



Decoding the Proteome of In-Vitro Fertilization Ovarian Follicular Fluid for Women Over 35 Years

35 Yaş Üzeri İn-Vitro Fertilizasyon Over Follikül Sıvı Proteomların Çözümlemesi

The Proteome of In-Vitro Fertilization Ovarian Follicular Fluid

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Özet

Amaç: Proteomik teknikleri kullanılarak folliküler sıvı içeriğinin anlaşılması ve gebelik sonuçlarına etkisinin anlaşılmasına yardımcı olabilir. 35 yaş ve üstü kadınlarda folliküler sıvı proteinlerinin belirlenmesi ve dizelenmesi. **Gereç ve Yöntem:** Folliküler sıvı içeriği başarılı bir şekilde gebe kalan 11 kadın ve gebe kalamayan 10 kadından oluşan 21 kadından elde edildi. Numuneler, bir LTQ XL iyon tuzağı kütle spektrometresi ile in-line nano-sprey iyonizasyon kütle spektrometresi ile birlikte çok boyutlu kromatografi ile analiz edildi. Gebe kalan ve kalamayan gruplarda protein bileşenleri tanımlamak için PDQuest yazılım Biomarker Analizi Programı kullanılan. **Bulgular:** toplamda, 1024 protein örneği belirlendi. Belirlenen proteinler deney boyunca incelenen örnekler ile uyumlu idi. **Tartışma:** Folliküler sıvı protein listelerinin oluşturulması oosit gelişiminde önemli proteinlerin belirlenmesi ve değerlendirilmesi konusunda uygun bir başlangıç olabilir; çalışmanın sonuçları folliküler-sıvı proteom konusunda önemli bir bilgi açığını kapatabilir.

Anahtar Kelimeler

İN-Vitro Fertilizasyon; Follikül Sıvısı; Proteom

Abstract

Aim: The study of follicular fluid using proteomic techniques could provide a useful tool for understanding follicular fluid components and their effect on pregnancy outcome. The aim of the study is to identify and catalog follicular fluid proteins in women 35 years of age or older. **Material and Method:** Follicular fluid was collected from 21 couples, of which 11 couples achieved successful pregnancy and 10 couples failed to get pregnant. Samples were analyzed by multidimensional chromatography coupled with in-line nano-spray ionization mass spectrometry on an LTQ XL ion trap mass spectrometer. We used the Biomarker Analysis Program from PDQuest software to identify protein constituents in pregnant and non-pregnant groups. **Results:** In total, 1024 protein specimens were identified. The proteins identified were consistent throughout the experiment and within each of the analyzed specimens. **Discussion:** A compiled listing of follicular fluid proteins could be a potential starting point for the identification and evaluation of important proteins involved in the development of oocytes; the results of our study may fill a noticeable knowledge-gap in the understanding of follicular fluid proteome.

Keywords

In-Vitro Fertilization; Follicular Fluid; Proteome

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Introduction

It is estimated that by the year 2000, more than 200,000 babies were born worldwide after use of assisted reproductive technology (ART) [1]. As more and more women enter the workforce and achieve higher education, an apparent trend of delayed childbearing until the fourth decade of life in some communities has emerged. This emerging category of women in their late 30s to early 40s who are seeking their first pregnancy; has evidenced a decline in fertility potential, also known as sub-fertility [2]. A growing pool of evidence supports the idea that age-related sub-fertility is most likely related to oocyte senescence rather than a simple number/age correlation [3]. Therefore, the quality of retrieved oocytes could be the determining factor in using any of the reproductive technologies successfully [4]. This clarified the craving demand of methodological advances to overcome the oocyte aging process and consequences or at least anticipating the oocyte fertility potential [5]. Most practicing embryologists consider morphological assessment of oocytes as a common method to determine embryo viability during IVF cycles. However, it seems that the rely on the morphological criteria for the oocyte to predict the potential of producing a fertilized oocyte and hence embryo seems to be inaccurate. This could be attributed partially to its modest predictive power or to the inherent inter and/or intra-observer variability [6,7]. The same concepts may be applied to studies on polarizing microscopy analysis [8], and studies that evaluate the genes of granulosa cells and/or in the oocyte itself such as the use of polar body biopsy for the identification of specific molecular markers of oocyte quality [9,10]. Most of these techniques are quite complicated, require extensive experience and dedicated time to complete procedures, and consequently are not currently applicable in clinical practice.

Reasonable consideration should be given to the state of the follicular fluid surrounding the future oocyte and its biochemical composition in determining the state of follicle maturation; its quality and its subsequent potential to achieve fertilization; and, embryo development. These factors enable us to predict the success of in-vitro fertilization and likelihood of a full-term pregnancy [11,12].

