Zygote

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Assessment of cell-free DNA and apoptosis in an oocyte microenvironment: promising biomarkers to predict intracytoplasmic sperm injection outcomes

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Research Article

Cite this article: Debbarh H *et al.* (2023) Assessment of cell-free DNA and apoptosis in an oocyte microenvironment: promising biomarkers to predict intracytoplasmic sperm injection outcomes. *Zygote*. **31**: 296–302. doi: 10.1017/S0967199423000126

Received: 15 February 2022
Revised: 1 February 2023
Accepted: 6 March 2023
First published online: 17 April 2023

Keywords
Cell-free DNA; Cumulus cells; DNA fragmentation index; Human follicular fluid; ICSI outcomes

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Summary

Cell-free DNA (cf-DNA) is defined as DNA fragments that are released into the body fluids from apoptosis or necrosis cells, including follicular fluid (FF), which can affect the microenvironment of the oocyte associated with infertility. We aimed to investigate a relationship between apoptosis of cumulus cells (CCs) and cf-DNA levels in FF and clinical outcomes of women undergoing intracytoplasmic sperm injection (ICSI). Therefore, 82 FF samples were collected, and the corresponding CCs were isolated for ICSI procedures. FF cf-DNA concentration was quantified using ALU-quantitative polymerase chain reaction (PCR), and CCs DNA fragmentation index (DFI) was evaluated by the terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labelling (TUNEL) method. We found that cf-DNA and DFI levels were significantly higher in FF and CCs samples related to the age of women ≥37 years compared with the age of women < 37 years. Moreover, in older and younger women, FF cf-DNA and CCs DFI levels were significantly lower when the anti-Müllerian hormone (AMH) level was > 1.1 ng/ml compared with when AMH ≤ 1.1 ng/ml. In addition, patients with a low number of retrieved oocytes ≤ 6 had significantly higher levels of CCs DFI and FF cf-DNA than women with a higher number of retrieved oocytes > 6. Additionally, we observed that higher levels of cf-DNA and DFI were associated with poor oocyte maturity and poor embryo quality. Finally, cf-DNA and DFI levels were significantly lower in pregnant women than in non-pregnant ones. We conclude that DFI and cf-DNA levels in the oocyte microenvironment could have potential use in evaluating oocyte and embryo developmental competence.

Introduction

During assisted reproductive technology (ART) procedures, oocyte quality is a crucial factor influencing embryo developmental competence and implantation rate, but the evaluation of oocyte and embryo quality mainly depends on assessing morphological criteria. This method has shown limitations in predicting successful pregnancy (Assou *et al.*, 2008; Aydiner *et al.*, 2010). Developing new analytical techniques and assays to improve these evaluation methods is necessary. Therefore, and given that the oocyte quality and its microenvironment affect early embryo development (Krisher, 2004), several studies have focused on investigating new testing and non-invasive biomarkers based on the analysis of the oocyte microenvironment components, follicular fluid (FF) and cumulus cells (CCs) to improve the embryo selection process (Salehi *et al.*, 2017; Liu *et al.*, 2019) and *in vitro* fertilization (IVF) clinical outcomes. The oocytes are surrounded by somatic cells, which are the granulosa cells (GCs) and CCs, and grow in FF which constitutes the microenvironment within which the cumulus–oocyte complex matures, and granulosa cells differentiate. FF is produced from plasma and contains factors produced locally by the follicle cells. The CCs establish a physical connection with the oocyte through gap junctions. As a consequence of this close molecular dialogue, CCs are thought to play an important role in oocyte maturation and fertilization, with signalling and regulation of physiologic function that depends on paracrine and autocrine cytokines in the ovarian microenvironment and reproductive hormones in peripheral blood (Hull and Harvey, 2014). It has been reported that the reduced number of CCs and disruption in the cell–cell communication might deprive the oocyte of nutrients and survival factors inside the preovulatory follicle and induce apoptosis in ovulated oocytes (Tripathi *et al.*, 2013). Apoptosis is defined as programmed cell death for homeostasis and is closely involved in most of the reproductive processes, including atresia and luteolysis, as well as decidualization and placentation during embryo implantation (Varras *et al.*, 2012). Moreover, the CC apoptosis could negatively affect live birth

