Unraveling the sperm proteome and post-genomic pathways associated with sperm nuclear DNA fragmentation

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Abstract

Purpose Sperm DNA fragmentation has been suggested as a marker for infertility diagnosis and prognosis. Hence, understanding its impact on male physiology and post-genomic pathways would be clinically important. We performed the proteomics and functional enrichment analyses of viable spermatozoa from ejaculates with low and high sperm DNA fragmentation to identify protein expression and pathways altered in association with sperm DNA fragmentation.

Methods Sperm DNA fragmentation using the Comet assay and the Komet 6.0.1 software was assessed in raw samples from 89 subjects from a human reproduction service. The Low and High sperm DNA fragmentation groups were formed according to the Olive Tail Moment variable. Spermatozoa proteins from these groups were pooled and analyzed by a shotgun proteomic approach (2D nanoUPLC-

ESI-MS^E). Differentially expressed proteins were used for a functional enrichment study.

Results Two hundred and fifty-seven proteins were identified or quantified in sperm from the Low and High sperm DNA fragmentation groups. Of these, seventy-one proteins were exclusively or overexpressed in the Low group, whereas twenty-three proteins were exclusively or overexpressed in the High group. One hundred and sixty-three proteins were conserved between these groups. We also functionally related the differentially expressed proteins in viable spermatozoa from the groups. Processes such as triacylglycerol metabolism, energy production, protein folding, response to unfolded proteins, and cellular detoxification were found to be altered in these cells.

Conclusions Sperm DNA fragmentation is associated with differential protein expression in viable spermatozoa. These proteins may potentially be used as biomarkers for sperm DNA integrity.

Capsule Sperm DNA fragmentation is associated with differential protein expression and biological functions in viable sperm. These proteins may be potentially used as biomarkers for sperm DNA integrity.

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Introduction

Infertility affects approximately 15 % of reproductive-aged couples and the male factor is present in 50 % of cases [1]. The mechanisms leading to male infertility are still poorly understood, but studies suggest an important involvement of sperm DNA fragmentation [2,3]. Although sperm are highly differentiated cells with a characteristic morphology that render their DNA compact and protected from surrounding toxic substances [4], sperm DNA can be damaged through three major mechanisms: (i) oxidative stress - the main cause of sperm DNA fragmentation in mature sperm [5], (ii) alterations in chromatin packing [6], and (iii) abortive apoptosis [7].

About 10 % of fertile men and 20–25 % of infertile men present high levels of sperm DNA damage [8], which is associated with reduced reproductive potential [9–12] and embryo quality [13] and elevated miscarriage rates [14]. Moreover, it increases the risk of iatrogenic transmission of genetic defects during assisted reproduction techniques (ART) [15]. Although the assessment of sperm DNA integrity is considered controversial in the routine clinical workup of the infertile male [16], it has been suggested as an independent marker of sperm function, providing further information regarding semen quality and male fertile potential [17–19]. Thus, while sperm DNA fragmentation is not a surrogate endpoint for male fertility, it is an important indicator of reduced fertility potential. However, despite the growing knowledge on sperm DNA damage itself, little is known towards the underlying post-genomic mechanisms.

The ejaculate is composed of a heterogeneous cell population in humans, with sperm in different steps of maturation and various functional traits [20]. Thus, the sperm subsets range from viable sperm that are able to undergo capacitation and acrosome reaction to non-viable, apoptosis-marked sperm [21]. While the pattern of spermatogenesis itself can help explain why different sites in the testis produce sperm at any given time [22], we cannot expect that a group of viable sperm which mature alongside apoptosis-marked sperm will provide a similar fertility potential as viable sperm from a more homogeneous cohort. The latter is demonstrated by a high observed correlation between sperm marked for initial apoptosis and sperm with DNA fragmentation in an ejaculate [23]. Under this rationale, even a viable subset from an ejaculate with a higher amount of sperm with DNA fragmentation is expected to contain proteomics alterations, because (i) oxidative DNA damage, which is responsible for the oxidation of sperm membrane proteins, can occur during the entire male reproductive process, i.e. during spermatogenesis, epididymal maturation, sperm transit or even after ejaculation [24], and (ii) the ejaculation of a subset of sperm which had its DNA fragmented in the testis might reveal a dysfunction in the selection mechanisms by Sertoli cells, suggesting that viable sperm with an altered proteome can also be ejaculated. The latter can be further supported by the fact that Fas may be found externalized to the membrane of mature sperm in the ejaculate [7,25]. These mechanisms will ultimately alter the sperm proteome through: (i) error in the translation of fragmented genes, because the sperm transcription and translation processes will become quiescent only after spermiogenesis [24], and (ii) different protein exchange between the sperm membrane and the epididymal fluid during sperm storage, maturation, and traffic. In normal conditions, the epididymal fluid and the sperm membrane undergo intense protein exchanges, including the removal of specific testicular proteins from the membrane and the addition of secreted epididymal proteins [26]. Therefore, sperm with

damaged DNA might present different testicular proteins associated with its membrane, which might not be removed in the epididymis, and/or suffer the addition of other epididymal proteins.

In this context, viable cells from an ejaculate containing high sperm DNA fragmentation rates will also be exposed to oxidative stress and to altered selection mechanisms, reducing their potential. We hypothesize this may be reflected in the sperm proteome of the viable subset from an ejaculate of lower quality. To test our hypothesis, we performed sperm DNA fragmentation analysis in ejaculated sperm of raw semen and performed a comparative proteomic analysis of the viable sperm subset. We expect our results ultimately to verify if indeed a differential sperm proteome is present, and if so, which cellular physiologic mechanisms are taking place under each condition.

