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Research Article

Analysis of Follicular Fluid Fatty Acids in Iraqi Women Undergoing Intracytoplasmic Sperm Injection

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Abstract

Background: Oocytes are susceptible to alterations in the various fatty acid contents of follicular fluid (FF), which may influence maturation and embryogenesis. Different fatty acids exert various effects on intracytoplasmic sperm injection (ICSI), which needs further studies to uncover the involved mechanisms. ***Objectives***: To assess FF fatty acids in women undergoing ICSI and to correlate them with ICSI parameters, namely the total count of aspirated oocytes, oocyte maturation rate, fertilization rate and percentage of good-quality embryos. ***Methods***: Fifty women undergoing ICSI were enrolled in this cross-sectional study. FF samples were collected during oocyte retrieval and were analyzed for fatty acids using gas chromatography. Fatty acids were calculated as percentages of the total fatty acids. ***Results***: The most common fatty acids found in the FF of women who underwent ICSI were palmitic acid, stearic acid, and oleic acid, with median (interquartile range) of 58.61%(21.66%), 26.27%(14.31%), and 20.13%(31.05%), respectively. Palmitic acid correlated inversely and significantly with oocyte maturation rate, fertilization rate, and percentage of good-quality embryos, with p=0.003, 0.037, and 0.028, respectively. Stearic acid correlated negatively and significantly with oocyte maturation rate (p=0.037) and fertilization rate (p=0.041). Furthermore, an inversely significant correlation was noticed between propionic acid and the percentage of good-quality embryos, as indicated by p=0.014. ***Conclusions***: Palmitic, stearic, and propionic acids in the FF might influence ICSI parameters; thus, they might be used as markers of oocyte developmental competence. Nevertheless, further research is warranted.

Keywords: Embryo quality, Fatty acids, Fertilization rate, Follicular fluid, Oocyte maturation rate.

تحليل الأحماض الدهنية في السائل الجريبي لدى النساء العراقيات اللواتي يخضعن لحقن الحيامن داخل السيتوبلازم

الخلاصة

الخلفية: البويضات عرضة للتغيرات في محتويات الأحماض الدهنية المختلفة للسائل الجريبي والتي قد تؤثر على النضج والتطور الجنيني. تمارس الأحماض الدهنية المختلفة تأثيرات مختلفة على حقن الحيامن داخل السيتوبلازم (ICSI) ، والتي تحتاج إلى مزيد من الدراسات للكشف عن الآليات المعنية. **الأهداف**: تقييم الأحماض الدهنية في السائل الجريبي لدى النساء اللائي يخضعن للحقن المجهري وربطها بمعايير الحقن المجهري، مثل العدد الإجمالي للبويضات المستحصلة، ومعدل نضج البويضة، ومعدل الإخصاب، والنسبة المئوية للأجنة ذات النوعية الجيدة. **الطريقة**: تم تسجيل خمسين امرأة خضعن للحقن المجهري في هذه الدراسة المقطعية. تم جمع عينات من السائل الجريبي أثناء استرجاع البويضات وتم تحليل محتواها من الأحماض الدهنية باستخدام كروماتوغرافيا الغاز. تم حساب الأحماض الدهنية كنسب مئوية من إجمالي الأحماض الدهنية الكلي. **النتائج**: كانت الأحماض الدهنية الأكثر شيوعا الموجودة في السائل الجريبي للنساء اللائي خضعن للحقن المجهري هي حمض البالمتيك وحمض الستياريك وحمض الأوليك، مع وسيط (نطاق ربعي) 58.61% (21.66٪) و 26.27% (14.31٪) و 20.13% (31.05٪) على التوالي. يرتبط حمض البالمتيك عكسيا ومعنويا بمعدل نضوج البويضة ومعدل الإخصاب والنسبة المئوية للأجنة ذات النوعية الجيدة. يرتبط حامض الستياريك سلبا ومعنويا بمعدل نضوج البويضة ومعدل الإخصاب، وعلاوة على ذلك، لوحظ وجود علاقة عكسية بين حمض البروبيونيك والنسبة المئوية للأجنة ذات النوعية الجيدة. **الاستنتاجات**: الأحماض النخيلية الشمعية والبروبيونية في السائل الجريبي قد تؤثر على معلمات الحقن المجهري. وبالتالي، يمكن استخدامها كعلامات على كفاءة نمو البويضة. ومع ذلك ، هناك ما يبرر إجراء مزيد من البحث.

