



# Elucidating the processes and pathways enriched in buffalo sperm proteome in regulating semen quality

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## Abstract

Sperm carries a reservoir of proteins regulating the molecular functions to attain functional competence. Semen samples collected from buffalo bulls were assessed for sperm functional attributes ( $n = 11$ ) and proteome profiling ( $n = 6$ ). Sperm proteins were extracted and profiled by employing LC–MS/MS. Overall, the buffalo sperm contained 1365 proteins, of which 458 were common between the groups. The unique proteins were 477 and 430 in good and poor quality semen, respectively. In the whole proteome of buffalo sperm, sexual reproduction with phosphatidylethanolamine-binding protein1 (PEBP1), fetuin-B (FETUB) and acrosin (ACR) was the most enriched ( $p = 8.44E-19$ ) biological process, also with thermogenesis ( $p = 0.003$ ), oocyte meiosis ( $p = 0.007$ ) and vascular smooth muscle contraction ( $p = 0.009$ ) apart from metabolic pathways. In good quality semen, mesenchyme migration ( $p = 1.24E-07$ ) and morphogenesis ( $p = 0.001$ ) were abundant biological processes. In good quality semen, the fluid shear stress ( $p = 0.01$ ) and, in poor quality semen, valine, leucine and isoleucine degradation ( $p = 3.8E-05$ ) pathways were enriched. In good quality semen, 7 proteins were significantly ( $p < 0.05$ ) upregulated and 33 proteins were significantly ( $p < 0.05$ ) downregulated. On validating the abundantly expressed sperm proteins, serine protease inhibitor Kazal-type 2-like (SPINK2; 2.17-fold) and neddylin (NEDD8; 1.13-fold) were upregulated and YBX2 was downregulated (0.41-fold) in good quality semen as compared with poor quality semen (1-fold). The present findings revealed the importance of sperm proteins in oocyte maturation, fertilization process and early embryonic development. The variations in the proteomic composition can be used as potential markers for the selection of breeding bulls.

**Keywords** Buffalo · Sperm proteome · Sperm pathways · Semen quality · Fertility

## Introduction

Globally, the reproductive efficiency in humans and dairy animals has been declining for the past 50 years. The reasons for the low reproductive efficiency are multifactorial but male factors and idiopathic causes influence the fertility in approximately 50–60% of the breeding population (Agarwal et al. 2015). Breeding bulls are considered to be superior if the seminal biomolecules aid the sperm to attain functional competence for successful fertilization and embryonic development. Semen samples harbor signature proteome, transcriptome and metabolome for the reproductive success. The bio-molecular compositions of the sperm vary in quantity and quality among the bulls of different fertility status (Somashekar et al. 2017). In this regard, it is imperative to understand the composition and biological functions of all the biomolecules especially proteins in

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semen samples. Recent developments in new generation omics technologies coupled with bioinformatics have helped researchers in understanding the role of sperm biomolecules in the establishment of fertility.

Sperm are terminally differentiated, transcriptionally and translationally silent but are rich with proteins that determine the success of the sequential events of fertilization in the female reproductive tract. In humans (Jodar et al. 2016) and animals such as bulls (Somashekar et al. 2017), goat (Pinto et al. 2019), sheep (Zhu et al. 2020) and pig (Roca et al. 2020), proteomic profiling has been used for assessing male sperm functional competence and fertility status.

Successful breeding of buffalo is a major concern as there are wide variations in the semen quality among bulls (Verma et al. 2015). The selection of bulls that produce good quality semen is most important as one bull produces thousands of semen doses per year (Bhakat et al. 2011). Although a few buffalo sperm proteomic studies have been carried out in the recent past (Selvaraju et al. 2016a, b; Codognoto et al. 2018; Hou et al. 2019; Aslam et al. 2019; Fu et al. 2019), an in-depth quantitative proteomic profile has not been conducted concerning the semen quality. Only the differentially expressed protein spots between high and low fertile buffalo sperm (MALDI–TOF/MS) analysis revealed that the proteins associated with energy metabolism and capacitation factors are overexpressed in low fertile sperm (Aslam et al. 2019). The whole protein profiling of sperm is needed for elucidating the molecular processes that are important for the establishment of fertilization and pregnancy. Although in the normozoospermic buffalo, sperm protein profiles have been conducted and identified ODF2, AKAP4 and TUBB were the abundant proteins involved in transport, phosphorylation and macromolecule localization (Fu et al. 2019), the proteomic differences between the good and poor quality semen have not been recognize. Identification and quantification of such signature proteins in sperm elucidate not only the proteomic differences between the semen samples varying in quality and also the processes and pathways determining the success of the fertilization. Hence, the present study aims (i) to understand the functional proteomics of sperm proteins in general and (ii) to identify the signature proteins that are determining the sperm functional competence between the good and the poor quality buffalo semen samples.

## Materials and methods

### Sample collection and grouping

Neat and frozen semen samples were obtained from buffalo bulls (*Bubalus bubalis*,  $n = 11$ ), aged between 4 and 6 years (four ejaculates from each bull), maintained

at the Central Frozen Semen Production and Training Institute, Hessarghatta, Bengaluru, India. The animals were initially selected based on semen production capacity as determined by semen volume, sperm concentration per milliliter and total sperm production per ejaculate. From the neat semen samples, 0.5 mL was aliquoted for protein profiling. Another aliquot (0.25 mL) was subjected to sperm functional parameters analyses. The remaining semen sample was diluted with Tris-egg yolk extender, frozen using a programmable biological freezer.

In the neat semen samples, plasmalemma integrity (live), acrosomal integrity, mitochondrial membrane potential and chromatin distribution were assessed. In the post-thaw semen samples, sperm kinematics, plasmalemma integrity, acrosome integrity, mitochondrial membrane potential and chromatin distribution were assessed. Based on the sperm functional tests, the semen samples were scored. The samples were grouped according to the percentage of the positive cells obtained in each test. The semen samples with higher than average values for each functional parameter were scored as good quality semen ( $n = 6$ ) and less than average were scored as poor quality semen ( $n = 5$ ). The semen samples from three animals having the highest scores from the good quality and three animals having the lowest scores from the poor quality semen producers were selected for the LC–MS/MS study (Fig. 1).

