Introduction

Human endometrium undergoes a series of morphological and molecular changes during the transition from proliferative to secretory phase, under the influence of ovarian steroids [1,2]. After ovulation, endometrium is under the influence of progesterone which undergoes differentiation and other morphological changes e.g. appearance of vacuoles, and formation of spiral arteries take place to develop the secretory endometrium [3]. During secretory phase, endometrium becomes receptive for a short period of time (spanning between days 20 and 24 of the menstrual cycle) to allow the attachment of developing embryo [4]. Intricate network of signalling molecules (hormones, cell adhesion molecules, cytokines and growth factors etc.) is known to be involved in embryo-uterine interaction and plays critical role in embryo attachment [1,2]. Any alteration in cascade of these signalling mechanisms caused pregnancy loss, implantation failure or infertility [5].

Unexplained infertility accounts for approximately 25–30% of all known female infertility which is represented in a subset of infertile women having normal ovulatory cycles, normal hormonal profile and no evident reasons for infertility [6]. Compromised receptivity of the endometrium is a major cause of unexplained infertility, implantation failure and subclinical pregnancy loss. In women, unexplained infertility has been coupled with a range of...
cellular and molecular defects in the endometrium [7–9]. Several genomics and proteomics approach-based studies have revealed large number of differentially regulated genes/proteins by comparing pre-receptive (LH+2) and receptive phase (LH+7) endometrium of fertile women [10–13]. Although, these studies have provided a large number of molecular candidates that are important during endometrial receptivity and some of them have been established as receptivity markers for fertile endometrium but to date the causes of unexplained infertility are not well explored. A few genomics and proteomics approach-based studies have explored the molecular targets responsible for the cause of recurrent implantation failure and unexplained infertility [14–16]. Although, the outcome from these studies have broadened our knowledge to understand the molecular mechanism involved in unexplained infertility, actual causes of unexplained infertility remain largely unknown.

With a view to explore the molecular basis of unexplained infertility, the present study was undertaken to generate 2D map of endometrial proteins during secretory phase of women with unexplained infertility and to analyze whether proteomic profile of endometrium differs from early- (LH+2) to mid-secretory (LH+7) phase. To our knowledge this is the first study to report the differentially expressed endometrial proteins between early-secretory (LH+2) and mid-secretory (LH+7) phase in women with unexplained infertility. Results of this study might be helpful in understanding the molecular mechanism involved in unexplained infertility and defective endometrial receptivity.

**Materials and Methods**

**Ethics Statement**

Human endometrial biopsy samples were obtained for research after written consent from each patient and the study was approved by the Institutional Ethics Committee of King George’s Medical University, Lucknow, India.

**Sample collection and patient details**

Endometrial biopsies were taken by using pipelle catheters (Genetics, Belgium) under sterile condition and immediately frozen at −80°C until used. For proteomic study, 16 human endometrial biopsy samples were collected from different 16 women once during early-secretory (LH+2) (n = 8), mid-secretory (LH+7) (n = 8) and mid-secretory (LH+7) (n = 4) phases from infertile women with unexplained infertility. The detection of LH in morning urine (Donachek ovulacion; Novalab Iberica, S.A.L., Coslada, Madrid, Spain) was used to determine the day of the LH surge (day LH+0). Additional eight endometrial samples (4 from each group, collected once) were collected from different infertile women outside the sample cohort, for the validation by western blot and immunohistochemistry, to confirm the biological significance of the findings. Another eight endometrial biopsy samples were collected once from different fertile women, during early-secretory (LH+2) (n = 4) and mid-secretory (LH+7) (n = 4) phases of menstrual cycle. Histological dating of each sample was performed [17]. Serum progesterone levels as determined by immunoenzyme assay [18] were found to be 8.52±1.25 ng/ml for LH+2 stage and 18.16±1.32 ng/ml for LH+7 stage in infertile patients. A specific written consent was obtained from each patient, and the study was approved by the ethics committee of King George’s Medical University, Lucknow.

To determine the clinical state of infertile women, the complete examination and investigation of all infertile couples were carried out (Table S1). All women were aged 25–35 years and their cycle lengths were within normal range (27–29 days). The female patients had normal functioning fallopian tubes which were confirmed by HSG/SSG and if required diagnostic laparoscopy and hysteroscopy was done. Normal ovulatory function and absence of bacterial vagiunosis has been confirmed. The test for systemic diseases e.g. diabetes was found to be negative, endometrial biopsies were negative for tuberculosis. The male partner had a normal sperm count. The couples have been trying to conceive for at least one year. Thus, the infertility investigation had not revealed any cause of the infertility.

