Salivary Proteome Profile of Women during Fertile Phase of Menstrual Cycle as Characterized by Mass Spectrometry

Ganesan Saibaba[1,2], Durairaj Rajesh[1,3], Subramanian Muthukumar[4], Ganesan Sathiyanarayanan[5], Archunan Priya Aarthy[6], Govindaraju Archunan[1]*

[1]Department of Animal Science, Bharathidasan University, Tiruchirappalli, [4]Department of Biotechnology, School of Chemical and Biotechnology (SCBT), SASTRA Deemed University, Thanjavur, [5]School of Life Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, [6]Division of Obstetrics and Gynecology, Rabindra Nath Tagore Medical College, Udaipur- 313001, Rajasthan, India, [2]Department of Animal Science, Agricultural Research Organization, Volcani Center, Rishon LeTsiyon-7528809, Israel, [3]Department of Molecular Biology and Chemical Communication, Research Institute in Semiochemistry and Applied Ethology (IRSEA), 84400 Apt, France

Abstract

Objectives: Ovulation is such a critical physiological process that its noninvasive detection based on salivary constituents has several advantages in humans. Hence, the present study is proposed to identify the ovulatory-specific proteins in saliva in order to detect ovulation phase.
Materials and Methods: Samples were collected from women volunteers. The procedure adopted was approved by the Institutional Human Ethical Committee (DM/2014/101/38), Bharathidasan University. The saliva samples were collected from thirty healthy female volunteers, with a prior written consent. One-way analysis of variance was used to calculate protein concentration and band intensity using SPSS 16 software (SPSS Inc., Cary, NC, USA). The salivary protein expression pattern during different phases of menstrual cycle was analyzed using gel-based high resolution-liquid chromatography-mass spectrometry/mass spectrometry and matrix-assisted laser desorption ionization-time of flight/time of flight. Further, bioinformatics tools were adopted to annotate the proteins identified at various phases of menstrual cycle.
Results: As many as 530 proteins showed up in the saliva during ovulatory phase, whereas there were only 251 proteins identified during postovulatory phase. The functional annotation of salivary proteins revealed that the proteins got assigned to the class of "extracellular proteins" which are concerned with regulatory functions. The 16 unique and/or differentially expressed protein spots appeared during ovulatory phase, among which Cystatin-S, Prolactin-inducible protein, Cystatin-A, Cystatin-SN, BPI fold-containing family A member 2, Alpha-tubulin N-acetyltransferase 1, Carbonic anhydrase-6, Protein LEG1 homolog, Hemoglobin subunit beta, and Pancreatic alpha-amylase were identified.
Conclusion: Total salivary proteome profile has been listed with respect to various phases of menstrual cycle. Among the protein listed, Cystatin-S offers a biomarker protein and/or indicator of ovulatory phase. However, extensive validation is required before arriving to a candidate bio-marker protein.

Keywords: Biomarker, cystatin, gel electrophoresis, liquid chromatography-mass spectrometry/mass spectrometry, ovulation, protein, saliva, sodium dodecyl sulfate polyacrylamide gel electrophoresis

INTRODUCTION

The term saliva stands for secretions from the major (i.e., submandibular, sublingual, and parotid) and minor salivary glands, which contains molecules from blood and salivary epithelial protein combined.[1] The secretion of saliva is regulated by the autonomic nervous system via signal transduction systems that couple receptor stimulation to ion transport and protein secretory mechanisms.[2] Many salivary biomolecules arrive from the blood through passive intracellular diffusion and active transport or extracellular

Address for correspondence: Dr. Govindaraju Archunan, Department of Animal Science, Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India.
E mail: garchu56@yahoo.co.in

Supplementary material available online
Access this article online

Quick Response Code:

Website:
www.e-gmit.com

DOI:
10.4103/GMIT.GMIT_78_20

How to cite this article: Saibaba G, Rajesh D, Muthukumar S, Sathiyanarayanan G, Aarthy AP, Archunan G. Salivary proteome profile of women during fertile phase of menstrual cycle as characterized by mass spectrometry. Gynecol Minim Invasive Ther 2021;10:226-34.

ultrafiltration.[3] The levels of salivary components vary in respect to the spectrum of oral and general health. For example, low levels of lysozymes[4] and presence of lactoferrin[5] were observed in saliva under a condition of dental caries. In the beginning of menstruation and during ovulation, the protein content of saliva increases considerably, which turns out to be a rich source of nutrient to bacteria, the count of which may increase during menstruation and ovulation.[6] As diagnostic fluid, saliva and urine offer many advantages over blood, which include simple, noninvasive, and less protein concentration.[7]

Most of the animals have limited and short fertile period, but the external indications and attractiveness synchronize with ovulation maximize the chance of successful fertilization.[8,9] In human, females do not show corresponding cyclical changes that would be an indicator of ovulation, therefore it was felt essential to develop a method to identify the time of ovulation during the reproductive cycle.[10] The time of ovulation in humans is associated with the fertile phase of menstrual cycle. In mammals, it is generally accepted that many reproductive processes such as ovulation, menstruation, implantation, and parturition are linked with inflammation.[11] As the consequence of these processes, there is upregulation of inflammatory mediators, which include cytokines, growth factors, and lipid mediators.[12] Similarly, in response to inflammation in an ovary, there are specific pro- or anti-inflammatory cytokine/protein expressions in the body fluids.[13] Still there is not yet a reliable noninvasive modality to detect the time of ovulation. Hence, a method for accurate prediction or detection of the fertile phase during menstrual cycle has enormous significance in promoting or controlling fertility. Saliva, for the reasons mentioned above, has long been speculated to possess one or more biomarkers indicting important events in the reproductive cycle. Thus, we adopted a proteome-based approach to detect salivary biomarkers for fertile phase. The complex protein mixtures were analyzed qualitatively by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE),[14] which suggested that SDS-PAGE is an adequate technique to segregate the protein composition from the human saliva.[15] However, in the recent decade, two-dimensional (2D) gel-electrophoresis is used to separate complex protein mixtures of saliva based on different modifications and isoforms of the particular protein and recent advancements in mass spectrometry techniques are adopted to better facilitate protein biomarker identification in saliva.[3] In our preliminary study,[16] the ovulatory-specific proteins in saliva have been mapped. Hence, it was thought that the identification of an ovulatory-specific protein in saliva would help for future application in regard to ovulation detection. Therefore, the present study was directed towards identifying the proteins in human saliva in relation to phases in the menstrual cycle by adopting 1D and 2D gel

electrophoresis followed by mass spectrometry to discern the various proteins, and to map the ovulatory-specific proteins.