Materials and methods

Ethics

This study received Institutional Review Board Approval from the Sao Paulo Federal University (UNIFESP) Research Ethics Committee. Subjects included provided informed written consent.

Study design

A case–control cross-sectional study was performed on 89 prospectively and consecutively collected samples (over 6 months) from normozoospermic [27] men attending to the Andrology Laboratory of UNIFESP. Immediately (under 1 h) after liquefaction, three semen aliquots were produced: the first aliquot was used for semen analysis; the second aliquot was used for evaluation of sperm DNA fragmentation in the raw (pre-processing) sample, assuring that the evaluated damage was not an iatrogenic cause from centrifugation; and the third aliquot was processed by a density gradient for removal of cellular elements other than spermatozoa, in order to perform sperm proteomics analysis. Using the results from the sperm DNA fragmentation test, we were able to divide the patients into three groups: low DNA fragmentation, average DNA fragmentation, and high DNA fragmentation. In order to compare each extreme, sperm proteomics analysis was performed in the patients from the Low and High sperm DNA fragmentation groups. All the study design steps including definition of experimental groups are presented in Fig. 1. These protocols are described below. All reagents used in this study were purchased from Sigma (Sigma-Aldrich, St. Louis, Missouri, USA), unless otherwise described.

Fig. 1 Study design

Aliquot 1: Semen analysis

Semen samples were collected by masturbation following 2 to 5 days of ejaculatory abstinence. Semen analysis was performed according to World Health Organization criteria (WHO [27]). Sperm morphology was evaluated by Kruger's strict criteria [28].

Aliquot 2: Sperm DNA integrity and detailed experimental groups formation

Sperm nuclear DNA integrity was evaluated in raw semen samples by a modified alkaline single-cell gel electrophoresis, or Comet assay, as previously described [29]. A total of 60 sperm were analyzed using the Komet 6.0.1 software (Andor Technology, Ulster, UK) [30], and the Olive Tail Moment was calculated by the software for each sample. This variable is defined as the product of total DNA percentage in the comet tail and the distance between the centers of mass in comet head and tail (see Fig. 1) and is considered one of the main parameters to evaluate DNA migration [30].

A descriptive statistical analysis of the Olive Tail Moment variable was then performed to divide the patients into four quartiles. Thereafter, the first quartile (25 % of subjects with the lowest sperm DNA fragmentation) and the forth quartile

(25 % of subjects with the highest sperm DNA fragmentation) were used to form the experimental groups: Low and High sperm DNA fragmentation, respectively. The patients from the 2nd and 3rd quartiles (average sperm DNA fragmentation) were not assigned to either group and were excluded from proteomics analysis. A Student's T test for unpaired samples was applied to assess whether the groups were indeed statistically constituted ($p < 0.05$). The sperm samples selected to compose the groups were then used for proteomic analysis, as described below.

Aliquot 3: Sperm proteomic analysis

The remaining semen volume (aliquot 3) was centrifuged at $800 \times g$ for 30 min to separate the seminal plasma supernatant, used in other studies [31,32], and the cellular fraction. The resulting pellet containing the cellular fraction was submitted to discontinuous density gradient centrifugation. Briefly, the pellet was resuspended in 0.5 mL of Biggers Whitten Whittingan (BWW) media supplemented with 10 % Bovine Serum Albumin (BSA) and this resuspension was layered over a discontinuous two-layer (Percoll 45 % to 90 %, GE Healthcare, Amersham Place, UK) density gradient in a 15 mL conical tube and centrifuged at $1,300 \times g$ for 30 min. This centrifugation force was greater than forces used during regular sample treatment (WHO [27]) because our aim was to maximize post-processing sperm retrieval, without overtly affecting the sperm selection. Although it is possible that some non-viable sperm were collected after the processing, these represent only a small portion of the obtained pellet, not affecting subsequent proteomic analysis. Spermatozoa collected from the bottom layer (90 % layer) were washed twice by resuspension in 0.5 mL of BWW and centrifuged again at $1,300 \times g$ for 10 min. After the second centrifugation, the supernatant was removed and the pellet containing only spermatozoa was frozen without cryoprotectant, to avoid contamination with proteins from the cryoprotectant, and kept at -20°C until the proteomic analysis.

Prior to the beginning of proteomics analysis, the pellets containing spermatozoa were thawed and centrifuged at $3,000 \times g$ for 15 min to remove excess culture medium. Then, the samples were rapidly frozen in contact with liquid nitrogen, thawed at room temperature and homogenized in vortex. This step was performed three times. Each pellet received 0.1 mL of RapiGest SF surfactant 0.5 % (w:v) in water (RapiGest SF, Waters, Massachusetts, USA) and 0.01 mL of 1 mM Phenylmethylsulfonyl fluoride (PMSF) in methanol to inhibit endogenous proteases. After intense homogenization, the samples were incubated at 100°C for 5 min, vigorously agitated by vortex and kept at 100°C for 25 min. At the end of this step, 0.05 mL of a 50 mM NH_4HCO_3 solution was added and the samples were centrifuged at $16,100 \times g$ for 30 min at 4°C . Finally, an aliquot of

the supernatant was used for protein quantification and the remaining volume was frozen and kept at -20°C .

Total protein concentration of sperm cellular fractions obtained as above was evaluated using the Bicinchoninic Acid (BCA) protein assay (modified Lowry method, [33]). Each sample was quantified in triplicate and a quantification curve was generated in duplicate. Samples quantified with coefficients of variation over 10 % were quantified again in another run. Following protein quantification, the samples from different subjects were pooled according to the sperm DNA fragmentation status, taking care that each sample contributed with the same amount of total protein to the final pools. Due to low protein concentration, sperm sample pooling required protein concentration through volume concentration using AmiconUltra 3 kDa filter (Millipore, Carrigtwohill, Ireland). Therefore, two pools were formed: (i) sperm proteins from the Low sperm DNA fragmentation group and (ii) sperm proteins from the High sperm DNA fragmentation group. These pools were then quantified in triplicate using the BCA assay mentioned above. A comprehensive shotgun analysis was then performed using the pooled samples in an experimental design created along the lines published by Cho et al. [34] and Pan et al. [35], and four technical replicates of each pool were used.

