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Prediction of the Presence of Lipid Derivatives in Follicular Fluid and Reproductive Outcome among Infertile Women by MALDI Mass Spectrometry Method

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It has been documented that specific follicular fluid (FF) biochemical characteristics may be essential to determining oocyte quality. Lipid derivatives have the most important role in the fertilization process and embryonic development. MALDI mass spectrometry is used for the diagnosis of biomolecules in the FF and serum of infertile women. FF and blood samples from 13 infertile women (20-38 years old) undergoing intracytoplasmic sperm injection (ICSI) was obtained, stored frozen at -80 ºC, and later analyzed for Lipid Derivatives. Both FF and serum samples were compared and Mf_g was used as similarity index. For comparison between the FF and serum samples for one person, the mass spectrometry (MS) intensity ratio versus molecular weight, between the FF and serum samples were calculated. Out of 13 patients, three ongoing pregnancies were observed, so the percentage of pregnancy in the studied population was 25%. The patients who became pregnant after micro-injection had higher unsaturated fatty acids and omega-3 fatty acid compared to other samples, but Stearic and palmitic fatty acids were not high in these patients. A number of specific fatty esters and peptide derivatives in the FF and serum samples were found that may help to improve oocyte quality.

Keywords: Follicular fluid, Infertility, MALDI mass spectrometry, Lipid derivatives

INTRODUCTION

Follicular fluid (FF), as an endogenous medium, participates in transmitting nutrition signals to the cumulus-oocyte complex (COC). FF is easily available as it is aspirated together with the oocyte at the time of oocyte retrieval [1].

This fluid is a product of blood-plasma constituents that cross the blood follicular barrier and involved in the secretary activity of granulosa and theca cells. Therefore, specific FF biochemical characteristics may be essential to determine the oocyte quality and the probable achievement of fertilization and embryonic development.

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The analysis of FF components may also help in the determination of metabolic modification in blood serum, as the circulating biochemical milieu may be reflected in the composition of FF [2-4]. Although there are several factors affecting reproductive outcome, some studies have supported the important function of the chemical constituents of FF in determining oocyte quality [1,5,6].

They showed that serum metabolic changes are reflected in the FF, therefore, recent research has focused more on the complex type of molecular analysis, metabolomics, and the surrounding micro-environment of the ovarian follicle of infertile women undergoing *in vitro* fertilization (IVF) or intra-cytoplasmic sperm injection (ICSI).

It can provide useful information regarding the requirements for cells, and oocyte maturation. The

composition may be used as a provisional guide for formulating suitable culture media for in *vitro* cell culture and oocyte maturation in particular specie [7,8].

Fatty acids are important components in the surrounding micronutrient environment of the ovarian follicle. In an animal model study, exogenous saturated fatty acids such as stearic and palmitic acids, inhibited the proliferation of various cells, as well as the fertilization and development of embryos cultured *in vitro*. Unsaturated fatty acids, such as oleic acid promoted blastocyst formation, had a positive effect on oocyte maturation and embryo development [9].

Only few studies have focused on how body mass index (BMI; kg m^{-2}) or dietary lipids of humans may affect follicular fluid or ovarian cell lipid composition. However, differences in body composition are associated with distinct alterations [10,11]. Furthermore, elevated BMI in women attending an infertility clinic was associated with increased levels of follicular fluid triglyceride which indicates changes in the blood, but the fatty acid compositions of the human follicular fluid are not tightly correlated with BMI [12].

In an early study by Jungheim [13], it was reported that alpha-linolenic acid levels were negatively associated with embryo implantation rates and chance of pregnancy in women undergoing IVF. Biomarkers are useful tools, which may provide helpful information in the early diagnosis of human diseases.

Numerous studies have shown worse outcomes in assisted reproduction techniques (ART) in patients with endometriosis and poly cystic ovarian syndrome (PCOS) due to lower quality of oocyte. The lower fertilization rate and poorer embryo quality in these cases may be explained by oxidative stress in infertile women. An imbalance between prooxidant intermediates and antioxidants in the FF could be responsible for the abnormal developmental competence of oocytes, which would result in poor embryo quality [14,15]. Other studies failed to demonstrate the differences in IVF and ICSI outcomes in these patients [16-18] .

