Caracterització proteòmica de l'espermatozoide humà. Proteïnes diferencials trobades en pacients astenozoospèrmics.

Per Juan Martínez Heredia

8.- Annexos

Annex 1.

En aquest annex es presentaran els càlculs ja fets per als volums més usuals de totes les solucions descrites a la tesis. Així tot resultarà més còmode a l'hora de repetir els experiments.

Mètode físic de decapitació (pàgina 52)

Basat en treballs previs (Van Blerkom et al., 1995)

- Solució 1. Sense PMSF ni Tritó X100, que s'afegeixen al moment.

Per a 100 mL, els volums i pesos necessaris són:

PIPES → 1.814 g

HEPES → 0.596 g

EGTA → 0.380 g

$MgCl_2 \cdot 6H_2O$ → 0.04066 g

S'ha d'ajustar després el pH a 7.4 amb HCl.

La solució de treball és de 1 mL/mostra, i es aquí on s'afegeix el Tritó X100 i el PMSF.

Tritó X100 → 10 μL

PMSF → 40 μL de PMSF 100 mM

- Solució 2. Sense PMSF, que s'afegeix al moment.

Per a 100 mL, els volums i pesos necessaris són:

PIPES → 1.814 g

HEPES → 0.596 g

EGTA → 0.380 g

$MgCl_2 \cdot 6H_2O$ → 0.04066 g

S'ha d'ajustar després el pH a 7.4 amb HCl.

La solució de treball és de 1 mL/mostra, i es aquí on s'afegeix el PMSF.

PMSF → 40 μL de PMSF 100 mM

- PBS (Phosphate Buffered Saline).

Per a 1 L, els volums i pesos necessaris són:

NaCl → 8 g

KCl → 0.2 g

Na_2HPO_4 → 1.44 g

KH_2PO_4 → 0.24 g

Això en 800 mL d'aigua destil·lada. S'ajusta el pH a 7.4 amb HCl. S'enrasa a 1L.

Mètode químic de decapitació (pàgina 53)

Basat en treballs previs (Jassim et al., 1992)

- Solució 1. Sense PMSF ni Tritó X100, que s'afegeixen al moment.

Per a 10 mL, els volums i pesos necessaris són:

DTT 1M → 0.2 mL

Tris HCl pH 8.8 1M → 0.5 mL

La solució de treball és de 1 mL/mostra, i es aquí on s'afegeix el Tritó X100 i el PMSF.

Tritó X100 → 10 μL

PMSF → 40 μL de PMSF 100 mM

- Solució 2.

Per a 10 mL, els volums i pesos necessaris són:

DTT 1M → 0.2 mL

Tris HCl pH 8.8 1M → 0.5 mL

Urea → 2.4024 g

La solució de treball és de 1 mL/mostra, i es aquí on s'afegeix el PMSF.

PMSF → 40 μL de PMSF 100 mM

- Tris HCl pH 8.8 1M

Per a 0.5 mL, els volums i pesos necessaris són:

Tris → 60.57 g

S'ajusta després el pH a 8.8 amb HCl.

- PBS (Phosphate Buffered Saline).

Per a 1 L, els volums i pesos necessaris són:

NaCl → 8 g

KCl → 0.2 g

Na_2HPO_4 → 1.44 g

KH_2PO_4 → 0.24 g

Això en 800 mL d'aigua destil·lada. S'ajusta el pH a 7.4 amb HCl. S'enrasa a 1L.

Extracció àcida de proteïnes. (pàgina 54)

Segons de Yebra et al., 1993.

En aquest protocol no es preparen solucions estoc, ja que les solucions es preparen al moment (exceptuant el Tris/EDTA).

PMSF → 15 µL de PMSF 100 mM (per a 1.5 mL)

EDTA → 0.4 mL EDTA 0.5M (per a 10 mL)

Tris → 1 ml de Tris 1M pH 8 (per a 10 mL)

Hidroclorur de Guanidina → 0.29659 g (per a 0.5 mL)

DTT → 0.2875 mL de DTT 1M (per a 0.5 mL)

Preparació de gels àcids (pàgina 57)

Per a un gel petit (tires de 7 cm) els volums i pesos necessaris són els següents:

H_2O mQ → 4.3 mL

Àcid Acètic → 0.771 mL

Urea → 2.25 g

Acrilamida/Bisacrilamida (29:1) → 6.76 mL

El APS i el TEMED s'afegeixen a l'últim moment:

APS (10%, 0.1g/mL) → 0.8 mL

TEMED → 0.08 mL

Per a un gel gran (tires de 17 cm), els volums i pesos es multipliquen per 3:

H_2O mQ → 13.017 mL

Àcid Acètic → 2.313 mL

Urea → 6.75 g

Acrilamida/Bisacrilamida (29:1) → 20.28 mL

El APS i el TEMED s'afegeixen a l'últim moment:

APS (10%, 0.1g/mL) → 2.4 mL

TEMED → 240 μL

Tampó d'electroforesis. (pàgina 58)

Per a gels àcids i 1L de tampó acètic, els volums són els següents:

Àcid Acètic glacial → 51.374 mL

Extracció total de proteïnes. (pàgina 56)

Segons Pixton et al., 2004.

Per a 10 mL, els volums i pesos necessaris són:

Urea → 4.2 g

Tiourea → 1.52 g

CHAPS → 0.1 g

N-octilglucopiranòsid → 0.1 g

IPG buffer → 50 μL

Blau de bromofenol → 0.0005 g

La solució de treball que es prepara acostuma a ser de 1 mL/mostra, i es aquí on s'afegeix el PMSF i el DTT.

DTT → 18 μL

PMSF → 24 μL

Tampó d'equilibració per a tires. (pàgina 60)

Per a 12 mL (volum necessari per als dos tampons d'una sola tira), els volums i pesos necessaris són:

Urea → 4.32 g

Tris HCl → 3 mL Tris 1.5M

Glicerol → 2.4 mL

SDS → 0.24 g SDS

Després, cada tampó es suplementa amb DTT (tampó I) o iodoacetamida (tampó II). Els pesos per a 12 mL (dos tires) són els següents:

DTT → 0.24 g

Iodoacetamida → 0.3 g

Gel d'acrilamida al 12%. (pàgina 61)

Per a un gel petit (tires de 7 cm) els volums i pesos necessaris són els següents:

H_2O mQ → 4.3 mL

Tris HCl 1.5 M pH 8.8 → 2.76 mL

SDS 10% → 120 μL

Acrilamida/Bisacrilamida (29:1) → 2.4 mL

Duracryl → 2.4 mL

El APS i el TEMED s'afegeixen a l'últim moment:

APS (10%, 0.1g/mL) → 56 μL

TEMED → 15.6 μL

Per a un gel gran (tires de 17 cm), els volums i pesos es multipliquen per 4:

H_2O mQ → 17.2 mL

Tris HCl 1.5 M pH 8.8 → 11.04 mL

SDS 10% → 480 μL

Acrilamida/Bisacrilamida (29:1) → 9.6 mL

Duracryl → 9.6 mL

El APS i el TEMED s'afegeixen a l'últim moment:

APS (10%, 0.1g/mL) → 224 μL

TEMED → 62.4 μL

Tampó d'electroforesis. (pàgina 63)

Per a gels de SDS i per a 2L de tampó 10x, els volums i pesos són els següents:

Tris → 60.06 g

Glicina → 288.4 g

SDS → 20 g

Tinció de plata. (pàgina 63)

Per a 500 mL, els volums i pesos són els següents:

Fixador:
- Etanol → 150 mL
- Àcid Acètic → 50 mL

Rentats:
- Etanol → 150 mL

Pretractament:
- $Na_2S_2O_3$ → 5 mL estoc 2%

Tinció:
- $AgNO_3$ → 1 g

Solució de velat:

Na_2CO_3 → 30 g
Na_2SO_4 → 200 μL
Formaldehid 37% → 250 μL

Annex 2.