The protein samples were then reduced in the presence of 2.5 μL of 100 mM dithiothreitol at 60°C for 30 min, alkylated with 2.5 μL of 300 mM iodoacetamide at room temperature and enzymatically digested at 37°C overnight with trypsin (Sequencing Grade Modified Trypsin, Promega, Wisconsin, USA) at 1:100 (w/w) enzyme:protein ratio. Next, 10 μL of 5 % trifluoroacetic acid was added to the digestion mixture in order to hydrolyze the RapiGest used in the sample preparation step and the samples were incubated at 37°C for 90 min. The tryptic peptide solution was then centrifuged at $16,000\times g$ for 30 min at 6°C and the supernatant was combined with 10 μL of the internal standard (Yeast Alcohol Dehydrogenase at 1 pmol/ μL) [31,32].

Ten microliters of each digested replicate (corresponding to 2.5 μg of total protein digests) were injected into a system composed by a nanoACQUITY ultrahigh pressure liquid chromatography (nanoUPLC) and Synapt Q-TOF G1 MS mass spectrometer equipped with a nanolockspray ion source (Waters, Manchester, UK). Samples were concentrated and desalted in a Symmetry C18 trapping cartridge (5 μm , $180\ \mu\text{m}\times 20\ \text{mm}$) at a flow rate of 10 $\mu\text{L}/\text{min}$. Peptides were fractionated by a SCX 300 $\text{\AA}$ column (5 μm , $180\ \mu\text{m}\times 23\text{-mm}$) using 8 subsequent injections of crescent concentrations of ammonium formate (50–200 mM) and acetonitrile (5–30 %). Each SCX fraction were subsequently separated in a HSS T3 column (1.8 μm , $75\ \mu\text{m}\times 10\ \text{cm}$) using a binary gradient from 2 to 40 % of acetonitrile in 0.1 % formic acid at a flow rate of 400 nL/min. For all measurements, the mass spectrometer was operated in the ‘V’ mode with a typical

resolving power of at least 12,500. All analysis were performed using nanoelectrospray ionization in the positive ion mode nanoESI(+) in a NanoLockSpray source (Waters, Manchester, UK). The lock mass channel was sampled every 30 s. The mass spectrometer was calibrated with a GFP solution (*Human [Glu¹]-fibrinopeptide B*) (200 fmol/mL) delivered through the reference sprayer of the NanoLockSpray source. The doubly-charged ion ($[\text{M}+2\text{H}]^{2+}$) was used for initial single-point calibration (L_{teff}) and MS/MS fragment ions of GFP were used to obtain the final instrument calibration. Data-independent scanning (MS^E) experiments were performed with a Q-TOF Synapt MS mass spectrometer (Waters, Manchester, UK), which was automatically planned to switch between standard MS (3 eV) and elevated collision energies MS^E (15–50 eV) applied to the trap ‘T-wave’ CID (collision-induced dissociation) cell with argon gas; the transfer collision cell was adjusted for 1 eV, using a scan time of 0.6 s, both in low energy and in high-energy CID orthogonal acceleration time-of-flight (oa-TOF) MS^E from m/z 50 to 2,000. The RF offset (MS profile) was adjusted such that the LC/ MS^E data were effectively acquired from m/z 300 to 2,000, which ensured that any ions observed in the LC/ MS^E data less than m/z 300 were known to arise from dissociations in the collision cell [32].

Protein identification and quantitative data packaging were generated by the use of dedicated algorithms and searching against a species-specific database. The databases were randomized ‘on-the fly’ during the database queries and appended to the original database to access the false-positive rate of identification, set up to 4 %. For spectra processing and database searching, a ProteinLynx Global Server v.2.4 (PLGS) with an Expression^E informatics v.2.4 license installed was used. A UniProtKB/Swiss-Prot Release 2011_10 database was used and the search conditions were based on taxonomy [*Homo sapiens* (human)], maximum missed cleavages by trypsin allowed up to 1, fixed modification by carbamidomethylation (C) and variable modifications by acetyl N-terminal and oxidation (M). Proteins were organized by the PLGS into a list corresponding to unique proteins for each condition (exclusively expressed in sperm fractions from Low or High sperm DNA fragmentation groups) and proteins present in sperm fractions from both groups. Only proteins present in at least two replicates were included for statistical analysis.

For statistical analysis of quantitative data, a descriptive analysis of proteins present in both groups was initially performed for calculating the mean of the four replicates and the fold-change (ratio between the mean of each group). Positive fold-change values represent a greater protein expression in the High sperm DNA fragmentation group, while negative values show the greatest expression in the Low sperm DNA fragmentation group. Experimental groups were

compared using a Mann–Whitney test ($p<0.05$). Proteins statistically significant were termed “overexpressed”, and proteins not significant, “conserved”.

