
GYNAECOLOGY

Mass Spectrometry-based Serum Proteome Pattern Analysis in Prophylactic Oophorectomy

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ABSTRACT

Background: Mass spectrometry (MS) is an analytical chemistry technique that measures the mass-to-charge ratio (m/z). A plot of the ion signal in term of the m/z ratio is called mass spectrum. Matrix-assisted laser desorption/ionization (MALDI) is an ionization technique used in MS. The technique determines the mass of a molecule such as a peptide from within a heterogeneous mixture. Besides two well-known reproductive functions, producing of oocytes and reproductive hormones, the ovaries would play a vital role in other undiscovered aspects.

Aim: To study role of ovary reflecting from serum proteomic pattern changes before and after prophylactic oophorectomy

Materials and Methods: A prospective cohort study with 20 premenopausal women diagnosed benign gynecologic condition but not ovarian abnormalities and scheduled for prophylactic oophorectomy was conducted. Blood was drawn and serum was then centrifuged and kept at -80 degree Celsius before and at 6 weeks after oophorectomy. All serum specimens were prepared and then analyzed by MALDI-TOF.

Results: Age, BMI and duration of follow up were 49.30 ± 1.59 years, 24.56 ± 4.07 kg/m² and 45.70 ± 6.13 days, respectively. A comparison of mean mass spectrum intensity between before and after oophorectomy showed that five m/z 1882, 2467, 8766, 8923 and 9136 were statistically significant different (7.96 and 16.93, $P < 0.001$, 2.42 and 8.57, $P = 0.01$, 4.12 and 6.04, $P = 0.005$, 16.06 and 18.40, $P = 0.002$, 8.54 and 12.59 $P = 0.016$, respectively).

Conclusions: MS revealed a significant change of serum proteomic pattern from women undergone prophylactic oophorectomy. The protein identification study is currently ongoing in order to identify the ovarian function.

Keywords: mass spectrometry, serum proteomic, prophylactic oophorectomy

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Introduction

The ovary, ovum-releasing organ, functions as both gonad and endocrine gland. In terms of endocrine gland, ovary secretes estrogen, testosterone, progesterone, inhibin, and others substances. As women age, the ovarian function and fertility decrease. There is also a parallel express in short and long-term consequences⁽¹⁻³⁾.

The changes during peri- and menopausal period can tremendously affect women's quality of life. Physical and psychological disturbances were reported in more than 80% of women⁽⁴⁾. Physical symptoms include vasomotor symptoms, hot flashes and night sweats, insomnia, vaginal dryness, and urinary incontinence. The decreased sex hormone levels cause vasomotor symptoms, vaginal dryness and vaginal atrophy⁽⁴⁻⁶⁾. Several psychological symptoms related menopause include difficulty concentrating, depression, anxiety and sadness^(7, 8). Most women have at least one of these symptoms while they pass the menopause period^(7, 9). The endocrine changes observable with reproductive aging and especially the entry into the menopausal transition appear to result from the progressive decrease in ovarian follicle numbers⁽¹⁰⁾.

Prophylactic or preventive bilateral salpingo-oophorectomy (BSO) is the surgical removal of the ovaries aimed to reduce risk of ovarian cancer, benign ovarian tumor, pelvic pain or painful endometriosis as well as breast cancer⁽¹¹⁾. However, study of prophylactic BSO showed adverse consequences after surgery such as an increased risk of cardiovascular disease⁽¹²⁾ and salt-sensitive hypertension⁽¹³⁾. Moreover, oophorectomy in pre-menopausal women before 45 years old increases risk of osteoporosis⁽¹⁴⁾. Psychomotor disturbances such as dementia, cognitive defect, and neurological abnormalities specifically Parkinson's disease had been reported more often in surgical

menopause than in natural menopausal women⁽²⁾. The sudden onset of hot flashes, poor quality sleep, headaches, depression and urogenital atrophy are related to the hypo-estrogenic state caused by oophorectomy⁽¹⁵⁾.

The large-scale study of protein by proteomic profiling determines gene expression reflecting the global function of the cell. The advantages of the technique include ability to analyze protein expression levels, study post-translational modifications as well as protein-protein interactions⁽¹⁶⁾. Mass spectrometry (MS) is an emerging analytical technique to study of whole proteins and peptides by weighing mass of a molecule. Principle of MS is the conversion of sample or mass into ions and measurement of their mass-to-charge ratio (m/z). Each MS consists of three main components; i) the source, which converts ions from a sample, ii) a mass analyzer, which separates peptide ions according to their m/z ratio, and iii) a detector which measures ion abundance for each m/z ion and generates a mass spectrum^(17,18). Matrix-assisted laser desorption/ionization (MALDI) using laser to fragment proteins, peptides, and polymers into ions is one of several MS methods. A mass analyzer that is frequently coupled with MALDI MS is called time-of-flight (TOF). It separates the ions of mass by accelerating the ions through the electric field under the same potential. The lighter ions but the same ion charge travel faster than the heavy ones. The advantages of MS over traditional methods of protein study such as western blot analysis or immunohistochemistry include its robustness and high throughput^(17,19).