## Assessment of sperm functional parameters

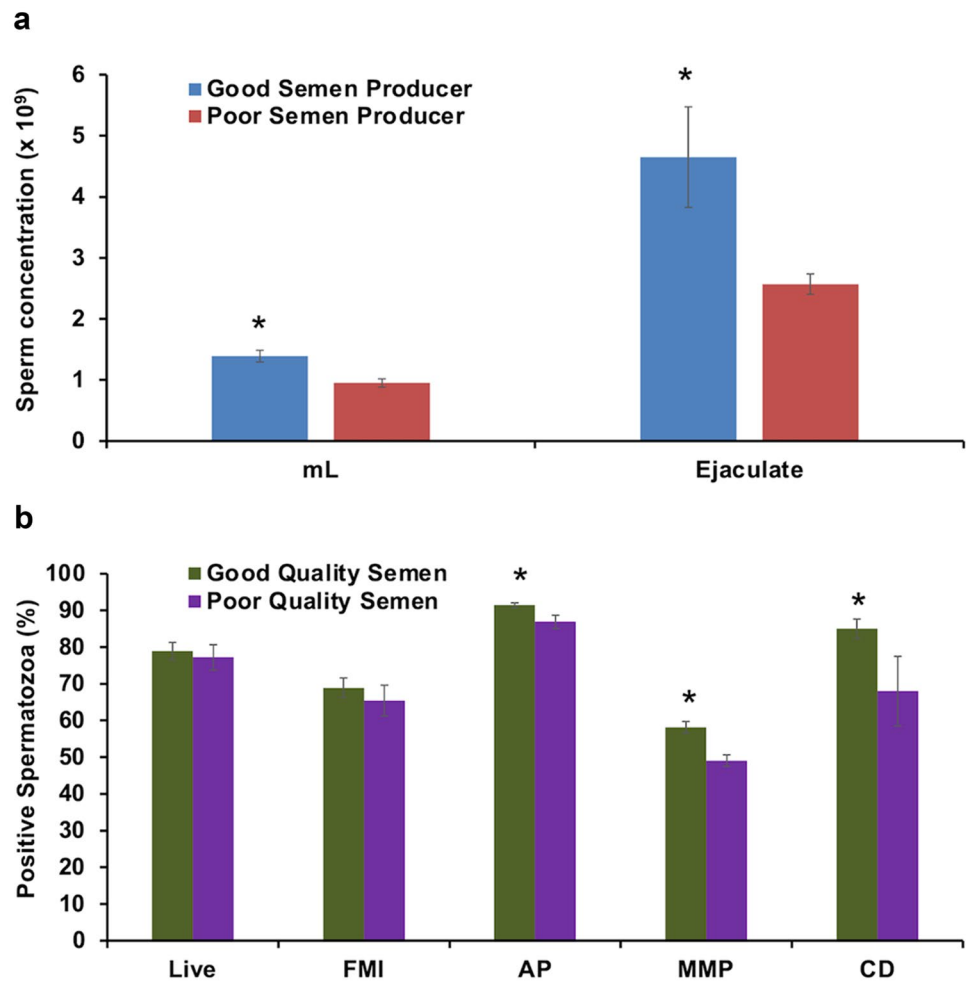
### Sperm kinematic analysis

Sperm kinetic parameters were analyzed using CASA (Sperm Class Analyzer; Microptic, Barcelona, Spain) as described (Selvaraju et al. 2016b). In brief, the cell concentration was maintained at  $7 \times 10^6/\text{mL}$  in Tris buffer (Tris, 274.1 mM; glucose, 55.5 mM; citric acid, 87.08 mM; pH, 6.8) for analysis. The diluted sample (8  $\mu\text{L}$ ) was placed on a prewarmed glass slide (37 °C) and covered with an 18  $\times$  18 mm coverslip. At least 10 different fields with a minimum of 500 sperm in each field were analyzed with the following set parameters: Speed type - VCL, cell size between 5 and 70  $\mu\text{m}^2$  and sperm with  $< 10$ , 10–35, 35–60 and  $> 60 \mu\text{m/s}$  were classified as immotile, slow, medium and fast motile, respectively. Total motile -  $> 10 \mu\text{m/s}$ , progressive forward motility -  $> 60\%$  straightness index and circular -  $< 50\%$  linearity index. All the parameters were analyzed after deleting the non-sperm objects in each field.

### Plasmalemma integrity

The percentage of plasma membrane intact cells was examined by using eosin (5%)–nigrosin (10%) stain (Selvaraju et al. 2016a). In brief, the sperm sample and

**Fig. 1** Selection of good and poor semen producer bulls. **a** The bulls were selected based on sperm production capacity. The sperm production capacity of the selected bulls differs significantly ( $p < 0.05$ ). **b** Sperm functional parameter analyses in neat semen and classification of good and poor semen samples. The sperm functional parameters such as live, functional membrane integrity (FMI), acrosomal integrity (AP), mitochondrial membrane potential (MMP) and chromatin distribution (CD) were assessed. The percentages of sperm positive for acrosomal integrity, mitochondrial membrane potential and chromatin distribution were significantly ( $p < 0.05$ ) higher in good as compared with poor semen samples



stain were mixed in a 1:1 ratio, allowed to react for 30 s and then smeared onto a glass slide and 200 sperm were assessed from different fields under 40 $\times$  phase contrast objective (Eclipse 80i, Nikon, Japan). The unstained sperm were considered as a structural membrane intact and stained sperm were considered as membrane-damaged cells.

### Functional membrane integrity

The percentage of sperm positive for functional membrane integrity was assessed using the HOS-G test as described earlier (Selvaraju et al. 2008). In brief, 50  $\mu$ L of the semen samples was added to 450  $\mu$ L of hypo-osmotic solution (150 mOsm) and 300 mOsm (control). The samples were incubated at 37  $^{\circ}$ C for 30 min; then, a drop of semen sample was kept on a glass slide and 200 sperm were assessed from different fields under 40 $\times$  phase contrast objective (Eclipse 80i, Nikon, Japan). A sperm was considered as functional membrane intact if the hairpin bend in the principal piece of the tail was present otherwise considered as functional membrane lost cells.

### Acrosome integrity

The semen sample was washed in 1 mL of Tyrode's albumin lactate pyruvate (SP-TALP) by centrifugation at 1000 rpm for 5 min and the sperm were smeared on the glass slide immediately. The slides were air-dried and fixed with methanol for 10 min, followed by washing thrice (2 min each) in freshly prepared phosphate-buffered saline with 0.6% bovine serum albumin. The staining stock was prepared by adding 8  $\mu$ L of fluorescein isothiocyanate conjugated with pisum sativum agglutinin (FITC-PSA; 5  $\mu$ g/mL) and 2  $\mu$ L of propidium iodide (PI; 75  $\mu$ g/mL) and volume was made up to 2 mL. The smear was overlaid with 50  $\mu$ L of freshly prepared staining solution and incubated in the dark for 20 min. Two hundred sperm were counted in a fluorescent microscope (Eclipse 80i, Nikon, Japan) with an excitation filter of 510–560 nm and a barrier filter of 505 nm. The sperm with evenly distributed acrosome were considered as acrosome intact and cells with unevenly distributed acrosome were considered as acrosome lost. The percentage of acrosome intact sperm was calculated from the total cells counted.

## Mitochondrial membrane potential

The mitochondrial membrane potential was assessed using JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethyl benzimidazolyl carbocyanine iodide) stain. One microliter of JC-1 (1.53 mM in dimethyl sulfoxide) was added to 50  $\mu$ L of Tris buffer (pH 6.8) and mixed well until the stain dissolved completely. Subsequently, 10  $\mu$ L of the semen sample was added to the stain mixture and incubated for 30 min in the dark at 37 °C. The samples were smeared onto the slide and the midpiece was observed in a fluorescent microscope (Eclipse 80i, Nikon, Japan) fitted with a barrier filter of 505 nm and excitation filter 510–560 nm. The sperm with yellow to orange color at the midpiece was considered as positive for mitochondrial membrane potential and with green color was considered as negative (Selvaraju et al. 2008).

## Chromatin distribution

Chromatin distribution was assessed by Feulgen's staining procedure (Selvaraju et al. 2008). In brief, the semen samples were smeared onto the glass slide and air-dried for 10 min, fixed in 10% neutral buffered formal saline (sodium phosphate monobasic, 33.3 mM; disodium hydrogen phosphate anhydrous, 45 mM; formaldehyde, 37%–100 mL; distilled water, 900 mL) for 30 min and followed by washing in running water. The slides were air-dried and incubated in 5 N HCl for 30 min, washed in running water twice for 5 min each and followed by incubation in Schiff's reagent for 30 min in the dark. The slides were then washed thrice with freshly prepared sulfite water (5 mL of 10% potassium meta bisulfite and 5 mL of 1 N HCl in 100 mL distilled water) for 2 min each. Two hundred sperm were counted under 1000 $\times$  in a phase-contrast microscope (Eclipse 80i, Nikon, Japan). Sperm with evenly stained chromatin were considered normal and the unevenly stained ones were considered abnormal.

## Protein isolation

The aliquot of neat semen samples designated for protein isolation was immediately added with phenyl methyl sulfonyl fluoride (100 mM) and centrifuged at 7000 $\times$ g for 10 min at 4 °C to separate the sperm from seminal plasma. The seminal plasma was removed and the sperm pellet was washed twice with TC buffer (Tris; 3.99 mM, CaCl<sub>2</sub>; 2.61 mM, sodium azide; 1.53 mM, pH 7.2) and resuspended in protein extraction buffer (TC buffer with 0.1 M phenyl methyl sulfonyl fluoride and 0.1% Triton X-100). The sperm-TC buffer with Triton X-100 was incubated at 4 °C for 1 h, with intermittent vortexing for a brief period at 5 min intervals. In the end, the samples were centrifuged at 1764 $\times$ g for 30 min at 4 °C (D'amours

et al. 2012; Somashekar et al. 2017); the supernatant containing protein was recovered and stored at – 80 °C for further LC/MS–MS work.