Functional enrichment analysis

The set of exclusively or overexpressed proteins in each group ($p<0.05$) and of conserved proteins were used in Cytoscape 3.0 software [36]. Protein-protein interaction of each group of proteins was performed using the BisoGenet 1.41 plugin, which compiles and integrates data from multiple public sources that include interactions between genes/proteins (DIP, BIND, HPRD) [37]. Functional enrichment analysis was performed with the ClueGO 1.4 plugin, which calculates statistically hyper-represented Gene Ontology (GO) terms in each group and builds an enrichment map for (i) conserved + exclusively or overexpressed proteins from the Low sperm DNA fragmentation group, and (ii) conserved + exclusively or overexpressed proteins from the High sperm DNA fragmentation group. Subsequently, the software overlaps both enrichment maps, and it is possible to highlight the conserved functions between the Low and High sperm DNA fragmentation groups and the specific enriched functions from each group. The results are shown as GO functions statistically hyper-represented ($p<0.05$) in three major categories: (i) cellular component, (ii) molecular function and (iii) biological process. It is important to note that although some functions may be conserved between the two groups, protein expression related to these functions may be different, because one single protein may not statistically enrich a function, and because different proteins in the two groups may enrich a similar function.

Results

A total of 89 patients were initially included in the study during the collection period. Of these, 18 subjects were statistically assigned to the Low sperm DNA fragmentation group (1st quartile, bottom 25 %) and 18 subjects to the High sperm DNA fragmentation group (4th quartile, top 25 %), based on their sperm DNA fragmentation values (aliquot 2). The remaining 53 patients (2nd and 3rd quartile) were not included in the groups and were excluded from proteomics analysis. Due to final sample sperm count, protein extraction was not possible in all samples from the Low and High sperm DNA fragmentation groups. Therefore, of the initial 18 sperm samples in each group we managed to extract proteins from 11 samples of the Low group and from 6 samples of the High group. The proteins from these samples were used to form the pools. The results (mean $\pm$ standard deviation) for the Olive Tail Moment were 0.194 ± 0.04 for the Low sperm DNA fragmentation group ($n=11$) and

1.116 ± 0.183 for the High sperm DNA fragmentation group ($n=06$, $p<0.0001$).

A total of 257 proteins were identified or quantified from sperm protein pools derived from the Low and High sperm DNA fragmentation groups. Of these proteins, 24 were overexpressed and 47 were exclusively expressed in the Low sperm DNA fragmentation group (Table 1), whereas 11 were overexpressed and 12 were exclusively expressed in the High sperm DNA fragmentation group (Table 2). The remaining 163 proteins were not quantitatively different between these groups (Online Resource 1). The protein-protein networks of exclusively or overexpressed proteins from the Low and High sperm DNA fragmentation groups and of conserved proteins are presented in Fig. 2. Furthermore, an overview of the enriched functions related to each group of proteins can be found in Figs. 3 and 4.

Discussion

To the best of our knowledge, this is the first proteomic study to evaluate the sperm proteome associated with sperm DNA fragmentation on viable sperm. For this purpose, we performed a high-throughput proteomic technique to identify the differential protein expression between viable sperm from ejaculates with Low and High sperm DNA fragmentation, in an unbiased and untargeted manner. It is important to note that our goal was not to explain the causes of sperm DNA fragmentation, but rather to elucidate the underlying post-genomic mechanisms involved with its negative impact on sperm function. Our hypothesis was that a viable subset within an ejaculate with a lower amount of sperm DNA integrity would be exposed to oxidative stress and altered selection mechanisms, which could be reflected in its proteome.

Shotgun proteomics studies coupled with interactome and functional enrichment analyses are of great value because they enable highlighting processes relevant to a particular biological condition [38]. Furthermore, it is possible to identify the co-functional proteins of these processes, which have a greater potential to be selected as possible biomarkers within the proteomics data [39]. These non-invasive infertility biomarkers would help improve management of the infertile man, which is currently based only on physical and seminal analyses, rendering more specific and individualized diagnostics and treatments [40]. This is even more important in idiopathic male infertility, which affects 25 % of infertile men, and is possibly related to functional alterations in sperm [41].

We identified 257 sperm proteins. Of these, 71 proteins were exclusively or overexpressed in the Low sperm DNA fragmentation pool and 23 proteins in the High sperm DNA fragmentation pool. Furthermore, we showed the alteration

Table 1 Exclusively or overexpressed proteins in viable sperm from the Low sperm DNA fragmentation group

Symbol	Uniprot AC	Mean (ng/column)		Fold-change ^a	p
		Low	High		
ACPP	P15309	2.701	0.797	−3.4	0.021
ACRV1	P26436	0.743	0.453	−1.6	0.021
ACTB	P60709	1.570	1.061	−1.5	0.021
BANF1	O75531	0.134	0.086	−1.6	0.021
CABYR	O75952	0.745	0.475	−1.6	0.021
CKB	P12277	0.639	0.247	−2.6	0.021
CLU	P10909	5.454	3.528	−1.5	0.021
DEFA1	P59665	0.160	0.061	−2.6	0.034
DLAT	P10515	0.391	0.248	−1.6	0.021
EEF1A1	P68104	1.454	1.173	−1.2	0.021
GAPDH	P04406	0.682	0.427	−1.6	0.021
HSP90AA1	P07900	3.218	2.026	−1.6	0.021
HSP90B1	P14625	1.451	0.851	−1.7	0.021
HSPA5	P11021	1.194	0.878	−1.4	0.021
PDIA3	P30101	0.727	0.582	−1.3	0.043
PGK2	P07205	1.131	0.850	−1.3	0.021
PIP	P12273	1.757	0.979	−1.8	0.021
ROPN1	Q9HAT0	0.422	0.329	−1.3	0.043
SEMG1	P04279	11.445	5.498	−2.1	0.021
SPANXA	Q9NS26	0.195	0.054	−3.6	0.021
SPANXB	Q9NS25	0.582	0.186	−3.1	0.021
SPANXC	Q9NY87	0.033	0.011	−3.1	0.021
SPINK2	P20155	0.177	0.142	−1.2	0.034
SPNAXD	Q9BXN6	0.100	0.014	−7.2	0.050
ACADS	P16219	0.130	-	Exclusive from Low	-
ACE	P12821	0.597	-	Exclusive from Low	-
AK1	P00568	0.037	-	Exclusive from Low	-
ANXA5	P08758	0.140	-	Exclusive from Low	-
C20orf3	Q9HDC9	0.127	-	Exclusive from Low	-
CCIN	Q13939	0.257	-	Exclusive from Low	-
CCT8	P50990	0.119	-	Exclusive from Low	-
COX5A	P20674	0.080	-	Exclusive from Low	-
CYCS	P99999	0.053	-	Exclusive from Low	-
DLD	P09622	0.224	-	Exclusive from Low	-
DPY19L2	Q6NUT2	0.972	-	Exclusive from Low	-
ENO2	P09104	0.027	-	Exclusive from Low	-
ENO3	P13929	Identified	-	Exclusive from Low	-
FAM205A	Q6ZU69	0.273	-	Exclusive from Low	-
FAM290A	Q5JX71	0.147	-	Exclusive from Low	-
FASN	P49327	0.930	-	Exclusive from Low	-
GANAB	Q14697	0.434	-	Exclusive from Low	-
GOT2	P00505	0.116	-	Exclusive from Low	-
GSTP1	P09211	0.073	-	Exclusive from Low	-
HK1	P19367	0.307	-	Exclusive from Low	-
HSPB1	P04792	0.204	-	Exclusive from Low	-
KIAA1683	Q9H0B3	0.531	-	Exclusive from Low	-
KLK3	P07288	0.256	-	Exclusive from Low	-