**Exclusion criteria**

The women having uterine abnormalities like leiomyomas, polycystic ovarian syndrome, endometriosis, hydrosalpinx, acute infection PID, vaginitis, male factor infertility were excluded from the study. Those who had received steroid hormone therapy in the last six months were also excluded from the study (Table S2).

The fertile women all had proven parity and were presenting for tubal ligation or assessment for reversal of tubal ligation.

Biopsy samples from only women with normal cycle and hormonal profile were collected. All patient groups were age matched and cycling (Table S3).

**Sample preparation for two dimensional polyacrylamide gel electrophoresis**

10% homogenate was prepared in sample lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS (3-(3-Cholamidopropyl) dimethylammonio)-1-propanesulfonic acid) and 20 mM Tris–HCl (pH 8.5), 1 mM EDTA, containing 50 mM DTT) in ice cold condition [19–22]. The whole tissue homogenate contained luminal epithelium, glandular epithelium, stroma, and vessels. Tissue homogenate was centrifuged and protein estimation was done [23].

**Isoelectric focusing**

IPG strips (11 cm) were rehydrated with proteins (200 μg), mixed in rehydration buffer (8 M urea, 2% w/v CHAPS, 130 mM DTT, 0.002% bromophenol blue) and IPG buffer (11 μl) in a total volume of 210 μl before subjecting to IEF [23,24]. The rehydrated strips ([pH 3–10] were focused at 50 μA per strip in a multiphor-II electrophoresis unit (GE Healthcare, BUCKS, UK) up to 14000 V h at 20°C (500 V for 30 min linear gradient, 1000 V for 10 min, 8000 V for 3 h, 8000 V for 6 h and 8000 V for 7 h).

**SDS-PAGE**

Following focusing, the strips were incubated in equilibration buffer (50 mM Tris/HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue) for 45 min placed over the resolving gels, covered with sealing gel (0.5% agarose in SDS electrophoresis buffer) and SDS-PAGE was carried out on 12.5% polyacrylamide gels overnight in a Bio-Rad Dodeca cell (Richmond CA). Proteins were visualized by silver staining as reported previously [24,25]. In brief, gels were fixed in methanol: acetic acid: water in the ratio of 50: 5: 43, for 2 h. Gels were rinsed with double distilled water (DDW) twice and kept in DDW for 1 h. Gels were then incubated with sensitizing solution (0.02% sodium thiosulphate) for 1–2 min with constant shaking. Further, the gels were incubated in 0.1% AgNO₃ solution in dark for 30 min and spots were developed by a solution of 2% Na₂CO₃ and 35% HCHO for 5 min. 1% Acetic acid was used immediately to avoid the over staining of gels [24,25]. Coomassie brilliant blue (R-250) staining was also performed to confirm the differentially expressed protein spots which were observed in silver stained gel. Equal amount of protein was loaded in each experiment. Similar
Disulfide bonds were cleaved by incubating the samples for 60 min at 56°C. The gel pieces were dehydrated by adding 500 μl of 25 mM ammonium bicarbonate in 50% acetonitrile for 60 min. The gel pieces were digested with trypsin and cut into size of 1 mm, washed with 500 μl of 25 mM ammonium bicarbonate buffer. Alkylation of cysteines was performed by the addition of 200 μl of 55 mM iodoacetamide in a 100% acetonitrile. Gel bands were washed with 25 mM ammonium bicarbonate buffer and dehydrated with 500 μl of acetonitrile. Gel pieces were covered with trypsin solution (10 ng/μl in 25 mM ammonium bicarbonate buffer). After 30-min incubation on ice, the remaining trypsin solution was removed, and 25 μl of 25 mM ammonium bicarbonate was added. Proteolysis was performed overnight at 37°C and stopped by adjusting the samples to 5% formic acid. Further, these peptides were analyzed by electrospray ionization mass spectrometry using the Ultimate 3000 nano HPLC system (Dionex) coupled to a 4000 Q TRAP mass spectrometer (Applied Biosystems). Tryptic peptides were loaded on to a C18 PepMap100, 3 μM (LC Packings) and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v/v) at flow rate of 0.3 μl/min and gradient elution time was 45 minutes. Data were analyzed using Mascot sequence matching software (Matrix Science) with Ludwig NR database and taxonomy set to human [26–29]. Changes were deemed significant at a false discovery rate (FDR) corrected p-value of <0.05 using a standard Benjamini-Hochberg correction [30–33]. Details of LC-MS data have been given in Table S5, Figures S1 to S9. Standard search parameters were: type of search, MS/MS ion search; enzyme, trypsin; external calibration, 100 ppm; variable modifications, oxidation (M); mass values, monoisotopic; protein mass, unrestricted; peptide mass tolerance, ±1.2 Da; fragment mass tolerance, ±0.6 Da, and up to 1 missed cleavage was allowed.