En aquest annex, es mostren les cases comercials on s'han comprat els reactius utilitzats durant aquesta tesi.

Reactiu	Referència	Casa Comercial
PIPES	20426	USB
HEPES	H-3375	Sigma
EGTA	84.350	Merck
$MgCl_2 \cdot 6H_2O$	5833	Merck
Triton X100	X-100	Sigma
PMSF	P7626	Sigma
NaCl	1.06404	Merck
KCl	4933	Merck
Na_2HPO_4	6580	Merck
KH_2PO_4	4873	Merck
HCl	317	Merck
DTT	D9779	Sigma
Tris	75825	USB
Urea	161-0731	BioRad
EDTA	1.08418	Merck
Hidroclorur de Guanidina	16830	USB
Tiourea	1.07979	Merck
CHAPS	C-3023	Sigma

Reactiu	Referència	Casa Comercial
N-octilglucopiranòsid	494459	Calbiochem
IPG buffer	163-2094	BioRad
Blau de bromofenol	8122	Merck
Glicerol	US-16374	USB
SDS	428023	Calbiochem
Iodoacetamida	I 1149	Sigma-Aldrich
Acrilamida/bisacrilamida 29:1	161-0156	Bio-Rad
Duracryl	80-0148	Proteomics Solutions
APS	13375	Serva
TEMED	17-1312-01	PlusOne
Glicina	4201	Merck
Etanol	131086	Panreac
Àcid acètic	131008	Panreac
$Na_2S_2O_3$	1.06512	Merck
$AgNO_3$	1.01510	Merck
Na_2CO_3	1.06392	Merck
Na_2SO_4	1.06649	Merck
Formaldehid 37%	4003	Merck

Els següents reactius són exclusius per a DIGE.

Reactiu	Referència	Casa comercial
CyDye DIGE Fluor, Cy2 minimal dye	25-8010-82	Amersham
CyDye DIGE Fluor, Cy3 minimal dye	25-8010-83	Amersham
CyDye DIGE Fluor, Cy5 minimal dye	25-8010-85	Amersham
L-lysine	L 5626	Sigma
DMF (n,n dimetilformamida)	40228	Sigma
2D Quant kit	80-6483-56	Amersham
2D CleanUp Kit	80-6484-51	Amersham
PlusOne Urea	17-1319-01	Amersham
Tiourea	T 8656	Sigma
CHAPS	C 9426	Sigma
Blau de bromofenol	B 0126	Sigma
Destreak reagent	17-60003-18	Amersham
DTT	D 0632-25g	Sigma
Iodoacetamida	I 1149-25g	Sigma
0.5M Tris HCl pH 8.8	161-0798	Bio-Rad
APS	2015589	Sigma
SDS solution 20% (w/v)	161-0418	Bio-Rad
TEMED	161-0801	Bio-Rad
Agarosa IEF, 10g	17-0468-01	Amersham
Trizma Base	T 1503	Sigma
Glicerol	G 8773	Sigma
Glicina 99%	G 8898	Sigma
SDS	71729	Fluka

Annex 3.

En aquest annex es presenta una còpia de l'article publicat formant part del desenvolupament d'aquesta tesis.

Martinez-Heredia J, Estanyol JM, Ballesca JL and Oliva R (2006). Proteomic identification of human sperm proteins. Proteomics 6(15), 4356-69.

DOI 10.1002/pmic.200600094

Proteomics 2006, *6,* 4356–4369

RESEARCH ARTICLE

Proteomic identification of human sperm proteins

Juan Martínez-Heredia[1, 2], Josep Maria Estanyol[3], José Luis Ballescà[4] and Rafael Oliva[1, 2]

[1] Human Genetics Research Group, IDIBAPS, Faculty of Medicine, University of Barcelona, Barcelona, Spain
[2] Genetics Service, Hospital Clínic i Provincial, Barcelona, Spain
[3] Unitat de Proteòmica, Faculty of Medicine, University of Barcelona, Barcelona, Spain
[4] Institut Clínic de Ginecologia, Obstetricia i Neonatología. Hospital Clínic i Provincial, Barcelona, Spain

Conventional 1-DE has in the past provided a wealth of information concerning the major sperm proteins. However, so far there are relatively few reports exploiting the potential of the present proteomic tools to identify and to study additional yet-unidentified important proteins present in human spermatozoa. In the present work, 2-DE of proteins extracted from human normozoospermic spermatozoa led to the resolution of over 1000 spots. Subsequent excision from the gels of 145 spots and MALDI-TOF MS analysis allowed the identification of 98 different proteins. The function of these proteins turned out to be energy production (23%), transcription, protein synthesis, transport, folding and turnover (23%), cell cycle, apoptosis and oxidative stress (10%), signal transduction (8%), cytoskeleton, flagella and cell movement (10%), cell recognition (7%), metabolism (6%) and unknown function (11%). As many as 23% of the proteins identified have not been previously described as being expressed in human spermatozoa. The present data provide an important clue towards determining the function of these proteins and opens up the possibility to perform additional experiments.

Received: February 6, 2006
Revised: March 31, 2006
Accepted: April 3, 2006

Keywords:
2-DE / Human / Proteome / Spermatozoa /Spermatozoon

1 Introduction

The human spermatozoon is a highly specialized cell with extremely marked compositional, morphological and functional differences, as compared to other somatic or germinal cells [1–4]. For example, the sperm nucleus is condensed by protamines into a highly compact and hydrodynamic sperm head, and the cell is provided with a large flagellum to allow its motility [1–4]. In addition to the importance of the study of the sperm proteins to understand the fundamental aspects of reproduction, it is also relevant towards the identification of the causes of human infertility.

Correspondence: Dr. Rafael Oliva, Human Genetics Research Group, Faculty of Medicine, University of Barcelona, Casanova 143, 08036 Barcelona, Spain
E-mail: roliva@ub.edu
Fax: +34-9340-5278

Many efforts to identify sperm proteins have been directed to the nuclear proteins [1, 5–8], to identify the proteins required for sperm motility [9–10], and in the understanding of the membrane proteins required for capacitation, egg interaction and fertilization [11]. So far, most of the fundamental knowledge on the sperm protein composition has been gained using conventional protein purification and identification strategies [1–6, 12, 13]. Anomalies of the expression of the most abundant nuclear sperm proteins have been found associated to infertility [6, 14–21]. However, the majority of causes of male infertility still remain unknown. This is at least in part due to the relative lack of knowledge concerning the protein composition of spermatozoa.

The mature male germ cell proteome has been studied in model species such as plants [22] or worms [23]. In man, specific approaches to target subcellular organelles or compartments have been successfully used [11, 24–28]. A 2-D protein map of the human sperm head has been described, but without MS identification [29]. The human sperm proteome has also been analyzed after separation of the proteins

by LC and MS/MS, although the individual proteins identified have not been reported [30]. A previous case report has already provided some evidence of the potential of the present proteomic tools to identify proteins involved in infertility [3]. More recently, the PTMs during sperm maturation in the rat have also been studied using fluorescent 2-DE [31]. We initiated the present work to characterize the most abundant proteins extracted from human sperm cells fractionated on Percoll gradients, through 2-DE and subsequent MS (MALDI-TOF and nano ESI IT) identification.

2 Materials and methods

2.1 Subjects and sample collection

This project was approved by the bioethics committee of the hospital and informed consent was obtained from the participants. Eleven normozoospermic sperm samples (ejaculates) were included in this study. Samples were collected in specific sterile containers after at least 3 days of sexual abstinence and were allowed to liquefy. After liquefaction of the semen, the sperm parameters (volume, sperm concentration, percentage of motility and motion characteristics) were evaluated according to published recommendations [32] using a computer–assisted semen analyzer (CASA, Photolux) and a Makler counting chamber (Sefi Medical Instruments, Hainfa, Israel). Sperm morphology was evaluated using strict Kruger criteria [33], and at least 100 cells were examined per slide. None of the semen samples had significant numbers of round cells or leukocytospermia as per WHO guidelines [32]. The average sperm count obtained by CASA in the 11 samples was 91.4 millions /mL, the average volume was 3.4 mL, the average proportion of normal forms was 24%, the average rapid progressive motility was 31.2%, the slow motility was 29.2%, the non-progressive motility was 19.9% and the non-motile spermatozoa were 19.7%.