Mendoza [19] revealed that FF concentrations of growth hormone (GH), Luteinizing hormone (LH) and interleukin-1 (IL-1) are associated with faster post- fertilization development cleavage speed and better embryo morphology.

However, only few studies have highlighted how such differences in metabolically important serum parameters are reflected in the follicular fluid and how this may affect the oocyte's developmental capacity in infertile women. Studies have analyzed the free fatty acids and follicular fluid biomarkers of human and animals, using the GC-MS method [20,21]. However, few other studies applied the MALDI- MS method [22,23].

Mass spectrometry is an analytical method based on ionization and mass analysis of compounds, to determine the chemical composition and structure of molecules. This method can play a major role in the detection of biomolecules and can be one of the most effective diagnostic methods for biological and non-biological samples [24].

Liu *et al*. [23] revealed the direct analysis of tryptic digests of FF samples using MALDI-TOF MS for the investigation of biomarkers. They described a promising approach in the comparison of peptide patterns of HFF containing oocytes at different growth stages. In a study using MALDI-MS, Cataldi *et al*. [25] identified a difference in glycerol phospholipids ion abundance in the FF of women with poor ovarian response and normal women. They showed that species from five glycerol lipid subclasses (PC, PE, PI, DAG and PG) are potential biomarkers for poor ovarian responders and concluded that alterations in the glycerol phospholipids balance, might impact ovarian hormonal responses. Based on the MALDI spectra, one can predict the fatty esters in the FF and serum samples.

The MALDI-MS method is a fast and sensitive tool for identification of some biomarkers in the FF and serum samples. This procedure needs a small volume of the sample and can be providing a distribution pattern of the biomolecules in it. The present study demonstrates that MALDI-MS is a feasible tool for the diagnosis of biomolecules in the FF of infertile women. In this study, we have identified some specific peptide derivatives and fatty esters in the FF and serum samples.

MATERIALS AND METHODS

Patients

From March 2015 to July 2015 a total of 13 women, 22-

38 years of age, who undergone ICSI and embryo transfer at Fatemeh Zahra Infertility and Reproductive Health Research Center, Babol University of medical sciences, Iran**,** were included in the study.

Oocyte Retrieval

All patients had a transvaginal ultrasound (TVS) on the third day of menstruation (5 MHz probe Fokuda , Japan) to rule out ovarian cysts. Following ovarian suppression by the subcutaneous injection of 0.1 mg gonadotropin releasing hormone analog (triptorelin, Diphereline, Ipsen Pharma Biotech, France) from the midluteal phase of the preceding cycle, controlled ovarian stimulation was started with a dose of 75-150 IU recombinant human follicle stimulating hormone (rFSH, 75 IU GONAL-f, merck Serono, Germany) (HMG, Fostimon75 IU/Ampule IBSA, Switzerland) were given daily until the average diameter of the leading follicle reached 18-20 mm.

Then, intramuscular HCG (Karma, Germany) at a dose of 10,000 IU was administered. Under ultrasound guidance, oocyte retrieval was performed 36 h after HCG administration. Sample of venous blood was collected on the day of oocyte retrieval. After removal of the oocyte, FF and coagulated blood were immediately centrifuged (1000 g, min) and stored at -80 °C until analysis.

A written consent was obtained from each patient for the use of FF and the study design was approved by the Ethical Review Committee of Babol University of Medical Sciences.

MALDI-MS ANALYSES

All chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted and were used without further purification. For MALDI-MS analyses, 0.5 µl of matrix, (CHCA, 7 mg ml^{-1}, 50% acetonitrile, 0.2% TFA) was spotted and dried on a stainless steel MALDI plate (Bruker Daltonics). FF and serum samples (0.5 µl) were spotted on dried spots of CHCA. Collected spectra were summed from 500 laser shots (355 nm Nd: YAG Smart beam laser, UltraflexTreme MALDI time-of-flight (TOF)/TOF mass spectrometer, Bruker Daltonics, Bremen, Germany). All MALDI-MS experiments were performed in positive ion mode.

