Human Reproduction Vol.19, No.6 pp. 1438–1447, 2004
Advance Access publication April 22, 2004

DOI: 10.1093/humrep/deh224

Sperm proteome mapping of a patient who experienced failed fertilization at IVF reveals altered expression of at least 20 proteins compared with fertile donors: Case report

Katherine L.Pixton[1], Emma D.Deeks[1], Frits M.Flesch[1], Fleur L.C.Moseley[1,3], Lars Björndahl[1,3], Peter R.Ashton[2], Christopher L.R.Barratt[1,3,4] and Ian A.Brewis[1,3]

[1]The Reproductive Biology and Genetics Group, Division of Medical Sciences, School of Medicine, [2]School of Chemical Sciences, The University of Birmingham, Birmingham B15 2TT and [3]The Assisted Conception Unit, Birmingham Women's Hospital, Birmingham B15 2TG, UK

[4]To whom correspondence should be addressed. E-mail: c.l.barratt@bham.ac.uk

The aim of this study was to compare the sperm protein expression profile (proteome map) from a patient who experienced failed fertilization at IVF with fertile controls. One patient and three fertile donor sperm samples were characterized using two-dimensional electrophoresis. Differences in protein expression were established using gel analysis software before attempted protein identification. Gel analysis of the fertile donor proteome maps revealed excellent reproducibility as well as very low intra-donor and inter-donor variability in the presence of protein spots. In the patient samples, we have noted 20 consistent differences in protein expression (six spots missing, three additional spots, four less abundant, seven more abundant) compared with the controls. Two proteins that were more intense in the patient have been conclusively identified as secretory actin-binding protein and outer dense fibre protein 2/2. In conclusion proteome variation between different fertile donors was very low. In contrast, the patient proteome exhibited 20 differences compared with controls, which we believe is an underestimate. These proteins merit further investigation to determine whether failed fertilization at IVF might be caused by abnormalities in their expression. This case report represents a proof of principle that proteomics may be useful to study defects in sperm function.

Key words: infertility/IVF/proteomics/spermatozoa/unexplained infertility

Introduction

Male factor infertility, primarily diagnosed by abnormal semen parameters, is the single most common defined cause of infertility (Hull *et al.*, 1985; World Health Organization, 1999). Although the multiple underlying causes of male infertility are poorly understood, treatment using ICSI is very effective and routinely used for men with mild and severe forms of infertility. Consequently, conventional IVF is now reserved for men with normal or nearly normal semen parameters (mild male factor infertility).

With the general elimination of men with sperm dysfunction, it is therefore surprising that total fertilization failure is still a relatively common occurrence at IVF (Ola *et al.*, 2001; Tournaye *et al.*, 2002). For example, a randomized controlled trial comparing ICSI and IVF in mild male factor infertility reported that fertilization failure occurred in 25% of the IVF cycles (Tournaye *et al.*, 2002). Flexible clinical and laboratory protocols, such as insemination of higher numbers of sperm, reduce this number to ~5%, but failed fertilization is still apparent (Tournaye *et al.*, 2002). This emphasizes the fact that sperm dysfunction not apparent with conventional semen analysis is a significant cause of fertilization failure.

Until recently, we knew very little about the detailed clinical nature of sperm dysfunction in so-called 'hidden' male factor infertility (Mackenna *et al.*, 1993). However, there is accumulating data in humans to show that sperm defects, such as defective zona binding or the zona-induced acrosome reaction, are a significant cause of poor fertilization and total fertilization failure in assisted conception (Franken *et al.*, 1993; Oehninger *et al.*, 1997; Liu and Baker, 2000, 2003; Esterhuizen *et al.*, 2001; Liu *et al.*, 2001; Bastiaan *et al.*, 2003). As well as these proposed defects, it is likely that this is a very heterogeneous phenotype and that there are other factors involved in failed fertilization that have yet to be established. These may also prove to be significant causes of sperm dysfunction in men with apparently normal semen or mild male factor infertility.

At present, there is no information regarding what the molecular nature of these defects in spermatozoa might be or whether they represent defects in individual proteins involved

in these mechanisms. In animals, there is evidence that individual protein knockouts can affect zona binding, for example SED-1 (Ensslin and Shur, 2003), and so it is possible that individual protein defects in the human might also cause fertilization failure. In addition, there are also data to suggest that certain molecular mechanisms during fertilization may be different in humans compared with rodents, for example fertilin (Frayne *et al.*, 1999) and the molecular composition of the zona pellucida (Hughes and Barratt, 1999). Therefore, it is important to examine these events in the human rather than animal models.

Proteomics (the study of proteins in a genome) is a key area of emerging research in the post-genomic era (Brewis, 1999; Aebersold and Mann, 2003; Tyers and Mann, 2003). As proteins, or more correctly protein–protein interactions, are responsible for cellular function, it is critical that a comprehensive and systematic identification and quantification of proteins expressed in cells and tissues is undertaken to gain new insights into these processes. In contrast to the genome, the proteome is not a fixed feature of an organism, but differs, for example due to disease state (Banks *et al.*, 2001). Advances in two-dimensional electrophoresis (2DE) for the separation of proteins, and mass spectrometry (MS) in particular for peptide sequencing to facilitate protein identification has led to this field rapidly expanding throughout biomedical research (Blackstock and Weir, 1999; Pandley and Mann, 2000). Spermatozoa should be a good cell type for proteomic studies as they are transcriptionally inactive and hence there is not the consideration of new protein synthesis that exists in mature cells (Brewis and Wong, 1999).

There are a number of groups that have studied spermatozoa using proteomic approaches to enable the visualization of many proteins at the same time. In several studies, Herr and co-workers have used proteomics coupled with tandem mass spectrometry (MS/MS) to discover possible target proteins for the development of contraceptive vaccines primarily using antisperm antibodies (Naaby-Hansen *et al.*, 1997; Shetty *et al.*, 1999, 2001; Shibahara *et al.*, 2002). Examining those proteins recognized by antisera from immune infertile patients has also been investigated by other groups (Primakoff *et al.*, 1990; Bohring and Krause, 1999, 2003), and Bohring *et al.* (2001) have published work identifying some of these proteins using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS. Other than these, there have been very few studies that have employed proteomic approaches to examine male infertility. An exception to this are two papers investigating the role of A-kinase anchoring protein (AKAP) in fibrous sheath defects (dysplasia of the fibrous sheath and stump tail spermatozoa) (Turner *et al.*, 2001a,b). In both cases, no role for defects in the AKAP genes or their resulting proteins in these infertility conditions was found. Proteomics has also been applied in several studies to examine different aspects of normal sperm function. Examples have included calcium-binding proteins and proteins that are tyrosine phosphorylated (Naaby-Hansen *et al.*, 2002; Ficarro et al, 2003).

The aim of this study was to employ proteomic strategies potentially to identify, objectively and without preconceptions, sperm protein defects that might be responsible for failed fertilization at IVF. Specifically, we were interested in identifying differences in sperm protein expression between control (fertile) men and a patient with spermatozoa that failed to fertilize at IVF. We have taken into consideration possible variation between different ejaculates from the fertile donors that were assessed in order to confirm that any differences observed in the patient sample were real. This study has focused on one patient and should be thought of as a proof of principle that proteomics may be used to study such defects.

