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Research Article

Correlation between Follicular Fluid Fatty Acids and Cell-Free Mitochondrial DNA in Women Undergoing Intra-Cytoplasmic Sperm Injections

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Abstract:

Background: Beta-oxidation of fatty acids takes place in the mitochondria to produce energy. This process is linked to the formation of free radicals. Previous researches propose that some fatty acids may be related to mitochondrial dysfunction, as they induce oxidative stress.

Objectives: To examine the correlation between follicular fluid fatty acids and relative cell-free mitochondrial DNA in the follicular fluid in women experiencing intra-cytoplasmic sperm injection (ICSI).

Methods: Fifty women subjected to ICSI participated in this cross-sectional research. Follicular fluid samples were obtained during oocyte pick-up. The samples were assessed for fatty acids, utilizing gas chromatography, and for relative cell-free mitochondrial DNA, real time polymerase chain reaction (PCR) was used.

Results: There was a strong significant positive correlation between follicular fluid margaric acid and follicular fluid relative cell-free mitochondrial DNA, as the correlation coefficient was 0.869, and the *P* value was 0.025. In addition, a strong significant inverse correlation was noticed, in women with diminished ovarian reserve, between follicular fluid oleic acid and relative cell-free mitochondrial DNA in the follicular fluid, as indicated by a correlation coefficient = - 0.9 and a *P* value = 0.037.

Conclusion: Margaric acid correlated positively with the relative cell-free mitochondrial DNA, which might reflect mitochondrial dysfunction, due to aggravation of oxidative stress. Whereas, oleic acid in women with diminished ovarian reserve, correlated negatively with relative cell-free mitochondrial DNA. However, more studies are required in this area of research.

Keywords: Follicular Fluid; Fatty acids; Intracytoplasmic Sperm injection; Mitochondrial DNA; Oxidative Stress.

Introduction:

Infertility is the inability to reproduce after twelve months of frequent and unprotected sexual activity (1). Male infertility can be due to decreased sperm count, motility, or normal morphology (2). Nevertheless, the actual reason can be unknown (3). In females, polycystic ovary syndrome (PCOS), a frequent endocrine disorder, may affect them during reproductive years (4). It may occur due to numerous factors (5). It can cause hyperandrogenism, irregular menses, infertility, obesity, and metabolic abnormalities (6-10). Diminished ovarian reserve is the reduction in the oocyte count and quality, which affects the reproductive potential negatively (11). Unexplained infertility is considered the reason for subfertility when all tests performed are normal (1). Fatty acids, comprising the carboxylic acid group and the hydrocarbon chain, are regarded as the primary building units of lipids (12). There are saturated, monounsaturated, and polyunsaturated fatty acids (13). Evidence states that fatty acids are a source of energy for sperm and oocytes (14,15). Oocytes need a high amount of energy to resume meiosis. In addition, fatty acids influence ovarian follicle growth by affecting prostaglandin and steroid synthesis in the granulosa cells (15). However, studies have shown discrepancies concerning the effects of fatty acids on oocytes and embryos (16, 17). Mitochondrial DNA (mtDNA) is a double-stranded DNA, circular in shape, located in the mitochondrial matrix, near the respiratory chain, which makes it liable for oxidation and mutations (18). Research has documented that the mtDNA copy numbers show a marked increment during oocyte maturation, due to the need for a considerable amount of energy (19). This energy, derived from the mitochondria, is crucial not only for growth of the oocytes but also for proper early embryonic development as glycolysis is blocked until embryos reaching morula- blastocyst stage. Mitochondrial dysfunction of the oocytes has been linked to energy deficiency and redox imbalance. Evidence showed that mitochondrial supplementation might improve oocyte quality as proposed (19) which possibly enhances fertility outcomes in assisted reproductive techniques (ART). Nonetheless, studies have cited contradictory findings about the

