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Proteomic Identification and Analysis of Human Endometrial Proteins Associated with Unexplained Infertility

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Abstract

Unexplained infertility (UI) represents about 25-30% of all known types of female infertility. To date, UI remains an unsolved problem in a subgroup of infertile but otherwise healthy women and the molecular causes of UI are not yet known. In the current study, we have compared and analyzed the proteomic profile of receptive phase (LH+7) endometrium from fertile and infertile women, with a view to identify the appropriate protein target signatures that may be responsible for defective endometrial receptivity as a cause of UI. 12 Differentially expressed proteins (8 up-regulated and 4 down-regulated) were identified by Liquid Chromatography-Mass Spectrometric analysis. These differentially expressed proteins were involved in immunological response, glycolytic pathway, lipid metabolism, blood agglutination, protein synthesis, molecular chaperone, antioxidant system, mitochondrial ATP generation, and Ca^{+2} signalling. The expression of four differentially expressed proteins such as HSPβ-1, Apolipoprotein-A1, IGK@ protein, and RPLP2 were further validated by immunoblotting and immuno-histochemical analysis in separate biopsy samples, and also in *in-vitro* experimental model of decidualization of human endometrial stromal cells (hESCs). These proteins may have functional significance as regards the initiation and maintenance of the window of receptivity. Results of this study might be helpful in understanding the molecular basis of endometrial defects in a subset of infertile women having normal ovulation and hormonal profile. To our understanding, this is the first study to demonstrate the differential endometrial protein profiling in women with endometrium based-unexplained infertility.

Keywords: Apolipoprotein A1; Endometrial receptivity; Infertility

Introduction

Human endometrium is a highly dynamic tissue and remodelled during menstrual cycle under the influence of steroidal hormones. Corresponding to mid-secretory phase of the cycle on day 20 to 24 or seven to nine days after ovulation, endometrium becomes receptive to the blastocyst [1,2]. This 'endometrial receptivity' is regulated via an intricate network of various signalling. Any alteration at the molecular level during receptive phase of endometrium, leads to infertility, implantation failure and early pregnancy loss [3]. There are several known reasons for infertility such as ovulation disorder, endometriosis, uterine fibroid, tubal blockage, polycystic ovarian syndrome, and male factor infertility [4]. Reported data have shown that unexplained infertility represents about 25-30% of all known types of female infertility [5]. The extent to which endometrial receptivity is perturbed in infertile patients without clear pathology is as yet not well defined [6].

In order to find out the actual molecular factors responsible for unexplained infertility, only a few investigations have been carried out so far. By using Visualization and Integrated Discovery functional analysis, a substantial number of dysregulated genes in the endometria of infertile women, involved in cellular localization, transport, transporter activity, with major localization in extracellular regions, have been detected [7]. Furthermore, the proteomic analysis of timed endometrial samples from fertile women and patients with unexplained recurrent implantation failure (RIF) yielded a distinct proteomic fingerprint that may discriminate between receptive and un-receptive endometrium [8]. In order to get more relevant causes of unexplained infertility, the proteomic profiling of uterine lavage provided a new understanding about the role of (antithrombin III and alpha-2-macroglobulin) whose altered expression appeared to be involved in the development of unexplained infertility [9]. However, the causes of unexplained infertility and the factors responsible for implantation failure still remain largely unknown.

In the current study, with an aim to gain knowledge about appropriate protein targets that are responsible for defective endometrial receptivity as a cause of unexplained infertility, the endometrial proteomic profile of women with unexplained infertility has been analyzed and compared with that of fertile women during receptive phase i.e. mid-secretory phase (LH+7) of the menstrual cycle. The study has explored various proteins that may have functional significance in endometrium and may be responsible for defective endometrial receptivity as a cause of unexplained infertility.

Material and Methods

Patient details

Human endometrial biopsies were collected during receptive phase (LH+7 i.e. 7 days after the LH peak occurred) from fertile and infertile women with unexplained infertility, aged 23-36 year, in the operating room of the Department of Obstetrics and Gynecology, King George's

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Medical University, Lucknow. Only infertile women having normal ovulation and normal hormonal profile were considered in this study. A specific written consent was obtained from each patient, and the study was approved by the Institutional Ethics Committee.

All the women (fertile/ infertile) involved in this study had normal ovulation cycle and had normal hormonal profile as determined by immunoenzymatic assay [10]. Hormonal profile was determined at receptive phase (LH+7) of fertile and infertile women The serum hormonal profile of fertile women was: E2=101 ± 2.54 pg/ml, P4=14.35 ± 1.29 ng/ml; whereas the hormonal profile of infertile women was : E2=98.58 ± 2.10 pg/ml, P4 =16.04 .2 ± 2.74 ng/ml.

