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Proteomic analyses reveal lower expression of TEX40 and ATP6V0A2 proteins related to calcium ion entry and acrosomal acidification in asthenozoospermic males

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ABSTRACT

Aims: Idiopathic nature of male infertility disorder needs to be investigated by different horizons of molecular biology for its treatment and to device male contraceptive. Further, it can also aid in advancement of assisted reproductive technology (ART), as nowadays the failure and disquiets of ART are consistent. Herein, we have attempted to find out proteins responsible for male infertility by comparing proteome profile of sperms collected from normal control and asthenozoospermic (AS) males.

Main methods: Differential proteome profiles were studied by 2-dimensional differential gel electrophoresis (2D-DIGE) and mass spectrometry. The confirmation of proteome profiling results was done by western blotting and ELISA. Quantitative reverse-transcription-PCR was also performed in an independent cohort of AS and normal individuals to investigate the transcriptional regulation of proteins.

Key findings: Although seven differentially regulated proteins were identified, highpoints of the study were two proteins, TEX40 and ATP6V0A2. Lower expression of a crucial sperm motility related protein, TEX40 is reported for the first time in clinically diagnosed AS males in the present investigation. Most likely with reference to previous findings the down regulation of TEX40 leads to fewer entries of calcium ions in the sperm and lower expression of ATP6V0A2 is responsible for acrosomal de-acidification.

Significance: Conclusively, the down regulation of these two proteins in AS males might result in diminished sperm motility. The findings can be worthwhile for male contraception and ART management besides their use for male infertility therapy.

1. Introduction

Male infertility is a state characterized by etiological ambiguity as the information for its molecular basis is pretty limited. Accumulating epidemiological evidences conclude that male infertility is associated with nearly 40% of infertility cases [1]. Sperm quality especially in terms of sperm motility forms the keystone to male fertility, and is the major parameter of semen analysis for fertility determination [2]. Both progressive motility of the sperm and their number remain to be obligatory preconditions for the successful fertility outcome being it natural or assisted [3]. Inadequate understanding of the precise molecular events leading to reduced sperm motility has categorized AS as an idiopathic syndrome.

AS is the most predominant cause of male infertility, with a prevalence of about 81% [4,5]. A study conducted in South Indian population accounted a decrease in sperm motility by 22.92% [6]. In another retrospective analysis from Central India, asthenoteratozoospermia (low sperm motility and abnormal shape) is found to be prevalent in 19.35% of cases [7]. Recently, declining trends of semen quality and sperm functional parameters have been reviewed [8]. A modest approach to deduce the underlying causes of AS is the identification of the proteins involved in the sperm motility, as sperm is both a transcriptionally and translationally silent entity [9]. Several studies from the literature quote sperm proteins (chaperons, cytoskeletal, energy metabolism and structural) in AS condition [10–12]. Pertaining to the low significance level in the reports due to lack of reproducibility of

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results, no concrete consent has been formed. Consequently, there is a need to decode the role of proteins in sperm motility.

Proteomics holds the calibre of scrutinising thousands of proteins at the same time, thus proving to be a proficient tool to unravel the regulatory mechanisms controlling sperm motility. Furthermore, the non-invasive easy accessibility of the spermatozoa to purify in higher concentrations in both native and functional states has made it the material of choice for proteomic analysis [9,13]. Albeit, the numerous reports, a concrete model of the myriad mechanisms is beyond the scope.

Amongst the literature cited in support to the involvement of multitude of proteins for sperm motility, is a group categorized as energy-metabolism and signalling-transport related proteins. Herein, we did comparative proteomic analysis between the AS males and normal individuals using 2 dimensional differential gel electrophoresis (2D-DIGE) coupled with matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) identification. Notable findings of the study illustrated two distinct differentially expressed proteins, viz., TEX40 and ATP6V0A2. TEX40 is promisingly related to signalling and transport, and is a newly identified zeta (ζ) subunit of Ca^{2+} channels (CatSper) [14]. Later is an ATP dependent proton pump regulating the acidification of intracellular compartment and is thus required for the normal spermatogenesis and maturation of sperm [15]. Furthermore, the aforementioned proteins were validated at protein level by immunoassays (western blot and ELISA). To examine if the alterations at the protein level are duplicated at the gene/mRNA level quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was also performed.

2. Materials and methods

2.1. Subjects and sample collection

The study was approved from the Institute Ethics Committee (IEC/NP-465/2013 RP-26/2013). Human semen samples from normal (n = 70) and AS individuals (n = 70) were collected from the Department of Laboratory Medicine, All India Institute of Medical Sciences (AIIMS), New Delhi after the written informed consent from the donors (Table 1). After semen collection the samples were allowed to liquefy at room temperature and within 1 h of collection routine analysis was performed to assess sperm parameters (number, motility, volume), in accordance with the WHO, 2010 guidelines (≤32% progressive sperm motility for AS and ≥40% progressive motility for normal; > 20 million/ml sperm count for both) [16]. Exclusion criteria included HIV positive samples and those diagnosed with other sexually transmitted diseases. Also, samples contaminated with blood were omitted from the study.

2.2. Sample preparation for proteomic analysis

Age matched samples were pooled from normal as well as AS group to obtain desired protein amount for experiments. The pooled samples were centrifuged at 1300*g* for 15 min at 4 °C to separate the sperm fraction from the seminal plasma. Pellet obtained was further processed for 2D-DIGE experiments as described. Briefly, the sperm pellet obtained was washed thrice with ice cold phosphate buffer saline (PBS) pH 7.0 followed by centrifugation at 1300*g* for 5 min at 4 °C. Finally, the total sperm pellet was solubilised in lysis buffer containing 2 M thiourea and 7 M urea, 4% w/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and protease inhibitor cocktail. Samples were incubated in the lysis buffer for 1 h, followed by centrifugation at 5000*g* for 10 min at 4 °C to separate cell debris. The solubilised samples were aliquoted and kept at −20 °C till further use. Protein quantity was assessed by Bradford method using BSA as standard and equal amounts were used for 2D-DIGE experiments.

Table 1
Clinical details of the AS patients and normal controls.

