Molecular Human Reproduction, Vol.16, No.7 pp. 452–462, 2010
Advanced Access publication on March 18, 2010 doi:10.1093/molehr/gaq009

MHR Basic science of reproductive medicine

ORIGINAL RESEARCH

Proteomics-based study on asthenozoospermia: differential expression of proteasome alpha complex

Archana Bharadwaj Siva [1], Duvvuri Butchi Kameshwari [1], Vaibhav Singh [1], Kadupu Pavani [1], Curam Sreenivasacharlu Sundaram [1], Nandini Rangaraj [1], Mamata Deenadayal [2], and Sisinthy Shivaji [1,*]

[1]Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India [2]Infertility Institute and Research Centre, Hyderabad, India

*Correspondence address. Tel: +91-40-27192504; Fax: +91-40-27160311; E-mail: shivas@ccmb.res.in

Submitted on September 19, 2009; resubmitted on January 20, 2010; accepted on January 30, 2010

ABSTRACT: With a view to understand the molecular basis of sperm motility, we have tried to establish the human sperm proteome by two-dimensional PAGE MALDI MS/MS analysis. We report identification of 75 different proteins in the human spermatozoa. Comparative proteome analysis was carried out for asthenozoospermic and normozoospermic patients to understand the molecular basis of sperm motility. Analysis revealed eight proteins (including one unidentified) with altered intensity between the groups. Differential proteins distributed into three functional groups: 'energy and metabolism' (triose-phosphate isomerase, glycerol kinase 2, testis specific isoform and succinyl-CoA:3-ketoacid co-enzyme A transferase 1, mitochondrial precursor); 'movement and organization' (tubulin beta 2C and tektin 1) and 'protein turnover, folding and stress response' (proteasome alpha 3 subunit and heat shock-related 70 kDa protein 2). It was interesting to note that although the proteins falling in the functional group of 'energy and metabolism' are higher in the asthenozoospermic patients, the other two functional groups contain proteins, which are higher in the normozoospermic samples. Validation of results carried out for proteasome alpha 3 subunit by immunoblotting and confocal microscopy, confirmed significant changes in intensity of proteasome alpha 3 subunit in asthenozoospermic samples when compared with normozoospermic controls. Significant positive correlation too was found between proteasome alpha 3 subunit levels and rapid, linear progressive motility of the spermatozoa. In our understanding, this data would contribute appreciably to the presently limited information available about the proteins implicated in human sperm motility.

Key words: asthenozoospermia / human sperm proteome / MALDI MS/MS / proteasome

Introduction

Sperm motility is crucial for male fertility but despite this accepted fact information on the molecular basis of sperm motility is rather scarce. One simple approach to address this question would be to identify proteins involved in sperm motility by conventional studies of inhibiting motility and then identifying the protein involved. But this approach is not always free of ambiguities since the inhibition of sperm motility may not necessarily be due to the effect on a single protein and may involve a cascade of proteins ultimately manifesting as inhibition in sperm motility. Such studies have recognized and implicated proteins such as tektins (Wolkowicz *et al.*, 2002; Roy *et al.*, 2007) and outer dense fiber proteins (Jassim *et al.*, 1992) in sperm motility. Nature has provided us an alternate approach, the asthenozoospermic patients, to unravel the molecular basis of sperm motility at the protein level since in these individuals, <50% of the spermatozoa are motile. The lack or decrease in motile spermatozoa in asthenozoospermic individuals has been attributed to factors such as functional deficiency of the epididymis or other accessory sex glands (Comhaire *et al.*, 1999) and structural defects in the sperm-tail protein components (Ryder *et al.*, 1990).

It is in this context that attempts to establish human sperm proteome seems extremely relevant (Naaby-Hansen *et al.*, 1997; Baker *et al.*, 2007; deMateo *et al.*, 2007; Johnston *et al.*, 2005; Martínez-Heredia *et al.*, 2006; Lefièvre *et al.*, 2007; Li *et al.*, 2007; Brewis and Gadella, 2010) since these studies have lead to the identification of proteins involved in sperm motility, sperm capacitation (Secciani *et al.*, 2009) and fertilization ability (Bohring and Krause, 1999;

© The Author 2010. Published by Oxford University Press on behalf of the European Society of Human Reproduction and Embryology. All rights reserved.
For Permissions, please email: journals.permissions@oxfordjournals.org

Downloaded from https://academic.oup.com/molehr/article/16/7/452/1226950 by U.S. Department of Justice user on 17 August 2022

Bohring *et al*., 2001; Bohring and Krause, 2003; Pixton *et al*., 2004). As yet studies directed towards proteome analysis of spermatozoa from asthenozoospermic individuals are limited to three (Zhao *et al*., 2007; Martínez-Heredia *et al*., 2008; Chan *et al*., 2009) and there is a need to extend these studies to get a consistent view on the specific proteins involved in this defect associated with sperm motility. The present study besides establishing the human sperm proteome, proposes to identify qualitative and quantitative changes in proteins in spermatozoa from normozoospermic and asthenozoospermic individuals, with the view to understand the molecular basis of sperm motility.

Materials and Methods

Chemicals

Reagents for two-dimensional (2D) electrophoresis, MALDI MS/MS and Coomassie staining were from Sigma (St. Louis, MO, USA). IEF strips were from Bio-Rad Laboratories (CA, USA). Acetonitrile was from Merck (Darmstadt, Germany) and Percoll was from GE Healthcare Biosciences (Uppsala, Sweden). All other reagents were of analytical or protein sequencing grade and were purchased from local vendors (Qualigens, Mumbai, India; Genetix, New Delhi, India; Genei, Bangalore, India; HiMedia, Hyderabad, India). Anti-20S proteasome alpha antibodies were from Millipore, USA and the secondary conjugates were from Sigma, USA) and GE Healthcare biosciences (Uppsala, Sweden).

Patients and sample collection

Semen samples were collected from patients who were recruited at The Infertility Institute and Research Center, Hyderabad, India during September 2006 to July 2008. We included in this study, 17 asthenozoospermic patient sperm samples and 20 sperm samples from normozoospermic patients attending the clinic. Normozoospermic samples were defined as those with more than 50% motility or more then 25% Rapid linear progression (RLP) (Grade a) and asthenozoospermic samples were defined as those with <25% RLP (Grade a) (WHO laboratory manual, 1999). All the donors were free from sexually transmitted diseases, including human immunodeficiency virus (HIV), in accordance with recommended guidelines. Samples, in accordance with WHO guidelines (WHO laboratory manual, 1999) had >30% ideal morphological forms. The basic semen parameters of the patients are described in Supplementary Table SI.

Sample preparation for proteome analysis

Samples were collected by masturbation in 10 ml sterile containers after at least 3 days of sexual abstinence and were allowed to liquefy for 30 min at 37°C (95% CO_2 in air). Subsequently, the entire semen sample was layered on a discontinuous Percoll gradient prepared in medium M (10× concentration having 1.37 M NaCl, 25 mM KCl, 200 mM HEPES and 100 mM glucose) as described by Pixton *et al*. (2004). The 100% isotonic Percoll was prepared by adding Percoll with medium M (10× concentration) in a ratio of 10:1 (v/v). Percoll fractions of 30 and 70% were then made by diluting 100% Percoll with medium M (1×), having the final concentration of 137 mM NaCl, 2.5 mM KCl, 20 mM HEPES and 10 mM glucose. A density gradient of Percoll was prepared in a Falcon tube (70% fraction under the 30% fraction, 1 ml of each) and overlaid with semen (1 ml). The sample was then centrifuged (800*g* for 40 min), the pellet recovered, washed once with medium M (1×) and once with 30 mM Tris–HCl (pH 7.4), to remove excess salt by spinning at 1000 g for 10 min. Prior to the 30 mM Tris–HCl wash, sperm concentration was evaluated by Computer Assisted Semen Analyzer (CASA; Hamilton Thorne Research, ver 12.0L, MA, USA) on a Makler counting chamber. The pellet was either stored immediately at −70°C for future use or processed immediately. Spermatozoa were solubilized in 125 µl of lysis buffer containing 7 M urea, 2 M thiourea, 1% CHAPS (w/v), 18 mM dithiothreitol, 0.5% (w/v) IPG buffer (pH 3–10) and 1% (w/v.) *n*-octyl glucopyranoside (Rabilloud *et al*., 1997). This was followed by sonication (two pulses of 5 s each). After sonication, the samples were kept for 2 h at 4°C on a rotor shaker and then subjected to centrifugation at 131 000*g* to remove cell debris. The supernatant was recovered and either used immediately for isoelectric focusing (IEF) or stored at −70°C for future use.

