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Identification of proteomic differences in asthenozoospermic sperm samples

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BACKGROUND: Asthenozoospermia is one of the most common findings present in infertile males, but its aetiology remains unknown in most cases. Present proteomic tools now offer the opportunity to identify proteins which are differentially expressed in asthenozoospermic semen samples and potentially involved in infertility. METHODS: We compared the expression of 101 sperm protein spots in 20 asthenozoospermic samples to that of 10 semen donor controls using two-dimensional proteomic analysis. RESULTS: Seventeen protein spots have been identified at different amounts in the asthenozoospermic samples compared with controls. These are cytoskeletal actin-B, annexin-A5, cytochrome C oxidase-6B, histone H2A, prolactin-inducible protein and precursor, calcium binding protein-S100A9 (2 spots), clusterin precursor, dihydrolipoamide dehydrogenase precursor, fumarate hydratase precursor, heat shock protein-HSPA2, inositol-1 monophosphatase, 3-mercapto-pyruvate sulfurtransferase/dienoyl-CoA isomerase precursor, proteasome subunit-PSMB3, semenogelin 1 precursor and testis expressed sequence 12. The detected amount of these proteins enabled the grouping of asthenozoospermic sperm samples in an unsupervised clustering analysis. CONCLUSIONS: We have identified several proteins present at different amount in asthenozoospermic sperm samples. These proteins could be candidates towards the development of diagnostic markers, and open up the opportunity to gain further insight into the pathogenic mechanisms involved in asthenozoospermia.

Keywords: 2D-PAGE; human; proteome; spermatozoa; spermatozoon

Introduction

The molecular basis of asthenozoospermia is not yet fully understood at present despite the fact that it is a very common finding in infertile patients (Martinez *et al*., 2000; Turner, 2003; Baccetti *et al*., 2005; Luconi *et al*., 2006). This is most likely due to a relative lack of knowledge of the proteins involved in the normal physiology of the spermatozoa and due to the tools so far available to identify the corresponding proteins (Oliva and Dixon, 1991; Kierszenbaum and Tres, 2004; Rousseaux *et al*., 2005). Anomalies in the expression of sperm proteins have been found in patients using conventional protein purification and identification strategies (Oliva, 2006; Sautière *et al*., 1988; Oliva and Dixon, 1991; de Yebra *et al*., 1993; O'Bryan *et al*., 1994; Balhorn *et al*., 1988; Mengual *et al*., 2003; Torregrosa *et al*., 2006).

At present, the availability of two-dimensional gel electrophoresis techniques coupled to the identification of sperm proteins using mass spectrometry offers the opportunity to compare the proteome maps of independent sperm samples (Pixton *et al*., 2004; Tan *et al*., 2004; Baker *et al*., 2005; Johnston *et al*., 2005; Martinez-Heredia *et al*., 2006; Capkova *et al*., 2007; de Mateo *et al*., 2007). A recent study has already provided some evidence of the potential of this proteomic approach in the identification of proteins involved in asthenozoospermia (Zhao *et al*., 2007). Also tandem mass spectrometry (MS/MS) has been used to identify proteins present in human spermatozoa (Baker *et al*., 2007; Lefievre *et al*., 2007). However, the full potential of the present proteomic tools to gain insight into the fundamental aspects of the human sperm motility and the causes of asthenozoospermia has not yet been fully explored. Therefore, in the present study, we have performed a comparison of the expression of the 101 most abundant protein spots identified in two-dimensional electrophoresis in sperm samples from 20 asthenozoospermic patients and 10 sperm donor controls. Of relevance, we report the identification of several proteins present at a different amount in the asthenozoospermic samples.

Material and Methods

Subjects and sample collection

The bioethics committee of the hospital approved this project and informed consent was obtained from the participants. We included in this study 20 asthenozoospermic patient sperm samples, and 10 sperm samples form healthy semen donors from our assisted reproduction unit semen donor bank. The patient samples corresponded to consecutive asthenozoospermic patients diagnosed as part of the clinical and laboratory evaluation of infertile couples seeking assisted reproduction at our unit during the period comprised between 2005 and 2006. All patients and controls were from Catalonia (North-East of Spain). The semen donor samples corresponded to normozoospermic semen donors undergoing the clinical and laboratory evaluation at our assisted reproduction unit. The sperm samples were collected by masturbation in specific sterile containers after at least 3 days, but no longer than 7 days, of sexual abstinence. At least two samples were collected from each patient and control with an interval comprised between 7 days and 3 weeks. The samples were kept warm (22–37°C) and evaluated in the laboratory within 1 h of collection. After liquefaction of the semen, the sperm parameters (volume, sperm count, percentage of motility and motion characteristics) were evaluated according to published recommendations (ESHRE Andrology special Interest Group, 1998; Krause and Viethen, 1999; World Health Organization, 1999; Mortimer, 2000) using a computer–assisted semen analyser (CASA, Photolux S.L., Barcelona, Spain). The CASA system had been previously validated through quality control of aliquots of the samples blindly assessed in an external andrology laboratory (Puigvert Foundation, Barcelona, Spain), and through video recordings and slow motion analysis of the recorded images. Between 7 and 8 fields were assessed per CASA chamber and at least 200 spermatozoa were assessed. During the CASA sample assessments, the observer manually checks and eliminates particles and debris not corresponding to sperm cells, and takes into account crossing sperm paths. Asthenozoospermic samples were defined as those with $<25\%$ progressive motility (grade a) or $<50\%$ motile sperm (grades a + b) (World Health Organization, 1999). Sperm morphology was evaluated using strict criteria and at least 100 cells were examined per slide (Kruger *et al*., 1987). The basic sperm and semen parameters in patients and controls are described in Table I. None of the semen samples had significant numbers of round cells or leukocytospermia as analysed per published guidelines (Kruger *et al*., 1987; World Health Organization, 1999).