	Mean ± SD
Asthenozoospermia (AS) (n = 70)	
Age (yrs)	30.01 ± 4.43
Sperm count (×10^6 sperm/ml)	87.59 ± 22.17
Semen volume (ml)	4.02 ± 0.93
Progressive motility (%)	10.63 ± 5.56
Normal controls (n = 70)	
Age (yrs)	29.97 ± 3.84
Sperm count (×10^6 sperm/ml)	94.08 ± 31.64
Semen volume (ml)	3.78 ± 1.01
Progressive motility (%)	67.25 ± 5.55

2.3. Cy dye labelling of proteins

Equal amounts of sperm protein (AS and normal) were labelled with the respective Cy dyes for analytical gels. Labelling was done with Cy3, Cy5 and Cy2 fluors (GE Healthcare) using minimal dye labelling method according to manufacturer's protocol. Briefly, 50 μg of protein each from AS and normal control was used for labelling with Cy3 and Cy5 respectively. In case of minimal dyes a system bias at low spot volumes is used to be observed owing to different fluorescence characteristics of acrylamide at different wavelengths of excitation for Cy2, Cy3 and Cy5 dyes. In order to minimize this, system bias dye swap approach was employed by labelling normal controls with Cy3 and AS with Cy5 in another experimental setup. An internal standard (50 μg) consisting of equal amounts of samples from both groups was labelled with Cy2 dye. Internal standard was used to normalize the protein patterns across different gels thereby negating inter-gel variation. Labelling was done at 4 °C in dark for 45 min and the reaction was stopped by the addition of 1 μl of 10 mM lysine for 10 min at 4 °C in dark.

2.4. Rehydration of the IPG strips and isoelectric focusing (IEF)

Immobiline Dry Strips pH 3–10 were rehydrated overnight in dark with the Cy dye labelled protein samples. Analytical gels were run with maximum protein concentration of 50 μg. The labelled samples of AS, normal and internal standard were pooled altogether and the total volume was adjusted to 250 μl with the rehydration buffer (8 M urea, 0.5% CHAPS, 0.2% DTT, 0.2% Pharmalyte pH 3–10). The rehydrated pH strips were then isoelectrically focused in IPGphorTM IEF System (GE Healthcare Bio-Sciences) to a total of 50,000 V-h, using the standardized conditions as follows: 100 V for 1 h, 200 V for 2 h, 500 V for 1 h, 1000 V for 2 h, 2000 V for 2 h, 5000 V for 3 h, 6600 V for 5 h.

2.5. Two dimensional gel electrophoresis

Followed by focusing, the pH strips were equilibrated into two incubation steps; 15 min in reducing equilibration buffer (1% w/v DTT) followed by alkylating buffer (2.5% w/v iodoacetamide) for another 15 min. The equilibrated strips were then subjected to 12.5% w/v acrylamide gel for second dimensional protein separation. Following 2D electrophoresis, the DIGE gels were scanned using Typhoon Variable mode Imager. Images scanned were then analyzed using image analysis software DeCyder (GE Healthcare).

2.6. Image acquisition and analysis

Following 2D electrophoresis, the DIGE gels were scanned using Typhoon Variable mode Imager, Typhoon 9400 scanner (GE Healthcare). The labelled protein spots were visualized at the respective wavelength of the Cy dyes. The scanned images were analyzed for the differential protein spots amongst the experimental groups using Progenesis Same Spot Software (Non Linear Dynamics, USA). After

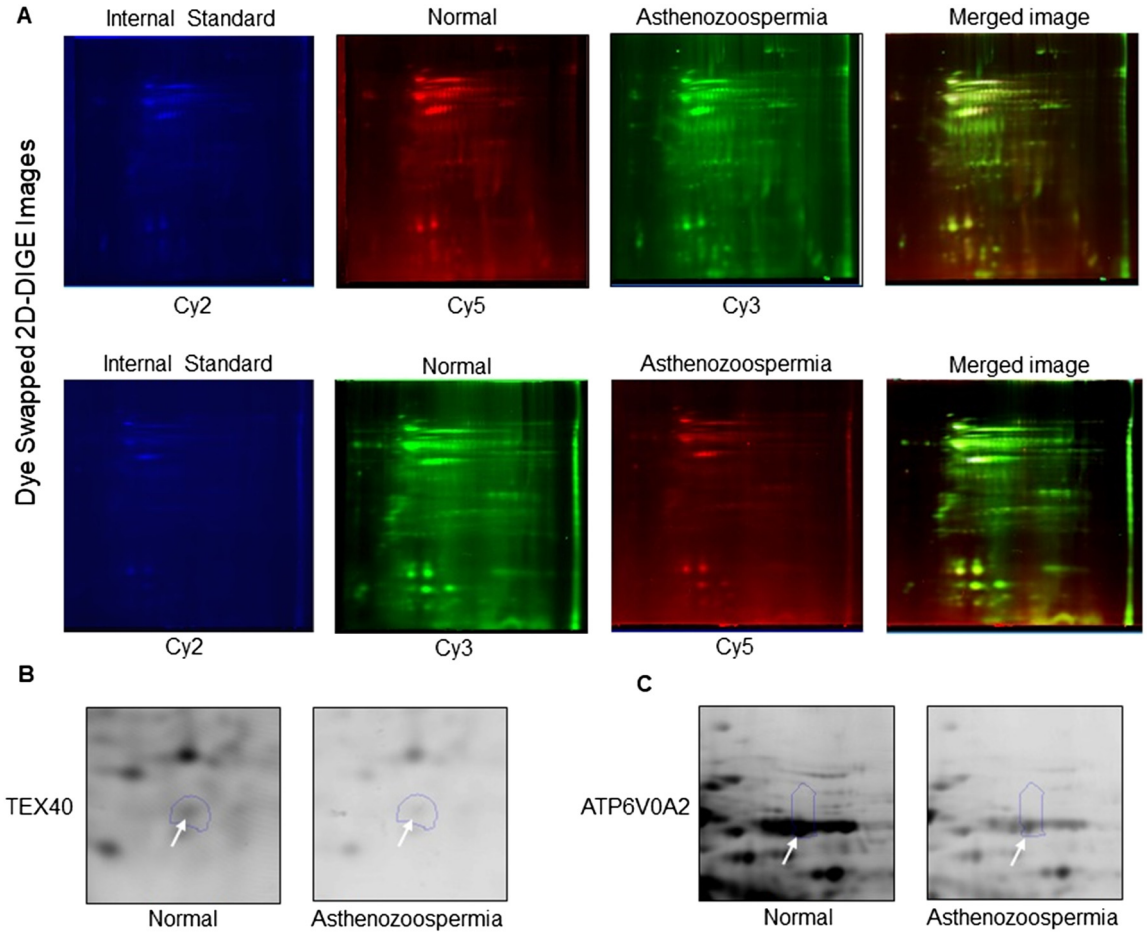

Fig. 1. Differential protein expression. Five age matched samples were pooled from the respective group for each set of experiments. (A) Replicate 2D-DIGE images after dye swapping. Sperm proteins from two groups (normal and AS) labelled with Cy5 (red, normal) and Cy3 (green, AS) in the upper panel gels. In the lower panel after dye swapping two groups labelled with Cy3 (green, normal) and Cy5 (red, AS) respectively. An internal standard (mixture of equal amounts of normal and AS) labelled with Cy2 (blue). Sections of gel showing spots identified as TEX40 (B) and ATP6V0A2 (C) in normal and AS individuals. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

detection, matching and normalization of the spots, differentially expressed protein spots between the experimental groups were reviewed statistically and those having $p \leq 0.05$, fold change ≥ 2.0 were excised from the gels and further subjected to mass spectrometry identification.