**Western Blot Analysis**

The whole tissue lysate of human endometrial samples from pre-receptive (LH+2) (n = 4), receptive phase (LH+7) (n = 4) endometrium of fertile women were prepared by homogenizing in RIPA buffer described by Awasthi et al (2007) [34]. 30 μg of protein/lane were run on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred on polyvinylidene difluoride membrane and blocked with 5% skimmed milk. Membranes were incubated overnight with primary antibody anti-Sorcin (39 M, sc-100859, Santacruz), anti-Ran (A7, sc-271376, Santacruz), anti-Apo-A1 (B-10, sc-376818, Santacruz) anti-Cofilin-1 (H-12, sc-32138, Santacruz) and anti-β-actin (N-21, sc-130656, Santacruz) of human origin and followed.
**Table 1.** Details of differentially expressed proteins identified by LC-MS analysis and comparison thereof with previous reports.

| S. No. | Sample ID | Protein ID | Protein identified by LC-MS analysis | Protein score | Matched peptide | Coverage | MW (KDa/PI) | Expression and fold change | Biological function | Reports from previous studies |
|--------|-----------|------------|-------------------------------------|---------------|-----------------|----------|-------------|---------------------------|---------------------|-----------------------------|
| 1      | 0         | IPI00306413| Tubulin-polymerization promoting protein family member 3 | 1086          | 488             | 0.579    | 19.14/10.07 | Down (1.65 fold)          | Cytoskeletal protein  | -                           |
| 2      | 1         | IPI00015148| Ras-related protein Rap-1b          | 713           | 426             | 0.152    | 21.03/5.39  | Up (1.58 fold)            | GTPase activity      | Up-regulated in endometriosis (Matsuzaki et al., 2005) |
| 3      | 7         | IPI00218733| Superoxide dismutase [Cu-Zn]        | 717           | 224             | 0.558    | 16.15/6.07  | Down (1.52 fold)          | Antioxidant system   | Up-regulated in mid-secretory phase of fertile women (Sugino et al., 1996); Up-regulated in LH+7 phase of fertile women (Riesewijk et al., 2003); Up-regulated in LH+7 phase of fertile women (Li et al., 2011) |
| 4      | 8         | IPI00027175| Sorcin                             | 229           | 223             | 0.186    | 21.94/5.21  | Down (2.97 fold)          | Ca<sup>2+</sup> signaling | Up-regulated in secretory phase of fertile women (Chen et al., 2009) |
| 5      | 9         | IPI00012011| Cofilin-1                          | 642           | 208             | 0.355    | 18.71/8.29  | Up (1.85 fold)            | Cytoskeletal protein  | Down-regulated in secretory phase of fertile women (DeSouza et al., 2005); Decreased secretion in uterine fluid of mid-secretory phase (Scotchi et al., 2009) |
| 6      | 10        | IPI00291922| Proteasome subunit alpha type-5     | 119           | 60              | 0.241    | 26.56/4.45  | Down (2.59 fold)          | Protein degradation   | Up-regulated in secretory phase of fertile women (DeSouza et al., 2005) |
| 7      | 11        | IPI00025252| Protein disulphide isomerase A3     | 1148          | 1354            | 0.316    | 57.14/6.28  | Up (1.50 fold)            | Molecular chaperone   | Down-regulated in secretory phase of fertile women (Rai et al., 2009) |
| 8      | 18        | IPI00643041| RAN GTP-binding nuclear protein (Ran) | 512           | 302             | 0.310    | 24.57/7.59  | Up (2.94 fold)            | Nucleo-cytoplasmic transport | Reported in proliferative and secretory phase endometrium in fertile women (Borthwick et al., 2003) |
by incubation with secondary horseradish peroxidase conjugated antibodies. Bands were detected using enhanced chemiluminescence detection system (Amersham Biosciences). Quantitation of band intensity was performed by densitometry using Quantity One software (v. 4.5.1) and a Gel Doc imaging system (Bio-Rad).

