LETTERS IN BIOMATHEMATICS
An International Journal

RESEARCH ARTICLE

OPEN ACCESS

Detection of granulosa cell apoptosis level in the ovarian follicular fluid as a fertility marker in a sample of Iraqi women

Mohammed Abdalmalek Ali Al-Bedhawi[1], Riyam Basim Ali[2]

[1]Institute of genetic engineering and biotechnology, University of Baghdad, Iraq
Email: bedhanimah@uobaghdad.edu.iq, Orcid ID: 0000-0003-1441-8339
[2]Institute of genetic engineering and biotechnology, University of Baghdad, Iraq
Email: rayam.bassem1100a@ige.uobaghdad.edu.iq, Orcid ID: 0009-0003-5751-829X

Summary

Infertility is a worldwide health issue affecting more than10% of couples, with female-related causes representing more than half of cases. Ovarian disfunctions such as polycystic ovary syndrome (PCOS), diminished ovarian reserve (DOR), tubal factor, and unexplained infertility, are closely linked to granulosa cell (GC) health and mitochondrial function. Markers like cell-free mitochondrial DNA (cf-mtDNA) in follicular fluid (FF), primarily released from apoptotic GCs, has recently emerged as a potential biomarker for mitochondrial integrity, oocyte competence, and reproductive outcomes. This study investigated the levels of cf-mtDNA in FF from 154 Iraqi women undergoing intracytoplasmic sperm injection (ICSI) in the period between November 2023 and June 2024. Patients were devided into five groups: male factor infertility, which represent the control, n=35), PCOS (n=30), DOR (n=28), unexplained infertility (n=34), and tubal factor infertility (n=27). FF was collected at oocyte retrieval and analyzed for cf-mtDNA using quantitative PCR targeting the mitochondrial ND4 gene , with nuclear β-globin as an internal nuclear DNA reference. Results demonstrated a consistent reduction in cf-mtDNA levels across all infertility groups compared to controls. The most pronounced deficits occurred in tubal factor (mean 0.38) and DOR (mean 0.37) cases, indicating significant mitochondrial disfunction in GCs. PCOS and unexplained infertility also showed reduced levels (means 0.52 and 0.67 , respectively), though with greater variability in the unexplained group. Results also revealed that patients averaged 34 years of age, had a median BMI of around 29.5, exhibited moderate oocyte yield and embryo quality. The current study findings suggested that mitochondrial deficiency is a commn pathological factor in different infertility disorders. cf-mtDNA in FF may serve as a sensitive and non-invasive biomarker for GC apoptosis with oocyte metabolic potential. Including cf-mtDNA analysis into infertility diagnostics could improve prognostic assessment and monitor personalized ICSI strategies.

ARTICLE HISTORY
Received September 17, 2026
Accepted October 28, 2026

KEYWORDS
Infertility, cf-mtDNA, follicular fluid, granulosa cells, oocyte competence, ICSI.

1. Introduction

Infertility as global problem, affects approximately 10–15% of spouses, female-related factors representing over 50% of cases (1,2). Among these, ovarian disfunction—such as polycystic ovary syndrome (PCOS), diminished ovarian reserve (DOR), tubal factor infertility, and idiopathic causes—plays a central role in this issue. The ovarian follicle, particularly its follicular fluid (FF), that supports oocyte development by providing a metabolically active environment enriched with hormones, cytokines, and nucleic acids (3). Recently studies focus have shifted toward cell-free DNA (cf-DNA), especially cell-free mitochondrial DNA (cf-mtDNA), as a potential biomarker of granulosa cell (GC) apoptosis and mitochondrial malfunction, processes closely linked to follicular atresia and/or reduced fertility(4). FF composition mainly reflects the follicular health. In PCOS for example, FF exhibits elevated oxidative stress and inflammatory markers, while in DOR the imbalance of amino acids content and lipids affects the quality of oocytes (5,6). GC apoptosis, mainly driven by mitochondrial pathways (e.g., Bax/Bcl-2 imbalance), which was exacerbated in aging and PCOS, impairing follicular viability(7). cf-mtDNA, essentially derived from apoptotic GCs, that serve as a sensitive indicator of mitochondrial distress. Elevated cf-mtDNA levels correlate with advanced maternal age, lower anti-Mullerian hormone (AMH) levels and poor blastocyst formation(8,9). Subtypes of infertility exhibit distinct FF profiles. PCOS shows elevated TNF-α and androgens; DOR displays impaired energy metabolism; while tubal factor infertility allows assessment of intrinsic oocyte quality via FF analysis. Unexplained infertility may harbor subclinical mitochondrial or apoptotic dysfunction undetectable by conventional means(10). This study aims to evaluate cf-mtDNA levels in various

infertility types and explore its potential as a non-invasive biomarker among Iraqi women.