2.7. Preparative 2D gels for spot picking

After the 2D-DIGE analysis, preparative gel (n = 5 pooled samples from respective group) using 500 μg of protein was run for spot picking. IPG strip was rehydrated with equal amounts of protein from both the groups by adjusting the volume to 300 μl with the rehydration buffer followed by IEF and SDS-PAGE, using standard protocol. The gel was finally stained with colloidal coomassie G-250 staining solution [1% (w/v) CBB, 12% (w/v), $(NH_4)_2SO_4$, 2% (v/v) phosphoric acid, 18% (v/v) ethanol] for 2 h. Gel was destained and the differentially expressed proteins were excised manually and further subjected to MALDI-TOF-MS for protein identification.

2.8. In gel digestion and mass spectrometry

After analyzing DIGE results, coomassie staining was performed and protein spots with significant fold change (p-value ≤ 0.05, fold change ≥ 2) were excised manually from a preparative gel. Trypsin digestion was performed as described previously [17]. Briefly, the destained spots were washed with 50 mM ammonium bicarbonate and acetonitrile in 1:1 ratio and further dehydrated with 100% acetonitrile for 1 h. The shrunk spots were reduced with 20 mM β-mercaptoethanol (56 °C, 30 min) followed by alkylation with 55 mM iodoacetamide (20 °C, 20 min, in dark). Digestion was performed by incubating the spots at 37 °C for 16–18 h with 30 μl of trypsin (0.02 μg/μl, modified porcine TPCK treated sequencing grade, SIGMA, USA).

The digested peptides were dried and mixed with 1 ml matrix solution (50% acetonitrile and 0.1% TFA) and spotted on the MALDI plate. MALDI-TOF-MS was done on AB Sciex TOF/TOF™ Series Explorer™ 7000 followed by matching of peptides mass spectra against protein databases using Mascot search engine by Matrix Sciences, London, UK. All searches were accomplished by using NCBInr database with a taxonomy parameter set to *Homo sapiens*. The criteria used were: peptide charge state: 1+, maximum missed cleavages: 2, peptide mass tolerance; 0.5 Da, fixed modification: carbamidomethyl (C), variable modification: oxidation (M).

2.9. RNA isolation and quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was extracted from sperm (n = 10, each group) using RNeasy Mini Kit (QIAGEN, Germany) and converted into cDNA with the help of iScript cDNA synthesis kit (Bio-Rad, USA). Oligonucleotide primers were designed for TEX40 (Forward primer 5′-GACGAGGAGA GCGTGCATAG-3′ and reverse primer 5′-GCGGGTCTTGCTAATGT TGC-3′), and previously reported primers were taken for ATP6V0A2 and GAPDH [18,19]. GAPDH was used as normalization control. Three independent biological replicates were used for qRT-PCR on CFX96

Real Time System (Bio-Rad, USA) and the threshold cycle (Ct) value for each gene was used to calculate fold change in gene expression by $\Delta\Delta C_T$ data analysis method.

2.10. Western blotting (WB) and ELISA

The validation of the mass spectrometry results was performed by WB and ELISA. Isolated protein (30 μg) was loaded and separated on 12% SDS gel followed by electroblotting on nitrocellulose membrane. Membranes were probed overnight at 4 °C with primary antibodies of TEX40 (1:100; polyclonal, ab123745, Abcam, UK) and ATP6V0A2 (1:500, polyclonal, PA5-29271, Thermo Fischer Scientific, USA) and processed further with HRP-conjugated secondary antibody. Blots were developed for protein bands using ECL detection kit (GE Healthcare). Densitometric quantification of the bands was done by Image J software (National Institutes of Health, Bethesda, MD). Concentrations of ATP6V0A2 were assessed using ELISA kits (Cusabio Inc., China) following the manufacturers protocol (n = 20, each group).

2.11. Statistical analysis

The qRT-PCR and WB data were analyzed by Student's *t*-test. GraphPad Prism software (version 7.04) was used to analyse the ELISA data and p-value ≤ 0.05 was considered as statistically significant comparison between the normal and AS groups.

3. Results

3.1. Down-regulation of TEX40 and ATP6V0A2 in AS patients

2D-DIGE was performed to study the human sperm proteome profiles. Five age matched samples were pooled from the respective group for each set of experiments and a total of twenty samples were used for four set of DIGE experiments. One set of DIGE experiment resulted in six images showing uniform distribution of spots across the gels (Fig. 1A). Comparative proteomic analysis using DIGE followed by MALDI-TOF-MS resulted in seven differentially expressed proteins ($p \leq 0.05$, fold change ≥ 2.0; Table 2), SERPINB9, TEX40, ATP6V0A2, K2C1, PSA, AJUBA and POGK. The MASCOT score for each identified protein is provided in the supplementry data. Amongst them, SERPINB9, TEX40, ATP6V0A2 and PSA were found relevant to male fertility. Interestingly, two proteins TEX40 and ATP6V0A2 explicitly related to sperm motility showed lesser protein expression in the AS patients compared to normal individuals (Fig. 1B and C). The electrophoretograms displayed ≥2 fold decreased level in terms of % volume, TEX40 (normal vs. AS: 2.59 vs. 1.19) and ATP6V0A2 (normal vs. AS: 6.08 vs. 2.07).

3.2. No change in mRNA expression of TEX40 and ATP6V0A2

To check whether TEX40 and ATP6V0A2 expression is regulated at transcriptional level, we evaluated the differential mRNA expression of these genes in normal and AS individuals. qRT-PCR was done in an independent cohort (n = 10 for each group). Results showed no significant change at the mRNA level of TEX40 and ATP6V0A2 genes between normal and AS individuals (Fig. 2).