IEF, 2D gel electrophoresis and PD-Quest image analysis

Equal concentration of sperm protein (250 µg) was diluted in a rehydration buffer (composition same as lysis buffer), loaded on to a commercially available IPG strip. Seven centimetre, pH 5–8 IPG strips were used in the study for a good resolution (Bio-Rad, CA, USA). Passive rehydration was carried out for 12 h and the strip was then subjected to IEF at 50 mA/IPG strip at 4000 V for 20 000 VH using a Protean IEF cell (Bio-Rad, CA, USA) as described earlier (Kota *et al*., 2009). After the IEF run the strips were equilibrated and the second dimension electrophoresis was performed on 10% SDS polyacrylamide gels (8 × 8 × 0.15 cm) (Amersham Buckinghamshire, UK). The 2D gels were stained with Fast Coomassie Brilliant Blue R-250 (Fast CBB) staining procedure (Wong *et al*., 2000).

The stained 2D gels were then scanned using Fluor-S^{TM} Multi-Imager (Bio-Rad Laboratories, Hercules, CA, USA) and the resulting images were analyzed by PD-Quest (version 8.0.1; Bio-Rad, Hercules, CA, USA). Staining intensities of the protein spots were determined after normalization using the accurate 'Local regression model' available in PD-Quest and the qualitative and quantitative changes were assessed between the normo- and the asthenozoospermic samples. Differences in normalized expression ≥1.5-fold were considered and the significance of differences in expression levels was assessed using non-parametric Mann–Whitney test setting the significance threshold at $P < 0.05$. Changes in the intensity of protein(s) was further validated by immunoblotting and immunofluorescence. Samples from different individuals were used for each experiment ($n = 9$ from each category for immunoblotting, $n = 3$ from each category for confocal microscopy, $n = 5$ from each category for PD-Quest analysis).

Protein identification by MALDI MS/MS

For MALDI MS/MS analysis, spermatozoa from normozoospermic patients were used ($n = 3$). In gel digestion and identification of proteins was performed as described by us recently (Kota *et al*., 2009). In brief, the protein spots were excised, destained, vacuum-dried, trypsin digested, spotted on a MALDI plate and identified by MALDI MS and MS/MS using Applied Biosystems 4800 Proteomics Analyzer. Peptide mass calibration was performed with external mass standard (Calmix 5; Applied Biosystems, Foster City, CA, USA). The spectra were analyzed using in-house GPS Explorer$^{(TM)}$ software, version 3.5 with fixed carbamidomethylcysteine and variable methionine oxidation and MASCOT integration. The database used was Human Swiss-Prot 2009, release 15.0, March 24, 2009 (http://www.expasy.org/sprot/).

The criteria used for MS/MS peak filtering were: a mass tolerance of 50 ppm and the number of accepted missed cleavage sites was set to one. The experimental mass values were monoisotopic. No restrictions on protein molecular weight and pI values were applied. Total ion score cut-off of 28 (for Swiss-Prot 2009) and %CI of >95% for MS/MS analysis was the criteria used. Functional annotation for the identified proteins was done based on the information available for each one of them in the Human Swiss-Prot 2009 database.

Downloaded from https://academic.oup.com/molehr/article/16/7/452/1226950 by U.S. Department of Justice user on 17 August 2022

Immunoblotting

Sperm proteins (30 μg) that were separated by SDS–PAGE were electro-transferred onto a nitrocellulose membrane at 100 V for 1 h using the wet transfer system (Hoefer Scientific Instruments, San Francisco, CA, USA) (Kota *et al*., 2009). Subsequently, the membrane was stained with 0.1% Ponceau S to check equal loading of the proteins. Membranes were then blocked with 5% (w/v) non-fat milk in TBST [10 mM Tris, 150 mM NaCl containing Tween 0.1% (v/v)] for 1 h at room temperature, washed and incubated overnight at 4°C with the primary antibody, anti-proteasome alpha complex antibody (1:750, Millipore, MA, USA) prepared in TBST plus 1% BSA. After the incubation period, the membranes were washed three times (10 min each wash with TBST) and incubated in the appropriate secondary antibody prepared in TBST containing BSA 1% (w/v) for 1 h at room temperature. Goat anti-mouse IgG linked to horseradish peroxidase (1:10 000) was used as the secondary antibody (SIGMA, St. Louis, MO, USA). The blots were developed using 3,3′,5,5′-tetramethyl-bezidine as a substrate (Calbiochem, Germany; Zheng *et al*., 2004). Blots were scanned with Fluor-S^{TM} Multi-Imager (Bio-Rad Laboratories, Hercules, CA, USA) and the bands of interest were quantified using GeneTools version 3.06.04 from SynGene (Cambridge, England). Sperm lysates (30 μg) from various patients was resolved on 10% SDS–PAGE and the gel was stained with Coomassie Brilliant Blue R-250 (CBB). The stained gel was scanned and the total intensity of CBB staining in each lane was then quantified using GeneTools version 3.06.04 from SynGene (Cambridge, England). CBB stained gel was utilized for normalization purpose, where ratio of proteasome alpha complex intensity to CBB stain intensity was calculated and compared between the normo- and asthenozoospermic patients.

Immunofluorescence

Sperm smears from different normo- and asthenozoospermic patients and fertile donors were utilized for immunolocalization of proteasome alpha complex in human spermatozoa. Confocal microscopy was carried out and the Region of Interest (ROI) was quantified off-line using the LAS AF software (version 1.81, Leica Microsystems, GmBH, Germany) to get an idea about quantitative differences between the various samples in terms of proteasome alpha complex expression. The ROI values obtained were analyzed by non-parametric Mann–Whitney test and considered significantly different at $P < 0.05$. The comparisons were done with at least three patients in each category. ROI for proteasome localization was the connecting piece of the human spermatozoa.

The sperm on the coverslips were initially air-dried and then permeabilized by dipping in ice-cold methanol for 20 s. They were subsequently blocked with 5% BSA in TBS, followed by incubations with the primary antibody, anti-proteasome alpha complex antibody (1:50), overnight at 4°C. After 3 × 10 min washes in TBS, the smears were incubated with 1:200 donkey anti-mouse Cy3 antibody as the secondary antibody (Molecular probes, Invitrogen, Carlsbad, CA, USA) that contained 1% BSA for 1 h at room temperature. After immunostaining, the coverslips were mounted on clean glass slides using Vectashield (Vector Laboratories, Burlingame, CA, USA) as the mounting medium and viewed under a Confocal Laser Microscope (Leica Microsystems, GmBH, Germany) at 100× under oil immersion.

Statistical analysis

Non-parametric Mann–Whitney test (for comparing two groups) were performed to analyze the results statistically using the software Graph Pad, Prism, version 3.02. *P*-values of <0.05 were considered significant.

Results

Identification of human spermatozoal proteins by MALDI MS/MS analysis

2D PAGE of proteins extracted from human normozoospermic spermatozoa led to the resolution of over 400 spots in the pI range of 5–8 and molecular weight of 20–97 kDa (7 cm IPG strip). MALDI MS/MS analysis of human sperm proteome gave reliable identities for 120 protein spots out of 200 protein spots that were excised and analyzed. A representative gel picture of human spermatozoa is shown in Fig. 1, with the MALDI MS/MS identified proteins encircled. These spots corresponded to 75 different proteins. The details of the identified proteins are tabulated in Supplementary Table SII (for 114 spots), although the remaining six spots, which were identified as differentials are mentioned in Table I.