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relationship between the mtDNA and intra-cytoplasmic sperm injection (ICSI) outcomes (20,21). Fatty acid beta-oxidation occurs in the mitochondria and it produces reactive oxygen species (ROS) (22,23). It has been proposed that some fatty acids may aggravate the redox imbalance, a disturbed balance between free radicals and their scavengers (24), in the mitochondria (25). A link between fatty acids and mitochondrial dysfunction has been suggested as free fatty acids might potentiate ROS formation thus aggravating the oxidative stress damage. Furthermore, they can decrease mitochondrial membrane potential, they can also increase mitochondrial permeability and mtDNA expression causing mitochondrial dysfunction (25,26). Additionally, some fatty acids, namely, saturated fatty acids might activate apoptosis process (16) as a result of excessive ROS production. The need for ART has been increased (27,28). Assessing the follicular fluid (FF), which contains various substances, may be useful for finding markers that predict ICSI outcomes (29,30), as well as for a better understanding of the environment in which oocytes develop. This study was performed, due to the possible association between fatty acids and cf-mtDNA and the importance of understanding the microenvironment of the oocytes. This research aims to explore the correlation between fatty acids and relative cf-mtDNA in the FF of women, who experienced ICSI.

Patients and Methods:

This cross-sectional research involved fifty women who underwent ICSI at the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University, Baghdad in the period between December 2022 and May 2023. The study subjected included 11 cases with male factor infertility, 11 cases with PCOS, 10 cases with diminished ovarian reserve, 10 cases with unexplained infertility and 8 cases with tubal factor infertility. The inclusion criteria were women subjected to the ICSI program for various reasons of infertility. However, women having genital malformations, systemic diseases such as diabetes mellitus, thyroid gland disorders, renal or liver diseases, and females taking fibrates and statins (due to the potential impact of these medicines on fatty acid levels) were excluded. Male subfertility was diagnosed based on seminal fluid analysis (3). Tubal factor infertility was confirmed by hysterosalpingography (1). Cases of PCOS were diagnosed depending on the Rotterdam criteria (31-33), and cases of diminished ovarian reserve were diagnosed based on the Bologna criteria (34). Unexplained infertility was determined when all infertility investigations were normal (1). Body mass index (BMI) was calculated as weight in kilograms per height in square meters (35-37). Detailed history was obtained, full examination was carried out and hormone values were acquired from the women. On the 2nd day of the menstrual period, recombinant follicle stimulating hormone (r-FSH) (Gonal F, Merk Serono, Germany) injections were commenced with a dose of 150 to 300 IU per day based on the patient's clinical status. A flexible start protocol was used for the administration of gonadotropin releasing hormone (GnRH) antagonist, namely, Cetrorelix acetate (Cetrotide®, Merk, Switzerland), 0.25 mg per day. It was started when the leading follicles' size reached 13 to 14 mm. Serial ultrasound evaluations and frequent serum estradiol (E2) quantifications were performed to monitor ovarian follicles' size and number. Once the size of 3 follicles reached 17 mm, subcutaneous human chorionic gonadotropin (hCG) injection (Ovitrelle®; Merck international, Italy) was given to provoke the release of the oocytes. 35 to 36 hours after giving hCG, oocyte picking up was done via a single lumen ovum aspiration needle by Wallace (CooperSurgical, California, USA) under transvaginal ultrasound monitoring.

Assessment of oocytes and embryos:

Removal of cumulus cells was done before ICSI to assess oocytes' maturity. Oocytes were categorized into germinal vesicle (GV), metaphase I (MI) oocytes, and metaphase II (MII) oocytes depending on their maturity status. 18 to 20 hours after ICSI, evaluation of fertilization was done and fertilization rate was computed as the ratio of oocytes with 2 pronuclei to oocytes that were injected (38). Cleavage-stage embryos were categorized into grade 1, grade 2, and grade 3 based on the Istanbul Consensus Workshop (39).

Collection of the follicular fluid samples:

During oocyte retrieval, FF (containing no flushing media) was collected (pooled from multiple follicles) and divided into 2 parts. One part was centrifuged at 1500xg for 10 minutes and the supernatant was then used for assessing fatty acids. The other part was centrifuged at 3000xg for 15 minutes and the supernatant was then used for DNA extraction and relative cf-mtDNA evaluation. The supernatant samples were transferred into sterile tubes and kept at – 20 C till assessment time. FF samples that were cloudy or stained with blood were not included.

