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Research Article

Proteomic Pattern of Implantative Human Endometrial Fluid *In Vitro* Fertilization Cycles

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Abstract

The aim of this study is to assess whether there are proteins in Endometrial Fluid Aspirate (EFA) that predict implantation.

The population under study consisted of 285 women undergoing Embryo Transfer (ET) underwent endometrial fluid aspiration immediately before ET. Results of proteomic analysis of EFA were compared between 33 who achieved pregnancy and 33 who did not. Samples were analyzed by 2D electrophoresis and mass spectrometry. Blood samples were studied by ELISA Pregnancy rates and maternal complications were compared with those in women refusing aspiration.

We found 23 proteins differentially expressed in the EFA in conception cycles: 19 down-regulated (FC=0.31 to 0.78) and 4 up-regulated. Among the five studied proteins that were differentially expressed in EFA, none was differentially expressed in serum. The aspiration procedure had no impact on pregnancy rate. We found a very different protein profile in implantative cycles, the majority of proteins being down-regulated. This probably reflects a different endometrial functional status, more favourable to implantation. EFA proteomic analysis could be a useful tool in the planning ET strategies.

Proteomic analysis of EFA could be a useful tool in the planning ET strategies (freezing embryos/oocytes, cancelling ET, or adjusting the number of embryos to be transferred).

Keywords: Endometrium; Implantation; IVF; Endometrial fluid; Proteomics

Introduction

Embryo implantation is one of the most inefficient steps in assisted reproduction techniques [1]. A number of procedures have been developed to differentiate good-prognosis from poor-prognosis embryos. In contrast, the endometrial side of implantation has received much less attention. Currently, endometrial ultrasound is the only universally accepted tool to study the influence of the endometrium on implantation [2]. Endometrial tissues have been studied by histological, histochemical, and biochemical methods in the last two decades. A large number of proteins and other molecules, which are expressed in the endometrium in a cycle-dependent manner, have been described [3]. However most techniques to investigate endometrial receptivity require an endometrial biopsy, which precludes their use in the same cycle as Embryo Transfer (ET).

In recent years, some works have been directed to the analysis of uterine cavity [4] or even directly endometrial fluid with no lavage [5,6]. Uterine fluid is a protein-rich histotroph that contains secretions from the endometrial glands and cleavage products of both the secreted proteins and the glycocalyx (the glycoprotein mucin-rich layer coating the endometrial apical cell surface) [7]. The secretions are derived from two principal sources: a serum transudate arising from the rich capillary plexus surrounding the glands; and specific proteins, carbohydrates and other metabolites synthesized within the glandular cells [8]. Glandular secretions are known to be essential for implantation in sheep [9] and mice [10].

Our research group has developed a non-invasive technique for analyzing endometrial proteins in Endometrial Fluid Aspirate (EFA) obtained during the window of implantation. We have previously reported that more than 800 proteins can be detected in this fluid by proteomic techniques [5]. Further, we have shown with two-dimensional (2D) electrophoresis that some of these proteins change according to endometrial status allowing differentiating between women with and without endometriosis [6]. Proteomic analysis, that is, the study of the proteins in the sample, provides more physiologically relevant information than genomic analysis, since there are many regulatory steps between the transcriptome and functional proteome: indeed, there is often only a weak correlation between an mRNA and its protein derivative in the endometrium [11,12].

Most previous studies on endometrial markers have been performed using genomic or proteomic techniques in endometrial cells [13,14]. Only a few have used proteomic or genomic approaches to analyze endometrial cavity lavage fluid [4], and as far as we know, only one endometrial fluid, by lipidomics [15]. Most researchers take as gold standard the "receptive endometrium", that is, the endometrium when it is developmentally competent for implantation [7]. It is not clear, however, whether 1) a receptive endometrium, when receiving a good quality embryo, always produces a pregnancy; or 2) the timing of the endometrium being receptive is the same in different women or in different cycles of the same woman. In addition, controlled ovarian stimulation used in IVF cycles considerably alters the endometrium

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[16,17], resulting in both inadequate receptivity and/or changes in its timing.

In our opinion, the gold standard for studying the endometrium, from a reproductive point of view, should be the "implantative" endometrium, that is, the endometrium where the implantation occurs in the very same cycle. The hypothesis of the present study is that, in IVF cycles, a different level of endometrial development might yield a different protein secretion pattern, and that the implantation outcome might be associated with some of these proteins. The knowledge of such patterns could be of great interest allowing different alternatives: in poor prognosis cases, cancellation of the ET (freezing oocytes or embryos) or even increasing the number of embryos to be transferred, and in good prognosis, reducing the number of embryos transferred.