Besides two well-known reproductive functions, production of oocytes and reproductive hormones, the ovaries would play a vital role in other undiscovered aspects. In the present study we conducted the research to answer the hypothesis that the changes of proteomic,

peptide profiling, will represent the ovarian function by comparing the pattern of peptide before and after oophorectomy. Therefore, the objective of this study was to determine serum proteomic pattern comparing between before and after surgical prophylactic oophorectomy.

Materials and methods

The prospective cohort study was conducted at the Department of Obstetrics and Gynecology Ramathibodi Hospital from 30 April 2013 to 30 June 2014. The study was approved by the Ethical Committee on Human Rights Related to Researches Involving Human Subjects at the Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Thailand.

The outcomes were serum proteome pattern and signal intensity or relative abundance of ions comparing between before and after surgical prophylactic oophorectomy. A mass spectrometer determines the mass of a molecule by measuring mass-to-charge (m/z) ratio. MS works by ionizing protein, polypeptides, and chemical compounds and the results display on mass spectrum. The m/z represents mass of a molecule divided by charge number. A mass spectrum is a vertical bar graph, in which each bar represents an ion having a specific m/z and height of the bar indicates the signal intensity.

Subjects

A pilot study ($N=10$) was conducted to test the equipment and the data acquisition method. The mean mass intensity of peptide ions at 1882 from the pilot study was used to calculate the sample size. Mean mass intensity of M/Z 1882 before and after prophylactic salphingo-oophorectomy were 8.53 (+4.45 SD) and 13.6 (+6.69 SD), respectively. Assuming that there was significant proteomic change in bi-direction; therefore, 16 subjects were required to obtain an 80% of power with a 5% level of significance. After adding 20% to the sample size to compensate for data loss, 20 subjects were required.

The inclusion criteria were premenopausal women age 45-55 years old who were scheduled for

hysterectomy and prophylactic bilateral oophorectomy, had regular menstrual period and willing to participate in the study. The indications for uterine removal were benign gynecologic disorders; for example, uterine fibroid, adenomyosis and, endometrial hyperplasia. Exclusion criteria were gynecologic cancer, ovarian pathology, oral hormonal use such as oral contraceptive pill, cyclic progestin and oral estrogen therapy within 3 months before surgery, Depot medroxyprogesterone acetate (DMPA) or GnRH analogue within 3 months before the surgery. We also excluded the women who either received blood transfusion or donated their blood during the previous 4 months. The participants who could not complete follow up were also excluded. After an informed consent was obtained, a serum sample was collected from each participant before the operation and at 6 weeks after the operation. Baseline demographic data including age, underlying disease, BMI, FSH level before and after operation and duration of follow up were recorded.

Serum samples

Five milliliter blood samples were collected in a 6 ml pro-coagulation Tube (IMPROVACUTER), incubated for 30 minutes at room temperature to allow clotting, and then centrifuged at 1,000 g for 10 minutes to separate the serum which was then stored at -80 degree Celsius.

Sample preparation and peptide extraction for MALDI-TOF MS analysis

Sample were purified by 2 methods using ZipTip with C4 resin and ZipTip with C18 resin in order to separate peptide with mass range between 3,000-60,000 Da and 700-7,000 Da, respectively. All purified processes were done according to the manufacturer's protocols. Briefly, equilibration of the ZipTip pipette tip for sample binding was done by aspirating the wetting solution 10 ul and dispensing to waste for ten times. Secondly, binding and washing the peptides or proteins was performed. The peptides were bound to the equilibrated ZipTip pipette tip by performing 20-30 aspirate-dispense cycles of the entire sample. 10 μ L of

washing solution was aspirated into the ZipTip pipette tip, and then dispensed to waste. Finally, the bound peptides were eluted by firstly aspirating 5 µL of elution solution through the ZipTip pipette and then dispensed the solution out. The aspiration-dispense were done for 30 cycles. The eluted purified samples were collected for further analysis.