Evaluation of the follicular fluid samples using gas chromatography:

FF samples (1.5 ml for each) were vortexed for three minutes. Then, 3 ml of cold acetone was added to separate the proteins from the solution. The samples were shaken for seconds and were put at – 20˚C for fifteen minutes. The samples were centrifuged, and then the supernatant of each sample was mixed with three ml aliquots of hexane (Thomas Baker, India) and water. Following that, horizontal shaking of samples was done for 5 minutes. To isolate the solvent phase from the aqueous phase, centrifugation was carried out another time. The top layers (hexane) were taken into sterile tubes. A 0.25 ml aliquot of buffer (pH= 9), made by mixing 0.1 M Na3PO4 with 0.1 M Na2HPO4 in water, and 0.25 ml iodomethane (Fluka, Switzerland) in dichloromethane (Central drug house, India) (1:10 vol: vol) were added as well. Finally, shaking of samples was done for 5 minutes via the vortex to produce the fatty acid methyl esters (FAME) (16,40).

Identification of FAME was done by gas chromatography (7820A, Agilent Technologies, USA) equipped with the analytical column (Agilent HP-5ms ultra inert, USA) having dimensions of 30 m length, 0.250 mm inner diameter, and 0.25 µm film thickness. Helium (99.99 %) was the carrier gas. The beginning temperature was 60˚C (for 3 minutes), elevated to 180˚C (7˚C/ minute), then was raised to 280˚C (8˚C/ minute), which was kept for 3 minutes. Recognition of FAME was done depending on their retention times, and levels of fatty acids were calculated as the weight percentage of the whole fatty acids found (41).

Relative cell-free mitochondrial DNA evaluation:

FF samples were centrifuged again at 16000xg for 10 minutes before cell-free DNA extraction. After that, the supernatants of FF samples were transferred into new Eppendorf tubes. Each FF sample (200 µL) was processed for extracting cell-free DNA by the usage of the AddPrep Genomic DNA Extraction Kit (ADD BIO INC, Daejeon, Republic of Korea), corresponding to the guidelines of the producer.

Specific primers for the β-globin gene (represents the nuclear DNA) and ND1 gene (represents mitochondrial DNA) were used for the amplification; (Macrogen Co., Ltd., Republic of Korea) designed the primers. The following primers: 5′-CCCTAAAACCCGCCACATCT-3′ (forward) and 5′-GAGCGATGGTGAGAGCTAAGGT-3′ (reverse), which amplify a 69 base pair DNA piece, identified ND1. The primers: 5′-AAAGGTGCCCTTGAGGTTGTC-3′ (forward) and 5′-TGAAGGCTCATGGCAAGAAA-3′ (reverse), that amplify a 77 base pair DNA segment, were used for the detection of β-globin (20).

Relative quantification of cf-mtDNA was achieved by real time quantitative polymerase chain reaction (PCR) (Rotor-Gene Q, QIAGEN, Germany). 20 µL was used as a total volume to perform the reaction, which consists of the extracted DNA (2 µL), sense, and anti-sense primers (10 µM). SYBR Green master mix (10 µL) (PowerUp SYBR Green Master Mix, Applied Biosystems, Thermo Fisher Scientific Baltics UAB). PCR circumstances were 94 C (2 minutes), 40 cycles of 95 C (10 seconds), then 60 C (30 seconds).

Relative cf-mtDNA copy numbers were estimated via the Delta Delta CT method (Livak method), and fold changes were calculated using the equation: $2^{-\Delta\Delta Ct}$ (42).

Statistical Analysis:

Data analysis was accomplished utilizing Statistical Package for the Social Sciences (SPSS) version 29 (Chicago, IL, USA). Normally distributed data were expressed as mean ± standard deviation and non-normally distributed values were presented as median (interquartile range). Pearson correlation test was applied to test for correlations in normally distributed data. Spearman correlation test was utilized for data that are not normally distributed. The finding was assumed statistically significant when the *P* value is less than 0.05.

