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Cell-free DNA in Human Follicular Fluid as Biomarker for Intracytoplasmic Sperm Injection Procedure Outcome

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Abstract

BACKGROUND: Follicular fluid (FF) is considered as an important microenvironment for oocyte development. Cellfree DNA (cfDNA) fragments that are found in this fluid and are released from cell apoptosis and/or necrosis.

AIM: This study aimed to quantify the level of cfDNA, in the FF, and to assess any relationship between the level of cfDNA in this fluid with women's age, duration of infertility, cause of infertility, and her ovarian reserve values.

METHODS: Eighty-nine women were prospectively included in this study FF cfDNA, which was determined by conventional real-time PCR-SYBR green detection approach which quantified by ALU-specific primers.

RESULTS: Cell-free DNA (cfDNA) level in FF samples of Iraqi women level was cfDNA (Mean ± SD, 0.916 ± 0.106 ng/µl). There was no significant relation between cfDNA and pregnancy outcome, but very low level and very high level cfDNA were related to negative pregnancy outcome, cfDNA was the second most important predictive factor of pregnancy outcome after fertilization rate, but both not statistically significant p-values were 0.622 and 0.241, respectively.

CONCLUSION: The present study notices that cfDNA in the FF may mainly reflect the cellular activity and the balance between programmed apoptosis and cell necrosis.

Introduction

Despite the advancement in treatment techniques, infertility remains a struggle for many doctors to treat couples in their reproductive years. Many doctors prefer a single embryo transfer as an important step in the management, but the selection methods for the embryo with the highest chance for implantation still rely on embryo morphology alone; however, there are limitations to these evaluations and selections [1], [2]; researcher focused on non-invasive biomarkers based on oocyte microenvironment studies to upgrade embryo selection process [3]. New "omics" technology includes biomarkers and assays for oocyte and embryo selection *in vitro* fertilization (IVF/ICSI). It is a new procedure that depends on different body fluids and tissues, genomics, transcriptomics, proteomics, and metabolomics [4], [5]. How uses follicular fluid (FF) produced from plasma with a product from granulosa cells. It is significant since it is the environment, in which the oocyte grows [2], [6]. The oocyte is the functional unit of the ovary, surrounded by somatic cells, which are the granulosa cells. It is the female gamete (germ cell) that is participating in the fertilization process. The environment for oocyte growth is FF produced from plasma and contains factors secreted by granulosa cells (Gc). It is used as a non-invasive practical method for predicting oocyte quality in assisted reproductive techniques [7], [8]. The most recent method in IVF is a genomics analysis of cell-free DNA (cfDNA). Mandel and Metais discovered the cfDNA in the body's tissues and fluids in 1948 and named it cfDNA (cell-free DNA). It also included extracellular DNA molecules, such as those present in serum, plasma, and other body fluids [9], [10]. DNA was shown to be the inheritance's material byWcatson and Crick in 1953, the researchers demonstrate its presence in the plasma of healthy and ill individuals [11]. These discoveries enhanced scientists in other fields, who began looking for non-host DNA in the plasma and serum. Lo *et al*. in 1997 first reported that the existence of fetal derived Y chromosomal DNA sequence in maternal circulation [12] was initially reported by which later made a non-invasive prenatal testing feasible and early given the high detection rate for trisomy 21 [13]. It has recently been shown that it is also higher among people who have pre-eclampsia, pre-term labor, and other complications during pregnancy [14], [15]. Scientists are looking into the existence of cfDNA in several fields of reproductive medicine, the levels of plasma cfDNA during ovarian