Analysis of purified peptide by MALDI-TOF

The 1 µl purified sample was mixed with 9µl HCCA matrix solution (70% ACN and 0.1% TFA). This mixture was then deposited onto position on the 600 µm Anchor Chip target plate (Bruker Daltonics). This essential step removed the majority (up to 80%) of albumin and other abundant high-molecular weight proteins from the serum samples

MALDI-TOF-MS screening of differential peptides

In order to identify serum peptide mass range between 3,000-60,000 Da, we used MALDI-TOF MS with linear mode with using 1,000 laser shots. However, MALDI-TOF MS with reflectron mode with using 200 laser shots were used in order to identify serum peptide mass range between 700-7,000 Da. Mass calibration

was performed after every eight samples using protein Calibration Standard I (Bruker Daltonics). Spectra were analyzed with FlexControl 3.4 and FlexAnalysis 3.4 software. Spectra comparison between pre-operative and post salpingo-oophorectomy for individual participant was performed using ClinproTools V. 3.0

Statistical and Data Analysis

The statistical analysis was done by SPSS version 16. We determined the distribution of data by Kolmogorov-Smirnov test. Either pair T test or Wilcoxon signed-rank was used to compare two related samples. A probability value of 0.05 was considered statistically significant.

Results

Twenty participants were recruited. The demographic characteristics were demonstrated in Table 1. There were 7 participants with hypertension, 3 with dyslipidemia, 2 with thyroid disease, 1 with diabetes and asthma. The indications for hysterectomy and prophylactic oophorectomy were adenomyosis (9 cases) and leiomyoma (11 cases). The mean duration of follow up was 45.7 (+6.13 SD) days.

Table 1. Characteristic of the patients.

Characteristics (n=20)	Mean ± SD
Age (y)	49.3 ± 1.59
BMI (kg/m ²)	24.56 ± 4.07
FSH level (mIU/ml)	
- Pre operative	11 ± 7.97
- Post operative	72.06 ± 24.5
Duration of follow up (d)	45.70 ± 6.13

MALDI-TOF MS profiling of serum samples revealed 16 m/z peaks which were significantly different between before and after oophorectomy in each individual participant. Of these, five peptide peaks m/z 1882, 2467, 8766, 8923 and 9136 were statistically significant different after 6 week oophorectomy among

the whole group. All these five peptide peaks were up regulated. (Table2)

A comparison between the number of women with underlying disease and healthy women focusing on each peptide change showed no statistically significant difference. (Table 3)

Table 2. MALDI-TOF differential peptide peaks between before and after BSO. Mean intensity of 5 m/z peptide peak showed statistically significant different comparing between before and after oophorectomy. All these peaks were up-regulated.

m/z	Before BSO		After BSO		P
	Mean	SD	Mean	SD	
1882	7.96	3.87	16.93	7.96	< 0.001
2467	2.42	1.41	8.57	4.68	0.010
8766	4.12	2.49	6.04	3.70	0.005
8923	16.06	7.05	18.40	8.84	0.002
9136	8.54	3.45	12.59	7.01	0.016

Table 3. Participants distribution according to 5 significant differential m/z ions. Comparison of serum peptide profiling between women with underlying diseases and healthy women showed that differential m/z ions were not statistically different among both groups.

Significant differential peaks (M/Z)	No. of women with underlying disease (N%)	No. of healthy women (N%)	P
1882 Up regulated	25	35	0.65
1882 Unchanged	25	15	
2467 Up regulated	15	5	0.58
2467 Unchanged	35	45	
8766 Up regulated	30	20	0.64
8766 Unchanged	15	25	
8766 Down regulated	5	5	
8923 Up regulated	25	10	0.35
8923 Unchanged	25	40	
9136 Up regulated	10	15	0.77
9136 Unchanged	30	30	
9136 Down regulated	10	5	