MATERIALS AND METHODS
Volunteers' information and ethical statement
Samples were collected from women volunteers, and the procedure adopted was approved by the Institutional Human Ethics Committee (DM/2014/101/38), Bharathidasan University, Tiruchirappalli, India. The saliva samples were collected between 8.00 and 9.00 AM from 30 healthy female volunteers (age, mean = 24, range = 19–30), with a prior written consent. The volunteers were instructed not to consume food and/or soft drink for 10 h before the sample collection. The volunteers were also asked to brush the teeth 30 min before sample collection of saliva so as to prevent contamination.

Sample collection and process
The saliva sample was collected by spitting method. The duration of collection of saliva was about 10 min and the saliva secretion over the 1st min was discarded. The collected samples were kept in an ice box and brought to the laboratory without any time delay. The samples were centrifuged at 16000 ×*g* for 15 min to remove insoluble materials and cells, if any. The samples were stored at −80°C until further analysis. The saliva samples were segregated among three phases, viz., preovulatory (day 6–12), ovulatory (day 13 and 14) and postovulatory (day 15–26), according to the pattern of salivary hormones and fern pattern analysis, as was done in our previous study.[16]

Protein precipitation and estimation
The salivary proteins were concentrated by trichloroacetic acid (TCA)-acetone precipitation method.[17] The samples were mixed with TCA: Acetone (TCA-20% W/V; Acetone-90% V/V) in 1:1 ratio and 20 mM dithiothreitol (DTT) then incubated overnight at −20°C. After incubation, the samples were centrifuged at 5000 ×*g* at 4°C for 30 min. The pellets were washed twice with ice cold acetone by centrifugation at 5000 ×g at 4°C for 30 min. Finally, the pellets were air-dried and re-suspended in UTC (6 M urea, 3 M thiourea, and 8% CHAPS) buffer. The protein concentration was determined adopting the modified protocol of Bradford.[18]

One-dimensional – Gel electrophoresis
To resolve the salivary proteins, SDS-PAGE was carried out on 12% gel and 5%–15% gradient gel (Bio-Rad) by adopting the modified method of Laemmli.[19] The salivary protein preparation from each volunteer (30 µg) was thoroughly mixed with 1x sample buffer (50 mM Tris-Cl [pH 6.8], 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 100 mM β-mercaptoethanol) and kept for 1 min at 100°C for complete denaturation of proteins.

Two-dimensional – Gel electrophoresis

Protein samples were mixed with an equal volume of UTC buffer (6M urea, 3M thiourea, 8% CHAPS, 100 mM DTT, and 2% IPG buffer (GE, Amersham), and incubated for 30 min in ice. The content was then diluted to the required volume using rehydration buffer (7M urea, 2M thiourea, 4% CHAPS, 0.5% ampholytes, 50 mM DTT, 1% IPG buffer (GE, Amersham), and 0.004% bromophenol blue). The strips (IPG Strips-pH 3-10NL) were then focused in IPGphor III after 16 h of passive rehydration. Consecutively, the strips were subjected to reduction and alkylation. For reduction, the strips were incubated in SDS-equilibration buffer I (6 M urea, 50 mM Tris-Cl, 30% glycerol, 2% SDS, 0.004% bromophenol blue, and 1% DTT) for 15 min in a gel rocker. For alkylation, the strips were incubated in SDS-equilibration buffer II (6 M urea, 50 mM Tris-Cl, 30% glycerol, 2% SDS, 0.004% bromophenol blue, and 2.5% iodoacetamide) for 15 min in a gel rocker. The strips were then placed on top of 12% polyacrylamide gel and sealed with overlay of 0.5% agarose solution. The electrophoresis conditions were 0.5 W for 45 min and 2 W for 5–6 h until the tracking dye reached the bottom of the gel plate. After electrophoresis, the gels were stained according to Dyballa and Metzger.[20] Digital images of 2D-gels were acquired using ChemiDoc™ XRS imaging system (Bio-Rad) with internal calibration.

High-resolution-liquid chromatography-mass spectrometry/mass spectrometry

The 1D protein spots were analyzed using 6550 i-Funnel quadrupole-time-of-flight liquid chromatography-mass spectrometry/mass spectrometry (QTOF-LC-MS/MS) coupled with 1260 Infinity Nano pump and 1260 Cap pump along with 1260 Chip-cube (Agilent Technologies). The peptides were fractionated along with Solvent A (0.1% formic acid in milliQ water) and Solvent B (90% acetonitrile +0.1% formic acid +10% milliQ water). The proteins were identified by mass comparison with the SWISS-PROT database entries. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository[21] with the dataset identifier PXD004511. The article has been registered as preprint at Research Square (10.21203/rs.2.13524/v2).

Matrix-assisted laser desorption ionization-time of flight/time of flight analysis

2D protein spots were processed using an automated gel cutter and processor (Shimadzu, Xcise™). The gel spots were washed and destained with 50% ACN and 50 mM NH4HCO3, and subjected to in-gel digestion with 30 μL of trypsin solution for 2 h at 37°C. ZipTips (C18) were wetted and conditioned with 50% ACN and 0.05% TFA and 0.1%

TFA, respectively. Cleaved peptides bound to the C-18 resin were desalted using 0.1% TFA. The peptides were then eluted and spotted with 2.5 μL of Solvent (5 mg/mL of CHCA in 50% ACN and 5 mM of NH_4CHO_3) onto a 384-well matrix-assisted laser desorption ionization (MALDI) plate. Finally, samples were identified using MALDI-time of flight/time of flight (MALDI TOF/TOF) (AB Sciex 4800). The acquired mass spectra were processed using DataExplorer® software, and the mono-isotopic peptide masses were assigned and utilized for the database search. The proteins identification was searched against *Homo sapiens* protein sequence in MASCOT database.

Functional annotation

The salivary proteins of the ovulatory phase were further analyzed to decipher their cellular location, molecular function and biological process by STRAP 1.5 online database.[22]

Molecular functional ontology

The prominent salivary proteins showing up during the ovulatory and postovulatory phases were further classified based on their cellular component, biological process, and molecular function in the UniProt database. The GO entries were used to depict the percentage of proteins through Interproscan analysis in BLAST2GO. The retrieved GO ID's of protein entries, with their enrichment values, were used to generate a scatter plot by adopting Reduce Visualize Gene Ontology (REViGO) web server.

Statistical analysis

The protein concentration and band intensity values corresponding to ovulatory, preovulatory, and postovulatory phases were represented as mean ± standard deviation and analysed using one-way analysis of variance using SPSS 16 software (SPSS Inc., Cary, NC, USA).