stimulation, as well as the relationship between cfDNA concentration and pregnancy rates in women undergoing IVF-embryo transfer were investigated in several prospective studies [16]. Moreover, these studies discovered that plasma cfDNA levels were higher in women who did not conceive than in those who did, and the author linked this finding to the presence of factors that may interfere with the embryo implantation, as a result, they decided that cfDNA analysis in individual FF samples could be a useful biomarker for predicting the best quality embryo [2], [5] cell-free DNA: Are circulating free cell DNA are degraded DNA fragments which are non-capsulated genetic materials, cfDNA refers to nuclear or mitochondrial DNA fragments. Nuclear strands are typically 150–180 base pairs (bp) in length, have an average half-life of fewer than 2 h, where macrophages rapidly phagocytized it in healthy individuals who have a low level of cfDNA [17], [18]. Its formation is most likely related to one of three events: Apoptosis, necrosis, and active secretion. Healthy people get their cfDNA primarily from apoptotic cells, which are considered the principal source of this cf-DNA as there is 50-70 billion cells die via programmed cell death. These cells are primarily phagocytosed by macrophages shortly after being released into circulation. This cfDNA can be quantified in the FF and investigated as a non-invasive simple method to qualify the oocyte's microenvironment and predict the success of treatment in the ICSI cycle [19], [20]. In this study, we aimed to quantify the level of cfDNA, in the FF, and to assess any relationship between the level of cfDNA in this fluid with women's age, duration of infertility, cause of infertility, and her ovarian reserve values then we searching to found any relations between the types of protocol, several oocytes, embryos, and success of ICSI outcomes by assessing the clinical pregnancy results. Trying to find a new non-invasive parameter that may reflect the oocyte microenvironments development and that reflects the quality of oocytes and can expect the response to the stimulation, quality of embryo developed. Moreover, see whether this new parameter may improve the infertility treatments and results.

Materials and Methods

In this prospective study, the data from 89 patients undergo *in vitro* fertilization cycles conducted at the Reproductive Medical Centers for Fertility and IVF cycle, in High Institute of Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University, and AL-Nada IVF private center (Baghdad/Iraq), between October 2020 and August 2021, under the supervision of consultant genetic researchers from Forensic DNA Center for Research and Training (AL-Nahrain University). After informing the participants about the objectives and methods, their consent was obtained on the day of oocyte retrieval. Arab Focality approved this study for medical specialization. The patient's characteristics are detailed in Table 1. Women ages ranged from 19 to 42 years (Mean ± SD 30.9 ± 6.1), their body mass index (BMI) was between 19 and 33 (Mean ± SD 25.96 ± 3.48 kg/m^2), and infertility duration 1–17 years (Mean ± SD 6.9 ± 4.4). Cause of infertility was a malefactor in 30.3%, hormonal assessment in day 3 of the cycle, and AFC evaluated day 5 of the cycle. Patients received standard ovarian stimulation with recombinant FSH (r-FSH), two methods are used either under pituitary suppression with a GnRH agonist, the GnRH agonist (Decapeptyl 0.1 mg Ferring, Kiel Germany) subcutaneously in the mid-luteal phase of the previous cycle usually day [21], with a daily dose, after check pituitary desensitization then ovarian stimulation was achieved by administration of 150–225 IU/day of r-FSH (Gonal-F, Merck Serono SA Aubonne Branch, Swiss Confederation). FSH was administered on an individual basis according to the

Table 1: The demographic and biochemical features of the study group

Variable	No.	%	Mean ± SD	Range
Wife age (years)				
<20 years	1	1.1	30.9 ± 6.1	19–43
20–29	37	41.6		
30–39	42	47.2		
≥40 years	9	10.1		
BMI (kg/m^2)				
Normal (18.5–24.9)	34	38.2	25.96 ± 3.48	19–33
Overweight (25–29.9)	47	52.8		
Obese (≥30)	8	9.0		
Duration of infertility				
<2	4	4.5	6.9 ± 4.4	1–17
2–4	27	30.3		
≥5 years	58	65.2		
Cause of infertility				
Endometriosis	8	9.0		
Hypogonadotropic hypogonadism	3	3.4		
Male	27	30.3		
Mixed	10	11.2		
PCOS	6	6.7		
Poor reserve	12	13.5		
Tubal	11	12.4		
Unexplained	12	13.5		
First ICSI				
Yes	31	34.8		
No	58	65.2		
Type of protocol				
Antagonist	83	93.3		
Agonist	6	6.7		
FSH (U/L)				
<10	76	85.4	6.57 ± 3.06	0.10–16.3
≥10	13	14.6		
LH (U/L)				
<3	13	14.6	7.79 ± 8.31	0.10–35.9
3–5	38	42.7		
>5	38	42.7		
AMH				
≤1	26	29.2	2.04 ± 1.34	0.24–7
>1	63	70.8		
AFC				
<10	28	31.5	13.28 ± 7.14	3–38
≤10	61	68.5		
17 b-estradiol (E2; pg./ml)				
<1000	30	33.7	1459.0 ± 805.3	236.1–
1000–2000	40	45.0		3500
>2000	19	21.3		
Prolactin			20.85 ± 11.73	1.2–56.66
TSH			1.97 ± 0.99	0.51–6.00
Peak E2 level (pg./ml)			40.14 ± 19.07	1.0–87.97
Duration of stimulation (days)				
7–10	23	25.6	11.1 ± 1.3	8–13
>10	66	74.4		
Total dose				
<3000	58	65.2	2487.3 ± 780.4	800–4500
≥3000	31	34.8		