The protein IDs mentioned were obtained from the human database, human Swiss-Prot 2009. Functional (Fig. 2A) and organelle (Fig. 2B) distribution of the identified proteins from spermatozoa of normozoospermic patients is represented as a pie chart.

Comparison of the intensity of proteins in the spermatozoa of normo- and asthenozoospermic individuals

Of the 120 proteins spots identified, we could unambiguously match 100 spots in the sperm proteome of all the normozoospermic ($n = 5$) and asthenozoospermic ($n = 5$) individuals for PD-Quest analysis. Comparison of the protein amounts in the two categories analyzed by the software resulted in the detection of eight proteins with a significant difference ($P < 0.05$) in intensity (Table I and Fig. 1). In asthenozoospermic patients, five proteins namely proteasome subunit alpha type 3 (PSMA3), heat shock 70 kDa related protein (HSPA2), tubulin beta-2C chain (TUBB2C), tektin 1 (TEKT1) and a protein of Mr ~ 33 kDa and pI ~ 5.7 were lower and three proteins, namely a pI isoform (pI 6.1) of triose-phosphate isomerase (TPIS), testis specific glycerol kinase 2 (GKP2) and succinyl-CoA:3-ketoacid co-enzyme A transferase 1, mitochondrial precursor (OXCT1) were higher than in normozoospermic samples. The average fold difference in the relative amounts ranged between 1.7 and 6.4 (Table I). Representative differential protein spots are shown in Fig 3.

Lower levels of proteasome alpha complex in asthenozoospermic men by immunoblot analysis

Monoclonal antibody against the whole 20S proteasome alpha complex (clone MCP231) was used for detection of proteasome alpha 3 subunit, since individual antibodies against the proteasome alpha 3 subunit were not available. Moreover, since the alpha subunits are very similar in sizes (~29–31 kDa), it was considered best to use the signal from the proteasome alpha complex as a whole for quantification and comparison purpose. Immunoblot analysis revealed that asthenozoospermic samples ($n = 9$) had significantly lower levels of the proteasome alpha complex ($n = 9$, 30 521 ± 7693) when compared with the normozoospermic men ($n = 9$, 45 269 ± 20 777, Fig. 4B). Normalization was carried out for samples by taking a ratio of the proteasome alpha complex intensity (by immunoblotting,

Downloaded from https://academic.oup.com/molehr/article/16/7/452/1226950 by U.S. Department of Justice user on 17 August 2022

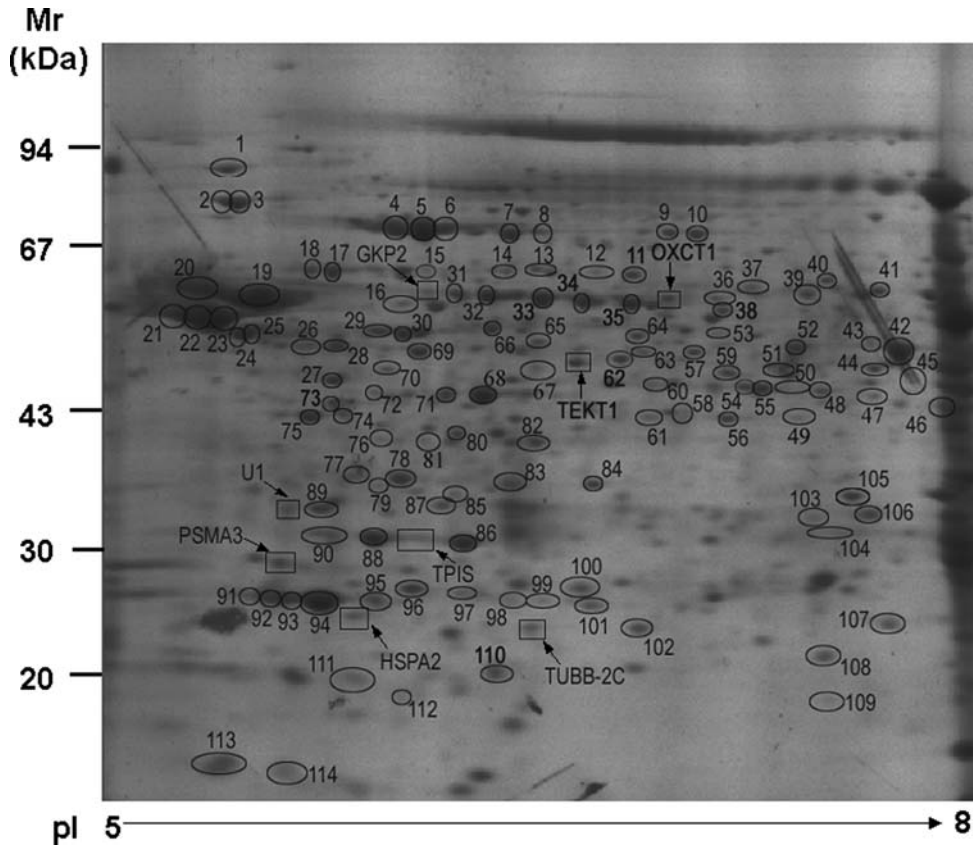

Figure 1 Human sperm proteome of a normospermic individual resolved following IEF on pH 5–8 IPG strip (7 cm) and 2D gel electrophoresis on 10–15% gradient SDS–PAGE. The resolved proteins were visualized by Fast CBB staining. The protein spots identified by MALDI MS/MS analysis are encircled and annotated by numbers. Details of these proteins are mentioned in Supplementary Table SII. Differential proteins highlighted by black squares are referred to by their protein IDs. Details of these proteins are tabulated in Table I.

Fig. 4A) and the corresponding CBB stain intensity of the sample (in gel lane Fig. 4A′). After normalization, the normozoospermic patients, showed a ratio of 5.3 ± 1.6, although asthenozoospermic patients had a ratio of 2.1 ± 0.4 ($n = 3$, $P < 0.05$; Fig. 4C). A representative immunoblot is also shown in Fig. 4A. It is important to highlight that the whole proteasome alpha complex was lower in asthenozoospermic patients. A significant correlation was also observed between proteasome alpha complex levels (shown in Fig. 4B) and rapid linear progressive motility of spermatozoa (Fig. 4D; Spearman $r = 0.58$, $P < 0.01$).

Lower levels of proteasome alpha complex in asthenozoospermic patients by immunofluorescence

Confocal microscopy was used to localize proteasome alpha complex in normo- and asthenozoospermic sperm samples ($n = 3$) by immunofluorescence using anti-proteasome alpha complex antibodies. The results indicated that proteasome alpha complex is localized in the connecting piece of the human spermatozoa (Fig. 5A and B, white arrow). No labeling was found in the mid piece or the remainder of the flagellum. Off-line quantification of the ROIs (here the connecting piece) revealed that asthenozoospermic samples showed lower amount of proteasome alpha complex as compared with the normozoospermic patients (Fig. 5C, $P < 0.01$). The mean value for the asthenozoospermic sperm samples ($n = 3$) was 42.1 ± 11.4 a.u., although the normozoospermic sperm samples ($n = 3$) revealed a mean of 55.6 ± 14.1 a.u.