The second part of our study was focused on as certaining whether the protein markers of implantation detected in the EFA could also be detected and validated in a paired blood sample also obtained at the time of ET.

Material and Methods

The population under study consisted of 285 women undergoing IVF at the Reproductive Unit of Cruces University Hospital (University of the Basque Country).

The inclusion criteria were: i)age under 40 years, ii) fresh ET, iii) no more than two previous IVF cycles, iv) ET performed on day 2-3, v) absence of polyps, myoma, andhydrosalpinx, vi) absence of infectious risk, vii) no requirement foroocyte donation, preimplantation genetic diagnosis or testicular biopsy, and viii) easy previous mock transfer. We obtained approval from the Institutional Review Board (CEIC 09/54 and CEIC 11/45) and informed consent from participants.

In the 285 women recruited, endometrial fluid was aspirated using the catheter used for ET (Frydman, Instrumentos Médicos Estériles SA, Spain) connected to a 10 mL syringe, just prior to ET, under abdominal ultrasound guidance. Sample extraction was performed by gentle manual application of negative pressure with the syringe. To prevent contamination with cervical mucus, aspiration was interrupted at the internal cervical os. Special care was taken in the collection procedure to avoid touching the uterine fundus or injuring the cervix, and to minimize sample contamination with blood and endometrial tissue. In cases with excessive vaginal secretions, the vagina was cleaned with saline solution. Aspirated samples were expelled into standard cryogenic tubes and immediately frozen at-80°C until processed. Aspirate volumes ranged from 5 up to 50 μL. Five minutes after ET, a blood sample was taken by venipuncture (10 mL) and serum was obtained by routine centrifugation. Serum samples were also frozen (-80°C) until processing.

Of the 285 EFAs obtained, 35 were discarded due to insufficient sample volume [10] or visually evident blood contamination. From the remaining 250, the first consecutive 33 samples corresponding to ET resulting in pregnancy were selected for proteomic analysis, while he control group was composed of 33 samples from women in whom ET did not result in pregnancy, immediately following each pregnancy case. Biochemical pregnancies and ectopic pregnancies were excluded. The remaining 194 samples have not yet been analyzed.

For the safety analysis, we also included 200 oocyte donors who underwent conventional ovarian stimulation, from whom EFA was obtained on day 3 after oocyte pick-up.

Ovarian cycle management in our IVF patients has been described previously. Briefly, it consisted in either a long agonist protocol or a conventional antagonist protocol, ovarian stimulation being performed in women≤ 35 years with only recombinant FSH, and in women aged 36-39 with recombinant FSH plus hMG or with combined recombinant FSH and LH. Recombinant HCG was given i.m. at a dose of 250 mcg when at least three follicles were observed to have reached a mean diameter of 18.5 mm. Transvaginal ultrasound-guided follicular aspiration was scheduled 36 hours after HCG injection [1]. The oocyte donor protocol consisted of recombinant FSH with antagonist short protocol and triggering with 0.2 mg of triptorelini.

At the moment of the study, the ET policy consisted of transferring, when available, two embryos in good prognosis cases (woman age < 37 years, good quality embryos) and three embryos in poor prognosis cases (woman age ≥ 38 years, poor quality embryos, third IVF cycle).

Phases of the Study

Our study was divided into four different phases: 1) preliminary safety analysis; 2) EFA analysis; 3) serum sample analysis; and 4) overall safety analysis.

Preliminary safety phase

This safety study (approved by the Institutional Board Review, ref. CEIC 09/54) was designed as a non-inferiority trial. The purpose was to analyze 30 cases and compare them with matched controls (considering age, infertility diagnosis, ovarian stimulation protocol, estradiol level, and numbers of oocytes obtained, top quality embryos, and embryos transferred) treated over the same period of time.

EFA sampling was to be considered safe if the Pregnancy Rate (PR) was the same or higher in the study group. If the PR were≥10% lowers in the study group, the aspiration would be considered unsafe and the study would be halted. If the PR were between 0.1 and 9.9% lower, a further 30 cases would be studied, and the study would be halted if the total PR were> 1% lower.

Once the non-inferiority had been demonstrated in the preliminary safety study, the investigational study was undertaken. The investigational study was approved by our Institutional Review Board (code CEIC 11/45). Informed consent was obtained from all participating women (regardless of whether their data were used for the safety or the investigational phase).

