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

Archana Bharadwaj Siva, Duvvuri Butchi Kameshwari, Vaibhav Singh, Kadupu Pavan, Curam Sreenivasacharlu Sundaram, Nandini Rangaraj, Mamata Deenadayal, and Sisinthy Shivaji

1Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India 2Infertility 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 (Comhair 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; Martinez-Heredia et al., 2006; Leflèvre 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;
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 than 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 S1.

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% CO2 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 diluted by 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 layer with semen (1 ml). The sample was then centrifuged (800g 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 Sperm 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 000g 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/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™ 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/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™ 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 pl values were applied. Total ion score cut-off of 28 (for Swiss-Prot 2009) and %ID 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.
**Immunoblotting**

Sperm proteins (30 µg) that were separated by SDS–PAGE were electrotransferred 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-benzidine as a substrate (Calbiochem, Germany; Zheng et al., 2004). Blots were scanned with Fluor-S™ Multi-Imager (Bio-Rad Laboratories, Hercules, CA, USA) and the bands of interest were quantitated using GeneTools version 3.06.04 from SynGene (Cambridge, England). CBB stained gel was utilized for normalisation. CBB 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 reincubated 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.

**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 proteosome 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 pl ~ 5.7 were lower and three proteins, namely a pl isofrom (pl 6.1) of triose-phosphate isomerase (TPIS), testis specific glyceraldehyde 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,
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.
<table>
<thead>
<tr>
<th>Protein ID (gel annotation)*</th>
<th>Access-ion No. b)</th>
<th>Experimental Mol wt (kDa)/pI</th>
<th>Av. normalized intensity ± S.D</th>
<th>Fold difference*</th>
<th>MS identification MOWSE score/sequence coverage (%)</th>
<th>MS identification Matched peptides/total peptides e-Value e)</th>
<th>Matched peptide; sequence/ion score e)</th>
<th>MS/MS identification Matched peptide; sequence/ion score e)</th>
<th>Sequence coverage (%) ± CI f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteasome subunit alpha type 3 (PSMA3)</td>
<td>P25788</td>
<td>29/5.65</td>
<td>16.5 ± 7.9</td>
<td>6.2 ± 4.5</td>
<td>−2.6</td>
<td>67/34</td>
<td>8/42</td>
<td>0.004</td>
<td>2/28</td>
</tr>
<tr>
<td>Heat shock 70 kDa related protein (HSPA2)</td>
<td>PS4652</td>
<td>24.5/5.85</td>
<td>25.7 ± 17.5</td>
<td>4.0 ± 4.9</td>
<td>−6.4</td>
<td>58/11</td>
<td>5/8</td>
<td>0.011</td>
<td>3/64</td>
</tr>
<tr>
<td>Tubulin beta-2C chain (TUBB2C)</td>
<td>P68371</td>
<td>24/6.55</td>
<td>30.0 ± 12.6</td>
<td>10.8 ± 4.8</td>
<td>−2.8</td>
<td>60/22</td>
<td>13/65</td>
<td>0.021</td>
<td>7/480</td>
</tr>
<tr>
<td>Tektin 1 (TEKT1)</td>
<td>Q969V4</td>
<td>49/6.7</td>
<td>14.3 ± 1.7</td>
<td>8.7 ± 2.6</td>
<td>−1.7</td>
<td>76/17</td>
<td>9/22</td>
<td>0.0005</td>
<td>–</td>
</tr>
<tr>
<td>Triose phosphate isomerase (TPIS)</td>
<td>P60174</td>
<td>31/6.1</td>
<td>26.0 ± 15</td>
<td>13.4 ± 11.2</td>
<td>+3.5</td>
<td>59/25</td>
<td>7/41</td>
<td>0.027</td>
<td>4/152</td>
</tr>
<tr>
<td>Glycerol kinase, testis specific 2 (GKP2)</td>
<td>Q1-4410</td>
<td>61/6.15</td>
<td>5.7 ± 5.0</td>
<td>19.9 ± 21.3</td>
<td>+5.3</td>
<td>92/32</td>
<td>12/53</td>
<td>1.4e-005</td>
<td>4/120</td>
</tr>
<tr>
<td>Succinyl-CoA:3-Ketoacid co-enzyme A transferase 1 (OXCT1)</td>
<td>PSS509</td>
<td>60/7.0</td>
<td>2.3 ± 1.7</td>
<td>12.1 ± 10</td>
<td>+1.8</td>
<td>58/26</td>
<td>6/20</td>
<td>0.032</td>
<td>2/151</td>
</tr>
<tr>
<td>Unidentified (UI)</td>
<td>–</td>
<td>33/5.7</td>
<td>7.8 ± 3.9</td>
<td>14.1 ± 3.4</td>
<td>−1.9</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*aAnnotation as in Fig. 1.

bAccession numbers are from Swiss-Prot 2009 database.

cDown-regulated protein spots in asthenozoospermic subjects are denoted by ‘−’ and up-regulated ones are denoted by ‘+’.

dOnly the sequence of two peptides with the highest ion score as identified by MALDI MS/MS are given.

e-Value e) and % CI f refer to the significance level of the protein scores obtained in MALDI MS AND MS/MS, respectively.
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-CoA3-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,
Figure 3  Protein spots showing changes in intensity in the spermatozoa of normozoospermic 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 (Lefèvre 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 glyceralddehyde 3-phosphate during glycolysis. We identified four pl isoforms of TPIS (pl 5.75, 5.95, 6.1 and 6.25) in the human spermatozoa, of which the isoform with pl 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 pl 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 pl 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 acetoacetoyl 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
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’

**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.
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 patient samples. The different trend seen for proteasome activity depends on ATP. Proteasomes have been detected in sperm from numerous species and its involvement in several sperm functions, including mobility has been addressed (Inaba and Morisawa, 1992; Inaba et al., 1993; Inaba et al., 1998; Pizarro et al., 2004; 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 proteasome complex that is increased or decreased is not identical. For instance Zhao et al. (2007) showed that the 26S protease regulatory subunit 7 is increased in patient samples (a trend seen for the proteasome alpha complex in this study) whereas in contrast Martinez-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 proteasome 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


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