rates in IVF (Lee *et al.*, 2001). It has been shown that CC apoptosis correlates with poor oocyte and embryo fragmentation (Bosco *et al.*, 2015). Alternatively, the apoptotic and/or necrosis cells are considered the principal source of circulating cell-free DNA (cf-DNA) (Aucamp *et al.*, 2018). The latter refers to nuclear or mitochondrial DNA fragments and can be detected in any body fluid, including FF (Scalici *et al.*, 2014). cf-DNA can be released passively in the blood from apoptotic or necrotic cells (Schwarzenbach *et al.*, 2011) and phagocytized by macrophages, and its level remains low in physiological conditions (Pisetsky and Fairhurst, 2007), whereas increased levels of cf-DNA have been associated with many diseases, such as certain types of cancer and inflammatory conditions and fetal anomalies (Jylhävä *et al.*, 2013; Cheng *et al.*, 2017). Alternatively, various studies have associated elevated levels of cf-DNA in FF with gynaecological and obstetric disorders. They have considered them non-invasive biomarkers in the early detection and/or prognosis (Traver *et al.*, 2014). Preliminary studies have demonstrated that cf-DNA in follicles was found as a biomarker for embryo quality in IVF (Scalici *et al.*, 2014).

Based on the fact that FF composition affects oocyte development and consequently has a strong influence on embryo quality (Baka and Malamitsi-Puchner, 2006). We believe there is an association between CC apoptosis and oocyte development and that high cf-DNA levels in FF may lead to the apoptosis of CCs, which in turn affects the dialogue with the oocyte necessary for its normal development. Therefore, cf-DNA quantification in FF samples could represent a non-invasive biomarker approach complementary to morphological criteria for embryo selection.

In this paper, we report a prospective cohort study to investigate if apoptosis in CCs and cf-DNA levels in FF samples from women undergoing intracytoplasmic sperm injection (ICSI) could be related to their age, AMH level, number of oocytes retrieved, oocyte maturity, embryo quality and pregnancy outcomes.

Material and methods

Patients' characteristics

This prospective study included 82 women undergoing ICSI procedures at the Fertilization Center IRIFIV in Casablanca, Morocco. Written informed consent was obtained for using FF and CCs samples during oocyte collection. The women's age ranged from 23 to 43 years. Infertility duration was between 1 and 5 years. In this study, female infertility was the cause of the consultation for 28 couples; male factors for 19 (include only oligospermia 'fewer than 15 million/ml of semen, or fewer than 39 million in total ejaculate' with DFI fewer than 30%), mixed infertility for 12, and unexplained infertility for 23. Enrolled patients met the following inclusion criteria: (i) no endocrine disorders and or history of ovarian surgery affecting the ovaries or gonadotropin secretion, and (ii) no current hormone therapy, no metabolic syndrome, no polycystic ovary syndrome, no pelvic surgery, no ovarian tumours, no morbid obesity and no autoimmune disease. The samples with high DNA fragmentation (>30%) and abnormal morphology and motility (based on WHO criteria) were excluded from the study.

Ovarian stimulation procedure

All patients were stimulated with antagonist protocol using the follicle-stimulating hormone (FSH) (Orgalutran 0.25 IU and Gonal-F). FSH was administered (Gonal-F; Serono Laboratories, Saint Cloud, France) by daily subcutaneous injection (150–225 IU/day) or (¼ 300 IU/day). The FSH dose was based on the woman's age, antral follicle count (AFC) on days 2–3 of the cycles and AMH concentration was maintained constant for 5 days and adjusted according to usual follicle growth parameters determined using ultrasound monitoring. A potent, third-generation gonadotrophin-releasing hormone (GnRH) antagonist, Ganirelix (Orgalutran VR, MSD Schering-Plough, France), was injected subcutaneously once daily starting on day 6 of FSH administration. A subcutaneous injection of human chorionic gonadotrophin (HCG; chorionic gonadotrophins, Ovitrelle VR, Merck Serono) was performed when triggering criteria reached ≥ 3 follicles of 17 mm.