2. Materials and methods

Sampling

The study was approved by the Institutional Review Board (from institute of genetic engineering and biotechnology university of Baghdad in addition to the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University, Baghdad. All participants provided written informed consent.

The current study included 154 women undergoing intracytoplasmic sperm injection (ICSI) at the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University, Baghdad, from November 2023 to June 2024. Participants were grouped based on infertility causes: male factor (35 women) which representing the control group, polycystic ovary syndrome (PCOS) (30 women), diminished ovarian reserve (28 women), unexplained infertility (34 women), and tubal factor infertility (27 women). Women with systemic diseases, genital malformations, or certain medications were excluded. Standard diagnostic criteria, such as Rotterdam and Bologna were used. All participants underwent ovarian stimulation with recombinant FSH and a GnRH antagonist protocol. Ovulation was triggered by hCG, followed by transvaginal oocyte retrieval. Oocytes were assessed for maturity, fertilization, and embryo grading based on international standards. Follicular fluid (FF) was collected during retrieval, pooled, and processed for cf-mtDNA evaluation. The contaminated FF samples were excluded from the current study.

3. Molecular diagnosis

Relative Cell-Free Mitochondrial DNA Levels

The samples of follicular fluid (FF) underwent secondary centrifugation at 3000 × *g* for 10 minutes to pellet residual cellular debris, which mainly from granulosa cells prior to cell-free DNA (cfDNA) extraction. The supernatants were transferred to fresh microcentrifuge tubes. cfDNA was isolated from 200 µL aliquots of each clarified FF sample using the AddPrep Genomic DNA Extraction Kit (ADD BIO INC, Daejeon, Republic of Korea), strictly adhering to the manufacturer's protocol.

Amplification targeted specific genomic regions: a 359-base pair (bp) fragment of the mitochondrial ND4 gene (representing mtDNA) and a 77-bp fragment of the nuclear β-globin gene (representing nuclear DNA), employing the following primers synthesized by Macrogen Co., Ltd. (Republic of Korea):ND4 (mtDNA): Forward 5'- TACTAGTCTTTGCCGCCTGC-3', Reverse 5'-TGGATAAGTGGCGTTGGCTT-3' (359 bp). β-globin: Forward: 5'-AAAGGTGCCCTTGAGGTTGTC-3', Reverse: 5'-TGAAGGCTCATGGCAAGAAA-3' (11). Relative quantification of cell-free mitochondrial DNA (cf-mtDNA) was performed by real-time quantitative polymerase chain reaction (qPCR) using a Rotor-Gene Q instrument (QIAGEN, Germany). Reactions (30 µL total volume) contained 3 µL of extracted DNA template,1 µL of 10 µM of each primer, and 15 µL of PowerUp SYBR Green Master Mix (Promega) 11 µL of nucleases free water. Thermocycling conditions comprised an initial denaturation at 94°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds and combined annealing/extension at 60°C for 30 seconds. Relative cf-mtDNA levels, expressed as fold change, were calculated using the comparative Ct (ΔΔCt) method (Livak method) with β-globin as the reference gene, according to the formula $2^{\Delta\Delta Ct}$ (12).

4. Statistical Analysis

Data analysis was conducted employing statistical software, including SAS (v9.4, SAS Institute) and R (v4.3.x, R Foundation). Results for continuous data are presented as mean ± standard deviation for normally distributed variables and median (interquartile range) for non-normally distributed variables. Correlations were evaluated using Pearson's test for normally distributed data and Spearman's test for non-normally distributed data. A p-value below 0.05 was considered statistically significant.

