Protein repertoire of human uterine fluid during the mid-secretory phase of the menstrual cycle

T. Parmar1, G. Sachdeva1,5, L. Savardekar2, R.R. Katkam1, S. Nimbkar-Joshi1, S. Gadkar-Sable1, V. Salvi3, D.D. Manjramkar4, P. Meherji2 and C.P. Puri1

1Primate Biology Division, National Institute for Research in Reproductive Health, Jehangir Merwanji Street, Parel, Mumbai 400012, Maharashtra, India; 2Division of Clinical Research, National Institute for Research in Reproductive Health, Jehangir Merwanji Street, Parel, Mumbai 400012, Maharashtra, India; 3Department of Obstetrics and Gynecology, Seth G.S. Medical College and King Edward Medical Hospital, Parel, Mumbai 400012, Maharashtra, India; 4Experimental Animal Facility, National Institute for Research in Reproductive Health, Indian Council of Medical Research, Jehangir Merwanji Street, Parel, Mumbai 400012, Maharashtra, India

BACKGROUND: This study is an attempt to construct a repository of polypeptide species in human uterine fluid during the mid-secretory phase of menstrual cycle. This information is essential to generate alternative and less invasive tools for the assessment of uterine functions. METHODS: Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) and mass spectrometric analysis were used to resolve and identify the major components of human uterine fluid. RESULTS: Uterine fluid collected during the mid-secretory phase (n = 6) demonstrated ca. 590 polypeptide spots in the linear range of pH 4–7 after 2D PAGE. Mass spectrometric analysis revealed the presence of heavy and light chains of immunoglobulins, alpha-1 anti-trypsin precursor, anti-chymotrypsin precursor, haptoglobin, apolipoprotein A4, apolipoprotein A1 fragment, beta-actin fragment, heat shock protein 27, hemopexin precursor and transferrin precursor. 2D protein profile of fluid collected during the proliferative phase (n = 5) revealed ca. 433 polypeptide spots, of which 279 could be paired with mid-secretory phase protein spots on the basis of their coordinates (isoelectric point and molecular weight) in 2D gels. Apolipoprotein A4, apolipoprotein A1 fragment and alpha-1 anti-trypsin precursor were 2–3-fold more abundant in uterine fluid collected during the mid-secretory phase as compared with that in the proliferative phase. Further, 86 uterine fluid polypeptides were conserved across species, being detected in human, rat and bonnet monkeys. CONCLUSIONS: The molecular repertoire of the mid-secretory phase human uterine fluid, when compared with that of the proliferative phase uterine fluid, is broadened due to differential expression of proteins. Further, some of the mid-secretory phase proteins were conserved across species.

Keywords: endometrium; two-dimensional gel electrophoresis; receptivity; uterine fluid; protein maps

Introduction

Endometrial receptivity, triggered by ovarian hormones and sustained by an intricate network of autocrine, juxtacrine and paracrine factors in uterine cavity, is a self-limiting period of 4–5 days in the post-ovulatory phase of every menstrual cycle. This phenomenon precedes the event of implantation and if goes awry, contributes to significant percentages of infertility. The molecular mechanisms, which equip the endometrium with receptivity, are still not explicit, owing to the multiplicity of events involved. The advent of genomics tools has made possible to investigate this phenomenon as an integrated whole. Sufficient evidences have been gathered using microarray, differential display RT–PCR, subtractive hybridization etc. to demonstrate that this phase bears distinct molecular signature and is marked by differential expression of several genes (Ace et al., 1994; Patil et al., 2005; Horcajadas et al., 2007). Although micro array studies have generated high throughput data on the molecular participants involved in endometrial receptivity, these data need to be integrated with the protein expression profiling of endometrial tissue as well as of uterine secretions.