The oocyte maturity rate was calculated based on the ratio of the number of metaphase II oocytes to the total oocyte number. A pregnancy test was performed 2 weeks after the embryo transfer, and pregnancy was confirmed when fetal heart activity was detected on transvaginal ultrasound 4 weeks after embryo transfer. The oocyte maturation rates were divided into two groups, group I had an oocyte maturity rate of < 60% and group II contained the FF with an oocyte maturity rate of ≥ 60%. Anti-Müllerian hormone (AMH) was assessed for each patient on the third day of the menstrual cycle. Levels of AMH < 1.1 ng/ml are considered to reflect a reduced ovarian reserve, and levels ≥1.1 ng/ml are normal ovarian reserve (Gnoth *et al.*, 2008). Three days after oocyte retrieval, embryo quality was graded from A to D according to the following morphological criteria: (i) number of blastomeres, (ii) blastomere regularity and (iii) fragmentation rate. An embryo was considered of top quality (grades A and B) if 6–8 blastomeres of regular size with < 25% fragmentation were observed.

Follicular fluid samples

Collection and preparation

Follicular fluid samples were collected, and the corresponding CCs were isolated for ICSI procedures. FF samples were obtained from mature follicles at the time of oocyte retrieval. To avoid any blood contamination, only clear FF samples were included and purified using a Ficoll-based protocol (3 ml), as described by Ferrero *et al.*, 2012, and then immediately stored at −20°C.

DNA extraction and quantification using ALU-qPCR

Free DNA was extracted from the purified FF using the SaMag™ STD DNA Extraction Kit according to the manufacturer's instructions. The total free DNA was quantified using qPCR, and ALU 115 primers (Umetani *et al.*, 2006). Each ALU-qPCR reaction included 2 µl extract of FF and added to the reaction mixture was 0.25 µl of each ALU 115-5′-CCTGAGGTCAGGAGTTCGAG-3′ (forward) and -5′-CCCGAGTAGCTGGGATTACA-3′ (reverse) and 5 µl of Luna Universal QPCR Mix (containing the enzyme *Taq* DNA polymerase, nucleotides and free SYBRGreen™ fluorescence intercalator). Cycling conditions were as follows: 95°C for 60 s, then 40 cycles of 95°C for 15 s, 58°C for 20 s and 60°C for 30 s. All reactions were performed in duplicate by Sacace biotechnologies. Cell-free DNA concentration in FF pools was determined using a standard curve obtained by successive dilutions of genomic DNA (Umetani *et al.*, 2006). A negative control (without the template) was integrated into each qPCR plate.

https://doi.org/10.1017/S0967199423000126 Published online by Cambridge University Press

Table 1. Relationship of cell-free DNA and DNA fragmentation levels in human follicular fluid and cumulus cells with age and anti-Müllerian hormone (AMH) combined with age

	Age (years)		AMH (ng/ml)			
			< 37 (years)		≥ 37 (years)	
	< 37; N = 50	≥ 37; N = 32	> 1.1; N = 33	≤ 1.1; N = 17	> 1.1; N = 12	≤ 1.1; N = 20
FF cf-DNA; mean ± SD (pg $\times 10^{-5}$)	0.74 ± 0.15	2.26 ± 1.33	0.37 ± 0.16	1.55 ± 1.49	0.32 ± 0.27	3.42 ± 2.93
P-values	0.05 S		0.005 S		0.04 S	
CCs DFI%; mean ± SD	67.90 ± 12.22	73.06 ± 11.15	65.54 ± 11.00	72.31 ± 13.85	67.91 ± 9.60	74.15 ± 12.01
P-values	0.02 S		0.04 S		0.05 S	

Values are in mean ± standard deviation (SD).
CCs: cumulus cells; cf-DNA: cell-free DNA; DFI: DNA fragmentation index; FF: follicular fluid.
Statistical significance was defined as $P < 0.05$; S: significant.