2.2 Sample preparation

The samples were centrifuged through a 50% step Percoll gradient at $800 \times g$ for 20 min. at 4°C to eliminate potentially contaminating cells present in semen [20]. The 50% Percoll was used instead of a higher percentage of Percoll so that the resulting 2-D gels would be representative of the average spermatozoon present in the normozoospermic sperm samples, rather than represent a highly selected subpopulation of the fittest spermatozoa. Essentially, a solution of 100% isotonic Percoll was initially prepared with 8.7 parts of Percoll (Amersham Pharmacia Biotech AB, Uppsala, Sweden), 0.3 parts of 7.5% $NaHCO_3$ and one part of Ham's F10 10 × (Biological Industries, Israel). Subsequently, to prepare the 50% Percoll, the 100% Percoll was diluted with Ham's F10 1 × (Gibco BRL, Life Technologies Ltd, Paisley, UK) with 3% of $NaHCO_3$. Spermatozoa from the sediment, clean from potentially contaminating cells (as checked by phase contrast microscopy), were re-suspended in Ham's F10 1 ×, counted using Makler counting chamber and then washed twice with Ham's F10 1 ×.

Solubilization of the sperm cells was performed essentially as described [3], with variations as follows. Spermatozoa from the pellet were solubilized in lysis buffer containing 7 M urea, 2 M thiourea, 1% CHAPS, 1% n-octyl-glucopyranoside, 0.5% IPG buffer, 18 mM DTT, 2.4 μM PMSF, and 0.005% bromophenol blue. The volume of lysis buffer was calculated to obtain a concentration of lysate corresponding to 230×10^6 sperm/mL. The samples were then incubated for 1 h at room temperature to allow solubilization, and finally centrifuged at $3000 \times g$, 5 min at 4°C to eliminate potentially insolubilized debris. The solubilized samples were divided into aliquots and stored at −30°C for subsequent analysis.

2.3 IEF and SDS-PAGE

Solubilized protein, 300 μL equivalent to 69×10^6 sperm cells, were then placed in the rehydration tray from the Protean IEF cell with 17-cm IPG (pH 5–8) linear strips (Bio-Rad) and rehydrated for at least 12 h, following the commercial instructions. Strips were focused at 20°C with the following program: 15 min with a rapid ramp (0–250 V), 2 h with a slow ramp (250–10 000 V), 45 000–60 000 Vh, rapid ramp, 10 000 V, 10 hours, 50 V, slow ramp.

Once the IEF was completed, the strips were equilibrated in 6 M urea containing 0.375 M Tris-HCl pH 8.8, 20% glycerol, 2% SDS, 0.01% bromophenol blue and 2% DTT for 10 min, followed by the same buffer without DTT and supplemented with 2.5% iodoacetamide for 10 min. SDS-PAGE was performed using in a DodecaCell (Bio-Rad) or a Protean II XL (Bio-Rad). Three replicas from each sample were made following this procedure. The second dimension was run at 300 V for approximately 3 h until the bromophenol blue front just started to migrate off the lower end of the gels.

2.4 Silver staining

After electrophoresis, the gels were stained with silver as previously described [34], with slight modifications as follows. The gels were fixed in 30% ethanol and 10% acetic acid, for at least 1 h and washed three times in 30% ethanol (20 min each wash). The gels were then pre-treated for 1 min with 0.02% $Na_2S_2O_3$ followed by three washes with Milli-Q water and staining in 0.2% $AgNO_3$ for 20 min. Subsequently, three washes with Milli-Q water were done to remove the excess of silver, and the proteins were visualized by developing the gels in 6% Na_2CO_3, 0.0004% $Na_2S_2O_3$ and 0.05% formaldehyde (37%) for 90 s followed by two quick washes with water to remove excess of developing solution. The reaction was finally stopped in a solution with 30% ethanol and 10% acetic acid during 10 min.

2.5 Scanning and computer analysis of the 2-DE

For image analysis, silver-stained gels were scanned in transmission scan mode using a high-resolution calibrated scanner (GS-800, Bio-Rad). Raw 2-DE gels were analyzed using the PDQuest 7.1.1. software (Bio-Rad). Protein spots were characterized by their M_r and p*I* using internal 2-DE standards (Bio-Rad). To corroborate the reproducibility of the results, at least three electrophoresis runs were performed from each sperm sample.

2.6 In-gel digestion, PMF and database searching

Punches (2-mm diameter) of the 2-DE were taken containing the protein spots selected on the basis of high intensity and resolution in the gels; 145 spots were excised out from a total of four independent gels. Eighty five spots were excised only once. In addition, 30 spots were excised twice (the same spot picked from two independent gels) because of a poor MALDI result after the first analysis. The excised protein spots were then digested with 100–150 ng trypsin (Promega) at 37°C overnight using a Montage In-Gel Digest$_{ZP}$ (Millipore) using the standard manufacturer's protocol. Peptides were dried and resuspended in water 0.1% TFA. Subsequently, 0.5 μL of suspension was mixed with same amount of CHCA (3 mg/mL) and peptides were analyzed by a MALDI-TOF Voyager DE Pro mass spectrometer (Applied Biosystems) operated in delayed extraction reflector mode at 20 kV as accelerating voltage, 90 ns of pulse delay time, 75% of a grid voltage, and a guide wire voltage of 0.005%. Spectra were accumulated for 100 laser shots obtained over a mass range of 750–3500 Da. and were analyzed using Protein Prospector MSFit software (version 3.2.1; University of California San Francisco). All mass spectra were calibrated externally using a standard peptide mixture [angiotensin II (1046.5), angiotensin I (1296.7), substance P-amide (1347.7), bombesin (1619.8), ACTH fragment 1–17 (2093.1), ACTH fragment 18–39 (2465.2), domatostatin 28 (3147.5); Bruker].

The non-redundant databases from Swiss-Prot and the National Center for Biotechnology Information (NCBI, NIH, Bethesda, MD, USA) were used as the protein sequence database and to obtain information of their expression and function. A mass tolerance of 50–75 ppm and one incomplete cleavage were allowed. Acetylation of the N terminus, alkylation of cysteine by carbamidomethylation, and oxidation of methionine were considered as possible modifications. The criteria used to accept identifications included the extent of sequence coverage of the theoretical sequences matched within a mass accuracy of 50–75 ppm with internal calibration, the number of peptides matched, and the probabilistic score (MOWSE score), as indicated from each peptide in Table 1. Spectra with a MOWSE cut-off lower than 1000 were accepted if peaks were clean and better that those corresponding to trypsin. If necessary, peptides were excluded if their masses corresponded to those for trypsin, human keratins, or to other irrelevant proteins. Out of the 30 spots excised twice because of a poor result in the first analysis, there was a marked improvement in 22 cases after the second analysis.

3 Results and discussion

In the present study we have been able to resolve over 1000 spots in 2-DE corresponding to the sperm proteins extracted from normozoospermic human samples (Fig. 1). We excised from the gels a total of 145 spots all ranging from a molecular mass of 10–70 kDa and a p*I* of 5–8. After MALDI-TOF MS analysis we were able to identify 98 different proteins (Table 1, Figs. 1 and 2). We found that approximately 96% of the identified spots were reproducible across the 11 independent individual samples. Within the three replicas from each sample the variation was only 1%. These results are comparable to those previously described [3].

The functional distribution of the 98 identified proteins is shown in Fig. 2. The most abundant group corresponds to the proteins involved in energy production (23%; Fig. 2A). Within this group, tricarboxylic acid and beta oxidation accounted for 67% of the proteins followed by 17% of the proteins involved in glycolysis and gluconeogenesis. This observation is also consistent with the fact that 20% of the proteins identified are located in the mitochondria (Fig. 2B). The abundance of proteins involved in energy production is consistent with the high energy requirements for the tail movement in the spermatozoon.