Table I. Basic parameters in patients and controls.

	Mean	Standard deviation	Minimum	Maximum
Asthenozoospermic patients				
Age of patients (year)	33.5	5.2	26	43
Sperm count ($\times 10^6$ sperm/ml)	73.7	38.1	15.4	159.4
Volume (ml)	3.6	1.8	0.8	8.0
Motility a (Rapid progressive, %)	9.4	7.1	0.8	22.6
Motility b (Slow or sluggish, %)	21.3	6.6	10.3	32.2
Motility c (Non-progressive, %)	19.6	8.5	3.0	41.2
Normal morphology (%)	14.8	4.9	3	22
Controls				
Age of donors (year)	25.2	4.3	18	32
Sperm count ($\times 10^6$ sperm/ml)	75.7	30.4	33.6	115.6
Volume (ml)	3.9	1.6	2.1	7.0
Motility a (Rapid progressive, %)	36.3	17.7	16.5	60.4
Motility b (Slow or sluggish, %)	26.3	2.6	22.3	28.8
Motility c (Non-progressive, %)	14.8	6.8	5.2	20.7
Normal morphology (%)	28.2	5.2	18	36

Sample preparation for proteomic analysis

For proteomic analysis, a single ejaculate was processed from each participant. The seminal plasma and other potential contaminating cells present in semen were removed, as previously described, through centrifugation in a 50% step Percoll gradient in culture media (Mengual *et al*., 2003; Martinez-Heredia *et al*., 2006). Fifty per cent Percoll was used instead of a higher percentage of Percoll so that the resulting 2D gels would be representative of the average spermatozoon present in each of the sperm samples, rather than representive of a highly selected subpopulation. This decision was based on previous work from our laboratory where we examined the morphology of spermatozoa in different Percoll fractions (Mengual *et al*., 2003). 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 (Sefi Medical Instruments, Hainfa, Israel; ~200 cells were counted per sample) and then washed twice with Ham's F10 1×. Solubilization of the sperm cells was performed essentially as described (Pixton *et al*., 2004; Martinez-Heredia *et al*., 2006), 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 mM PMSF and 0.005% bromophenol blue. The volume of lysis buffer was calculated to obtain a concentration of lysate corresponding to $\sim 230 \times 10^6$ sperm/ml in a volume of 300 μl per Isoelectric focusing (IEF) strip. The samples were then incubated for 1 h at room temperature to allow solubilization, and finally centrifuged at 3000*g*, 5 min at 4°C to eliminate potentially insolubilized debris. The solubilized samples were divided into aliquots and stored at −20°C for subsequent analysis.

Isoelectric focusing strip and SDS–PAGE

The solubilized protein, 300 μl equivalent to $\sim 69 \times 10^6$ sperm cells, was 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 V h with a rapid ramp (10 000 V), 10 h with a slow ramp (50 V).

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 and 2% DTT for 10 min, followed by the same buffer without DTT and supplemented with 2.5% iodoacetamide for 10 min. A 1% agarose solution, supplemented with 0.01% bromophenol blue, was laid on the top of gels to protect the strip. SDS–PAGE was performed using 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 ~3 h until the bromophenol blue front just started to migrate off the lower end of the gels.

Staining of the 2D electrophoresis, scanning and computer analysis

After electrophoresis, the gels were stained with silver as previously described (Martinez-Heredia *et al*., 2006). For image analysis, the

silver-stained gels were scanned in transmission scan mode using a high-resolution calibrated scanner (GS-800, Bio-Rad). Raw 2D electrophoresis gels were analysed using the PDQuest 7.1.1 software (Bio-Rad). Protein spots were characterized by their molecular weight (Mr) and iso-electric point (pI) using internal 2D electrophoresis standards (Bio-Rad). To corroborate the reproducibility of the results, at least three electrophoresis runs were performed from each sperm sample.