Proteomic techniques involve a comprehensive quantitative descriptive study of the total protein expression and their subsequent changes in a system. These techniques offer a more focused analysis and places the protein in its biological context [13,14]. A comprehensive examination of follicular fluid through proteomic techniques which represent the link between genes-protein and physiological/pathological processes is a very attractive field of research and will provide a novel perspective for understanding the follicular fluid constituents and their effect on pregnancy outcome [15].

Cell proteins and their respective metabolites represent the by-products of cell regulatory processes; and therefore, reveal the response of biological systems to a variety of genetic, nutrient or environmental influences. Our proposed specific aims to: Identify and catalogue follicular fluid proteins for women of 35 years of age or older.

Material and Method

A prospective cohort of 165 heterosexual couples which inclu-

ding females, < 35 years of age with a diagnosis of tubal factor infertility were approached from February 2010 to June 2011 at the Nashville Fertility Center, Nashville TN and The Center for Reproductive Health, Nashville TN. Couples were recruited prior to their first in vitro fertilization (IVF) cycle. Approximately 20% of those approached (31 couples) agreed to participate in our study. Out of those that agreed to participate (21 couples) matched our inclusion criteria, and signed an informed consent document. The Institutional Review Board of Meharry Medical College, Nashville TN oversaw the study and approved both the study protocol and the informed consent document.

Two matched groups of participants were identified the first group of participants "Successful Group" (11 women) was defined as women with a proven clinically positive pregnancy result by both serum β hCG (more than 5 mIU/ml) 14 days after embryo transfer and ultra-sonography detection of fetal cardiac pulsations. A second group of participants "Failure Group" (10 women) was defined as women who failed to become pregnant after two successive negative readings of β hCG (less than 5 mIU/ml) after 14 days and 16 days of embryo transfer respectively.

Inclusion/Exclusion Criteria:

Inclusion Criteria:

- Women over 35 years of age with a diagnosis of only tubal factor infertility

Exclusion Criteria:

- Women less than 35 years of age.
- Women who have previously undergone in-vitro fertilization.
- Women diagnosed with endometriosis, diminished ovarian reserve, ovulatory dysfunction, uterine factors, male factors and unexplained factors.
- Women receiving donor semen or donor eggs, gestational carriers.

Follicular Fluid Collection: During the mid-luteal phase of the menstrual cycle, the enrolled subjects were stimulated with a long down-regulation protocol [16] using leuprolide acetate 0.1 mg (Lupron; TAP Pharmaceuticals, Deerfield, IL). Subjects then underwent a controlled ovarian hyper-stimulation with follicular stimulating hormone (FSH) (Follistim/Gonal f; MERK & CO., Inc., NJ, USA). The initial dose of FSH was based on each subject's clinical condition. Dosages thereafter, were determined and adjusted according to the subject's response. Cycle monitoring was conducted by trans-vaginal ultrasound and serial E2 hormone starting from CD3 of the stimulated cycle until the day of the hCG trigger using 10,000 IU hCG (Profasi; Serono, Rockland, MA) intramuscularly when the mean follicle diameter of ≥ 2 follicles ≥ 18 mm in diameter. Oocyte retrieval was performed approximately 36 h after hCG injection. All sample collections were performed by one of two physicians in both centers to minimize surgical variability. Oocytes and embryos were cultured in the same type of media (LifeGlobal® Media, LGGF 100). All embryos were transferred on Day 3 after assigned a unique identifying number.

Follicular fluid was obtained from the largest follicle (>18 mm) before using any flushing medium and only consisted of fluid from one follicle. This follicle was aspirated with a 17-gauge Cook needle attached to 100 mm Hg pump-operated aspirator and was the first puncture of the oocyte retrieval. After collec-

tion of the ova the remaining follicular fluid was placed into a 15-mL conical tube and centrifuged at low speed (200 g) for 15 min. Supernatant was placed in a clean storage tube, and aliquoted into 2 mL tubes, labeled with the subjects' unique identifier, and frozen at -80° Celsius till time of analysis.

Protein Digestion: Prior to the Multidimensional Protein Identification Technology (MudPIT) analysis: Samples were aliquoted into 2.5µl Allocating 2.5µL of the sample. Assuming 40µg/µL that makes 100µg of sample, Added 7.5µL of 100mM Tris. Then added 10µL of Tri-fluoroethanol (TFE). This made the total volume of 20µL; to be reduced with 10 mM TCEP (tris(2-carboxyethyl)phosphine) (Add 0.4µL of stock 0.5 M) for 30 min at room temperature. Alkylate with 20 mM Iodoacetamide (Add 0.8µL of 0.5 M) for 30 min in the dark. Add 76 µL of 100 mM Tris. Add 2µL of 1µg/µL Trypsin (2 µg). pH Checked that Should be about 8-9. Incubate overnight in a 37°C oven. Removed from oven. Split the sample evenly between 2 tubes (50 µg per tube). Freeze in -80°C freezer until ready to load on MudPIT column.