The next most abundant group of proteins identified corresponds to those involved in transcription, protein synthesis and turnover, protein transport and folding (23%). This finding has been somehow surprising as the spermatozoon was thought to be a transcriptionally inert cell with the only purpose of delivering the highly condensed "inactive" paternal genome complexed with protamines [1]. Thus, in this scenario it is intriguing to know what could be the function of MYCBP, a protein that has been described to stimulate the activation of E-box-dependent transcription of C-myc proto-oncogene [35]. The same can be argued for the product of the PARK7 gene encoding an RNA-binding regulatory subunit of oncogene DJ1 [36]. The notion that perhaps the spermatozoon delivers something more than just the genomic DNA to the oocyte started to change with the identification of RNAs that are also transferred to the ova [37]. In addition, it has been shown that proteins such as those present in the human sperm centrosome are essential for normal syngamy and early embryonic development [38]. Furthermore, it has been recently shown that mammalian sperm translate nuclear-encoded proteins by mitochondrial-type ribosomes [39]. Thus, there are several potential explanations for the proteins identified involved in RNA binding and in transcription in the present work. One is that some of the proteins present in the human spermatozoon could be leftovers of the spermatogenesis process. Another is that they could play a role in the normal physiology of the spermatozoon. Finally, they could have important, yet unknown, functions upon fertilization and initial stages of development.

Table 1. Human sperm proteins identified by MS

Spot name	Protein name	Accession no.	MOWSE score	Masses match (%)	% protein covered	Mol. mass/p*I*		Function
						Determined	Expected	
Metabolism								
ACYP1	Acylphosphatase, organ-common type isozyme	P07311	249	7/187 (3)	85	16.8/6.44	11/9.52	Its physiological role is not yet clear. May be involved in phosphate metabolism
ACYP2	Acylphosphatase, muscle type isozyme	P14621	183	13/187 (6)	95	16.8/6.44	11/9.52	Same as above
GLUL	Glutamine synthetase	P15104	3.16e+003	6/60 (10)	21	44.8/6.97	42/6.43	Belongs to the glutamine synthetase family; amino acid metabolism
HIBADH	3-Hydroxy- isobutyrate dehydrogenase precursor	P31937	5.78e+003	8/18 (44)	28	31.7/6.30	35/8.38	Dehydrogenase; involved in valine metabolism
HFLJ	Hypothetical protein FLJ11342	XP_416633	1.49e+009	14/106 (13)	48	28.5/7.63	34/8.80	Predicted hydrolase involved in amino acid transport and metabolism
PRPSAP1	Phosphoribosyl pyrophosphate synthetase-associated protein 1	Q14558	1.16e+003	8/140 (5)	17	20.8/7.59	391/6.73	Negative regulatory role in PRPP synthesis and forms part of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
Energy								
Glycolysis and gluconeogenesis								
ENO1	Alpha enolase	P06733	5.62e+008	13/29 (44)	36	50.2/7.84	47/7.01	Glycolysis, growth control, hypoxia tolerance, allergic responses, transcriptional repressor
GAPDHS	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	O14556	2.05e+008	14/37 (37)	49	50.3/7.78	44/8.39	May play a role in regulating the switch between different pathways for energy production during spermiogenesis and in the spermatozoon
PGAM1	Phosphoglycerate mutase 1	P18669	3.21e+006	9/238 (3)	51	30.0/7.67	29/6.68	Interconversion of 2,3-bisphosphoglycerate
TPI1	Triosephosphate isomerase 1	CAG46503	4.13e+006	11/30 (36)	51	31.3/5.95	27/6.45	Plays an important role in several metabolic pathways (included glycolysis)
Oxidation of fatty acid								
ACADM	Acyl coenzyme A dehydrogenase, C-4 to C-12 straight chain	P11310	4.27e+005	11/20 (55)	28	48.5/7.74	47/8.61	Involved in primary stages of mitochondrial fatty acid beta-oxidation pathway
CYB5R3	Cytochrome b5 reductase b5R2	P00387	5.14e+004	6/44 (13)	31	28.7/7.77	31/8.49	Denaturation and elongation of fatty acids, cholesterol synthesis and drug metabolism
DECR1	2–4 Dienoyl CoA reductase 1 precursor	Q16698	1.29e+006	9/44 (20)	32	28.7/7.77	36/9.35	Auxiliary enzyme of beta-oxidation which participates in the metabolism of unsaturated fatty enoyl-CoA.

Table 1. Continued

Spot name	Protein name	Accession no.	MOWSE score	Masses match (%)	% protein covered	Mol. mass/p*I*		Function
						Determined	Expected	
ECH1a	Delta 3,5-delta 2,4-dienoyl-CoA isomerase precursor	Q13011	9.01e+003	6/51 (11)	22	33.2/6.69	36/6.61	Isomerization of 3-trans,5-cis-dienoyl-CoA to 2-trans,4-trans-dienoyl-CoA; Fatty acid beta-oxidation cycle
ECH1b	Peroxisomal enoyl-coenzyme A hydratase like	NP_001389	4.03e+004	7/40 (17)	33	33.2/7.26	36/6.61	This enzyme functions in the auxiliary step of the fatty acid beta-oxidation pathway
SCAD	Acyl-CoA dehydrogenase	P16219	1.19e+011	18/60 (30)	62	44.8/6.97	44/8.13	Mitochondrial fatty acid beta-oxidation system; first step
Anaerobic glycolysis								
LDHC	L-Lactate dehydrogenase C	P07864	1.49e+005	11/123 (8)	41	33.2/7.66	37/7.08	Anaerobic glycolysis; final step, testis specific
Tricarboxylic acid cycle								
DLD	Dihydrolipoamide dehydrogenase precursor	P09622	3.73e+005	11/55 (20)	25	61.7/7.71	54/7.59	Dihydrolipoyl dehydrogenase is a component of the mitochondrial pyruvate and alpha-ketoglutarate dehydrogenase
			5.98e+005	10/38 (26)	26	63.7/7.22	54/7.59	
ETFA	Electron transfer flavoprotein alpha subunit	P13804	6.62e+008	11/78 (14)	46	31.8/7.59	35/8.62	Transfers the electrons to the main mitochondrial respiratory chain via ETF-ubiquinone oxidoreductase
FH	Fumarate hydratase precursor	P07954	1.60e+007	12/46 (26)	34	49.4/7.78	55/8.85	Enzymatic component of the tricarboxylic acid cycle
PDHB	Pyruvate dehydrogenase E1 component beta subunit precursor	P11177	2.06e+010	13/34 (38)	50	33.8/6.07	39/6.21	The pyruvate dehydrogenase complex catalyzes the overall conversion of pyruvate to acetyl-CoA
Respiration								
ATP5A1	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit isoform 1	P25705	6.67e+003	5/37 (13)	15	50.3/7.78	60/9.16	Mitochondrial ATP synthase. This gene encodes the alpha subunit of the catalytic core
ATP5B	ATP synthase beta chain, mitochondrial precursor	P06576	2.46e+013	20/53 (37)	55	54.5/5.43	56/5.26	Mitochondrial ATP synthase. The beta chain is the catalytic subunit
ATP5H	ATP synthase H transporting mitochondrial F0 complex subunit d	O75947	4.08e+004	8/78 (10)	47	19.9/5.58	18/5.21	Mitochondrial ATP synthase. This gene encodes d subunit of the F0 complex
			6.91e+003	6/140 (4)	46	20.8/5.59	18/5.21	
COX6B	Cytochrome c oxidase subunit Vib	Q7L1R4	7.76e+003	5/132 (3)	66	12.2/6.51	10/6.54	Electron transport
UQCRC1	Ubiquinol cytochrome c reductase core 1	P31930	7.95e+011	21/59 (35)	53	51.4/5.96	53/5.94	Part of mitochondrial electron transport chain
Other pathways								
ASRGL1	Asparaginase-like 1 protein	Q7L266	4.77e+003	9/59 (15)	34	16.0/6.92	32/5.84	Glycoprotein catabolism.
			1.27e+003	4/20 (20)	13	22.4/6.26	32/5.84	
NIT1	Nitrilase homolog 1	Q86X76	1.07e+005	8/40 (20)	36	33.2/7.26	36/7.91	This protein have an hydrolase domain