It is anticipated that when compared with endometrial tissue, uterine secretions will be less complex in terms of protein repertoire and may serve as a pool of biomarkers for endometrial function or dysfunction. Uterine secretions may perform an important role in feto-placental development, besides acting as a source of nutrients. Contrary to the earlier belief that nutrition of the mammalian conceptus is essentially histiotrophic before implantation and hemotrophic after the implantation, data are now accumulating to suggest that
uterine secretions may play a major role in early human pregnancy and their malfunction could be a factor in early pregnancy loss. Burton et al. (2002) have also shown that histiotrophic nutrition pathway remains active until at least 10 weeks of pregnancy. This emphasizes the critical role of uterine fluid components in female reproduction and warrants extensive investigations on identification and functional characterization of uterine fluid proteins.

Some efforts have been made previously to analyse the protein repertoire of uterine fluid using two-dimensional polyacrylamide gel electrophoresis (2D PAGE); however, these studies did not reveal the identity of uterine fluid proteins (Maclaughlin et al., 1986). The present study is an attempt toward generating a 2D map of polypeptides from human uterine fluid samples collected during the mid-secretory phase, when the endometrium is most receptive to an incoming embryo. The study also investigates whether some of the proteins present in uterine fluid are evolutionarily conserved.

Materials and Methods

The study was approved by the Animal Ethics Committee of the Institute and the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India. The study was also approved by the Institute’s ethics committee for collection of samples from healthy women.

**Human subjects**

Women of reproductive age (21–35 years) with a history of regular, monthly menses and at least one live birth were enrolled in the study. Exclusion criterion included subjects using any hormonal contraceptive methods and women with symptoms of reproductive tract infections or polycystic ovary syndrome, uterine fibroids or luteal phase defect. All women gave informed consent to participate in the study. Uterine fluid samples were collected from women (n = 6) during the mid-secretory phase, the period when the endometrium is ready to accept an embryo. Samples were also collected from women (n = 5) during their pre-ovulatory period (proliferative phase).

Ovulation was monitored by serial ultrasonography (USG) to ascertain the follicular collapse. The first USG was done on day 6 or day 7 of the menstrual cycle, depending on length of the last menstrual cycle, the second USG on day 8 or day 9 and then it was done daily until the follicular rupture was observed. Uterine fluid samples were collected on day 6 following the follicular rupture and categorized as mid-secretory samples. Pre-ovulatory or proliferative samples were collected on day 9 or 10 of cycle from another group of subjects. In these subjects also, USG was continued till the follicular rupture was observed. This was done to determine the day of sample collection with respect to the day of ovulation. Samples collected 2 day prior to ovulation were included in the analysis.

Method of sample collection: A bivalve Cusco’s speculum was used to visualize the cervix and excess cervical discharge was cleaned by sterile swab. Anterior lip of cervix was held with vulsellum. A flexible infant feeding tube no. 10 was cut and a uterine stillete was inserted into it to provide rigidity and facilitate easy passage through the cervical canal. At the extreme upper end of the infant feeding tube (which entered the uterine cavity), two to three openings were made in the sidewalls. With traction on the anterior cervical lip, the tube was inserted into the uterine cavity without touching the sidewalls of the vagina. The stillete was then withdrawn. Sterile saline (1.5 ml) was flushed through a 5 ml syringe attached to the outer end of the infant feeding tube and aspirated quickly to minimize loss of the fluid instilled. The fluid was collected without contamination by vaginal secretions. Samples were centrifuged at 9500 g for 30 min after which the supernatants were collected in presence of 1× protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA) and stored at −20°C till processed.

Following uterine irrigation, endometrial biopsies were obtained from the uterine cavity using a probe. The tissue was immediately fixed into 10% buffered formalin for endometrial dating. Histological dating of the endometrial biopsies was assessed according to Noyes’ criteria (Noyes et al., 1950). Histological features of endometrial samples correlated well with the features, characteristic of the phase of cycle during which samples were collected. Blood samples were also collected to determine serum levels of progesterone on the day of biopsy collection to ascertain luteal sufficiency. Progesterone levels in serum were estimated by radioimmunoassay.