RESULTS

Validation of ovulatory phase

It is necessary to assess the ovulatory phase after appropriate screening of the menstrual cycle. Those women who exhibited normal 28–30 day cycle length through the 5 or more cycles were chosen to be the volunteers, and then saliva sample was collected. The ovulatory phase was confirmed from direct fertility marker, namely, status of the follicle adopting ultrasonography [Supplementary Figure 1a], and biophysical fertility markers such as basal body temperature and fern pattern in saliva [Supplementary Figure 1b].[23]

One-dimensional – Gel electrophoresis

The total proteome was fractionated by 12% SDS-PAGE [Figure 1a]. The protein profile of ovulatory phase saliva was compared to that of postovulatory phase. Put together,
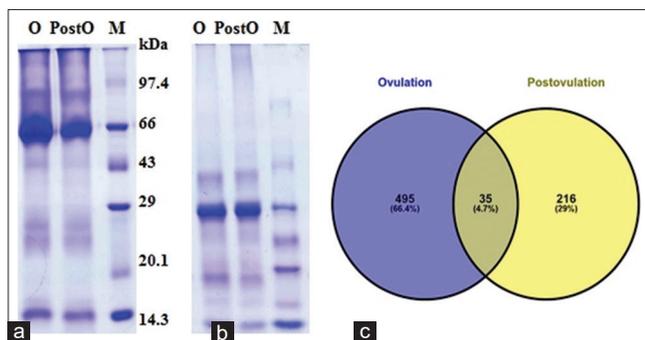
Figure 1: (a) Salivary protein profile in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. O – Ovulatory phase, PostO – postovulatory phase, M – Protein reference markers. (b) Salivary protein profile in gradient gel (5%–15%). O – Ovulatory phase, PostO – Post-ovulatory phase, M – Protein reference markers. (c) Total identified salivary proteins. In ovulatory phase 530 proteins showed up whereas 251 proteins were observed during postovulatory phase

during the two phases, a total of 12 distinct protein bands appeared in the CBB-stained gels. Further, the proteins pattern was verified in the gradient gel [Figure 1b] and it was found to be similar to that revealed in 12% SDS-PAGE. Among the various bands 66, 43, and 14.5 kDa were in the highest intensity during the ovulatory phase compared to postovulatory phase.

High-resolution-liquid chromatography-mass spectrometry/mass spectrometry analysis

In order to identify the salivary proteins, the SDS-PAGE protein profiles of each phase were excised equally into six separate pieces, which were individually subjected to trypsin digestion followed by mass spectrometry analysis. In total, 781 proteins were identified combining ovulatory and postovulatory phases of menstrual cycle. During ovulatory phase 530 proteins were found, whereas 251 proteins were found during postovulatory phase. Among these proteins, 35 were common to both ovulatory and postovulatory phases [Figure 1c].

Ovulatory-specific proteins

Several functionally important ovulatory phase-specific proteins were identified and listed. Table 1 shows the list of thirty functionally important proteins related to reproduction during ovulatory phase. Cystatin-S, Disintegrin, Metalloproteinase domain-containing protein 7, TANK-binding kinase 1 and Exportin 7 appeared to be predominant, having more number of peptide identifications [Table 1].

Gene ontology

The GO entries were mapped using GI number of proteins in UNIPROT database. After refinement of datasets, it was seen that saliva of ovulatory and postovulatory phases contained 154 and 117 GO entries, respectively. The gene ontology clearly revealed more UNIPROT entries and greater percentage of annotation during the ovulatory phase than the other phases.

Functional annotation

The salivary proteins identified during the ovulatory and postovulatory phases were subjected to functional annotation using STRAP online database. The results revealed that the ovulation phase had more number of GO terms and greater percentage of annotations than during the post-ovulation phase [Supplementary Figure 2].

Molecular functional ontology

The GO entries were used to depict the percentage of proteins at the molecular functional level through Interproscan analysis in BLAST2GO. The cloud tag image confirmed that the proteins identified in saliva of ovulatory phase are essentially those with binding property and catalytic activity. Particularly, the ovulatory phase salivary proteins showed up higher number of binding proteins (41.6%) and metal ion binding proteins (16.1%) compared to other phases [Supplementary Figure 3]. Further, the molecular functional network was constructed using GO terms of salivary proteins. The integrated network map revealed the proteins identified during ovulatory phase have roles on glycoprotein binding, ion binding, and immunoglobulin binding, and receptor activity [Figure 2a]. Additionally, molecular network analysis revealed the major interaction between the identified proteins.

Scatter plot for binding proteins

Overall, molecular function analysis expounded most of the proteins thus identified to possess binding property. The cluster of proteins during ovulatory phase corresponds, to the receptor-, protein complex-, peptide-, and GABA-binding and MAP kinase activities as depicted in yellow bubbles. The GPCR-, heat shock-, hyaluronic acid-, lipid phosphatase-, and ligase-binding, and motor activity proteins are shown in linear green bubbles. GABA-A receptor activity and receptor activity proteins are denoted in orange bubbles [Figure 2b]. These proteins might be having some functional significance during ovulatory phase. Toll-like receptor-, fatty acid-, and lipid-binding proteins, and protein transporter activity proteins during postovulatory phase are shown as separate clusters [Figure 2c].

Two-dimensional – Gel electrophoresis

The salivary protein expression profiles were analysed during preovulatory, ovulatory, and postovulatory phases of menstrual cycle by 2D gel electrophoresis. The protein spots were present in the pI range 4–7 and the molecular weight between 14 and 97 kDa [Figure 3a-c]. The gel analyses carried out during different phases using PDQuest software (Bio-Rad) showed nearly 50 spots in each phase. Particularly, there were more spots during ovulatory phase, and many of them had higher densities compared to the other phases. Additionally, we found that the quantitative differences in the spots were high in 14.5–21 kDa region of ovulatory phase proteins compared to the other phases. Though the ovulatory phase had a different protein expression compared