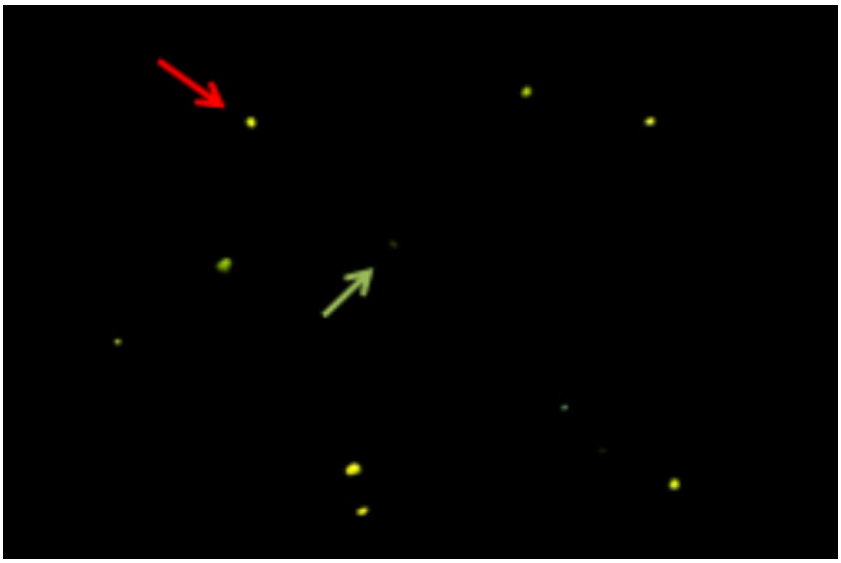

Figure 1. DNA fragmentation detection using the TUNEL assay in cumulus cells observed under a microscope (EUROStar Germany) equipped with a reflected light fluorescence attachment in a ×20/0.40 objective. Cumulus cells with DNA fragmented show intense yellow fluorescence (red arrow), whereas normal cells appear with no colour (green arrow).

Cumulus cell samples

Collection and preparation

After oocyte pick-up, the CCs were mechanically released by gently pipetting with a 100-micron denuding pipette, then put in a buffered culture medium GYNEMED© at pH 7. Cumulus cell samples were collected in a test tube containing hyaluronidase enzyme GYNEMED 80 IU/ml with HEPES (20 mM) followed by two centrifugations for 7 min at 800 rpm, and then fixed in 3.7% paraformaldehyde for 1 h for apoptosis analysis.

Fluorescence in situ *TUNEL assay*

The TUNEL technique was performed using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche®, Germany), according to the manufacturer's instructions. After fixation, CCs were permeabilized on ice in 0.1% Triton X-100 and 0.1% sodium citrate in phosphate-buffered saline (PBS), then washed three times in PBS at room temperature. CCs were then incubated for 45 min at 37°C in 5 µl of terminal transferase (TdT) enzyme. CCs were observed under a microscope (EUROStar Germany) equipped with a reflected light fluorescence attachment in a ×20/0.40 objective (Figure 1).

Statistical analysis

The results are expressed as the mean ± standard deviation (SD). Differences between groups were compared using the Mann–Whitney *U*-test (Statistical Package for the Social Sciences) software; statistical significance was defined as $P \leq 0.05$.

Results

This study included 82 infertile women under ICSI treatment whose age was between 23 and 43 years. The outcomes of age, infertility length, maturity rate, embryos quality, the number of oocytes, age combined with AMH and pregnancy were divided into lower and higher groups based on the statistical analysis data with cell-free DNA (cf-DNA) and DNA fragmentation index (DFI) in oocyte microenvironment composed to FF and CCs.