Discussion

Approximately 15–20% of human couples are affected by infertility and about half of these cases of infertility can be attributed to men (Moore and Reijo-Pera, 2000). In men, the main causes of infertility are oligozoospermia (low sperm production), asthenozoospermia (poor sperm motility), teratozoospermia (abnormal sperm morphology) and azoospermia (lack of sperm production), which account for 20–25% of the infertility cases. In the present study attempts have been made to identify qualitative and quantitative changes in sperm proteins in asthenozoospermic individuals using proteomics. In this report, 120 protein spots (75 different proteins) in the human spermatozoa have been consistently identified by MALDI MS/MS identification (Supplementary Table SII) and majority of the proteins have been functionally categorized as those involved in sperm movement and structural organization (34%), energy and metabolism

Downloaded from https://academic.oup.com/molehr/article/16/7/452/1226950 by U.S. Department of Justice user on 17 August 2022

Downloaded from https://academic.oup.com/molehr/article/16/7/452/1226950 by U.S. Department of Justice user on 17 August 2022

Table I Proteins showing changes in levels in the spermatozoa of normo- and asthenozoospermic individuals.

Protein ID (gel annotation)[a]	Access-ion No.[b)]	Experimental Mol wt (kDa)/ pI	Av. normalized intensity ± S.D			MS identification			MS/MS identification		
			Normo	Astheno	Fold difference[c)]	MOWSE score/ sequence coverage (%)	Matched /total peptides	e-Value[d]	Matched peptides /total ion score	Matched peptide; sequence/ion score[e]	Sequence coverage (%)/% CI[f]
Proteasome subunit alpha type 3 (PSMA3)	P25788	29/5.65	16.5 ± 7.9	6.2 ± 4.5	−2.6	67/34	8/42	0.004	2/28	SLADIAREEASNFR/22; HVGMAVAGLLADAR/5	10/95
Heat shock 70 kDa related protein (HSPA2)	P54652	24.5/5.85	25.7 ± 17.5	4.0 ± 4.9	−6.4	58/11	5/8	0.011	3/64	VHSAVITVPAYFNDSQR/36; PTPSYVAFTDTER/17	6/100
Tubulin beta-2C chain (TUBB2C)	P68371	24/6.55	30.0 ± 12.6	10.8 ± 4.8	−2.8	60/22	13/65	0.021	7/480	GHYTEGAELVDSVLDVVR/152 GHYTEGAELVDSVLDVVRK/99	21/100
Tektin 1 (TEKT1)	Q969V4	49/6.7	14.3 ± 1.7	8.7 ± 2.6	−1.7	76/17	9/22	0.0005	–	–	–
Triose phosphate isomerase (TPIS)	P60174	31/6.1	26.0 ± 15	13.4 ± 11.2	+3.5	59/25	7/41	0.027	4/152	VPADTEVVCAPPTAYIDFAR /51; HVFGESDELIGQK /49	23/100
Glycerol kinase, testis specific 2 (GKP2)	Q14410	61/6.15	5.7 ± 5.0	19.9 ± 21.3	+5.3	92/32	12/53	$1.4e^{-005}$	4/120	FEPQIQATESEIR/61; TAAVGPLVGAVVQGTNSTR /41	12/100
Succinyl-CoA:3-Ketoacid co-enzyme A transferase 1 (OXCT1)	P55809	60/7.0	2.3 ± 1.7	12.1 ± 10	+1.8	58/26	6/20	0.032	2/151	GLTAVSNNAGVDNFGLGLLLR /91 QYLSGELEVELTPQGTLAER/61	7/100
Unidentified (UI)	–	33/5.7	7.8 ± 3.9	14.1 ± 3.4	−1.9	–	–	–	–	–	–

[a]Annotation as in Fig. 1.
[b]Accession numbers are from Swiss-Prot 2009 database.
[c]Down-regulated protein spots in asthenozoospermic subjects are denoted by '-' and up-regulated ones are denoted by '+'.
[e]Only the sequence of two peptides with the highest ion score as identified by MALDI MS/MS are given.
e-Value[d] and % CI[f] refer to the significance level of the protein scores obtained in MALDI MS AND MS/MS, respectively.

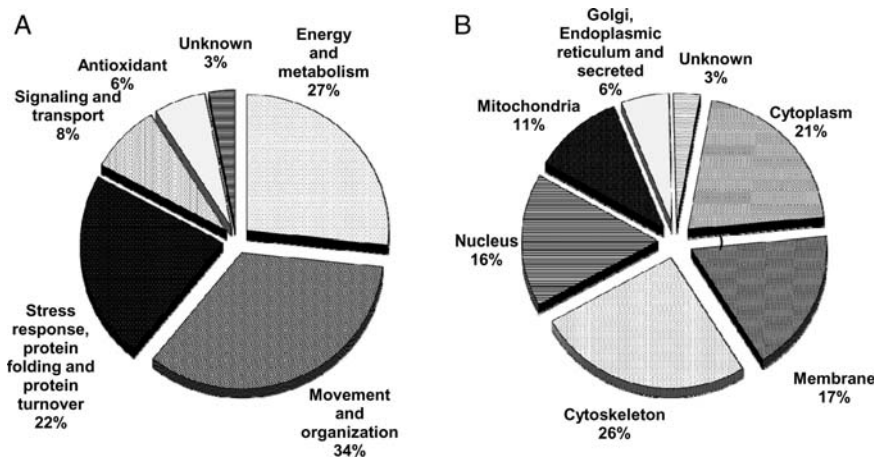

Figure 2 Pie charts showing categorization of the human sperm proteome (Supplementary Table SII) based on their function (**A**) and organelle distribution (**B**). Functional annotation for the identified proteins was done based on the information available for each one of them in the Human Swiss-Prot 2009 database.

(27%), stress response, protein folding and protein turnover (22%), signaling and transport (8%) and antioxidant activity (6%). The function of the remaining 3% of the proteins was not known. There have been so far few studies on the characterization of the complete human sperm proteome (Johnston *et al*., 2005; Baker *et al*., 2007; Martínez-Heredia *et al*., 2006; Secciani *et al*., 2009). These studies differed from the present one in that the sperm protein identities were based either on MALDI MS analysis (Martínez-Heredia *et al*., 2006; Secciani *et al*., 2009) or 1-D SDS–PAGE followed by LC-MS/MS (Johnston *et al*., 2005; Baker *et al*., 2007) although the present study, provides information about sperm proteome based on 2D SDS–PAGE followed by MALDI MS/MS analysis for the first time. In the present study, we have identified 75 different proteins, out of which 23 were consistent with the earlier report of Martínez-Heredia *et al*. (2006). Comparison of the present study with that of the earlier study (Martínez-Heredia *et al*., 2006) indicated that majority of the proteins identified by us (57%) are in the high molecular weight range (>50 kDa) whereas only 16% of the proteins reported by Martínez-Heredia *et al*. (2006) lie in this range; thus implying the identification of additional high molecular weight proteins in the human spermatozoa. It is important to highlight here that the proteome analysis can be made more exhaustive by altering the approach used. In this regard, Brewis and Gadella (2010) mention that the main approach of proteome analysis, 2DE [where proteins are separated on the basis of charge (IEF) and molecular weight (SDS–PAGE)], has certain limitations when it comes to protein solubilization of integral membrane proteins or multiprotein complexes or resolution of highly charged or large proteins (Rabilloud, 2009); and such shortcomings can be overcome by using techniques like LC-MS/MS. Brewis and Gadella (2010), however, also mention that these limitations are not very rigid and this is what is seen in the present study, where proteins have been identified in some of the categories mentioned above such as Voltage-dependent anion-selective channel protein 2, an integral membrane protein and proteasome subunits are a part of a large multiprotein complexes (Supplementary Table SII).

Studies on the sperm proteome from asthenozoospermic patients till date have revealed nearly 34 proteins (10 by Zhao *et al*., 2007; 17 by Martínez-Heredia *et al*., 2008 and 7 in the present study (1 being unknown)], which are found to be differentially expressed in these samples when compared with the normozoospermic individuals. The number and identity of differentially expressed proteins reported in these three studies is different indicating that the list of proteins potentially important for human sperm motility is on the increase. Further analysis of these three studies reveals that TPIS (Zhao *et al*., 2007) and heat shock-related 70 kDa protein 2 (Martínez-Heredia *et al*., 2008) were the two proteins, common between the present and the earlier studies, whereas semenogelin I precursor was the protein common between the studies by Zhao *et al*. (2007) and Martínez-Heredia *et al*. (2008). Our results are more in agreement with Zhao *et al*. (2007) than with Martínez-Heredia *et al*. (2008). At this moment, however, whether this observation can be attributed to biological variation in various ethnic groups (present study and study by Zhao *et al*., 2007 have worked on Asian population and Martínez-Heredia group has worked on European population) would be speculative, since only studies on a larger population would be required to do so (Pan *et al*., 2007).