The identification of the same spots in different gels was performed by the standard computer-aided alignment (PD-Quest) and manual confirmation. The spots included in the analysis had all been already identified as part of a previous proteomic project (Martinez-Heredia *et al*., 2006; Fig. 1). When the match was confirmed and finished, the values were normalized following the 'total quantity in valid spots' method according to manufacturer procedure (Bio-Rad). These normalized values were then analysed with SPSS statistical software. Subsequently the non-parametrical Mann–Whitney test was performed to search for differences in the expression of the different proteins in the asthenozoospermic samples when compared with the sperm donor control samples (Table II). A few representative examples of proteins detected as increased or decreased are shown in Fig. 2.

The protein expression data corresponding to the proteins detected as increased or decreased in the asthenozoospermic samples were then used to run an unsupervised clustering of the sperm donor controls and asthenozoospermic samples using the Cluster and TreeView software (Eisen *et al*., 1998). The complete linkage clustering with uncentered correlation method was used. The TreeView software was used to generate the visual representations of the clusters (Fig. 3).

Immunocytochemistry with anti-Clusterin antibodies

An aliquot of the semen samples was washed once in PBS, pH 7.4 and re-suspended into a final concentration of 1×10^6 cells/ml. The cells were then smeared onto a glass slide treated with poly-L-lysine and allowed to air-dry. After fixation with 2% PFA, the slides were washed with PBS-T (PBS-0.05% Triton X-100) and blocked 1 h at 37°C with blocking-buffer with Triton (PBS-T, 5% non-fat dry milk powder) and NGS (10%). The slides were incubated overnight (4°C) with 100 ml of blocking-buffer with Triton, NGS (10%) and anti-Clusterin (1/100) primary antibody (monoclonal mouse, Abcam, ab16077), and washed with PBS-T and PBS. The slides were then blocked for 30 min at 37°C with blocking-buffer without Triton and NGS (10%) and incubated 2 h at 37°C in the dark, with blocking-buffer without Triton, supplemented with NGS (10%) and the secondary antibody labelled with Alexa Fluor 488 (goat anti-mouse, 1/300). Finally, the slides were washed with PBS and counter-stained with DAPI (Hoescht 33342; Sigma) diluted in PBS (1 mg/ml, 1:3000) and cover-slipped using ProLong Gold anti-fade solution. The immunolabelled proteins were detected in an epifluorescence microscopy (Olympus DX-50) with the Alexa 488 filters and the digital image captured using Cell Software (Olympus) and analysed with the Image-J software (Abramoff *et al*., 2004).