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INTRODUCTION

Infertility is the inability to achieve pregnancy after one year of repeated sexual intercourse without using any contraceptive modality [1]. The pathogenesis of subfertility is complex, as it might be related to a male factor, a female factor or both [2]. Male infertility might be due to a reduction in sperm count, a decline in sperm motility or abnormal sperm morphology [3]. However, the real cause of male infertility might be unknown [4]. In females, many disorders can result in infertility. Polycystic ovary syndrome (PCOS), the most prevalent endocrine disorder affecting females of childbearing age [5,6], has probably multifactorial origins [7]. It might cause anovulation, hyperandrogenism, menstrual irregularities, infertility, obesity and metabolic disorders [8–12]. Diminished ovarian reserve is a

decline in oocytes' quantity and quality, which can result in a reduction in reproductive potential and unfavorable fertility outcomes [13]. Numerous factors can influence ovarian reserve, including hormonal factors, metabolic factors, environmental effects, diseases, drugs, and others [14]. The term unexplained infertility refers to cases in which all tests related to male and female infertility are normal [15]. Fatty acids are carboxylic acids with aliphatic chains of various lengths and saturation degrees. Saturated fatty acids, like palmitic acid, carry single bonds between their carbon atoms, while unsaturated fatty acids, like oleic acid and linoleic acid, contain one or more double bonds [16]. Fatty acids, the main constituents of lipids, perform numerous functions in body systems. They are the principal component of cell membranes, they regulate enzyme functions and inflammatory processes, and they also serve as an important energy source [17]. Research has proposed that fatty acids provide energy to the oocytes via beta oxidation since oocytes require a high energy supply for resuming meiosis. They also promote follicle growth and oocyte quality by influencing the production of steroids and prostaglandins in granulosa cells and the corpus luteum, as well as the lipid components of the follicular fluid (FF), which reflect the status of granulosa cells and theca cells. Furthermore, fatty acids supply the embryos with energy, so they may have an impact on their quality and ability to implant [18]. Studies showed controversial results concerning the impacts of fatty acids on reproductive potential. Researchers have noticed that excessive amounts of palmitic acid and stearic acid in the FF negatively affect oocyte maturation and implantation. Oleic acid has been shown to exert favourable outcomes on the oocytes [19]. Nonetheless, another study concluded that oleic acid at a high level might compromise fertility by decreasing steroidogenesis [20]. Thus, further research in this field is mandatory. The demand for assisted reproductive technologies has been expanded worldwide [21]. Analysis of FF, which contains metabolites, ions, and hormones [22], might be helpful in improving the results of in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) by finding markers that can predict the outcomes [23,24]. The aim of this study is to evaluate the fatty acids present in the FF of a sample of Iraqi women undergoing ICSI, and to assess the correlation between the percentages of these fatty acids and the parameters of ICSI.

METHODS

Characteristics of the participants

This cross-sectional study included 50 patients undertaking ICSI at the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies/Al-Nahrain University from December 2022 to May 2023. The causes of infertility in this research were male factor infertility (11 cases), PCOS (11 cases), diminished ovarian reserve (10 cases), unexplained infertility (10 cases) and tubal factor infertility (8 cases).

Inclusion criteria

The inclusion criteria include women undertaking ICSI program for various reasons of subfertility. However, the exclusion criteria include females with genital malformations, diabetes mellitus, thyroid gland abnormalities, liver or renal diseases, and women taking lipid-lowering drugs (statins and fibrates).

Diagnosis and interventions

Diagnosing PCOS in women was in accordance with the Rotterdam criteria [25–27]. Diminished ovarian reserve patients were diagnosed depending on the Bologna criteria [28]. A thorough history was taken, a physical examination was performed and hormonal levels were obtained from the participants. Body mass index (BMI) was computed as weight (kilograms) divided by height (square meters) [29–31]. Ovarian stimulation was initiated by administration of recombinant follicle stimulating hormone (rFSH) (Gonal-F®, Merck Serono, Germany) injections that started on day 2 of the menses with a dose of 150–300 IU per day, depending on the woman's underlying medical state. Cetrotide acetate injection at a dose of 0.25 mg (Cetrorelix®, Merck, Switzerland) was begun using a flexible protocol when the dominant follicles achieved diameters of 13–14 mm. Ovarian follicles were monitored by frequent ultrasonography examinations. Successive evaluations of serum estrogen were also performed to follow up the ovarian response. The trigger of ovulation was performed by the administration of a human chorionic gonadotropin (hCG) injection (Ovitrelle®; Merck International, Italy) when the size of three follicles reached more than 17 mm. After hCG injection, 35 to 36 hours, oocyte retrieval was carried out utilizing a single lumen ovum aspiration needle by Wallace (CooperSurgical, California, USA) under trans-vaginal ultrasound guidance.