Materials and methods

Chemicals

Unless otherwise stated, all of the reagents for 2DE and silver staining were from Amersham Biosciences, Chalfont St Giles, Bucks, UK. Sequencing grade modified trypsin (V5111) was from Promega, Southampton, UK. Percoll, CHAPS, HEPES, urea, 2,5-dihydroxybenzoic acid (DHB), acetonitrile, iodoacetamide, the molecular weight markers (M4038 and M3788) and thiourea (specifically Fluka Product Number 88810) were from The Sigma Chemical Company, Poole, Dorset, UK. Unless otherwise stated, all other chemicals were at least Analar grade and from Sigma.

Donors and patient

The research donors were recruited at The Assisted Conception Unit, Birmingham Women's Hospital [Human Fertilization and Embryology Authority (HFEA) Centre 0119] in accordance with HFEA Code of Practice. All of the samples were screened and shown to be free from sexually transmitted diseases, including human immunodeficiency virus (HIV), in accordance with recommended guidelines (British Andrology Society, 1999). These donors were normozoospermic in accordance with WHO guidelines (World Health Organization, 1999) with >14% ideal morphological forms (by strict criteria) and no antisperm antibodies. Additionally, all donors had a sperm concentration >60 $\times$ 10^6 sperm/ml. They were of proven fertility either with their own partner or through the use of their cryopreserved semen in donor insemination treatment cycles.

The incidence of failed fertilization at IVF is <4% in our clinic. However, it is rare that the female presents no symptoms that might themselves contribute to the failed fertilization. Such factors might include age, polycystic ovarian syndrome or low numbers of eggs (<5) produced from superovulation. In reviewing our database, 20 patients exhibited failed fertilization from 604 IVF cycles over the last 2 years. Fifteen patient couples had defined female factors that might contribute to fertilization failure or the semen sample was either not normozoospermic according to WHO criteria (World Health Organization, 1999) or contained antisperm antibodies. In total, only five patients were therefore deemed suitable for this study using these criteria. There were no examples of repeated cycles of failed fertilization at IVF as ICSI is invariably followed in subsequent treatment cycles. The patient that we selected fulfilled these criteria, and sperm preparation during the treatment cycle resulted in a good yield of progressive sperm that were diluted to 200 000 progressive spermatozoa in 1 ml. In total, nine eggs were produced that all failed to be fertilized in one IVF cycle. The female partner had no other complicating factors that might obviously influence fertility and there were no other unusual circumstances during the treatment cycle.

Initial assessment of the patient revealed no evidence of physiologically significant levels of antisperm antibodies, >14% normal sperm morphology, a total of 220 $\times$ 10^6 sperm and 50% forward progressive motility. The patient was re-tested immediately prior to the analyses and again found to be apparently normozoos-

permic. Two standard semen analyses were carried out on separate occasions showing: sperm concentration 35×10^6 and 44×10^6/ml; volume 4.5 and 3.5 ml; 62 and 32% forward progressive motility; and on both occasions >14% morphologically normal forms. Spermatozoa were also subjected to the Kremer test (performed in hyaluronic acid) as described in Ivic *et al.* (2002), and this was shown to be within normal limits.

Approval was obtained from the Local Research Ethics Committee (LREC 5570) for this particular study, and the patients and donors all gave informed consent for the work. Semen was collected by masturbation after 2–3 days of sexual abstinence, and allowed to liquefy for 30 min at 37°C (95% CO_2 in air). Both the patient and one of the donors produced samples at the same time to enable the two samples to be analysed in parallel.

Sample preparation

Fast progressively motile spermatozoa were purified by a Percoll gradient technique (Moohan and Lindsay, 1995). The 100% isotonic Percoll was prepared by adding Percoll with M medium (10×; 1.37 mol/l NaCl, 25 mmol/l KCl, 200 mmol/l HEPES, 100 mmol/l glucose) in a ratio of 12:1 (v/v). Percoll fractions of 90 and 45% were then made by diluting the 100% Percoll with M medium (1×; 137 mmol/l NaCl, 2.5 mmol/l KCl, 20 mmol/l HEPES, 10 mmol/l glucose, osmalality adjusted to 285–300 mOsm/kg with NaCl). A density gradient of Percoll was prepared in a Falcon tube (90% fraction under the 45% fraction, 1 ml of each) and overlaid with semen (1 ml). Following centrifugation (800 *g*, 20 min), the pellet was aspirated, washed and resuspended in M medium. Fast progressive motility (>10 μm/s) of the vast majority (>90%) of the cells was confirmed using a Hamilton-Thorne Research IVOS version 10.9, USA with standard set-up procedures (ESHRE Andrology Special Interest Group, 1998). Sperm concentration was determined using an improved Neubauer haemocytometer.

2D electrophoresis (2DE)

Percoll-prepared cells were centrifuged (1000 *g*, 10 min) and resuspended in lysis buffer [7 mol/l urea, 2 mol/l thiourea, 1% (w/v) CHAPS, 1% (w/v) *n*-octyl glucopyranoside, 18 mmol/l dithiothreitol (DTT), 0.5% (w/v) IPG buffer pH 3–10, 0.005% (w/v) bromophenol blue, 2.4 μmol/l phenylmethylsulphonyl fluoride (PMSF); 400 μl] (Rabilloud *et al.*, 1997). The sample was rotated for 1 h at room temperature and then centrifuged (20 000 *g*, 10 min) to remove non-solubilized material. The supernatant (350 μl equivalent to 5×10^6 cells) was added to an IPG strip holder and overlaid with an IPG strip (18 cm, pH 3–10 linear) and subjected to isoelectric focusing with an IPGphor [Amersham Biosciences; rehydration (12 h), 500 V (1 h), 1000 V (1 h), 2000 V (1 h), 4000 V (1 h), 6000 V (1 h) 8000 V (4 h)].

The focused strip was rotated in 65 mmol/l DTT in equilibration buffer [50 mmol/l Tris–HCl (from stock 1.5 M Tris–HCl, pH 8.8), 6 mol/l urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.005% (w/v) bromophenol blue] 10 ml for 15 min, and subsequently in 135 mmol/l iodoacetamide in equilibration buffer (10 ml for 15 min). Proteins were separated by SDS–PAGE using pre-cast gradient gels (ExcelGel XL SDS 12–14) and a horizontal Multiphor gel system (Amersham Biosciences; 1000 V, 20 mA for 45 min followed by 1000 V, 40 mA for 160 min) (Craven *et al.*, 2002). Molecular weight markers were also run on each gel (0.5 μl) and pI values assigned from known proteins and using carbamylated protein markers (Amersham Biosciences) as internal standards on a reference gel.

Gel analysis

Proteins were visualized by silver staining using a commercially available kit (Amersham Biosciences) with the standard protocol except that glutaraldehyde was omitted from the sensitizing step and formaldehyde was excluded from the silver solution but increased in the developing step. The silver staining was performed using an automated staining machine (Processor Plus, Amerham Biosciences) with identical times for each of the steps including final development. Gels were initially fixed [40% (v/v) ethanol, 10 (v/v) acetic acid, 30 min] and then sensitized [0.2% (w/v) sodium thiosulphate, 30% (v/v) ethanol, 0.83 mol/l sodium acetate, 30 min]. Following three washes in distilled water, the gel was subjected to the silver reaction [0.25% (w/v) silver nitrate, 20 min] and subsequently washed a further twice. The gels were developed [0.24 mol/l sodium carbonate, 0.015% (w/v) formaldehyde] for 1 min and then 3 min with a fresh solution. Development was quenched with 50 mmol/l EDTA (10 min) and the gel was stored in 1% (v/v) acetic acid (modified from Shevchenko *et al.*, 1996; Yan *et al.*, 2000).