## Table 1. List of functionally important salivary proteins identified during ovulatory phase of menstrual cycle.

Swiss-Prot acc. no.[a]	Name	Function[b]	pI[c]	MW[d]	Length[e]
P01036	Cystatin-S	Strong inhibitor	4.83	14.18	141
Q9H2U9	Disintegrin and metalloproteinase domain-containing protein 7	Role in Reproduction	5.92	65.09	754
Q9UHD2	TANK-binding kinase 1	Regulating inflammatory responses	6.32	83.64	729
Q9UIA9	Exportin 7	Export of proteins	5.91	123.9	1087
P29122	Proprotein convertase subtilisin/kexin type 6	Endoprotease activity	7.96	106.4	969
Q14031	Collagen alpha-6(IV) chain	Major structural component	9.31	163.8	1691
Q5FYB1	Arylsulfatase I	Displays arylsulfatase activity at neutral pH	8.82	64.03	569
P02768	Serum albumin	Binding capacity for water, Ca (2+), Na(+), K(+), fatty acids, hormones, bilirubin and drugs	5.92	69.36	609
Q5XXA6	Anoctamin-1	Chloride conductance	8.76	114.07	986
Q9Y5E6	Protocadherin beta-3	Potential calcium-dependent cell- adhesion protein	4.88	86.77	796
P28566	5-hydroxytryptamine receptor	G-protein coupled receptor	9.09	41.68	365
Q496J9	Synaptic vesicle glycoprotein 2C	Positively regulates vesicle fusion	4.92	82.34	727
Q8WXI7	Mucin-16	Protective, lubricating barrier	-	-	22152
Q969D9	Thymic stromal lymphopoietin	Antimicrobial peptide in the oral cavity	9.75	18.14	159
Q8TE04	Pantothenate kinase 1	Role in the physiological regulation	7.51	64.33	598
P50453	Serpin B9	Protease inhibitor	5.61	42.40	376
Q8TD43	Transient receptor (TRPM4)	Mediates transport of monovalent cations	8.49	134.3	1214
Q3YEC7	Rab-like protein 6	May enhance cellular proliferation	5.11	79.54	729
Q13733	Sodium/potassium-transporting ATPase subunit alpha-4	Hydrolysis of ATP coupled with the exchange of sodium and potassium ions	6.23	114.1	1029
Q8N587	Zinc finger protein 561	Transcriptional regulation	9.09	55.19	486
Q02790	Peptidyl-prolyl cis-trans isomerase FKBP4	Interaction with heat-shock protein 90 (HSP90)	5.35	51.80	459
P28325	Cystatin-D	Cysteine proteinase inhibitor	6.71	16.08	142
Q8WYA1	Aryl hydrocarbon receptor nuclear translocator-like protein 2	Component of the circadian clock	7.01	70.88	636
O75177	Calcium-responsive transactivator	Transcriptional activator which is required for calcium-dependent dendritic growth	5.96	42.99	396
Q9H6R6	Palmitoyltransferase ZDHHC6	Palmitoylates calnexin (CALX), which is required for its association with the ribosome translocon complex and efficient folding of glycosylated proteins.	8.81	47.66	413
Q05JX5	Apolipoprotein B	Ion binding	8.41	46.48	384
P16112	Aggrecan core protein	Resist compression in cartilage	4.10	250.1	2399
Q5EK51	Lactotransferrin	Anti microbial response	8.56	78.32	711
Q8N4F0	Bactericidal/permeability-increasing protein-like 1	Lipid binding	8.82	49.17	458
O00154	Acyl-CoA thioesterase 7	Hydrolysis of acyl-CoAs	8.85	41.79	380
P12273	Prolactin-induced protein	Protein binding	8.26	16.57	146
P23284	Peptidylprolyl isomerase B	Accelerate the folding of proteins	9.42	23.74	216
P23280	Carbonic anhydrase 6	Unknown	6.51	35.36	308
P49895	Deiodinase	Hormone biosynthetic process	8.90	28.92	249
Q59FP8	Neogenin	Unknown	6.56	123.9	1130

[a,e]Proteins having at least one identified peptide in ovulation phase saliva are listed with their Swiss-Prot/TrEmbl accession numbers and length. [b]Functions were retrieved using the STRAP online database bioinformatics resource. [c,d]Theoretical pIs and monoisotopic molecular weights were calculated using the Swiss-Prot website

to pre- and post-ovulatory phases, 16 spots were identified as unique and highly expressed during this phase [Supplementary Figure 4a]. These protein spots expressions were also validated in the Melanie 3D viewer [Supplementary Figure 4b].

Identification of proteins from selected spots

The differentially expressed 16 spots were identified as: Cystatin-S, Prolactin-inducible protein, Cystatin-A, Cystatin-SN, BPI fold-containing family A member 2,
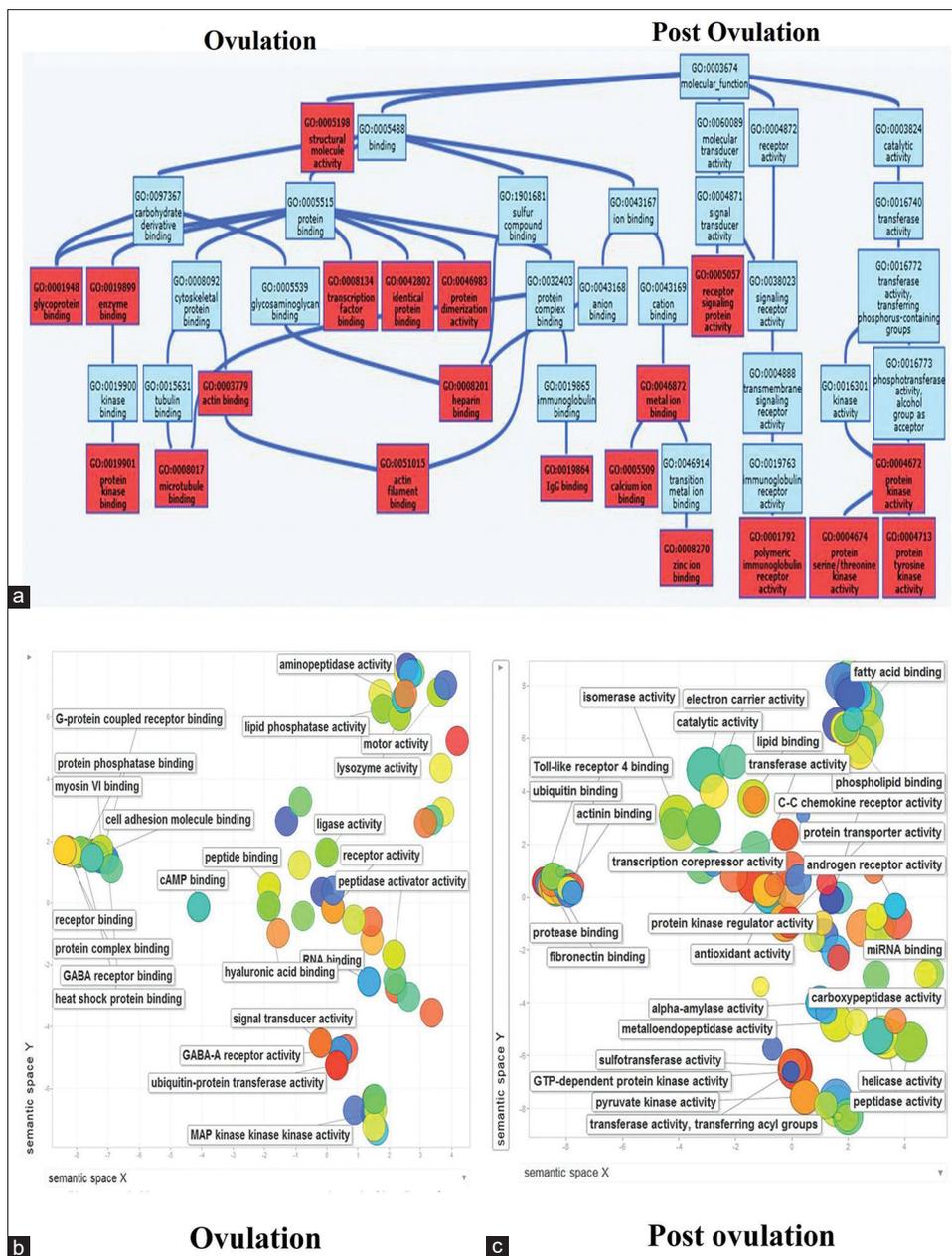
Figure 2: (a) Molecular functions of proteins. The scatter plots show the clusters of proteins with their functional activity in (b) ovulatory and (c) postovulatory phase. The ovulatory plot has the higher number of binding and lesser activity protein compared to postovulatory phase