Table 1. Continued

Spot name	Protein name	Accession no.	MOWSE score	Masses match (%)	% protein covered	Mol. mass/p*I*		Function
						Determined	Expected	
OXCT1	Succinyl CoA 3 ketoacid coenzyme A transferase 1 precursor	P55809	9.55e+006	11/98 (11)	32	61.3/6.78	56/7.13	Key enzyme for ketone body catabolism (catabolyzes the first step) and transfers the CoA moiety from succinate to acetoacetate
Transcription, protein synthesis and turnover								
Transcription								
H2A	Histone H2A	Q93077	243	3/55 (5)	11	16.3/5.86	14/10.86	Forms parts of the nucleosome
H4A	Histone H4	P62805	1.02e+003	6/43 (13)	43	12.8/6.20	11/11.36	Forms parts of the nucleosome
MYCBP	C-myc binding protein 1	Q99417	1.38e+003	3/65 (4)	46	13.0/6.06	12/5.71	May control the transcriptional activity of c-Myc
PARK7	RNA-binding regulatory subunit of oncogene DJ1	Q99497	2.36e+005	7/104 (6)	38	20.7/6.01	20/6.33	Acts as positive regulator of androgen receptor-dependent transcription and may function as redox-sensitive chaperone and as sensor for oxidative stress
			2.69e+008	11/175 (6)	60	21.0/6.44	20/6.33	
RUVBL1	RuvB like 1	Q9Y265	1.03e+010	18/86 (20)	50	55.8/7.18	50/6.02	ATPase and DNA helicase activities and component of the NuA4 histone acetyltransferase
RUVBL2	RuvB like 2	Q9Y230	4.13e+009	18/58 (31)	44	57.3/5.96	51/5.49	Same as above and forms a multimeric complex with RUVBL1
Protein turnover								
ACRBP	Acrosin binding protein	Q8NEB7	1.24e+006	9/41 (21)		11.5/ 7.46	18/5.21	Involved in packaging and condensation of the acrosin zymogen
ACRBPpre	Proacrosin binding protein sp32 precursor	Q8NEB7	6.54e+003	6/89 (6)	16	29.8/7.36	61/5.09	Same as ACRBP (see above)
			1.05e+005	8/39 (20)	19	30.5/5.68	61/5.09	
LAP	Cytosol aminopeptidase	P28838	1.59e+013	27/119 (22)	57	53.9/7.21	53/6.29	Turnover of intracellular proteins.
PSMA2	Proteasome subunit alpha type 2	P25787	2.71e+007	9/46 (19)	51	26.7/7.76	26/6.91	Subunit of the proteasome with a potential regulatory effect
PSMB2	Proteasome subunit beta type 2	P49721	7.53e+003	8/125 (6)	33	22.3/7.54	23/6.52	Subunit of the proteasome
PSMB3	Proteosome beta 3 subunit human	P49720	1.01e+004	9/15 (60)	47	25.4/6.68	23/6.14	Same as above
PSMB4	Proteasome subunit beta type 4	P28070	3.78e+010	12/40 (30)	39	22.6/6.25	29/5.72	Same as above
PSMB7	Proteosome beta 7 subunit	Q99436	4.47e+003	6/92 (6)	23	25.4/6.45	30/7.58	Same as above
UAP1	Sperm-associated antigen 2	Q16222	224	4/92 (4)	9	25.5/6.29	59/5.92	Converts UDP and GlcNAc-1-P into UDP-GlcNAc, and UDP and GalNAc-1-P into UDP-GalNAc
UBA52	60S ribosomal protein L40	P62987	1.49e+003	6/63 (9)	46	10.0/7.41	15/9.87	Fusion protein consisting of ubiquitin at the N terminus and ribosomal protein L40

Table 1. Continued

Spot name	Protein name	Accession no.	MOWSE score	Masses match (%)	% protein covered	Mol. mass/p*I*		Function
						Determined	Expected	
Protein folding								
APCS	Serum amyloid P-component precursor	P02743	249	4/33 (12)	21	26.8/6.11	25/6.10	Can interact with DNA and histones and may scavenge nuclear material released from damaged circulating cells
HSPD1	Mitochondrial matrix P1	P10809	2.36e+011	18/35 (51)	46	65.5/5.68	61/5.70	Mitochondrial protein import, assembly and folding of imported proteins
HSPA8	Heat shock-related 70 kDa protein 8 isoform 1	P11142	1.29e+003	7/26 (26)	12	71.5/5.97	70/5.38	Belongs to the HSP70 family which binds to nascent polypeptides to facilitate correct folding and also functions as an ATPase in the disassembly of clathrin-coated vesicles
Protein transport								
DBI	Diazepam binding inhibitor	P07108	7.77e+003	4/80 (5)	42	11.6/6.31	12/5.41	Displaces ligands bound to the beta-carboline/benzodiazepine recognition site and is involved in molecular transport
NUTF2	Nuclear transport factor 2	P61970	164	3/61 (4)	29	13.7/5.30	14/5.10	Facilitates protein transport into the nucleus and interacts with the nucleoporin p62 and with Ran
RAB2	RAB 2, member RAS oncogene family	P61019	71.7	4/29 (13)	21	10.3/6.35	23/6.08	Required for protein transport from the endoplasmic reticulum to the Golgi complex
hVDAC2	Voltage-dependent anion-selective channel 2	P45880	7.47e+005	9/31 (29)	46	33.4/7.73	39/6.32	Form a channel between mitochondria and cytoplasm
Signal transduction								
ARHGDIB	Rho GDP dissociation inhibitor beta	P52566	9.23e+003	9/130 (6)	65	24.0/5.57	23/5.1	Regulates the GDP/GTP exchange reaction of the Rho proteins
HINT1	Histidine triad nucleotide-binding protein 1	P49773	1.15e+005	5/86 (5)	51	13.8/7.13	14/6.43	Hydrolyzes adenosine 5′-monophosphoramidate substrates and forms part of a signal transduction pathway
			8.05e+003	4/168 (2)	51	13.8/7.16	14/6.43	
IMPA1	Inositol-1 (or 4)-monophosphatase	P29218	2.88e+004	8/17 (47)	37	29.8/5.46	30/5.16	Key enzyme of the phosphatidyl inositol signaling pathway
PDIA3	58 kDa glucose regulated protein	P30101	2.52e+010	17/31 (54)	38	62.8/6.18	57/5.99	Catalyzes the rearrangement of -S-S- bonds in proteins. Forms part of a signal transduction pathway.
PRKG1	Protein kinase cGMP dependent type 1	P14619	1.65e+003	8/122 (6)	13	18.8/6.33	78/5.28	Kinase; involved in some transcriptional pathways
SLC9A8	Sodium/hydrogen exchanger 8	Q9Y2E8	1.06e+003	4/72 (5)	9	26.8/6.90	65/6.07	Involved in pH regulation and also a role in signal transduction