It is interesting to note that the seven proteins which exhibited quantitative changes in spermatozoa of asthenozoospermic individuals compared with spermatozoa from normozoospermic individuals could be categorized into three main functional groups namely: proteins involved in 'energy and metabolism' [TPIS, glycerol kinase 2, testis specific isoform (GKP2) and succinyl-CoA:3-ketoacid co-enzyme A transferase 1, mitochondrial precursor (OXCT1)], 'sperm movement and structural organization' [tubulin beta 2C (TUBB2C) and TEKT1] and 'protein turnover, protein folding and stress response' (PSMA3,H-SPA2). The observed changes are not surprising since the above functional groups of proteins are crucial for sperm structural organization and motility. It is interesting to note that although the proteins falling in the functional group of 'energy and metabolism' are higher in the asthenozoospermic patients [may be because of incomplete maturation,

Downloaded from https://academic.oup.com/molehr/article/16/7/452/1226950 by U.S. Department of Justice user on 17 August 2022

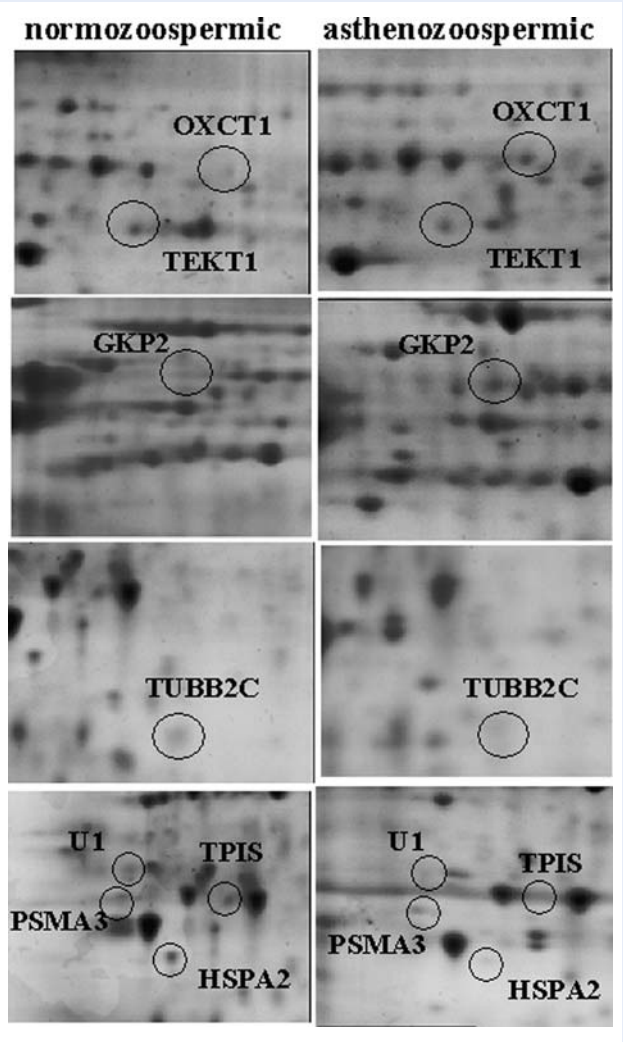

Figure 3 Protein spots showing changes in intensity in the spermatozoa of normo- and asthenozoospermic individuals.

spermatozoa may contain redundant cytoplasm (droplets) and hence more cytosolic proteins], the other two functional groups contain proteins, which are higher in the normozoospermic samples. Earlier studies on asthenozoospermic samples also observed that majority of the differential proteins could be divided into the above mentioned three main categories (Zhao *et al*., 2007; Martínez-Heredia *et al*., 2008).

Since sperm functions are regulated to a large extent by protein post-translational modifications (PTMs), interpreting the observations from this perspective seemed essential. Protein tyrosine phosphorylation and S-nitrosylation are two well-established PTMs implicated in sperm motility (Leclerc *et al*., 1996; Lewis *et al*., 1996; Si and Okuno 1999). In fact, a recent paper by Chan *et al*. (2009) establishes the importance of protein PTMs, where they describe 12 proteins which display differential phosphorylation (serine, threonine and tyrosine phosphorylation) between normozoospermic and asthenozoospermic samples and further validate the expression of gamma-tubulin complex associated protein 2. Importance of protein tyrosine phosphorylation in sperm motility has been demonstrated unequivocally (Visconti *et al*., 1995a, b) and in fact, it has been shown that asthenozoospermic sperm samples exhibited lower levels of sperm-tail tyrosine phosphorylation and hyperactivation as compared with normozoospermic patients during capacitation (Yunes *et al*., 2003). Some of the identified differential proteins are known to be tyrosine phosphorylated, namely GKP2 (Kota *et al*., 2009) and TUBB2C (Baker *et al*., 2006) in spermatozoa and PSMA3 (Rush *et al*., 2005) and HSPA2 (Rikova *et al*., 2007) in other cell types. In fact, using a predictive software NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/), it was found that all these proteins were putatively tyrosine phosphorylated as reflected by the high scores obtained-PSMA3 (four sites, scores −0.939, 0.843, 0.765, 0.620); HSPA2 (seven sites, scores −0.987, 0.947, 0.850, 0.817, 0.705, 0.548, 0.529); TEKT1 (three sites, scores −0.962, 0.853, 0.816); TPIS (one site, score −0.937); OXCT1 (four sites, scores −0.980, 0.761, 0.705, 0.731); GKP2 (three sites, scores −0.917, 0.852, 0.787), TUBB2C (six sites, scores −0.897, 0.879, 0.878, 0.678, 0.649, 0.593). Out of these seven proteins, 4 (57%) of them have been already reported to be tyrosine phosphorylated in cells, as mentioned above, although further experimental validation in sperm is required. One such protein, Dihydrolipoamide dehydrogenase that has a high score of 0.816 in NetPhos 2.0 was found to be tyrosine phosphorylated in hamster spermatozoa (Mitra and Shivaji, 2004); strengthening the predictive value of NetPhos 2.0.

Protein S-nitrosylation too is known to affect sperm motility in several species (Lewis *et al*., 1996; Kameshwari *et al*., 2003). Four of the seven differential proteins (57%), namely TUBB2C, TPIS, HSPA2 and PSMA3, have been reported to get S-nitrosylated in human sperm during capacitation (Lefièvre *et al*., 2007), suggesting an involvement of these proteins in various sperm functions during capacitation, including sperm motility.

Contribution of metabolic/energy pathways to sperm motility is obvious, since 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); which is derived from the metabolic pathways namely glycolysis and Kreb's cycle. TPIS catalyzes the isomerization of dihydroxyacetone phosphate to glyceraldehyde 3-phosphate during glycolysis. We identified four pI isoforms of TPIS (pI 5.75, 5.95, 6.1 and 6.25) in the human spermatozoa, of which the isoform with pI 6.1 was found to be up-regulated in the asthenozoospermic patient samples (Fig. 1, TPIS). The reason for this increase in the intensity of pI 6.1 isoform of TPIS is not known but this isoform may be a functional isoform, which gets up-regulated in the patient sample as a compensatory mechanism to pH or oxidative stress in the cells. TPIS is known to shift between different pI isoforms in response to stress (Yamaji *et al*., 2004; Stigliano *et al*., 2008).