Results:

Fifty females were involved in the present research, 33 women complained of primary infertility, and 17 women experienced secondary infertility. Out of those 50 women, thirty-eight women performed no previous trials of ICSI, 10 women had failed one ICSI trial previously, 1 female had failed two previous trials and 1 participant underwent a successful previous trial. Characteristics of all patients concerning demographic data and ICSI parameters are illustrated in Table 1.

Table 1: Demographic data and ICSI parameters of the participants

Patients' characteristics (N= 50)	The value
Age (years)	32 ± 5.4
Body mass index (kg/m^2)*	28.8 (4.1)
Follicle stimulating hormone (mIU/ml)	6.4 ± 1.7
Luteinizing hormone (mIU/ml)	5.8 ± 2.9
Estradiol (pg/ml)	38.6 ± 14.7
Total count of collected oocytes*	11 (11)
Oocyte maturity rate (%)	65.1 ± 21.2
Fertilization rate (%)	67.1 ± 22.9
Percentage of high quality embryos (%)*	50 (48.7)

Data are reported as mean ± standard deviation, in normal distribution, or median (interquartile range), in non-normal distribution. ***** refers to variables in which the median was used. ICSI: Intra-cytoplasmic sperm injection; N: Number of patients.

In terms of correlations between fatty acids and relative cf-mtDNA in the FF, Table 2 shows the correlations between saturated fatty acids and relative cf-mtDNA. The correlation between margaric acid and cf-mtDNA was significant as illustrated by a *P* value less than 0.05.

Table 2: Correlations between saturated fatty acids and relative cf-mtDNA in the FF

Saturated fatty acids in the FF %	Relative cf-mtDNA in the FF	
Palmitic acid % (N= 50)	rho = 0.096	*P* = 0.509
Stearic acid %(N= 41)	rho = 0.212	*P* = 0.184
Margaric acid % (N= 6)	r = 0.869	*P* = 0.025
Acetic acid % (N= 5)	r = 0.426	*P* = 0.474
Propionic acid % (N= 19)	rho = 0.049	*P* = 0.842

Pearson correlation and Spearman correlation tests are used according to data distribution. cf-mtDNA: Cell-free mitochondrial DNA; FF: Follicular fluid; N: Number of patients.

Concerning the correlations between FF unsaturated fatty acids and relative cf-mtDNA in the FF, all correlations were non-significant as observed in Table 3.

Table 3: Correlations between unsaturated fatty acids and relative cf-mtDNA in the FF

Unsaturated fatty acids in the FF %	Relative cf-mtDNA in the FF	
Oleic acid % (N= 21)	rho = - 0.313	*P* = 0.167
Linoleic acid % (N= 8)	rho = 0.548	*P* = 0.160
Palmitoleic acid % (N= 6)	rho = 0.086	*P* = 0.872

Spearman correlation test is applied. cf-mtDNA: Cell-free mitochondrial DNA; FF: Follicular fluid; N: Number of patients. When it comes to the correlations between FF fatty acids and FF relative cf-mtDNA in different causes of subfertility, it has been shown that palmitic acid correlated positively with relative cf-mtDNA in women having reduced ovarian reserve and in cases of male infertility as rho = 0.564, *P* = 0.090 and rho = 0.545, *P* = 0.083, respectively. A positive, non-significant correlation was also identified between stearic acid and relative cf-mtDNA, in male factor infertility, since rho was 0.612 and *P* value was 0.060. It was found that oleic acid in the diminished ovarian reserve patients correlated significantly and negatively with cf-mtDNA in the same group as the correlation coefficient (rho) was -0.9 and the *P* value was 0.037.

Discussion:

This study aims to correlate FF fatty acids and relative cf-mtDNA in the FF of women undergoing ICSI.