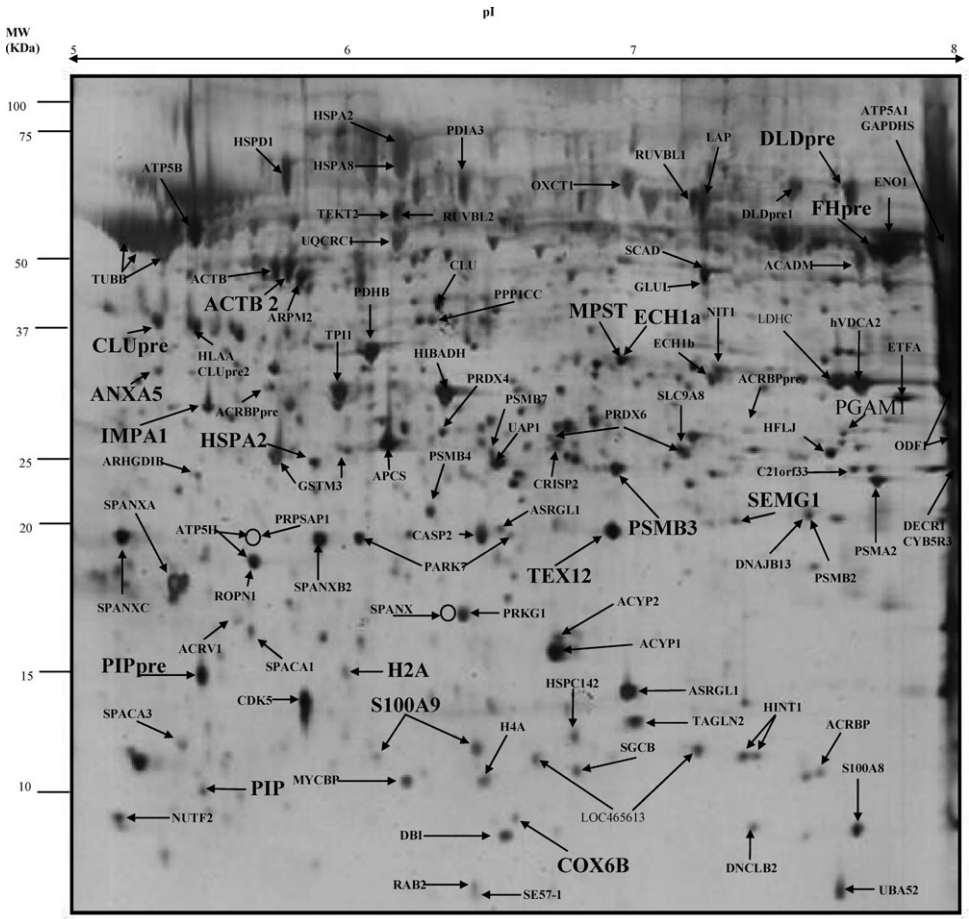

Figure 1: Two-dimensional gel electrophoresis of sperm proteins identified by mass spectrometry and considered for analysis in the present work. The proteins detected with an increased (or decreased) intensity in the asthenozoospermic patient samples are indicated in larger font size

Table II. Proteins with a significantly higher or lower expression in asthenozoospermic samples.

Spot name	Protein name	Protein accession number	Protein in patient samples	Average protein expression		*P* (Mann–Whitney)	Function
				Control	Astheno		
ACTB	Cytoskeletal actin	P60709	↓	23 074	14 063	<0.01	Cell motility, structure and integrity
ANXA5	Annexin A5	P08758	↓	9318	6456	<0.05	Membrane fusion and exocytosis. cell signalling pathways
COX6B	Cytochrome c oxidase subunit 6B	Q7L1R4	↓	2681	1703	<0.05	Electron transport
H2A	Histone H2A	Q93077	↓	13 842	5751	<0.05	Forms parts of the nucleosome
PIP	Prolactin induced protein	P12273	↓	27 352	8894	<0.001	Involved in some kind of actin binding function but not yet clear
PIPpre	Prolactin-inducible protein precursor	P12273	↓	87 770	53 574	<0.05	Precursor of PIP (see above)
S100A9 (spots 1 and 2)	S100 calcium binding protein A9	P06702	↓	3947	2051	<0.05	Regulate migration of phagocytes via modulation of tubulin polymerization
			↓	4582	1077	<0.05	
CLUpre	Clusterin precursor	P10909	↑	11 743	23 843	<0.05	Not yet clear. associated with programmed cell death (apoptosis) and fertility
DLDpre	Dihydrolipoamide dehydrogenase (DLD) precursor	P09622	↑	7555	13 679	<0.01	DLD is a component of the mitochondrial pyruvate and alpha-ketoglutarate dehydrogenase
FHpre	Fumarate hydratase precursor	P07954	↑	8599	14 971	<0.05	Enzymatic component of the tricarboxylic acid (TCA) cycle
HSPA2	Heat shock-related 70 kDa protein 2	P54652	↑	2909	7114	<0.001	Involved in spermatid development and male meiosis
IMPA1	Inositol-1(or 4)-monophosphatase	P29218	↑	3196	6756	<0.01	Key enzyme of the phosphatidyl inositol signalling pathway
MPST/ ECH1pre	3-mercapto-pyruvate sulfurtransferase/Delta 3,5-delta 2,4-dienoyl-CoA isomerase precursor	P25325/Q13011	↑	2076	8581	<0.001	MPST is involved in toxin response/ECH1 is involved in beta-oxidation of fatty acids
PSMB3	Proteosome beta 3 subunit human	P49720	↑	10 399	14 714	<0.05	Subunit of the proteasome with a potential regulatory effect
SEMG1pre	Semenogelin I protein precursor	P04279	↑	3079	5220	<0.05	Predominant protein in semen involved in the formation of a gel matrix
TEX12	Testis-expressed sequence 12 protein	Q9BXU0	↑	6968	13 370	<0.001	Seems to be part of synaptonemal complex

Results

Detection of protein differences

We included in the analysis 101 human sperm protein spots previously identified using mass spectrometry as part of our ongoing proteomic sperm identification project (Fig. 1; Martinez-Heredia *et al*., 2006). The comparison of the protein amounts in the asthenozoospermic samples with the sperm donor control samples resulted in the detection of 17 proteins with an increased or with a decreased amount (Table II and Fig. 2). The average fold differences in the relative amount ranged between 1.4 and 4.5 (Table II).