The women with primary infertility were considered for this study. The complete examination and investigation of all infertile couples were carried out. All infertile women were aged 23-35 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 vaginosis 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

Primary infertility is an inability to have any live birth, whereas, secondary infertility is an inability to have an additional live birth. All infertile women, presented with primary infertility were screened for uterine abnormalities like leiomyomas, polycystic ovarian syndrome, endometriosis, hydrosalpinx, acute infection PID, vaginitis, male factor infertility or those who had received steroid hormone therapy in the last six months, were excluded.

The fertile women had proven parity and were presenting for tubal ligation (n=7) or assessment for reversal of tubal ligation (n=5). All patient groups were age matched and cycling (Supplementary Table-1).

Sample collection

The detection of LH in morning urine (Donacheck ovulacion; Novalab Iberica, S.A.L., Coslada, Madrid, Spain) was used to determine the day of the LH surge (day LH+ 0) and then after 7 days biopsies were collected.

Endometrial biopsies were taken using pipelle catheters (Genetics, Belgium) under sterile condition (procedure takes 30 seconds) and immediately frozen at -80°C until used. Samples were collected from fertile (n=12) and infertile women (n=12) at LH+7 day of cycle for proteomic analysis. Additional 32 endometrial biopsy samples (16 from fertile women and 16 from infertile women) were collected for the validation of proteomic findings by immunoblotting and immunohistochemical analysis. However, these 32 endometrial biopsy samples were not used in proteomic analysis. Hence, these 32 endometria biopsy samples were termed as "additional endometrial biopsies". The proteomic analysis data was from initial LH+7 samples in the 24 patients (12 form fertile women and 12 from infertile women) and data of validation of 4 proteins viz. IGK@protein, Apo-A1, HSPβ-1 and RPLP2 were form additional 16 +16 biopsies. Histological dating of each sample was performed in order to confirm the menstrual cycle stage [11].

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-pH 8.5), 1 mM EDTA, containing 50 mM DTT). The whole tissue homogenate contained luminal epithelium, glaundular epithelium, stroma, and vessels. Tissue homogenate was centrifuged and protein estimation was done [12].

Isoelectric focusing

IPG strips (11 cm) were rehydrated with proteins (400 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 [13,14]. 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 [13,15]. Gels were fixed in methanol: acetic acid: water in the ratio of 50: 5: 45, for 2 h. Gels were rinsed with double distilled water (DDW) twice and left 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% $AgNO_3$ solution in dark for 30 min and spots were developed by a solution of (2%) Na_2CO_3 and 35% HCHO for 5 min. 1% Acetic acid was used immediately to avoid the over staining of gels. Coommassie 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 procedure for analysis/staining was used to minimize the variations arising due to 'sample loading', 'staining procedures' and 'run-to-run' variability.

Image capture and analysis

The silver stained 2-D gels of fertile (n=12; in duplicates) and infertile (n=12; in duplicates) endometrium were scanned and analyzed by using image master-2D-Platinum software version 7. The pooling of samples for proteomics minimizes the internal variations. With this view, a total of six independent experiments with pooled samples were performed. All gel images were aligned properly, calibrated and normalized using Image Master 2D platinum software (Amersham Bio- sciences) to allow for quantitative comparison between gels. Total 12 gels were prepared (six for each group) and six set of experiments were run. The volume of each spot was normalized against the sum total of volume of all detectable spots in the 2D gel, this normalization was performed by Image Master 2D platinum software that corrects for any minor differences in protein loading among replicate gels. The background in silver-stained gels has been subtracted at the time of gel analysis with the help of Image Master 2D platinum software to avoid any possible error over protein spot intensity. The volume of all the spots from independent sets of experiments were taken. To calculate fold change, the spot volume of fertile group was divided with spot

volume of infertile group. Only protein spots that changed ≥ 1.5-fold were consistently altered in the same manner in all six experiments were considered to be differentially altered. Experimental setup has been shown in Supplementary Table 2.

Protein identification by liquid chromatography-mass spectrometric analysis (LC-MS)

The gel pieces were digested with trypsin and cut into size of 1 mm, washed with 500 µl of H_2O followed by 500 µl of 25 mM ammonium bicarbonate in 50% acetonitrile for 60 min. The gel pieces were dehydrated by the adding 500 µl of acetonitrile. Disulfide bonds were cleaved by incubating the samples for 60 min at 56°C with 200 µl of 10 mM DTT in 25 mM ammonium bicarbonate buffer. Alkylation of cysteines was performed by the addition of 200 µl of 55 mM iodoacetamide in 25 mM ammonium bicarbonate buffer and incubation of the samples for 45 min at room temperature in darkness. 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). Spectra were analyzed to identify the proteins of interest (Supplementary Figures 1-4) using Mascot sequence matching software (Matrix Science) with Ludwig NR database and taxonomy set to human [16,17]. 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 of receptive phase from fertile (n=16; in duplicate), infertile groups (n=16; in duplicate) and from primary culture of human endometrial stromal cells (hESCs) were prepared by homogenizing in RIPA buffer as described earlier [18]. 30-40 µg of protein/lane were run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred on polyvinylidene difluoride membrane and blocked with 5% skimmed milk. Membranes were incubated with primary antibody (1:1000) i.e. mouse monoclonal HSP-27 (G3.1, sc-59562, Santacruz), mouse monoclonal apolipoproteinA1 (B-10, sc-376818, Santacruz), mouse monoclonal IGK@protein (MEM-09, sc-51637, Santacruz), rabbit polyclonal RPLP2 (ab103103, Abcam), and mouse monoclonal Prolactin (6F11, MA1-10597, Thermo Scientific) followed by HRP-conjugated secondary antibodies. Bands were detected using ECL-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).