3.3. Confirmation of down-regulation of TEX40 and ATP6V0A2 in AS patients

Confirmation of low abundance of TEX40 and ATP6V0A2 in AS group was done by WB using suitable antibodies (Fig. 3A). Total ten samples were used for each group. Finally, two normal and two AS samples were prepared by pooling five samples from each group to obtain desired amount of protein for experiment. WB results for the two proteins in both the groups are graphically illustrated in Fig. 3B and C. The results were in concert with the MS findings of these two proteins with both TEX40 and ATP6V0A2 down regulated in the AS.

3.4. Evaluation of ATP6V0A2 protein expression by ELISA

The evaluation of ATP6V0A2 protein expression to discriminate the normal individuals from the AS patients was performed by ELISA in normal controls (n = 20) and AS patients (n = 20). Non-availability of ELISA based assay for TEX40 as it is a newly identified protein, rendered us to proceed further with ATP6V0A2 only. Concisely, ELISA results revealed that ATP6V0A2 levels were low in AS individuals as compared to normal controls ($p < 0.01$) (Fig. 3D).

4. Discussion

Clinical fertility tests available in the market include the analysis of various sperm related factors like motility, concentration and morphology. Although, detailed evaluation of all still leaves nearly 50% cases of infertility with no known cause, thus making the discovery of infertility sensitive diagnostic tools obligatory. In the present scenario proteomics has emerged as a crucial research field in biology and medicine having the potential to classify and authenticate potent targets, at the molecular level. Protein markers may help in elucidating the unknown causes of male infertility, thus simultaneously paving the path for therapeutic solutions to the problem. Cited literature describes the cases as idiopathic in nature, thus quoting no specific credited cause responsible for the condition [20–22]. Customarily, two major pathways related to sperm motility are described i.e. Ca^{2+}signalling and cAMP/protein kinase A pathway [23]. The plethora of proteins referred in support to their involvement in sperm motility can be categorized as energy-metabolism and signalling-transport related proteins [9,24–26]. Herein, attempts were made to study 2D-DIGE based differential sperm proteome profile, which revealed seven differentially expressed protein spots that were further identified by MALDI-TOF-MS (Table 2).

In the present investigation, we identified SERPINB9, TEX40, ATP6V0A2 and PSA as the most relevant proteins related to male fertility. The study suggests that low levels of these sperm proteins could be a signature for low motility state. Although, highlight of the study

Table 2
List of differentially expressed sperm proteins identified by MALDI-TOF/MS having a fold change of ≥2.

Protein name[a]	Accession no.	MW (kDa)	Fold change (p-value)	Function
K2C1	P04264	66	−1.7 (0.034)	Regulator of kinases
AJUBA	Q96IF1	56.9	−1.9 (0.033)	Scaffold protein, cell-cell adhesion, transcription repression
TEX40	Q9NTU4	23	−2.1 (0.023)	Auxiliary component of the CatSper complex involved in Ca^{2+} entry
POGK	Q9P215	69.4	−2.9 (0.043)	DNA binding protein, probable transposon
ATP6V0A2	Q9Y487	99.1	−2.93 (0.044)	Proton pump involved in acrosome acidification
SERPINB9	P50453	43	−3.1 (0.035)	Serine protease inhibitor
PSA	P07288	28.7	−4.9 (0.012)	Serine protease involved in seminal liquefaction

[a] K2C1 (keratin, type II cytoskeletal 1), AJUBA (LIM domain containing protein), TEX40 (testis expressed protein 40), POGK (Pogo transposable element with KRAB domain), ATP6V0A2 (a2 isoform of vacuolar ATPase), PSA (prostate-specific antigen).

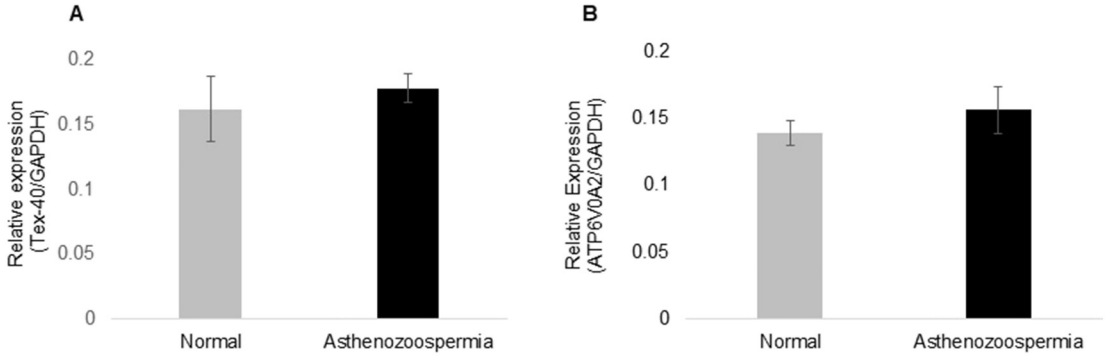

Fig. 2. Differential mRNA expression (n = 10 for each group). Relative expression of TEX40 (A) and ATP6V0A2 (B) genes at mRNA level in normal and AS individuals. GAPDH was used as internal control.