Evaluating oocytes and cleavage stage embryos

Cumulus cells were stripped away prior to ICSI in order to examine the maturity of the oocytes, as only the oocytes that expelled the first polar body were considered mature. So, according to their maturity, oocytes were classified into germinal vesicles, metaphase I oocytes and metaphase II oocytes. Following ICSI by 18–20 hours, assessment of fertilization was done based on the presence of pronuclei, and fertilization rate was identified by calculating the ratio of the number of oocytes with two pronuclei to the quantity of total oocytes that were injected [32]. Cleavage stage embryos were graded into grade 1 (good) embryos, grade 2 (fair) embryos, and grade 3 (poor) embryos in accordance with the Istanbul Consensus Workshop [33].

Collection of the follicular fluid

During ovum pick-up, FF (with no flushing media) was collected, centrifuged at 1500 x g for 10 minutes, and the upper layer was then frozen at -20 ºC until analysis time. Clear FF samples were used, whereas cloudy and blood-stained samples were not included.

Analysis of follicular fluid by gas chromatography

1.5 ml of each FF sample was shaken using a vortex device (Fanem, Brazil) for 3 minutes. Following that, 3 ml of ice-cold acetone was added to precipitate proteins. Then, the samples were shaken another time for seconds and they were kept at – 20˚C for fifteen minutes. Centrifugation of the samples was done. After that, the upper layer was taken and mixed with 3 ml of equal portions of hexane (Thomas Baker, India) and water. Afterwards, the tubes were closed tightly and shaken horizontally for five minutes. Centrifugation was done again to separate the solvent and the watery phases. The top layer (the hexane) was then put in sterile tubes. A 0.25 ml portion of buffer having a pH of 9 (produced by combining 0.1 M Na_3PO_4 and 0.1 M Na_2HPO_4 with water) and 0.25 ml of iodomethane (Fluka, Switzerland) in dichloromethane (Central Drug House, India) (1:10 v/v) were also added. Lastly, the samples were shaken in the vortex for five minutes to form fatty acid methyl esters (FAME) [19,34]. FAME were splitted and detected via gas chromatography (GC) (7820A, Agilent Technologies, USA) using the analytical column Agilent HP-5ms ultra-inert (USA) with dimensions of 30 m length x 0.250 mm inner diameter x 0.25 µm film thickness. Helium 99.99% was the carrier gas. The injection volume was 1µl, and the pressure was 11.933 psi. The GC inlet line temperature was 250˚C, whereas the auxiliary heater temperature was 300 ºC. The injector temperature was 250˚C, with a scan range of 25–1000 m/z, and the injection type was splitless. The temperature of the oven was initially 60 °C, which was kept for 3 minutes; it was then increased to 180 °C at a rate of 7 °C per minute. After that, the temperature was elevated to 280 °C at a rate of 8 °C per minute; subsequently, 280 °C was held for 3 minutes. FAME were recognized based on their retention times, and the concentrations of fatty acids were computed as a weight percentage of the total fatty acids available [35].

Ethical consideration

Approval of the study was issued by the ethical committee at the University of Baghdad, College of Medicine (Certificate number 195, on October 9, 2023). Informed consent was obtained from the participating females.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) version 29 (Chicago, IL, USA) was used for data analysis. The mean ± standard deviation was utilized to report data with a normal distribution and the median (interquartile range) was applied to present variables with a non-normal distribution. Pearson correlation and Spearman correlation tests were used to carry out the correlations according to the normality of the distribution of the variables. A *P* value below 0.05 was supposed to be statistically significant.

RESULTS

The current study involved a total of fifty women, with thirty-three of them experiencing primary infertility and seventeen reporting secondary infertility. Table 1 displays the attributes of the female participants included in this study.

Table 1: Characteristics of the enrolled patients (n=50)

Variables	Value
Age (year)	32.20±5.35
BMI (kg/m^2)	28.83(4.12)
Duration of infertility (year)	6.00(7.00)
FSH (mIU/ml)	6.39±1.66
LH (mIU/ml)	5.76±2.87
E2 (pg/ml)	38.64±14.66
Prolactin (ng/ml)	18.17±5.75
TSH (mIU/l)	2.00±0.83
AMH (ng/ml)	2.54(1.86)

Data with normal distribution are presented as mean ± standard deviation, whereas non-normally distributed results are reported as median (interquartile range). n: number of patients; BMI: Body mass index; FSH: Follicle stimulating hormone; LH: Luteinizing hormone; E2: Estradiol; TSH: Thyroid stimulating hormone; AMH: Anti-Müllerian hormone.