**Bonnet monkeys (macaca radiata)**

The conditions for maintenance of adult bonnet monkeys in the animal house facility of the Institute have been mentioned previously (Rosario et al., 2005). Cyclicity of the animals was monitored by vaginal smears. Serum estradiol (E2) and progesterone concentrations were measured by specific radioimmunoassays as described previously (Rosario et al., 2005). Regularly cycling female bonnet monkeys (n = 4) with normal hormonal profiles (peak levels-E2: 300–600 pg/ml; progesterone: 3–6 ng/ml) were admitted into the study.

Uterine fluids were collected on day 8 post-E2 peak. In brief, oviducts were clamped to avoid backflow of saline into oviducts. Base of the uterus was also clamped to avoid escape of saline into cervix. Saline (5 ml) was then infused into the upper region of endometrial cavity using a 22G needle connected to a syringe. The fluid was simultaneously collected from the lower region of endometrial cavity through a 22G needle connected to a sterile syringe. Eighty percentage of the infused saline could be recovered. The fluid was collected in presence of 1× protease inhibitor cocktail (Sigma-Aldrich). Samples were centrifuged at 13 709 g for 30 min at 4°C and supernatants were stored at −20°C. Tissue biopsies were also obtained to date the endometrium as described previously (Patil et al., 2005).

**Rats**

Adult female Holtzman rats weighing >50 gm were housed in individual cages on a 14-h light, 10-h dark lighting schedule at ambient temperature between 23 and 25°C. Vaginal smears were taken daily and only those animals showing at least three consecutive 5-day estrous cycles were used. Animals in estrous phase were anesthetized with 20–30 mg/kg i.p. ketamine hydrochloride. After skin preparation and draping, a midline incision was made to enter to abdominal cavity. Uterine horns were ligated at the tip of the two horns and also at the conjoined portion of the two horns. Associated fat tissue was removed from the uterine horns and sterile saline (approximately 2 ml) infused into one end of the horn. The fluid was collected from the other end of the individual horns. Samples from 5 rats were centrifuged at 13 709 g for 30 min at 4°C. Supernatants were collected and pooled. Samples were stored at −20°C for proteomic analysis.

**Histological analysis**

Endometrial tissues fixed in 10% buffered formalin were washed in 70% vol/vol ethanol for 24 h and embedded in paraffin. Paraffin sections were cut at 5 μm and stained with hematoxylin/eosin.
2D gel electrophoresis and protein identification

Proteins were isolated from uterine fluid samples using 2D clean up kits as per the manufacturer’s instructions (Amersham Pharmacia Biotech, Uppsala, Sweden). The protein rich pellets were resolubilized in rehydration buffer containing 9 M urea, 10 mM dithiothreitol (DTT) and 0.5% CHAPS. Bradford method was used to determine the protein concentration (Bradford, 1976). Immobiline pH gradient strips of 11 cm length in the linear range of pH 4–7 were actively rehydrated with protein sample (200 µg) at 50 V for 12 h at 20°C. Isoelectric focusing (IEF) was carried out using an IEF cell (BioRad, Richmond, CA, USA) for 30 000 Volt hours. Following IEF, the strips were first reduced and then alkylated in buffer containing 50 mM Tris HCl (pH 8.8), 6 M urea, 30% glycerol, 65 mM DTT (for reduction) or iodoacetamide (for alkylation), 2% sodium dodecyl sulphate (SDS) for 30 min each at room temperature. Second dimension separation was carried out using 10% SDS-PAGE gels. Each sample was run three times to ensure reproducibility. Thirty-three gels were run for 11 human uterine fluid samples. Four gels were run for the pooled rat uterine fluid sample. Uterine fluid samples from four bonnet monkeys were run individually, each in triplicate. Three human serum samples collected during the mid-secretory phase were also run individually in triplicate. Proteins were stained using a silver staining method compatible with mass spectrometry (Mortz et al., 2001). Silver stained gels were scanned using gel scanner (Amersham Pharmacia Biotech). Spot detection and determination of densitometric intensity was carried out using Image Master 2D Platinum Software (Amersham Pharmacia Biotech). Spots were detected after filtering the noise and background on each gel. Gels to be compared were aligned by auto matching using the landmarks and then normalized for total gel spot intensity. The reference gels were generated for the mid-secretory phase as well as proliferative phase to represent the spots detected in all samples within each group. Protein spots were considered as differentially expressed if 2-fold or more difference was observed in their intensities between mid-secretory and proliferative gels.