MudPIT (Multidimensional Protein Identification Technology): Samples were analyzed by multidimensional chromatography coupled in line to nano-spray ionization mass spectrometry on an LTQ XL ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Biphasic columns were packed in house into Self-Pack (Pico Frit®) columns (uncoated; 100 µm inner diameter; tip, 15 µm) with 12 cm of reverse-phase resin (BioBasic, C18, 5 µm, 300 Å) and 6 cm of polysulfoethyl, a strong cation exchange (SCX) matrix (5 µm, 300 Å, Poly LC). Samples were initially loaded onto a micro-precolumn cartridge (5 µm, 100 Å, LC Packing) at a flow rate of 20 µl/min and desalted for 3 min with buffer A (0.1% formic acid in HPLC-grade water). Samples were resolved from the peptide trap and loaded onto the SCX part of the biphasic column using a 40 min gradient of 0–40% buffer B (0.1% formic acid in HPLC-grade acetonitrile) followed by a 10-min gradient of 40–98% buffer B.

The flow rate was adjusted to 250 nl/min with a splitter. Peptides were step-eluted from the SCX phase onto the reverse phase of the biphasic column using 2-min salt pulses of 7.5, 15, 20, 25, 30, 35, and 40% buffer C (500 mm ammonium acetate, 5% acetonitrile, and 0.1% formic acid), respectively, or a 7-min gradient of 40–100% buffer C. Peptides were resolved from the reverse phase using a 60-min gradient of 2–25% buffer B followed by a 40-min gradient of 25–55% buffer B. Tandem spectra were acquired using a data dependent scanning mode in which one full MS scan (m/z 400-2000) was followed by 9 MS-MS scans. Tandem spectra were searched against the human subset UniProt human Protein knowledgebase (version 2013_03, with 150600 entries) using the SEQUEST algorithm (U.S. patents 6,017,693). All proteins identified by less than two peptides were eliminated, resulting in false positive rates of <1%. The Sequest output was also filtered using IDPicker using a false positive ID threshold (default is 0.05 or 5% false positives) based on reverse sequence hits in the database.

Results

Protein samples and quantification

Our aim was to identify and profile the follicular fluid proteome in women over 35 years old. To-date, there are no publis-

Table 1. The clinical characteristics and ultrasound measurements of the study group.

Characteristics	Study Group (N=21)
Mean age (year) ± SD	38.50 ± 1.5
BMI (kg/m2) ± SD	23.0 ± 3.69
Basal FSH (IU/mL) ± SD in previous cycle	6.97 ± 2.1
Mean ovarian volume ± SD	5.2 ± 0.03
Mean Endometrial thickness (mm) ± SD in previous cycles	0.82 ± 0.25

Table 2. The hormonal profile and the stimulation cycle characteristics in the study group

Hormonal Profile US Measurements	Study Group (N=21)
CD3 LH (IU/L)	3.63 ± 2.02
CD3 E2 (ng/ml)	16.83 ± 10.01
CD3 Progesterone (ng/mL)	1.04 ± 0.07
Starting dose of FSH	453.84 ± 41.28
Total dose of FSH	4967.45 ± 874
Treatment duration (days)	10.0 ± 1.50
No of retrieved oocytes	7.0 ± 3.0
No. of MII oocytes	6.0 ± 2.0
No of fertilized eggs	5.0 ± 2.0

hed works which provide an itemized comprehensive listing of over 35 years old whole protein content of the follicular fluid. We identified 1024 consistent proteins in samples of both study groups; Table (3) and Appendix (I).

Table 3. A sample representing the profiling of the follicular fluid proteome of women over 35 years old (Full list at appendix I).

