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Evaluation of Cell Free DNA in Follicular Fluid and Embryo Quality in Poly Cystic Ovarian Syndrome of Iraqi Women

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Abstract
Background: *Poly cystic ovary syndrome (PCOS) is still considered as the most controversial disease that affected females at reproductive age. It represents about (5-10%) of females worldwide. Cell free DNA (cf-DNA) is found normally in blood as a result of apoptosis. Lately it is found to be present in follicular fluid as a result of apoptosis of follicular cell. Studies has been conducted to explore the ability to use it as an indicator of the quality of oocyte and embryo in the in vitro fertilization procedures (IVF) to have a better and higher success rate, since IVF is considered as an expensive procedure and it is the last hope for families to have children .*
Methods: *(60) samples of women had been involved in this study.(32) of them were having polycystic ovary (PCOS), and (28) were not (having un-explained infertility), and considered as control. This prospective study was done in AL-Samaray Teaching Hospital for infertility and artificial reproductive technology (ART) in Baghdad during the period from January to March 2017.*
Results:*It found that there is no significant difference in the amount of cf-DNA between control and PCOS patient was (2.49±1.41 ng/µl vs 2.15±1.31ng/µl respectively) .cf-DNA levels are significantly lower (p ≤o.oo1) in good quality of oocyte than bad ones (3.58±0.68 ng/µl vs 1.04±0.5ng/µl respectively) in control and (3.17±0.77ng/µl vs 0.89±0.65ng/µl) in PCOS patients. 17 ß-estradiol level measured at time of oocyte collection was found to have negative correlation (non significant) with cf-DNA(r= - 0.033 and p=0.869 in control and r= -0.0238 and p=0.18 for PCOS). Good quality of embryos was found to have significantly lower cf-DNA than bad ones and the correlation between them was significantly negative (r= - 0.874 ,p=<0.001 for control and r= -912 ,p=<0.001 for PCOS).*
Conclusion*: There is no difference in quantity of cf-DNA in the follicular fluid between control and PCOS females. Cell free DNA was higher in Poor quality of embryos, while it was low in top quality embryo.*
Keywords *: cf-DNA, embryo quality, estradiol ,PCOS ,IVF*

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I. Introduction

Infertility can be defined as the inability to become pregnant in spite of regularly, unprotected intercourse for (1) year (WHO, 2000). There are different causes of infertility which can be male or female caused. Male causes accounts (19-57%) while female cause account about (21-32%),while un explained infertility accounts (8-30%), and for both male and female factors (34%)(Maheshwari, *et al*.2008 & Wilkes, *et al*.2009).

Poly cystic ovary syndrome is considered as the main cause of ovulatory dysfunctio\, it causes (75%) of inovulatory infertility cases (Frank,*et al*.2008& Anwen,*et al*.2006).

For promoting fertility, in vitro fertilization(IVF) is considered as the best method but it has an invasive and costly aspect, for this reason selection of embryo in this procedure is important to increase success rate by finding the most cost effective and accurate ways for selection of embryos (Czamanski-Cohen *et al* .,2013). Many researchers have been used molecular-level examination of embryo media so that they can find out a biomarker which improves their ability to expect embryo implantation potential and at the same time improves IVF treatment outcome. Many of these parameters have focused on different body fluids and tissues beside embryonic culture media. Besides all of them are still under investigation, but it should be kept in mind that these methods have a promising improvement and predictive value in assisted reproduction (Assou *et al*., 2010).Time lipase technology have a promising way to describe embryo morphokinitic data that will help in embryo selection (Herrero and Meseguer, 2013). Follicular fluid (F.F) is considered as a micro- environment for the oocyte that reflects the degree of its development and follicular maturation (Hashemitabar *et al.,* 2014). It is derived mainly from plasma and secreted from granulose cell , many researchers studied it as a biomarker

(Lédéé *et al*., 2013). Cell free DNA are fragment that result from apoptosis and\or necrosis process, it was detected in plasma and other body fluid including F.F. (Scalici *et al*., 2014). Normally they present in blood in low amount because of process of phagocytosis done by macrophages, their amount is increased in presence of other disease as cancers (Schwarzenbach *et al*., 2011). Since the appearance of cell free fetal DNA as a non invasive prenatal test, it carried a promising future in obstetric and gynecological study (Liao *et al*., 2014).