OXCT1 enzyme is involved in ketone metabolism (Tanaka *et al*., 2004a, b) and it catalyses the formation of acetoacetyl CoA by transfer of a CoA moiety from succinyl-CoA to 3-oxo acid, which is further broken down to two acetyl CoA molecules capable of entering the tricarboxylic acid cycle (Solomon and Jencks, 1969). OXCT1 precursor is higher in spermatozoa of asthenozoospermic patients meaning that the actual functional protein OXCT1 is likely to be lesser in these samples. One plausible effect of compromised OXCT1 activity is ketoacidosis, a problem associated with OXCT1 deficiency (Tildon and Cornblath, 1972). It is, however, not clear at present what implications ketoacidosis would have on sperm motility. Another effect of

Downloaded from https://academic.oup.com/molehr/article/16/7/452/1226950 by U.S. Department of Justice user on 17 August 2022

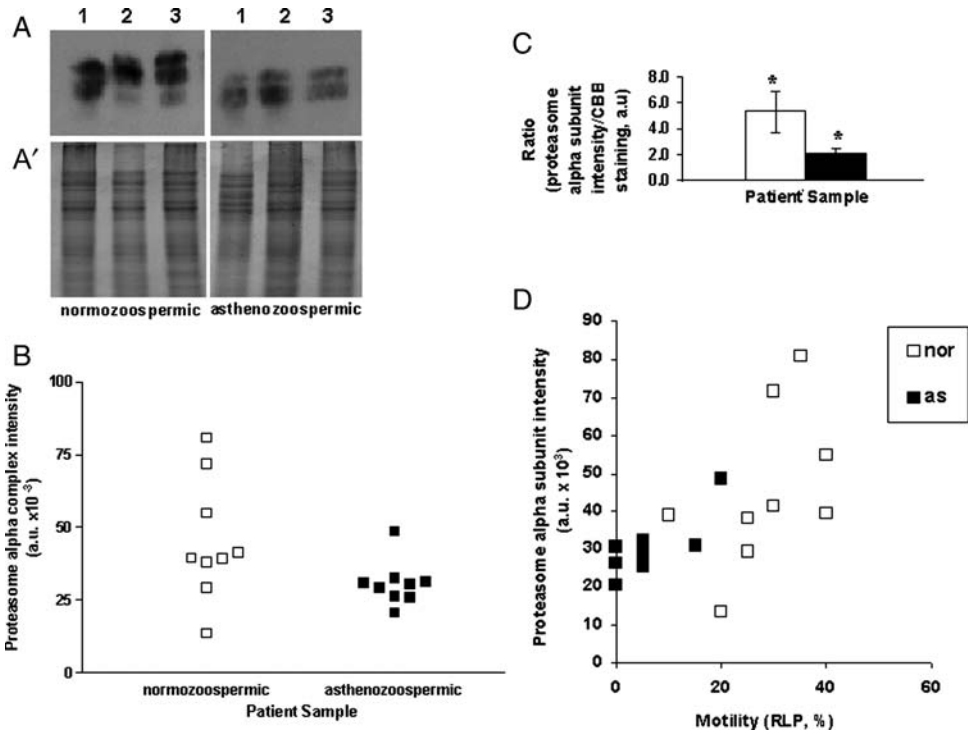

Figure 4 Immunoblot analysis of proteasome alpha complex in spermatozoa of normospermic and asthenozoospermic individuals. Spermatozoa from asthenozoospermic individuals showed lower intensity of proteasome alpha complex compared with normospermic individuals as demonstrated by immunoblot analysis (**A**) and also based on band intensities (**B**). (**C**) Differences are also represented as a ratio of intensities of proteasome alpha complex/CBB staining (shown in **A**′). (**D**) The correlation between intensities of the proteasome alpha complex and rapid linear motility (Grade a motility) is shown. All the data were obtained using samples from nine normospermic and nine asthenozoospermic individuals except in A and C where the data for only three individuals is shown. Values with asterisk differ significantly at $P < 0.05$. Normozoospermic samples are represented by open squares/bar and asthenozoospermic samples are shown in solid squares/bars.

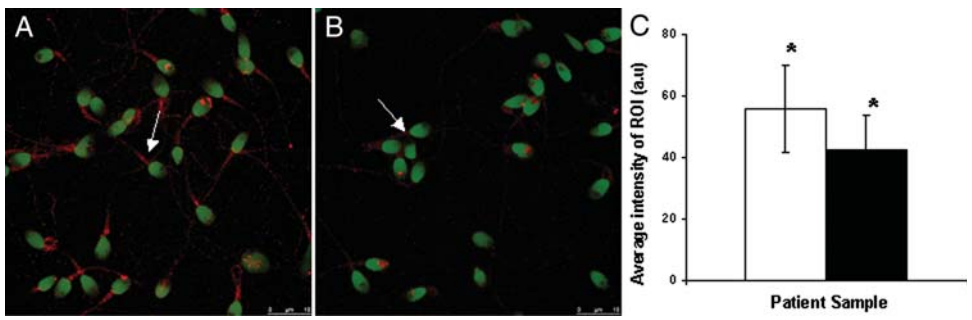

Figure 5 Immunofluorescent localization of proteasome alpha complex in human spermatozoa. In both normozoospermic (**A**) and asthenozoospermic (**B**) sperm samples proteasome alpha complex is localized only in the connecting piece. But, in asthenozoospermic sperm samples (B) the intensity of proteasome alpha complex compared with normozoospermic sperm patients (A) was significantly reduced. The ROI intensities calculated off-line showed significant differences in the intensities (a.u) of proteasome alpha complex in the two categories (**C**). Values with asterisk differ at $P < 0.05$. Nuclear staining has been represented by the green pseudocolor. Bars = 10 μm. Number of samples used in each category were three.

OXCT1 deficiency in sperm could be a consequential compromised glycolysis, as has been seen in fibroblast cells (Tildon *et al.*, 1971).

Importance of structural proteins such as AKAP (Moretti *et al.*, 2007) and tektins (Tanaka *et al.*, 2004a, b; Cao *et al.*, 2006; Roy *et al.*, 2009) in sperm motility has been demonstrated by several studies and thus getting a lower level of TEKT1 in asthenozoospermic subject is not surprising. TUBB2C is a major cytoskeletal protein present in the head and the flagellum of mammalian spermatozoa and already well implicated in sperm motility (Cosson *et al.*, 1996; Fujinoki *et al.*, 2004). 'Sperm movement and structural organization'

Downloaded from https://academic.oup.com/molehr/article/16/7/452/1226950 by U.S. Department of Justice user on 17 August 2022

category is also supported by earlier studies which have indicated that spermatozoa in asthenozoospermic individuals are defective in motility due to structural defects in the sperm-tail (Ryder *et al.*, 1990) like dysplasia of the fibrous sheath, defective centrosome and absence of the dynein arms (Afzelius *et al.*, 1975; Nagy, 2000).

Within the 'stress response, protein folding and protein turnover', category, we have detected PSMA3 and HSPA2 in lower amounts in different asthenozoospermic patients. HSPA2 protein is a heat shock protein and has been shown to be a marker for sperm maturity (Ergur *et al.*, 2002) with lower expression in oligoteratozoospermic men (Cedenho *et al.*, 2006).

The proteasome is a complex multienzymatic threonine protease that is composed of a proteolytic core complex termed the 20S proteasome, which is associated with the PA700 proteasome activator (19S cap), composed of several ATPases and regulatory proteins, to constitute the 26S proteasome (2000 kDa). The 26S proteasome regulates the traffic of proteins that need to be degraded or processed and its activity depends on ATP. Proteasomes have been detected in sperm from numerous species and its involvement in several sperm functions, including motility has been addressed (Inaba and Morisawa, 1992; Inaba *et al.*, 1993; Inaba *et al.*, 1998; Pizarro *et al.*, 2004; Sutovsky *et al.*, 2004). In sea urchins, proteasomes have also been implicated in acrosome reaction and fertilization (Yokota and Sawada, 2007). A recent study on human spermatozoa, discusses the involvement of proteasome in human capacitation (Kong *et al.*, 2009). The main function of the proteasome is to degrade cytosolic and nuclear proteins previously labeled with ubiquitin molecules (Sutovsky *et al.*, 2003). Low levels of proteasome might result in higher ubiquitinated proteins in the cells. Such accumulation of ubiquitinated proteins has been correlated well with sperm parameters including motility (Sutovsky *et al.*, 2004).