Q6PIL8_HUMAN	Q68CN4_HUMAN
Q6MZV7_HUMAN	IGHG2_HUMAN
Q6PYX1_HUMAN	Q6MZX7_HUMAN
Q6MQZ6_HUMAN	Q5EBM2_HUMAN
Q6N094_HUMAN	Q6N030_HUMAN
A8K008_HUMAN	Q56917_HUMAN
Q6GMX6_HUMAN	Q96K68_HUMAN
Q7Z351_HUMAN	Q8NCL6_HUMAN
Q5EFE5_HUMAN	Q6MZW0_HUMAN
Q6N096_HUMAN	Q6ZW64_HUMAN
Q6N097_HUMAN	Q6N092_HUMAN
Q6GMX0_HUMAN	Q96DK0_HUMAN
Q0KKI6_HUMAN	A4F255_HUMAN
Q8TCD0_HUMAN	Q6N090_HUMAN
Q6P558_HUMAN	Q9NPP6_HUMAN
Q7Z3Y4_HUMAN	Q6MZV6_HUMAN
Q5EFE6_HUMAN	Q7Z374_HUMAN
Q6N095_HUMAN	Q6ZVX0_HUMAN
Q6MZU6_HUMAN	Q6P089_HUMAN
Q6N093_HUMAN	Q6MZX9_HUMAN

Discussion

In this study, we analyzed human follicular fluid whole proteome in women 35 years old of age or older undergoing IVF/ICSI in order to profile/catalog the whole follicular fluid protein content within this age group given the noticeable gap in knowledge regarding this particular age group.

We chose to conduct this study among women of this age group

(over 35 years old) because it is within this reproductive decade in which women typically show the poorest outcomes in achieving satisfactory ovulation, furthermore, this age group of women seems to have inherited the reputation of decreased oocyte quality and hence are less likely to achieve pregnancy. To the best of our knowledge there are no data to-date that has cataloged the whole proteome of women among this age group. The clinical characteristics and ultrasound measurements of our participants showed clearly that the un-intended homogeneous randomly selection of the study sample. Same findings that shown regarding the hormonal profile and the stimulation cycle characteristics

The finding that directly correlates with number of embryos amenable for transfer to the uterus which in turn affects the pregnancy rate after in vitro fertilization-embryo transfer (IVF-ET) [17]

In order to discuss profiling/cataloging of the whole follicular fluid human proteome, we used the collected follicular fluid after centrifugation and proper protein digestion, then we analyze the collected fluid using multidimensional chromatography coupled in line to nano-spray ionization mass spectrometry technique as previously described. All experimental procedures were planned with the intent to analyze the follicular fluid in a direct way with minimum of sample treatment not to alter the follicular fluid proteomic pattern to avoid protein loss and or artifacts aiming to identify all the possible unknown follicular fluid protein content.

Using the LTQ-XL, 1024 proteins were identified in each analyzed sample (Appendix I). Interestingly, the pattern was consistent throughout the experiment and within each of the analyzed specimen. In conducting a literature search for relevant articles, regarding identified follicular fluid proteome we used the following key words: proteomics, follicular fluid, human follicular fluid, human proteome using the search engines: PubMed, The National Library of Medicine, and the National Institutes of Health.

Several publications on proteomic analyses using human follicular fluids are available. Mukherjee et al., recently published a study using the Liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique in an effort to broaden their understanding as to the process of folliculogenesis. They identified 480 proteins within their follicular fluid samples, of which 320 had not been previously described [13]. In addition, Missmer et.al, performed a proteomic evaluation of follicular fluids through two-dimensional polyacrylamide gel electrophoresis followed by the Liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique to compare between normo-responders versus poor-responders in matched pairs of IVF patients; however, samples were collected among women 32 years of age or less. In their analysis, they described 11 potential protein candidates for prediction of oocyte normal response [18]. Furthermore, Hanrieder et al., identified 73 unique proteins (significance level of 95%), including acute phase proteins [19], while using an alternative approach of bottom-up technique. Conclusive results identified approximately 69 proteins of which 32 had not been previously reported [20]. Consequently, Demmers and his group utilized a setup based on extensive pre-fractionation of proteolytic peptides and nanoflow reversed-

phase LC-MS/MS and identified 246 specific proteins, the majority of which were involved in coagulation- and immune response pathways [13]. In 2006, Angelucci, Ciavardelli et al. and his group used the MALDI-TOF-MS analysis (Matrix-assisted laser desorption-ionization/ time-of-flight mass spectrometer) to assign 183 Human follicular fluid/plasma matched spots and 27 human follicular fluid/plasma unmatched spots. A large number of acute-phase proteins, were identified in human follicular fluid in relatively high concentrations. For well over a decade, there has been curiosity in studying the follicular fluid protein through the proteomic analysis, [21].

After reviewing these reports and others, we noticed that our resultant data was similar to the afore mentioned studies, with the data gathered from our study providing a complete complement concerning the full (to the best of the available techniques and results) follicular fluid profiling. However, our study is novel in that it provides the first data set of more than 1000 different proteins which catalogs the whole follicular fluid proteome. Comparing the available different proteomic techniques and previously published results of follicular fluid proteomic analyses we concluded that by using the Liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique we were able to identify a greater number group of proteins at very low concentrations.

Competing interests

The authors declare that they have no competing interests.

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