II. Materials and methods

I- Samples

Sixty women had been participated in this study, all were chosen from AL-Samurai Teaching Hospital for infertility and artificial reproductive technology (ART) in Baghdad during the period from October 2016-March 2017. Those females have been sub grouped to (32) of them were having polycystic ovary (PCOS), and (28) were not (having un-explained infertility), and considered as control.

To measure the amount of cf-DNA levels in follicular fluid (FF) samples from women with PCOS undergoing in vitro fertilization (IVF) programs, and relate it to the ovum and embryo quality it consider as anon invasive biomarker**.** After taking thorough history and full examination, blood samples were drawn from them, and they were given ovarian stimulation protocol (antagonist protocol). At time of oocytes collection follicular fluid had been collected, DNA was extracted, and cf-DNA had been calculated.

(The age range of both PCOS and control groups was 32-20 years vs 20-40 years respectively).Polycystic ovary syndrome in the study group was diagnosed according to Rotterdam criteria:

1-Oligo-ovulation or an ovulation .

2- Clinical or biochemical hyper-androgenisim and\or

3- poly cystic ovary by ultra-sound.(Rotterdam, 2004)

Couples with male factor infertility have been excluded also females with any endocrine disease (daibetes mellitus ,thyroid disease ,liver disease ,renal disease ,etc) and females with Moderate to severe hyperprolactnemia .

After taking full history and doing examination , blood samples have been drawn at day 2 of the menstrual cycle. Leiutinizing hormone, follicle stimulating hormone, prolactin hormone, estradiol hormone (E2)have been measured. Ovarian stimulation protocol was started to females according to the antagonist protocol (AL-Inany, *et al* .2007). It included giving the woman follicular stimulating hormone injection (r-FSH)(Puregon, MSD, Courbevoie, France or Gonal –F Merck-Serono, Switzerland) twice daily. At day (5) of cycle E2 was estimated and ultrasound was done to evaluate the number and size of follicles and accordingly the dose of FSH is adjusted . Cetrotide (Merek Serono,Switzerland) (antagonist) was given in a dose of 0.25 daily till the day of hCG injection (human chorionic gonadotrophin) (Ovitrelle, Merck Serono, Lyon, France , at dose of 6000 I.U), that is at the time of gaining at least 3 follicles measuring 17 mm and above. Estradiol is remeasured again. (32-36) hours later, oocytes were collected under ultrasound guide. Oocytes were stored in culture media (Gain 1), which is (Bicarbonate-buffered balanced salt solution with 10mg\liter Gentamicin and (3.5g\l) human serum albumin).

After preparation of sperm, intra cytoplasmic sperm injection was done .Twenty hours later examination of fertilized ovum was performed for the presence of 2 pronucli which normally appears after(12) hours of fertilization. Transfer of embryos were done at day (2 or 3) after fertilization. All women were given progesterone supplementation in the form of Dauphston tablet (Dydrogesteron 10 mg , Abbot ,Netherland)and Cyclogest supposteris (Progesteron 400 mg, Actavis , UK). βhCG (β-Human chorionic gonadotrophin) hormone is estemated in blood after (14) day of embryo transfer for pregnancy confirmation.

Follicular fluid was collected at time of ovum pickup. Any F.F which was contaminated with blood or does not contain oocyte were discarded. These samples were freezed directly at (-20C°) ,till the time of measurement, then about (1ml) of F.F was centrifuged for estimation of E2 by ELISA test .

Extraction of the DNA is done according to the kit used, DNA extraction was proved by agarose gel electrophoresis (Sambrook *et al* .,1989).