Sperm proteome studies on asthenozoospermia samples have revealed that some component of the proteasome complex is differentially expressed, suggesting an importance of the proteasome complex in sperm motility. However, this study and the two earlier studies differ in that the component of the proteosome complex that is increased or decreased is not identical. For instance Zhao *et al.* (2007) showed that the 26S protease regulatory subunit 7 is decreased in patient samples (a trend seen for the proteasome alpha complex in this study) where as in contrast Martínez-Heredia *et al.* (2008) report that proteasome subunit-PSMB3 is increased in patient samples. The different trend seen for proteasome subunit-PSMB3 is, however not clear and may be due to ethnic differences, as discussed earlier. There are incidents of asthenozoospermia being related to ultrastructural defects in the centriole region of the sperm, visible solely by electron microscopy (Afzelius *et al.*, 1975; Nagy, 2000) and therefore, such defects go undetected in normal semenology procedures. It is likely that the asthenozoospermic patients in the study may have connecting piece defects leading to loss of centriolar proteins like proteasome and this is being reflected in the proteome analysis of these samples.

Using MALDI MS/MS 120 protein spots corresponding to 75 different proteins have been identified consistently in the human sperm proteome. Comparison of the sperm proteome of normospermic and asthenozoospermic individuals has led to the identification of eight proteins which show changes in intensity and interestingly many of these proteins can be implicated in sperm motility. One of these proteins, the proteosome alpha complex level was also validated by immunoblot analysis, and its localization was also studied by immunofluorescence. This study reinforces the validity of the proteomic approach in carrying out comparative analysis between normal and pathological patients.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

Acknowledgements

We greatly acknowledge the help of Ms Anita and Mr Simhachalam in semen collection. Scientific inputs from Ms Priyanka Rai, Mr Kota Venkatesh and Dr Swapna are also greatly appreciated.

Funding

The work was funded by Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India and Council of Scientific and industrial Research (CSIR), Government of India.

References

Afzelius BA, Eliasson R, Johnsen O, Lindholnier C. Lack of dynein arms in immotile human spermatozoa. *J Cell Biol* 1975;**66**:225–232.

Baker MA, Hetherington L, Aitken RJ. Identification of SRC as a key PKA-stimulated tyrosine kinase involved in the capacitation-associated hyperactivation of murine spermatozoa. *J Cell Sci* 2006;**119**:3182–3192.

Baker MA, Reeves G, Hetherington L, Muller J, Baur I, Aitken RJ. Identification of gene products present in Triton X-100 soluble and insoluble fractions of human spermatozoa lysates using LC-MS/MS analysis. *Proteomics Clin Appl* 2007;**1**:524–532.

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

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

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

Brewis IA, Gadella BM. Sperm surface proteomics: from protein lists to biological function. *Mol Hum Reprod* 2010;**16**:68–79.

Cao W, Gerton GL, Moss SB. Proteomic profiling of accessory structures from the mouse sperm flagellum. *Mol Cell Proteomics* 2006;**5**:801–810.

Cardullo RA, Baltz JM. Metabolic-regulation in mammalian sperm – mitochondrial volume determines sperm length and flagellar beat frequency. *Cell Motil Cytoskel* 1991;**19**:180–188.

Cedenho AP, Lima SB, Cenedeze MA, Spaine DM, Ortiz V, Oehninger S. Oligozoospermia and heat-shock protein expression in ejaculated spermatozoa. *Hum Reprod* 2006;**21**:1791–1794.

Chan C-C, Shui H-A, Wu C-H, Wang C-Y, Sun G-H, Chen H-M, Wu G-J. Motility and protein phosphorylation in healthy and asthenozoospermic sperm. *J Proteome Res* 2009;**8**:5382–5386.

Comhaire FH, Mahmoud AM, Depuydt CE, Zalata AA, Christophe AB. Mechanisms and effects of male genital tract infection on sperm quality and fertilizing potential: the andrologist's viewpoint. *Hum Reprod Update* 1999;**5**:393–398.

Downloaded from https://academic.oup.com/molehr/article/16/7/452/1226950 by U.S. Department of Justice user on 17 August 2022

Cosson J, White D, Huitorel P, Eddé B, Cibert C, Audebert S, Gagnon C. Inhibition of flagellar beat frequency by a new anti-beta-tubulin antibody. *Cell Motil Cytoskeleton* 1996;**35**:100–112.

deMateo S, Martínez-Heredia J, Estanyol JM, Domiguez-Fandos D, Vidal-Taboada JM, Ballesca JL, Oliva R. Marked correlations in protein expression identified by proteomic analysis of human spermatozoa. *Proteomics* 2007;**7**:4264–4277.

Ergur AR, Dokras A, Giraldo JL, Habana A, Kovanci E, Huszar G. Sperm maturity and treatment choice of in vitro fertilization (IVF) or intracytoplasmic sperm injection: diminished sperm HspA2 chaperone levels predict IVF failure. *Fertil Steril* 2002;**77**:910–918.

Fujinoki M, Kawamura T, Toda T, Ishimoda-Takagi T, Hideki O, Shimizu N, Okuno M. Identification of 66 kDa phosphoprotein associated with motility initiation of hamster spermatozoa. *Reprod Med Biol* 2004; **3**:133–139.

Inaba K, Morisawa M. Chymotrypsin-like protease activity associated with demembranated sperm of chum salmon. *Biol Cell* 1992;**76**:329–333.

Inaba K, Akazome Y, Morisawa M. Purification of proteasomes from salmonid fish sperm and their localization along sperm flagella. *J Cell Sci* 1993;**104**:907–915.

Inaba K, Morisawa S, Morisawa M. Proteasomes regulate the motility of salmonid fish sperm through modulation of cAMP-dependent phosphorylation of an outer arm dynein light chain. *J Cell Sci* 1998; **111**:1105–1115.

Jassim A, Gillot DJ, Al-Zuhdi Y, Gray A, Foxon R, Bottazzo GF. Isolation and biochemical characterization of the human sperm tail fibrous sheath. *Hum Reprod* 1992;**7**:86–94.

Johnston DS, Wooters J, Kopf GS, Qiu Y, Roberts KP. Analysis of the human sperm proteome. *Ann N Y Acad Sci* 2005;**1061**:190–202.

Kameshwari DB, Siva AB, Shivaji S. Inhibition of in vitro capacitation of hamster spermatozoa by nitric oxide synthase inhibitors. *Cell Mol Biol (Noisy-le-grand)* 2003;**49**:421–428.

Kong M, Diaz ERS, Morales P. Participation of the human sperm proteasome in the capacitation process and its regulation by protein kinase A and tyrosine kinase. *Biol Reprod* 2009;**80**:1026–1035.

Kota V, Dhople VM, Shivaji S. Tyrosine phosphoproteome of hamster spermatozoa: role of glycerol-3-phosphate dehydrogenase 2 in sperm capacitation. *Proteomics* 2009;**9**:1809–1826.

Leclerc P, deLamirande E, Gagnon C. Cyclic adenosine 3′,5′ monophosphate-dependant regulation of protein tyrosine phosphorylation in relation to human sperm capacitation and motility. *Biol Reprod* 1996;**55**:684–692.

Lefièvre L, Chen Y, Conner SJ, Scott JL, Publicover SJ, Ford WCL, Barratt CLR. Human spermatozoa contain multiple targets for protein S-nitrosylation: An alternative mechanism of the modulation of sperm function by nitric oxide? *Proteomics* 2007;**7**:3066–3084.

Lewis SEM, Donnelly ET, Sterling ESL, Kennedy MS, Thompson W, Chakravarthy U. Nitric oxide synthase and nitrite production in human spermatozoa: evidence that endogenous nitric oxide is beneficial to sperm motility. *Mol Hum Reprod* 1996;**2**:873–878.