5. Results

The results demonstrated relative evaluation of granulosa cells apoptotic rate using cf-mtDNA levels in various infertility types of over 150 women and explore its potential as a non-invasive biomarker among Iraqi women. Starting with a correlation analysis of the demographic data with ICSI parameters of the participants as presented in (table 1), which summarizes key fertility data for 154 women (average age 34), which were undergoing ICSI treatment. Their median BMI was 29.5, indicating overweight. Hormone levels are typical for patients. A median of 9 oocytes were retrieved, with 65.1% mature and 67.1% fertilized. Almost half of the resulting embryos were high quality.

Table 1: Patient characteristics and ICSI treatment details (N = 154)

Characteristic	Value
Age (years)	34 ± 6.4
Body mass index (kg/m²)*	29.5 (4.3)
Follicle stimulating hormone (mIU/ml)	6.2 ± 2.1
Luteinizing hormone (mIU/ml)	5.4 ± 2.5
Estradiol (pg/ml)	35.7 ± 13.5
Total count of collected oocytes*	9 (9)
Oocyte maturity rate (%)	65.1 ± 21.2
Fertilization rate (%)	67.1 ± 19.7
Percentage of high quality embryos (%)*	50 (48.7)

• Values shown are either median (IQR) or mean ± SD.

6. Molecular findings

The results exhibited specific primers detection patterns of amplification and melting of PCR products revealing specificity and reliability of primers and PCR results as shown in (figure 1). The data of (table 2), derived from Livak method (delta-delta Ct analysis) indicated that all four patient groups recorded a significantly lower mean apoptotic factor (CF Mt DNA) compared to the control group (Mean = 1).

A lower value indicates a reduce mitochondrial DNA in apoptotic cells, which then interpreted as a lower mitochondrial content. This suggests a fundamental metabolic deficiency in the granulosa cells of these infertile women, even before the event of apoptosis occurs. The most mitochondrial DNA deficiency were observed in the Tubal Factor and Diminished Ovarian Reserve (DOR) groups, with means of approximately 0.38 and 0.37, respectively. This suggests severely compromised energy production in their follicular environment, which is crucial for oocyte development and competence.

Mitochondrial content was lower in the Unexplained and PCOS groups compared to controls (means of 0.67 and 0.52), though not as low as in the Tubal Factor and DOR groups. Notably, the Unexplained group showed high variability (SE = 1.22), implying it was a less homogenous patient group. These results imply that impaired mitochondrial biosynthesis or function in granulosa cells may be a common pathological pathway associating with infertility in these patients in an independent factor of their primary diagnosis.

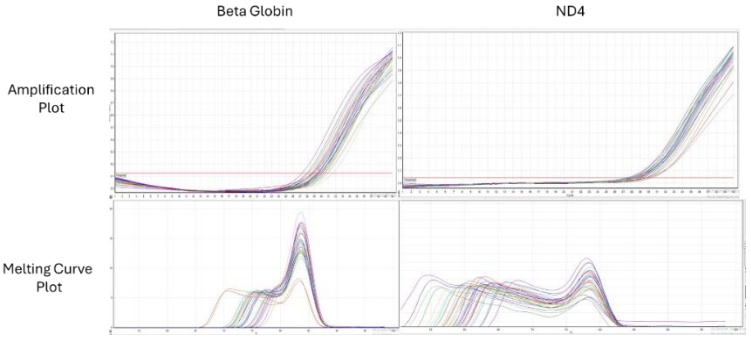


Figure (1). Amplifications and melting curves plots of Beta Globin (reference gene) and ND4 (mitochonderial gene) in a sample of patients groups

Table (2). The ratios of apoptosis of granulosa cells calculated by Cf-Mt DNA in the collected follicular fluids

Cases	Mean of Apoptotic factor (CF DNA)	SE
Control (male Factor)	1	2.372861
Tubal factor infertility	0.377945	0.134702
Unexplained case	0.665649	1.223854
polycystic ovary syndrome	0.516438	0.623226
Diminished ovarian reserve	0.370894	0.266387