P-value from Chi-square

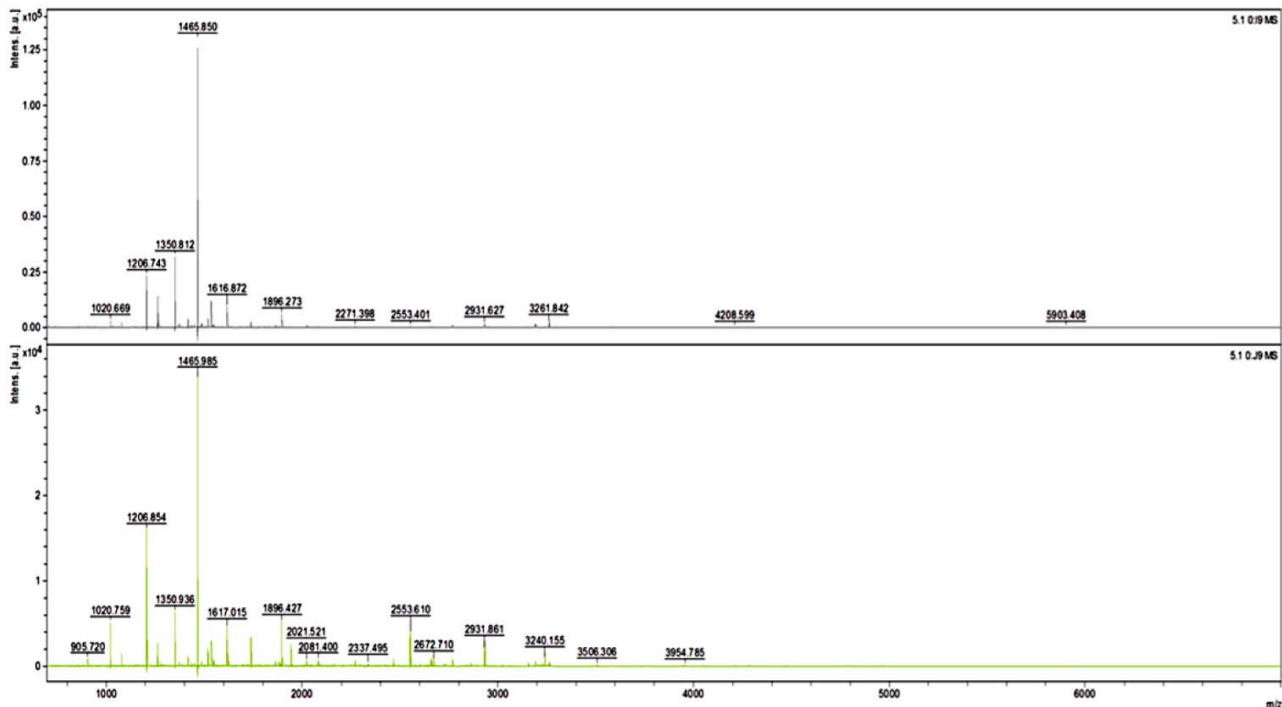


Fig. 1. MALDI-TOF differential peptide intensity peak between before and after BSO

Discussion

MALDI-TOF MS, a novel proteomic technique, is able to identify the proteins and polypeptides that are undetectable by the conventional methods, and thus being considered more advantageous than two-dimensional electrophoresis and the conventional techniques used in protein research. MALDI-TOF MS could detect proteins in a selective way which reduces the complexity of the protein contents for the samples to be detected. Being characterized by the rapid detection, easy operation, and small sample consumption, the new technique could analyze both qualitative and quantitative changes in the protein contents of the serum samples that were obtained from the patients⁽¹⁷⁾.

We have shown statistically significant different changes of 5 ion peaks at m/z 1882, 2467, 8766, 8923 and 9136 before and after BSO. None of these ion peaks showed the significantly different proportion between normal participants and the ones with underlying disease; therefore, the underlying medical

diseases demonstrated no effect on the proteomic change.

After a literature review, the peptide with a molecular mass at 8923 would probably be complement component 3a (C3a) which is one of the proteins formed by the cleavage of complement component 3⁽¹⁷⁾. Component C3 is the most abundant complement protein in the serum (~1.2 mg/ml). Its main source is the hepatocyte⁽²²⁾ but macrophages⁽²³⁾ and, with less efficiency, endothelial cells⁽²⁴⁾ can also secrete this crucial complement component. The C3 molecule belongs to the α 2-macroglobulin family, whose members like C4 and the proteinase inhibitor α 2-macroglobulin, contain an internal thioether group and plays an important role in the immune response. The half life of C3 is approximately 1 hour⁽²⁴⁾. Not only C3a triggers immune response but it also plays an important role in chemotaxis, and anaphylatoxin. Additionally, there is a literature reporting the association of C3 with pre-existing severe coronary artery disease and new vascular events in women⁽²⁵⁾. Consequently, the present

study proposes the probability of supplemental crucial ovarian function as well as the proclaimed increased risk of various diseases after oophorectomy.

No data are available for the other peptides m/z 1882, 2467, 8766, and 9136. In order to figure out type and function of these peptides or proteins, their peptide sequences are required to identify peptide/protein against protein database. The study is ongoing investigated by other MS techniques and approaches. These unknown peptides may play significant roles in reproductive function.