**Immunohistochemistry**

Immunohistochemical analysis was performed with minor modifications as described previously [34]. In brief, formalin-fixed and paraffin-embedded endometrial biopsies sections were deparaffinized and rehydrated. The sections were treated with 0.5% H₂O₂ in deionized water for 5 min to block endogenous peroxidase activity and were blocked with 5% bovine serum albumin along with normal goat serum for 2 h. followed by primary antibodies having reactivity in human 1:500, anti-Sorcin (39 M, sc-100859, Santacruz), anti-Ran (A7, sc-271376, Santacruz), anti-Apo-A1 (B-10, sc-376818, Santacruz) and anti-Cofilin-1 (H-12, sc-32158, Santacruz) for 48 h at 4°C temperature. For the negative control, mouse/goat IgG (Santacruz) was used in place of primary antibody. These sections were incubated with biotin labelled anti-mouse/anti-goat secondary antibodies (1:1000) (Sigma Aldrich) for 2 h at room temperature, followed by Streptavidin (1:1000) (Invitrogen) incubation for 1 h. Staining was achieved with 3, 3- diaminobenzidine (Sigma-Aldrich) for 5 min and counterstained by hematoxylin for 1 min after washing with double distilled water, slides were mounted with DPX (Sigma-Aldrich). The staining intensity of all these proteins in glandular epithelium, luminal epithelium and stromal compartment were quantified by image analysis software Image-Pro Plus 4.0 (Maryland, USA) and results were expressed as % image analysis score [34].

**Statistical analysis**

One-way ANOVA along with unpaired ‘t’ test was used to determine the significance of data between the two groups. The data are expressed as means ± SE, p value less than 0.05 was considered as significant.

**Results**

Proteomic profile of early-secretory (LH+2) and mid-secretory phase (LH+7) endometrium of women with unexplained infertility

Comparison of proteomic profile of early-secretory (LH+2) and mid-secretory phase (LH+7) endometrium of women with unexplained infertility were performed by 2D-PAGE analysis. Whole proteins of human endometrial tissue from early-secretory and mid-secretory phase, following 2D-PAGE, were resolved into a number of proteins in the molecular weight range of 15–130 kDa and pI of 3–10 (Figure 1). A total of 78 protein spots were observed and among them 18 protein spots were aligned in all gels. The proteins displaying significant (p<0.05) expression changes across all the gel images were considered to have altered expression levels having ≥1.5 fold change in their expression. Only 9 proteins showed significant abundance changes (FDR corrected p-value ≤0.05 [30]) between early- and mid-secretory endometrium (5 increased and 4 decreased in the mid- versus early-secretory phase) by using Image master-2D- platinum software version 7 (Figure 1).

These 9 consistently appearing protein spots were excised from the silver stained 2D gels and processed for identification by LC-MS analysis. Details of these differentially expressed proteins have been shown in Table 1 and Figure 2. The 5 increased proteins were identified as Ras-related protein Rap-1b, Protein disulfide isomerase A3, Apolipoprotein-A1 (Apo-A1), Cofilin-1 and RAN

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**Table 1. Cont.**

| Sample ID | Protein ID | Protein name | Protein score | Matched peptide Coverage | MW (kDa)/P | Expression and fold change | Biological function | Reports from previous studies |
|-----------|------------|--------------|---------------|-------------------------|------------|---------------------------|---------------------|-------------------------------|
| 9         | 23         | Apolipoprotein A1 | 2947          | 0.835                   | 30.7/55.5 | Up (1.7 fold)             | Lipid metabolism | Up-regulation in RIF patient during mid-secretory phase (Brosens et al., 2010); Decreased secretion in uterine fluid of mid-secretory endometrium of infertile women (Hannan et al., 2010); Decreased secretion in uterine fluid of mid-secretory endometrium of fertile women (Scotchi et al., 2009) |

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Figure 2. Various differentially expressed proteins. Bar diagram showing 9 differentially expressed proteins between early-secretory (LH+2) and mid-secretory (LH+7) phase endometrium from women with unexplained infertility. Values were expressed as mean ± SE, n = 8. P values are (b) \( p < 0.01 \) and (c) \( p < 0.05 \) versus early-secretory phase (LH+2). ES = early-secretory phase, MS = mid-secretory phase, LH+2 = 2 days after luteinizing hormone surge, LH+7 = 7 days after luteinizing hormone surge.