Table 1 (continued)

Symbol	Uniprot AC	Mean (ng/column)		Fold-change ^a	p
		Low	High		
LAP3	P28838	0.169	-	Exclusive from Low	-
LDHLA6B	Q9BYZ2	0.208	-	Exclusive from Low	-
LPL	P06858	0.313	-	Exclusive from Low	-
PAEP	P09466	0.061	-	Exclusive from Low	-
PGAM2	P15259	0.118	-	Exclusive from Low	-
PGAM4	Q8N0Y7	Identified	-	Exclusive from Low	-
PKLR	P30613	Identified	-	Exclusive from Low	-
POT EI	P0CG38	Identified	-	Exclusive from Low	-
POT EJ	P0CG39	Identified	-	Exclusive from Low	-
PRDX6	P30041	0.150	-	Exclusive from Low	-
PRKAR2A	P13861	0.150	-	Exclusive from Low	-
S100A9	P06702	0.179	-	Exclusive from Low	-
SLC25A31	Q9H0C2	0.134	-	Exclusive from Low	-
SLC2A14	Q8TDB8	Identified	-	Exclusive from Low	-
SLC2A3	P11169	0.583	-	Exclusive from Low	-
SPA17	Q15506	0.289	-	Exclusive from Low	-
SPESP1	Q6UW49	0.256	-	Exclusive from Low	-
STOML2	Q9UJZ1	0.039	-	Exclusive from Low	-
TMCO2	Q7Z6W1	0.191	-	Exclusive from Low	-
TMED9	Q9BVK6	0.046	-	Exclusive from Low	-
TMEM190	Q8WZ59	0.077	-	Exclusive from Low	-
TUBAL3	A6NHL2	Identified	-	Exclusive from Low	-
VAR S	P26640	0.449	-	Exclusive from Low	-
VCP	P55072	0.238	-	Exclusive from Low	-

^a Positive fold-change (expression rate) values represent a greater expression in the High sperm DNA fragmentation group, while negative values show the greatest expression in the Low sperm DNA fragmentation group

in several biological functions in viable sperm from the High sperm DNA fragmentation group.

Conserved functions

The conserved sperm functions observed in this study are: spermatid development (spermiogenesis), outer dense fibers assembly, de novo posttranslational protein folding, L-lactate dehydrogenase activity, triglyceride catabolism, single fertilization, cytoplasmic membrane-bounded vesicle lumen, and extracellular vesicular exosome.

Spermiogenesis is a complex process and involves, among other events, the flagellum formation, which depends on the development of outer dense fibers (ODF) [42]. Failures in ODF assembly can lead to flagellum alteration and thus to male infertility [43]. ODF provide a stable and elastic structure to the sperm flagellum, supporting its beat movement and protecting it during epididymal transit and ejaculation [42]. Interestingly, among the proteins related to these

processes, only Outer Dense Fiber Protein 2 (ODF2) was conserved between the Low and High sperm DNA fragmentation groups, whereas Outer Dense Fiber Protein 1 (ODF1) was exclusively expressed in the High group.

ODF2 seems to be essential to ODF assembly [44], and an overexpression of this protein was related to defective sperm function [45] and loss of cell integrity [46]. ODF1 is assigned to the small Heat Shock Protein family (sHSP), as ODF1/HSPB10 [47]. sHSPs are related to protection against cellular stress, including oxidative stress [48], negative regulation of apoptosis [49], and aggregation of unfolded proteins and its refolding [50]. Although ODF1 was not expected to be exclusively expressed in the High group, we suggest that it might play an important role in protecting the viable sperm subset against possible oxidative damage in the ejaculate from this group, as a response to surrounding injury.