Table 1. Continued

Spot name	Protein name	Accession no.	MOWSE score	Masses match (%)	% protein covered	Mol. mass/p*I*		Function
						Determined	Expected	
S100A8	S100 calcium binding protein A8	P05109	2.31e+002	4/9 (44)	26	12.0/7.72	11/6.51	May function in the inhibition of casein kinase, as a cytokine and interact with components of the intermediate filaments
S100A9	S100 calcium binding protein A9	P06702	2.65e+006	8/28 (28)	72	14.5/6.04	13/5.71	Same as above
		P06702	6.44e+003	5/151 (3)	48	13.8/6.07	13/5.71	
Cytoskeleton, flagella and cell movement								
ACTB	Cytoskeletal actin	P60709	1.55e+007	11/34 (32)	36	47.4/5.65	42/5.29	Actins are highly conserved proteins that are involved in cell motility, structure and integrity
			6.57e+007	13/26 (50)	46	47.6/5.68	42/5.29	
ARPM2	Actin-related protein M2	Q8TDY3	2.19e+007	12/30 (40)	37	44.7/5.70	42/5.28	Belongs to the actin family with synthesis in late spermatids and localized in the calyx
DNCL2B	Dynein cytoplasmic light polypeptide 2B	Q8TF09	6.05e+003	5/144 (3)	61	11.9/7.36	11/6.91	May be involved in assembly and motor function of dynein, which a role in cell division and intracellular transport
		Q8TF09	7.13e+003	5/49 (10)	61	11.9/7.36	11/6.91	
ODF1	Outer dense fiber of sperm tails 1	Q14990	2.26e+004	10/39 (25)	35	29.9/7.98	28/8.47	Component of the outer dense fibers of spermatozoa
			2.26e+004	10/39 (25)	35	29.9/7.98	28/8.47	
			2.67e+003	6/44 (13)	28	28.7/7.77	28/8.47	
PIP	Prolactin induced protein	P12273	5.98e+003	5/86 (5)	50	12.7/5.51	17/8.26	Involved in some kind of actin binding function but not yet clear
PIPpre	Prolactin-inducible protein precursor	P12273	4.40e+005	7/32 (21)	56	16.3/5.51	17/8.26	Same as above
SEMG1	Semenogelin I protein precursor	P04279	3.82e+004	9/82 (10)	25	21.8/7.32	52/9.30	Predominant protein in semen involved in the formation of a gel matrix.
SGCB	Sarcoglycan beta	Q16585	1.34e+002	3/17 (17)	9	13.5/6.78	35/8.86	Forms a link between the F-actin cytoskeleton and the extracellular matrix
TEKT2	Tektin 2	Q9UIF3	1.55e+007	10/58 (17)	41	57.3/5.96	50/5.39	Family of proteins that are co-assembled with tubulins to form
TUBB	Tubulin beta subunit	Q13509	1.18e+011	21/45 (46)	64	53.8/5.22	50/4.79	Tubulin is the major constituent of microtubules
			2.64e+008	15/26 (57)	53	52.9/5.25	50/4.79	
			1.19e+010	18/24 (75)	64	52.1/5.34	50/4.79	
Cell cycle , apoptosis and oxidative stress								
Cell cycle								
CDK5	Cyclin dependent kinase 5	Q00535	37.3	3/13 (23)	15	15.4/5.83	33/7.57	Probably involved in the control of the cell cycle
HSPA2	Heat shock-related 70 kDa protein 2	P54652	8.2e+004	9/67 (13)	21	27.1/5.86	70/5.56	Involved in spermatid development and male meiosis
			5.26e+009	18/26 (69)	36	71.5/5.97	70/5.56	

Table 1. Continued

Spot name	Protein name	Accession no.	MOWSE score	Masses match (%)	% protein covered	Mol. mass/p*I*		Function
						Determined	Expected	
PPP1CC	Serine/threonine protein phosphatase 1, catalytic subunit, gamma isoform	P36873	3.13e+004	9/54 (16)	34	38.2/6.23	37/6.12	Essential for cell division, and participates in the regulation of glycogen metabolism, muscle contractility and protein synthesis. Involved in regulation of ionic conductance and long-term synaptic plasticity
Apoptosis								
CASP2	Caspase 2 isoform 3	P42575	678	5/124 (4)	54	22.4/6.26	12/7.88	Member of the cysteine-aspartic acid protease (caspase) involved in apoptosis
CLU	Clusterin precursor	P10909	922	7/55 (12)	14	35.8/5.36	52/5.89	Not yet clear. It has been associated with programmed cell death (apoptosis)
			922	7/55 (12)	16	35.4/5.50	52/5.89	
			6.79e+003	6/51 (11)	14	38.2/6.23	52/5.89	
DNAJB13	Testis spermatocyte apoptosis-related protein	P59910	2.61e+003	5/125 (4)	23	21.2/7.54	36/7.68	May be involved in inhibiting testis spermatogenesis apoptosis
Protection against oxidative injury and toxins								
GSTM3	Glutathione S-transferase Mu 3	P21266	3.14e+008	17/63 (26)	57	26.8/5.97	26/5.37	Belongs to the *mu* class of enzymes involved in the detoxification of carcinogens, drugs, toxins and products of oxidative stress
			3.01e+006	12/21 (57)	50	26.3/5.64	26/5.37	
MPST	3-mercapto-pyruvate sulfurtransferase	P25325	5.14e+005	8/51 (15)	42	33.2/6.69	33/6.13	Transfer of a sulfur ion to cyanide or to other thiol compounds and may be involved in response to toxins
PRDX4	Thioredoxin peroxidase	Q13162	2.31e+007	11/66 (16)	53	27.7/6.12	30/5.86	Involved in redox regulation of the cell
PRDX6	Peroxiredoxin 6	P30041	6.41e+005	12/43 (27)	63)	27.4/7.10	25/6.00	Involved in redox regulation of the cell and regulation of phospholipid turnover and protection against oxidative injury
			3.65e+004	9/91 (9)	45	27.1/6.47	25/6.00	
Sperm-egg interaction and cell recognition								
HLAA	HLA class I antigen A-31 alpha chain precursor	P16189	2.32e+003	6/89 (6)	21	37.5/5.43	41/6.02	Involved in the presentation of foreign antigens to the immune system
ACRV1i	Acrosomal vesicle protein 1 isoform i	P26436	1.03e+003	5/60 (6)	37	18.5/5.62	16/6.79	May be involved in sperm-zona binding or penetration
ACRV1j	Acrosomal vesicle protein 1 isoform j	P26436	1.23e+003	5/60 (8)	46	18.5/5.62	13/8.58	Same as above
CRISP2	Cysteine rich secretory protein 2	P16562	6.17e+004	5/91 (5)	29	27.1/6.47	27/6.09	Intra-acrosomal protein in fresh sperm; relevant for sperm-oocyte interaction

Table 1. Continued

Spot name	Protein name	Accession no.	MOWSE score	Masses match (%)	% protein covered	Mol. mass/p*I*		Function
						Determined	Expected	
ROPN1	AKAP-binding sperm protein ropporin	Q9HAT0	1.42e+006	9/79 (11)	54	19.9/5.58	24/5.52	Porin activity; involved in fusion of sperm to egg plasma membrane, acrosome reaction and another pathways
SPACA1	Sperm acrosome associated 1	Q9HBV2	1.17e+003	5/110 (4)	18	17.5/5.66	32/4.57	Involved in sperm-egg interaction
SPACA3	Sperm acrosome associated 3	Q8IXA5	1.81e+003	417 (23)	24	14.4/5.45	23/8.26	Involved in sperm-egg interaction
Unknown function								
C21orf33	ES1 protein homolog mitochondrial precursor	P30042	5.52e+004	5/120 (4)	41	27.2/7.70	28/8.50	Unknown
HSPC142	HSPC142 protein	CAG38557	2.12e+003	7/108 (6)	23	14.8/6.72	Unknown	Unknown
LOC465613	Similar to dJ54B20.3	XP_521044	5.55e+002	4/104 (3)	30	14.0/6.60	18/5.94	Unknown
			3.8e+003	5/84 (5)	33	13.6/6.42	18/5.94	
TAGLN2	Transgelin-2	P37802	1.82e+003	7/76 (9)	37	15.0/6.92	22/8.41	One of the earliest markers of differentiated smooth muscle of unknown function
SE57-1	CTCL tumor antigen se57-1	NP_079490	207	6/29 (20)	16	10.3/6.35	39/8.78	Unknown
SPANXA1	Sperm protein associated with the nucleus X chromosome (SPANX) A1	Q9NS26	81.7	3/21 (14)	32	18.8/5.41	18/4.97	Unknown
SPANXA2	SPANX A2	Q5JWI0	133	4/47 (8)	53	18.8/5.41	11/5.04	Unknown
SPANXB2	SPANX B2	NP_663697	1.42e+003	5/71 (7)	50	18.9/5.85	12/5.92	Unknown
			1.42e+003	5/71 (7)	50	20.8/5.88	12/5.92	
			1.42e+003	4/30 (13)	42	18.5/6.27	12/5.92	
			1.07e+003	3/13 (23)	31	20.7/6.38	12/5.92	
SPANXC	SPANX C.	Q9NY87	133	3/21 (14)	24	18.8/5.45	11/5.07	Unknown
SPANXD	SPANX D	Q9BXN6	133	3/30 (10)	24	19.0/6.19	11/5.87	Unknown
SPANXE	SPANX E	Q8TAD1	134	3/30 (10)	24	19.0/6.19	11/5.22	Unknown