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Figure 3. Validation of Sorcin, Cofilin-1, Apolipoprotein-A1 (Apo-A1) and Ran GTP-binding nuclear protein (Ran) in infertile women. Expression of Sorcin, Cofilin-1, Apo-A1 and Ran were analyzed by western blot analysis in early-secretory (LH+2) and mid-secretory phase (LH+7) endometrium of infertile women, which had not been previously analyzed in the 2D-PAGE analysis. The results matched the findings of 2D-PAGE analysis. Representative images (upper panel) of immunoblot of Sorcin, Cofilin-1, Apo-A1 and Ran have been shown. \( \beta \)-actin served as a loading control for normalization. Quantification of band intensity (lower panel) was performed by densitometric analysis by using Quantity One software (v. 4.5.1) and a Gel Doc imaging system (Bio-Rad). P values are (b) \( p < 0.01 \) and (c) \( p < 0.05 \) versus early-secretory phase (LH+2). Values are expressed as mean ± SE; n = 4. ES = early-secretory phase, MS = mid-secretory phase, LH+2 = 2 days after luteinizing hormone surge, LH+7 = 7 days after luteinizing hormone surge.

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GTP-binding nuclear protein (Ran). Apart from this, the 4 decreased proteins were identified as Tubulin polymerization promoting protein family member-3, Superoxide dismutase [Cu-Zn], Sorcin, and Proteasome subunit alpha type-5.

To validate protein differences attained by 2D-PAGE, western blot analysis was performed for Sorcin, Cofilin-1, Apo-A1 and Ran GTP-binding nuclear protein (Ran) in another biopsy samples of early-secretory (n = 4) and mid-secretory phase (n = 4) endometrium from women with unexplained infertility. Expression of Sorcin was found significantly (p < 0.01) down-regulated in mid-secretory phase (LH+7) as compared to early-secretory phase endometrium. The expression of Cofilin-1, Apo-A1 and Ran were significantly (p < 0.01, p < 0.05) up-regulated in mid-secretory phase (LH+7) endometrium as compared to early-secretory phase (LH+2) endometrium from the women with unexplained infertility (Figure 3). The expression pattern of these proteins was found to be similar as observed in 2D-PAGE analysis.

In the endometrium of fertile women, the expression of Sorcin was significantly up-regulated in mid-secretory phase as compared to early-secretory phase whereas, the expression of Cofilin-1 and Apo-A1 was significantly down-regulated in mid-secretory phase. However, the expression of Ran was unaltered in both the phases of endometrium in fertile women (Figure 4).

Immunohistochemistry of Sorcin, Cofilin-1, Apolipoprotein-A1 and Ran GTP-binding nuclear protein

To validate the observed protein abundance changes between early-secretory (LH+2) and mid-secretory phase (LH+7) endometrium from infertile women, immunohistochemistry of Sorcin, Cofilin-1, Ran GTP-binding nuclear protein (Ran) and Apo-A1 were carried out on individual endometrial biopsies following fixation in formalin. Image analysis revealed the down-regulated expression of sorcin in stroma (3.33 fold), luminal epithelium (LE) (1.6 fold) and glandular epithelium (GE) (2 fold) of mid-secretory phase (LH+7) as compared to early-secretory phase (LH+2) endometrium of infertile women (Figure 5A-D). In case of Cofilin-1, up-regulated expression was observed in stroma (2.4 fold), LE (1.9 fold) and GE (2 fold) of mid-secretory phase (LH+7) as compared to early-secretary phase (LH+2) endometrium of infertile women (Figure 5E-H). Expression of Ran was up-regulated 2.32 fold in stromal cells, 1.86 fold in LE and 1.66 fold in GE of mid-secretary phase as compared to early-secretary phase endometrium of infertile women (Figure 5I-L). The expression of Apo-A1 was up-regulated in stromal cells (2.6 fold), LE (1.66 fold), and GE (1.57 fold) of mid-secretary phase as compared to early-secretary phase endometrium of infertile women (Figure 5M-P). The magnitude of difference in the expression of sorcin, cofilin-1, Ran and Apo-A1 were higher in
stroma than in LE and GE. No staining was observed in negative controls where primary antibody was substituted by control IgG (see inset in Figure 5A, E, I and M).