Among the functions of HSPs already described, protein folding stands out, because it was also conserved between

Table 2 Exclusively or overexpressed proteins in viable sperm from the High sperm DNA fragmentation group

Symbol	Uniprot AC	Mean (ng/column)		Fold-change ^a	p
		Low	High		
ACR	P10323	0.824	1.208	1.5	0.021
ELSPBP1	Q96BH3	0.100	0.153	1.5	0.034
GSTM3	P21266	0.623	1.024	1.6	0.021
H3F3A	P84243	0.857	1.402	1.6	0.021
HIST1H2AH	Q96KK5	0.060	0.081	1.4	0.050
LTF	P02788	0.770	1.631	2.1	0.021
TGM4	P49221	0.841	2.251	2.7	0.021
TUBA8	Q9NY65	0.358	2.868	8.0	0.050
TUBB3	Q13509	0.018	0.165	9.3	0.034
VDAC2	P45880	0.296	0.422	1.4	0.021
VDAC3	Q9Y277	0.270	0.423	1.6	0.021
APOC3	P02656	-	0.098	Exclusive from High	-
CALM	P62158	-	0.166	Exclusive from High	-
CRISP1	P54107	-	0.218	Exclusive from High	-
CS	O75390	-	0.164	Exclusive from High	-
FN1	P02751	-	3.613	Exclusive from High	-
HSP90AA4P	Q58FG1	-	Identified	Exclusive from High	-
IPO5	O00410	-	0.195	Exclusive from High	-
ODF1	Q14990	-	0.076	Exclusive from High	-
PGC	P20142	-	0.231	Exclusive from High	-
SDHA	P31040	-	0.088	Exclusive from High	-
SPACA4	Q8TDM5	-	0.099	Exclusive from High	-
ZBPB2	Q6X784	-	0.082	Exclusive from High	-

^a Positive fold-change (expression rate) values represent a greater expression in the High sperm DNA fragmentation group, while negative values show the greatest expression in the Low sperm DNA fragmentation group

Fig. 2 Protein-protein interaction networks including exclusively or overexpressed proteins from the Low and High sperm DNA fragmentation groups and conserved proteins. Identified proteins are represented as “V”s and quantified proteins, as triangles

Fig. 3 Overview of the main enriched Gene Ontology functions. **a** Conserved functions between the Low and High sperm DNA fragmentation groups; **b** Enriched functions in the Low sperm DNA fragmentation group; **c** Enriched functions in the High sperm DNA fragmentation group

the groups. Protein folding is important for protein stabilization, function and protection against degradation [51]. Refolding of unfolded or misfolded proteins by the endoplasmic reticulum is especially important during spermiogenesis and depends fully on the production of large quantities of HSPs [52]. The disruption of this process can lead to male infertility [53]. In our study, three HSP were overexpressed in the Low group and were involved with these processes: HSP90AA1, HSP90B1, and HSPA5.

Other HSPs were observed in this study, of which we highlight the conserved Heat Shock-Related 70 kDa Protein 2 (HSPA2). HSPA2 was suggested as an important protein for sperm-egg recognition and fusion during fertilization [54]. Lima et al. [55] demonstrated that adolescents with varicocele and unaltered semen when compared to age-matched controls presented an overexpression in the HSPA2 gene, not observed in adolescents with varicocele and altered semen. Furthermore, a recent review of Kovac et al. [40] suggests the HSPA2 protein as a fertility biomarker. These studies and the conservation of this protein in the High sperm DNA fragmentation group suggest that HSPs may indeed play a protective responsive role in male fertility, maintaining the fertilization potential.

This is further supported by the conservation of fertilization function, which is in accordance with compelling evidences showing that sperm DNA fragmentation does not

affect fertilization [12,13,56]. Two proteins involved with fertilization were uniquely expressed in the Low group: Sperm Surface Protein Sp17 (SPA17) and Sperm Equatorial Segment Protein 1 (SPESP1). On the other hand, the Zona Pellucida Binding Protein 2 (ZPBP2) was exclusively expressed in the High group, whereas other proteins were overexpressed in this group, such as Cysteine-rich Secretory Protein 1 (CRISP1), Epididymal Sperm-Binding Protein 1 (ELSPBP1), and Acrosin (ACR). This differential protein expression in the High group may act as a compensatory mechanism of the possible damages and alterations suffered by viable cells from an ejaculate of lower quality, allowing sperm fertilization capacity to remain intact. Furthermore, the conserved proteins A-Kinase Anchor Protein 4 (AKAP4) and Sperm Acrosome Membrane-Associated Protein 3 (SPACA3), involved with this process, were recently suggested as fertility biomarkers [40]. AKAP4 is present in the fibrous sheath of sperm flagellum and is involved with sperm motility and morphology and in GPDHS binding to fibrous sheath [57]. SPACA3 is an acrosome protein and is important to sperm-egg fusion during fertilization [58].

Fertilization function can be related to prostasomes, the main extracellular vesicular exosome in males, which can be found in large amounts in human semen. Prostasomes interact directly with sperm, modulating its function [59–61] and

Fig. 4 Detailed enriched Gene Ontology functions. **a** Conserved functions between the Low and High sperm DNA fragmentation groups; **b** Enriched functions in the Low sperm DNA fragmentation group; **c** Enriched functions in the High sperm DNA fragmentation group

protecting them against oxidative stress [62]. Human prostasomes can also be involved with sperm metabolism, because these vesicles express glycolytic enzymes [63]. All proteins observed in this study related to extracellular

vesicular exosome were conserved between Low and High groups, except Actin, cytoplasmic 1 (ACTB), Annexin A5 (ANXA5), and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which were differentially expressed in the Low

group. Thus, our results suggest that the presence of prostasomes in the High sperm DNA fragmentation group, as well as the presence of ODF1, might have a role in protecting viable sperm against oxidative stress.