As shown in Table 1, FF cf-DNA and CCs DFI levels were significantly lower in patients whose age was less than 37 years compared with those whose age was equal to or greater than 37 years (respectively $P = 0.05$; $P = 0.02$). While, in older and younger women, FF cf-DNA and CCs DFI levels when AMH rate was > 1.1 ng/ml were significantly less than in those with AMH ≤ 1.1 ng/ml.

https://doi.org/10.1017/S0967199423000126 Published online by Cambridge University Press

According to the data presented in Table 2, we observed that cf-DNA and DFI levels were significantly higher in FF and CCs patients who had been trying to conceive for more than 5 years compared with women who had tried only for ≤ 5 years ($P = 0.02$ and $P = 0.01$, respectively). In addition, CCs and FF pools from patients with a low number of retrieved oocytes (≤6) had significantly higher levels of DFI and cf-DNA than those from women with a higher number of retrieved oocytes (>6). Indeed, we also noted a higher level of cf-DNA and DFI in the group with a maturity rate < 60% compared with the group with a maturity rate ≥ 60% ($P = 0.02$ and $P = 0.02$, respectively)

Furthermore, we noticed significantly higher cf-DNA and DFI levels in FF and CCs samples related to oocytes that generated poor-quality embryos (grades C and D) compared with those related to top embryos (grades A and B). Finally, cf-DNA and DFI levels were significantly higher in women who had not been pregnant compared with women who had been pregnant ($P = 0.02$ and $P = 0.02$, respectively; Table 2).

Discussion

The oocyte microenvironment is considered to directly affect the differential oocyte developmental capacity. Therefore, FF composition strongly influences oocyte quality, its developmental competence, and subsequent embryo quality (Baka and Malamitsi-Puchner, 2006). Therefore, many studies have considered them potential non-invasive biomarkers for oocyte and embryo quality prediction (Baka and Malamitsi-Puchner, 2006; Borowiecka *et al.*, 2012). In fact, CCs play a physiological role in antral follicles contributing to metabolic support and maintaining meiotic arrest in the growing oocyte (Coticchio *et al.*, 2015; Monniaux, 2016). However, there has been mounting evidence that, under certain circumstances, the higher incidence of apoptosis in CCs is associated with an increased rate of empty follicles and fewer oocytes retrieved, poor oocyte and embryo quality and low conception and pregnancy rate (Nakahara *et al.*, 1997; Saito *et al.*, 2002).

In the follicular microenvironment, the cf-DNA levels reflect the proportion of apoptotic and necrotic cell damage (Snyder *et al.*, 2016). Furthermore, Scalici *et al.*, 2014 showed that ~85% of FF cf-DNA is derived from cell apoptosis. Alternatively, data from Traver *et al.*, 2015 have indicated that FF cf-DNA could be used to predict the clinical pregnancy outcome.

In the current study, we report a prospective cohort study to assess apoptosis in CCs and cf-DNA levels in FF and relate these findings to clinical parameters and pregnancy outcomes of women undergoing ICSI.

Our study has shown that cell-free DNA (cf-DNA) and DFI levels were significantly lower in FF and CCs of patients whose age was < 37 years compared with those whose age was ≥ 37 years. This finding is consistent with research that has noted increased levels of CC apoptosis with advanced age (Fujino *et al.*, 1996; Tesarik *et al.*, 2021). The shortened telomeres in human oocytes are associated with reproductive age (Kalmbach *et al.*, 2013). Several studies have been interested in evaluating CC telomere length as a function of advanced age. They have shown that the short telomeres fail to protect the chromosomal ends from being recognized as DNA double-stranded breaks, lead to genomic instability, and activate DNA repair pathways, which finally generate cellular senescence or apoptosis (Wellinger, 2014). Moreover, in our previous study, we showed that 37 years of reproductive ageing was accompanied by a change in redox status imbalance in FF that impaired reactive oxygen species (ROS) scavenging efficiency