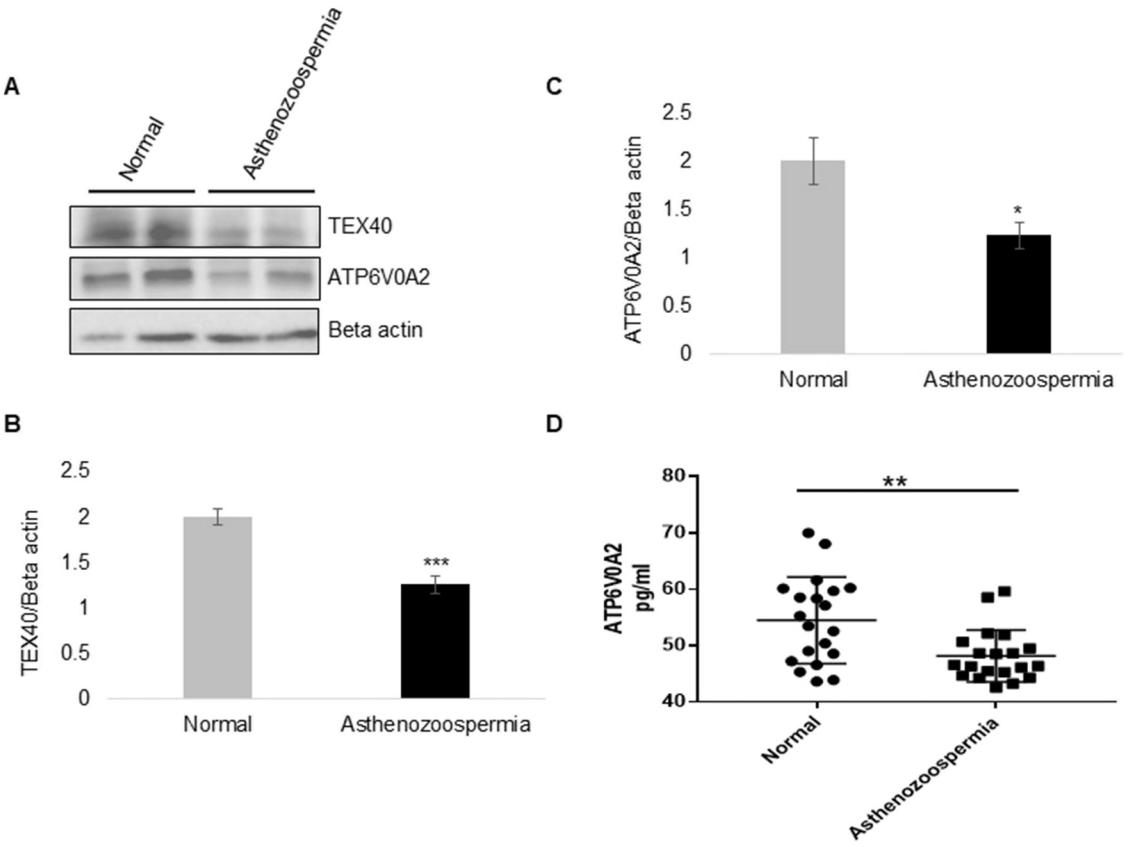

Fig. 3. Validation of differentially expressed proteins. (A) Representative immunoblots showing expression of TEX40 and ATP6V0A2 proteins in normal and AS individuals (each band corresponds 5 pooled samples from the respective group). Beta actin was used as an internal control. (B and C) Graphical illustration showing quantitative expression of TEX40 and ATP6V0A2. (D) Evaluation of ATP6V0A2 protein expression by ELISA (n = 20 individual samples from each group). Data expressed as mean ± SD. *** indicates a significant difference $P \leq 0.001$, ** significant difference $P \leq 0.01$, and * significant difference $P \leq 0.05$.

were two proteins, TEX40 and ATP6V0A2, which could be assorted in the class of energy-metabolism (ATP6V0A2) and signalling-transport (TEX40) related proteins. The above alterations are not surprising as these functional groups are imperative for the proper functioning and motility of the sperm. Nonetheless, the two proteins are attention drawers as their lowered expression in clinically diagnosed AS individuals is reported for the first time. Also, the above mentioned proteins are not listed in a recently published report of human sperm proteins identified by 2-dimensional electrophoresis [27]. Probably, the reason being less sensitivity associated with 2-dimensional electrophoresis as compared to flour dye based DIGE.

SERPINB9 is a 43 kDa serine protease inhibitor, which inhibits Granzyme B a serine protease present in the cytotoxic T cells. A report unveils that SERPINB9 is abundantly expressed in human testicular sertoli cells, where it may contribute to immune privilege by giving protection against maternal or self-reactive cytotoxic lymphocytes produced granzyme B [28]. Evasion of the immune system is the finest mechanism opted by cancer cells to survive and proliferate. Granzyme B initiates apoptosis mediated tumor cell death. Higher expression of SERPINB9 in prostate cancer suggests a protective effect of later on prostate cancer cells and thus, its deregulation may play a protective role early in cancer progression [29]. A recent report examining the exome sequence of two azoospermic brothers revealed homozygous splice mutation in SPINK2, encoding another serine protease inhibitor which targets sperm acrosomal protease, acrosin [30]. The results of this study clearly define that deficiency of this protein induce a pathological state ranging from oligoasthenoteratozoospermia in heterozygotes to azoospermia in homozygotes [30]. Similarly, a mice model

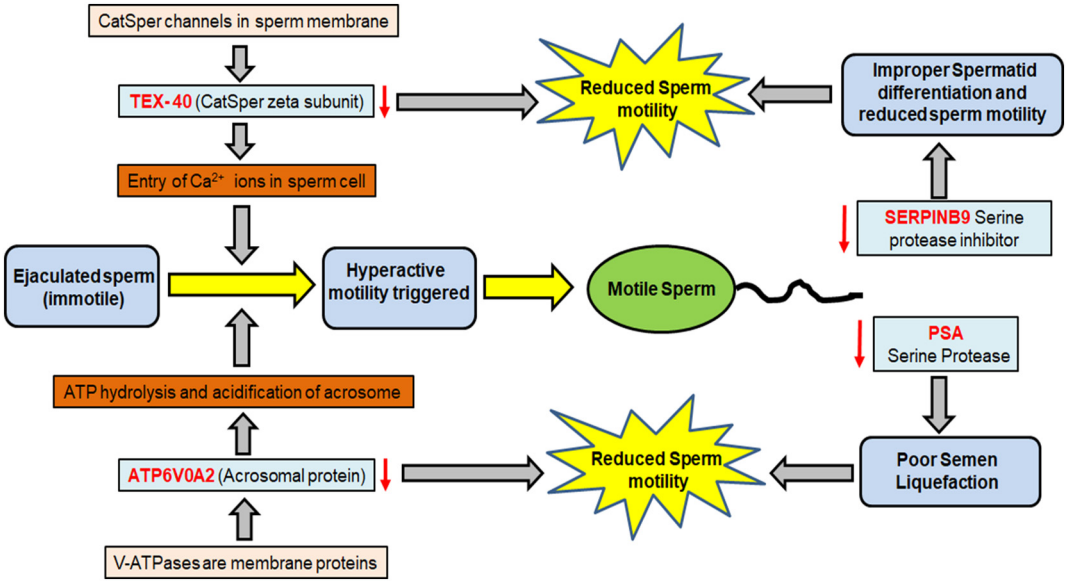

Fig. 4. Schematic diagram depicting probable role of TEX40, ATP6V0A2, SERPINB9 and PSA in sperm motility. Down-regulation of TEX40 leads to fewer entries of Ca^{2+} in sperm causing retarded motility. Lower expression of ATP6V0A2, responsible for acrosomal deacidification results in diminished sperm motility. SERPINB9 mechanism has not been much explored and probably works alike other serine protease inhibitors causing improper spermatogenesis and reduced sperm motility. PSA down regulation causes poor semen liquefaction resulting in lessened sperm motility.

study has revealed that mutation in Spink2 gene, abundantly expressed in testes causes impaired spermatogenesis and fertility. Authors have also suggested that the protein is also involved in maintenance of normal spermatogenesis, and possibly regulates serine protease-mediated apoptosis in male germ cells [31]. Above mentioned studies are aptly parallel with our present findings discussing down regulation of SERPINB9 in AS Indian males. It is thus hypothesised that SERPINB9 works in a similar way as other serine protease inhibitors.