Gels were scanned to a high resolution (500 d.p.i.) with a high specification scanner (ImageScanner, Amersham Biosciences) and saved as tiff images. Detailed gel analysis subsequently was performed using Phoretix 2D Version 5.1 and later using Phoretix 2D Evolution Version 2003.03 (NonLinear Dynamics Ltd, Newcastle, UK). This software has been demonstrated previously to be an objective and accurate method of gel analysis and to be effective in this type of study (Mahon and Dupree, 2001). The software automates the identification and quantification of gel spots by normalizing spot volumes and excluding background noise spots by having a minimum area parameter. Pairs of 2D gels can be compared with the software which matches spots between gels by comparing their location (selected by determining the centre of optical density within the spot). The more advanced Phoretix Evolution has much improved detection and matching algorithms, and additionally allows images to be viewed in 3D to assist user analysis. With both versions of software, it was necessary additionally to confirm manually that each spot had been identified correctly by the software and validate the results for spot comparison between gels. This was achieved by studying enlarged on-screen images of the gels. In this study, we were interested in the absence of spots in one gel compared with the other or a marked difference in spot intensity. The marked difference in spot intensity was arbitrarily set as a >4-fold difference following advice from the software manufacturers (Nonlinear Dynamics Ltd, personal communication).

In-gel protein digestion

In-gel trypsin digestion of manually excised spots was performed using an automated 96-well plate protocol plate modified from Shevchenko *et al.* (1996). Spots were dehydrated with acetonitrile (80 μl, 5 min), de-stained [1:1 (v/v) ratio of freshly made 30 mmol/l $K_3Fe(CN)_6$, 100 mmol/l $Na_2S_2O_3.5H_2O$; 50 μl per spot, 15 min shaking occasionally], rinsed 2–3 times with 25 mmol/l NH_4HCO_3 (50 μl) and the supernatant removed. Acetonitrile was added (50 μl, 15 min), the supernatant removed, the gel rehydrated in 25 mM NH_4HCO_3 (50 μl, 10 min), the supernatant removed and acetonitrile added (50 μl, 15 min). The samples were dried to completeness in an oven (60°C, 30–45 min) and 10 mmol/l DTT in 25 mmol/l NH_4HCO_3 added (25 μl, 56°C for 1 h). The sample was cooled to room temperature, the supernatant removed and 55 mmol/l iodoacetamide in 25 mmol/l NH_4HCO_3 added (25 μl, 45 min at room temperature in the dark). Supernatant was removed and the gel plugs were washed with 25 mmol/l NH_4HCO_3 (25 μl; 10 min). The supernatant was removed and acetonitrile was added (50 μl, 15 min). The supernatant was removed and the plugs were rehydrated with 25 mmol/l NH_4HCO_3 (50 μl, 10 min). Following further dehydration in acetonitrile (50 μl, 15 min) and removal of the supernatant, the spots were dried to completion in an oven (60°C, 30–45 min).

Table I. Assessment of variation of the sperm proteome by automated gel analysis

Experimental comparison	Total no. of spots detected in reference gel	No. of spots with increased or decreased expression on one gel compared with the other (percentage of total spot number)	No. of spots absent from one gel compared with the other (percentage of total spot number)
Same ejaculate	1087	58 (5.3)	14 (1.3)
Different ejaculates, same donor	1175	84 (7.1)	16 (1.4)
Different donors	882	67 (7.6)	16 (1.8)
Fertile donor and patient (same gels as Figure 1)	963	36 (4.0)	48 (5.0) (28 unique to control; 20 unique to patient)

Experimental, intra-donor, inter-donor and patient versus control variation was assessed by gel analyses of four gel pairs using Phoretix Evolution software. Increased and decreased expression was defined as a >4-fold difference in spot intensity between the two gels.

Sequencing grade modified trypsin was prepared as described by the manufacturer and made to a final concentration of 6.25 ng/µl in 50 mmol/l NH_4HCO_3. This trypsin solution was added to the gel plugs (10 µl on ice until the gel had fully rehydrated). Once fully rehydrated (~20 min), the plug was covered with a minimal volume of 25 mmol/l NH_4HCO_3 (10 µl) to keep the gel wet during digestion, and incubated at 37°C overnight. The digestion buffer (10 µl) that surrounds the gel plug was removed and placed in a well in a clean plate. The buffer was dried to completion in an oven (60°C) and the remaining peptides resuspended in 1% (v/v) formic acid (6 µl) for MS/MS or in 0.1% (v/v) trifluoroacetic acid (TFA; 0.5 µl) for MALDI-TOF MS.

Protein identification

MS/MS was performed on the sample (5 µl) using a nanoESI Q-Tof mass spectrometer (Q-Tof Ultima GLOBAL, Micromass Ltd, Manchester, UK) following separation of peptides using capillary liquid chromatography (Waters Ltd, Elstree, Hertford, UK) and a PepMap C18 column [75 µm i.d. × 15 cm; catalogue no. 160396, Dionex (UK) Ltd, Camberley, Surrey, UK]. Following MS/MS, the raw data were processed using MassLynx 3.5 (Micromass). The tryptic peptide sequences were then compared with the NCBI non-identical protein sequence database using MASCOT software (Matrix Science Ltd, London, UK). Following matching of a peptide to a protein, the quality of the raw MS/MS data was validated (this required the presence of good quality y-ion data). Finally, each of the peptides was used to BLAST search to confirm that the protein identified by MASCOT was the only relevant match in the non-redundant protein database for a particular peptide sequence (Kinter and Sherman, 2000).

Samples (0.5 µl) subjected to MALDI-TOF MS were mixed with an equal volume of DHB matrix. This was prepared by mixing 5 mg of DHB in 1 ml of 1:2 (v/v) acetontrile:0.1% (v/v) TFA solution. This was mixed well and spotted onto an AnchorChip target plate (Bruker Daltonics Ltd, Coventry, UK) keeping the droplet centred on the anchor spot (400 µmol/l target selected). This was allowed to dry, and MALDI mass spectra were obtained using a Biflex IV MALDI-TOF MS (Brucker Daltonics) with a nitrogen laser at 337 nm following routine calibration. The singly charged peptide fingerprint was assigned monoisotopic peptide masses using Biotools software (Brucker Daltonics). These data were then used to search the NCBI non-identical protein sequence database using MASCOT software (Matrix Science), and statistically significant hits were recorded together with the number of peptides and percentage coverage of the protein.

Results

Assessment of variation in the proteome of normozoospermic fertile donors

It was important to assess the level of variation in performing 2DE on human spermatozoa. Such variation might be caused by problems of reproducibility with sample preparation and/or performing the 2DE (experimental variation). It might also be caused by differences in proteins in the control samples either from different ejaculates from the same donor (intra-donor variation) or from ejaculates from different donors (inter-donor variation). To address this issue provisionally, detailed gel analyses using Phoretix Evolution software on four paired experiments were performed and the results are presented in Table I.