Limitation of MALDI-TOF MS for protein and peptide mass determination is that it can detect certain range of peptide mass. Although no upper analytical limit for MALDI-TOF MS, it provides the optimal result for m/z between 1,000 to 5,000 Da⁽²⁰⁾ since resolution and mass accuracy decline if m/z increases. Therefore, if peptides and proteins of interest are larger than 4,000 Da such as FSH and LH, molecular weight is 35,500 and 30,000 da, respectively; different separation methods to purify peptides/proteins and MS approach would probably play roles⁽²¹⁾.

The mean duration of follow up was 45.7 days. The longest half life of recognized plasma protein is 15-19 days which belongs to albumin. Consequently, the conclusion can be drawn that all proteins detected postoperatively were newly secreted after oophorectomy.

The present study is the pioneer work to determine the serum proteomic pattern changes before and after surgical prophylactic oophorectomy. The result has revealed that there were five proteomic changes after prophylactic oophorectomy. However, the novel data is not completed. Lack of clinical benefit is the drawback of the present study. Further researches are required to identify other specific peptides and proteins. The clinical application may be obviously seen if we know the significant change of the protein/peptide caused from oophorectomy.

Conclusion

There were significant proteomic changes after prophylactic-oophorectomy by MALDI-TOF MS analysis. The outcome may reflect the essential and the other undiscovered aspects of the ovary.

Acknowledgement

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การศึกษาซีรัมโปรตีนโดยเครื่อง Mass spectrometry ในสตรีผู้ที่เข้ารับการผ่าตัดรังไข่แบบป้องกัน

ลัดนา พร้อมวัฒนาพันธุ์, อารีย์พรรณ โสภณสฤษฏ์สุข, ญัฐพงศ์ อิศรางกูร ณ อยุธยา

วัตถุประสงค์และวิธีการ: ศึกษาซีรัมโปรตีนในสตรีวัยก่อนหมดประจำเดือนที่เข้ารับการผ่าตัดรังไข่แบบป้องกัน เปรียบเทียบก่อนผ่าตัดและหลังผ่าตัด

วัสดุและวิธีการ: การวิจัยทำในสตรีที่ได้รับการวินิจฉัยภาวะโรคทางนรีเวช ซึ่งได้รับการผ่าตัดรังไข่แบบป้องกัน ที่โรงพยาบาลรามาริบัติระหว่าง วันที่ 30 เดือนเมษายน 2556 ถึง วันที่ 30 เดือนมิถุนายน 2557 จำนวน 20 ราย เจาะเก็บเลือด จำนวน 10 มิลลิลิตร จากสตรีในกลุ่มการศึกษา แบ่ง 5 มิลลิลิตร เพื่อส่งตรวจ Follicular stimulating hormone, FSH อีก 5 มิลลิลิตร ปั่นแยกซีรัม เก็บแช่ตู้เย็นที่ -80 องศาเซลเซียส เมื่อได้ตัวอย่างครบจำนวน ทำการตรวจหาโปรตีนในเลือด โดยใช้ MALDI-TOF mass spectrometry คำนวณค่าเฉลี่ย Peak intensity ของโปรตีนที่มวลต่อประจุ (m/z) นำมาเปรียบเทียบความแตกต่างของความเข้มข้นมวลต่อประจุ (differential ions peak) ก่อนและหลังการผ่าตัดด้วย Kolmogorov-Smirnov test, pair T test หรือ Wilcoxon signed-rank

ผลการวิจัย: พบว่าอายุเฉลี่ย, ดัชนีมวลกาย, ระยะห่างเฉลี่ยของการเจาะเลือดก่อนและหลังผ่าตัด ของผู้เข้าร่วมวิจัย คือ 49.30 ± 1.59 ปี, 24.56 ± 4.07 kg/m² และ 45.70 ± 6.13 วัน ตามลำดับ เมื่อเปรียบเทียบค่าเฉลี่ยความเข้มข้นมวลต่อประจุก่อนและหลังตัดรังไข่แบบป้องกัน พบว่ามีมวลต่อประจุ 5 ชนิด ที่มีความแตกต่างอย่างมีนัยสถิติ ดังนี้ มวลต่อประจุที่ 1882, 2467, 8766, 8923 และ 9136 ตามลำดับ (7.96 and 16.93, $P < 0.001$, 2.42 and 8.57, $P = 0.01$, 4.12 and 6.04, $P = 0.005$, 16.06 and 18.40, $P = 0.002$, 8.54 and 12.59 $P = 0.016$)

สรุป: MS แสดงผลความแตกต่างของซีรัมโปรตีนของกลุ่มผู้ป่วยที่เข้ารับการผ่าตัดรังไข่แบบป้องกัน ซึ่งการศึกษาต่อไประหว่างดำเนินการเป็นภาระของโปรตีนที่มีความสำคัญในการสะท้อนหน้าที่ของรังไข่