EFA analysis

Protein extraction: Samples were processed as described previously [6]. Briefly, samples were resuspended in 500 μl of PBS and purified using the Vivapure Anti-HSA/IgGKit (Vivascience AG, Hannover, Germany). After this step, samples were precipitated with 15% w/v trichloroacetic acid for1hour at 4°C, followed by centrifugation (10 min, 16000 x g, 4°C). Pellets were washed with 1 ml of pre-chilled acetone and centrifuged. After drying the pellets at room temperature for 20 min, they were rehydrated with 470 μl

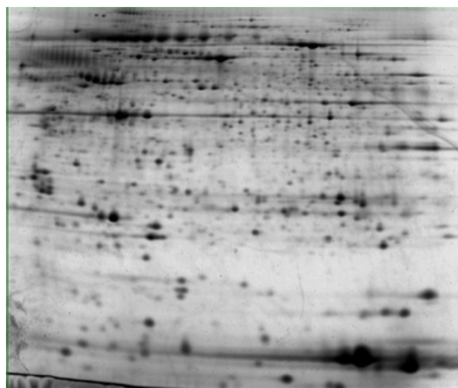

Figure 1: A representative 2D image of an endometrial fluid aspirate sample.

of solution containing urea 7 M, thiourea 2 M and CHAPS 2%. The protein content of the resuspended samples was determined by the Bradford method using Bio-Rad Protein Assays (Bio-Rad) following the manufacturer's instructions.

2D electrophoresis analysis: To perform the 2D electrophoresis, DeStreak Rehydration solution 1.2% (Ref. 17-6003-18, GE Healthcare, Little Chalfont, UK), bromophenol blue 0.002% and IPG buffer pH 3-10 NL 0.5% (product code: 7-6000-88, GE Healthcare) were added to 200 µg of each EFA sample to obtain a final volume of 450 µl of rehydration solution. This mixture was loaded into immobilized pH gradient strips (ImmobilineDryStrip;24-cm gels, pH 3-10 NL; product code: 17-6002-45, GE Healthcare) and first-dimension isoelectric focusing was performed in an Ettan™ IPGphor™ 3 System (GE Healthcare) following the manufacturer's instructions to resolve proteins by their charge (isoelectric point) in a pH range of 3 to10. The voltage applied was as follows: active rehydration of the strips at 50 V for 11 hours, 250 V for 15 minutes, increasing the voltage to 10,000 V by limiting the current to 50 µA per strip, and stopping the focusing when the voltage reached >90,000 Vht.

Next, the strips were equilibrated and the second dimension electrophoresis (sodium dodecyl sulphate polyacrylamide gel electrophoresis, SDS-PAGE) was performed to separate proteins in acrylamide gels according to their molecular weight. For this purpose, 12.5% acrylamide gels (dimensions: 26 x 20 cm) were polymerisedusing the EttanDALT Twelve Gel Caster (GE Healthcare). Strips were held on top of the gels and were run in the EttanDALT twelve Large Format Vertical System following the manufacturer's instructions until the electrophoresis reached the bottom of the gels.

Then, gels were stained using Flamingo Fluorescent gel stain (Bio-Rad), and scanned on a Typhoon Trio scanner (GE Healthcare) for subsequent image analysis of the protein spots. For some samples, more than one 2Dgel was run. A representative 2D image obtained from an endometrial aspirate sample is shown in Figure 1.

Analysis of the protein spots: Digitalized 2D proteomes were analyzed using the Progenesis PG240 version 2007 software (Nonlinear Dynamics, Newcastle upon Tyne, UK) as described previously [6]. A data matrix, corresponding to the protein expression intensity foreach spot, was exported for subsequent statistical analysis.

Statistical analysis

Statistical analysis was performed using IBM SPSS (IBM, Armonk, NY, USA). Differences in each of the biomarkers between the groups achieving and not achieving pregnancy were assessed with the Mann-Whitney U test. A p value of 0.05 was considered the threshold for statistical significance. The Fold Change (FC) of in the expression level of each protein between the two groups was obtained by comparing the medians of the expression intensity values obtained from the progenesis software. The differential spots were checked on the 2D images to rule outmatching errors and when necessary, the matching was corrected and the statistical analysis was repeated. Pregnancy was defined as the visualization of a gestational sac 4 weeks after embryo transfer.

Protein identification

A preparative 2D gel was run and stained using silver nitrate (Silver Staining Kit, ref 17-1150-01, GE Healthcare). Protein spots of interest, namely those corresponding to differentially-expressed proteins, were cut out of the gel for mass spectrometry analysis. Proteins were reduced, alkylated and then further digested with trypsin. Resulting peptides were analyzed bymatrix-assisted laser desorption/ionization time of flight mass spectrometry in an Autoflex Smart beam System (Bruker Daltonics). Proteins were identified by a combination of peptide mass and peptide fragment fingerprinting, using the Mascot search engine (Matrix Science) against data in the Uniprot database.