2D spot excision was performed manually and spots were washed four times with sterile water by centrifugation at 13 709 g for 10 min at 4°C and then stored at −20°C till processed for matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis at the Central Instrumentation Facility, Jawaharlal Nehru University, New Delhi, India; where mass spectra of trypsin digested gel spots were generated on Biflex III MALDI-TOF-mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). Peptide fingerprints were analysed using BioTools (Bruker Daltonik) and Mascot software (Matrix Science, London, UK) and the Mass Spectrometry Protein Sequence Database (MSDB). MSDB is a composite database built from several primary source databases such as PIR1, PIR2, PIR3, PIR4, TrEMBL, Genbank and Swissprot. For confirmation, post-source decay analysis was performed to obtain sequence information of a few selected peptides. The peptide mass fingerprinting of the proteins were scored with the Mowse score. The criteria used for a positive identification was significant Mowse score. Mowse scores greater than or equal to 64 were considered as significant (P < 0.05).

Western blotting: One-dimensional (1D) SDS-PAGE protein gels loaded with 20 µg uterine fluid protein or 2D SDS-PAGE gels loaded with 200 µg uterine fluid protein were blotted to methanol activated polyvinylidene difluoride (PVDF) membranes (Hybond P, Amersham Pharmacia Biotech) in transfer buffer (25 mM tris, 92 mM glycine, 20% methanol). Electroblotting was carried out for 2 h at 100 V at 4°C. The blots were reactivated, blocked in 2% blocking reagent and probed with different dilutions of antisera against human placental protein 14 (PP14), leukemia inhibitory factor (LIF), insulin like growth factor (IGF) or epidermal growth factor (EGF) at 4°C overnight. Antibodies against human PP14 were gift from Dr Anjali Karande, IISc, Bangalore. Other antibodies were procured from R&D Systems, MN, USA. After washing with PBST (0.05% tween 20 in phosphate-buffered saline (PBS)) six times for 10 min each, blots were incubated with goat anti-rabbit antibodies coupled to horse-radish peroxidase, diluted at 1:1000 in PBS. After washing with PBST, signals were detected using advanced enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech) by autoradiography. For dot blot, 0.01–10 µg of uterine fluid protein and 0.1–100 ng of respective recombinant antigens (PP14/LIF/EGF/IGF-1) were spotted on PVDF membranes and probed as mentioned above.

Results

Human uterine fluid in the mid-secretory phase demonstrated presence of at least 590 polypeptides, when resolved in the pH range of 4–7, whereas samples collected during the proliferative phase demonstrated presence of at least 433 polypeptides. About 279 spots could be paired in the mid-secretory phase and proliferative phase proteomes on the basis of their coordinates with respect to molecular weight and isoelectric point (pI) (Fig. 1). Data obtained by mass spectrometric
analysis of spots excised from the mid-secretory phase human proteome (Fig. 2) are shown in Table I. Table I includes only those spots where mass spectrometry sequence coverage was found to be $>30\%$.

Haptoglobin, anti-chymotrypsin precursor, alpha-1 antitrypsin precursor and immunoglobulins were distributed in more than one spot. This implies that there exist different forms of these proteins in human uterine fluid. Densitometric analysis, after normalizing the total spot intensities of 2D gels, demonstrated 2-fold increase in the intensities of apolipoprotein A4 and apolipoprotein A1 fragment and $>2.5$-fold increase in that of alpha-1 anti-trypsin precursor in the mid-secretory phase samples when compared with that in the proliferative phase samples.

Eighty-six proteins were found to be common in the mid-secretory phase proteomes of human, monkey and rat uterine fluid with respect to their coordinates in 2D gels (Fig. 3). These proteins included anti-chymotrypsin precursor, transferrin precursor, hemopexin precursor, haptoglobin and alpha-1 antitrypsin precursor. Interestingly haptoglobin spots differed in their pI as indicated by the shifts in their location in 2D gels for monkey and rat samples.