Seven of the proteins were detected with a lower amount in the asthenozoospermic samples when compared with the sperm donor control samples (Table II). These proteins are cytoskeletal actin-B (ACTB), annexin-A5 (ANXA5), cytochrome-C oxidase subunit-6B (COX6B), histone H2A, prolactin-inducible protein (PIP) and prolactin-inducible protein precursor (PIPpre), and calcium binding protein-S100A9 (2 spots). Eight proteins were detected with a higher amount in the asthenozoospermic samples when compared with the sperm donor control samples (Table II). These proteins are clusterin precursor (CLUpre), dihydrolipoamide dehydrogenase precursor (DLDpre), fumarate hydratase precursor (FHpre), heat shock protein-HSPA2 (HSPA2), inositol-1 monophosphatase (IMPA1), proteasome subunit-PSMB3 (PSMB3), semenogelin 1 precursor (SEMG1pre) and testis expressed sequence 12 (TEX12). Finally, a single spot corresponding to two different proteins (3-mercapto-pyruvate sulfurtransferase/dienoyl-CoA isomerase precursor; MPST/ECH1pre) was also detected with a higher amount in the asthenozoospermic samples when compared with the sperm donor control samples (Table II). It should be noted that many of the sperm proteins have not been analysed since we have included for analysis only the 101 proteins identified from over 1000 spots detected in the 2D gels (Fig. 1). Thus, it could be expected that future proteomic studies targeting the additional proteins will identify new proteins over or under expressed in asthenozoospermic sperm samples.

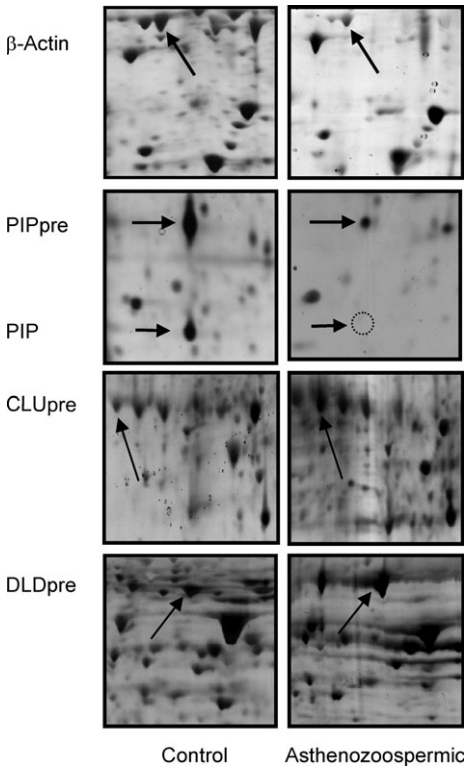

Figure 2: Proteins detected as increased or decreased in asthenozoospermic patients when compared with sperm donor control samples. Representative examples are shown of the 2D gel regions corresponding to four different proteins (five protein spots) detected in a different amount in control samples and asthenozoospermic samples

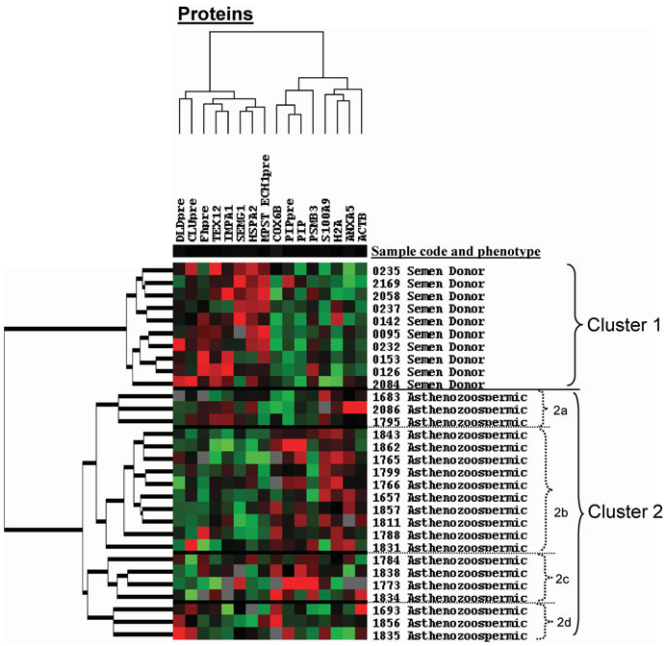

Figure 3: Hierarchical clustering of the samples according to the protein amount data of the proteins detected as differentially increased or decreased. Red colour indicates increased quantity as compared to the normalized value. Green colour indicates decreased quantity of the protein when compared with the normalized value. Black indicates no differences in quantity. Grey indicates no data available. Two major clusters appear. In addition, within the cluster corresponding to the asthenozoospermic group, different proteomic profile patterns appear (subclusters 2a–2d)

Clustering of the asthenozoospermic and sperm donor control samples according to the proteomic profile

The result of an unsupervised clustering (Cluster program) of the control and asthenozoospermic samples, obtained according to the protein quantity data of the proteins detected with an increased or with a decreased quantity, is shown in Fig. 3. The clustering results indicate that the sperm donor's samples (controls) cluster together at one branch of the tree and separate from the asthenozoospermic patient group (Fig. 3). The distribution of the control and asthenozoospermic samples in the two clusters is not random ($P < 0.0000001$; χ^2). It is also interesting to note that different patterns of protein profiles (Subclusters 2a–2d) are evident within the asthenozoospermic group (Fig. 3). However, we have not detected any significant correlation with the motility of the asthenozoospermic samples.