Owing to the relationship between the level of cf-mtDNA in body fluids and oxidative stress (43), and the correlation between some fatty acids and oxidative stress (44), we proposed that there might be a correlation between fatty acids and cf-mtDNA in the FF of females subjected to ICSI. In our study, a significant positive correlation has been detected between FF margaric acid and FF relative cf-mtDNA. This may be attributed to the relationship between margaric acid, being a saturated fatty acid, and cf-mtDNA with oxidative stress, which indirectly causes this positive correlation between margaric acid and cf-mtDNA. In a previous study, a positive correlation was shown between plasma mtDNA and the level of H2O2 (which reflects the level of ROS) and illustrated a possible link between elevated mtDNA and oxidative stress (43). It has been documented that ingestion of saturated fat, in women with PCOS, can result in the induction of oxidative stress; the researchers have noticed that ROS production, $p47^{phox}$ gene expression, and circulating thiobarbituric acid-reactive substances increase after consumption of a diet rich in saturated fat (45). Likewise, saturated fatty acids may lead to severe apoptosis, which probably modifies the constitution of the mitochondrial membranes, altering the mitochondrial function (46). Endoplasmic reticulum stress may be induced by lipotoxicity, as well (47). Besides that, free fatty acids have a role in lowering mitochondrial membrane potential, elevating mitochondrial permeability, and mtDNA expression (25). Furthermore, accumulation and increment of excess fatty acids might result in impairment of mitochondrial function by increasing the production of toxic metabolites and elevating oxidative stress, thus affecting mitochondrial performance (48). This might be another explanation of the positive correlation between margaric acid and cf-mtDNA. In addition, impaired mitochondrial function can occur secondary to insulin resistance that might happen due to ingestion of high amount of saturated fat (for example, margaric acid), therefore, raised saturated fatty acids might be associated with increased cf-mtDNA due to mitochondrial dysfunction (48).

FF palmitic acid in diminished ovarian reserve and male factor subfertility, and FF stearic acid, in male factor infertility show a positive correlation with FF relative cf-mtDNA in the current research. This can reflect mitochondrial dysfunction due to exacerbating oxidative stress, as agreed to in numerous studies (46-48). Although these correlations have not reached statistical significance, they are approaching that stage. This study detected a significant inverse correlation between oleic acid and relative cf-mtDNA in the FF of women with low ovarian reserve. This finding was in accordance with that mentioned in a recent study about the effects of monounsaturated fatty acids as they promoted mitochondrial oxidation, elevated antioxidant ability, and reduced inflammatory and peroxidation markers (49). This probably explains their favorable effects on mitochondrial function and illustrates that oleic acid, being a monounsaturated fatty acid, might have a protective role by improving the metabolic profile (49). Meanwhile, the excess level of FF cf-mtDNA possibly reflected the mitochondrial functional impairment of ovarian granulosa cells that led to the elevation of FF cf-mtDNA, secreted from these cells (50). Therefore, these opposite effects of oleic acid, suggested to have a protective role, and cf-mtDNA (linked to oxidative stress and mitochondrial dysfunction) might explain the significant negative correlation that was found between them. In the current research, no correlation was identified between linoleic acid and cf-mtDNA. This might be due to the small number of participants. This finding disagreed with another study done by Xu et al. (2019) which revealed that omega-6 polyunsaturated fatty acids, owing to their pro-oxidative effects, might cause mitochondrial dysfunction (26).

Limitations:

Some limitations that should be addressed are the small number of participants, the sample size especially for certain fatty acids with very small numbers, the cross-sectional design of the study, and the lack of involvement in the exact nutritional status, and the eating habits of the women included in the study. It is recommended to include more participants and to take into consideration the nutritional condition in future studies. Moreover, our suggestion for future research is exploring the exact role of oxidative stress biomarkers in conjunction with fatty acid profiles or cf-mtDNA levels.

Conclusion:

This research demonstrated a possible link between fatty acids and relative cf-mtDNA in the FF. Margaric acid showed a significant direct correlation with relative cf-mtDNA reflecting mitochondrial dysfunction due to oxidative stress. Oleic acid correlated significantly and inversely with relative cf-mtDNA in patients with diminished ovarian reserve. Therefore, different fatty acids exert various effects, which might be related to their degree of saturation.