## Sample preparation by tryptic in-solution digestion and mass spectrometry analysis

The procedure employed for LC–MS/MS analysis of trypsin digested peptide fragments of ethanol precipitated protein sample was carried out as per the established procedure (Shevchenko et al. 2007). In brief, 20  $\mu$ g of the lyophilized sample was reconstituted in 100 mM ammonium bicarbonate buffer with 10 mM dithiothreitol followed by alkylation with 50 mM iodoacetamide (in the dark). The protein was then subjected to in-solution digestion with sequencing grade trypsin (50 ng/ $\mu$ L in 25 mM ammonium bicarbonate containing 10% acetonitrile). These samples were incubated at 37 °C for 16 h. The digested peptides were recovered by vacuum drying and reconstituted in 20  $\mu$ L of 0.1% (v/v) formic acid in 2% acetonitrile. One microliter of this sample was subjected to nano-UHPLC–MS/MS analysis. The ion source was electron spray ionization (ESI, nano-spray), fragmentation mode was collision-induced dissociation (CID, y and b ions), MS scan mode was FT-ICR/Orbitrap and the MS/MS scan in the range from 500 to 200 *m/z* using linear ion trap. For collision-induced dissociation (CID) MS/MS analysis, the doubly or triply charged ions were selected. Twenty femtomoles of standard BSA digest were also analyzed in parallel to the sequence to check the performance of the instrument.

## Protein identification

Peptide mass fingerprinting was performed on a proteome discoverer (Thermo Fusion V 1.4). For fixed and variable modifications, carbamidomethylation of cysteine residues and oxidation of methionine residues were selected, respectively. The percent mass error tolerance and fragment mass error tolerance were set to 12 ppm and 0.8 Da, respectively. The data were searched against the UniProt *Bubalus bubalis* database (a non-redundant database with reviewed proteins) and *Bos taurus* from NCBI. Proteins showing 1 or more than 1 peptide were considered for analysis. The false discovery rate (FDR) was kept very stringent (0.8%), which was verified manually. The corresponding search against the database was obtained for all the three animals from each group with a list of proteins along with its peak area-based quantification values, score, coverage, number of unique peptides, number of peptides, number of peptide sequence matches, molecular weight and the calculated pI.



## Label-free protein quantification

Raw LC–MS/MS data for all the replicates were imported into Progenesis QI for Quantitative Proteomics (Nonlinear Dynamics Limited, UK). The imported runs were quality-controlled by visual inspection of the 2D representations and automatically aligned to the most suitable reference run identified by the software. Considering the good initial alignment quality, the data set was not subjected to any further manual correction such as vector editing. Relative quantitation of all peptides was done with default settings of the software. After successful alignment, no further filtering was applied for subsequent quantification steps. After peak picking, no further filtering was applied so that all detected features were normalized to a reference run identified by the software. For peptide identification, data were searched against the combined target–decoy database specified above with the following parameters: digest reagent, trypsin; maximum missed cleavages, three; fixed modifications, carbamidomethyl; and variable modifications, deamidation N, deamidation Q and oxidation M. Search tolerance parameters were as follows: peptide tolerance,  $\pm 1.2$  Da; fragment tolerance,  $\pm 0.6$  Da; and FDR,  $< 1\%$ . Default settings (i.e., no protein grouping and quantitation from non-conflicting features only) were used for protein building.

## Bioinformatic analysis

To understand the role of proteins on sperm functions, total proteins observed in both groups were subjected to GO analysis and enrichment studies. To identify the functions of unique proteins in good and poor quality semen, the proteins present in at least two animals (to avoid the individual animal error) were selected. The functional profiling of total and unique proteins in sperm was carried out using PANTHER (<https://www.pantherdb.org>), whereas the enriched functions and pathways were analyzed using ShinyGo (v0.61) and STRING (version 11) with *Homo sapiens* as background. Enrichment analysis is calculated based on hypergeometric distribution followed by FDR correction. FDR less than 0.05 was considered as statistically enriched.

## Validation using western blot

Semen samples from different sets of buffalo bulls ( $n = 6$ ) were used for the validation study. Semen samples having  $> 90\%$  acrosome intact sperm in neat semen were considered as good ( $n = 3$ ), otherwise as poor ( $n = 3$ ) quality semen samples. Acrosome integrity was considered for validation study as this parameter was significantly different in neat and post-thaw samples of good and poor semen quality. Sperm proteins from good and poor quality

semen were isolated for western blotting. The abundantly as well as consistently expressed proteins in good quality sperm, namely SPINK2 and NEDD8 and significantly downregulated protein, YBX2, were validated using western blotting experiments. Since the sequences of the selected proteins were found to have similarities between the *Homo sapiens* and *Bubalus bubalis*, the antibodies raised against the proteins of *Homo sapiens* were used for the present study. The percentages of similarity between human and water buffalo proteins were 100%, 90.3% and 73.1% for NEDD8, YBX2 and SPINK2, respectively. For the SPINK2 protein, the epitope specificity was more than 80%. The protocol followed for western blotting of the sperm proteins is as described earlier (Somashkar et al. 2017). In brief, 20  $\mu\text{g}$  of protein from each animal was electrophoresed in 12% SDS gel. The gel was equilibrated for 20 min in a blotting buffer before electro-transfer and the proteins were transferred to PVDF membrane (0.2 microns) at 20 V, 70 mA at 4 °C for 3 h. After transfer, the membrane was washed with Tris buffer saline with Tween-20 (TBS-T) for 2 min and then incubated in a blocking buffer containing 5% bovine serum albumin at 4 °C for 1 h. The membrane was washed with TBS-T and incubated with primary antibodies such as mouse anti- $\beta$ -actin (DSHB, Cat. no.: 8-7A5, 1:1000 dilution, raised in mouse), rabbit anti-SPINK2 (Sigma, Cat. no.: HPA026813, 1:1000 dilution), rabbit anti-NEDD8 (Enzo Cat. no. ENZABS376, 1:2000 dilution) and mouse anti-YBX2 (DSHB, 1:1000 dilution) at 4 °C with constant rocking overnight. After incubation, the membrane was washed with TBS-T thrice within 10 min. Then, the membrane was incubated in HRP conjugated secondary antibodies such as goat anti-mouse for  $\beta$ -actin (1:2000 dilution), goat anti-rabbit for SPINK2 (1:2000 dilution), mouse anti-rabbit IgG for NEDD8 (1:3000 dilution) and goat anti-mouse IgG for YBX2 (1:2000 dilution) at room temperature (28 °C) for 1 h, followed by three washes (5 min each) in TBS-T. Before detection with immunocomplex, the membrane was washed with TBS and then, immunocomplex was detected by using the ECL-HRP substrate (SuperSignal West Femto, Thermo Scientific, USA). The membrane was incubated with a substrate for 5 min in the dark and the signal was captured by the chemi-documentation system (GBOX, iChemiXR, Syngene, UK). The bands were analyzed in ImageJ software (<https://imagej.nih.gov/ij/>). The  $\beta$ -actin (42 kDa) was used as normalizing control to obtain the relative expression of SPINK2, NEDD8 and YBX2 proteins in sperm.

## Statistical analysis

The sperm functional parameters were arcsine transformed before being subjected to statistical analysis. The level of

significance ( $p < 0.05$ ) was determined using the Student  $t$  test. The values are expressed as mean  $\pm$  SEM.