Alpha-tubulin N-acetyltransferase 1, Carbonic anhydrase 6, Protein LEG1 homolog, Hemoglobin subunit beta, and Pancreatic alpha-amylase. The spots 1, 3, 4, 7, and 10 were identified as Cystatin-S and these spots might be isoforms/variants of Cystatin-S proteins. Notably, spot 1 identified as cystatin-S family protein having 71% sequence coverage with eight peptides matches [Figure 3d and e].

DISCUSSION

Saliva is an important diagnostic biological fluid, which reflects the physiological as well as biochemical changes in the body.[24] Human saliva has been subjected to proteomic analysis by extensive proteomic technique, namely, 2D gel-electrophoresis followed by MALDI-TOF/MS and Q-TOF/MS[25] as well as comparative proteomic analysis on intra-and inter-person variability of whole saliva using LC/ESI-TOF/MS.[26] Even though, detailed salivary proteomic studies during menstrual cycle adopting the 2D-based mass spectrometry have not been conducted. However, very recently studies have been carried out to comprehensively catalog the salivary proteome with regard to cellular localization, biological processes and molecular
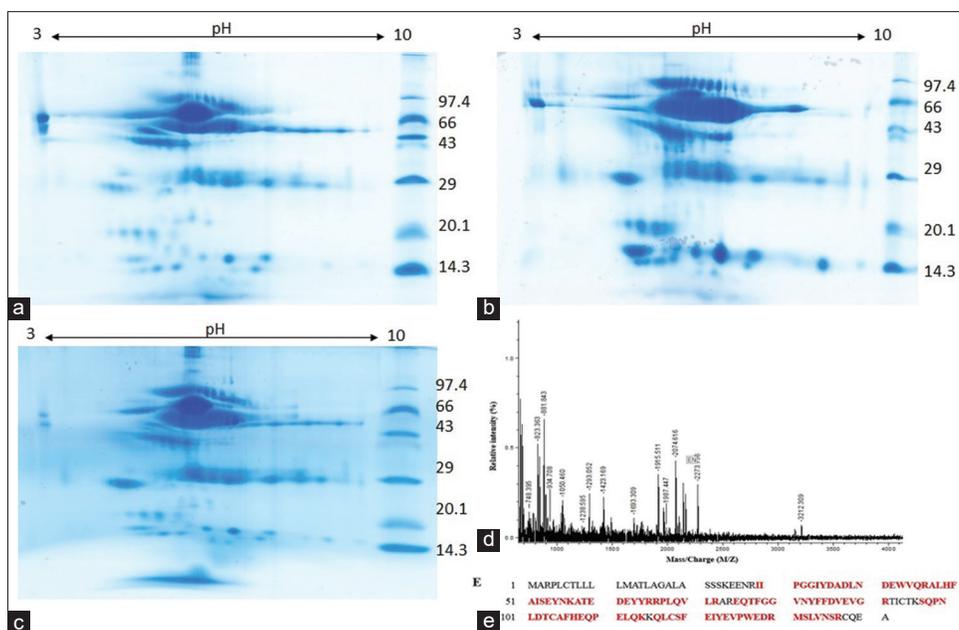
Figure 3: The two-dimensional gel electrophoresis of salivary proteins (a) preovulatory phase, (b) ovulatory phase, (c) postovulatory phase. The 11 cm IPG strips were used with 3–10 pH range, and gel run with the 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. (d) Two-dimensional protein spot 1 was subjected to in-gel tryptic digestion, and the spectrum was collected form matrix-assisted laser desorption ionization-time of flight mass spectrometry/mass spectrometry . The number of the mass spectrum gives precise m/z (M + H) values for detected peptide ion signals. (e) Single letter coded of cystatin-S sequence was obtained from mascot search. The matched 71% sequence coverage is highlighted in bold red color

functions.[27] In our previous study, we have reported that the 14.5 kDa protein band is consistently present and we projected it as the ovulatory phase-specific protein. Analysis of this band revealed that the Cystatin-S expression was significantly higher during the ovulatory phase, which was further validated adopting immunoblotting, confirming that Cystatin-S is the predominant protein during ovulation.[16]

In the present study, a complete proteomic catalogue on human saliva has been mapped with as much as 781 proteins during various phases of menstrual cycle. Among, 495 proteins are ovulatory-specific, and 216 proteins are postovulatory-specific, and 35 proteins were common in both phases. Many proteins reported in human saliva have been previously identified in human in different physiological conditions. The functionally important proteins associated with reproduction such as Cystatin-S, Disintegrin and metalloproteinase domain-containing protein 7 (ADAM7), TANK-binding kinase 1, Anoctamin-1, Carbonic anhydrase 6, and so on, appeared during ovulatory phase. More specifically, Carbonic anhydrase is present during all phases of animals exhibiting estrous cycle. Indeed, there is a significant expression of Carbonic anhydrase in cattle, camel, and goat.[28] In the human, recent studies have confirmed that secretions of specific peptides/proteins are differentially expressed in the pediatrics compared to adults.[29]

The functional annotation of the identified proteins showed that these proteins are associated with binding property and

regulatory function. In addition, these extracellular proteins would play an important role during ovulation. It is to be noted that proteins having binding property are widely present along with volatiles in body fluids and facilitate chemical communication during the estrus phase.[30,31] A more recent report showed that the binding proteins are abundant during estrus compared to the other phases in estrous cycling mammals.[32] The binding proteins may have a significant role in increasing the stability of other proteins.[33]