Immunohistochemical analysis

Immunohistochemical analysis was performed with minor modifications as described previously [18]. In brief, formalin-fixed and paraffin-embedded endometrial biopsies sections were de-paraffinized and rehydrated. The sections were treated with 0.5% H_2O_2 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 2h followed by primary antibodies having reactivity in human 1:500 i.e. mouse monoclonal HSP-27 (G3.1, sc-59562), mouse monoclonal apolipoproteinA1 (B-10, sc-376818), mouse monoclonal IGK@ protein (MEM-09, sc-51637) (Santacruz), and rabbit polyclonal RPLP2 (ab103103) (Abcam) for 48h at 4°C temperature. For the negative control, mouse/rabbit IgG (Santacruz) was used in place of primary antibody. These sections were incubated with biotin labelled anti-mouse/anti-rabbit secondary antibodies (1:1000) (Sigma Aldrich) for 2 h at room temperature, followed by Streptavidin (1:1000) (Invitrogen) incubation for 1h. Staining was achieved with 3, 3-diaminobenzidine (Sigma-Aldrich) for 5 min and counterstained by hematoxylin for 1 min and washing with double distilled water, slides were mounted with DPX (Sigma-Aldrich). The captured images were analysed using colour discriminating software (Image Pro Plus 4.0, Maryland, USA). Scoring of immunostaining was done on the digitalized images. Five fields were analysed and measured individually in each section for stromal cells, luminal epithelium, and glandular epithelium from each group for analysis. Using software, the total area of positively stained nuclei (brown) was calculated and expressed as a ratio of the total area of the cell nuclei (brown + blue) and results were expressed as % image analysis score [18].

Primary human endometrial stromal cell isolation, culture and *in-vitro* decidualization of human endometrial stromal cells (hESCs)

For the isolation of stromal cells, endometrial biopsies were collected from pre-menopausal women (n=3), aged 35-40 years, undergoing hysterectomy for benign indications (prolapsed of uterus) who had not been on any hormonal therapies for a minimum three months prior to surgery. Isolation and culture of human endometrial stromal cells (hESCs) were performed as described previously [19]. The purity of the stromal preparations were confirmed by positive cellular staining for vimentin (Supplementary Figure 5). *In-vitro* decidualization was performed as described previously [20]. Briefly, after 80% confluence, cells were rinsed and treated with 2.5% charcoal-stripped fetal bovine serum in the presence of estradiol (E2) (10 ng/ml, 36.7 nM)), and progesterone (P4) (100 ng/ml, 318 nM). Culture media were changed every 3rd day. Control hESCs were cultured in parallel over 9 days without hormonal treatment. Expression of decidual marker prolactin was checked by immunoblotting. All results from hESCs in *in-vitro* studies were analyzed by using primary hESCs obtained from at least three independent biological replicates (n=3).

Statistical analysis

Statistical approach was applied for determining significance of data between the two groups by using One-way ANOVA along with unpaired t test. The data were expressed as means ± SE, and 'p' values less than 0.05 were considered as significant.

Results

Expression of receptivity marker, β3 integrin

β3 integrin has been used as a well-known endometrial receptivity marker in several studies and abnormal expression of β3 integrin caused unexplained infertility and recurrent pregnancy loss in women [21-23]. Endometrial biopsy samples were checked for expression β3 integrin in fertile and infertile women. Results showed that β3 integrin

expression was found to be significantly down regulated in infertile women (Supplementary Figure 6).