## Results

### Sperm functional parameters

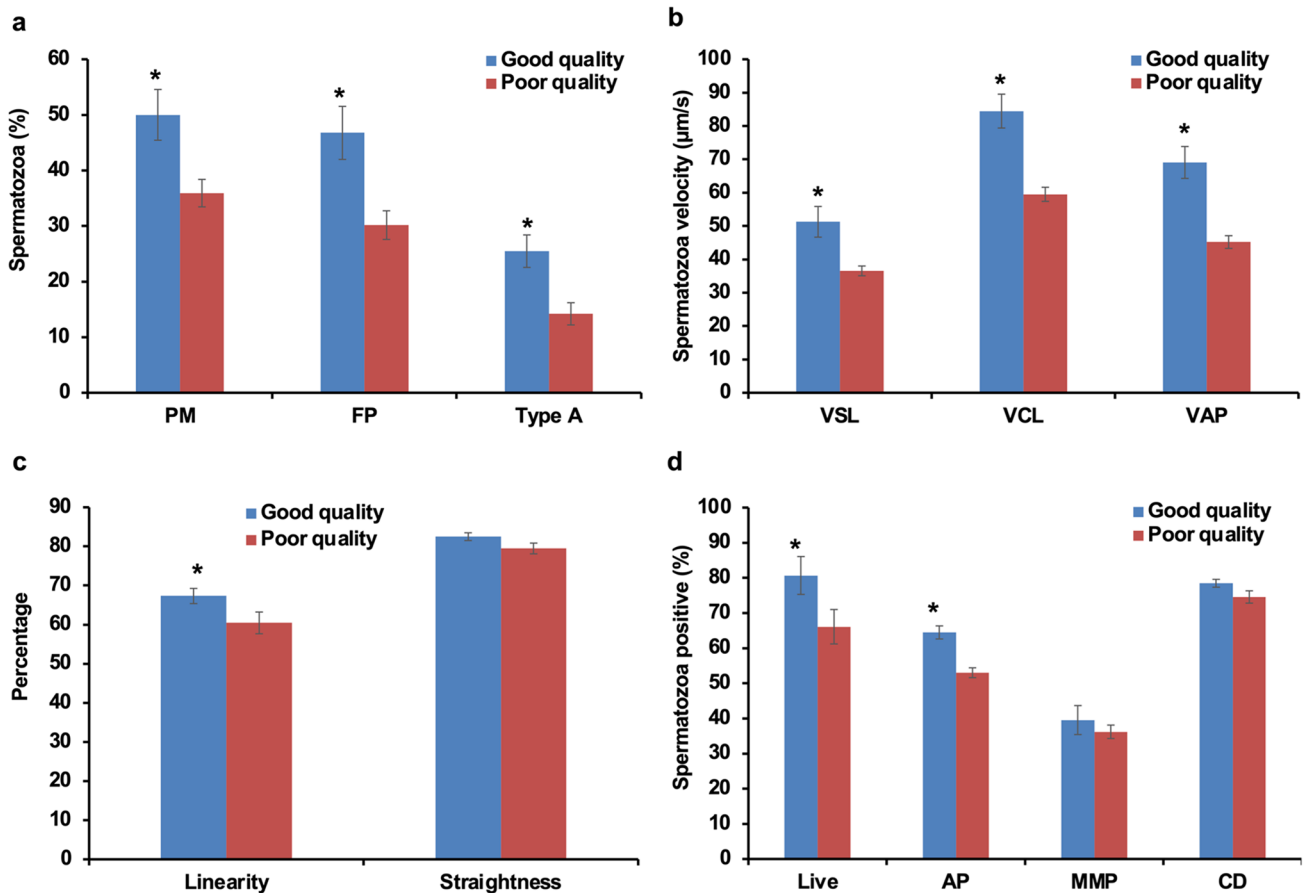
The sperm concentration and total semen production capacity were significantly higher in good as compared with poor semen producers (Fig. 1a). In the neat semen samples, the sperm positive for acrosome integrity, high mitochondrial membrane potential, and normal chromatin distribution were significantly ( $p < 0.05$ ) higher in good as compared with poor quality samples (Fig. 1b).

In the post-thaw semen samples, the percentages of progressive and type-A (Fig. 2a), curvilinear velocity and average path velocity (Fig. 2b) and linearity (Fig. 2c) were significantly ( $p < 0.05$ ) higher in good than poor quality samples. The percentage of straightness did not

differ significantly between groups (Fig. 2c). Though the percentages of sperm having high mitochondrial membrane potential and normal chromatin distribution did not differ significantly between groups, the percentage of acrosome intact sperm was significantly ( $p < 0.05$ ) higher in good as compared with poor quality semen samples (Fig. 2d).

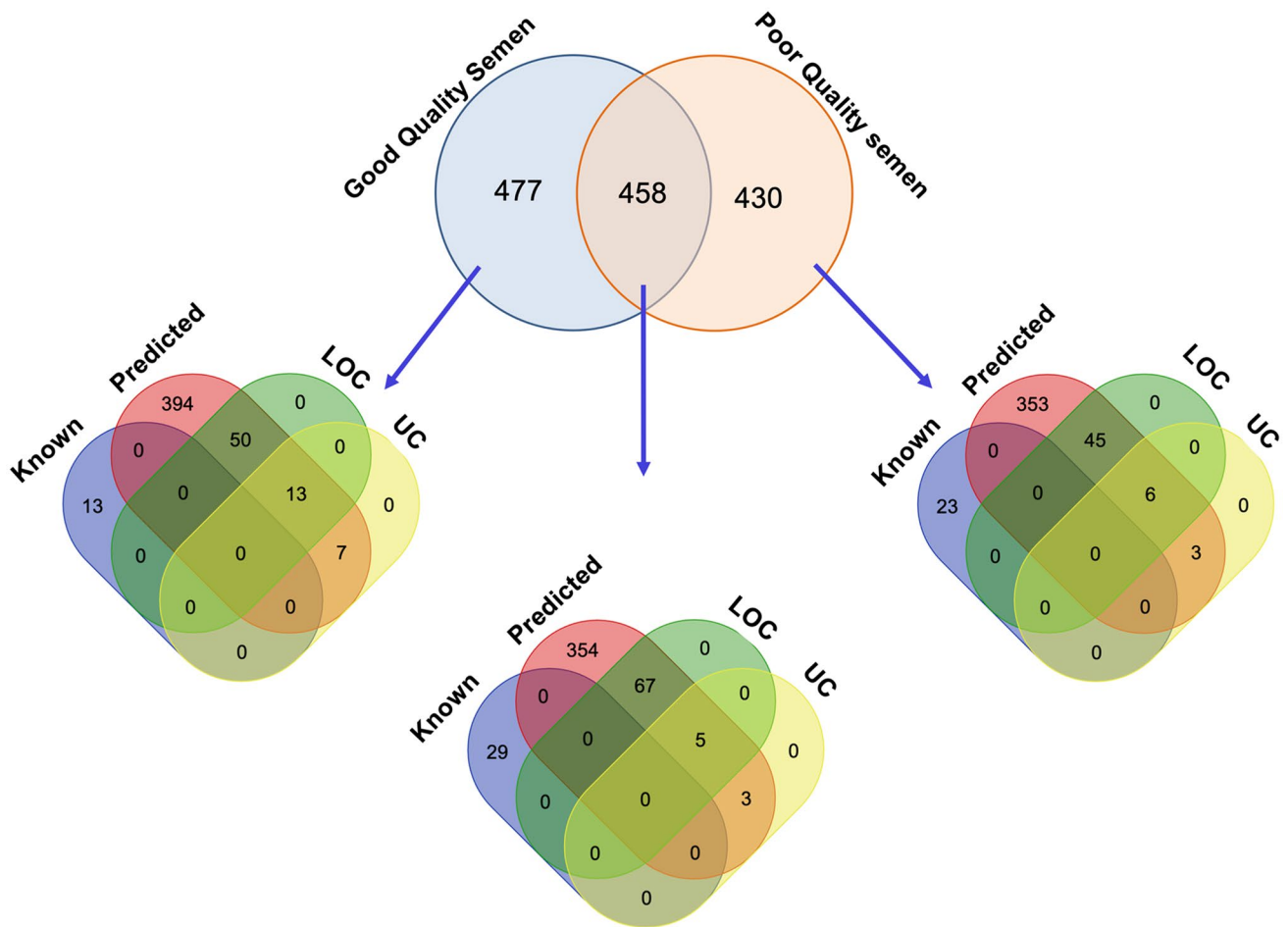
### Mass spectrometry analysis and protein identification

In total, 1365 proteins were identified from buffalo sperm, of which good and poor quality sperm had 935 and 888 proteins, respectively (Fig. 3). Out of 477 unique proteins in good quality sperm (Table 1), 13, 464 and 63 proteins were found to be known, predicted and LOC numbers, respectively (Supplementary data set 1). Correspondingly, out of 430 unique proteins in poor quality sperm (Table 2), 23, 407 and 51 proteins were found to be known, predicted and LOC numbers, respectively (Supplementary data set 2).