Table 2 presents the ICSI outcomes, which include the number of aspirated oocytes, the rate of oocyte maturation, the rate of fertilization, and the percentage of good-quality embryos.

Table 2: The ICSI parameters of the enrolled patients (n=50)

ICSI parameter	Value
Aspirated oocytes	11.00(11.0)
Oocyte maturation rate (%)	65.06±21.24
Fertilization rate (%)	67.06±22.86
Good quality embryos (%)	50.0(48.69)

Normally distributed data are demonstrated as mean ± standard deviation, whereas results with non-normal distribution are shown as median (interquartile range). n: number of patients; ICSI: Intracytoplasmic sperm injection.

Table 3 presents the proportions of fatty acids identified in the FF of the female participants in this investigation.

Table 3: Percentages of fatty acids in the follicular fluid

FF Fatty acids	Value
Palmitic acid (n= 50)	58.61(21.66)
Stearic acid (n= 41)	26.27(14.31)
Oleic acid (n= 21)	20.13(31.05)
Linoleic acid (n= 8)	9.13±6.64
Palmitoleic acid (n= 6)	16.40±15.13
Margaric acid (n= 6)	7.88±4.77
Acetic acid (n= 5)	13.40±10.93
Propionic acid (n= 19)	9.52(26.80)

Data having normal distribution are described as mean ± standard deviation, whereas non-normally distributed results are mentioned as median (interquartile range). n: number of patients.

The present research findings indicate significant negative correlations between palmitic acid and oocyte maturation rate, fertilization rate, and percentage of good-quality embryos in the context of

the correlations between the percentage of each fatty acid and each parameter of ICSI (Table 4). In addition, there was a significant and negative correlation between stearic acid and both oocyte maturation rate and fertilization rate, as indicated in Table 4. In addition, Table 4 showed a substantial inverse association between propionic acid and high-quality embryos.

Table 4: Correlations between fatty acids in the follicular fluid and ICSI parameters

Follicular Fluid FA		Aspirated oocytes (n)	Oocyte maturation rate (%)	Fertilization rate (%)	Good quality embryos (%)
Palmitic acid % (n= 50)	r_s	0.032	- 0.410	- 0.296	- 0.311
	p	0.827	0.003	0.037	0.028
Stearic acid % (n= 41)	r_s	0.029	- 0.327	- 0.320	0.036
	p	0.857	0.037	0.041	0.825
Oleic acid % (n= 21)	r_s	0.070	0.162	0.184	0.069
	p	0.764	0.484	0.424	0.768
Linoleic acid % (n= 8)	r_s	0.156	0.239	- 0.071	- 0.144
	p	0.713	0.569	0.867	0.734
Palmitoleic acid % (n=6)	r_s	- 0.038	0.342	0.462	0.094
	p	0.942	0.507	0.356	0.859
Margaric acid % (n= 6)	r_s	- 0.199	- 0.451	- 0.265	0.360
	p	0.706	0.370	0.612	0.483
Acetic acid % (n= 5)	r_s	0.050	- 0.757	- 0.415	- 0.342
	p	0.936	0.139	0.488	0.573
Propionic acid % (n=19)	r_s	- 0.006	- 0.104	0.177	- 0.552
	p	0.980	0.672	0.469	0.014

Pearson correlation test is used in cases of normally distributed variables, Spearman correlation test is utilized in cases of non-normally distributed variables. n: number of a variable; ICSI: Intracytoplasmic sperm injection.