Table 2. Relationship between cell-free DNA and DNA fragmentation levels in human follicular fluid and cumulus cells with infertility length, number of oocytes and ICSI outcomes

	Infertility length; (years)			Number of oocytes			Maturity rates			Embryos quality			Pregnancy		
	≤5; N = 37	>5; N = 45		>6; N = 40	≤6; N = 42		≥60%; N = 52	<60%; N = 30		A, B; N = 56	C, D; N = 26		Positive; N = 28	Negative; N = 54	
FF cf-DNA mean ± SD (pg × 10^{-5})	0.44 ± 0.43	2.80 ± 2.47	0.02S	0.54 ± 0.40	2.16 ± 1.80	0.04S	0.32 ± 0.24	1.84 ± 1.45	0.02S	0.81 ± 0.28	2.47 ± 1.09	0.04S	0.38 ± 0.37	2.12 ± 1.10	0.02S
CCs DFI % mean ± SD	66.13 ± 10.28	72.78 ± 12.33	0.01S	68.09 ± 10.08	71.87 ± 12.85	0.05S	68.58 ± 12.80	74.06 ± 9.76	0.02S	67.91 ± 11.44	74.30 ± 12.27	0.01S	66.86 ± 11.11	72.02 ± 12.49	0.02S

Values are in mean ± standard deviation (SD).
CCs: cumulus cells; cf-DNA: cell-free DNA, DFI: DNA fragmentation index, FF: follicular fluid.
Statistical significance was defined as $P < 0.05$; S: significant.

https://doi.org/10.1017/S0967199423000126 Published online by Cambridge University Press

(Debbarh *et al.*, 2021). ROS overproduction can damage mitochondrial DNA (mtDNA). It can reduce ATP production, which increases vulnerability to apoptotic signalling (Ventura-Clapier *et al.*, 2017), therefore causing a decrease in oocyte quality and interfering with embryonic development (Wang *et al.*, 2021). The apoptosis of CCs with reproductive ageing may induce unfavourable environments for follicular oocyte development. This could cause the significant release of cf-DNA related to advanced age, which results in the deterioration of oocyte quality, thereby reducing fertilization rates and embryo development (Chaube *et al.*, 2014).

Age-dependent AMH production in antral follicles starts in females in the 36th week of gestation. It reaches a peak in puberty, after which there is a continuous decrease until the AMH serum level reaches undetectable levels with menopause. Therefore, AMH is a marker for predicting ovarian reserve (Gnoth *et al.*, 2008). Our data showed that, in young women, cf-DNA content in FF and DFI of CCs were significantly lower when AMH was > 1.1 ng/ml than when the AMH ≤ 1.1 ng/ml. These observations agreed with those of Ebner *et al.* (2014) providing evidence that patients with lower AMH levels produce CCs of reduced quality showing strand breaks in their DNA. This result can be explained by the ovarian reserve decline via the apoptosis of the GCs responsible for the secretion of AMH, therefore promoting a significant release of the fragment-free DNA in FF (Jayaprakasan *et al.*, 2010). Furthermore, patients with low AMH rates and diminished ovarian reserve have shown a subexpression in tropomyosin-related kinase, thought to mediate a survival signal that maintains viable CCs (Buyuk *et al.*, 2011), therefore promoting apoptosis in these patients.

We also noted that cf-DNA and DFI levels were significantly higher in patients who had been trying to conceive for > 5 years compared with those who tried only for ≤ 5 years. Various studies have suggested that a long period of infertility is associated with increased psychological stress in infertile couples (Chiba *et al.*,1997; Lynch *et al.*, 2014), which could lead to follicular cell apoptotic events and the release of cf-DNA (Czamanski-Cohen *et al.*, 2014).

We also observed that CCs and FF pools from patients with a low number of retrieved oocytes ≤ 6 had significantly higher levels of DFI and cf-DNA than those from women with a higher number of retrieved oocytes > 6. It is largely recognized that, during the process of ovarian stimulation, the apoptotic pathway can be activated in many recruited oocytes (Tilly, 1997). The higher the free DNA in the FF, the greater the apoptotic cascade, resulting in either apoptotic oocytes or a limited number of oocytes (Dimopoulou *et al.*, 2014).