To examine experimental variation, two gels were analysed from the same ejaculate of a donor of proven fertility prepared and processed simultaneously. This revealed a low variation, with only 14 spots (1.3% of the total number of spots identified) missing from one gel compared with the other. Different ejaculates from the same donor were compared (two gels) as were ejaculates from two different donors (two gels). These experimental samples were prepared at different times but electrophoresed simultaneously. This revealed intra- and inter-donor variation to be 16 spots in each case (corresponding to 1.4 and 1.8% of the total number of spots identified, respectively). The number of spots showing increased or decreased expression were 58, 84 and 67 for experimental, intra-donor and inter-donor variation, respectively (Table I).

Altered protein expression in the patient sample compared with fertile controls

Finally, the same donor sample used as the reference gel in the above study was compared with a gel from the patient. These two gels were derived from one experiment where the control and patient sample had been prepared in parallel to minimize experimental variation. In the first instance, this revealed 28 spots missing in the patient sample but present in the control sample, and 20 spots missing in the control sample but present in the patient sample. This corresponded to 5% of the total

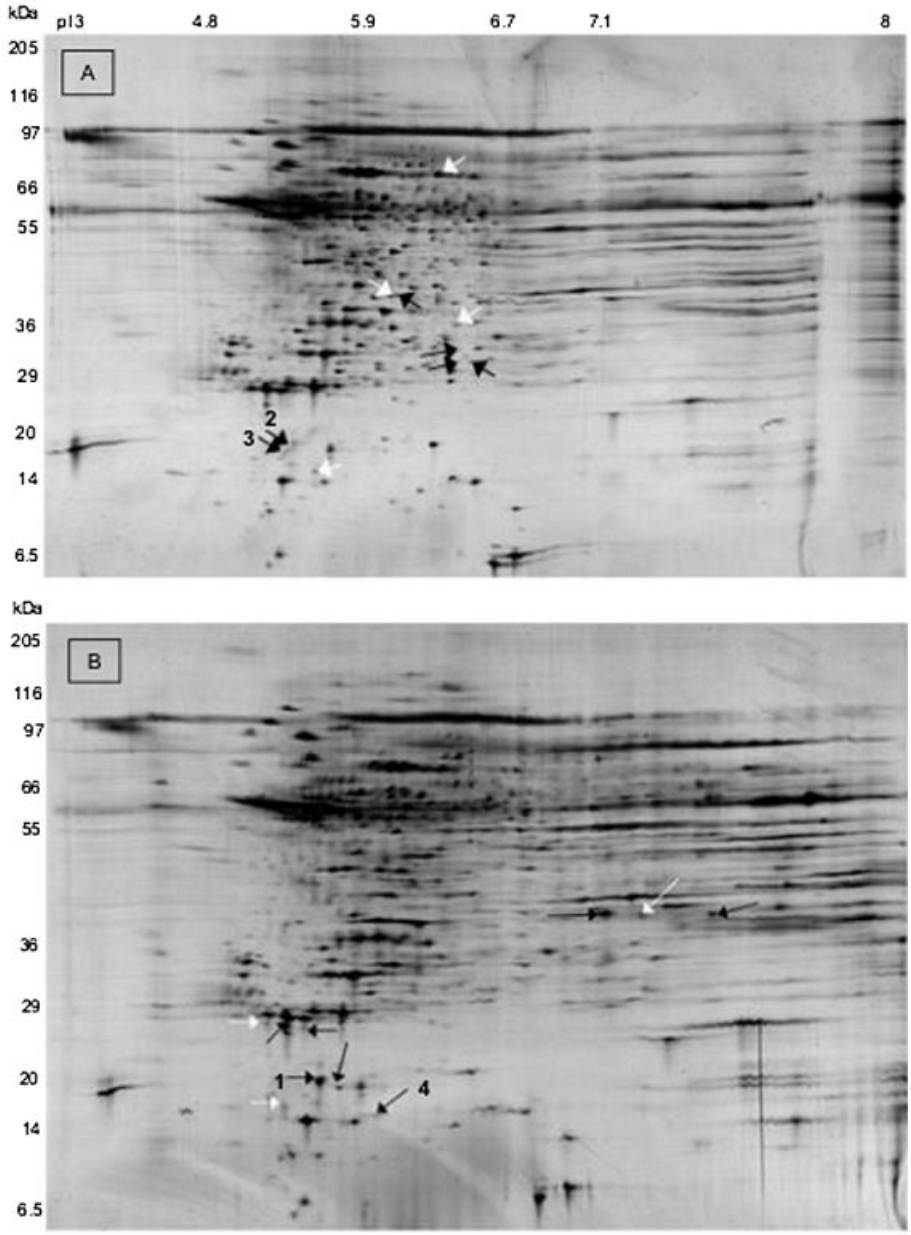

Figure 1. 2D electrophoretic separation of sperm proteins from the patient and a fertile donor. (A) Fertile control sample. (B) Patient sample. These samples were prepared and separated in parallel and this experiment was performed in triplicate. Proteins from 5×10^6 cells were loaded onto each gel. The apparent molecular weights are indicated down the side, and the pI values across the top of the gels. The 20 protein spots highlighted are those that consistently displayed altered expression between the patient and control samples following detailed analysis of all of the gels including other control samples (see text). (**A**) Spots that were unique to the control gels are indicated by black arrows. Spots showing increased expression in the control gels compared with the patient gels are indicated by white arrows. (**B**) Spots that were unique to the patient gels are indicated by white arrows. Spots showing increased expression in the patient gels compared with the control gels are indicated by black arrows. Conclusive or provisional protein identification was achieved for spots 1–4 (see Table II).

number of spots missing from one gel compared with the other. In this comparison, the number of increased or decreased spots (≥4-fold change in intensity) was also detected, and revealed 29 spots with increased expression and seven spots with decreased expression in the patient sample compared with the control.

However, this represented only one experiment. To examine this variation between the control and the patient in detail, this experiment was performed on three separate occasions with different ejaculates each time. The patient and control sample were examined in parallel each time. Two gels (one control and one patient sample) from the same experiment are shown in Figure 1 (these are the same gels analysed in Table I). Differences noted on each occasion were only examined further if spots were missing/additional or significantly changed in all patient samples (ejaculates) compared with all donor gels in these three experiments. These data were then compared with six further gels (two different ejaculates from three different donors), and again only those spots that were consistently different in every gel were noted.