PSA is another imperative protein involved in semen liquefaction thereby releasing sperm for active motility. Degradation of semenogelins yields numerous polypeptides with varied biological functions, such as hyperactivated motility [32]. It has been suggested that in addition to facilitating coagulum liquefaction, PSA might activate a motility-activating peptide [33]. These reports accurately rationalize the reduced level of PSA found in the present study, which may cause diminished hyperactive sperm motility.

In a de novo murine model study, TEX40 is identified as a 23 kDa protein and denoted as zeta (ζ) subunit of CatSper channel complex [14]. The same group initially stated that CatSper ion channel is required for sperm motility and fertility [34]. Further, they identified it as a complex containing seven proteins (CatSper1–4, β, γ and δ), of which four form a pore for entry of Ca^{2+} ions and three accessory proteins probably participating in hyperactive motility [35]. Recently, two new accessory proteins have been added in the list as ε and ζ related to this channel by the same group of investigators [14]. They have stated that genetic disruption of mammalian specific Catsper ζ results in severe subfertility. Also, the study has illustrated that both mouse and human sperm share a common structural organisation of the CatSper complex [14]. Mammalian sperm displays two types of physiological motility, activated (in ejaculated sperm) and hyper activated (sperm recovered from fertilisation site) [36]. Hyper activated motility triggered by CatSper, is the key requisite for normal sperm navigation, rheotaxis, and zona pellucida (ZP) penetration [37,38]. TEX40 is an accessory protein of CatSper complex required for its linear quadrilateral distribution along the flagellum, and mutation in this leads to fragmented distribution of CatSper strips. Altogether, this obstructs Ca^{2+} influx through the channel thus affecting the sperm motility by making it more rigid [14]. In view of TEX40, Chung et al. investigation is the first and recent of its kind supporting the fact that TEX40 is crucial for the motility of the sperm. Low expression of TEX40 obtained herein is another experimental proof, which testifies the clinical state of affairs of the AS individuals. Furthermore, the WB results support the MALDI-TOF-MS findings. Our results at the protein level explain a similar pattern of expression (DIGE and WB).

Herein, another protein ATP6V0A2 was found down-regulated in AS individuals. Furthermore, WB and ELISA results were in agreement with the proteome profiling discoveries. The a2 isoform of vacuolar ATPase (ATP6V0A2) is a 99 kDa ATP dependent multi-subunit proton pump required for the acidification of the intracellular compartments as well as extracellular milieu [15]. The reports suggest the acrosomal location of ATP6V0A2 in the spermatozoa from murine model [39]. Higher acrosomal expression of ATP6V0A2 in capacitated murine sperm than non-capacitated spermatozoa from caudal epididymis attests its alliance with male infertility. Also, ATP6V0A2 is involved in cytokine secretion, which is essential for successful implantation [40]. Similar findings in the human semen demonstrating link between ATP6V0A2 expression and cytokine secretion are observed [41]. Another major functional outcome of ATP6V0A2 protein is in spermatogenesis by regulating testicular immune response [42] and apoptotic pathways [15]. Abnormal spermatogenesis due to unhinged chemokine/cytokine network and apoptotic pathways results in reduced sperm motility and poor sperm production. Also, paternal inhibition of ATP6V0A2 reduces the pregnancy outcomes in the females as reported by others [42]. Our results presenting low expression of ATP6V0A2 in AS infertile male postulate that deregulated inflammatory response and apoptotic pathways root low sperm motility in this group. In support of the above assumption, our previous study has reported the higher expression of interleukin-1 family member 10 (IL-1F10) cytokine in seminal plasma of AS individuals from the same population [43]. At the transcriptomic level, the results exhibited no changes in the mRNA expression of TEX40 and ATP6V0A2, concluding that supposedly their expression might not be regulated at the transcriptional level. The role of some epigenetic factors (miRNA) or post-translational protein degradation mechanisms in down-regulating protein expression can be further explored.

In a nutshell, investigation states that down regulation of TEX40, a Ca^{2+} signalling sperm motility related protein is reported for the first time in clinically diagnosed AS males. Supporting the fact, a recent

report also suggests that compromised Ca^{2+} signalling is likely to affect capacitation, regulation of acrosome reaction and sperm viability [44]. Another important protein is ATP6V0A2, which has also been reported earlier in a mixed population of infertile males [41]. Validation experiments further confirmed their lower expression. A schematic representation illustrates the role of TEX40, ATP6V0A2, SERPINB9 and PSA in sperm motility (Fig. 4).

5. Conclusion

The proteome stock in sperms of normal and AS males was studied in the present investigation so as to elucidate the idiopathic nature of male infertility. The two proteins identified, work in a sequential and coordinated manner to provide hyperactive motility to the ejaculated sperm. The simultaneous down regulation of both proteins found herein can be further evaluated in a large cohort of clinically diagnosed males with low sperm motility. The down regulation of both could have a deleterious effect on sperm hyperactive motility and could be a plausible explanation for idiopathic male infertility. Also, in future these proteins can be further utilized as contraceptive targets.

Competing interests

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2018.12.016.