Proteomic analysis of endometrium during receptive phase, from fertile and infertile women with unexplained infertility

Comparison of proteomic profile of receptive phase (LH+7) endometrium between fertile and infertile women was performed by 2D-PAGE. Only proteins displaying significant expression changes ($p < 0.05$) across all gel images were considered to be present at altered levels. These altered proteins had ≥ 1.5 fold change (up-regulated or down-regulated) in their expression between the two groups (Table 1). Whole proteins of human endometrial tissue from fertile and infertile women, following 2D-PAGE was resolved into a number of proteins in the molecular weight range of 10-170 kDa and pI between 3-10. A total of 12 differentially altered protein spots (8 up-regulated and 4 down-regulated) were identified by Image Master-2D- Platinum 7 software (GE Healthcare) (Figure 1). These 12 consistently appearing protein spots were excised from the gels and analyzed for identification by LC-MS. Details of these 12 differentially expressed proteins were shown in Table 1. 8 Up-regulated proteins were identified as triose phosphate isomerase, heat shock protein beta-1, apolipoprotein A1, cDNA clone FLJ60461, highly similar to peroxiredoxin-2, cDNA FLJ33589 fis, clone BRAMY2012536, highly similar to human asparaginase like 1 (ASRGL1), cDNA, FLJ96792, highly similar to human calmodulin 2 (phosphorylase kinase, delta) (CALM2), mRNA, hemoglobin subunit beta, and IGK@ protein. Apart from this, 4 down-regulated proteins were identified as ATP synthase subunit d, Cold agglutinin FS-2 L-chain (Fragment), Uncharacterized protein, and 60S acidic ribosomal protein P2 (RPLP2) (Figure 2).

Western blot analysis of IGK@Protein, heat shock protein beta-1, Apolipoprotein-A1, and 60S acidic ribosomal protein P2

To validate protein differences analyzed by 2D-PAGE analysis, immunoblot analysis was performed for randomly selected differentially expressed proteins such as, IGK@Protein, HSPβ-1, Apo-A1 and RPLP2 in samples from fertile and infertile women outside the sample cohort (Figure 3). β-Actin was used as a loading control to normalize protein abundance in all the experiments. Expression of IGK@Protein, HSPβ-1, Apo-A1 protein were found significantly up-regulated ($p<0.05$) whereas expression of RPLP2 was significantly down-regulated ($p<0.01$) in infertile women as compared to fertile women. The patterns of the expression of these proteins were also similar as observed in 2D-PAGE.

Immuno-histochemistry of IGK@Protein, heat shock protein beta-1, Apolipoprotein-A1, and 60S acidic ribosomal protein P2

To validate the observed protein abundance changes between fertile and infertile endometrium immunohistochemical analysis of IGK@

Spot ID	MS job run and PI-number	Protein identified by LC- MS analysis	Expression and fold change	P value versus fertile group	Mol. Wt. and pI	Moscot score	Matched peptide	Coverage (%)	Biological function	Existing information	Reference
0	111207-2626A	ATP synthase subunit d	Down (1.51 fold)	p<0.05	18.4 kDa, 5.21	206	11	65	Mitochondrial ATP generation	Down-regulated in endometriosis	[30]
3	111207-2626B	Triose phosphate isomerase	Up (1.56 fold)	p<0.05	26.6 kDa, 7.36	635	39	-	Glucose metabolism	Secreted in uterine fluid of early and mid-secretory endometrium of fertile women	[28]
4	111207-2626C	Heat shock protein beta-1	Up (1.61 fold)	p<0.05	22.7 kDa, 5.98	317	21	49	Molecular chaperone	Altered secretion in uterine fluid from early to mid-secretory endometrium of fertile women Increased expression in mid-secretory phase as compared to mid-proliferative phase endometrium of fertile women Down regulated mRNA expression during the window of implantation	[28] [29] [27]
5	111207-2626D	Cold agglutinin FS-2 L-chain (Fragment)	Down (1.53 fold)	p<0.05	25.7 kDa, 5.94	147	18	-	Blood agglutination	-	
6	111207-2626E	Apolipoprotein A1	Up (2.67 fold)	p<0.01	30.7 kDa, 5.56	793	71	78	Lipid metabolism	Up-regulated in the endometrium of RIF patients Down-regulated in uterine lavage in women with unexplained infertility	[8] [9]
8	111207-2626F	cDNA FLJ60461, highly similar to Peroxiredoxin-2	Up (1.53 fold)	p<0.05	20.0 kDa, 8.90	209	15	49	Antioxidant system	Down-regulated in endometriosis Decreased secretion in the uterine fluid from early to mid-secretory endometrium of fertile women	[30] [28]
11	111207-2626G	Uncharacterized protein	Down (1.50 fold)	p<0.05	22.7 kDa, 8.51	95	5	20	-	-	
13	111207-2626H	cDNA clone BRAMY2012536, highly similar to Homo sapiens asparaginase like 1 mRNA	Up (1.57 fold)	p<0.05	32.0 kDa, 5.75	108	4	16	Amino acid metabolism	-	
15	111207-2626I	cDNA, FLJ96792, highly similar to Homo sapiens calmodulin 2 (CALM2),mRNA	Up (1.55 fold)	p<0.01	16.7 kDa, 4.09	99	5	41	Ca^{+2} signaling	-	
18	111207-2626J	Hemoglobin subunit beta	Up (1.76 fold)	p<0.05	16.0 kDa, 6.75	528	41	74	Oxygen transport	Less secretion in the uterine fluid from early to mid-secretory endometrium of fertile women	[28]
19	111207-2626K	IGK@ protein	Up (2.07 fold)	p<0.01	26.0 kDa, 5.94	187	11	22	Immune system	Decreased secretion in the uterine fluid from early to mid-secretory endometrium of fertile women	[28]
23	111207-2626L	60S acidic ribosomal protein P2	Down (2.21 fold)	p<0.01	11.6 kDa, 4.42	150	5	60	Protein synthesis	-	