Serum analysis validation of biomarkers

Serum was extracted from blood samples by centrifugation (10 min, 3000 rpm). Five proteins selected from the mass spectrometry analysis were quantitatively detected in serum using commercial Enzyme-Linked Immunosorbent Assay (ELISA) tests following the manufacturer's instructions: annexin A2 (ANXA2), capping protein (actin filament) muscle Z-line, beta (CAPZB), cofilin 1 (non-muscle)

Table 1: Demographic and clinical parameters of the groups studied in the pre-study safety analysis.
Comparison of IVF outcome and clinical parameters in women who underwent endometrial fluid aspiration at embryo transfer compared with those who underwent conventional embryo transfer. None of the differences were significant.

	Endometrial fluid aspiration at embryo transfer	Control group
Mean age, years	36.2±2.8	36.4±2.7
Infertility duration, years	3.1±1.6	2.9±1.8
Body mass index, kg/m^2	26.3±3.9	27.5±3.9
Oocytes obtained	9.6±3.1	9.4±2.9
Embryos transferred	2.6±0.4	2.5±0.5
Per transfer pregnancy rate	40 (12/30)	36.7 (11/30)
Infectious or haemorrhagic complications	0 (0/30)	0 (0/30)

Table 2: Proteins differentially expressed in endometrial fluid aspirate from conception and non-conception cycles.
P<0.05 in all cases. In some cases, the same protein was identified in two or more different spots, corresponding to different isoforms or post-translational modifications of the protein.

Spot	Biomarker	Name	Uniprotcode	Median (conception cycle) (N=33)	Median (non conception cycle) (N=33)	Foldchange (conception/non-conception)
436 *	ALBU	Serumalbumin	P02768	2.56	1.79	1.43
537 *	TRFE	Serotransferrin	P02787	0.61	0.32	1.92
624 *	TRFE	Serotransferrin	P02787	0.59	0.4	1.45
706	HSP7C	Heat shock cognate 71 kDa protein	P11142	0.35	0.6	0.59
720	HSP71	Heat shock 70 kDa protein 1A/1B	P08107	1.04	1.4	0.74
933	PLSL	Plastin-2	P13796	0.6	0.87	0.69
938	PDIA3	Protein disulfide-isomerase A3	P30101	0.58	0.91	0.63
1424	ARG1	Arginase-1	P05089	0.04	0.11	0.36
1524	CAZA1	F-actin-capping protein subunit alpha-1	P52907	0.73	0.94	0.78
1744	ACTBM	Putative beta-actin-like protein 3	Q9BYX7	0.24	0.42	0.59
1824	ACTB	Actin, cytoplasmic 1	P60709	0.13	0.31	0.44
1898 *	KV302	Ig kappa chain V-III region SIE	P01620	2.52	1.3	1.94
1911	PSB4	Proteasome subunit beta type-4	P28070	0.2	0.37	0.53
1944	PARK7	Protein DJ-1	Q99497	0.07	0.19	0.37
2031	SODM	Superoxide dismutase [Mn], mitochondrial	P04179	0.13	0.24	0.55
2048	CDC42	Cell division control protein 42 homolog	P60953	0.15	0.23	0.65
2230	CFL1 or COF1	Cofilin-1	P23528	0.24	0.51	0.46
2304	STMN1	Stathmin	P16949	0.47	0.84	0.56
2433	MYDGF	Myeloid-derived growth factor	Q969H8	0.18	0.33	0.54
2435	TBCA	Tubulin-specific chaperone	O75347	0.17	0.31	0.56
3171	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	P04406	1.09	1.93	0.56
3173	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	P04406	1.32	3.26	0.4
3319	ACTB	Actin, cytoplasmic 1	P60709	0.24	0.54	0.45
3325	ACTB	Actin, cytoplasmic 1	P60709	0.24	0.35	0.67
3353	CAPZB	F-actin-capping protein subunit beta	P47756	0.06	0.2	0.31
3374	ANXA2	Annexin A2	P07355	0.44	0.82	0.53
3375	ANXA2	Annexin A2	P07355	0.96	1.63	0.59
3418	ACTB	Actin, cytoplasmic 1	P60709	0.81	1.07	0.75
3469	CATA	Catalase	P04040	0.67	0.42	1.58
3470	CATA	Catalase	P04040	0.74	0.48	1.52

* = proteins which could have a serum or unspecific origin

(CFL1), Parkinson protein 7 (PARK7) and stathmin 1(STMN1) (catalogue numbers: ABIN1113424, ABIN829266 and ABIN1568789, ABIN1114233, ABIN366580ABIN1874419 and ABIN1117229, respectively, antibodies-online GmbH, Germany).