BMI: Body mass index, ICSI: Intracytoplasmic sperm injection, AMH: Anti-Mullerian hormone, AFC: Antral follicle count, E2: 17 b-estradiol, TSH: Thyroid-stimulating hormone.

ovarian response, assessed by sequential transvaginal ultrasonography and serum estradiol measurements. Or using the GnRH antagonist program, by a starting daily dose of 200–225 IU, rFSH (Gonal F Merck Sernono, or Follitrope LG Life Science) was started on day 2 of the menstrual cycle, women received the GnRH antagonist cetrorelix acetate (Cetrotide; Asta Medica AG, Frankfurt, Germany) at a dose of 0.25 mg/day from the day when the dominant follicle reached a mean diameter ≤14 mm until the day of HCG administration. In the stimulation period, rFSH daily dose was adjusted individually. In all groups, the trigger of the oocyte by either Ovitrelle 250 μg/0.5 ml, Merck Serono S.P.A-Italy, or Pregnel (HCG) 5000 iu/ampMSD, both adjusted according to the BMI of patients, in patients with a high risk of OHSS trigger done by decapeptide (0.2 mg) SC, to avoid OHSS, oocyte aspiration was performed (35–36 h) after HCG injection. Intracytoplasmic sperm injection was performed using standard procedures and the embryos were transferred 3–5 days later. Maturation and morphological features of oocytes were investigated immediately before ICSI, fertilization rate, early embryo development, and transfer were documented and studied. All FF samples were collected from the patients with clear appearance, 10 ml was centrifuged for 10 min at 3000 g, later on, filtered with 0.45 μm filters paper then stored at −8°C, until all collected samples were completed and ready to analyzed cfDNA. Fertilization of oocyte which is then cultured in universal IVF media (Orogio, Denmark), the fertilization 18 h examined, and on days 2–3. On days 4–5, quantitative of ALU repeats: Cell-free DNA extraction and quantification by ALU-qPCR FF pools were prepared for cfDNA quantification as previously reported [20]. We use gSYNC (DNA) Extraction Kit, with a cat. No. GS100 and Lot No. FG32501 (Geneaid company). Add 400 μl of W1 buffer to the GS Colum and put the tube in a centrifuge at 14,000 g for 30 s, discard the flow-through, place the GS Colum. Add 600 μl of wash buffer and put the tube in a centrifuge for 30 s discard the flow-through, put the tube in a centrifuge without adding any buffer because dry the tube, take 30 for ELUTION buffer to GS Colum and let stand for at least 3 min, and put the tube in a centrifuge at 14,000 g for 1 min, elute the purified DNA. The total cfDNA was quantified by qPCR, using ALU 115 primers. Each ALU-qPCR reaction included 1 μL of PK-digested FF pool and a reaction mixture containing 0.25 μM of forward and reverse ALU 115 primers and 5 μL of 2X LightCycler480 SYBR Green I master mix (Roche Applied Science, Germany). CfDNA concentration in FF pools was determined using a standard curve obtained by successive dilutions of genomic DNA [21]. A negative control (without template) was integrated into each qPCR plate and each FF pool was analyzed in quadruplicate. To determine which proportion of cfDNA was released from necrotic or apoptotic cells, cfDNA was also quantified using qPCR with ALU 247 primers. These primers amplify only larger fragments that result from necrosis. This allows the calculation of DNA integrity using the Q247/Q115 ratio [21], which represents the proportion of cfDNA generated by necrosis over total cfDNA. The mean of Q247/Q115 ratio in FF was cfDNA (Mean ± SD, 0.91 ± 60.106 ng/μl), suggesting that the cfDNA analyzed mainly originated from cellular apoptotic events.

Statistical analysis of data was carried out using the available statistical package of SPSS-27 (Statistical Packages for the Social Sciences – version 27). Data were presented in simple measures of frequency, percentage, mean, standard deviation, and range (minimum-maximum values).