**Discussion**

We have for the first time compared the protein profile between early-secretory (LH+2) and mid-secretory phase (LH+7) endometrium of women with unexplained infertility (UI) by using 2D-PAGE followed by LC-MS analysis. Nine consistent differentially expressed proteins were identified in mid-secretory phase (LH+7) endometrium as compared to early-secretory phase (LH+2) endometrium of women with unexplained infertility (Figure 1, Table 1). We have also compared our findings with previously published reports on cyclical changes in various endometrial proteins from ‘pre-receptive to receptive’ phase as well as from ‘proliferative to secretory’ phase in normal fertile women (Table 1).

Ras-related protein Rap-1b is a small GTPase and member of Ras superfamily. Rap-1b serve as a molecular switch and regulate several cellular processes, such as cell growth, cell cycle, angiogenesis, and differentiation [35,36]. The increased expression of Rap-1b has been found in women with endometriosis as compared to early secretory phase endometrium from fertile...
women and, is regulated via RAS/RAF/MAPK and PI3K pathways [37]. In our study, endometrial expression of Rap-1b was increased during mid-secretory phase (LH+7) when compared to early-secretory phase (LH+2) in women with unexplained infertility. The increased expression of Rap-1b in mid-secretory phase of fertile women may be because of altered- or dysregulated- progesterone signalling and might also involve RAS/RAF/MAPK and PI3K pathways. However, the regulation of Rap-1b protein in relation to progesterone signalling is a matter of further investigation.

RAN GTP-binding nuclear protein (Ran), is also a member of Ras superfamily with GTPase activity and known to involved in nucleo-cytoplasmic transport of various protein and RNA across the nuclear pore [38,39]. Higher level of Ran has been reported during progression and development of ovarian cancer cells [40]. In a genomic profile based study, presence of Ran has been found in proliferative as well as secretory phase endometrium of fertile women [11]. However, cyclic changes in Ran expression and hormonal regulation thereof is notexplores as yet. In our study, the endometrial expression of Ran was found to be increased in mid-secretory phase as compared to that in early-secretory phase in unexplained infertility and the highest magnitude of change was observed in stromal cells. However, the expression of Ran was unaltered in fertile women in early- and mid-secretory phase endometrium. The increased expression of Ran during mid-secretory phase of fertile women might have a role in altering the progression and development of stromal cells of the endometrium.

Protein disulphide-isomerase A3 (PDIA3) is involved in the proper folding of proteins [41,42]. The regulation of PDIA3 via IL-11/STAT3 signalling in rat uterine stromal cells has been found [43]. Whereas, the down-regulation of PDIA3 by siRNA resulted in decreased activation of STAT3 which is involved in cell proliferation and growth [44]. In our study, the expression of PDIA3 was increased in mid-secretory phase as compared to early-secretory phase endometrium of women with unexplained infertility. In a report, the decreased expression of PDIA3 was found in the endometrium of fertile women during secretory phase as compared to proliferative phase [19]. However in our study, the increased expression of PDIA3 during receptive phase/mid-secretary phase endometrium of infertile women might induce IL-11/STAT3 signalling pathway, which in turn cause proliferation and growth of endometrial cells. Nevertheless, more functional studies are required to support our hypothesis.

Apolipoprotein-A1 has anti-inflammatory property, and has ability to inhibit the synthesis of inflammatory mediators and cell adhesion molecules that might play crucial role at the time of implantation [15,45,46]. Previous report revealed decreased secretion of Apo-A1 in the uterine fluid of mid-secretory endometrium as compared to early-secretory endometrium of fertile women [47]. In the current study, the expression of Apo-A1 was found to be increased in mid-secretory phase as compared to early-secretory phase endometrium in fertile women. Significantly increased immunoreactivity of Apo-A1 was found in stromal compartment, followed by LE and GE, in mid-secretory phase endometrium of infertile women. However, the expression of Apo-A1 in mid-secretory phase of fertile women was down-regulated over the early-secretary phase endometrium in case of fertile women. This indicates that the down-regulated expression of Apo-A1 might be responsible for decidualization of stromal cells in the normal fertile women. Furthermore, Brosens et al., (2010) have revealed higher expression of Apo-A1 in the mid-secretory phase endometrium which might be a cause for recurrent implantation failure [15]. In contrary, the decreased expression of Apo-A1 was found in uterine lavage of women with unexplained infertility [16]. This difference in the expression pattern of Apo-A1 may be because of variation in experimental sample i.e. uterine lavage and the endometrial tissue. Another study carried out on genomic profiling at the time of embryo implantation in women with unexplained infertility, has also revealed the differential (up-regulated as well as down-regulated) expression of genes involved in lipid metabolism [14]. We hypothesize that up-regulated expression of Apo-A1 during receptive/mid-secretary phase of endometrium might have altered the normal level of inflammatory as well as cell adhesion molecules which may be responsible for inadequate progression of stromal cells to decidual cells thus causing infertility.