II- Quantitation of DNA concentration:

Purity and concentration of DNA solution extracted from fluid sample were determined by measuring the absorbance of DNA solution at 260 nm (A260) and 280nm(A280) by using Nano drop spectrophotometer

DNA purity =A260\ A280 this ratio for pure DNA range between 1.8-2.0

III- CF-DNA concentration:

Preparation of buffer containing (25 ml\L tween 20 , 50 mmol\l Tris and 1mmol\l EDTA) (Umetani *et al* . 2006) about(10 μl) of buffer with(10μl) of F.F. DNA digested in(8 μg) of proteinase K (Qiagen) mix with pipatte then the mixture was put at (50^{o} C) for (20 min) followed by heat inactivation and in solubilisation at

(95°C) for (5 min) then sample was taken and distilled water (970ml) was added to the mixture to be ready for reading by Spectrophotometer at (260 and 280) .
The two reading were taken and then the difference were calculated then an equation was applied

$$= \frac{(260\text{-}280) \text{ X dillusion factor X DNA(sample)}}{1000}$$

The result represent the amount of nucleic acid (cell free DNA) that present in the sample. Estradiol estimation was done by bacteriologist by using human ELISA kit (Germany).

III. Results

For comparing the age for the PCOS and control patients, it was found that the control patients are significantly older (p= 0.001) than PCOS ones (Figure 1)**.**

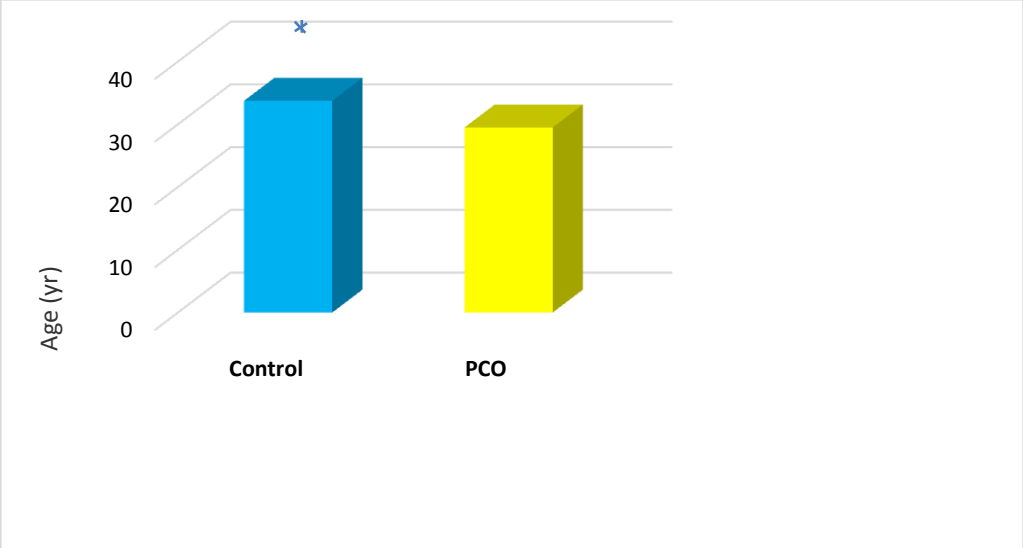

Fig (1) comparing age between PCOS and control females.

The duration of the PCOS disease has been correlated with the amount of cf-DNA and it appears that there is negative no significant correlation between them i.e. the longer the duration of the disease the less cf-DNA amount (Table- 1).

Table (1) correlation between duration of PCOS and cf- DNA:

	Group	**Duration (yr)**	
		r	**P**
Cf- DNA	**PCO**	**-0.082**	**0.657**

P=<0.05

Table (2) shown a negative correlation between cf-DNA and F.F.E2 but it is of no significance.

Table (2) Correlation of FF E2 level with cf- DNA in PCO and control groups

	Group	**FF E2**	
		r	**P**
cf-DNA	PCO	-0.238	0.189

P=<0.05

After assessing the quality of eggs, it appears that good quality eggs have low level of cf-DNA , while bad quality ones have high level of cf- DNA, and there is significant difference between them in both studied groups (Table 3).

Table (3) Comparison of cf- DNA level according to egg quality in PCOS and control groups

	Egg quality		
Group	**Bad Mean±SD**	**Good Mean±SD**	**P value**
PCOS	3.17±0.77 (N=17)	0.98±0.65* (N=15)	< **0.001**
Control	3.58±0.68 (N=16)	1.04±0.5* (N=12)	< **0.001**

* P ≤ 0.05 is significant.