Among proteins involved in protein turnover, five correspond to proteasome subunits (Table 1). This observation is consistent with the evidence presented for the presence of proteasomes in the spermatozoon [40]. Also using a MS/MS proteomic approach, different proteasome subunits have been recently identified in human spermatozoa [30].

Other important categories of proteins identified are involved in cell recognition and signal transduction (Fig. 2A). These proteins are consistent with the functions required for sperm capacitation, interaction with the zona pellucida, acrosomal reaction and oocyte penetration. In addition, two specific acrosomal proteins have been detected; the acrosomal binding protein ACRBP and proacrosin binding protein sp32 precursor (Table 1 and Fig. 2).

An important number of proteins (11%) are involved in protection against oxidative injury and stress, or are involved in apoptosis or cell cycle (Fig. 2A). An interesting protein detected in this category is peroxiredoxin 6 (PRDX6). This protein can reduce H_2O_2 and cellular hydroperoxides, and may play a role in oxidative injury [41]. There is now substantial evidence for oxidative injury in spermatozoa of infertile patients, which in turn leads to increased injury of the genome [42]. Another interesting protein that could have been included in this category is the product of PARK7, which we have included under the protein synthesis category (see above). This protein many also function as a redox-sensitive chaperone and as a sensor for oxidative stress, and has been related to male fertility and Parkinson's disease [36].

Cytoskeletal and flagellar proteins involved in cell movement account for 10% of the proteins identified (Fig. 2), which is also consistent with the highly specialized shape of the spermatozoon and its large flagella.

Among nuclear proteins, histones H2A and H4 have been detected (Table 1). However, most of the spermatozoal DNA is complexed to protamines rather than histones. The latest data provide substantial evidence for the presence of only up to 15% of the DNA complexed to histones and 85% of the DNA complexed to protamines [4, 7]. It was once questioned whether histones were present at all in the human sperm nucleus, and their function is still not fully understood at present [4–7]. The

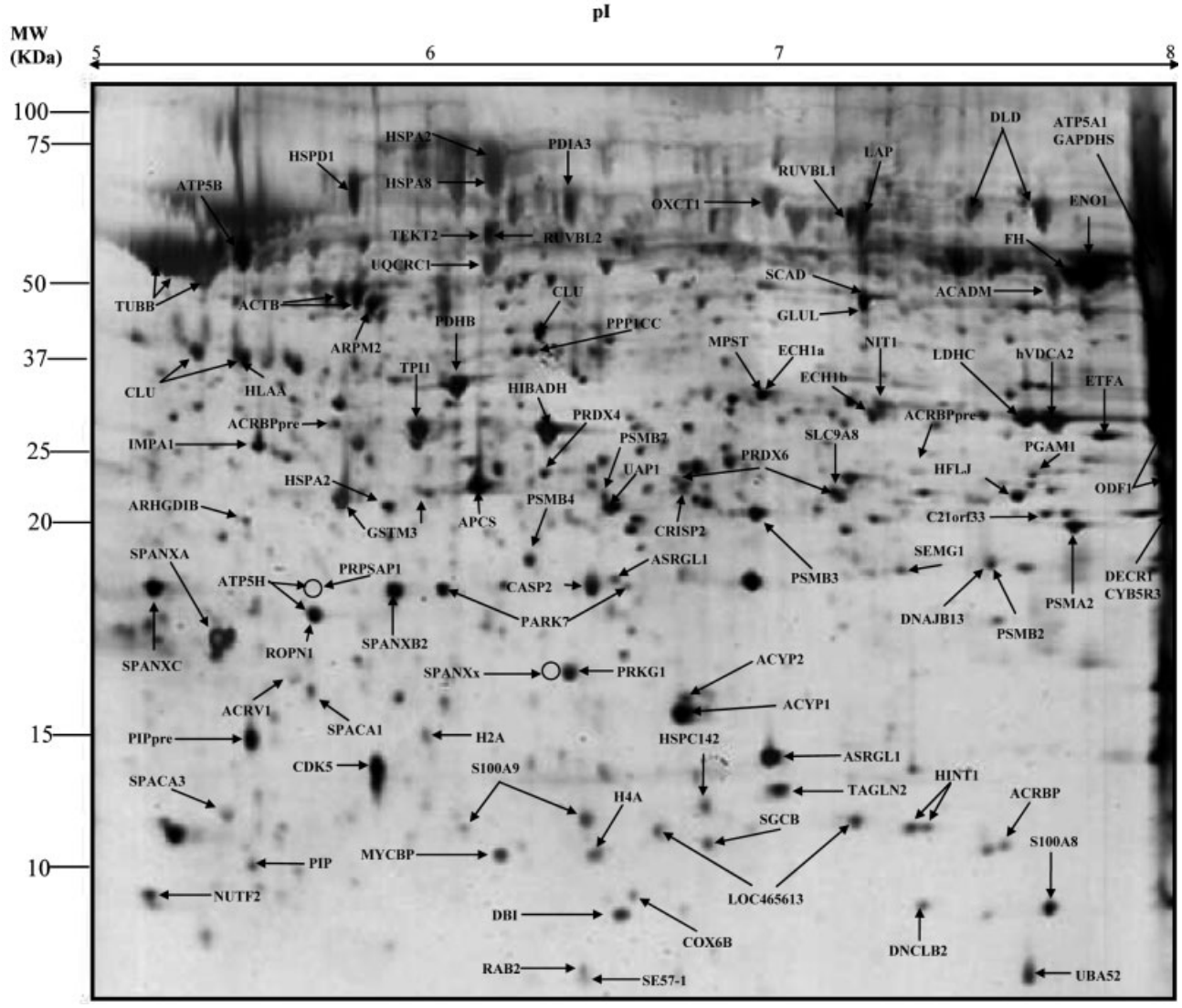

Figure 1. 2-DE map of normozoospermic human sperm proteins. The first dimension was performed by IEF on pH 5–8 IPG strips, the second dimension on 12% SDS-PAGE gels and the proteins were visualized by silver staining. The indicated spots were excised from the gel and identified by MS. Proteins are mapped and annotated by their gene name, according to HUGO database. For these abbreviations and complete information see Table 1. The two circles indicate the position of two spots absent in this particular 2-DE gel.

detection in the present work of histones by mass spectroscopy further provides unequivocal evidence that histones are indeed present in human normozoospermic spermatozoa. As we anticipated, in the present work we have not detected the protamines, and indeed the detection of protamines was not one of our goals. Human protamines contain approximately 50% arginine residues, representing the most basic proteins known in nature [1, 5, 6, 8, 43]. Because of their highly positive charge, they are focused at one end of the IEF strip, thus out of the range of p*I* that we decided to analyze in the present work. In addition, protamines are insoluble in the presence of SDS, so little protein could be expected to enter the second SDS-PAGE dimension. Protamine identification and analysis has been an extensively studied issue both in normal spermatogenesis and in spermatozoa from infertile patients [6, 14–21, 43]. Recently an approach to specifically extract, separate and identify human protamines by MS has been described [8]. Other proteins not identified in the present work are A kinase anchoring proteins (AKAPs), possibly because their molecular weight is out of the range studied in the present work [11].