L-lactate dehydrogenase activity and triglyceride catabolism were also conserved between both groups. L-lactate dehydrogenase activity is involved with gluconeogenesis and anaerobic glycolysis. All proteins related to this function were conserved between the groups, with the exception of L-lactate dehydrogenase A-like 6B (LDHAL6B), expressed uniquely in the Low group. Our finding regarding gluconeogenesis function is interesting given the lack of reports of this process in mammalian spermatozoa. Indeed, although one study described the presence of gluconeogenesis in dog sperm to support motility and capacitation [64], there is a lack of reports in other species, including human. Mukai and Okuno [65] suggest the presence of gluconeogenesis in mouse sperm, however, the results were not conclusive. On the other hand, Marin et al. [66] showed the absence of gluconeogenesis in boar spermatozoa.

Nevertheless, anaerobic glycolysis seems to be the most important source of energy to human sperm, and is important to sperm motility [67,68], capacitation [69], and fertilization ability [67]. Triacylglycerols are a source of fatty acids, which are also important for sperm metabolism [70,71]. Amaral et al. [72] have shown the association between the inhibition of a variety of enzymes involved with fatty acid beta oxidation and decreased human sperm motility, demonstrating its importance to sperm motility. In our study, the conserved protein Glyceraldehyde-3-Phosphate Dehydrogenase, testis-specific (GAPDHS), a glycolytic enzyme of the fibrous sheath involved with sperm motility [73], was involved with this function, in addition to spermatid development. This protein was suggested as a fertility biomarker by Kovac et al. [40]. Therefore, our results suggest the maintenance of gluconeogenesis, anaerobic glycolysis and triacylglycerol catabolism in viable cells of the High sperm DNA fragmentation group.

Therefore, the conserved functions demonstrate that basic processes for sperm functions, such as sperm metabolism, protection against oxidative damage, and fertilization, remain intact in viable sperm from an ejaculate with high sperm DNA fragmentation and are not involved in the associated male infertility.

Enriched functions in the low sperm DNA fragmentation group

The main enriched functions revealed in this group are glycolysis, gluconeogenesis, bisphosphoglycerate mutase activity, cytochrome-c oxidase activity, pyruvate metabolism, sperm flagellum and capacitation, regulation of proteasomal ubiquitin-dependent protein catabolic process, protein folding, response

to unfolded protein, regulation of interleukin-1 beta production, NuA4 histone acetyltransferase complex, protein kinase regulation, and protein import into mitochondrial membrane.

The hyper-representation of glycolysis, gluconeogenesis, and the related bisphosphoglycerate mutase activity was expected, because these processes were also observed among the conserved functions. Cytochrome-c oxidase activity and pyruvate metabolism are involved with mitochondrial oxidative phosphorylation. Although its specific role for ejaculated sperm energy generation is not yet defined, some studies suggest that aerobic respiration is involved with normal sperm function [74], spermatogenesis [75], capacitation [76], and maintenance of motility [77]. However, Narisawa et al. [78] demonstrated that if this pathway is defective in mice, fertilization is not altered, and motility is reduced, but not suppressed. Furthermore, Ferramosca et al. [79] suggest that mitochondrial oxygen uptake during respiration may be correlated with sperm DNA integrity, which can be supported by our results, because this pathway was not observed in the High sperm DNA fragmentation group. The exclusive protein Solute Carrier Family 2, facilitated glucose transporter member 14 (SLC2A14), and the overexpressed protein Phosphoglycerate Kinase 2 (PGK2) were related to these processes and previously suggested as fertility biomarkers by Kovac et al. [40]. SLC2A14 is a glucose transmembrane transporter that may act specifically during spermatogenesis, being important for glucose intracellular metabolism [80]. PGK2 is important for glycolysis and was observed in the seminal plasma from fertile men by Batruch et al. [58].

Additionally to aerobic respiration, pyruvate metabolism is also involved with triacylglycerols biosynthesis. This process is important for sperm membrane remodeling during capacitation [70]. Therefore, our results indicate that although viable sperm from the High sperm DNA fragmentation group can obtain energy through anaerobic glycolysis and fatty acid beta oxidation, the oxidative phosphorylation and triacylglycerol turnover are defective. This can be a consequence of oxidative stress occurring in the ejaculate from this group, which can alter sperm mitochondrial activity [81]. Because mitochondria are usually the first site for alterations triggered by oxidative stress [81], this can be occurring in the High group even in the presence of some proteins involved with sperm protection, such as ODF1 and prostasomal proteins. These impaired processes might affect sperm function, especially regarding capacitation, leading to the related negative impact on human reproduction. This can be confirmed by the hyper-representation of sperm capacitation and flagellum in the Low group. The overexpressed protein Prolactin-inducible Protein (PIP), an acrosomal protein that plays an important role in fertilization [82], was overexpressed in fertile men [83] and has been suggested as a potential fertility biomarker [40].

Proteasomal ubiquitin-dependent epididymal protein degradation is important for spermatogenesis [84], fertilization [85–87], and removal of DNA-damaged spermatozoa [88]. Furthermore, the response to unfolded proteins not only includes the protein refolding, a function conserved between the Low and High groups, but also the degradation of unfolded proteins by the ubiquitin system [89]. Clusterin (CLU) was the main overexpressed protein in the Low group related to response to unfolded proteins and protein folding. CLU is involved with sperm maturation in rats [90], apoptosis [91], cell-cell interaction in rams [92], and complement inhibition [93], with consequent inhibition of sperm lysis [94]. Furthermore, CLU also prevents protein precipitation [95] and the agglutination of altered sperm [96]. Thacker et al. [83] observed overexpression of this protein in sperm from fertile men. CLU was suggested as a fertility biomarker in bulls [95], stallions [97], and humans [40]. Taken together, these results suggest that protein ubiquitination might be altered in the High sperm DNA fragmentation group, leading to defective removal of sperm with damaged DNA and incomplete response to unfolded proteins. Because altered response to unfolded proteins can lead to sperm apoptosis [53], we can suppose that viable sperm from an ejaculate with higher sperm DNA fragmentation can (i) initiate the following apoptosis process, but suffer abortive apoptosis and be ejaculated, or (ii) be affected with the apoptosis of surrounding spermatids during spermiogenesis, because spermatids are interconnected [52].