Detection of clusterin using immunocytochemistry

The immunocytochemistry using anti-Clusterin antibodies and quantification of the fluorescence using the Image-J software resulted in the detection of a 'Mean Grey Value' per cell which ranged from 0.0 to 125.4. A cell was considered as positive for staining with the anti-Clusterin antibody if the 'Mean Grey Value' was 10.0 or over. The results indicated that only 8% of the spermatozoa from normozoospermic samples were positively stained, whereas a significantly higher proportion of stained sperm cells (26%; $P < 0.005$; χ^2) could be detected in the asthenozoospermic samples (Fig. 4). In addition, the average 'Mean Grey Value' per cell was also lower in the control samples (20 ± 15; mean $\pm$ SD) when compared with the asthenozoospermic samples (42 ± 24).

Discussion

In the present work, we have measured the amount of the major proteins extracted from asthenozoospermic and from normozoospermic control sperm donor samples and have found that 17 protein spots are detected as either increased or decreased in the asthenozoospermic group (Figs 1 and 2, Table II). All of these proteins, with the exception of semenogelin and clusterin, are reported for the first time as deregulated in asthenozoospermic sperm samples. Semenogelin has recently been described as having an increased quantity in asthenozoospermic samples, in good agreement with our present proteomic results (Zhao *et al.*, 2007). Our immunocytochemistry result also indicates that clusterin is detected in an increased quantity in asthenozoospermic samples (Fig. 4). In addition, clusterin precursors are also detected at a higher amount in asthenozoospermic samples as derived from our proteomics results (Table II). These results are also in good agreement with the previous results obtained using conventional approaches (O'Bryan *et al.*, 1990; Ibrahim *et al.*, 2000).

It is interesting to note that the function of 14 of the 17 proteins identified fall into three main functional groups: 'energy production', 'structure and movement' and 'cell signalling

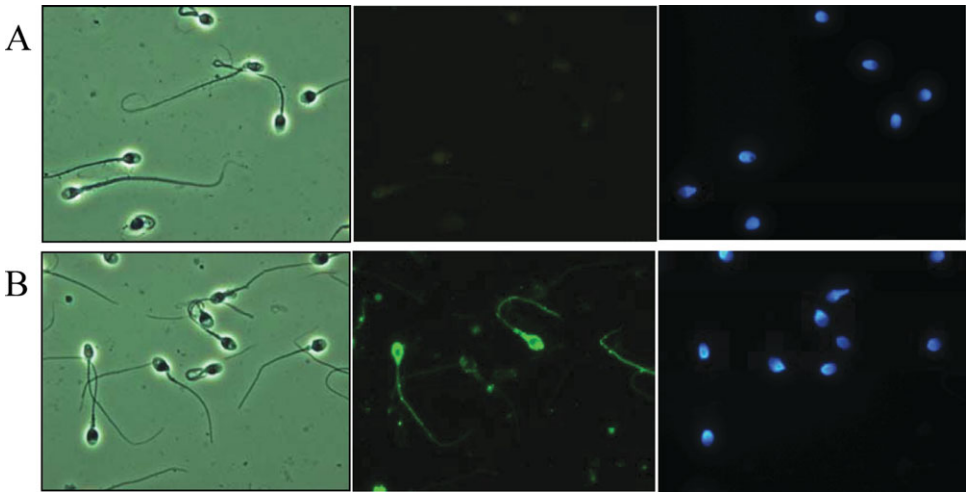

Figure 4: Immunocytochemical detection of Clusterin. (**A**) Sample corresponding to a normozoospermic patient. In the left panel, a phase contrast microphotograph is shown. In the central panel, the result of the fluorescence immunocytochemistry with the clusterin antibody is shown. In the right panel, the staining of the DNA with DAPI is shown. (**B**) Sample corresponding to an asthenozoospermic patient. In the left panel, a phase contrast microphotograph is shown. In the central panel, the result of the fluorescence immunocytochemistry with the clusterin antibody is shown. In the right panel, the staining of the DNA with DAPI is shown

and regulation'. The COX6B, DLDpre, FHpre and ECH1pre constitute the 'energy production' group. The 'structure and movement' group is constituted by ACTB, H2A, PIP, PIPpre and SEMG1 proteins. The 'cell signalling and regulation' group is constituted by ANXA5, S100A9 and IMPA1. It is also of potential importance to note that 6 of the 17 proteins identified are precursor forms (PIPpre, CLUpre, DLDpre, FHpre, ECH1pre and SEMG1pre) of mature proteins. It is interesting to speculate that the accumulation of precursors in asthenozoospermic patients could indicate the existence of a generalized post-translation processing problem of these proteins. The accumulation of protamine 2 precursors in the sperm cells of some infertile patients has already been documented (de Yebra *et al*., 1998; Torregrosa *et al*., 2006). The accumulation of the precursor forms could also result in the deregulation of some of the downstream functions related to these proteins.