Global safety phase

The pregnancy rate obtained among the 285 women who underwent endometrial fluid aspiration was compared with a matched population (considering age, infertility diagnosis, ovarian stimulation protocol, estradiol level, and the numbers of oocytes obtained, top quality embryos, and embryos transferred) treated over the same period of time who declined to participate in the study. The rates of infection and of haemorrhage after ET were also compared between the two aforementioned populations. Infection/hemorrhage rates were also studied among the 200 oocyte donors who had also undergone endometrial fluid aspiration.

Results

Pre-study safety analysis

The pregnancy rate among the studied 30 patients was unaffected by the collection of the endometrial fluid sample when compared with the control group of patients of similar characteristics assisted during the same period of time. Pregnancy rates were similar in the two groups: 40.0% in the study group (12/30) *vs*. 36.7% (11/30) in the control group. There were no significant differences between both

Table 3: Demographic and clinical parameters of the groups studied in the whole population who underwent endometrial fluid aspiration compared with the control population.
Comparison of IVF outcome and clinical parameters in patients who underwent endometrial fluid aspiration at embryo transfer and those who received conventional embryo transfer. Differences were not significant.

	Endometrial fluid aspiration at embryo transfer	Control group
Mean age, years	37.3±2.9	37.5±2.8
Infertility duration, years	3.4±1.8	3.1±1.9
Body mass index, kg/m^2	26.7±4.2	27.4±4.1
Oocytes obtained	9.5±3.2	9.5±3.0
Embryos transferred	2.6±0.3	2.4±0.5
Per transfer pregnancy rate	35.4 (101/285)	34.4 (98/285)
Infectious or haemorrhagic complications*	0 (0/485)	0 (0/285)

*= oocyte donors included.

groups in regards to demographic and clinical parameters (Table 1).

EFA analysis

Table 2 lists the proteins for which the spot intensity was significantly different ($p<0.05$), proteins subsequently being identified by mass spectrometry. Each spot number is represented along with the corresponding protein acronym and Uniprot code. The FC in expression level of each protein between the groups studied is also included. FC> 1 indicate that the protein was over expressed and FC< 1 that the protein was down-regulated. In some cases, the same protein was identified in two or more different spots, corresponding to different isoforms or post-translational modifications of the same protein.

Of the approximately 800 observed spots in the 2D gels, we found 23proteins that were differentially expressed among women achieving and not achieving pregnancy. Most of the proteins were down-regulated (n= 19), with FC ranging from0.31 to 0.78(heat shock cognate 71kDa protein; heat shock 70 KDa protein; plastin- 2; protein disulfide-isomerase A3; arginase-1; F-actin-capping protein subunit alpha-1; putative beta actin like protein 3; actin, cytoplasmic 1; proteasome subunit beta type 4;protein deglycase DJ-1, also known as Parkinson disease protein 7; superoxide dismutase [Mn], mitochondrial; cell division control protein 42 homolog; Cofilin-1; stathmin; myeloid- derived growth factor; tubulin- specific chaperone A; glyceraldehyde-3-phosphate dehydrogenase; and F-actin-capping protein subunit beta; annexin A2). Just four proteins were up-regulated: catalase, serum albumin, serotransferrinand Ig kappa chain V, with FC ranging from 1.43 to 1.92. The last three should be considered as having a blood origin or non-specific source.

Serum analysis

ELISA analysis of blood samples obtained 5 minutes after collection of endometrial fluid was used to study five proteins: cofilin-1; stathmin, annexin-2, CAPZβ, and PARK7.No significant differences in any of these five proteins were found between conception and non-conception cycles (Figure 2). ELISA kits used failed to detect CAPZβ.

EFA and serum sample proteins and clinical parameters

None of the 23proteins differentially expressed in EFA were associated with any of the clinical parameters investigated (maternal age, body mass index, estradiol levels, recovered oocytes, mature oocytes, numbers of fertilized, top quality, and transferred embryos, day of ET, and number of gestational sacs) (data not shown). None of the five proteins studied by ELISA in blood samples was associated with any of the aforementioned parameters.

Over all safety

During the study period, 285 procedures were performed to aspirate endometrial fluid at the moment of ET. The pregnancy rate in these cases was similar to that the control population (matched for age, infertility diagnosis, ovarian stimulation protocol, estradiol levels, and number of oocytes obtained, top quality embryos, and embryos transferred) (Table 3).