Cofilin-1, a critical modulator of the actin cytoskeleton, is associated with cell adhesion, cell invasion and angiogenesis [48]. Dysregulation of actin reorganization is suggested to be one of the causative factors in endometriosis [49]. Earlier proteomic -based study by DeSouza et al., (2005) have revealed the down-regulated expression of Cofilin-1 in fertile women during secretory phase as compared to proliferative phase [50]. Decreased secretion of Cofilin-1 was reported in uterine fluid of mid-secretary endometrium as compared to early-secretory endometrium of fertile women [47]. In our study, the endometrial expression of Cofilin-1 was up-regulated in mid-secretary phase as compared to early-secretary phase in case of unexplained infertility. Immunolocalization experiments revealed the up-regulated expression of Cofilin-1 in stroma, GE and LE in mid-secretary phase as compared to early- secretory phase endometrium of women with unexplained infertility. Interestingly, the expression of Cofilin-1 was observed to be down-regulated in mid-secretary phase as compared to early-secretory phase endometrium in case of fertile women. The low expression of Cofilin-1 during mid-secretary phase might be necessary for proper endometrial responsiveness to progesterone. During the secretory phase of fertile women, expression of several cytoskeletal proteins becomes altered [19]. The increased expression of Cofilin-1 in receptive phase endometrium of infertile women might be because of improper actin polymerization and depolymerization [48]. Apart from this, another cytoskeletal protein, Tubulin polymerizing protein family member 3 (TPPP3) was also identified with decreased expression in mid-secretary phase endometrium than in early-secretary phase in these infertile women. The detailed investigation of role of tubulins in development and differentiation of secretory phase of the endometrium is further warranted.

Sorcin, a calcium-binding protein modulates ryanodine receptor function and intracellular calcium release [51]. Earlier proteomic approach- based study revealed the up-regulated expression of sorcin in secretory phase as compared to mid-proliferative phase in endometrium of fertile women [20]. In our study, the expression of Sorcin was down-regulated in mid-secretary phase as compared to early-secretary phase as observed in women with unexplained infertility. Immunolocalization experiment showed the down-regulated expression of Sorcin in stroma in mid-secretary phase as compared to early-secretary phase in case of infertile women. In contrast, the expression of Sorcin was up-regulated in the mid-secretory phase as compared to early-secretary phase in fertile women. The altered regulation of Sorcin might cause imbalance in Ca^{2+} levels [32] and might result in inadequate functioning of endometrium leading to infertility. Whereas in fertile samples the expression of Sorcin was found to be up-regulated suggesting its essential role in receptivity. Thus, Sorcin might be required in the development of secretory endometrium and might play crucial role during the embryo implantation. However, further studies are required to establish the functional role of Sorcin in embryo implantation.
Superoxides play important role in reproductive processes involving embryonic development and implantation [33,34]. In our study, in women with unexplained infertility, the expression of superoxide dismutase [Cu-Zn] was decreased during mid-secretory phase as compared to early-secretory phase endometrium. The earlier study in fertile women, revealed the higher activity of superoxide dismutase [Cu-Zn] during mid-secretory phase [55]. In a genomic profiling- based study, the increased expression of mitochondrial superoxide dismutase-2, was observed in receptive phase (LH+7) endometrium as compared to pre-receptive phase (LH+2) endometrium of fertile women [10]. In a proteomic study, up-regulation of superoxide dismutase (Mn) was found in the receptive phase as compared to pre-receptive phase endometrium of fertile women [13]. Furthermore, the reduced fertility rate has been found in female mice lacking superoxide dismutase [Cu-Zn] [56,57]. It appears that reduced level of superoxide dismutase [Cu-Zn] during mid-secretory phase endometrium may be responsible for dysfunctional antioxidant system in infertile women. This suggested the possibility of endometrial receptivity improvement via augmenting antioxidant defences.