Several salivary biomarkers such as lactoferrin, beta-2-microglobulin, and cystatin for Sjögren's syndrome, C-erbB-2, and epidermal growth factor for breast cancer,[34] and Lactoferrin for periodontitis and type 2 diabetes mellitus[35] have been listed using MS-based proteomic techniques. The 2D gel electrophoresis (2-DE) is capable of providing for better proteins separation (based on both charge and mass) and therefore, expounds a candidate biomarkers. In most of the studies, 2DE is used as the first step for protein separation, followed by tandem MS (MS/MS). The whole saliva separated by isoelectric focusing showed most distinctive proteins at different pIs.[36] The outcome of the present study strongly agrees with the previous reports for the IEF separation between the pI 4 and 7. Recently, the 2DE analysis in periodontitis patients evidenced 15 altered spots out of 128.[37] Likewise, the present study revealed that 16 spots out of 62 were altered during the ovulatory phase of menstrual cycle. Since the composition of salivary proteins is

influenced by physiological and environmental factors, they have the potential to access and monitor the diseases and physiological status.[24,38] Indeed, the differences in protein expression have been well documented in pathological conditions such as cystic fibrosis, dental caries, and periodontitis.[39,40] A comparative salivary proteomic study was undertaken between goats and sheep,[28] and goats and cattle.[41] Likewise, salivary proteomics is a promising tool for the discovery of biomarkers for various diseases. Currently, researchers are interested in developing biochemical-based and/or protein-, peptide-based marker from saliva for the detection of ovulatory phase in the human.

Recent proteomic platforms have showed about 3000 differentially expressed proteins and peptides in human saliva, many of which are of microbial origin.[42] Similarly, in the present study most of the proteins present in saliva are antimicrobial and defensive proteins. The proteins around 14.5 kDa, such as Cystatin-S, Prolactin-inducible protein, Cystatin-A, Cystatin-SN, BPI fold-containing family A member 2, Alpha-tubulin N-acetyltransferase 1, Carbonic anhydrase-6, Protein LEG1 homolog, Hemoglobin subunit beta, Pancreatic alpha-amylase are express at high levels during ovulatory phase. Among these proteins, Cystatin-S protein was highly expressed as five isoforms falling in different pI ranges during ovulatory phase. Further, the expression of Cystatin-S affirms the previous studies.[16] Likewise, Cystatin-A and cystatin isoforms were reported in gingival crevicular fluid in periodontal patients and chicken egg white during embryogenesis, respectively.[43,44] It is suggested that salivary glands respond to inflammation stimuli to secrete more Cystatin-C into saliva[43] and it has been considered as a biomarker candidate of renal function.[45] Alterations of serum Cystatin C was considered as early marker for hyperthyroidism, cancer, renal function in diabetic patients, and cardiovascular diseases.[46-49] Cystatin S is a promising tumor biomarker for early cancer diagnosis and treatment evaluation.[50] In the present study, the variations in cystatin-S expression indicate that physiological changes influence the protein secretion in saliva. Hence, the specific expression of Cystatin-S in saliva during the ovulatory phase would lead to bring up an important biomarker for ovulation detection in human.

Conclusions

The protein expression patterns in human saliva were analysed during different phases of menstrual cycle. Total salivary proteome profile is listed, and as many as 530 proteins appeared during ovulatory phase as compared to 251 proteins of the postovulatory phase. The unique and differentially expressed protein spots during ovulatory phase were identified as follows: Cystatin-S, Prolactin-inducible protein, Cystatin-A, Cystatin-SN, BPI fold-containing family A member 2, Alpha-tubulin N-acetyltransferase 1, Carbonic anhydrase-6, Protein LEG1 homolog, Hemoglobin subunit beta, Pancreatic alpha-amylase are highly expressed during ovulatory phase. Among the protein listed, Cystatin-S offers as a biomarker protein and/or indicator of ovulatory phase. Further study to characterize Cystatin-S to bring up a suitable indicator to detect ovulatory phase in human has been initiated.

Conflicts of interest

There are no conflicts of interest.

References

1. Humphrey SP, Williamson RT. A review of saliva: Normal composition, flow, and function. J Prosthet Dent 2001;85:162-9.
2. Dodds MW, Johnson DA, Yeh CK. Health benefits of saliva: A review. J Dent 2005;33:223-33.
3. Lee YH, Wong DT. Saliva: An emerging biofluid for early detection of diseases. Am J Dent 2009;22:241-8.
4. Mass E, Gadoth N, Harell D, Wolff A. Can salivary composition and high flow rate explain the low caries rate in children with familial dysautonomia? Pediatr Dent 2002;24:581-6.
5. Azevedo LF, Pecharki GD, Brancher JA, Cordeiro CA Jr, Medeiros KG, Antunes AA, *et al.* Analysis of the association between lactotransferrin (LTF) gene polymorphism and dental caries. J Appl Oral Sci 2010;18:166-70.
6. Prout RE, Hopps RM. A relationship between human oral bacteria and the menstrual cycle. J Periodontol 1970;41:98-101.
7. Pfaffe T, Cooper-White J, Beyerlein P, Kostner K, Punyadeera C. Diagnostic potential of saliva: Current state and future applications. Clin Chem 2011;57:675-87.
8. Martinez AR. Prediction and detection of the fertile phase of the menstrual cycle: An overview. Adv Contracept 1997;13:131-8.
9. Baird DT. Prediction of ovulation, biophysical, physiological and biochemical coordinates. In: Jeffcoate SL, editor. Ovulation Methods for its Prediction and Detection. Chichester, USA: John Wiley & Sons; 1983. p. 1-17.
10. Albertson BD, Zinaman MJ. The prediction of ovulation and monitoring of the fertile period. Adv Contracept 1987;3:263-90.
11. Goswami B, Rajappa M, Sharmaand M, Sharma A. Inflammation: Its role and interplay in the development of cancer, with special focus on gynecological malignancies. Int J Gynecol Cancer 2008;18:591-9.
12. Jabbour HN, Sales KJ, Catalano RD, Norman JE. Inflammatory pathways in female reproductive health and disease. Reproduction 2009;138:903-19.
13. Saibaba G, Archunan G. Does salivary protein(s) act an ovulation indicator for women? A hypothesis. Med Hypotheses 2015;84:104-6.
14. Westermeier R. Electrophoresis in Practice. 3rd ed. Weinheim: Wiley-VCH; 2001.

15. Schwartz SS, Zhu WX, Sreebnyl LM. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of human whole saliva. Arch Oral Biol 1995;40:949-58.

16. Saibaba G, Rajesh D, Muthukumar S, Sathiyanarayanan G, Padmanabhan P, Akbarsha MA, *et al.* Proteomic analysis of human saliva: An approach to find the marker protein for ovulation. Reprod Biol 2016;16:287-94.

17. Gehrke CW. Total Protein Extraction for IEF and 2-D SDS-PAGE; 2006. Available from: http://proteomics.missouri.edu/protocols.php. [Last accessed on 2016 Nov 23].

18. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.

19. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.

20. Dyballa N, Metzger S. Fast and sensitive colloidal coomassie G-250 staining for proteins in polyacrylamide gels. J Vis Exp 2009;30:1431.