No infectious or haemorrhagic complications occurred after ET in the EFA group or controls. Similarly, there were no infectious or haemorrhagic complications in the 200 oocyte donors who underwent endometrial fluid aspiration.

Discussion

In humans, embryo implantation occurs in the mid-secretory phase of the endometrial cycle, which is characterized by a number of changes in the endometrial epithelium and stroma, and especially by the development of endometrial glands. Endometrial secretions are essential for sustaining the concept us prior to implantation [9,10].

Despite the clear relevance to endometrial function, little is known about the identity of proteins secreted by the endometrium [4]. Since the early work of Noyes et al. [18], a number of studies have focused on endometrial changes during the ovarian cycle. However, most endometrial histological or biochemical studies require an endometrial biopsy, precluding their performance in IVF cycles close to the moment when ET is to be carried out, as it could have a detrimental effect on implantation. In addition, the results of the biopsy from the stimulated cycle might not be the same as in a previous non-stimulated cycle, or even in a new stimulated cycle. Ovarian stimulation has been shown to be associated with an advancement of endometrial maturation regardless of the protocol used [12]. In contrast, endometrial fluid aspirationis an atraumatic procedure that, even if performed immediately prior to embryo transfer, does not affect implantation [19,20]. We have previously shown how proteomic analysis of EFA detects more than 800 proteins [5,6], and how the EFA proteomic pattern differs between patients with and without endometriosis, as well as between those with advanced and non-advanced endometriosis [6].

A number of studies have been performed focused on the so-called

Table 4: Differentially-expressed proteins and their biological function.

ID	Biomarker	Sub cellular location	Molecular Class	Molecular function	Biological Process	MW
Actin, cytoplasmic 1	ACTB	Cytoplasm, extracellular exosome	Cytoskeletal protein	Structural constituent of cytoskeleton	Cell growth and/or maintenance	42052
Annexin A2	ANXA2	Secreted	Calcium binding protein	Calcium ion binding	Signal transduction; Cell communication	38808
Arginase-1	ARG1	Cytoplasm	Enzyme: Hydrolase	Hydrolase activity	Metabolism; Energy pathways	34713
Catalase	CATA	Cytoplasm	Enzyme: Oxidoreductase	Oxidoreductase activity	Metabolism; Energy pathways	59947
Cell division control protein 42 homolog	CDC42	Cytoplasm	GTPase	GTPase activity	Cell communication: signal transduction	21245
Cofilin-1	CFL1 o COF1	Cytoplasm	Cytoskeletal associated protein	Cytoskeletal protein binding	Cell growth and/or maintenance	18491
F-actin-capping protein subunit alpha-1	CAZA1	Cytoplasm	Cytoskeletalprotein	Actin capping	Barbed-end actin filament capping	33073
F-actin-capping protein subunit beta	CAPZB	Cytoplasm	Cytoskeletalprotein	Structural constituent of cytoskeleton	Cell growth and/or maintenance	31331
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Cytoplasm, extracellular exosome	Enzyme: Dehydrogenase	Catalytic activity	Metabolism; Energy pathways	36030
Heat shock 70 kDa protein 1A/1B	HSP71	Cytoplasm	Chaperone	Chaperone activity	Protein metabolism	70294
Heat shock cognate 71 kDa protein	HSP7C	Cytoplasm	Heat shock protein	Heat shock protein activity	Protein metabolism	71082
Ig kappa chain V-III region SIE	KV302	Extracellular, plasma membrane, exosome	Immunity protein	Antigenbinding	Immune response	11882
Myeloid-derived growth factor	MYDGF	Secreted	Growth factor	Growth factor activity	Cell communication: signal transduction	18783
Plastin-2	PLSL	Cytoplasm	Calcium binding protein	Calcium ion binding	Cell communication: signal transduction	70814
Proteasome subunit beta type-4	PSB4	Cytoplasm	Ubiquitin proteasome system protein	Ubiquitin-specific protease activity	Protein metabolism	29242
Protein disulfide-isomerase A3	PDIA3	Cytoplasm	Enzyme: Isomerase	Isomerase activity	Protein metabolism	56747
Protein DJ-1	PARK7	Cytoplasm	RNA binding protein	RNA binding	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	19878
Putative beta-actin-like protein 3	ACTBM	Cytoplasm	ATP binding protein	ATP binding	Blood coagulation; Platelet function	42331
Serotransferrin	TRFE	Secreted	Transport/cargo protein	Transporter activity	Transport	79294
Serumalbumin	ALBU	Secreted	Transport/cargo protein	Transporter activity	Transport	71317
Stathmin	STMN1	Cytoplasm	Structural protein	Signal transducer activity	Cell growth and/or maintenance; Signal transduction	17292
Superoxide dismutase [Mn], mitochondrial	SODM	Mitochondrialmatrix, extracelular exosome	Enzyme: Superoxide dismutase	Superoxide dismutase activity	Cell proliferation; Anti-apoptosis; Cell growth and/or maintenance	24878
Tubulin-specific chaperone A	TBCA	Cytoplasm	Chaperone	Chaperone activity	Protein metabolism	12904

