was done using unpaired t tests. A significant P value was considered as than 0.05. Correlations were done using Spearman correlation coefficient.

III. Results

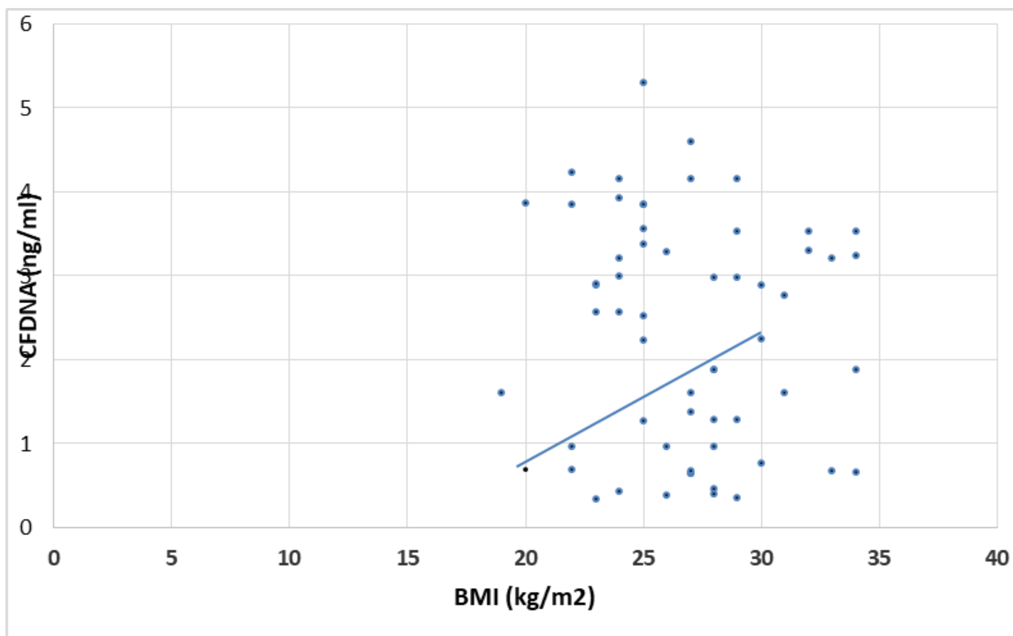
Table 2 shows the non significant difference between patients and control groups in age, and BMI [P>0.05].

Table [2]: Comparison between POCOS and control groups regarding age and BMI.

	PCOS	Control	P value
Age [years]	29.0 ± 5.3	33.0± 7.0	0.09
BMI [Kg/m^2]	25.3± 2.1	24.4± 2.5	0.12

P is Significant at ≤ 0.05

Figure 1 shows the correlation between body mass index and cell free DNA in all cases, which was non significant positive correlation.



r = -0.064, p = 0.6

Fig [1]: Correlation between body mass index of females and cell free DNA in follicular fluid in all cases.

Table 3 shows the non significant positive correlation between cfDNA and FSH level in both groups.

Table [3]: Correlation of FSH with cell free DNA in PCOS and control groups

	Group	FSH [mIU/ ml]	
		r	P
cfDNA [ng/ml]	PCOS	0.091	0.662
	Control	0.23	0.243

P is Significant at ≤ 0.05

The correlation between AMH and cfDNA was shown in table 4, which demonstrates the significant negative correlation between them in polycystic ovary group while it was not significant in control group

Table [4]: Correlation between AMH and cfDNA in both polycystic and control groups.

	Group	AMH [ng/ml]	
		r	P
cfDNA [ng/ml]	PCOS	-0.070	0.04*
	Control	-0.219	0.243

P is Significant at ≤ 0.05

In table [5] there is a significant difference in cf DNA levels according to the embryo quality being less in grade1 and 2 embryos and more in bad [G3,4] quality embryos in both groups.

Table [5]: Comparison of embryo quality according to the level of cfDNA in PCOS and control groups.

Group	cfDNA [ng/ml]				P. value
	Grade 1 Mean±SD	Grade 2 Mean±SD	Grade 3 Mean±SD	Grade 4 Mean±SD	
PCOS	0.55±0.17	1.8±0.65	2.99±0.69	4.09±0.54	< 0.001*
Control	0.69±0.27	1.29±0.49	3.45±0.76	3.86±0.38	< 0.001*

* P is Significant at ≤ 0.05

IV. Discussion

As the needs grow up for a better prediction of the good embryos to be obtained from IVF programs, this study was designed to take a role. In age matches polycystic ovary patients and control group [non PCOS], cfDNA in follicular fluid had positive correlation with BMI. This result is in agreement with that declared by Vora and his team, who found that the clearance of cfDNA is less efficient in obese women than non obese ones [15] and as it is known that follicular fluid is a combination of both plasma and granulosa cell secretion that's why cfDNA is high in follicular fluid in obese females [16].

The development of follicles in the ovary depends on the interaction of many hormones, like FSH and AMH [17]. A negative correlation had been demonstrated between cfDNA and FSH levels in blood of PCOS group, but positive one in control group. FSH has an important effect on the development and growth of the antral follicle [18]. The high concentrations of cfDNA are thought to stop the synthesis of new oocytes [19]. This effect makes FSH secreted in high amounts to stimulate more oocytes to grow, which appears in the positive correlation between cfDNA and FSH. CfDNA level is found to be higher in follicles from females with low ovarian reserve, as compared to females with normal ovarian reserve [20]. The high basal FSH levels [days 2-3 of the menstrual cycle] is associated with low production of oocytes and, so, the increase of cfDNA concentration may give indication of the number and the quality of oocytes pick up [5].

Cell free DNA was correlating negatively with AMH levels. This result is consistent with that found by another study that proved the correlation and reported that the decrease in AMH levels in serum is considered an indicator of a drop in the reserve of the follicles [17]. This result consolidate the role of the estimation of cfDNA in predicting ovarian reserve status.

As it is known that follicular fluid is considered as the microenvironment of the oocyte and eventually the subsequent embryo quality for this reason many tried to use follicular fluid as a biomarker to predict embryo quality as a non invasive procedure [8].

CfDNA quantity in the follicular fluid was negatively correlated with embryo quality. High cf-DNA in the follicular fluid was associated with poor embryo quality, which goes with the work done by Czamanski and his coworker who had found that the high cfDNA is associated with poor IVF outcome and low pregnancy rate. It was thought that cfDNA might increase due to the fact that the stress in females undergoing IVF leads to the increase in the level of cfDNA in these females which leads to poor IVF outcome, and after performing relaxation technique procedure in these females, it was found that level of cfDNA started to decrease which led to improvement of IVF results [21, 22]. Recently Salici, and his team had found that the low level of cfDNA was associated with good embryo quality and vice versa and this was due to the accumulation of negative signals that affect embryo division [10].

V. Conclusion

Cell free DNA in follicular fluid correlated negatively and significantly with the level of AMH in polycystic ovary syndrome patients, so the low levels of cfDNA indicate high ovarian reserve. The quality of embryos after intracytoplasmic sperm injection increased in embryos yielded from follicles with low levels of cfDNA.

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