Within the energy related group, COX6B has been identified. Testis specific isoforms of this enzyme have been identified (Huttemann *et al*., 2003). Cytochrome c oxidase is the terminal enzyme of the respiratory chain and catalyses the transfer of electrons to oxygen which is coupled to the translocation of protons and is necessary for ATP synthesis. Therefore, the decrease in COX6B could be related to lower ATP production which could lead to the decrease in motility present in the asthenozoospermic sperm samples. The DLD is one of the three catalytic proteins of the pyruvate dehydrogenase complex, which converts pyruvate to acetyl-CoA. In the present article, an increase in the precursor form (DLDpre) has been detected, which suggests a potential decrease in the mature protein. Thus, the potential deficiency of DLD could results in extensive metabolic disturbances, including lactic acidemia, Krebs cycle dysfunction and impaired branched-chain amino acid degradation (Robinson, 1995). The Krebs cycle dysfunction could be related to the decrease in the motility in asthenozoospermic patients. It is known that the beat frequency of the flagellum is directly related to the production rate of energy from ATP (Cardullo and Baltz, 1991). Another protein detected as increased is the fumarate hydratase precursor (FHpre). FH is an enzyme involved in the Krebs citric acid cycle (Coughlin *et al*., 1998). Again an increase in the precursor form suggests a potential decrease in the mature protein. So, in summary, we have found several proteins related to the major sources of ATP production in sperm, which could be related with the failure of tail movement in the spermatozoa from asthenozoospermic patients. Our results are consistent with the identification, using a different approach, of several proteins also related to energy production and differentially expressed in asthenozoospemic samples (Zhao *et al*., 2007). Our results together with those of Zhao *et al*. (2007) suggest the presence of a general deregulation in the metabolic pathways involved in energy production in asthenozoospermic patients.

Within the 'structure and movement' group, we have found actin (ACTB) which is one of the major cytoskeletal proteins and which participates in many important cellular functions, including cell motility, cytokinesis, vesicle and organelle movement, cell signalling and the establishment and maintenance of cell junctions and cell shape (Machesky and Install, 1999). Actin polymerisation may also represent an important regulatory pathway that is associated with tyrosine phosphorylation in sperm (Brener *et al*., 2003; Seligman *et al*., 2004). In this framework, a decrease in actin could explain a decrease in motility since, without cytoskeletal support, the flagellum might not beat correctly. Less information is available on prolactin-induced protein (PIP). PIP has been reported to be involved in some kind of actin binding function (Schaller *et al*., 1991; Caputo *et al*., 1999; Yoshida *et al*., 2003). It has also been found in the post-acrosomal zone, and it remains bound to the sperm surface after capacitation, so it could

have a role in fertilization (Bergamo *et al*., 1997). PIP is an aspartyl proteinase with specificity to fibronectin (Caputo *et al*., 2000). Previous reports have found that PIP specifically degrades the fibronectin molecule that is one of the major protein constituents of the seminal coagulum and constitutes at least 1% of seminal plasma proteins (Lilja *et al*., 1989; Autiero *et al*., 1991; Caputo *et al*., 2000). This suggests that PIP could contribute to fibronectin cleavage during liquefaction. Thus, the detected decrease of both PIP and PIPpre in the present study allows the hypothesis that an incomplete liquefaction of the ejaculate could lead to a more viscous seminal vesicular fluid representing an important restriction for movement of the spermatozoon. It has been previously reported that the beat frequency of the flagellum is inversely related to the viscosity of the surrounding medium (Cardullo and Baltz, 1991). Our results are consistent with data available for the SABP protein (a PIP homologue) where it was found using immunofluorescence, to have a higher expression in asthenozoospermic samples when compared with normozoospermic samples (Capkova *et al*., 2007). Another protein detected at an increased amount in the asthenozoospermic group is semenogelin. A sperm motility inhibitor function for the semenogelin 14 kDa fragment has been described (Yoshida *et al*., 2003). The identification in the present work of a 21 kDa fragment (Fig. 1), also in an increased amount in asthenozoospermic patients, and which has not been previously described as inhibitory of the sperm motility, suggests that this fragment also has this effect.