The embryo quality is correlated negatively and significantly with cf-DNA i.e. the worse the embryo the more cf-DNA table (4)

Table (1-4) Correlation of embryo quality with cf- DNA in PCO and control groups

	Group	**Embryo quality**	
		R	**P**
cf-DNA	PCOS	-0.912*	**< 0.001**
	Control	-0.874*	**< 0.001**

- P=<0.05

In Table (5) Comparison of biochemical pregnancy according to cf-DNA in control group,the table (6) Comparison of biochemical pregnancy according to cf-DNA in PCOS group.

Table (5) Comparison of biochemical pregnancy according to cf-DNA in control group

Group	**Biochemical pregnancy**	**Follicles with ↑ cf-DNA N=15**	**Follicles with ↓ cf-DNA N=13**	**P value**
Control	**Positive**	**0**	**6**	**0.005**
	Negative	**15**	**7**	

CFDNA cutoff value = 2.89 p≤0.05

Table (6) Comparison of biochemical pregnancy according to cf-DNA in PCOS group

Group	**Biochemical pregnancy**	**Follicles with ↑ cf-DNA N=10**	**Follicles with ↓ cf-DNA N=22**	**P value**
PCO	Positive	0	10	**0.010**
	Negative	10	12	

IV. Discussion

Polycystic ovary syndrome (PCOS) is a widespread diagnosis in females presenting with infertility. All the aspects of PCOS have not been completely explored concerning further results of egg and embryo quality after using ovarian stimulation protocols in IVF/ICSI cycles.

In the studied population, PCOS patients were younger than controls, and this age difference is sometimes cannot be avoided, and PCOS usually impacts young women in their reproductive age (Teede et al, 2010).There was a negative correlation of the amount of cfDNA with duration of PCOS , this result goes with that have been found by Sonntag who worked on hyper stimulated granulose cell of ovary and studied the viability of cells after Metformin treatment. They documented that there was reduced susceptibility of the granulose cell to undergo apoptosis with the increase of the duration of the disease (Sonntag , *et al.* 2005), and hence the patients in this study all had been taken Metformin of different periods according to duration of PCOS, this may explain the low amount of cf-DNA with long duration of PCOS unlike those with short duration of PCOS. Correlating cfDNA quantity to the oocyte quality, there is a significant negative correlation between them (p=0.001), this result goes with that of a research done by Lee,and his *et al.* whom found that apoptosis of cumulus cells of oocyte was high in bad quality one and this was associated with lower fertilization rate and lower pregnancy rate , while low rate of apoptosis of cumulus cells of the oocyte was associated with good quality oocyte and high rate of fertilization (Lee, *et al.*2001).

In this research it was proven that the high level cf-DNA is associated with poor embryo quality while low cf-DNA is accompanied by good embryo quality this is explained by the presence of high level of apoptosis in poor embryos and it is also as proven by Czamanski ,and his team who found that high level of cf-DNA in plasma one week after IVF is associated with high prediction of IVF failure since it is thought that the cf-DNA of the embryo may pass to the circulation of mother causing high level of cf-DNA in the circulation especially in the stress state (Czamanski-Cohen *et al.*, 2012). Salici and his co-workers used cf-DNA in F.F as a biomarker to predict embryo quality and he found that the high cf-DNA is associated with accumulation of negative signals which causes the increase of fragmentation rate and decrease the cleavage of embryos, but it does not affect the maturation of oocyte as its maturation continue even with the presence of high level of cf-DNA(Salici,*et al.*2014) . In this study, cutoff value was calculated to be (2.89),and it was found that pregnancy rate was higher in females with cf-DNA less than 2.89, while pregnancy rate decreased with higher cutoff value (more than 2.89) for both control and PCOS patient. This goes with the result of other research which found that detecting high level of cf-DNA can be used as a predictive value for IVF outcome (Czamanski-Cohen *et al.*, 2012). On the other hand, Hill and his team had found that there is no difference in cf-DNA between pregnant and non pregnant females (Hart, *et al*.2005). This difference can be explained by the fact that the present study was done in F.F. while Hill research had done their measurements in blood and one week after

embryo transfer. Other studies have found that high cf-DNA made hostile environment that may affect conceiving (Rodgers, et al. 2010).

As conclusion: The quantity of cf-DNA in the follicular fluid has no difference between control and PCOS females.Poor quality embryos has higher cf-DNA, while good quality embryos has low amounts. Pregnancy rate was higher in follicles with low cf-DNA.

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