**Fig. 2** Sperm functional parameter analyses in frozen-thawed good and poor semen samples. The sperm functional parameters such as (a): progressive motility (PM), fast progressive motility (FP) and Type A; (b): straight-line velocity (VSL), curvilinear velocity (VCL)

and average path velocity (VAP); (c): linearity and straightness; (d): plasmalemma integrity (live), acrosomal integrity (AP), mitochondrial membrane potential (MMP) and chromatin distribution (CD) were assessed



**Fig. 3** Composition of sperm proteins in good and poor semen samples in buffalo. In total, 1365 proteins were identified and 458 proteins were common to both good and poor semen samples. The

majority of the proteins were of predicted in nature, followed by LOC, known and uncharacterized (UC) proteins

The number of uncharacterized proteins was 20 and 9 in good and poor quality semen, respectively.

## Processes, functions and pathways

### Total sperm proteome

GO analysis of the whole proteome of buffalo sperm revealed that the proteins associated with sexual reproduction (PEBP1, FETUB and ACR; enrichment FDR  $8.44E-19$ ), generation of precursor metabolites and energy (MDH1, CS, and PFKP; enrichment FDR  $1.03E-18$ ), multi-organism reproductive process (PEBP1, FETUB and ACR; enrichment FDR  $2.04E-16$ ), etc. were observed to be the enriched biological processes (Fig. 4). The molecular functions of these proteins were nucleoside phosphate binding (HSPA5, PFKP and RHOA; enrichment FDR  $7.32E-19$ ), apart from cell adhesion molecule binding (VCL, HSPA5 and PKM; enrichment FDR  $1.05E-05$ ) and microtubule motor

activity (DYNLL1, DNAH7 and DNAI1; enrichment FDR  $3.33E-05$ ). The proteins were enriched to be localized in the extracellular exosome (LAP3, AK2 and RALA; enrichment FDR  $3.77E-38$ ), sperm part (SPA17, ACRBP and SPACA1; enrichment FDR  $1.52E-27$ ), cilium (PROM1, CATSPERG and CEP164; enrichment FDR  $2.41E-25$ ), etc. The KEGG pathways associated with sperm proteins were metabolic pathways such as carbon metabolism pathway (CS, DLD and DLST; enrichment FDR  $7.93E-29$ ), glycolysis (GALM, ENO1 and FBP1; enrichment FDR  $6.35E-17$ ) and insulin signaling pathways (HK1, HK2 and PPP1CC; enrichment FDR 0.001), apart from thermogenesis (COX5B, ACSL6 and NDUFA8; enrichment FDR 0.003), oocyte meiosis (YWHAQ, SMC1B and PRKACA; enrichment FDR 0.007; Fig. 5), vascular smooth muscle contraction (NPPC, PPP1CC and PRKACA;  $p = 0.009$ ; Fig. 6), etc. The reactome pathways also identified various metabolic pathways such as cilium assembly (AKAP9, ALMS1 and CCT2; enrichment FDR  $4.74E-08$ ), innate immune system

**Table 1** Unique and abundantly expressed (top 20) buffalo sperm proteins in good quality semen samples

| Sl. no. | GI_ID     | Protein name | Protein description   | Score  | M.Wt  | pI   |
|---------|-----------|--------------|---|--------|-------|------|
| 1       | 594107701 | ENO1         | Predicted: alpha-enolase isoform X2   | 285.26 | 47.40 | 6.46 |
| 2       | 594076725 | ANG          | Predicted: ribonuclease 4-like  | 122.17 | 16.93 | 8.85 |
| 3       | 594106854 | LOC102396050 | Predicted: 14-3-3 protein zeta/delta-like   | 98.82  | 27.59 | 4.78 |
| 4       | 594112703 | HSP90AA1     | Predicted: heat shock protein HSP 90-alpha isoform X2                                   | 87.18  | 83.82 | 5.12 |
| 5       | 594116783 | LOC102413388 | Predicted: clusterin-like, partial  | 84.13  | 25.15 | 5.55 |
| 6       | 594077690 | LCN1         | Predicted: lipocalin-1-like   | 81.75  | 19.13 | 4.70 |
| 7       | 594078858 | ACTA1        | Predicted: actin, alpha skeletal muscle   | 70.87  | 42.02 | 5.39 |
| 8       | 594104736 | ATP5F1A      | Predicted: ATP synthase subunit alpha, mitochondrial isoform X1                         | 70.08  | 59.65 | 9.19 |
| 9       | 594040651 | MMAB         | Predicted: cob(I)yrinic acid a,c-diamide adenosyltransferase, mitochondrial             | 65.86  | 26.50 | 9.04 |
| 10      | 594036061 | PDIA3        | Predicted: protein disulfide-isomerase A3   | 63.01  | 56.92 | 6.81 |
| 11      | 594035119 | PROCA1       | Predicted: protein PROCA1 isoform X3  | 61.85  | 39.12 | 6.89 |
| 12      | 594060957 | LOC102391307 | Predicted: protein SCO1 homolog, mitochondrial-like                                     | 59.50  | 33.57 | 8.47 |
| 13      | 594067512 | SIAE         | Predicted: sialate O-acetyltransferase isoform X1                                       | 58.26  | 59.59 | 6.99 |
| 14      | 594054168 | PPIA         | Predicted: peptidyl-prolyl cis-trans isomerase A-like                                   | 57.99  | 17.86 | 8.16 |
| 15      | 594065672 | EFHC1        | Predicted: EF-hand domain-containing protein 1 isoform X1                               | 51.41  | 74.03 | 5.99 |
| 16      | 594067723 | NDUFA8       | Predicted: NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 8                 | 50.30  | 20.09 | 7.90 |
| 17      | 594073022 | LOC102392644 | Predicted: disintegrin and metalloproteinase domain-containing protein 25-like, partial | 49.36  | 85.29 | 7.49 |
| 18      | 594059346 | LYZL2        | Predicted: lysozyme-like protein 1  | 49.32  | 16.78 | 7.83 |
| 19      | 594115921 | DPY30        | Predicted: protein dpy-30 homolog isoform X2  | 45.81  | 11.24 | 4.88 |
| 20      | 594034621 | DYNLRB2      | Predicted: dynein light chain roadblock-type 2-like                                     | 44.24  | 10.84 | 7.44 |