Li LW, Fan LQ, Zhu WB, Nien HC, Sun BL, Luo KL, Liao TT, Tang L, Lu GX. Establishment of a high-resolution 2-D reference map of human spermatozoal proteins from 12 fertile sperm-bank donors. *Asian J Androl* 2007;**9**:321–329.

Martínez-Heredia J, Estanyol JM, Ballescà JL, Oliva R. Proteomic identification of human sperm proteins. *Proteomics* 2006;**6**:4356–4369.

Martínez-Heredia J, deMateo S, Vidal-Taboada JM, Ballesca JL, Oliva R. Identification of proteomic differences in asthenozoospermic sperm samples. *Hum Reprod* 2008;**23**:783–791.

Mitra K, Shivaji S. Novel tyrosine-phosphorylated post-pyruvate metabolic Enzyme, Dihydrolipoamide dehydrogenase, involved in capacitation of hamster spermatozoa. *Biol Reprod* 2004;**70**:887–899.

Moore FL, Reijo-Pera RA. Male sperm motility dictated by mother's mtDNA. *Am J Hum Genet* 2000;**67**:543–548.

Moretti E, Scapigliati G, Pascarelli NA, Baccetti B, Collodel G. Localization of AKAP4 and tubulin proteins in sperm with reduced motility. *Asian J Androl* 2007;**9**:641–649.

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

Nagy ZP. Sperm centriole disfunction and sperm immotility. *Mol Cell Endocrinol* 2000;**166**:59–62.

Pan Q, Luo X, Chegini N. Genomic and proteomic profiling I: Leiomyomas in African Americans and Caucasians. *Reprod Biol Endocrinol* 2007;**5**:34.

Pixton KL, Deeks ED, Flesch FM, Moseley FLC, Björndahl L, Ashton PR, Barratt CLR, Brewis IA. 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. *Hum Reprod* 2004;**19**:1438–1447.

Pizarro E, Pasté C, Kong M, Morales P. Proteasomal activity in mammalian spermatozoa. *Mol Reprod Dev* 2004;**69**:87–93.

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

Rabilloud T. Membrane proteins and proteomics: love is possible, but so difficult. *Electrophoresis* 2009;**30**:S174–180.

Rikova K, Guo A, Zeng Q, Possemato A, Yo J, Haack H, Nardone J, Lee K, Reeves C, Li Y *et al.* Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. *Cell* 2007;**131**:1190–1203.

Roy A, Lin YN, Agno JE, DeMayo FJ, Matzuk MM. Absence of tektin 4 causes asthenozoospermia and subfertility in male mice. *FASEB* 2007; **21**:1013–1025.

Roy A, Lin YN, Agno JE, Demayo FJ, Matzuk MM. Tektin 3 is required for progressive sperm motility in mice. *Mol Reprod Dev* 2009;**76**:453–459.

Rush J, Moritz A, Lee KA, Guo A, Goss VL, Spek EJ, Zhang H, Zha XM, Polakiewicz RD, Comb MJ. Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. *Nat Biotechnol* 2005;**23**:94–101.

Ryder TA, Mobberley MA, Hughes I, Hendry WF. A survey of the ultrastructural defects associated with absent or impaired human sperm motility. *Fertil Steril* 1990;**53**:556–560.

Secciani F, Bianchi L, Ermini L, Cianti R, Sala GBL, Focarelli R, Bini L, Rosati F. Protein profile of capacitated versus ejaculated human sperm. *J Proteome Res* 2009;**8**:3377–3389.

Si Y, Okuno M. Role of tyrosine phosphorylation of flagellar proteins in hamster sperm hyperactivation. *Biol Reprod* 1999;**61**:240–246.

Solomon F, Jencks WP. Identification of an enzyme-gamma-glutamyl coenzyme A intermediate from coenzyme A transferase. *J Biol Chem* 1969;**244**:1079–1081.

Stigliano A, Cerquetti L, Borro M, Gentile G, Bucci B, Misiti S, Piergrossi P, Brunetti E, Simmaco M, Toscano V. Modulation of proteomic profile in H295R adrenocortical cell line induced by mitotane. *Endocr Relat Cancer* 2008;**15**:1–10.

Sutovsky P, Turner RM, Hameed S, Sutovsky M. Differential ubiquitination of stallion sperm proteins: possible implications for infertility and reproductive seasonality. *Biol Reprod* 2003;**68**:688–698.

Sutovsky P, Hauser R, Sutovsky M. Increased levels of sperm ubiquitin correlate with semen quality in men from an andrology laboratory clinic population. *Hum Reprod* 2004;**19**:628–638.

Tanaka H, Iguchi N, Toyama Y, Kitamura K, Takahashi T, Kaseda K, Maekawa M, Nishimune Y. Mice deficient in the axonemal protein Tektin-t exhibit male infertility and immotile-cilium syndrome due to impaired inner arm dynein function. *Mol Cell Biol* 2004a;**24**:7958–7964.

Tanaka H, Takahashi T, Iguchi N, Kitamura K, Miyagawa Y, Tsujimura A, Matsumiya K, Okuyama A, Nishimune Y. Ketone bodies could

Downloaded from https://academic.oup.com/molehr/article/16/7/452/1226950 by U.S. Department of Justice user on 17 August 2022

support the motility but not the acrosome reaction of mouse sperm. *Int J Androl* 2004b;**27**:172–177.

Tildon JT, Cornblath MJ. Succinyl-CoA: 3-ketoacid CoA-transferase deficiency. A cause for ketoacidosis in infancy. *Clin Invest* 1972;**51**:493–498.

Tildon JT, Cornblath M, Stevenson J. Abnormal carbohydrate metabolism in genetic ketoacidosis. *Fed Proc* 1971;**30**:78.

Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P, Kopf GS. Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development* 1995a;**121**:1129–1137.

Visconti PE, Moore GD, Bailey JL. Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development* 1995b;**121**:1139–1150.

WHO laboratory manual. *The Examination of Human Semen and Sperm–Cervical Mucus Interaction*, 4th edn. Cambridge, UK: Cambridge University Press, 1999.

Wolkowicz MJ, Naaby-Hansen S, Gamble AR, Reddi PP, Flickinger CJ, John CH. Tektin B1 demonstrates flagellar localization in human sperm. *Biol Reprod* 2002;**66**:241–250.

Wong C, Sridhara S, Bardwell JCA, Jakob U. Heating greatly speeds Coomassie blue staining and destaining. *BioTechniques* 2000;**28**:426–432.

Yamaji R, Fujita K, Nakanishi I, Nagao K, Naito M, Tsuruo T, Inui H, Nakano Y. Hypoxic up-regulation of triosephosphate isomerase expression in mouse brain capillary endothelial cells. *Arch Biochem Biophys* 2004;**423**:332–342.

Yokota N, Sawada H. Sperm proteasomes are responsible for the acrosome reaction and sperm penetration of the vitelline envelope during fertilization of the sea urchin Pseudocentrotus depressus. *Dev Biol* 2007;**308**:222–231.

Yunes R, Doncel GF, Acosta AA. Incidence of sperm-tail tyrosine phosphorylation and hyperactivated motility in normozoospermic and asthenozoospermic human sperm samples. *Biocell* 2003;**27**:29–36.

Zhao C, Huo R, Wang FQ, Lin M, Zhou ZM, Sha J-H. Identification of several proteins involved in regulation of sperm motility by proteomic analysis. *Fertil Steril* 2007;**87**:436–438.

Zheng L, Goddard JP, Baumann U, Reymond JL. Expression improvement and mechanistic study of the retro-diels-alderase catalytic antibody 10F11 by site-directed mutagenesis. *J Mol Biol* 2004;**341**:807–814.

Downloaded from https://academic.oup.com/molehr/article/16/7/452/1226950 by U.S. Department of Justice user on 17 August 2022