When compared with the proteome of human serum collected in the mid-secretary phase, some of the polypeptide spots such as heat shock protein 27 (hsp27) were found to be present only in uterine fluid. When normalized for the total protein amount, haptoglobin was found to be more abundant in human uterine fluids when compared with that in serum (Fig. 4).

Studies have been done to demonstrate the presence of PP14, LIF, EGF and IGF-1 in human uterine fluid (Li et al., 1993; Laird et al., 1997; Lighten et al., 1998; Taketani and Mizuno, 1998). This prompted us to locate the spots corresponding to these proteins on 2D gels by immunoblotting with respective antisera. However no signals were detected for these proteins on immunoprobed 2D gels. Immuno-dot-blot analysis also failed to detect these proteins in human uterine fluid in the secretory phase as well as in the proliferative phase (data not shown). No signals were present at the sites loaded with uterine fluid protein. Nonetheless strong signals were detected at the sites loaded with the respective recombinant antigens, suggesting that PP14, LIF, EGF and IGF-1 levels in uterine fluid were too low to be detected by immunoprobing of dot-blot/1D/2D PAGE western blots.

**Discussion**

It is well established that endometrium, the inner lining of the uterus, displays a distinct structural and molecular phenotype during day 6–9 post-ovulation in every menstrual cycle and this status endows the endometrium with an ability to accept the embryo. Significant data are available on the global gene expression profile in endometrial tissue during this period; however information on the global profile of proteins secreted into uterine lumen during the receptive phase in humans is not yet available.

Some attempts have been made to scan human uterine fluid for the presence of few proteins selected *a priori*, on the basis of their established presence or function in uterine tissue. These investigations revealed not only the presence but also the cyclic variations in levels of matrix metalloproteinase 2, cancer antigen 125, mucin 1, PP14 and LIF (Li et al., 1993; Hey et al., 1995; Laird et al., 1997, 1999; Hamilton et al., 2002). Among these proteins, PP14 levels in uterine fluid, and not in plasma, showed correlations with endometrial dating. Further, these levels were found to be lower in patients with unexplained infertility and recurrent spontaneous abortion (Li et al., 1993; Dalton et al., 1995). Similarly, LIF levels in flushings were found to be low around the time of implantation in women with unexplained infertility when compared with that in normal fertile women (Laird et al., 1997; Mikolajczyk et al., 2003). These studies collectively imply that there occur cycle dependent changes in the molecular repertoire of human uterine fluid. These studies thus offer a strong premise to generate a phase specific reference proteome of human uterine fluid. Availability of these protein maps may facilitate the discovery of biomarkers of endometrial function/dysfunctions.

Efforts made to characterize human uterine fluid at biochemical level demonstrated the presence of serum components and four uterine proteins (Wolf and Mastroianni, 1975). However, the identity of human uterine fluid proteins could not be established. This was not surprising, considering the limitations of mass spectroscopic techniques available in the pre-genomic era. Now with the advent of high throughput proteomics tools, it is feasible to identify the whole protein complement of various tissues/organs/biological fluids during any physiological or pathological state. The present study is an attempt to identify the major components of human uterine fluid, collected during a phase when the endometrium is expected to be the most receptive to the embryo.