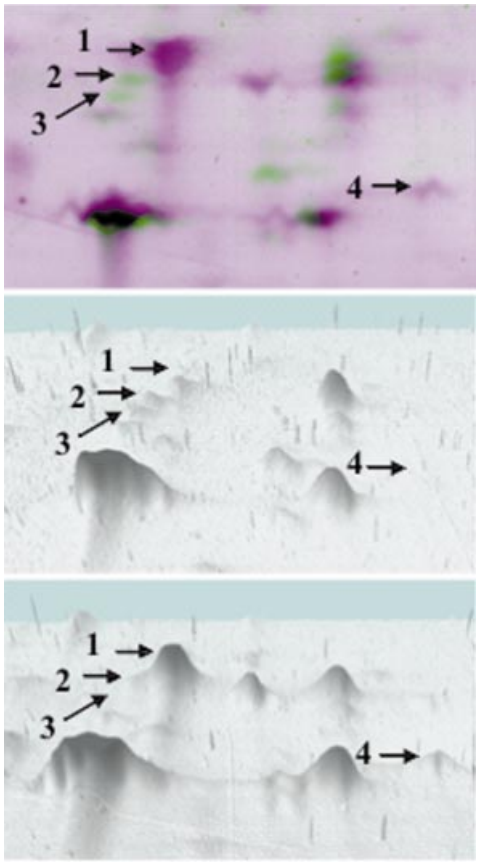

Figure 2. An example to demonstrate the results obtained from automated gel analysis. The area shown corresponds to the region of the gel where protein identification was successfully achieved. Gel analysis was performed using Phoretix Evolution software. Top panel: Phoretix overlay analysis of the patient sample (purple gel) compared with the fertile control (green gel). This allows analysis by observing differences in coloured areas. For example, spots that are absent (or significantly reduced) in the patent sample appear as green. Spots that appear as purple are absent (or significantly reduced) in the control. Where both colours are equally represented, they appear as black. Protein differences between gels can be viewed in 3D to ensure that a real spot is being detected rather than background noise. The middle panel corresponds to the control sample and the lower panel corresponds to the patient sample. Note that those spots (proteins) that have been identified (either conclusively or provisionally) are labelled: (1) secretory actin-binding protein (increased levels in patient); (2) DEAD/DEAH box helicase (missing in patient); (3) bromodomain PHD finger transcription factor (missing in patient); and (4) outer dense fibre protein 2/2 (increased in patient).

Based on these analyses, we have identified 20 differences between the control and patient samples that we can be confident represent true differences. There were six spots present in each of the fertile controls but absent in all of the gels from the patient, three spots present in the patient but absent in the control samples, seven spots more intense in the patient and four spots more intense in the control. These spots are highlighted in Figure 1.

Figure 2 has been included to demonstrate how Phoretix Evolution software is used. The reference (control) gel and the experimental (patient) gel are green and purple, respectively, and differences can be clearly seen. These can be assessed further using the 3D viewing feature where the third dimension is the pixel intensity of the image. The region of the gel presented corresponds to that region in Figure 1 where successful protein identification was achieved. Note, however, that this figure corresponds to only one experiment, and only those spots with consistent differences in all the analyses are annotated.

Protein identification

We have attempted to identify all of the proteins from those spots that showed consistently altered expression in the patient samples. However, we have only successfully achieved sequencing data for four spots to date. These data are summarized in Table II. Two proteins displayed increased expression in the patient sample compared with the fertile controls. One of these was secretory actin-binding protein which was identified from peptide mass fingerprinting of six peptides by MALDI-TOF MS. These peptides corresponded to 56% coverage of the protein. The other was outer dense fibre protein 2/2 which was conclusively identified from eight peptide sequences from MS/MS. Interestingly, these peptides corresponded to the protein sequence of only half of the protein, and the actual molecular weight of this protein on the gel was 18 kDa. Two proteins that were absent in the patient sample compared with the control were provisionally identified as DEAD/DEAH box helicase (DDX37) and a bromodomain PHD finger transcription factor. However, identification was achieved from only one and two peptides, respectively, and the MS/MS sequence data were not of sufficient quality for conclusive identification. The actual molecular weights of these spots was ~20 kDa.

Discussion

The systematic unbiased identification of proteins using proteomic strategies presently is the subject of much interest in biomedical research and has been shown to be a very powerful approach (Tyers and Mann, 2003). However, it is particularly complex, and much technical development and expertise is required to facilitate such studies for full protein identification. It is much more straightforward to perform 2DE, and a number of scientists have done this for human spermatozoa, with the best mapping project to date by Naaby-Hansen *et al.* (1997). However, no studies have examined the potential variability of the proteome in different semen samples. This was a particular concern in this study. It is important to discount the possibility that any differences might be accounted for by experimental variation due to sample preparation and 2DE or within the normal variation of the control samples.

In the first instance, extensive technical development was carried out optimizing sample preparation, in particular protein solubilization, and 2DE reproducibility. Several technical advances in recent years have facilitated 2DE reproducibility, including immobilized pH gradients in gel strips and pre-cast second dimension gels (Rabilloud *et al.*, 1997; Craven *et al.*, 2002). With these, we routinely achieve a very high level of

Table II. Summary of those proteins showing altered expression in the patient that have been identified by mass spectrometry

Gel spot	Difference in protein expression in patient compared with donors	Protein assigned from database search of peptides	Peptides identified and used for searching	Accession no.	Comments
1	Increased	Secretory actin-binding protein	SVRPNDEVTAVLAVQTELK TYLISSIPLQGAFNYK YTACLCDDNPK TVQIAAVVDVIR ELGICPDDAAVIPIK FYTIEILKVE	SQHUAC	Definitive protein identification using PMF (56% protein coverage). Protein mass and pI on gel was the same as previously reported.
2	Missing	DEAD/DEAH box helicase (DDX37)	SCLLSSPGTMLK	29029601	Provisional identification by MS/MS but data not conclusive. Lower molecular weight on gel than expected so possibly a fragment of this protein
3	Missing	Bromodomain PHD finger transcription factor	SHLLSSSDAEGNYR KPLIQEESDTIVSSSK	6683492	Provisional identification by MS/MS but data not conclusive. Lower molecular weight on gel than expected so possibly a fragment of this protein
4	Increased	Outer dense fibre protein 2/2	NYEGMIDNYK AEVEAIMEQLK LEADEVAAQLER LNQAHLEVQQLK TRLEADEVAAQLER LAECQDQLQGYER VTDLVNQQQTLEEK SEEYAEQLHVQLADK	2996006	Conclusive identification by MS/MS. Lower molecular weight on gel than expected and peptide only in one half of peptide sequence, therefore, this is a fragment of this protein.

Secretory actin-binding protein and outer dense fibre protein 2/2 were identified conclusively from the multiple peptides, whereas bromodomain PHD finger transcription factor and DEAD/DEAH box helicase represent provisional identifications.
PMF = peptide mass fingerprinting by MALDI-TOF mass spectrometry; MS/MS = tandem mass spectrometry.

reproducibility. A large database of gels from eight donors produced over a prolonged time has enabled us to establish confidence over the general patterns of spots displayed in the sperm proteome following 2DE. However, we wanted to examine this in a more objective manner by using gel analysis software. In the first instance, these analyses were performed using Phoretix 2D Elite. Whilst much better than manual analysis, there was still an element of subjectivity involved in performing these analyses due to limitations of the software. In addition, such analyses are time consuming (~8 h to perform a paired gel comparison and confirm the data). Recently, we have been able to use a new generation of gel analysis software (Phoretix Evolution). This is fully automated with more advanced spot identification and more matching algorithms than the older software. As such, it may be thought of as a non-biased form of analysis and requires only limited validation to confirm correct spot identification and spot comparisons between different gels.

Analysis of two gels with duplicate samples from the same ejaculate clearly demonstrated very low variation in the presence or absence of proteins in two samples prepared from the same donor ejaculate (Table I). Assessment of different ejaculates from a single donor and ejaculates from two different fertile donors (intra- and inter-donor variability, respectively) again revealed remarkably low levels of variation in relation to the absolute presence or absence of proteins even though these samples were prepared and subjected to 2DE at different times. Although many experiments would need to be performed to be able to carry out detailed statistical analyses, it is remarkable just how little variation there is in absolute spot differences. Whereas we believe these absolute differences in proteins to be unique to either the patient or control gels, it is more difficult to be certain about those proteins with increased or decreased expression. Although we have used a large change in expression (>4-fold difference), it is possible that such differences might be accounted for due to limitations in the staining procedure used or the gel analysis software. For example, silver staining is not ideally suited for densitometry due to its limited dynamic range. Further analyses would be required to determine mean values for such altered expression from a number of gels in order to probe this fully.