21. Vizcaíno JA, Deutsch EW, Wang R, Csordas A, Reisinger F, Ríos D, *et al.* ProteomeXchange provides globally coordinated proteomics data submission and dissemination. Nat Biotechnol 2014;32:223-6.

22. Bhatia VN, Perlman DH, Costello CE, McComb ME. Software tool for researching annotations of proteins: Open-source protein annotation software with data visualization. Anal Chem 2009;81:9819-23.

23. Alagendran S, Saibaba G, Muthukumar S, Rajkumar R, Guzman RG, Archunan G. Characterization of salivary protein during ovulatory phase of menstrual cycle through MALDI-TOF/MS. Indian J Dent Res 2013;24:157-63.

24. Hofman LF. Human saliva as a diagnostic specimen. J Nutr 2001;131:1621S-5S.

25. Huang CM. Comparative proteomic analysis of human whole saliva. Arch Oral Biol 2004;49:951-62.

26. Millea KM, Krull IS, Chakraborty AB, Gebler JC, Berger SJ. Comparative profiling of human saliva by intact protein LC/ESI-TOF mass spectrometry. Biochim Biophys Acta 2007;1774:897-906.

27. Helmerhorst EJ, Oppenheim FG. Saliva: A dynamic proteome. J Dent Res 2007;86:680-93.

28. Lamy E, da Costa G, Santos R, Capela E Silva F, Potes J, Pereira A, *et al.* Sheep and goat saliva proteome analysis: a useful tool for ingestive behavior research? Physiol Behav 2009;98:393-401.

29. Castagnola M, Scarano E, Passali GC, Messana I, Cabras T, Iavarone F, *et al.* Salivary biomarkers and proteomics: Future diagnostic and clinical utilities. Acta Otorhinolaryngol Ital 2017;37:94-101.

30. Vanmuylder N, Evrard L, Daeleman P, Van Reckand J, Dourov N. Expression of heat shock proteins in salivary gland tumors. Immunohistochemical study of HSP27, HSP70, HSP90, and HSP110: Apropos of 50 cases. Ann Pathol 2000;20:190-5.

31. Achiraman S, Archunan G. Urinary proteins and pheromonal communication in mammals. Indian J Exp Biol 2002;40:1077-8.

32. Muthukumar S, Rajkumar R, Rajesh D, Saibaba G, Liao CC, Archunan G, *et al.* Exploration of salivary proteins in buffalo: An approach to find marker proteins for estrus. FASEB J 2014;28:4700-9.

33. Caseiro A, Ferreira R, Padrão A, Quintaneiro C, Pereira A, Marinheiro R, *et al*. Salivary proteome and peptidome profiling in type 1 diabetes mellitus using a quantitative approach. J Proteome Res 2013;12:1700-9.

34. Streckfus CF, Dubinsky WP. Proteomic analysis of saliva for cancer diagnosis. Expert Rev Proteomics 2007;4:329-32.

35. Al-Tarawneh SK, Border MB, Dibble CF, Bencharit S. Defining salivary biomarkers using mass spectrometry-based proteomics: A systematic review. OMICS 2011;15:353-61.

36. Rudney JD, Staikov RK, Johnson JD. Potential biomarkers of human salivary function: A modified proteomic approach. Arch Oral Biol 2009;54:91-100.

37. Haigh BJ, Stewart KW, Whelan JR, Barnett MP, Smolenski GA, Wheeler TT. Alterations in the salivary proteome associated with periodontitis. J Clin Periodontol 2010;37:241-7.

38. Tsai H, Bobek LA. Human salivary histatins: Promising anti-fungal therapeutic agents. Crit Rev Oral Biol Med 1998;9:480-97.

39. Anil S, Remani P, Beena VT, Nair RG, Vijayakumar T. Immunoglobulins in the saliva of diabetic patients with periodontitis. Ann Dent 1995;54:30-3.

40. Tang X, Pan Y, Wang Z. Sodium dodecyle sulphate polyacrylamide gel electrophoresis of whole salivary proteins from patients with adult periodontitis. Hua Xi Kou Qiang Yi Xue Za Zhi 2003;21:98-100.

41. Mau M, Muller C, Langbein J, Rehfeldt C, Hildebrandt JP, Kaiser TM. Adhesion of bovine and goat salivary proteins to dental enamel and silicate. Arch Tierz 2006;49:439-46.

42. Grassl N, Kulak NA, Pichler G, Geyer PE, Jung J, Schubert S, *et al.* Ultra-deep and quantitative saliva proteome reveals dynamics of the oral microbiome. Genome Med 2016;8:44.

43. Blankenvoorde MF, Henskens YM, van der Weijden GA, van den Keijbus PA, Veerman EC, Nieuw Amerongen AV. Cystatin A in gingival crevicular fluid of periodontal patients. J Periodontal Res 1997;32:583-8.

44. Golab K, Gburek J, Gawel A, Warwas M. Changes in chicken egg white cystatin concentration and isoforms during embryogenesis. Br Poult Sci 2001;42:394-8.

45. Jiang R, Xu C, Zhou X, Wang T, Yao G. Detection of cystatin C biomarker for clinical measurement of renal disease by developed ELISA diagnostic kits. J Transl Med 2014;12:205.

46. Jayagopal V, Keevil BG, Atkin SL, Jennings PE, Kilpatrick ES. Paradoxical changes in cystatin C and serum creatinine in patients with hypo- and hyperthyroidism. Clin Chem 2003;49:680-1.

47. Ohara G, Miyazaki K, Kurishima K, Kagohashi K, Ishikawa H, Satoh H, *et al.* Serum levels of cystatin C in elderly lung cancer patients. Oncol Lett 2012;3:303-6.

48. Vega A, García de Vinuesa S, Goicoechea M, Verdalles U, Martínez-Pueyo ML, Chacón A, *et al.* Evaluation of methods based on creatinine and cystatin C to estimate glomerular filtration rate in chronic kidney disease. Int Urol Nephrol 2014;46:1161-7.

49. Svensson-Färbom P, Ohlson Andersson M, Almgren P, Hedblad B, Engström G, Persson M, *et al.* Cystatin C identifies cardiovascular risk better than creatinine-based estimates of glomerular filtration in middle-aged individuals without a history of cardiovascular disease. J Intern Med 2014;275:506-21.

50. Xiaojin Z, Yong Q, Yaling D, Yulong S, Weichen X, Zhen Y. Clinical significance of cystatin S in cancer diagnosis and treatment assessment. J Clin Oncol 2014;32:e22226.