**Table 2** Unique and abundantly expressed (top 20) buffalo sperm proteins in poor quality semen samples

| Sl. NO | GI_ID     | Protein name | Protein description  | Score  | M. Wt  | pI   |
|--------|-----------|--------------|--|--------|--------|------|
| 1      | 594077811 | TUBB4B       | Predicted: tubulin beta-4B chain isoform X2  | 277.66 | 50.09  | 4.86 |
| 2      | 594040972 | LETM1        | Predicted: LETM1 and EF-hand domain-containing protein 1, mitochondrial isoform X1 | 115.24 | 84.25  | 5.97 |
| 3      | 405113092 | HSP10        | 10 kDa heat shock protein, mitochondrial   | 112.61 | 10.94  | 9.23 |
| 4      | 594078319 | LOC102403160 | Predicted: uncharacterized protein LOC102403160                                    | 109.29 | 100.32 | 6.83 |
| 5      | 594056514 | TEX101       | Predicted: testis-expressed sequence 101 protein                                   | 105.56 | 27.35  | 6.18 |
| 6      | 594058952 | SOD1         | Predicted: superoxide dismutase [Cu-Zn] isoform X2                                 | 99.42  | 15.89  | 6.71 |
| 7      | 594115519 | LOC102396169 | Predicted: spermadhesin-1-like isoform X2  | 97.88  | 15.33  | 5.1  |
| 8      | 594095950 | GALM         | Predicted: aldose 1-epimerase isoform X2   | 87.52  | 37.6   | 6.52 |
| 9      | 594090763 | CATSPERG     | Predicted: cation channel sperm-associated protein subunit gamma isoform X1        | 83.35  | 145.9  | 6.42 |
| 10     | 595763350 | Lactoferrin  | Lactotransferrin precursor   | 74.73  | 77.6   | 7.99 |
| 11     | 594036745 | HSD17B10     | Predicted: 3-hydroxyacyl-CoA dehydrogenase type-2 isoform X2                       | 68.41  | 26.13  | 7.87 |
| 12     | 594093049 | LOC102405126 | Predicted: c-Myc-binding protein-like, partial                                     | 65.29  | 8.65   | 6.3  |
| 13     | 594094207 | CCT6A        | Predicted: T-complex protein 1 subunit zeta isoform X2                             | 65.27  | 53.72  | 6.81 |
| 14     | 594093848 | LOC102415514 | Predicted: nucleoside diphosphate kinase A 2-like isoform X3                       | 65.11  | 17.26  | 7.99 |
| 15     | 594036007 | LOC102400899 | Predicted: small conductance calcium-activated potassium channel protein 3-like    | 62.56  | 9.58   | 9.25 |
| 16     | 985567354 | FBP1         | Fructose-1,6-bisphosphatase 1  | 60.1   | 36.7   | 7.68 |
| 17     | 4699810   | LTF          | Chain A, structure of diferric buffalo lactoferrin at 2.5a resolution              | 60.03  | 75.7   | 7.94 |
| 18     | 594064812 | CCT8         | Predicted: T-complex protein 1 subunit theta isoform X2                            | 59.04  | 59.59  | 5.59 |
| 19     | 594037234 | EEF1G        | Predicted: elongation factor 1-gamma   | 59.02  | 50.31  | 6.74 |
| 20     | 594052951 | CRISP1       | Predicted: cysteine-rich secretory protein 1                                       | 58.25  | 28.12  | 6.86 |



(ABCA13, ACTB and AGA; enrichment FDR 4.45E–07), immune system (ABCA13, ALDOA and APOB; enrichment FDR 3.58E–05), cellular response to external stimuli (ATG4, CAPZA3 and CAPZB; enrichment FDR 5.15E–05), etc. as enriched in the sperm proteins.

It is also important to mention that the sperm carries proteins associated with Huntington's disease (COX5B, DNAH2 and DNAH8;  $p = 1.52E-05$ ), Alzheimer's disease (NCSTN, GAPDH and HSD17B10;  $p = 3.91E-05$ ) and Parkinson's disease (PARK7, HTRA2 and NDUFA8;  $p = 5.88E-05$ ).

### Unique protein analyses

The study revealed that mesenchyme migration (ACTA2, ACTA1 and ACTC1;  $p = 1.246E-07$ ), organelle organization (MTA2, RHOA and TIMM4;  $p = 5.917E-05$ ), tissue migration (RHOA, ATP5F1A and MACF1;  $p = 0.0013$ ), skeletal muscle satellite cell migration (RHOA and RHOC;  $p = 0.0013$ ), etc. were the unique enriched biological process in the good quality sperm (Table 3).

The good quality semen was enriched with the proteins involved in fluid shear stress (GSTM3, HSP90AA1 and RHOA;  $p = 0.01$ ), oxidative phosphorylation (NDUFA8, ATP5F1A and ATP5F1D;  $p = 0.02$ ), carbon metabolism (ENO1, HIBCH and IDH3A;  $p = 0.023$ ), vascular smooth muscle contraction (RHOA, ACTA2 and ACTG2;  $p = 0.023$ ) and thermogenesis (NDUFA8, ATP5F1A and ATP5F1D;  $p = 0.03$ ) pathways (Table 3). Importantly, the poor quality semen was enriched with proteins responsible for stress and the catabolic process. In the catabolic process, beta-oxidation of octanoyl-CoA to hexanoyl-CoA ( $p = 6.08E-08$ ) was significantly upregulated (Table 3). The pathways such as valine, leucine, and isoleucine degradation (HSD17B10, HADHA, HADH, ACADM;  $p = 3.80E-05$ ) and glycolysis (GALM, FBP1, PDHA1, PDHA2, PDHB;  $p = 6.9E-06$ ) were enriched in the poor quality semen (Table 3). In the good quality semen, the proteins responsible for the mitochondrial membrane organization, cilia development, microtubule-based process, antigen processing and thermogenesis were also enriched (Supplementary Table 1), whereas in the poor quality semen samples, such processes were not observed (Supplementary Table 2).

### Differentially expressed proteins

By employing the protein quantification methodology, a total of 497 proteins were differentially expressed (Supplementary Table 3). In good quality sperm, 136 proteins were upregulated and 361 proteins were downregulated. Among them, seven

proteins were significantly ( $p < 0.05$ ) upregulated and 33 proteins were significantly ( $p < 0.05$ ) downregulated (Table 4). The significantly upregulated proteins were functionally associated with spermatogenesis (SPINK2), motility (NIN), capacitation (PPP1CB) and other metabolic processes (ACAT1). While the proteins in good quality semen samples were associated with spermatogenesis and sperm function, the proteins involved in infertility (NCOA2), stress (HSP70) and glycolysis (SORDX1) were significantly downregulated (Table 4).