Table 1: Differentially expressed proteins identified by LC-MS analysis in the endometrium of infertile women.

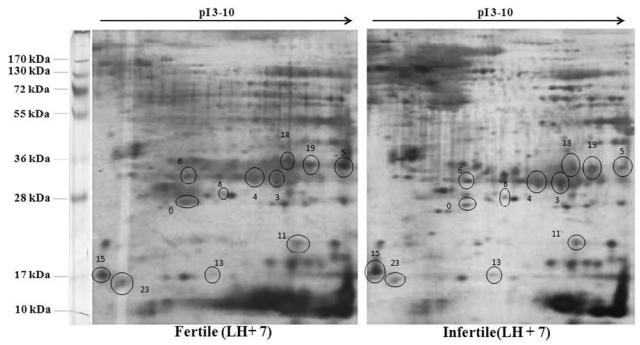

Figure 1: Two dimensional gel electrophoresis of human endometrium from fertile (LH+7) and infertile (LH+7) women. Representative gel images are shown. 12 differentially altered protein spots were identified by image master 2D platinum software and number denotes spot ID (0-23). The first dimension was performed by IEF on IPG strips over a range of pI 3-10, the second dimension on 12.5% SDS-PAGE gels and the proteins were visualized by silver staining.

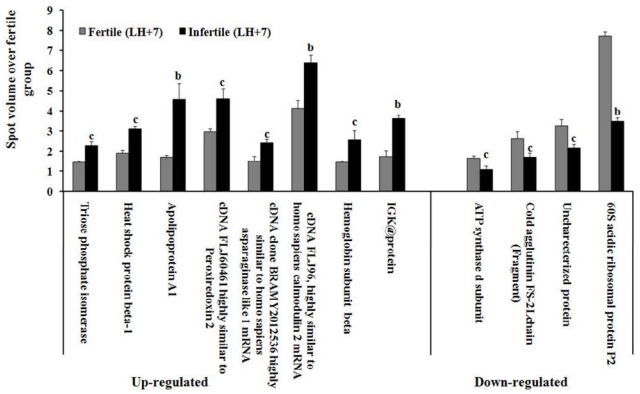

Figure 2: Bar diagram showing differentially expressed proteins between fertile (LH+7) and infertile (LH+7) human endometrium and clustered as up-regulated and down-regulated. P values are (b) p<0.01 and (c) p<0.05 versus fertile group. Values are expressed as mean ± SE, n=6.

Protein, HSPβ-1, Apo-A1, and RPLP2 were carried out on individual endometrial biopsies following fixing in formalin. Image analysis revealed that expression of IGK@protein was increased in stroma (2.63 fold), luminal epithelium (LE) (1.93 fold) and glandular epithelium (GE) (1.72 fold) of infertile women as compared to stroma, LE, and GE of fertile women (Figure 4 A-D). In case of HSPβ-1, increased expression was observed in stroma (1.82 fold), LE (1.53 fold) and GE (1.44 fold) of infertile women as compared to fertile women (Figure 4 E-H). Expression of Apo-A1 was enhanced 2.84 fold in stromal cells, 1.80 fold in LE and 1.63 fold in GE of infertile women as compared to fertile women (Figure 4 I-L). Reduced expression of RPLP2 was obtained in stroma (2.10 fold), LE (1.64 fold) and GE (1.55 fold) of infertile women as compared to that of fertile women (Figure 4 M-P). The magnitude of difference in the expression of IGK@protein, HSPβ-1, Apo-A1 and RPLP2 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 4 A, E, I and M).

Validation in *in-vitro* decidualization model

The validation of expression of IGK@Protein, Apolipoprotein-A1, and 60S acidic ribosomal protein P2 were also performed in *in-vitro* decidualization model of human endometrial stromal cells by immunoblotting and results were compared with that of non-decidualized hESCs. Immunoblot analysis revealed significantly increased ($p<0.001$) expression level of decidualization marker protein prolactin (PRL) after the hormonal treatment (Figure 5). The significantly less expression of IGK@Protein ($p<0.05$), and Apolipoprotein-A1 ($p<0.01$) was observed in decidualized hESCs than that in normal hESCs. However, the expression of RPLP2 was found to be significantly high ($p<0.01$) in decidualized hESCs as compared to that of control hESCs (Figure 5).