References

[1] K. Hwang, R.C. Walters, L.I. Lipshultz, Contemporary concepts in the evaluation and management of male infertility, Nat. Rev. Urol. 8 (2011) 86–94, https://doi.org/10.1038/nrurol.2010.230.
[2] D.R. Franken, S. Oehninger, Semen analysis and sperm function testing, Asian J. Androl. 4 (2012) 6–13, https://doi.org/10.1038/aja.2011.58.
[3] M. Tomlinson, S. Lewis, D. Morroll, Sperm quality and its relationship to natural and assisted conception: British Fertility Society guidelines for practice, Hum. Fertil. (Camb.) 16 (2013) 175–193, https://doi.org/10.3109/14647273.2013.807522.
[4] P. Thonneau, A. Spira, Prevalence of infertility: international data and problems of measurement, Eur. J. Obstet. Gynecol. Reprod. Biol. 38 (1991) 43–52, https://doi.org/10.1016/0028-2243(91)90206-Z.
[5] S.M. Curi, J.I. Ariagno, P.H. Chenlo, G.R. Mendeluk, M.N. Pugliese, L.M. Sardi Segovia, H.E. Repetto, A.M. Blanco, Asthenozoospermia: analysis of a large population, Arch. Androl. 49 (2003) 343–349, https://doi.org/10.1080/01485010390219656.
[6] S.K. Adiga, V. Jayaraman, G. Kalthur, D. Upadhya, P. Kumar, Declining semen quality among south Indian infertile men: a retrospective study, J. Hum. Reprod. Sci. 1 (2008) 15–18.
[7] A.R.C.N. Kumar, A.K. Singh, Prevalence of male factor infertility in last ten years at a rural tertiary care centre of Central India: a retrospective analysis, Indian J. Obstet. Gynaecol. Res. 2 (2015) 132–136.
[8] N. Kumar, A.K. Singh, Trends of male factor infertility, an important cause of infertility: a review of literature, J. Hum. Reprod. Sci. 8 (2015) 191–196, https://doi.org/10.4103/0974-1208.170370.
[9] A. Amaral, C. Paiva, C. Attardo Parrinello, J.M. Estanyol, J.L. Ballesca, J. Ramalho-Santos, R. Oliva, Identification of proteins involved in human sperm motility using high-throughput differential proteomics, J. Proteome Res. 13 (2014) 5670–5684, https://doi.org/10.1021/pr500652y.
[10] F.L. Liu, X. Liu, J.L. Han, Y.W. Wang, S.H. Jin, X.X. Liu, J. Liu, W.T. Wang, W.J. Wang, Aged men share the sperm protein PATE1 defect with young asthenozoospermia patients, Hum. Reprod. 30 (2015) 861–869, https://doi.org/10.1093/humrep/dev003.
[11] S. Shen, J. Wang, J. Liang, D. He, Comparative proteomic study between human normal motility sperm and idiopathic asthenozoospermia, World. J. Urol. 31 (2013) 1395, https://doi.org/10.1007/s00345-013-1023-5.
[12] P.P. Parte, P. Rao, S. Redij, V. Lobo, S.J. D'Souza, R. Gajbhiye, V. Kulkarni, Sperm phosphoproteome profiling by ultra performance liquid chromatography followed by data independent analysis (LC-MS(E)) reveals altered proteomic signatures in asthenozoospermia, J. Proteome 75 (2012) 5861–5871, https://doi.org/10.1016/j.jprot.2012.07.003.
[13] A. Sinha, V. Singh, S. Yadav, Multi-omics and male infertility: status, integration and future prospects, Front. Biosci. (Schol. Ed.) 9 (2017) 375–394, https://doi.org/10.2741/493.
[14] J.J. Chung, K. Miki, D. Kim, S.H. Shim, H.F. Shi, CatSperzeta regulates the structural continuity of sperm Ca^{2+} signaling domains and is required for normal fertility, Elife 6 (2017), https://doi.org/10.7554/eLife.23082.
[15] M.K. Jaiswal, V. Agrawal, G.K. Katara, S. Pamarthy, A. Kulshrestha, G. Chaouat, A. Gilman-Sachs, K.D. Beaman, Male fertility and apoptosis in normal spermatogenesis are regulated by vacuolar-ATPase isoform a2, J. Reprod. Immunol. 112 (2015) 38–45, https://doi.org/10.1016/j.jri.2015.07.003.
[16] D.o.R.H.a.R, WHO Laboratory Manual for the Examination and Processing of Human Semen, 5th ed., World Health Organization, Geneva, Switzerland, 2010.
[17] A.K. Tomar, B.S. Sooch, I. Raj, S. Singh, S. Yadav, Interaction analysis identifies semenogelin I fragments as new binding partners of PIP in human seminal plasma, Int. J. Biol. Macromol. 52 (2013) 296–299, https://doi.org/10.1016/j.ijbiomac.2012.10.011.
[18] M. Udono, K. Fujii, G. Harada, Y. Tsuzuki, K. Kadooka, P. Zhang, H. Fujii, M. Amano, S. Nishimura, K. Tashiro, S. Kuhara, Y. Katakura, Impaired ATP6V0A2 expression contributes to Golgi dispersion and glycosylation changes in senescent cells, Sci. Rep. 5 (2015) 17342, , https://doi.org/10.1038/srep17342.
[19] H. Chen, S.J. Shan, T. Mester, Y.H. Wei, R.S. Douglas, TSH-mediated TNFalpha production in human fibrocytes is inhibited by teprotumumab, an IGF-1R antagonist, PLoS One 10 (2015) e0130322, , https://doi.org/10.1371/journal.pone.0130322.
[20] D. Jaiswal, R. Sah, N.K. Agrawal, U.S. Dwivedi, S. Trivedi, K. Singh, Combined effect of GSTT1 and GSTM1 polymorphisms on human male infertility in north Indian population, Reprod. Sci. 19 (2012) 312–316, https://doi.org/10.1177/1933719111424451.
[21] A. Bracke, K. Peeters, U. Punjabi, D. Hoogewijs, S. Dewilde, A search for molecular mechanisms underlying male idiopathic infertility, Reprod. BioMed. Online 36 (2018) 327–339, https://doi.org/10.1016/j.rbmo.2017.12.005.
[22] F.F. Pasqualotto, A. Sundaram, R.K. Sharma, E. Borges Jr., E.B. Pasqualotto, A. Agarwal, Semen quality and oxidative stress scores in fertile and infertile patients with varicocele, Fertil. Steril. 89 (2008) 602–607, https://doi.org/10.1016/j.fertnstert.2007.03.057.
[23] C.J. Brokaw, Regulation of sperm flagellar motility by calcium and cAMP-dependent phosphorylation, J. Cell. Biochem. 35 (1987) 175–184, https://doi.org/10.1002/jcb.240350302.
[24] J. Martinez-Heredia, S. de Mateo, J.M. Vidal-Taboada, J.L. Ballesca, R. Oliva, Identification of proteomic differences in asthenozoospermic sperm samples, Hum. Reprod. 23 (2008) 783–791, https://doi.org/10.1093/humrep/den024.
[25] C. Zhao, R. Huo, F.Q. Wang, M. Lin, Z.M. Zhou, J.H. Sha, Identification of several proteins involved in regulation of sperm motility by proteomic analysis, Fertil. Steril. 87 (2007) 436–438, https://doi.org/10.1016/j.fertnstert.2006.06.057.
[26] A.B. Siva, D.B. Kameshwari, V. Singh, K. Pavani, C.S. Sundaram, N. Rangaraj, M. Deenadayal, S. Shivaji, Proteomics-based study on asthenozoospermia: differential expression of proteasome alpha complex, Mol. Hum. Reprod. 16 (2010) 452–462, https://doi.org/10.1093/molehr/gaq009.
[27] K. Nowicka-Bauer, M. Ozgo, A. Lepczynski, M. Kamieniczna, A. Malcher, W. Skrzypczak, M. Kurpisz, Human sperm proteins identified by 2-dimensional electrophoresis and mass spectrometry and their relevance to a transcriptomic analysis, Reprod. Biol. (2018), https://doi.org/10.1016/j.repbio.2018.02.003.
[28] C.E. Hirst, M.S. Buzza, V.R. Sutton, J.A. Trapani, K.L. Loveland, P.I. Bird, Perforin-independent expression of granzyme B and proteinase inhibitor 9 in human testis and placenta suggests a role for granzyme B-mediated proteolysis in reproduction, Mol. Hum. Reprod. 7 (2001) 1133–1142, https://doi.org/10.1093/molehr/7.12.1133.
[29] M. Ray, D.R. Hostetter, C.R.K. Loeb, J. Simko, C.S. Craik, Inhibition of granzyme B by PI-9 protects prostate cancer cells from apoptosis, Prostate 72 (2012) 846–855, https://doi.org/10.1002/pros.21486.
[30] Z.E. Kherraf, M. Christou-Kent, T. Karaouzene, A. Amiri-Yekta, G. Martinez, A.S. Vargas, E. Lambert, C. Borel, B. Dorphin, I. Aknin-Seifer, M.J. Mitchell, C. Metzler-Guillemain, J. Escoffier, S. Nef, M. Grepillat, N. Thierry-Mieg, V. Satre, M. Bailly, F. Boitrelle, K. Pernet-Gallay, S. Hennebicq, J. Fauré, S.P. Bottari, C. Coutton, P.F. Ray, C. Arnoult, SPINK2 deficiency causes infertility by inducing sperm defects in heterozygotes and azoospermia in homozygotes, EMBO Mol. Med. 9 (2017) 1132–1149, https://doi.org/10.15252/emmm.201607461.
[31] B. Lee, I. Park, S. Jin, H. Choi, J.T. Kwon, J. Kim, J. Jeong, B.N. Cho, E.M. Eddy, C. Cho, Impaired spermatogenesis and fertility in mice carrying a mutation in the Spink2 gene expressed predominantly in testes, J. Biol. Chem. 286 (2011) 29108–29117, https://doi.org/10.1074/jbc.M111.244905.
[32] N. Gupta, D.V.S. Sudhakar, P.K. Gangwar, S.N. Sankhwar, N.J. Gupta, B. Chakraborty, K. Thangaraj, G. Gupta, S. Rajender, Mutations in the prostate specific antigen (PSA/KLK3) correlate with male infertility, Sci. Rep. 7 (1) (2017 Sep 11) 11225, , https://doi.org/10.1038/s41598-017-10866-1.
[33] E. de Lamirande, G. Lamothe, Levels of semenogelin in human spermatozoa decrease during capacitation: involvement of reactive oxygen species and zinc, Hum. Reprod. 25 (7) (2010 Jul) 1619–1630, https://doi.org/10.1093/humrep/deq110.