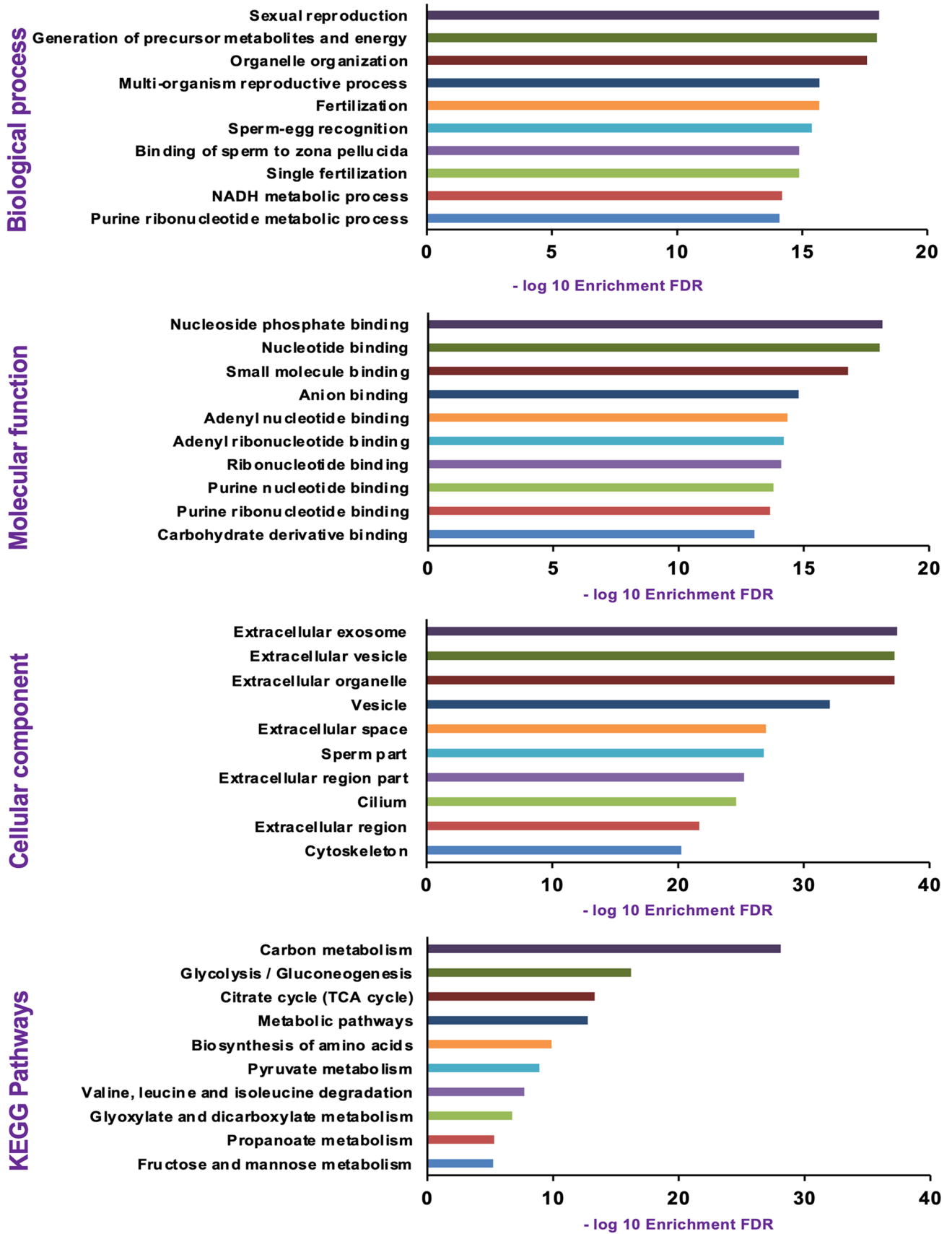
### Functional analysis of differentially expressed proteins

The upregulated proteins of the good quality semen were involved in the generation of precursor metabolites and energy (SDHA, ATP5F1 and ACAT1;  $p = 0.0008$ ), oxidation–reduction process (MDH2, QSOX1 and VAT1;  $p = 0.008$ ), fusion of sperm to egg plasma membrane involved in single fertilization (EQTN, IZUMO1 and LYZL6;  $p = 0.02$ ) and plasma membrane fusion (EQTN, IZUMO1 and LYZL6;  $p = 0.04$ ). Oxidoreductase activity (AKR1B1, SDHA and QSOX1;  $p = 0.02$ ) and adenylate kinase activity (AK1 and AK3;  $p = 0.014$ ) were the enriched molecular functions. The pathways such as calcium-dependent and PKA activations (PRKAR2A and PRKAR1B;  $p = 0.019$ ), fertilization (CATSPERG and IZUMO1;  $p = 0.025$ ) and post-translational protein phosphorylation (ALB, SPP1 and QSOX1;  $p = 0.03$ ) were enriched. The glycolysis (ALDOA, ENO1 and ENO2;  $p = 9.05E-15$ ), lysine catabolism pathways (ALDH7A1, DLD and DLST;  $p = 7.86E-05$ ) and fatty acid beta-oxidation (ACADM, ACOP13 and DDI;  $p = 4.4E-05$ ) were downregulated in these semen samples.

The poor quality semen samples were enriched with proteins involved in cellular component biogenesis (TEX11, DNALI1 and SRR) and the catabolic process. The protein–protein interaction study suggested that the proteins involved in the catabolic process were involved in valine, leucine and isoleucine degradation ( $1.54E-06$ ), lysine degradation ( $5.80E-05$ ), fatty acid degradation ( $3.82E-05$ ) and tryptophan metabolism ( $3.82E-05$ ). These proteins were identified to have protein domains such as the ubiquitin-like domain ( $8.11E-06$ ), 3-hydroxyacyl-CoA dehydrogenase and NAD binding domain ( $7.22E-05$ ).

### Validation with western blotting

Proteome analysis revealed that the proteins namely SPINK2 (10.34-fold) and NEDD8 (2.06-fold) were significantly ( $p < 0.05$ ) abundant in good quality semen (Fig. 7). SPINK2 and NEDD8 were upregulated (SPINK2 = 2.17-fold, NEDD8 = 1.13) and YBX2 was downregulated (0.41-fold) in good quality semen (Fig. 7).



**Fig. 4** Top 10 gene ontological functions and pathways enriched in the sperm proteins of buffalo. The majority of the proteins were associated with the reproductive process, metabolic function and located in the extracellular exosome. Among the pathways, carbon metabolism was enriched in the sperm proteins of buffalo

## Discussion

In the current study, buffalo sperm proteome was deciphered using LC–MS/MS coupled with bioinformatics. The whole sperm proteome analysis revealed that the sperm contains 1365 proteins. The existence of 477 and 430 unique proteins in good and poor quality semen, respectively, reflects the inherent differences in sperm functions that may influence the fertilizing capacity of the sperm and early embryonic development.

The sperm of both groups were enriched with proteins associated with spermatogenesis, fertilization and other reproductive processes apart from metabolic activities. The proteins involved in the biosynthesis of amino acids, glucagon, insulin signaling pathway and fatty acid degradation pathway were enriched in sperm indicating the essential role of energy metabolism for sperm kinematics and other functions. Sperm is a metabolically active cell requiring high energy for gaining motility to travel in the female reproductive tract and sequential events of fertilization especially penetration through cumulus cells and zona pellucida (Ruiz-Pesini et al. 2007). Glucagon-like peptide 1 receptor is expressed in human sperm and has a role in regulating sperm motility and cholesterol efflux (Parker et al. 2020). Insulin and leptin also improved sperm motility and acrosome reaction in mammals (Lampiao and Du Plessis 2008). The sperm motility, acrosomal reaction and sperm–oocyte fusion depend on the fatty acid profile (Collodel et al. 2020). Fatty acid degradation pathway occurs through an oxidation cycle and yields lower metabolites to generate energy for the sperm cellular function. However, further studies on the beneficial effects of different types of fatty acids and their metabolic pathways are warranted for assessing the type of fatty acid that undergoes denaturation during sperm metabolism (Collodel et al. 2020).

The sperm were also enriched to have proteins responsible for carbon metabolism, oocyte meiosis and vascular smooth muscle contraction. Carbon metabolic pathway aids in the transcriptional regulation via methylation. The role of the DNA methylation process in spermatogenesis is well known. Thus, carbon metabolism aids in the spermatogenesis and any mutations in the enzymes involved in the pathways are reported to cause DNA damage and affect normal fertility (Singh and Jaiswal 2013). In an earlier study from our group, we observed that the transcripts responsible for oocyte meiosis were also present in bovine sperm (Selvaraju et al. 2017). Though the sperm motility is important for the