In preliminary studies, we used direct swim-up procedures rather than density gradient centrifugation and saw more substantial variation between gels produced from the same donor from different ejaculates. Comparison between direct swim-up and density gradient centrifugation revealed much better reproducibility with the latter approach, and hence we have adopted this. Two of the most abundant proteins that were present in the swim-up but absent in the density gradient centrifugation fraction were identified as the abundant seminal plasma proteins prostate-specific antigen and prostatic acid phosphatase previously reported by Starita-Geribaldi *et al.* (2001) (data not shown). Hence it appeared that there might be seminal plasma contamination, even though the technique was performed with great care, and that this might be the cause of the greater variability following swim-up preparation. Rather than direct contamination with seminal plasma, it might also be the case that these seminal plasma proteins are adhered to the sperm and not lost with swim-up, but are removed by density gradient centrifugation through Percoll.

Although there is very little absolute variation in the proteome maps between different donors, the number of spots identified that were unique to one sample in the control versus patient gel comparison was higher (48 differences compared with only 16 between different controls). This experiment was repeated three times and only those differences consistently observed were noted. Finally, the differences were compared with a further six control gels (two different ejaculates from three donors) and again only the consistent differences were included in the final figure of 20 protein differences. In addition, differences in expression were only noted if these were high (>4-fold change in intensity). This therefore represents a very conservative estimate of the number of protein differences between this patient and the controls, and represents only those protein spots that we are confident from our studies are expressed differently in this patient compared with the fertile donors. We have performed enough experiments to confirm the absolute differences between the patient and control. We are more circumspect about the altered expression and have purposely set the level of altered expression as >4-fold in order to record only spots where there is a large and consistent change in expression. There are limitations with the 2DE approach that we have used. The broad range immobilized pH gradients do not produce enough resolution to separate all of the proteins, especially in some overloaded regions of the gel. This might be overcome by running multiple gels either with different levels of protein loading or by using multiple overlapping 'zoom gels' which have small pI ranges (Starita-Geribaldi *et al.*, 2003). The latter approach might also improve detection of the more minor proteins that may be expressed differently. In addition, the focusing strips used will fail to resolve the more acidic and basic proteins. Finally, some proteins, particularly hydrophobic membrane proteins, may not be solubilized during sample preparation.

We have successfully identified two of these 20 proteins with provisional identification for a further two proteins, and these data are summarized in Table II. The failed identification of the other proteins was due to their low abundance, which was beyond the detection limits of our technology at present rather than due to failed assignment of large numbers of peptides identified by MS/MS. Even with the state-of-the-art mass spectrometry facilities, where we can identify down to 2.5 fmol of peptide, faintly silver-stained protein spots still represent the limits of resolution of the technology. However, we will endeavour to address this in the future by, for example, pooling spots from multiple gels. There are very few groups studying spermatozoa that have successfully identified proteins by definitive mass spectrometry. Indeed only Herr, Visconti and co-workers have consistently identified low abundance sperm proteins from silver-stained gels (e.g. Naaby-Hansen *et al.*, 2002; Ficarro *et al.*, 2003).

Considering each of the identified proteins in turn, outer dense fibre protein 2/2 was identified conclusively. It has been been reported previously as one of a number of similar proteins in human, rat and mouse spermatozoa that are tail-specific cytoskeletal structures that surround the axoneme in the midpiece and principal piece (Brohmann *et al.*, 1997; Hoyer-Fender *et al.*, 1998, 1999). The fibres function in maintaining the elastic structure and recoil of the sperm tail as well as in protecting the tail from shear forces during epididymal transit and ejaculation. The identification of this protein is somewhat problematic in that the molecular weight of the protein resolved on the second dimension gel is lower than that predicted (18 kDa instead of ~70 kDa). In addition, only peptides corresponding to about half of the protein were identified. Therefore, this spot represents a fragment of the intact protein. It is possible to hypothesize that a raised level of this fragment might mean a reduced level of the intact protein which would have been missed in the analysis as it would localize to an overloaded area of the gel with poor resolution (70 kDa with a calculated pI 7.2–7.9). Defects in the outer dense fibre might lead to abnormal sperm morphology and infertility. However, the experiments were performed only with PMSF present as a protease inhibitor. It may well be the case that increased levels of this fragment are artefactual and due to proteolytic cleavage of the protein during sample preparation and cell lysis. Further work would be needed to confirm the reason for this result.

Secretory actin-binding protein was identified using MALDI-TOF, and the protein localized to a position on the gel that was comparable with that predicted in the literature. Secretory actin-binding protein was first identified and characterized by Schaller *et al.* (1991) from human seminal plasma. It is also known as gp17, and this seminal plasma protein has been shown specifically to interact with CD4 on T cells (Autiero *et al.*, 1995). Increased levels of this protein might therefore cause increased interactions with T cells and this might be responsible for failed fertilization. It is also possible to envisage that a raised level of such a protein that would interact with the key cytoskeletal protein actin might affect fertility. In support of this is a report where an anti-actin monoclonal antibody inhibited the zona pellucida-induced acrosome reaction and also hyperactivation but not zona binding in human spermatozoa (Liu *et al.*, 2002). It is possible that the raised levels of secretory actin-binding protein might also act in a similar fashion and affect zona binding.

BLAST analysis shows that the gene is located on chromosome 7q32 and that this protein is identical to prolactin-inducible protein (PIP; Myal *et al.*, 1991). PIP is an aspartyl proteinase that is found in benign and malignant breast tumours and in such normal exocrine organs as sweat, salivary and lacrimal glands (Myal *et al.*, 1991). Secretory actin-binding protein is in the same protein family and very closely related to seminal vesicle autoantigen (Huang *et al.*, 1999) that is present in the seminal plasma of mice. This 19 kDa secretory glycoprotein suppresses the motility of spermatozoa by interacting with phospholipids, and has also been shown to suppress other capacitation-related events, such as tyrosine phosphorylation (Huang *et al.*, 2000). It is tempting to suggest that secretory actin-binding protein might also have a similar role to seminal vesicle autoantigen in the human. However, in these experiments, we showed that the patient sperm prepared had significant levels of progressive motility. Nonetheless, it is possible that this raised level of secretory actin-binding protein might inhibit the development of hyperactivated motility or

other capacitation-related events which in turn might affect fertilization. These effects might be more marked in those spermatozoa not selected by this Percoll gradient technique and discarded. However, at this stage, these comments are only speculative and further work will be needed to assess the candidacy of this protein.

In two cases it was possible provisionally to identify proteins that were absent in the patient sample: DEAD/DEAH box helicase (DDX37) which is an ATP-dependent RNA helicase protein (Nagase *et al.*, 2000) and bromodomain PHD finger transcription factor (Jones *et al.*, 2000). With regard to DEAD/DEAH box helicase (DDX37), the anxiety in terms of definitive identification was that only one peptide was recovered that provided the match and the raw data were not fully convincing. Therefore, it is not possible to say conclusively that this protein is present. However, the data are stronger than for each of the two peptides for bromodomain PHD finger transcription factor. In both cases, the protein recovered was of a much lower molecular weight than the intact protein (in each case ~20 kDa not 130 and 313 kDa, respectively) and so these proteins at best represent cleavage products.