Table 1. The Similarity Index in Comparison of Serum and FF for Four Patients

Patients	Mf_g
K	67.12
E	76.70
D	59.48
M	46.67

COMPARISON OF THE SAMPLES

Alfassi [26] proposed a method to compare two mass spectra of the samples. The method calculates the Euclidean geometric distance between two spectra from the following equation:

$$Mf_g = \left[1 + \sum\left(\frac{u_i}{\sqrt{u_i^2}} - \frac{s_i}{\sqrt{s_i^2}}\right)\right]^{-1} \quad (1)$$

where, u_i and s_i are the *i*-th component of the intensity vector of samples *u* and *s,* respectively. In order to calculate the right geometrical distance both u and s vectors should be normalized separately, so that they have a unit length in hyperspace.

The percentage of differences between the MALDI samples are obtained from the following formula:

$$\text{Percentage of difference} = 100 \times Mf_g \quad (2)$$

Mf_g is providing from the MATLAB software according to the formula. Both FF and serum samples were compared, and Mf_g was used as the similarity index. In the other words, similarity indices were calculated with in serums and FF samples, and also between serums and FF samples, too. According to the formula 1, Mf_g is calculate for some patients and listed in Table 1.

In another point of view, for comparison between the FF and serum samples for one person, the MS intensity ratio versus molecular weight, between the FF and serum samples are calculate and plotted in Figs. 1-4. As seen, the

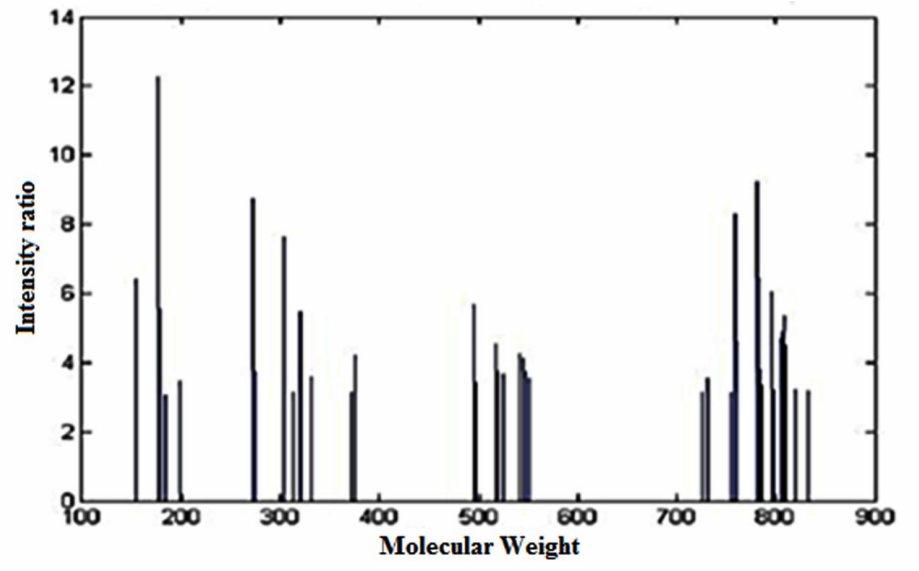

Fig. 1. The intensity ratio versus molecular weight between serum and FFsample of the patient K.