Nonetheless, further research is justified to unveil other unknown mechanisms and factors that explain the associations between fatty acids and cf-mtDNA in the FF.

Authors' declaration:
We confirm that all the Tables in the manuscript belong to the current study. Authors sign on ethical consideration's approval-Ethical Clearance: The project was approved by the local ethical committee in (the University of Baghdad/ College of Medicine) according to the code number (197) on (09/ October /2023).
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Authors' contributions:
Study conception & design: (Zainab M. Alawad and Hanan L. Al-Omary). Literature search: (Zainab M. Alawad). Data acquisition: (Zainab M. Alawad). Data analysis & interpretation: (Zainab M. Alawad and Hanan L. Al-Omary). Manuscript preparation: (Zainab M. Alawad). Manuscript editing & review: (Hanan L. Al-Omary).

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العلاقة بين الأحماض الدهنية والحمض النووي للمايتوكوندريا الخالي من الخلايا في السائل الجريبي لدى النساء اللاتي يخضعن للحقن المجهري

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الخلاصة:

الخلفية: أكسدة بيتا للأحماض الدهنية يحدث في المايتوكوندريا لإنتاج الطاقة. ترتبط هذه العملية بتكوين الجذور الحرة. تقترح الأبحاث السابقة أن بعض الأحماض الدهنية قد تكون ذات صلة بالاختلال الوظيفي للمايتوكوندريا لأنها تحفز الاجهاد التأكسدي.

الأهداف: لدراسة الارتباط بين الأحماض الدهنية في السائل الجريبي والحمض النووي النسبي للمايتوكوندريا الخالي من الخلايا في السائل الجريبي عند النساء اللاتي يخضعن للحقن المجهري.

المرضى والطرق: تمت مشاركة خمسين امرأة خضعت للحقن المجهري في هذا البحث المقطعي. تم الحصول على عينات السائل الجريبي أثناء التقاط البويضات. تم تقييم العينات للأحماض الدهنية باستخدام كروماتوغرافي الغاز وللحمض النووي النسبي للمايتوكوندريا الخالي من الخلايا تم استخدام تفاعل البلمرة المتسلسل في الوقت الحقيقي.

النتائج: كانت هناك علاقة ارتباط قوية معنوية موجبة بين حمض المارجريك في السائل الجريبي والحمض النووي النسبي للمايتوكوندريا الخالي من الخلايا في السائل الجريبي لأن معامل الارتباط كان 0.869 وكانت القيمة الاحتمالية 0.025. إضافة الى ذلك لوحظت علاقة قوية معنوية عكسية، في النساء اللاتي لديهن انخفاض في مخزون المبيض، بين حمض الأوليك في السائل الجريبي والحمض النووي النسبي للمايتوكوندريا الخالي من الخلايا في السائل الجريبي كما موضح بمعامل الارتباط = - 0.9 والقيمة الاحتمالية = 0.037.

الاستنتاج: من المحتمل ان تظهر الأحماض الدهنية المختلفة في السائل الجريبي ارتباطات مختلفة مع الحمض النووي النسبي للمايتوكوندريا الخالي من الخلايا في السائل الجريبي. هذا ممكن أن يحدد بمستوى التشبع لان حمض المارجريك ارتبط بشكل إيجابي مع الحمض النووي النسبي للمايتوكوندريا الخالي من الخلايا، الذي قد يعكس خلل المايتوكوندريا بسبب تفاقم الاجهاد التأكسدي. بينما، حمض الأوليك، في النساء اللاتي لديهن انخفاض في مخزون المبيض، ارتبط سلبا مع الحمض النووي النسبي للمايتوكوندريا الخالي من الخلايا. ومع ذلك هناك حاجة الى مزيد من الدراسات في هذا المجال البحثي.

الكلمات المفتاحية: السائل الجريبي، الأحماض الدهنية، الحمض النووي للمايتوكوندريا، الحقن المجهري، الاجهاد التأكسدي.