In summary, this study represents the first case where the sperm proteome map of a patient with a particular condition has been compared a number of fertile donor controls and should in part be thought of as a proof of principle that it is possible to probe infertility using 2DE of the entire sperm proteome as long as a considered approach is pursued. While we do not yet know the implications of the increased expression in the patient of outer dense fibre protein 2/2 or secretory actin-binding protein, this study represents a useful base for further investigations.

We have only examined one patient to date. Only when a number of patients have been studied might it be possible to conclude which differences in protein expression are specific for individual patients and which are more widespread. Ultimately, this will enable further elucidation of the molecular mechanisms involved in this particular condition and might shed further light on key sperm proteins involved in fertilization. It is also an important prerequisite to the development of diagnostic tests to identify these men in a clinical environment, and in the future might also be applied to the development of novel methods of sperm-targeted contraception.

Acknowledgements

We thank Nurse Evelyn Barratt for patient recruitment and also especially thank those patients who have participated in this study. Thanks to Dr Mathew Tomlinson and the clinical andrology staff for assistance with the provision of research sperm donors. We appreciate the assistance of Nonlinear Dynamics Ltd (Newcastle) with the gel analyses. We are indebted to Antony Jones (BBSRC Functional Genomics Laboratory, School of Biosciences, The University of Birmingham) for the automated trypsin digestion of the gel plugs. The Functional Genomics Laboratory was established and is maintained by BBSRC grant 6/JIF13209. This work was supported by Genosis Ltd (12–50 Kingsgate Road, Kingston KT2 5AA), The Lord Dowding Fund for Humane Research and Birmingham Women's Healthcare NHS Trust.

References

Aebersold R and Mann M (2003) Mass spectrometry-based proteomics. Nature 422,198–207.

Autiero M, Cammarota G, Friedlein A, Zulauf M, Chiapppetta G, Dragone V and Guardiola J (1995) A 17-kDa CD4-binding protein present in human seminal plasma and human breast tumour cells. Eur J Biochem 25,1461–1464.

Banks RE, Dunn MJ, Hochstrasser DF, Sanchez JC, Blackstock W, Pappin DJ and Selby PJ (2001) Proteomics: new perspectives, new biomedical opportunities. Lancet 356,1749–1756.

Bastiaan HS, Windt ML, Menkveld R, Kruger TF, Oehninger S and Franken DR (2003) Relationship between zona pellucida-induced acrosome reaction, sperm morphology, sperm–zona pellucida binding, and in vitro fertilization. Fertil Steril 79,49–55.

Blackstock WP and Weir MP (1999) Proteomics: quantitative and physical mapping of cellular proteins. Trends Biotechnol 17,121–127.

Bohring C and Krause W (1999) The characterisation of human spermatozoa membrane proteins—surface antigens and immunological infertility. Electrophoresis 20,971–976.

Bohring C and Krause W (2003) Immune infertility: towards a better understanding of sperm (auto)-immunity. The value of proteomic analysis. Hum Reprod 18,915–924.

Bohring C, Krause E, Habermann B and Krause W (2001) Isolation and identification of sperm membrane antigens recognised by antisperm antibodies and their possible role in immunological infertility disease. Mol Hum Reprod 7,113–118.

Brewis IA (1999) The potential importance of proteomics to research in reproduction. Hum Reprod 14,2927–2929.

Brewis IA and Wong CH (1999) Gamete recognition: sperm proteins that interact with the egg zona pellucida. Rev Reprod 4,135–142.

BritishAndrologySociety(1999) British Andrology Society guidelines for the screening of semen donors for donor insemination. Hum Reprod 14,1823–1826.

Brohmann H, Pinnecke S and Hoyer-Fender S (1997) Identification and characterisation of new cDNA encoding outer dense fibre proteins of rat sperm. J Biol Chem 272,10327–10332.

Byrjalsen I, Larsen PM, Fey SJ, Nilas L, Larsen MR and Christiansen C (1999) Two-dimensional gel analysis of human endometrial proteins: characterisation of proteins with increased expression in hyperplasia and adenocarcinoma. Mol Hum Reprod 5,748–756.

Craven RA, Jackson DH, Selby PJ and Banks RE (2002) Increased protein entry together with improved focussing using a combined IPGphor/Multiphor approach. Proteomics 2,1061–1063.

Ensslin MA and Shur BD (2003) Identification of mouse sperm SED1, a bimotif EGF repeat and discoidin protein involved in sperm–egg binding. Cell 114,405–417.

ESHRE Andrology Special Interest Group(1998) Guidelines on the application of CASA technology in the analysis of spermatozoa. Hum Reprod 13,142–145.

Esterhuizen AD, Franken DR, Lourens JGH and van Rooyen LH (2001) Clinical importance of zona pellucida-induced acrosome reaction and its predictive value for IVF. Hum Reprod 16,138–144.

Evans JP and Florman HM (2002) The state of the union: the cell biology of fertilization. Nature Cell Biol and Nat Med (Fertility Suppl) s57–s63.

Ficarro S, Chertihin O, Westbrook VA, White F, Jayes F, Kalab P, Marto JA, Shabanowitz J, Herr JC, Hunt DF and Visconti PE (2003) Phosphoproteome analysis of capacitated human sperm. J Biol Chem 278,11579–11589.

Franken DR, Kruger TF, Oehninger S, Coddington CC, Lombard C, Smith K and Hodgen GD (1993) The ability of the hemizona assay to predict human fertilization in different and consecutive in-vitro fertilization cycles. Hum Reprod 8,1240–1244.

Hoyer-Fender S, Petersen C, Brohmann H, Rhee K and Wolgemuth DJ (1998) Mouse Odf2 cDNAs consist of evolutionary conserved as well as highly variable sequences and encode outer dense fiber proteins of the sperm tail. Mol Reprod Dev 51,167–175.

Huang Y-H, Chu S-T and Chen Y-H (1999) Seminal vesicle autoantigen, a novel phospholipid-binding protein secreted from luminal epithelium of mouse seminal vesicle, exhibits the ability to suppress mouse sperm motility. Biochem J 343,241–248.

Huang Y-H, Chu S-T and Chen Y-H (2000) A seminal vesicle autoantigen of mouse is able to suppress sperm capacitation-related events stimulated by serum albumin. Biol Reprod 63,1562–1566.

Hughes DC and Barratt CL (1999) Identification of the true human orthologue

of the mouse Zp1 gene: evidence for greater complexity in the mammalian zona pellucida? Biochim Biophys Acta 1447,303–306.

Hull MG, Glazener CM, Kelly NJ, Conway DI, Foster PA, Hinton RA, Coulson C, Lambert PA, Watt EM and Desai KM (1985) Population study of causes, treatment and outcome of infertility. Br Med J 291,1693–1697.

Ivic A, Onyeaka H, Girling A, Brewis IA, Ola B, Hammadieh N, Papaioannou S and Barratt CLR (2002) Critical evaluation of methylcellulose as an alternative medium in sperm migration tests. Hum Reprod 17,143–149.

Jones MH, Hamana N and Shimane M (2000) Identification and characterisation of BPTF, a novel bromodomain transcription factor. Genomics 63,35–39.