[34] D. Ren, B. Navarro, G. Perez, A.C. Jackson, S. Hsu, Q. Shi, J.L. Tilly, D.E. Clapham, A sperm ion channel required for sperm motility and male fertility, Nature 413 (2001) 603–609.
[35] J.J. Chung, B. Navarro, G. Krapivinsky, L. Krapivinsky, D.E. Clapham, A novel gene required for male fertility and functional CATSPER channel formation in spermatozoa, Nat. Commun. 2 (2011) 153.
[36] R.M. Turner, Moving to the beat: a review of mammalian sperm motility regulation, Reprod. Fertil. Dev. 18 (2006) 25–38, https://doi.org/10.1071/RD05120.
[37] S. Ishijima, S.A. Baba, H. Mohri, S.S. Suarez, Quantitative analysis of flagellar movement in hyperactivated and acrosome-reacted golden hamster spermatozoa, Mol. Reprod. Dev. 61 (2002) 376–384, https://doi.org/10.1002/mrd.10017.
[38] S. Ishijima, Dynamics of flagellar force generated by a hyperactivated spermatozoon, Reproduction 142 (2011) 409–415, https://doi.org/10.1530/REP-10-0445.
[39] G.H. Sun-Wada, Y. Imai-Senga, A. Yamamoto, Y. Murata, T. Hirata, Y. Wada, M. Futai, A proton pump ATPase with testis-specific E1-subunit isoform required for acrosome acidification, J. Biol. Chem. 277 (2002) 18098–18105, https://doi.org/10.1074/jbc.M111567200.
[40] M.K. Jaiswal, T.M. Mallers, B. Larsen, J. Kwak-Kim, G. Chaouat, A. Gilman-Sachs, K.D. Beaman, V-ATPase upregulation during early pregnancy: a possible link to establishment of an inflammatory response during preimplantation period of pregnancy, Reproduction 143 (2012) 713–725, https://doi.org/10.1530/REP-12-0036.
[41] K. Ota, M.K. Jaiswal, S. Ramu, R. Jeyendran, J. Kwak-Kim, A. Gilman-Sachs, K.D. Beaman, Expression of a2 vacuolar ATPase in spermatozoa is associated with semen quality and chemokine-cytokine profiles in infertile men, PLoS One 8 (2013) e70470, , https://doi.org/10.1371/journal.pone.0070470.
[42] M.K. Jaiswal, G.K. Katara, T. Mallers, G. Chaouat, A. Gilman-Sachs, K.D. Beaman, Vacuolar-ATPase isoform a2 regulates macrophages and cytokine profile necessary for normal spermatogenesis in testis, J. Leukoc. Biol. 96 (2014) 337–347, https://doi.org/10.1189/jlb.3A1113-593RR.
[43] M. Saraswat, S. Joenvaara, T. Jain, A.K. Tomar, A. Sinha, S. Singh, S. Yadav, R. Renkonen, Human spermatozoa quantitative proteomic signature classifies normo- and asthenozoospermia, Mol. Cell. Proteomics 16 (2017) 57–72, https://doi.org/10.1074/mcp.M116.061028.
[44] M.C. Kelly, S.G. Brown, S.M. Costello, M. Ramalingam, E. Drew, S.J. Publicover, Martins Da Silva S. Barratt, Single-cell analysis of $[Ca^{2+}]_i$ signalling in sub-fertile men: characteristics and relation to fertilization outcome, Hum. Reprod. (2018), https://doi.org/10.1093/humrep/dey096.