DISCUSSION

This study aimed to assess the percentages of fatty acids in the FF of Iraqi females undergoing ICSI and to correlate these percentages with ICSI outcome. This study found palmitic acid to be the most common FF fatty acid, in agreement with other studies [35,36], followed by stearic acid and oleic acid. The percentages of FF fatty acids in the current work were relatively higher, apart from linoleic acid, compared with other studies [35,36]. Numerous factors, such as nutritional status and metabolic condition, significantly influence the composition of FF fatty acids, potentially explaining the high percentages of certain fatty acids in our study [37]. Various methodologies for evaluating fatty acids, different experimental designs of research studies, and different protocols and durations of ovarian stimulation may contribute to these differences across studies. Researchers postulate that elevated fatty acids not only impact oocyte development, but also impact embryo viability, metabolism, and quality. This leads to the formation of blastocysts, which exhibit increased apoptotic activity, decreased oxygen, glucose, and pyruvate expenditure, increased lactate utilization, and excessive amino acid metabolism [38]. Beta-oxidation of fatty acids creates reactive oxygen species. Too much of these can stop enzymes from working, damage lipids, and change DNA and proteins in ways that are bad for oocytes and embryos [39]. Numerous studies support the current study's inversely significant correlations between palmitic acid and oocyte maturation rate, fertilization rate, and the proportion of high-quality embryos. Chen et al. proposed that palmitic acid treatment of mice granulosa cells enhanced Caspase-3 activation and BAX expression, thereby inducing apoptosis [40]. Moreover, palmitic acid potentiated the expression of GRP78 and CHOP, suggesting an activation of the endoplasmic reticulum (ER) stress pathway. As a result, ER stress contributes to granulosa cell apoptosis triggered by palmitic acid [40]. Furthermore, studies have documented the alteration of embryonic IGF1 receptor (IGF1R) expression, increased glutamic pyruvate transaminase (GPT2) activity, and reduction of nuclei number in blastocysts exposed to high palmitic acid [41]. On the contrary, a study on gilts found positive effects of palmitic acid on oocyte quality [42]. Thus, further research is required. In the current study, stearic acid had a negative and significant correlation with oocyte maturation rate and fertilization rate. Earlier works were similar to our findings in terms of the correlation between stearic acid and metaphase II oocytes [19, 36]. Liu and co-workers demonstrated an inverse correlation between the proportion of stearic acid in FF samples of overweight and obese females and embryo quality parameters [43]. Mirabi *et al.* concluded that oleic acid is essential for oocyte maturation and may counteract the deleterious impacts of palmitic and stearic acids [19]. Furthermore, a previous study suggested positive impacts of oleic acid on oocyte developmental competence via several mechanisms, including the fact that oleic acid greatly boosts the partitioning of fatty acids towards adiposomes and works as the main determining factor for organizing structures in the cell membrane of follicular cells. Furthermore, oleic acid plays a crucial role as a metabolic contributor or coordinator of redox imbalance and signaling pathways within cells [44]. The present study also noticed positive correlations between oleic acid and ICSI outcomes. However, none of them reached statistical significance, and this may be due to the relatively small number of participants. Since linoleic acid is an essential fatty acid, individual dietary status primarily influences its amount in the FF [36], potentially explaining the relatively low percentage of linoleic acid in the FF of our research participants. Lee *et al.* reported that

linoleic acid has a positive impact on oocyte developmental ability because alpha-linoleic acid can improve oocyte nuclear maturation by decreasing oxidative stress [45]. Still, high levels of linoleic acid may be bad for oocyte maturation and embryogenesis because they lower the number of cumulus oocyte complexes with full cumulus cell expansion, metaphase II oocytes, and blastocyst rate [45,46]. We did not find any correlation between linoleic acid percentage and ICSI parameters. This could potentially be attributed to the relatively low percentage of linoleic acid in the follicular fluid of the participants, which may be a result of their dietary habits. This study found no significant correlations between palmitoleic acid, margaric acid, and ICSI parameters. A previous study mentioned a positive correlation between palmitoleic acid and germinal vesicle oocytes [19], which could mean that it has negative effects on ICSI parameters. The present study found no association between acetic acid and fertility parameters; however, a recent study on a rat model of PCOS revealed its protective role for ovarian function [47]. We observed a moderately inverse and significant association between the percentage of good-quality embryos and propionic acid. This could be attributed to the fact that propionic acid may potentially alter insulin action, leading to insulin resistance [48]. Investigators illustrated that unsaturated fatty acids can reduce the lipotoxicity of saturated fatty acids [49], so modifications to lifestyle and nutritional status might improve the fatty acid profile.

Study limitations

The study was characterized by a small sample size, a cross-sectional design, and the use of pooled FF rather than individual FF surrounding each oocyte. In addition, the analysis of FF fatty acids was carried out only in total lipids and not in various lipid factions that contain distinct fatty acid constituents, as suggested [50]. Furthermore, the study failed to consider the nutritional status of the participants.

Conclusion

The present study uncovered inverse correlations between some fatty acids, namely palmitic acid, stearic acid, and propionic acid, and ICSI parameters. Thus, these fatty acids are possibly considered markers for ICSI outcomes. However, more research is mandatory to reach a better explanation.

Recommendations

Suggestions for future work include increasing the sample size, using individual FF instead of pooled FF, evaluating FF fatty acids in different lipid fractions, and taking into account the nutritional state of the participants. As a result, nutritional counseling and diet modification are important to be implanted prior to the start of ICSI programs.

Conflict of interests

No conflict of interests was declared by the authors.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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