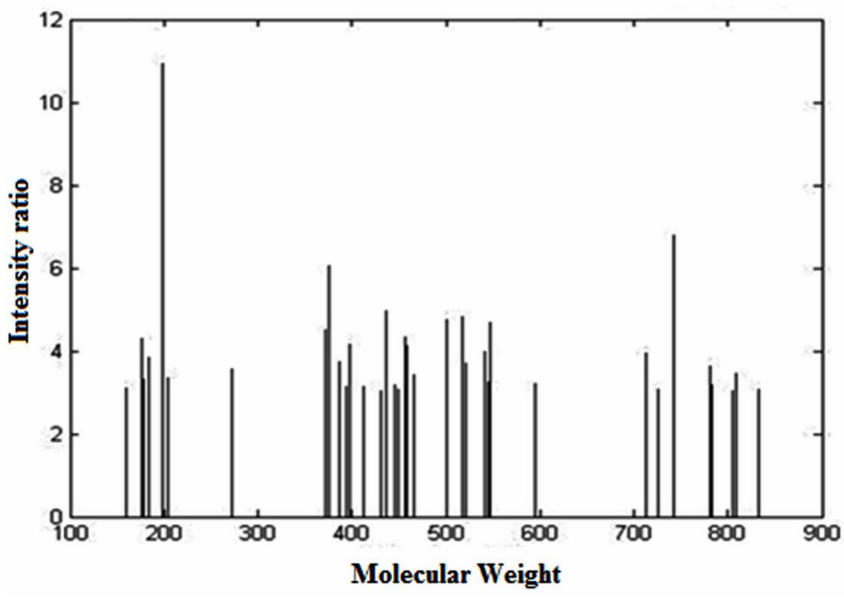

Fig. 2. The intensity ratio versus molecular weight between serum and FFsample of the patient D.

ratio of intensity above 6 is recognized, and may be informative for the comparison between the fertility situations of the person. For a molecular weight of about 200, the pattern is relatively similar in all samples, except one of them that no peak is observed in 500 (Patient M, Fig. 6). According to the data in Table 1, the calculated Mf_g for patient M is the lowest than that of the others. It seems that we can introduce Mf_g as well as the intensity ratio plots for comparison between the samples, and it can be used for rapid identification of differences between the patients.

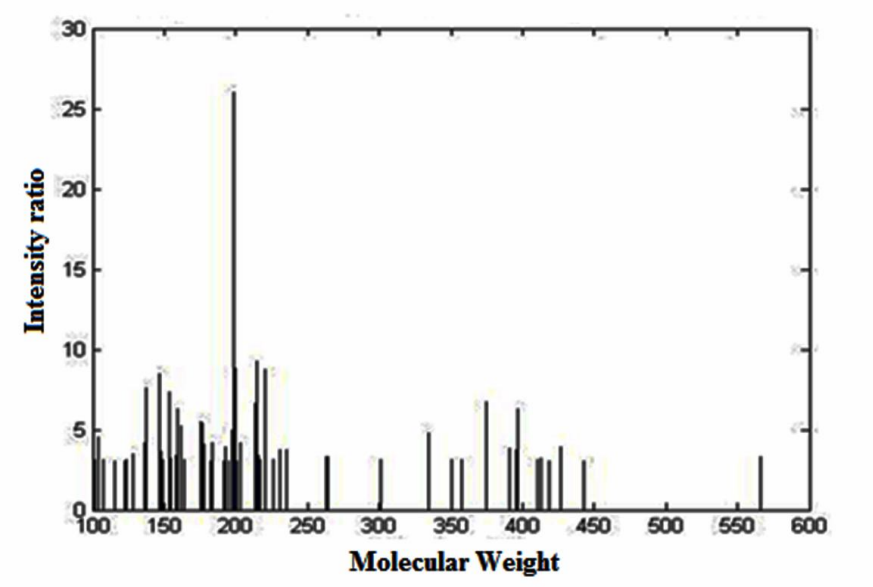

Fig. 3. The intensity ratio versus molecular weight between serum and FFsample of the patient M.

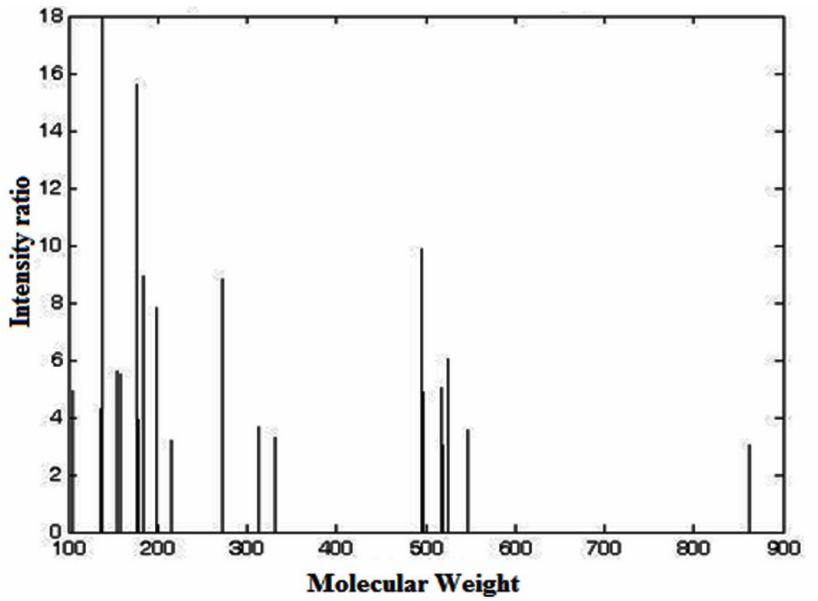

Fig. 4. The intensity ratio versus molecular weight between serum and FFsample of the patient E.