Kinter M and Sherman NE (2000) Protein Sequencing and Identification Using Tandem Mass Spectrometry. John Wiley, New York.

Krausz C, Bonaccorsi L, Maggio P, Luconi M, Criscuoli L, Fuzzi B, Pellegrini S, Forti G and Baldi E (1996) Two functional assays of sperm responsiveness to progesterone and their predictive values in in-vitro fertilization. Hum Reprod 11,1661–1667.

Liu DY and Baker HW (2000) Defective sperm–zona pellucida interaction: a major cause of failure of fertilization in clinical in-vitro fertilization. Hum Reprod 15,702–708.

Liu DY and Baker HW (2003) Disordered zona pellucida-induced acrosome reaction and failure of in vitro fertilization in patients with unexplained infertility. Fertil Steril 79,74–80.

Liu DY, Clarke GN, Martic M, Garrett C and Baker HW (2001) Frequency of disordered zona pellucida (ZP)-induced acrosome reaction in infertile men with normal semen analysis and normal spermatozoa-binding. Hum Reprod 16,1185–1190.

Liu DY, Martic M, Clarke GN, Grkovic I, Garrett C, Dunlop ME and Baker HW (2002) An anti-actin monoclonal antibody inhibits the zona pellucida-induced acrosome reaction and hyperactivated motility of human sperm. Mol Hum Reprod 8,37–47.

Mackenna A, Barratt CL, Kessopoulou E and Cooke ID (1993) The contribution of a hidden male factor to unexplained infertility. Fertil Steril 59,405–411.

Mahon P and Dupree P (2001) Quantitative and reproducible two dimensional gel analysis using Phoretix 2D full. Electrophoresis 22,2075–2085.

Moohan JM and Lindsay KS (1995) Spermatozoa selected by a discontinuous percoll density gradient exhibit better motion characteristics, more hyperactivation, and longer survival than direct swim-up. Fertil Steril 64,160–165.

Myal Y, Robinson DB, Iwasiow B, Tsuyuki D, Wong P and Shiu RP (1991) The prolactin-inducible protein (PIP/GCDFP-15) gene: cloning, structure and regulation. Mol Cell Endrocrinol 80,165–175.

Naaby-Hansen S, Flickinger CJ and Herr JC (1997) Two-dimensional gel electrophoretic analysis of vectorially labelled surface proteins of human spermatozoa. Biol Reprod 56,771–787.

Naaby-Hansen S, Mandal A, Wolkowicz MJ, Sen B, Westbrook VA, Shetty J, Coonrod SA, Klotz KL, Kim Y-H, Bush LA, Flickinger CJ and Herr JC (2002) CABYR, a novel calcium binding tyrosine phosphorylation-regulated fibrous sheath protein involved in capacitation. Dev Biol 242,236–254.

Nagase T, Kikono R, Ishikawa K-I, Hirosawa M and Ohara O (2000) Prediction of the coding sequences of unidentified human genes. The complete sequences of 100 new cDNA clones from brain which code for large proteins in vitro. DNA Res 7,143–150.

Oehninger S, Mahony M, Ozgur K, Kolm P, Kruger T and Franken D (1997) Clinical significance of sperm–zona pellucida binding. Fertil Steril 67,1121–1127.

Ola B, Afnan M, Sharif K, Papaioannou S, Hammadieh N and Barratt CL. (2001) Should ICSI be the treatment of choice for all cases of in-vitro conception? Hum Reprod 16,2485–2490.

Pandley A and Mann M (2000) Proteomics to study genes and genomes. Nature 405,837–846.

Petersen C, Fûzeli L and Hoyer-Fender, S (1999) Outer dense fibre proteins from human sperm tail: molecular cloning and expression analyses of two cDNA transcripts encoding proteins of ~70 kDa. Mol Hum Reprod 5,627–635.

Primakoff P, Lathrop W and Bronson, R (1990) Identification of human sperm surface glycoproteins recognised by autoantisera from immune infertile men, women, and vasectomized men. Biol Reprod 42,929–942.

Rabilloud T, Adessi C, Giraudel A and Lunardi J (1997) Improvement of the solubilization of proteins in two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 18,307–316.

Schaller J, Akiyama K, Kimura H, Hess D, Affolter M and Rickli EE (1991) Primary structure of a new actin-binding protein from human seminal plasma. Eur J Biochem 196,743–750.

Shetty J, Naaby-Hansen S, Shibahara H, Bronson R, Flickinger CJ and Herr JC (1999) Human sperm proteome: immunodominant sperm surface antigens identified with sera from infertile men and women. Biol Reprod 61,61–69.

Shetty J, Diekman AB, Jayes FC, Sherman NE, Naaby-Hansen S, Flickinger CJ and Herr JC (2001) Differential extraction and enrichment of human sperm surface proteins in a proteome: identification of immunocontraceptive candidates. Electrophoresis 22,3053–3066.

Shevchenko A, Wilm M, Vorm O and Mann M (1996) Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal Chem 68,850–858.

Shibahara H, Sato I, Shetty J, Naaby-Hansen S, Herr JC, Wakimoto E and Koyama K (2002) Two-dimensional electrophoretic analysis of sperm antigens recognized by sperm immobilizing antibodies detected in infertile women. J Reprod Immunol 53,1–12.

Starita-Geribaldi M, Poggioli S, Zucchini M, Garin J, Chevalier D, Fenichel P and Pointis G (2001) Mapping of seminal plasma proteins by two-dimensional gel electrophoresis in men with normal and impaired spermatogenesis. Mol Hum Reprod 7, 715–722.

Starita-Geribaldi M, Roux F, Garin J, Chevalier D, Fenichel,P and Pointis G (2003) Development of narrow immobilised pH gradients covering one pH unit for human seminal plasma proteomic analysis. Proteomics 3,1611–1619.

Tesarik J and Mendoza C (1992) Defective function of the nongenomic progesterone receptor as a sole sperm anomaly in infertile patients. Fertil Steril 58,793–797.

Tournaye H, Verheyen G, Albano C, Camus M, van Landuyt L, Devroey P and van Steirteghem A (2002) Intracytoplasmic sperm injection versus in vitro fertilization: a randomised controlled trial and a meta-analysis of the literature. Fertil Steril 78,1030–1037.

Turner RM, Foster JA, Gerton GL, Moss SB and Patrizio P (2001a) Molecular evaluation of two major fibrous sheath proteins, pro-hAKAP982 and hAKAP82, in stump tail sperm. Fertil Steril 76,267–274.

Turner RM, Musse MP, Mandal A, Klotz K, Jayes FCL, Herr JC, Gerton GL, Moss SB and Chemes HE (2001b) Molecular genetic analysis of two human sperm fibrous sheath proteins, AKAP4 and AKAP3, in men with dysplasia of the fibrous sheath. J Androl 22, 302–315.

Tyers M and Mann M (2003) From genomics to proteomics. Nature 422,193–197.

World Health Organization(1999) WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction, 4th edn. Cambridge University Press, Cambridge, UK.

Yan JX, Wait R, Berkelman T, Harry RA, Westbrook JA, Wheeler CH and Dunn MJ (2000) A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electrospray ionization-mass spectrometry. Electrophoresis 21,3666–3672.

Submitted on December 2, 2003; accepted on March 4, 2004