To characterize the major protein components of human uterine fluid, 2D proteomics approach was employed in the present study. Realizing that any inconsistencies or pitfalls in the methods employed to determine the time of sampling and also the method of collecting the samples may profoundly
<table>
<thead>
<tr>
<th>Spot identity</th>
<th>Proteins identified by mass spectrometric analysis and their accession number</th>
<th>Mowse score</th>
<th>Mr (kDa) of the matched protein entry</th>
<th>Isoelectric point (pI) of the matched protein entry *</th>
<th>Presence in endometrial tissue</th>
<th>Hormonal regulation in endometrial tissue (as reported by others)</th>
<th>Probable function of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>Anti-chymotrypsin precursor (validated)-human-gi 177933</td>
<td>240</td>
<td>46.8</td>
<td>5.2</td>
<td></td>
<td></td>
<td>Protease inhibitor</td>
</tr>
<tr>
<td>V2</td>
<td>Haptoglobin precursor allele 1-validated-human-HPHU1</td>
<td>122</td>
<td>38.72</td>
<td>6.1</td>
<td>Present</td>
<td>Higher in mid-secretory phase</td>
<td>Immunomodulator</td>
</tr>
<tr>
<td>V3</td>
<td>Haptoglobin-Homo sapiens-gi 3337390</td>
<td>179</td>
<td>38.72</td>
<td>6.1</td>
<td>Present</td>
<td>Higher in mid-secretory phase</td>
<td>Immunomodulator</td>
</tr>
<tr>
<td>V4</td>
<td>Alpha-1 anti-trypsin precursor (validated) human-ITHU</td>
<td>74</td>
<td>31.64</td>
<td>6.1</td>
<td>Present</td>
<td>Higher in mid-secretory phase</td>
<td>Immunomodulator</td>
</tr>
<tr>
<td>V5</td>
<td>Alpha-1 anti-trypsin precursor-ITHU</td>
<td>119</td>
<td>46.8</td>
<td>5.3</td>
<td>Present</td>
<td></td>
<td>Protease inhibitor</td>
</tr>
<tr>
<td>V6</td>
<td>Apolipoprotein A4 precursor-validated-human-LPHUA4</td>
<td>203</td>
<td>45.3</td>
<td>5.2</td>
<td>Present</td>
<td></td>
<td>Lipoprotein, plays a role in lipid transport and accumulation</td>
</tr>
<tr>
<td>V7</td>
<td>Hemopexin precursor (validated) human-OQHU</td>
<td>71</td>
<td>52.38</td>
<td>6</td>
<td>Present</td>
<td>Equally present in proliferative and mid-secretory phases</td>
<td>Iron transport</td>
</tr>
<tr>
<td>V8</td>
<td>Transferrin precursor (validated) human-TFHUP</td>
<td>125</td>
<td>79.2</td>
<td>7.0</td>
<td>Present</td>
<td>Equally present in proliferative and mid-secretory phases</td>
<td>Iron binding protein</td>
</tr>
<tr>
<td>V9</td>
<td>Immunoglobulin Kappa heavy chain precursor-Homo sapiens-CAA75030</td>
<td>97</td>
<td>52.65</td>
<td>9.6</td>
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<td>Immune response</td>
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<td>V10</td>
<td>APOA1 protein fragment-Homo sapiens (human)-CAA00975</td>
<td>206</td>
<td>28.06</td>
<td>5.1</td>
<td>Present</td>
<td></td>
<td>Lipoprotein, plays a role in lipid transport and accumulation</td>
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<td>22.88</td>
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<td>Immune response</td>
</tr>
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<td>V13</td>
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<td>11.59</td>
<td>9.7</td>
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<td></td>
<td>Immune response</td>
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<td>V14</td>
<td>Transthyretin chain A-human-2ROYA</td>
<td>146</td>
<td>13.3</td>
<td>5.2</td>
<td>Present</td>
<td>Transports thyroid hormone and vitamin A –</td>
<td></td>
</tr>
<tr>
<td>V15</td>
<td>Unnamed protein product-gi 16552732</td>
<td>156</td>
<td>21.9</td>
<td>5.1</td>
<td>Present</td>
<td>Reduces reactive oxygen species</td>
<td>Immunomodulator</td>
</tr>
<tr>
<td>V16</td>
<td>Haptoglobin isoform CRA-gi 47124562</td>
<td>83</td>
<td>31.64</td>
<td>6.3</td>
<td>Present</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The discrepancy in the pIs of proteins detected here with that of the protein entries in MSDB may arise because of post-translational modification.