Among nuclear proteins identified, one surprise has been the identification of RuvB-like 1 protein known to have ATPase and DNA helicase activities and to be part of the NuA4 histone acetyltransferase complex. Extensive histone hyperacetylation is known to take place during the nucleo-

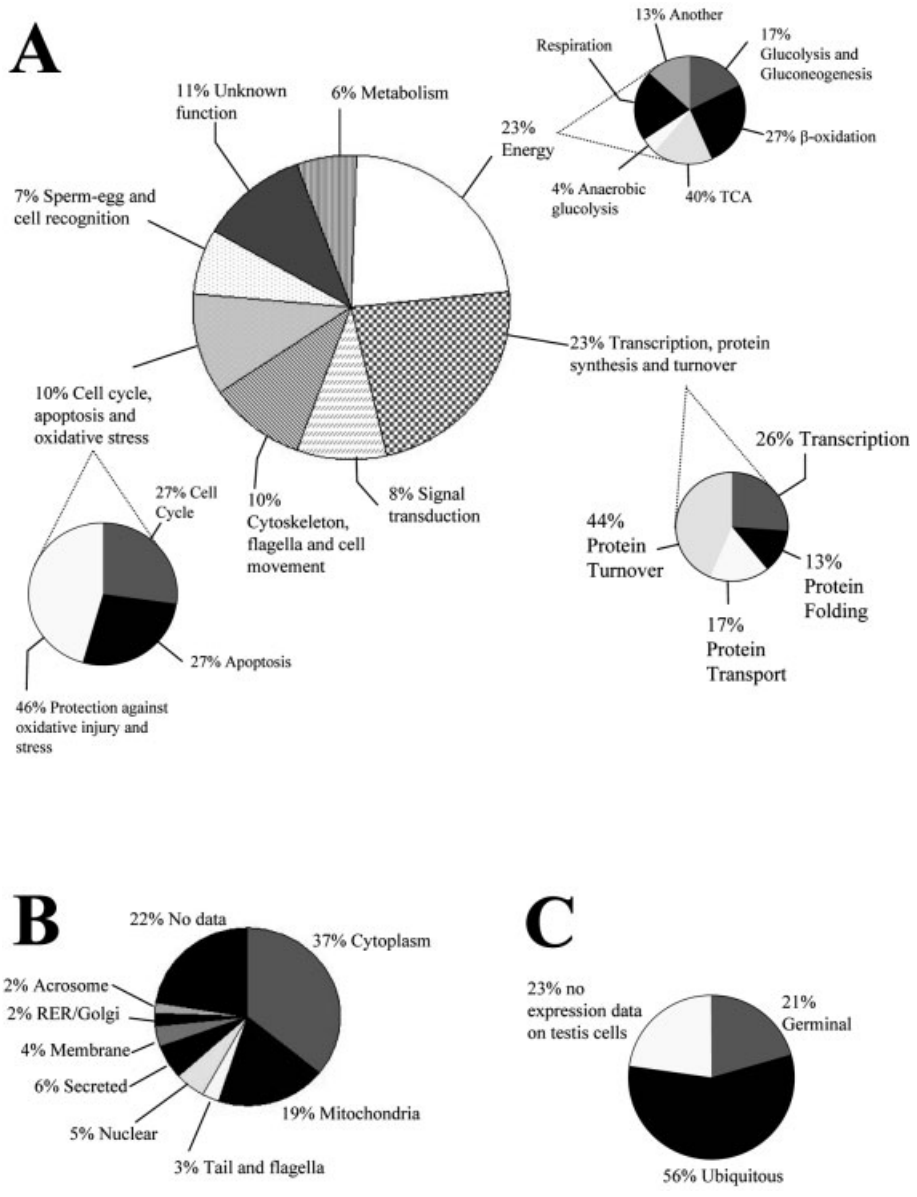

Figure 2. Functional distribution of the proteins isolated from the 2-DE gels and identified by MS in human normozoospermic samples. A: Functional distribution of the proteins identified is shown. B: Distribution of the proteins in the different compartments of the spermatozoon. C: Overall expression information available of the proteins identified.

histone to nucleoprotamine transition in spermatogenesis [44, 45]. Also, upon fertilization, a new wave of histone hyperacetylation takes place concomitant to the paternal nucleosome formation. This RuvB-like 1 protein is also known to interact with nucleoporin p62 and with Ran and to facilitate protein transport into the nucleus [46]. Thus, the identification of this protein in human sperm opens up the possibility of determining its function in human spermatozoa and whether it may be involved in the early nuclear stages upon fertilization.

Another important group of nuclear proteins identified are different SPANX family proteins [47, 48]. These proteins are mainly present in the sperm head, but interestingly have also been identified as cancer-associated proteins. The function of these proteins is not known at present. A non-nuclear protein identified is serum amyloid P-component precursor (APCS) known to interact with DNA and histones and to be involved in the scavenging of nuclear material released from damaged circulating cells. There is evidence that mechanisms of detection and elimination of damaged spermatozoa may be present during normal spermatogenesis. Thus detection of this APCS protein could be consistent with this function.

Finally, we have been able to detect up to 11% of proteins with a yet unknown function (Fig. 2A). For the abundant SPANX proteins, at least their expression in the testis and spermatozoa has already been well described [47, 48]. However, much less information is so far available for the rest of these 11% of proteins of unknown function. The ES1 or HES1 protein is a chromosome 21 gene encoded protein that has been

found to be increased in the fetal Down syndrome brain [49]. We have detected different independent testis EST IMAGE clones (4/533) in the GeneCards Database, which is consistent with the mass identification of the corresponding protein in the present work. Another protein with unknown function is HSPC142, which is a protein predicted from the genome sequencing project. We have detected different testis EST clones (10/623) in the GeneCards Database, which could also be consistent with the identification of this protein in spermatozoa. Another interesting protein identified of known function is the CTCL tumor antigen se57. Also corresponding ESTs (2/45) have been identified in GeneCards consistently with our mass data. The identification in the present work of all these proteins in spermatozoa may stimulate the design of further experiments towards determination of their function.

When considering the expression pattern of the proteins identified, we found that 21% of the proteins are specifically expressed in germinal cells, that 56% are ubiquitously expressed and that no expression data on testis cells is so far described for 23% of the proteins (Fig. 2C). Therefore, the identification of these 23% of the proteins in human normozoospermic spermatozoa now provides the first available experimental protein expression data.

With the approach followed in this work, we have been able to identify approximately 10% of the proteins visualized in the 2-DE gels. The general lysis and extraction procedure used has the advantage that it provides a general idea of the types of proteins that constitute the sperm proteome. Therefore, this approach should be complementary to other proteomic approaches focusing on specific compartments or organelles such as the cytoplasm, membrane, cytoskeleton, tail, acrosome or sperm head [8–11, 24–28].

4 Concluding remarks

In this article we have reported the proteomic identification of 98 proteins present in normozoospermic human spermatozoa. Because the function of many of the proteins identified is not fully understood, our data opens up the possibility to perform additional experiments. Conventional 1-DE has provided in the past a wealth of information concerning the major sperm proteins and their function in normal and in abnormal spermatozoa [1, 2, 5, 6, 13–21, 43]. In this article we have also demonstrated that the present proteomic approach is useful to detect additional proteins that had previously escaped identification using a more conventional approach. The reported proteomic identification of proteins in the present work now provides the basis for subsequent studies on the physiological and pathological aspects of germ cell differentiation and function.

Supported by grants from Ministerio de Ciencia y Tecnologia BMC2003-03937, fondos FEDER, Ministerio de Sanidad y Consumo V-2003-REDC07A-O and by Generalitat de Catalunya 2001SGR00382.

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