The significance of the difference of different means (quantitative data) was tested using Student's t-test for the difference between two independent means or the ANOVA test for difference among more than 2 independent means. The significance of the difference of different percentages (qualitative data) was tested using the Pearson Chi-square test (χ^2-test) with the application of Yate's correction or Fisher's exact test whenever applicable. Statistical significance was considered whenever $p \geq 0.05$ was considered. Receiver operating characteristic "ROC" curve technique was used to determine the use of any parameter as diagnostic or screening tool for disease and the ability to determine the "cutoff value" which of optimum sensitivity and specificity for diagnosing disease.

Results

The patient's characteristics are detailed in Table 1:

The reference level of cell-free DNA in the participant Iraqi women and its quantitate measured in 89 FF samples showed that its concentrations are varied from 0.676 to 1.401 ng/μl. Figure 1 clearly shows frequent distributions values lie between 0.8 and 0.9 ng/μl.

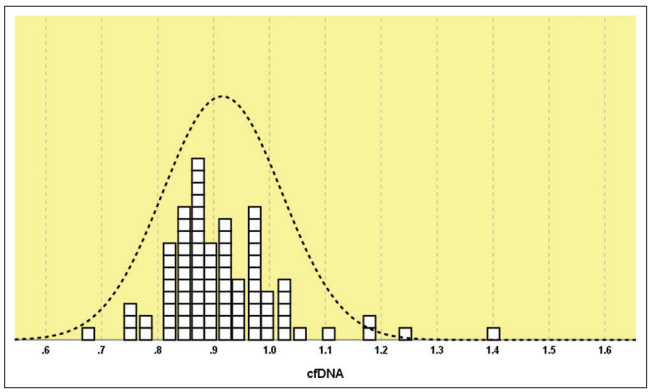

Figure 1: The distributions level of cfDNA in the follicular fluid among participants

CfDNA level in FF was not significantly associated with patients' parameters (Table 2), no statistically significant relations were found; however, we notice that there is a relation between a certain level of cfDNA and women age, for example, higher level of cfDNA in younger women, which may reflect higher cellular activity, but this is statistically not significant, p = (0.688), again the duration of infertility, that is, short duration of infertility associated with the low level of cfDNA (0.849 ± 0.028); however, this still statistically non-significant with p = 0.450. There was no association between ovarian reserve status, cause of infertility, and type of protocol used whether its agonist or antagonist: P = 0.588 with cfDNA level.

Table 2: Correlation between cell-free DNA level and different participant parameters

Variables	cfDNA		p-value
	No	Mean ± SD	
Wife age (years)			
19–29	38	0.926 ± 0.124	0.688
30–39	42	0.910 ± 0.081	
≥40 years	9	0.898 ± 0.135	
BMI (kg/m^2)			
Normal (18.5–24.9)	34	0.923 ± 0.077	0.626
Overweight (25–29.9)	47	0.916 ± 0.129	
Obese (≥30)	8	0.883 ± 0.059	
Duration of infertility			
<2	4	0.849 ± 0.028	0.450
2–4	27	0.919 ± 0.122	
≥5 years	58	0.919 ± 0.101	
Cause of infertility			
Endometriosis	8	0.924 ± 0.069	0.926
Hypogonadotropic hypogonadism	3	0.860 ± 0.177	
Male	27	0.907 ± 0.095	
Mixed	10	0.910 ± 0.065	
PCOS	6	0.915 ± 0.067	
Poor reserve	12	0.905 ± 0.134	
Tubal	11	0.953 ± 0.084	
Unexplained	12	0.925 ± 0.167	
First ICSI			
Yes	31	0.912 ± 0.088	0.821
No	58	0.917 ± 0.116	
Type of protocol			
Antagonist	83	0.917 ± 0.108	0.588
Agonist	6	0.893 ± 0.079	

#Significant difference between two independent means using Student's t-test at 0.05 level. ^Significant difference among over two independent means using ANOVA test at 0.05 level. BMI: Body mass index.

The study shows non-significant relation between cfDNA and hormonal analysis of participant, the same non-significant relations with AFC; however, analysis shows that a low level of cfDNA associated with a lower number of follicles than with the higher number of follicles cfDNA = (0.902 ± 0.103) and (0.922 ± 0.108), respectively, with p = 0.411.

Figure 2 shows correlations of cfDNA level and several embryos that are available for transfer.