"receptive endometrium". In most cases, the endometrium has been defined as receptive on the basis of histological, biochemical or genetic criteria. Nonetheless, the only way to ensure that an endometrium is receptive is that pregnancy has occurred in the very same cycleas when the analyzed sample was taken. Thus, to avoid such bias we have chosen to use the term "implantative" endometrium. Our embryos were not PGD tested, thus in our age range an aneuploidy rate close to 33% should be expected. Thus our study could under diagnose some cases where the endometrium could have been implantative if it had received aneuploid embryo.

We observed that 23 proteins were significantly differentially expressed when comparing the group achieving *vs.* non achieving pregnancy. After excluding non- specific proteins, we were left with 20 proteins clearly differentially expressed in conception and non-conception cycles. Just one of these was up-regulated: catalase, with FCof 1.52 to 1.58.All the others were down-regulated (n=19), with FC ranging from 0.31 to 0.67. Previous reports based on transcriptomic data of human endometrium have indicated that the majority of genes (60-94 %) were up-regulated [21-23], while three other studies found the same percentage of up- and down-regulated genes [24-26].

The majority of differentially expressed proteins we found were related to the biological process of cell growth and/or maintenance (actin, F-actin capping subunit beta, cofilin, superoxide dismutase, stathmin) (Table 4). There were also a number related to energy pathways (arginase-1, catalase, glyceraldehyde-3- phosphate dehydrogenase) or protein metabolism (heat shock cognate 71kDa and 70 kDa proteins, protein disulfide isomerase, proteasome subunit beta, tubulin- specific chaperone A), and lastly, some were related to cell communication (annexin 2, cell division control protein 42 homolog and plastin 2).

Previously, some authors have applied large-scale proteomic techniques to study human endometrial receptivity by means of endometrial biopsies. One group compared proliferative with secretory phase endometrium [27], another late proliferative with

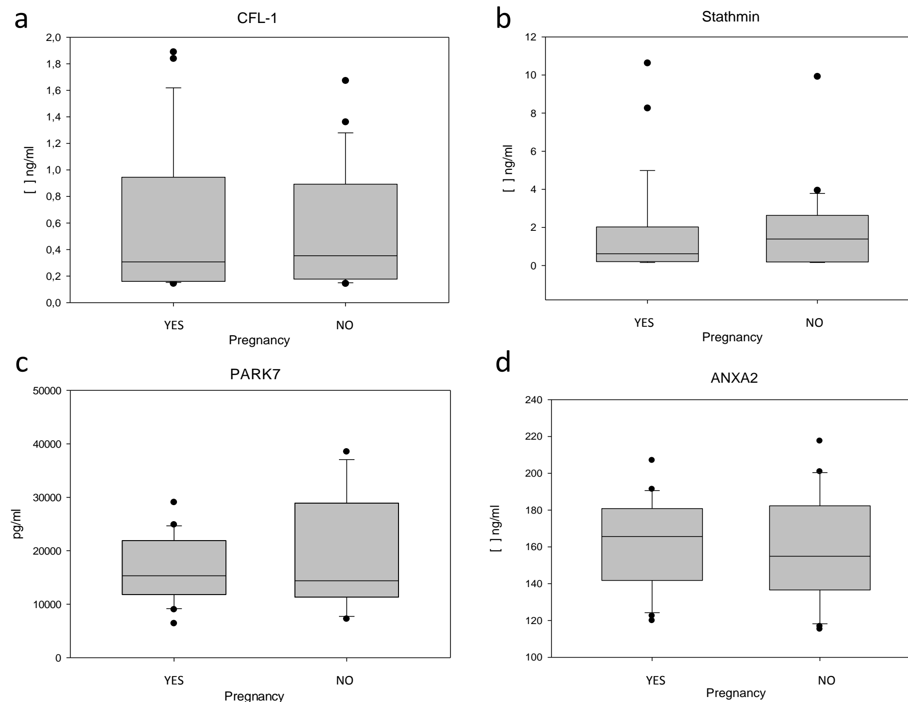

Figure 2: Comparison of the serum levels of four proteins significantly different in endometrial fluid aspirate (conception vs. no conception cycles). None of the differences were significant. The upper horizontal line of the box corresponds to the 75th percentile (Q3) and the lower horizontal line of box to the 25th percentile (Q1); the horizontal bar within box is the median. The upper horizontal bar outside box is calculated by this expression: Q3+1.5(Q3-Q1) and the lower horizontal bar outside box: Q1-1.5(Q3-Q1). Circles represent outliers.
CFL-1: cofilin 1; PARK7: Protein DJ-1; ANXA2: annexin 2.