Lipid derivatives have the most important role in the fertilization process. So, more focusing on the lipid compounds of the MALDI-MS spectra (24, 25 and 28) could be beneficial. Also the difference between the spectra and the ratio intensity plots are also related more to lipid derivatives. In Table 4, there are the list of predicted derivatives according to the m/z molecular weight found in the special software (*Lipidomics Gateway*) for comparison between two different samples, FF and serum one. As seen in this table, the most important listed compounds belong to

the lipid derivatives. For the precise recognition of the reagents in the samples with the special m/z, other experimental procedure and more information are required.

RESULTS AND DISCUSSION

A total of 13 women participated in this study. They ranged from 22-38 in age and had a mean age of 29.9 ± 5.66 years. Their mean BMI was 26.2 ± 4.50 kg m^{-2} and their mean duration of infertility was 6.7 ± 6.49 years. Their infertility diagnoses included fallopian tube abnormalities (n = 2), Polycystic Ovary Syndrome or PCOS (n = 2) and male factor infertility (n = 4). One of the participants had multiple infertility factors while four had unexplained infertility. The ultrasound results showed that one patient had a small ovary. Two patients had an FSH level higher than 10 mIU ml^{-1}, and three had hyperprolactinemia (prolactin > 20 ng ml^{-1}). The average number of *oocytes* retrieved was 9.71 ± 6.33 per cycle.

Only one of the 13 patients examined had no oocytes and the other 12 had embryo transfers (ET). Three of the subjects had ongoing pregnancies, making the percentage of pregnancy in the study population 25%. No significant differences were observed in age, BMI and the cause of infertility in the women who achieved pregnancy after IVF-ICSI compared to those who did not. Nonetheless, statistically significant differences were observed in the number of oocytes.

The results showed no significant differences between the serum and follicular fluid (FF) compositions in the other patients examined. The mass spectra analysis revealed several intense signals within the mass range of m/z between 100 and 800.

Figures 1 to 3 show the fatty acid pattern in the FF samples of the three patients who became pregnant after the ET. The results suggest the lack of significant differences between the serum and follicular fluid compositions in the other patients examined. Table 1 presents the similarity index for comparing the serum and follicular fluid compositions. The Mf_g was calculated for some of the patients according to Equation 1, and listed in Table 1. The MALDI-TOF MS method was used in this study on digested HFF to generate mass spectra, and a differential peak analysis was then performed to identify the biomarker candidates.

The reasons for choosing the MALDI method are:

1) An easy preparation and initiation process,
2) Requiring a small sample size for analysis,
3) The MALDI spectra can be obtained in a short time,
4) It recognizes many compounds in a short time.

Matorras *et al.* [27] assessed the fatty acid composition of 150 fertilization-failed human oocytes from 43 infertile women in IVF cycles analyzed using gas-liquid chromatography. The majority of the fatty acids (79.22%) were saturated in this study, 38.65% were stearic acids and 32.66% were palmitic acids, while only 9.7% of the cases were unsaturated fatty acids such as oleic acid.

Janghim *et al.* [13] studied the combination of free fatty acids in the blood and follicular fluid of a group of infertile women in Washington, US. Those with a poor morphology and a poor oocyte quality had high levels of saturated fatty acids such as Palmitic and Stearic acids (P = 0.027, OR = 3.3). The total free fatty acid concentration in the follicular fluid was reported 13% of the total number of oocytes with a low quality, and 32% of all oocytes containing a total fatty acid concentration higher than 0.232 mmol ml^{-1} were reported to be unable to fertilize less than 0.232 mmol ml^{-1}. Moreover, the differentiation between the embryonic stages and cleavage in the presence of higher levels of free fatty acids decreased significantly (P = 0.05).