Discussion

In this study, the proteomic approach was used to explore the novel endometrial proteins that are involved in unexplained infertility. The endometrial biopsy samples used for protein analysis contained epithelial cells, supportive stromal cells, glandular epithelium, and also the vessels that are not expected to be affected by the endometrial cycle. We have established endometrial proteomic profile at the time of embryo implantation in women with unexplained infertility by using 2D-PAGE followed by LC-MS analysis. A total of 12 proteins (8 up-regulated and 4 down-regulated) with altered expression have been identified in infertile women by comparative analysis with the profile of fertile women. These differentially expressed proteins were involved in immunological response, glycolytic pathway, lipid metabolism, blood agglutination, protein synthesis, molecular chaperone, antioxidant system, mitochondrial ATP generation, and Ca^{+2} signalling (Table 1). Among these, 4 proteins viz. heat shock protein beta-1, apolipoprotein A1, IGK@ protein and 60S acidic ribosomal protein P2 were randomly selected for further validation and expression with a view to illustrate and analyze the cellular location thereof.

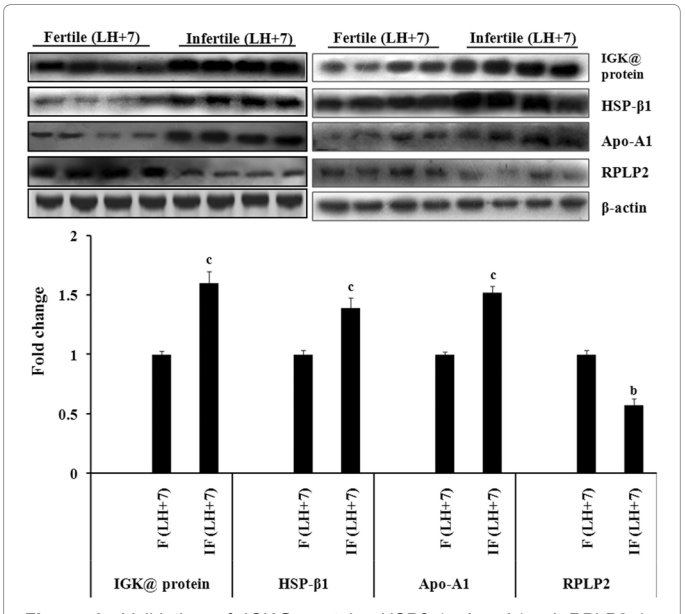

Figure 3: Validation of IGK@ protein, HSPβ-1, Apo-A1and RPLP2 by western blot analysis in fertile (LH+7) and infertile (LH+7) human endometrial biopsy samples which had not been previously analyzed in the 2D-PAGE analysis. The results matched the previous ones. Representative images (upper panel) of immunoblot of IGK@ protein, HSPβ-1, Apo-A1and RPLP2 have been shown here. β-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 fertile group. Values are expressed as mean± SE; n=8.