Proteasome subunit alpha type-5 (PSMA5) is a subunit of proteasome complex and participates in protein degradation [58,59]. Since efficient protein degradation is critical for cell cycle progression and apoptosis [60]. In fertile women, the phenomenon of apoptosis in endometrium is common during secretory phase [61]. There might be a possibility of involvement of proteasomal complex in degradation of several proteins involved at the time of apoptosis during secretory phase [62]. A 2.1 fold higher ratio of PSMA5 was found in endometrium at secretory phase over proliferative phase in case of fertile women [50]. These evidences suggest the possible role of PSMA5 in apoptosis during secretory phase. In our study, PSMA5 expression was decreased in mid-secretory phase as compared to early-secretary phase endometrium. It may be speculated that the reduced expression of PSMA5 in women with unexplained infertility, during mid-secretory phase of the cycle, slows down the process of apoptosis and might cause improper differentiation of endometrial cells. However, future in-depth studies are required to confirm the role of PSMA5 during apoptosis in human endometrium.

Thus, 2D-PAGE analysis followed by LC-MS analysis of the endometrial proteins in women with unexplained infertility explored the proteins that may have functional significance during transition from early-secretory (pre-receptive) (LH+2) to mid-secretary (receptive) phase (LH+7) of the menstrual cycle. However, further functional studies will be required to define those proteins important for explaining the molecular basis of infertility. To conclude, the analysis of endometrial proteins provided a general survey of changes in protein expression which represents the first step toward elucidating the definite molecular conditions during unexplained infertility. Differential expression of certain proteins during mid-secretory phase may be responsible for endometrial receptivity-related defects and/or alteration in the process of endometrial differentiation during pre-receptive to receptive phase transition. Taken together, the results of this study might be helpful in understanding and exploring the molecular mechanism involved in unexplained infertility. In addition, it opens avenues for search of role of these proteins as novel players in endometrial receptivity.

Supporting Information

Figure S1 Details of LC-MS analysis of Tubulin-polymerization promoting protein family member-3. (A) unique spectrum number, (B) unique peptide number, (C) Score delta (D), Charge, (E) fragment ion mass/Da, (F), peptide mass delta/Da.

Figure S2 Details of LC-MS analysis of Ras-related protein Rap-1b. (A) unique spectrum number, (B) unique peptide number, (C) Score delta (D), Charge, (E) fragment ion mass/Da, (F), peptide mass delta/Da.

Figure S3 Details of LC-MS analysis of Superoxide dismutase [Cu-Zn]. (A) unique spectrum number, (B) unique peptide number, (C) Score delta (D), Charge, (E) fragment ion mass/Da, (F), peptide mass delta/Da.

Figure S4 Details of LC-MS analysis of Sorcin. (A) unique spectrum number, (B) unique peptide number, (C) Score delta (D), Charge, (E) fragment ion mass/Da, (F), peptide mass delta/Da.

Figure S5 Details of LC-MS analysis of Cofilin-1. (A) unique spectrum number, (B) unique peptide number, (C) Score delta (D), Charge, (E) fragment ion mass/Da, (F), peptide mass delta/Da.

Figure S6 Details of LC-MS analysis of Proteasome subunit alpha type-5. (A) unique spectrum number, (B) unique peptide number, (C) Score delta (D), Charge, (E) fragment ion mass/Da, (F), peptide mass delta/Da.

Figure S7 Details of LC-MS analysis of Protein disulfide isomerase A3. (A) unique spectrum number, (B) unique peptide number, (C) Score delta (D), Charge, (E) fragment ion mass/Da, (F), peptide mass delta/Da.

Figure S8 Details of LC-MS analysis of RAN GTP-binding nuclear protein (Ran). (A) unique spectrum number, (B) unique peptide number, (C) Score delta (D), Charge, (E) fragment ion mass/Da, (F), peptide mass delta/Da.

Figure S9 Details of LC-MS analysis of Apolipoprotein-A1. (A) unique spectrum number, (B) unique peptide number, (C) Score delta (D), Charge, (E) fragment ion mass/Da, (F), peptide mass delta/Da.

Table S1 Inclusion criteria for selection of infertile women. (DOCX)

Table S2 Exclusion criteria during selection of infertile women subjects. (DOC)

Table S3 Details of infertile and fertile women. (TIF)

Table S4 Spot volume of differentially expressed proteins. (XLS)

Table S5 Details of LC-MS analysis of 9 differentially expressed protein spots. (XLS)

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Author Contributions

Conceived and designed the experiments: MM AD. Performed the experiments: MM VKS HK. Analyzed the data: MM VKS AD. Contributed reagents/materials/analysis tools: CDRI Proteomics. Wrote the paper: MM AD.

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