In a review article published in 2012, palmitic acid was reported to be the most abundant fatty acid in human follicular fluid, while stearic acid was the most common fatty acid in animal models; this finding shows that these two saturated fatty acids decrease fertility, cleavage and blastocyst. The increase in oleic and unsaturated fatty acids was found to be significantly linked to increased differentiation between the 2PN stage and the blastocyst stage, while the increase in palmitic acid was found to reduce the morula and blastocyst stages [9].

The patients who became pregnant after the micro-injection had higher levels of unsaturated fatty acids and omega-3 compared to the other samples, however, their stearic and palmitic fatty acid levels were not high. This finding is consistent with the results of other studies. The paired comparison of the patients showed a 99% similarity between *AF* and *E,* and the cause of infertility was unexplained in both cases; after the induction of ovulation,

Table 2. Similarity Index in Comparison of Serum Samples in Different Patients

Patient	K	E	D	MZ	KH	H
K	1	88.80	77.78	80.17	64.27	71.77
E		1	72.72	74.65	72.27	64.49
D			1	72.30	66.61	74.49
M				1	54.00	58.40
KH					1	70.74
H						1

Table 3. Similarity Index in Comparison of FF Samples between Patients

Patients	K	E	D	MZ	AF	A	EF	MP	M	kP	T
K	1	71.80	69.04	44.52	71.18	73.30	62.87	67.30	98.88	77.57	87.47
E		1	58.98	43.27	99.16	87.93	60.52	86.36	75.40	84.49	74.57
D			1	53.47	58.03	58.77	80.58	65.82	72.87	72.04	60.66
MZ				1	42.19	44.85	56.13	45.26	46.03	46.22	42.92
AF					1	84.95	60.11	85.45	74.56	82.73	74.19
A						1	57.16	79.70	75.90	81.99	78.26
EF							1	64.79	67.70	65.62	58.59
MP								1	71.93	92.63	71.64
M									1	81.68	87.62
KP										1	77.97
T											1

each had one oocyte (M1) and none became pregnant after the ET.

KH and *E* had more than 70% similarity, which is consistent with their clinical findings, as both had experienced about 20 years of primary infertility, and the reason for their infertility could not be explained in either of the cases. *K* and *MZ* had more than 80% similarity, and none became pregnant after the micro-injection, and no similarities were observed after assessing their clinical documents, as their causes of infertility were differed, which may be attributed to the inability to detect all the clinical factors used in the identification of infertility in the laboratory. There were probably some similarities between these two patients which caused their Mf_g number to exceed 80%. The difference in the type of infertility makes the Mf_g number different.

Given that MALDI samples were not available in the serum and follicular fluids of some of the patients, their percentage similarity could not be calculated; however, as anticipated in the other comparisons, the aforementioned samples had a high Mf_g. Tables 2 and 3 compare the patients' serum and follicular fluids.

The novelty of the present research lies in its use of a statistical model for comparing the MALDI peaks, and its introduction of a threshold level. Using the Mfg allows for a relatively fast comparison of the patients in terms of the complex MALDI peaks. There is no need for the detection of chemicals and bio-molecules in MALDI spectra. Mfg provides a number instead of investigating complex matrices in the MALDI spectra and thus accelerates the comparison of MALDI patterns. The MALDI-MS analysis was performed based on the method used for obtaining peaks from biological samples and this manuscript thus offers scientific patterns.

CONCLUSIONS

The MALDI method can contribute significantly to the detection of biomolecules and can be one of the most effective diagnostic methods used for biological and non-biological samples. The difference between serum and follicular fluid samples can be assessed from a biochemical point of view with this method. As a result, significant biochemical differences between important compounds in follicular fluid and serum can be used as an effective and powerful method of analysis. Since biomolecules in biological samples are sensitive to conventional chemical reactions during analysis, and since extraction processes are often time-consuming, exertional and little efficient, the MALDI method is a very suitable and effective alternative for achieving this purpose in very small volumes and concentrations. This method can also be used to detect the pattern of the distribution of molecules in samples. The Supplementary Information section presents the patients' fatty acid patterns and serum and follicular fluid compositions.

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CONFLICT OF INTEREST

None declared.

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