**Figure 3:** 2D protein profiles of uterine fluid collected from rat (A), bonnet monkey (*Macaca radiata*) (B) and human (C) in the mid-secretory phase of the endometrial cycle. Circles indicate spots which were matched in A, B and C.
misrepresent the 2D protein profile of uterine fluid, efforts were
taken to ensure that samples are dated appropriately according
to the day of ovulation in the biopsy cycle and collected
without injuring the cervical or endometrial lining. Serial
USG, estimation of circulatory progesterone levels and histo-
logical evaluation of endometrial samples were carried out to
document the ovulation, hormonal sufficiency and endometrial
responsiveness to progesterone, respectively. In the present
study, uterine fluid samples were collected using the lavage
method (i.e. by instilling saline into uterine cavity in controlled
fashion and retrieving the infused volume). Although there are
lesser chances of diluting the uterine cavity contents by
the method of aspiration (i.e. without instilling saline), the
amount of fluid expected to be recovered by aspiration was
much less, as the fluid volume in the uterine cavity is estimated
to be only 0.2 ml (Casslen, 1986). It has been reported by
Maclaughlin et al. (1986) that the method of collecting the
uterine contents by aspiration is superior to that of lavage; however, the samples compared by them were collected in
different phases of the menstrual cycle. Hence, it remains
difficult to ascertain from their investigation whether the 2D
protein profile of uterine fluid samples differed because of
the cyclic variations in the protein repertoire or if the differ-
ences appeared due to dissimilar efficiencies of the methods
of lavage and aspiration in retrieving the uterine luminal con-
tents. Nonetheless, we invariably experienced blood and cellular
contamination while aspirating the uterine contents. Therefore we opted to collect the uterine fluid samples by the
method of lavage, also used successfully by other investigators
(Dalton et al., 1998; Hamilton et al., 1998; Ng et al., 2004).

Our study demonstrated the presence of immunoglobulins,
transferrin, alpha-1 anti-trypsin precursor, anti-chymotrypsin
precursor, apolipoprotein, haptoglobin and hemopexin in
human uterine fluid. These proteins are known serum proteins and
evidences also exist to demonstrate localization of some of
these proteins in endometrium (DeSouza et al., 2005a,b; Fowler et al., 2007). In view of this, it is not easy to establish
whether these proteins appear in uterine fluid due to filtration
from serum or plasma across the endometrial epithelium or if
these are synthesized by endometrial cells and then secreted
into uterine lumen. Nonetheless, we believe that these proteins
are integral components of uterine fluid and did not originate
due to blood contamination while irrigating the uterine
cavity. First, uterine fluid samples collected were blood free.
This is evident from the fact that none of the 2D gels loaded
with uterine fluid showed detectable presence of two major
red blood cell proteins—super oxide dismutase and beta
subunit of hemoglobin. Secondly, even if it is assumed that
there were traces of blood contamination, it is hard to explain
the abundance of these proteins in human uterine fluid as
revealed by the comparison of serum and uterine fluid 2D
maps, normalized for total protein load.

Using 2D proteomics approach, we could detect at least 590
protein spots in mid-secretory phase human uterine fluid.
However there may be some, which could not be detected in
the present study, due to the limitations of 2D proteomics. For
example, despite loading high amount of uterine fluid protein
onto a high protein binding capacity membrane and using
advanced ECL chemiluminescent detection system; PP14,
LIF, IGF, EGF could not be detected in 1D/2D/dot blots
probed with specific antisera. Some of these proteins, such as
PP14 or LIF, could be detected in human uterine fluid using
only extremely sensitive techniques such as radio-immunoas-
says and two antibody based capture enzyme-linked immuno-
sorbent assay (Dalton et al., 1995; Mikolajczyk et al., 2003).

Ours is a first report to demonstrate the presence of hsp27 in
human uterine fluid. Further when we compared 2D proteomes
of uterine fluid and of serum collected in the same phase of
menstrual cycle, hsp27 was not detected in the serum pro-
tome. Thus, hsp27 does not seem to be a plasma exudate.
Further, there are evidences to suggest the presence of hsp27
in uterine tissue, implying that uterine cells could be a potential
source of secreted hsp27 (Ciocca et al., 1993).