7. Discussion

The resulted data presents that cell-free mitochondrial DNA (cf-mtDNA) levels in follicular fluid, which reflecting the mitochondrial content in granulosa cell and their apoptotic rate were markedly reduced in infertile women compared to male-factor controls. In addition, the demographic and clinical context (average age around 34, with median BMI about 29.5, moderate oocyte yield and embryo quality) associates with typical ICSI populations, thus establishing a reliable baseline for interpreting molecular findings. The significant reduction of cf-mtDNA across infertile groups specifically in Tubal Factor and DOR patient group suggests a critical mitochondrial deficiency within granulosa cells. Because mitochondria drive ATP production that is essential for folliculogenesis, oocyte maturation, and embryo development(13). This result supports with recent literature that demonstrating the ratios of cf-mtDNA/cf-nDNA in follicular fluid associate with the number of mature oocytes and might serve as a biomarker for oocyte competence, specifically with aging(14). Furthermore, lower cf-mtDNA levels in advanced-age women versus younger ones or PCOS women further underscore age-related mitochondrial shortages(14) .An emerging evidence from cf-mtDNA sequencing in follicular fluid reveals that ovarian stimulation can induce mtDNA mutations, affecting the mitochondrial D-loop region and such mutations correlate with lower oocyte outcome, poor embryo quality, and reduced IVF success rates (15). These findings suggest that oxidative stress is the major issue which might contributes to mitochondrial damage, compounding fertility challenges.

Earlier studies that affirming the promise of cf-mtDNA as a biomarker showed that relative cf-mtDNA content associated blastocyst development and maternal age of patients with higher cf-mtDNA, which had reduced blastocyst formation but increased age(16). These findings highlights that cf-mtDNA dynamics reflect both aging and developmental potential. On more extended cf- DNA evaluations, studies on total cf-DNA (nuclear-derived) support its value as a biomarker: when cf-DNA elevated in follicular fluid it was correlated with poor embryo quality and lower pregnancy rates across infertility factors, such as PCOS and endometriosis(17,18). Total cf-DNA levels correspond with embryo fragmentation and negative IVF outcomes(18). In an African cohort, cf-DNA studies in resource-limited settings echo these findings. elevated follicular fluid cf-DNA was linked to poor pregnancy outcomes post IVF/ICSI, reinforcing cf-DNA as a reasonable, non-invasive predictor, even outside high-resource contexts(19). Others linked this low outcome to regulatory factors like miR-210 and miR-1226 (20). However, a relatively successful attempts to overcome these low outcomes caused by PCOS using silymarin was suggested (21).

Collectively, these findings support a unifying pathophysiological model: mitochondrial dysfunction—manifested as reduced or mutated cf-mtDNA that contributes to impaired granulosa cell function, which might lead to compromised oocyte competence, particularly in conditions like DOR and Tubal Factor infertility.

8. Conclusion

These findings suggest that quantifying cf-mtDNA in follicular fluid has strong potential as a non-invasive biomarker for granulosa cell health and oocyte metabolic potential, in particular for Iraqi women where advanced diagnostic tools may be limited. Use of cf-mtDNA measurements could enhance prognosis beyond the common hormonal and imaging assessments, guiding personalized ICSI interventions.

References

Mascarenhas, M. N., Flaxman, S. R., Boerma, T., Vanderpoel, S., & Stevens, G. A. (2012). National, regional, and global trends in infertility prevalence since 1990: a systematic analysis of 277 health surveys. *PLOS Medicine, 9*(12), e1001356.

Inhorn, M. C., & Patrizio, P. (2015). Infertility around the globe: new thinking on gender, reproductive technologies and global movements in the 21st century. *Human Reproduction Update, 21*(4), 411–426.

Revelli, A., Delle Piane, L., Casano, S., Molinari, E., Massobrio, M., & Rinaudo, P. (2009). Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. *Reproductive Biology and Endocrinology, 7*(1), 40.

Santos, C., Soares, P., & Lima, J. (2022). cf-mtDNA: a biomarker of reproductive cell damage. *Reproduction, 163*(3), R77–R88.

Zhou, L., Xie, Y., Li, S., Liang, Y., Qiu, Q., Lin, H., & Zhang, Q. (2021). Proteomic analysis of follicular fluid from patients with polycystic ovary syndrome. *Frontiers in Endocrinology, 12*, 668143.

Lee, J. R., Kim, S. H., Kim, S. M., Jee, B. C., Ku, S. Y., Suh, C. S., & Kim, S. H. (2020). Metabolomics in the follicular fluid of women with diminished ovarian reserve. *Reproductive Biology and Endocrinology, 18*(1), 1–10.