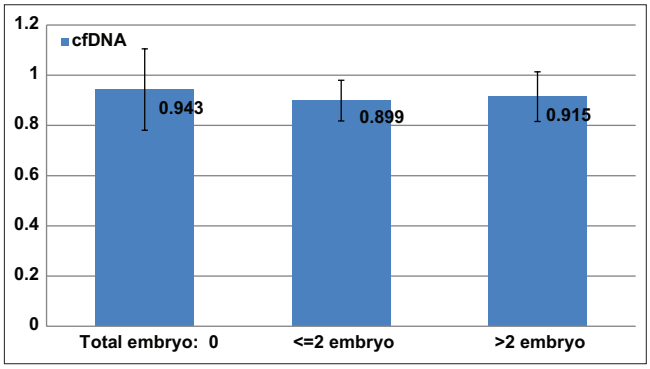

Figure 2: Correlations of cfDNA level and several embryos that are available for transfer

This figure shows the higher level of cfDNA detected when no embryo presents for transfer and also a high level detected with a high number of embryos, but it was non-significant statistically.

Pregnancy rate: There were no significant clear relations between PT test and cfDNA; however, cfDNA was lower in FF of pregnant women than in non-pregnant (0.900 ± 0.085 and 0.912 ± 0.099), respectively, and higher level when we decide to freeze all or when no embryo is present to be transferred, as shown in Table 3.

Table 3: Relationship of cfDNA with patient's hormonal and embryological results

Variables of patient	cfDNA		p-value
	No.	Mean ± SD	
FSH (U/L)			
<10	47	0.919 ± 0.093	0.302
≥10	8	0.883 ± 0.059	
LH (U/L)			
<3	8	0.892 ± 0.103	0.581
3–5	23	0.928 ± 0.087	
>5	23	0.915 ± 0.074	
AMH			
≤1	26	0.905 ± 0.101	0.554
>1	63	0.920 ± 0.109	
AFC			
<10	28	0.902 ± 0.103	0.411
≥10	61	0.922 ± 0.108	
Number of oocytes			
≤6	25	0.921 ± 0.102	0.765
>6	64	0.913 ± 0.109	
Total number of embryos			
0	13	0.943 ± 0.162	0.510
≤2	21	0.899 ± 0.081	
>2	55	0.915 ± 0.099	
Pregnancy test			
Positive	23	0.900 ± 0.085	0.282
Negative	55	0.912 ± 0.099	
Freeze all	8	0.952 ± 0.187	
No ET	3	1.010 ± 0.108	
Pregnancy test			
Positive	23	0.900 ± 0.085	0.613
Negative	55	0.912 ± 0.099	
Frozen E			
Yes	32	0.911 ± 0.123	0.725
No	56	0.920 ± 0.097	

Discussion

Study shows the reference level of cfDNA in the FF of Iraqi women participated in this study with Mean ± SD, 0.916 ± 0.106 ng/µl. There was non-significant relationship between cfDNA and patient demographic features such as age, BMI, her hormonal analysis, and laboratory results; however, low level of cfDNA notices in patients with short duration of infertility, <2 years, this is may be related to the fact that short duration of infertility is less stressful than long time of infertility [17], [18] (Table 2), same as mentioned in research done on patients undergo an IVF treatment [19], in the same time, this is proved that FF derived from serum and granulosa cell secretions and there is communication between FF and plasma [22]. Czamanski-Cohen *et al*. mentioned that maternal cell apoptotic leads high level of circulatory cfDNA, may be due that may be create a bad environment for pregnancy, and whether there is a pathological

process causing elevated cfDNA as well as causing her infertility [5], the same authors show that stress management by relaxation technique reduce plasma cfDNA and improved IVF outcome [23]. Although the level of cfDNA was higher in female infertility factor, whether ovarian or tubal, level not reached statistically significant value, still this value may affect the quantity and quality of oocyte retrieved; two important factors for embryo production and major influence in IVF success, as many authors recognized that FF microenvironment strongly influenced the competence of oocyte development [24], [25]. We think that there is a strong association and specific balance between granulosa cells apoptosis and oocyte development, the granulosa cells have specific programmed cells death that expresses by cfDNA in the FF neither high nor low level of cfDNA is important for oocyte development, this study shows that when we had no embryo to be transferred and when we in forced for freeze all strategy the cfDNA was higher, but the level was not statistically significant may be due to low sample size, FF composition is influenced oocyte quality.

Conclusion

The present study notices that cfDNA in the FF may mainly reflect the cellular activity and the balance between programed apoptosis and cell necrosis.

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