SUPPLEMENTARY MATERIAL

Supplementary Figure 1: Confirmation of ovulation. a) An ultrasound image of the dominant follicle size about ~ 20 mm of Left Ovary (LO). b) Salivary Fern Pattern
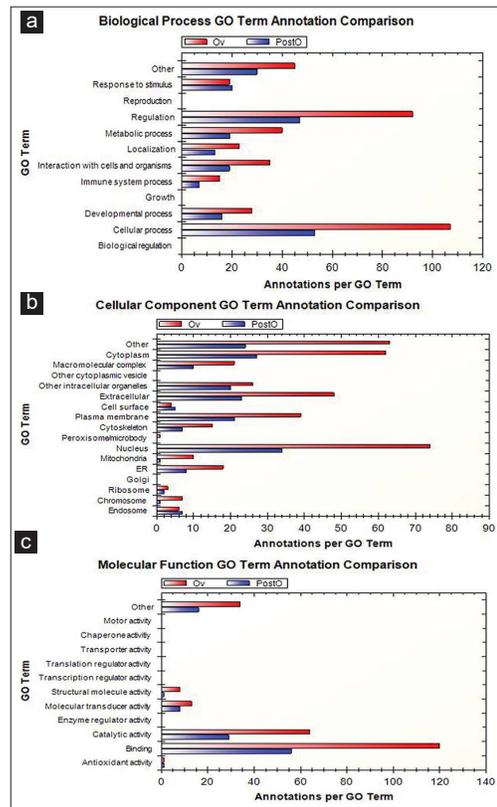
Supplementary Figure 2: Functional annotation of the salivary proteins. a) Biological process, b) Cellular component, c) Molecular function. Functional annotation was carried out by using STRAP 1.5 online database and found most of the identified proteins to be associated with binding property and regulatory function
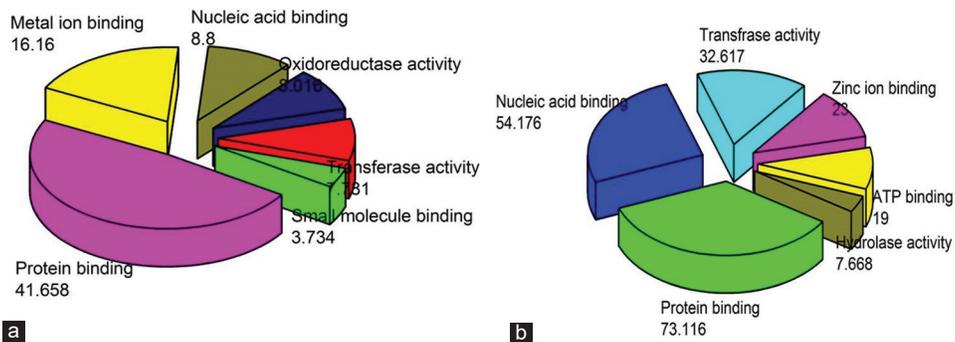
Supplementary Figure 3: Molecular functional ontology. a) Ovulation phase, b) Post-ovulation phase. Protein domain entries are used to depict the percentage of proteins through Interproscan analysis in BLAST2GO
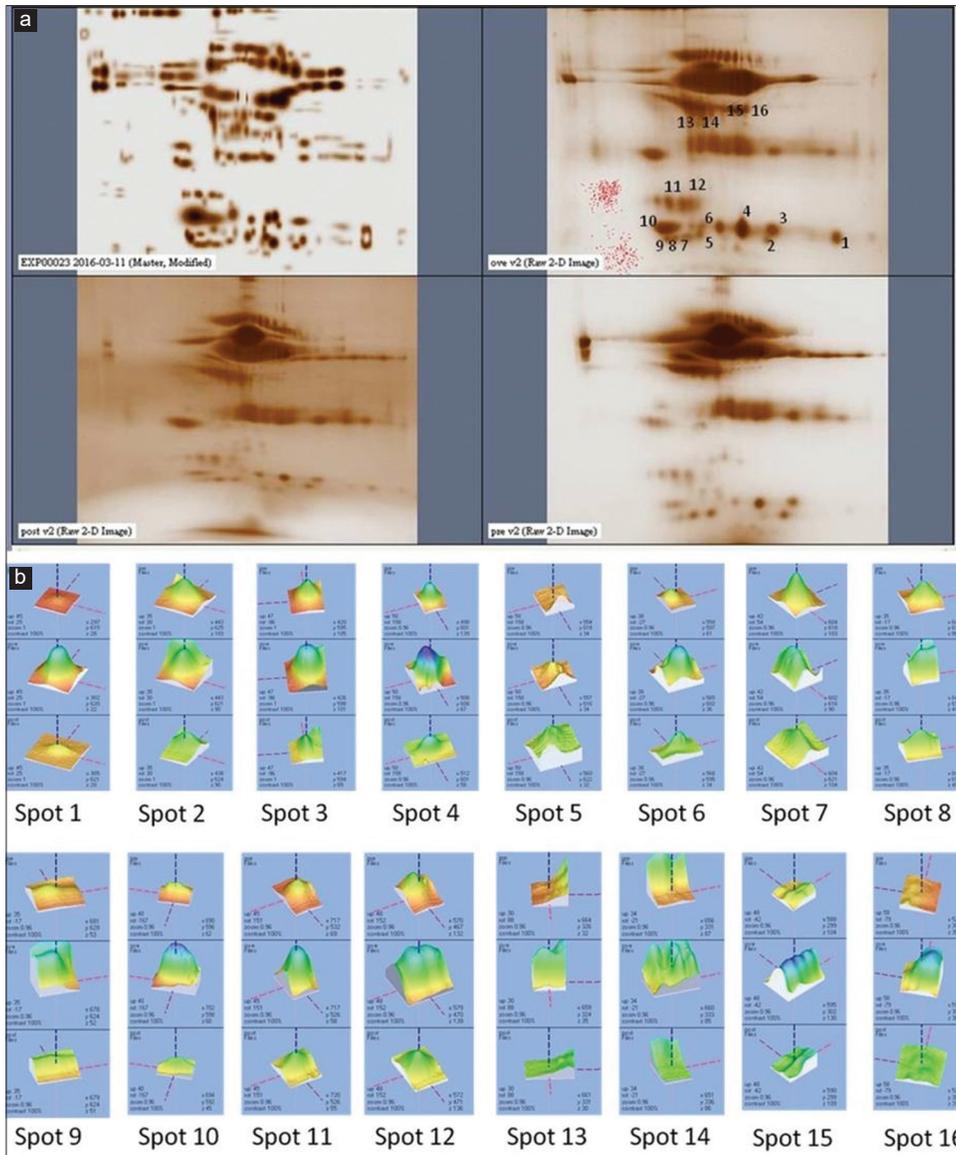
Supplementary Figure 4: Multi-channel image of 2D gel. a) The unique protein spots during ovulation phase. b) The differential expression level of 16 protein spots during pre-ovulation, ovulation and post-ovulation phases from the top respectively, by a Melanie 3D viewer