Liu, X., Wang, T., Wang, Y., Chen, X., & Tian, S. (2023). Bax/Bcl-2 signaling in ovarian aging and follicular apoptosis. *Frontiers in Endocrinology, 14*, 1177456.

Kawai, T., Yamada, T., Abe, K., Okamura, H., & Osuga, Y. (2019). Follicular fluid mitochondrial DNA as a predictor of embryo development. *Fertility and Sterility, 111*(3), 528–535.

He, Y., Wang, Y., & Liu, Y. (2022). Cell-free mtDNA as a biomarker in reproductive medicine. *Journal of Assisted Reproduction and Genetics, 39*(6), 1233–1242.

González-Fernández, R., Peña, O., Hernández, J., Martín-Vasallo, P., & Ávila, J. (2020). Mitochondrial dysfunction and apoptosis in reproductive disorders. *Biology of Reproduction, 103*(3), 506–518.

Alawad ZM, Al-Omary HL. Correlation between Follicular Fluid Fatty Acids and Cell-Free Mitochondrial DNA in Women Undergoing Intra-Cytoplasmic Sperm Injections. J Fac Med Baghdad. 2024 Dec. 31

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2^(-ΔΔCT) Method. *Methods, 25*(4), 402-408.

Benkhalifa, M., Ferreira, Y. J., Chahine, H., Louanjli, N., Miron, P., Merviel, P., & Copin, H. (2014). Mitochondria: participation to infertility as source of energy and cause of senescence. *International Journal of Biochemistry and Cell Biology, 55*, 60-64.

Tsirka, G., Zikopoulos, A., Papageorgiou, K., Kostoulas, C., Tsigkas, I., Moustakli, E., Kaltsas, A., Sarafi, E., Michaelidis, T. M., & Georgiou, I. (2023). The ratio of cf-mtDNA vs. cf-nDNA in the follicular fluid of women undergoing IVF is positively correlated with age. *Genes, 14*(7), 1504.

Ventayol-Guirado, M., Hernandez-Rodriguez, J., Florit, J., Llull-Alberti, M. V., Barragan, R., Ferragut, J. F., Martorell, J., Heine-Suñer, D., Martinez, I., Picornell, A., Torres-Juan, L., & Peralta, L. (2025). First follicular fluid cf-mtDNA sequencing unveils ovarian stimulation-induced mutations impacting oocyte quality and IVF success. *Journal of Assisted Reproduction and Genetics, 42*, 2375–2386.

Liu, Y., Shen, Q., Zhao, X., Zou, L., Wang, S., & Li, Y. (2019). Cell-free mitochondrial DNA in human follicular fluid: a promising bio-marker of blastocyst developmental potential in women undergoing assisted reproductive technology. *Reproductive Biology and Endocrinology, 17*(1), 54.

Alves, M. M. C., Oliani, L., Almeida, M., Cardoso, H. J., Oliani, A. H., Breitenfeld, L., & Ramalhinho, A. C. (2023). Cell-free DNA as a new biomarker of IVF success, independent of any infertility factor, including endometriosis. *Diagnostics, 13*(2), 208.

Alawad, Z. M., & Al-Omary, H. L. (2024). Analysis of Follicular Fluid Fatty Acids in Iraqi Women Undergoing Intracytoplasmic Sperm Injection. *Al-Rafidain Journal of Medical Sciences (ISSN 2789-3219)*, *7*(1), 153–158.

Jibrilla, K. T., Adeyemi, A. S., & Lawal, B. (2025). Follicular fluid cell-free DNA as a non-invasive biomarker of pregnancy outcomes in women undergoing IVF/ICSI: a study in Nigerian women. *[Regional Journal]*. (In press).

Israa M.Majeed, & M. Salih Almohaidi, A. (2025). Implantation outcome is Associated with Mucin-16, MiR- 1226 and MiR-210 Expression during IVF of Iraqi Females. *Iraqi Journal of Pharmaceutical Sciences* , *34*(4), 113-124.

A.Taher, M., A.Atia, Y., & K.Amin, M. (2017). Improving an Ovulation Rate in Women with Polycystic Ovary Syndrome by Using Silymarin. *Iraqi Journal of Pharmaceutical Sciences* , *19*(2), 11-18.