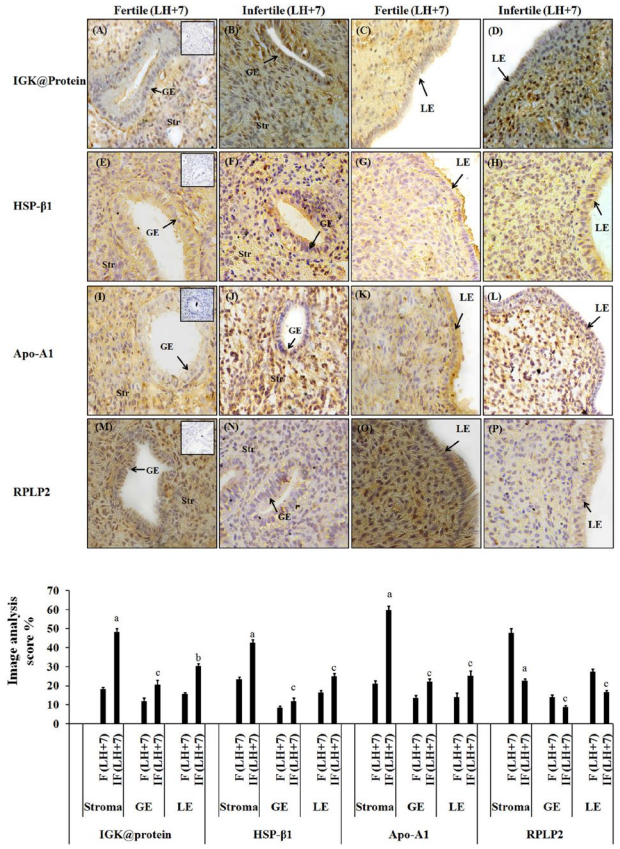

Figure 4: Representative images (upper panel) showing immunohistochemical localization of IGK@ protein, HSPβ-1, Apo-A1and RPLP2 proteins in fertile (LH+7) and infertile (LH+7) human endometrium. For negative controls (see inset in A, E, I, M) mouse-IgG/ rabbit IgG was used in place of primary antibody. Lower panel shows image analysis of IGK@protein, HSPβ-1, Apo-A1, and RPLP2 in fertile and infertile endometrium. Staining intensity of all these proteins were quantified by image analysis software Image-Pro Plus 4.0 (Maryland, USA). Str (Stroma), GE (glandular epithilium), and LE (luminal epithelium). Magnification X 400, bar=25 μm, n=5 in all the groups.

The higher expression of heat shock proteins increases cell survival by protecting and disaggregating stress-labile proteins [24,25]. In our study, the expression of HSPβ-1was found to be up-regulated in infertile women as compared to that in fertile women and the highest magnitude of change was observed in stromal cells. The altered level of HSPβ-1 might be responsible for development of refractory endometrium, leading to infertility. Interestingly, in normal fertile women, the decreased expression of HSP-27 was found on 7th day post -ovulation as compared to that on 2nd day post-ovulation of menstrual cycle [26]. Similarly, the global gene profiling of endometrium revealed the down-regulated mRNA expression of heat shock factor binding protein-1 (HSBP-1) in mid-secretory phase as compared to late proliferative phase of the cycle in fertile women [27]. The secretion of HSPβ-1 in the uterine fluid of early to mid-secretory phase was found to be altered in fertile women [28]. The increased expression of 4 isoforms of HSPB-1 in the mid-secretory phase as compared to mid-proliferative phase in fertile women has also been reported [29]. Since HSPβ-1 is regulated by estrogen in endometrium [25], it is probable that increased expression of HSPβ-1 leads to the proliferation of endometrial cells and these cells may not be further differentiated by progesterone and this situation might lead to infertility. No such information is available in endometrial defect and UI, except a recent report has suggested the up-regulated expression of HSP-27 during the progression of various stages of endometriosis [30].

Apo-A1 is the major constituent of high-density lipoprotein and primary acceptor for cholesterol in extra-hepatic tissues [31]. In this study, the expression of Apo- A1 was found to be up-regulated in infertile women as observed during the receptive phase of endometrium in fertile women. Significantly increased immunoreactivity of Apo-A1 was found in stromal compartment, followed by GE and LE, in infertile women. In *in-vitro* experiment, the decreased expression of Apo-A1 was found in decidualized cells as compared to non-decidualized stromal cells. Apo-A1 has anti-inflammatory property, and has the ability to inhibit the synthesis of inflammatory mediators and cell adhesion molecules that might play crucial role at the time of implantation [8,32,33]. Owing to its role in vascular biology, it is tempting to consider that aberrant endometrial secretion of this lipoprotein contributes to implantation failure. We hypothesize that up-regulation in Apo-A1expression might have role in altering the level of inflammatory and cell adhesion molecules which may be responsible for inadequate progression of stromal cells to decidual cells, thus causing infertility. Earlier study conducted on proteomic profiling of mid-secretory endometrium (LH+5 to LH+10) had revealed a 4.2-fold higher expression of Apo-A1 in RIF group than in control group, further strengthens this hypothesis [8]. Contrary to this, the proteomic study performed on uterine lavage of infertile women has shown down-regulation of apolipoprotein-A1 [9]. This difference in the expression of Apo-A1 may be because of variation in experimental samples i.e. uterine lavage and the endometrial tissue. Another study carried out on genomic profiling at the time of embryo implantation in women with UI, has also revealed the differential (up-regulated as well as down-regulated) expression of genes involved in lipid metabolism [7].