Within the 'cell signalling and regulation' group, we have detected an increased amount of IMPA1 in asthenozoospermic patients. IMPA1 is one of the enzymes involved in myo-inositol synthesis and is essential for embryonic development (Cryns *et al*., 2008). It is also known that the activity of this enzyme is high in the testes and that the concentration in myo-inositol seminiferous tubules is dramatically higher than levels found in serum (Chauvin and Griswold, 2004). A potential function has also been suggested for myo-inositol in osmoregulation in the seminal vesicular fluid. Both hypo- and hyper-osmotic media have been found to significantly decrease average sperm percentage progressive motility and velocities (Liu *et al*., 2006). Thus, it is possible that the detected increased amount of IMPA1 in the asthenozoospermic patients could be related, through this mechanism, to the reduction of sperm motility. It is also interesting to note that eight of the proteins found increased in the asthenozoospermic group (Table II; ACTB, SEMG1, ECH1pre, DLDpre, FHpre, HSPA2, PSMB3 and SEMG1pre) have been identified as S-nitrosylation targets in a recent MS/MS study, providing an alternative mechanism of the modulation of sperm function and motility by nitric oxide (Lewis *et al*., 1996; Lefièvre *et al*. 2007).

Within the proteins not included in any of the above functional groups, it is noteworthy to mention the HSPA2 and TEX12 proteins because of the statistical significance (Table II). HSPA2 was identified as the first chaperone of transition proteins TP1 and TP2 (Govin *et al*., 2006). The HspA2 gene has also been found to be down-regulated in sperm from infertile men (Cedenho *et al*., 2006). HSPA2 is also component of the synaptonemal complex. A relationship has also been found between HspA2 expression and events of late spermiogenesis, such as cytoplasmic extrusion and plasma membrane remodelling (Huszar *et al*., 2006). Thus, the detected overexpression of the HSPA2 protein in asthenozoospermic samples could be related to the dysfunction of motility through the alteration in any of these levels. It is interesting to note the identification the TEX12 protein which is also involved in the synaptonemal complex (Hamer *et al*., 2006).

The function of the identified clusterin precursor is not yet clear. The wide distribution and sequence conservation of clusterin suggest that this protein performs functions of fundamental importance both inside and outside cells. Focusing only in fertility related functions, clusterin is related to the prevention of damaging oxidative reactions (Reyes-Moreno *et al*., 2002), protein precipitation (Ibrahim *et al*., 2000), agglutination of abnormal spermatozoa (O'Bryan *et al*., 1990, 1994) and control of complement-induced sperm lysis (Jenne and Tschopp, 1989). The ability of clusterin to prevent oxidative damage might be of benefit to spermatozoa in the female reproductive as well as to sperm in an IVF system. In bulls, it was found that clusterin-positive spermatozoa are all abnormal ones, and its presence inversely correlated with motility (Ibrahim *et al*., 2000). In rams, rats and mice, an increment of testicular temperature has been shown to lead to a higher incidence of clusterin-positive spermatozoa (Clark and Griswold, 1997; Ibrahim *et al*., 2000). Also, clusterin has been described as present only in human abnormal spermatozoa (O'Bryan *et al*., 1990, 1994). In the present work, we have detected the clusterin precursor as significantly increased in asthenozoospermic samples when compared with normozoospermic control samples both using proteomic analysis (Table II, Fig. 2) and immunocytochemistry (Fig. 4). An interesting point to consider is whether the increase or decrease of the different proteins detected in the present work is due to over or under expression or instead it is due to altered import or export in the spermatozoa from the epididymal, seminal or prostatic fluid. At least the detected increase of semenogelin and clusterin could well be due to the hypothesis of an increased import. Future studies should further clarify to what extent the contact of the spermatozoa with the epididymal, seminal or prostatic fluid may be responsible for the different protein profiles detected in the asthenozoospermic patients.

Finally, we have used the proteomic information derived for the proteins detected in an increased or decreased level to determine if the protein expression signature of these proteins could predict asthenozoospermia using an unsupervised clustering approach (Fig. 3). The results indicate a clear-cut clustering of the normozoospermic sperm donor control group, separate from the asthenozoospermic patient group (Fig. 3). Thus, although none of the proteins alone is univocally associated with asthenozoospermia, the combination of the proteomic information of the different protein spots was sufficient to cluster the asthenozoospermic group separate from the control group. It is also interesting to point out that, within the asthenozoospermic patient group, several proteomic patterns can be identified (Fig. 3). Our interpretation of these results is that they may be pointing to important clues

towards the identification of potentially different aetiologies associated to asthenozoospermia.

In conclusion, in this work, we demonstrate the validity of the presented proteomic approach to identify proteins with altered amounts in the sperm cells of asthenozoospermic patients. Of importance, we report 17 proteins spots with an altered proteomic pattern in asthenozoospermic patients opening the door to further identification of the mechanisms involved in asthenozoospermia and to their potential assessment as novel biomarkers of infertility.

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