Furthermore, in our study, a higher level of FF cf-DNA and CCs DFI was noted in the group of women with a maturity rate of $<$ 60% compared with those with a maturity rate $\geq 60\%$. This observation confirms that increased levels of DNA fragmentation of CCs are associated with a higher number of immature oocytes (Ruvolo *et al.*, 2007; Bosco *et al.*, 2017). Therefore, we can assume that the apoptosis of CCs can reduce cAMP levels and steroid hormone biosynthesis (Chaube *et al.*, 2006), thereby generating hypoestrogenic conditions in the ovary and reducing the oocyte quality (Duffy *et al.*, 2005). Furthermore, the apoptotic cells release not only nuclear DNA but also mitochondrial DNA, and the oocytes with good quality have optimal mitochondrial numbers and adequate ATP levels (Van Blerkom *et al.*, 1995). ATP-generating capability is critical for the successful maturation of the cytoplasm and nucleus in preparation for fertilization and completion of meiosis II (St John *et al.*, 2010). It has been suggested that FF cf-DNA level is related to both retrieved oocyte quantity and quality, two key features for embryo production (Traver *et al.*, 2015).

In addition, we observed that CCs DFI and FF cf-DNA levels were significantly higher in samples related to oocytes that generated poor-quality embryos (grades C and D) compared with those related to top embryos (grades A and B). This result is consistent with other research that has reported a high level of DNA fragmentation of CCs that have generated poor-quality embryos (Salehi *et al.*, 2017; Emanuelli *et al.*, 2019). Indeed, the apoptosis of the GCs reduces the communication between these CCs and the oocytes, depriving oocytes of nutrients, cell signalling molecules and survival factors (Eppig *et al.*, 1982) which risks influencing oocyte and embryo quality. Furthermore, apoptosis is a fundamental process in releasing cell-free DNA (Aucamp *et al.*, 2018). The origin of cell-free mtDNA might be mitochondrial dysfunction. Indeed, the mature (MII) oocytes, fertilized oocytes, and early cleavage stage embryos depend on the function of the mitochondrial pool present at ovulation (Spikings *et al.*, 2006). Consequently, any adverse influence on mitochondrial function via accumulation of the mtDNA resulting from apoptosis will negatively affect the development of the preimplantation embryo. Various studies have suggested that a high level of cf-DNA generates a poor-quality embryo (Scalici *et al.*, 2014; Traver *et al.*, 2015).

We found that FF cf-DNA and CCs DFI levels were significantly higher in women who had not been pregnant compared with women who had been pregnant. These results are consistent with various reports suggesting that DNA fragments could come from massive apoptotic events that occur in the ovaries and that contribute to increasing cf-DNA levels in FF samples (Czamanski-Cohen *et al.*, 2013).

Furthermore, it has been reported that CCs are important in all processes of oocyte development, from maturation to embryo development. The CCs can prevent premature exocytosis of cortical granules and the hardening of the zona pellucida to avoid failure of sperm–oocyte recognition, allowing fertilization (Van Soom *et al.*, 2002).

All of the observations suggest that oocytes that develop in a cf-DNA-rich environment could have accumulated 'negative signals' causing harmful consequences on embryo quality and development, or a lack certain 'positive signals' normally transmitted by viable CCs.

In conclusion, the current study showed that DFI and cell-free DNA levels in the oocyte microenvironment could have potential use in assessing and predicting oocyte and embryo quality and clinical pregnancy outcomes complementary to the morphological embryo criteria.

Financial support. This research received no specific grant from any funding agency, commercial or not-for-profit sectors.

Conflict of interest. All the authors declare that they have no conflict of interest.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

https://doi.org/10.1017/S0967199423000126 Published online by Cambridge University Press

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