IGK@ protein is also known as immunoglobulin kappa locus

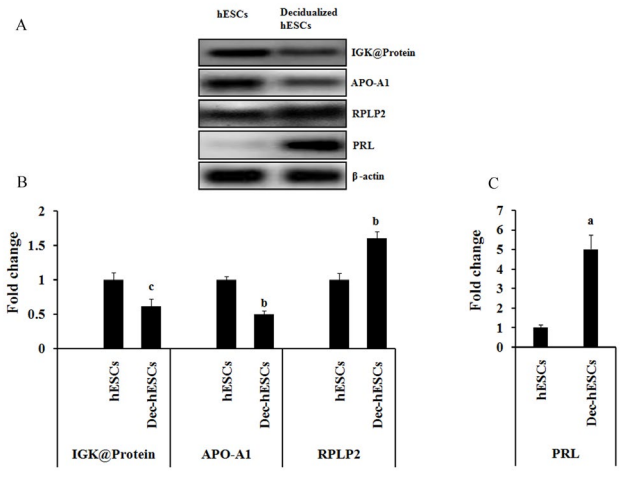

Figure 5: Western blot analysis of IGK@ protein, Apo-A1, RPLP2 and Prolactin during *in-vitro* decidualization of hESCs. Expression of decidual marker prolactin was checked in hESCs and decidualized hESCs (A). Representative images of immunoblot of IGK@ protein, Apo-A1and RPLP2 have been shown here (A). β-actin served as a loading control for normalization. Quantification of band intensity (B, C) 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 (a) p<0.001, (b) p<0.01 and (c) p<0.05 versus hESCs. hESCs, human endometrial stromal cells; Dec-hESCs, Decidualized human endometrial stromal cells. Values are expressed as mean ± SE; n=3.

that contains genes for the kappa (κ) light chains of antibodies [34]. Immunoglobulin kappa and immunoglobulin lambda light chain expressing cells have been detected in human endometrial biopsies by in situ hybridization [35]. In our study, the expression of IGK@ protein was been found to be increased by >2 fold in infertile women during receptive phase in the endometrium with highest increase in stromal cells. The higher expression of IGK@protein was observed in stroma followed by GE and LE in infertile women. These results were also substantiated by *in-vitro* experiment where non-decidualized stromal cells showed higher expression level which was decreased in decidualized hESCs. This supported our findings on infertile patient samples. Endometrial genomic profiling in UI have revealed the differential expression of genes involved in immune response at the time of implantation in infertile women with UI [7]. The report by Scotchie et al. [28], demonstrated the decreased secretion of IGK@ protein in uterine lavage from early to mid-secretory phase in fertile women. Also the expression of IG light chain was found to be higher in peritoneal fluid of infertile women with endometriosis [36,37]. Apart from this, earlier reports have suggested that women with UI have higher levels of $CD4^+$ cells and the lower levels of endometrial $CD8^+$ and $CD56^+$ cells, than that in fertile group [36]. On the basis of our findings, we speculate that $CD4^+$ cells might play a role in increasing the production of antibodies by B cells and ultimately enhancing the production of IGK light chain during unexplained infertility.

RPLP2 is a large 60S subunit of ribosome which is involved in protein synthesis. The ribosomal proteins exist in three forms P1, P2, and P0; all the three proteins are found in free state in the cytoplasm and they together form a pentameric complex known as P0 $(P1\text{-}P2)_2$ and create the stalk portion of ribosome. Therefore, the stalk can exist in multiple configurations and altering the P1/P2 composition of the stalk might hinder the activity of the ribosome which in turn affects protein biosynthesis [38]. The role of ribosomal proteins appears to be important for protein biosynthesis in endometrial cells during window of implantation [39]. In our study, the expression of RPLP2 was down regulated in endometrium of infertile cases as compared to normal fertile women. The decreased expression of RPLP2 in stroma in the tissue of infertile women was seen as compared to that of fertile women. Further, the higher expression of RPLP2 in stromal cells observed during *in-vitro* decidualization, also indicates the requirement of higher rate of protein synthesis that may well be associated with the increase in the size of the decidual cells**.** The decreased expression of 40S ribosomal protein SA (p40) between pre-receptive and receptive phase endometrium of fertile women has been observed [40]. However, other ribosomal proteins such as RPL7, RPL7p, RPL19 and YWHAZ were highly expressed in window of implantation in fertile women [39]. The dysregulated expression of endometrial RPLP2 in infertile patients observed in our study might be responsible for attenuating the synthesis of key proteins involved in endometrial receptivity and /or decidualization. However, the possibility of dysregulation of other ribosomal proteins P1 and P0 in the endometrium of infertile women, cannot be ruled out, and this needs to be explored.

Overall, our results showed various proteins which were differentially expressed in the endometrium of infertile women and were previously not known as regards to unexplained infertility. These differentially expressed proteins were involved in immunological response, glycolytic pathway, lipid metabolism, blood agglutination, protein synthesis, molecular chaperone, antioxidant system, mitochondrial ATP generation, and Ca^{+2} signalling. The up-regulated proteins are mainly known to be involved in growth and proliferation (Table 1). Since the endometrial biopsies used in the analysis are comprised of various cellular types each having specific function in implantation physiology, it will be essential to explore the cell-specific functions of these proteins in future. The detailed studies on regulation of these proteins might provide important clues to endometrial receptivity and implantation physiology. On the other hand, progesterone opposes oestrogen-induced proliferation of uterine epithelium, causes differentiation of endometrial stromal cells for successful implantation to take place, and is also known to induce the apoptosis of endometrial epithelial cells [41-43]. Thus, the correlation of identified proteins to the functional status of progesterone signalling and progesterone responsiveness during the period of endometrial receptivity would be an interesting area to be explored further. These functional studies will seek to define those proteins important for explaining the molecular basis of infertility.

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