Another interesting finding revealed herein was the enrichment of interleukin-1 production function. Indeed, there is a lack of knowledge about its production by differentiated spermatozoa and its function in reproduction. Huleihel et al. [98] demonstrated that germ cells at different stages of development and differentiation may produce and release cytokines to regulate spermatogenesis in a paracrine or autocrine manner. Furthermore, Austgulen et al. [99] suggest that sperm cells may be a source of cytokines production during embryo growth and implantation. These data suggest the presence of proteins involved in cytokines production in ejaculated sperm of subjects with Low DNA fragmentation. Thus, our results strongly indicate that the sperm with damaged DNA may have these set of functions altered, leading to an impaired sperm function.

The last hyper-represented function was NuA4 histone acetyltransferase complex, which is important prior to chromatin packaging in spermiogenesis. DNA packaging is an important step to protect sperm DNA against external damages [4]. Our results are in agreement with the hypothesis of chromatin compaction failure to explain the occurrence of sperm DNA fragmentation [6], because the acetyltransferase function was not observed in viable cells from the higher sperm DNA fragmentation ejaculate.

Therefore, here we suggest that viable sperm from an ejaculate with higher sperm DNA fragmentation may have some biological processes and important proteins altered, which might lead to several alterations in their function. All the differentially expressed proteins observed herein constitute potential fertility biomarkers, especially those previously suggested.

Enriched functions in the high sperm DNA fragmentation group

Hyper-represented functions in this group are porin activity, cellular detoxification, acrosome assembly, and aspartic-type endopeptidase activity. Proteins related to porin activity (VDAC2 and VDAC3) found overexpressed herein have been shown to be involved with inhibition of apoptosis [100], energy metabolism [101], bovine motility [102] and spermatogenesis, maturation and fertilization processes [101]. A recent study from Liu et al. [101] showed that the increase in VDAC2 expression compensates defects in sperm motility. Thus, the overexpression of these proteins in viable sperm from the High DNA fragmentation group suggests possible proteome alterations to compensate defects in sperm motility and/or maturation. Moreover, following the findings of Cheng et al. [100], this result suggests the inhibition of mitochondrial apoptosis pathway, which suggests an altered mechanism of sperm selection, explaining the ejaculation of sperm with damaged DNA. This corroborates the abortive apoptosis hypothesis for sperm DNA fragmentation occurrence [7].

Cellular detoxification is important for maintenance of cell redox homeostasis and, consequently, for sperm defense against oxidative stress [103,104]. Oxidative stress has been suggested as one of the major cellular mechanisms involved in male infertility [105]. Oxidative stress affects the sperm membrane through lipid peroxidation [106], leading to loss of membrane stability and selective permeability, which, in turn, can cause mitochondrial dysfunctions [81], defective acrosome reaction [107], and sperm DNA fragmentation [108]. Hence, our results clearly show that viable sperm from an ejaculate of lower quality possesses protective mechanisms against the effects of the surrounding oxidative stress. These mechanisms may be reduced in the sperm subset presenting DNA fragmentation.

The acrosome assembly function was related to the protein ZPBP2, exclusively expressed in this group and also involved with the conserved fertilization function, demonstrating its importance to the maintenance of sperm function in viable sperm from an ejaculate with high sperm DNA fragmentation. ZPBP2 is a soluble acrosomal protein, located at the region firstly exposed during acrosome reaction [109]. Lin et al. [109] demonstrated in mice that the loss of this protein can lead to sperm head alterations, and decreased, but not absent,

fertilization. Moreover, the authors have shown a structural role of this protein during spermiogenesis. Although this protein was not expected to be exclusively expressed in the High group, we hypothesized that it might be present in low, undetectable amounts in the Low group, being overexpressed and exclusively detected in the High group. This might be a compensatory mechanism to increase the affinity of sperm-zona pellucida interaction, and, thus, maintain the fertilization function [110].

Finally, the aspartic-type endopeptidase activity was hyper-represented due to the exclusively expressed protein Pepsinogen C (PGC). Although there are no reports of the presence of this protein in sperm, a few studies have identified PGC in human seminal plasma, originating from the prostate and seminal vesicles [111,112]. The function of PGC in semen was not yet elucidate, but it seems that the inactive PGC is converted into the active pepsin protein at the acidic pH of the vagina (below 5.0). This suggests a local function of PGC in ejaculated sperm, probably in the prevention of immune female responses [112]. Diamandis et al. [111] also found a correlation between seminal PGC levels, sperm motility, and glucose concentrations.

Finally, two study limitations which should be commented refer to protein extraction and results extrapolation. We were not able to extract protein satisfactorily from all the processed sperm samples, but only to about half of them. Improving protein extraction from these samples is of great importance if these results are to be further applied to a routine clinical environment. Moreover, as a hypothesis-driven shotgun proteomics experiment, our main results are further hypotheses (enriched functions) and protein targets for confirmatory studies. Therefore, results extrapolation should be made with this in mind.

In conclusion, the viable sperm subsets of ejaculates from low and high sperm DNA fragmentation rates indeed do present a differential protein expression. This differential proteome revealed to be associated with several alterations in post-genomic pathways, especially those associated with sperm metabolism, function, and protection against oxidative stress. The exclusively or overexpressed proteins related to the hyper-represented processes demonstrated herein, both in the Low and High sperm DNA fragmentations groups, are potential biomarkers of the surrounding damage associated with sperm DNA fragmentation in the raw sample.

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Conflict of interest The authors declare that they have no conflict of interest.

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