Haptoglobin was found to be one of the major protein com-
ponents of uterine fluid. The presence of haptoglobin in the
human uterus has been documented and its role in protecting

Figure 4: 2D gels depicting protein spots detected in uterine fluid sample (A) and serum (B) collected from same woman during the progesterone
dominant (mid-secretory) phase
Total proteins (200 μg) were resolved in linear range of pH 4–7 in the first dimension and 10% polyacrylamide gel in second dimension. Circles
indicate proteins which were highly abundant in the proteome of uterine fluid
the fetus from maternal allograft-like immune response has been postulated (Nadia et al., 2001). Further, the levels of haptoglobin were found to be higher in the secretory phase when compared with that in the proliferative phase endometrial tissue (Nadia et al., 2001). However, we did not find this pattern on comparison of mid-secretory and proliferative phase uterine fluid protein maps. It may be hypothesized that endometrial cells in mid-secretory phase tend to accumulate haptoglobin, instead of secreting it into the lumen. This conjecture agrees well with the report demonstrating high levels of haptoglobin in deciduas (Nadia et al., 2001).

Beta-actin fragment was also detected in human uterine fluid. Uterine flushings of pregnant sheeps also showed the presence of beta-actin fragment (Lee et al., 1998). It is likely that this protein is released in uterine fluid due to either constant apoptosis and remodeling of endometrial cells or exudation from plasma across endometrial epithelium. Interestingly human serum and endometrial cells also express beta-actin (Pieper et al., 2003).

Some other proteins, such as alpha-1 anti-trypsin precursor and apolipoprotein, were also detected in uterine fluid in the present study. Alpha-1 anti-trypsin precursor is a member of serpin (serine protease inhibitor) family, which regulates numerous intracellular and extracellular processes, including embryo implantation, blood coagulation, fibrinolysis, cell migration, cell differentiation, complement activation, tumor suppression and other functions (Potempa et al., 1998). We detected higher levels of alpha-1 anti-trypsin precursor in the mid-secretory phase when compared with that in the proliferative phase samples. This raises the possibility of the expression of alpha-1 anti-trypsin precursor being negatively regulated by estrogen. Interestingly, under-expression of alpha-1 anti-trypsin precursor has been reported in the endometrial cancer tissue homogenates. This not only indicates that the endometrial tissue is a source of alpha-1 anti-trypsin precursor but also implies a negative association between cellular proliferation and alpha-1 anti-trypsin precursor expression (DeSouza et al., 2005b).

Our results indicating the increase in apolipoprotein levels during the mid-secretory phase of cycle concur well with a report by Kao et al. (2002), which demonstrated significant increase in the levels of apolipoprotein transcripts during the mid-secretory phase. Considering the relevance of lipoproteins in embryonic development, it is likely that the accumulation of these proteins in uterine fluid occurs in the anticipation of an embryo arrival.

Comparison of uterine fluid protein profile from rat, monkey and human showed presence of 86 matched spots. Anti-chymotrypsin precursor, immunoglobulins, transferrin precursor and haptoglobin were among these conserved proteins. However, we observed significant shift in the pIs of haptoglobin in the 2D proteomes of rat and monkey uterine fluids, suggesting that haptoglobin undergoes species-specific post-translational modifications.

Estrogens and progestrone are known to control the vasculature of the endometrium (Niklaus et al., 2003). It is likely that any change in vascular permeability due to hormonal variations across the cycle may alter the ‘quantity’ or ‘quality’ of serum transudation across endometrial epithelium and hence consequently the uterine fluid protein profile across the cycle. This suggests that proteins in uterine flushings, even if of serum origin, may serve as markers of endometrial function.

The present study generates data which catalog major human uterine fluid proteins, on the basis of their molecular weight and pIs, during a phase when the endometrium achieves the receptivity to an incoming embryo. The study also demonstrates quantitative and qualitative variations in the 2D protein profiles of proliferative and mid-secretory phase uterine fluids.

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