mid-secretory phase endometrium [28] and a third mid-proliferative and mid-secretory phase endometrium [11]. Desouza et al. [27] comparing proliferative with secretory endometrial tissue, reported differential expression of some of the proteins we also observed differentially expressed in EFA: actin, cofilin, glyceraldehyde 3-phosphate dehydrogenase, heat shock cognate 71 kDa protein, and transferrin [27]. Parmar et al. [28] also identified heat shock protein β-1 (heat-shock protein 27) and transferr in to beup-regulated proteins in endometrial tissue, and present in uterine fluid and mid-secretory secretions.

Two previous studies have focused on "pre-receptive" and "receptive" endometrium [29,30]. Annexin A4 was found to be up-regulated in both cases, andannexinA2 only in one study [30]. Dominguez et al. [30] found a number of proteins to be differentially expressed, but only annexin A2 and stathmin I were consistently up-regulated in the two experiments they performed. It should be highlighted that in our study, both annexin 2 and stathmin 1 were significantly down-regulated. However, it is well known that changes in intracellular protein concentration do not necessarily reflect simultaneous changes in protein secretion. Indeed, since these specific biomarkers of the receptive endometrium in the aforementioned studies were identified under natural cycles, it could be that they are not representative of stimulated IVF cycles [12].

In a previous study, using broad-based proteomics (2D DIGE MS/MS), found at least two-fold changes in expression of 82 proteins between secretions from pre-receptive and receptive phase endometrium [4]. However, these authors analyzed endometrial lavage samples after flushing the uterine cavity twice with 4 mL of saline serum in 10 volunteers in a natural cycle comparing 4 *vs.* 9 days after LH surge. There was a remarkable variability in the number of protein species identified, with some gels identifying as few as 2 proteins and others as many as 104 proteins [4].

Some of our specific findings are similar to those of Scotchieet al [4]. They found variable expression of the transferrin protein in the mid-secretory phase compared with the early secretory phase: that is, expression increased in some cases and decreased in others. Like them, we found lower expression of cofilin-1, glyceraldehyde-3-phosphate dehydrogenase and transferrin, while levels of heat shock cognate 71kDa and 70 kDaproteins remained unchanged [4].

Some of the proteins we have found to be differentially expressed have also been described in the uterine fluid in the peri-implantation period in the cattle, namely, actin B, serotransferrin and HSP7C [31].

Annexin 2 is probably the most widely studied marker of implantation: it has been shown to increase in cultures of endometrial cells after interleukin 11 stimulation [32]. In a study investigating the endometrium of women using intrauterine devices for contraceptive purposes, annexin2 was shown to be up-regulated in the receptive compared to pre-receptive endometrium [30]. Numerous studies have shown that annexin 2 is involved in cell adhesion and actin cytoskeletal rearrangements [33,34], as well as increasing cell adhesion molecule production [35]. It has been suggested thatannexin2 could play a role in the remodelling of the apical pole of the luminal epithelium in the endometrium for cell-to-celladhesion [30]. It should be highlighted that annexin 2 was down-regulated in conception cyclesin our study.

Concerning stathmin, this protein has been reported to be down-regulated in endometrial cells in receptive endometrium in transcriptomic- [25] and proteomic- [30] based studies, with a FC similar to that observed in our study.

When we tried to detect some of the EFA implantation markers in blood samples, none of the five proteins studied was significantly different in women achieving and not achieving pregnancy. In our opinion, this is a consequence of the limited effect of small changes in endometrial fluid on peripheral blood. We should recall that blood volume is about 1000 times greater than the endometrial volume (5.5ml) [36], the volume of endometrial fluid being considerably lower.

Conclusion

Finally, concerning safety, no impact was seen on PR and no infectious/haemorrhagic complications were detected among the almost 500 women where EFA was performed.

Our findings show that endometrial fluid is aprotein-rich medium with a markedly different composition in conception and non-conception cycles, probably corresponding to a differential protein secretion that either facilitates embryo implantation and/or reflects a better endometrium quality. We conclude that a number of changes occur in protein composition of EFA in implantative cycles, most involving down-regulation, and measurement of these changes could constitute a useful tool for the ET planning.

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