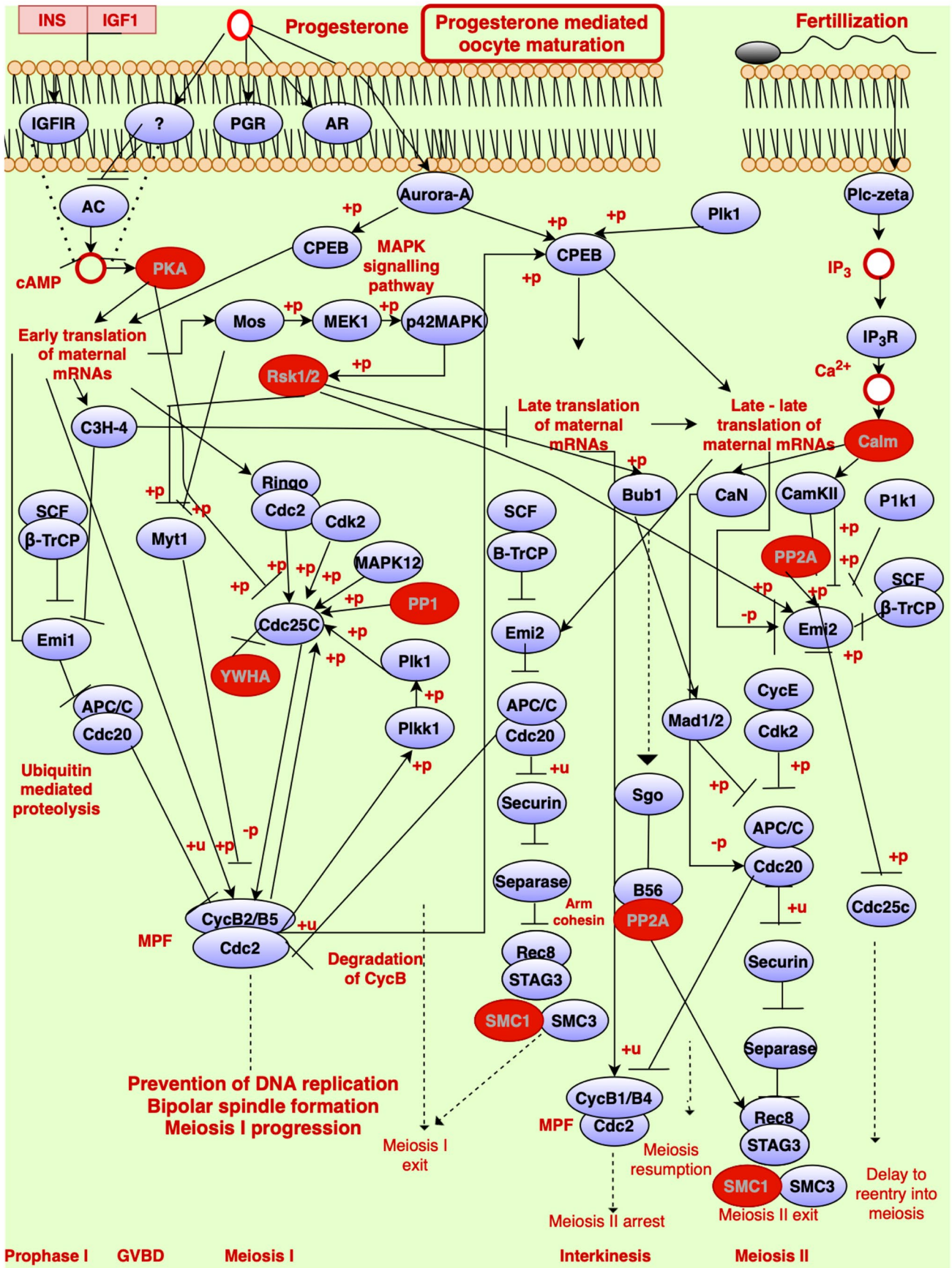
sperm fertilization process, maintenance and stimulation of sperm motility and ability to reach the site of fertilization are favored by contraction of vascular smooth muscles in the uterus and fallopian tube (Wanggren et al. 2008). In the present study, we observed that the proteins, such as NPPC present in the sperm, have the property of stimulating sperm motility and vascular smooth muscle contraction, which may facilitate the sperm to reach the site of fertilization. In mice, NPPC protein was also abundantly expressed in oviducts and attracts the sperm towards oocyte for fertilization (Kong et al. 2017). The sperm were also enriched with disease-associated proteins such as PARK7. PARK7 is a stress-responsive protein that protects the cells against ROS and mitochondrial damage (Sun et al. 2014) and as reviewed by Agarwal et al. (2020), a decrease in this protein expression is associated with oligozoospermia in humans. Reactome pathways also identified various metabolic pathways including the proteins responsible for immune regulation. A recent review (Archana et al. 2019) suggested that the proteins in semen and sperm provide immune tolerant mechanisms for protecting the sperm and developing an embryo from immunological reactions in the female reproductive tract. The molecular function indicates that the proteins are mainly involved in DNA or RNA binding. The proteins that bind to the DNA are transcription factors that can either act to repress gene activity or regulate biological functions. Thus, the majority of the sperm proteins were found to be involved in transcription and translation-related functions.

The localization of sperm proteins was mainly observed to be in extracellular exosomes. Exosomes, the nano-sized membrane vesicles, carry a cargo of proteins, lipids, microRNAs and mRNA. They are involved in intracellular communication. Exosomes transfer proteins to the sperm during their epididymal transit, help sperm in acquiring the fertilizing ability and protect it against the oxidative stress. Proteins associated with prostasomes were reported to regulate capacitation, thus avoiding the premature induction of acrosome reaction (Baskaran et al. 2020).

In the present study, mesenchyme migration was enriched in the unique proteins of good quality semen. Mesenchyme migration is an essential process during early embryonic development, wherein it migrates from the blastocyst to the endometrial layer for placental attachment. The presence of proteins associated with mesenchymal migration in sperm suggests that these proteins might be involved in early embryonic development (Hay 2005). Though the contribution of sperm protein on early embryo development has been reported, so far, no previous reports have suggested that sperm proteins are involved in mesenchymal migration during embryonic development.

In the good quality semen, fluid shear stress, vascular smooth muscle contraction, thermogenesis and amino





**Fig. 5** The oocyte meiosis pathway is enriched in buffalo sperm proteins. Sperm proteins, such as CALM and PP2A, may take part in the oocyte meiosis pathway. (The observed proteins in the sperm were highlighted in the red oval box; the pathways are re-created based on the KEGG database, Kanehisa Laboratories)

acid synthesis pathways were uniquely present. The fluid shear force can either positively or negatively affect sperm function. But, in the present study, the presence of fluid shear force proteins observed in the good quality semen suggests a positive influence on semen quality. The protein involved in fluid shear force, GSTM3, is secreted from the seminal vesicle (Westfalewicz et al. 2017) and the presence of GSTM proteins in the sperm head the reported to facilitate sperm–zona binding (Hemachand et al. 2002) and may improve fertility.

Importantly, biosynthesis of amino acids was enriched in good quality semen, whereas valine, leucine and isoleucine degradation pathways were enriched in the poor quality semen samples. These essential amino acids promote protein synthesis, signaling pathways and metabolism of glucose. The degradation of these amino acids may lead to oxidative stress and poor semen motility (Bahadorani et al. 2019; Huang et al. 2019). The expression of proteins in the amino acid degradation pathway might also be attributed to the spontaneous acrosome reaction in sperm resulting in poor fertilizing ability (Moos et al. 1993).

The upregulated differentially expressed proteins of the good quality semen were involved in sperm metabolic activity such as the generation of precursor metabolites and the energy oxidation–reduction process, apart from the fusion of sperm to egg plasma membrane involved in single fertilization, wherein equatorin and IZUMO1 proteins are involved. The equatorin protein present in the sperm head equatorial region is involved in sperm–oocyte binding (Toshimori 1998). IZUMO1 is present in the acrosome membrane and translocated to the plasma membrane after the acrosome reaction (Satouh et al. 2012) and the sperm from the IZUMO1 knockout males had a fusion defect with the vitelline membrane (Inoue et al. 2005). Besides, the proteins related to oxidoreductase activity and pathways such as PKA activation and post-translational protein phosphorylation were enriched indicating the importance of these proteins for sperm metabolism and competence in counteracting the stress. The post-translational protein phosphorylations are essential for the capacitation process apart from controlling sperm motility, cytoskeleton assembly, ionic current regulation and receptor regulation (Visconti and Kopf 1998). One of the proteins involved in post-translational protein phosphorylations, the SPP1, stimulates cell–cell adhesion, increases cell–extracellular matrix communication, promotes migration of immune cells and decreases cell death by reducing reactive oxygen species

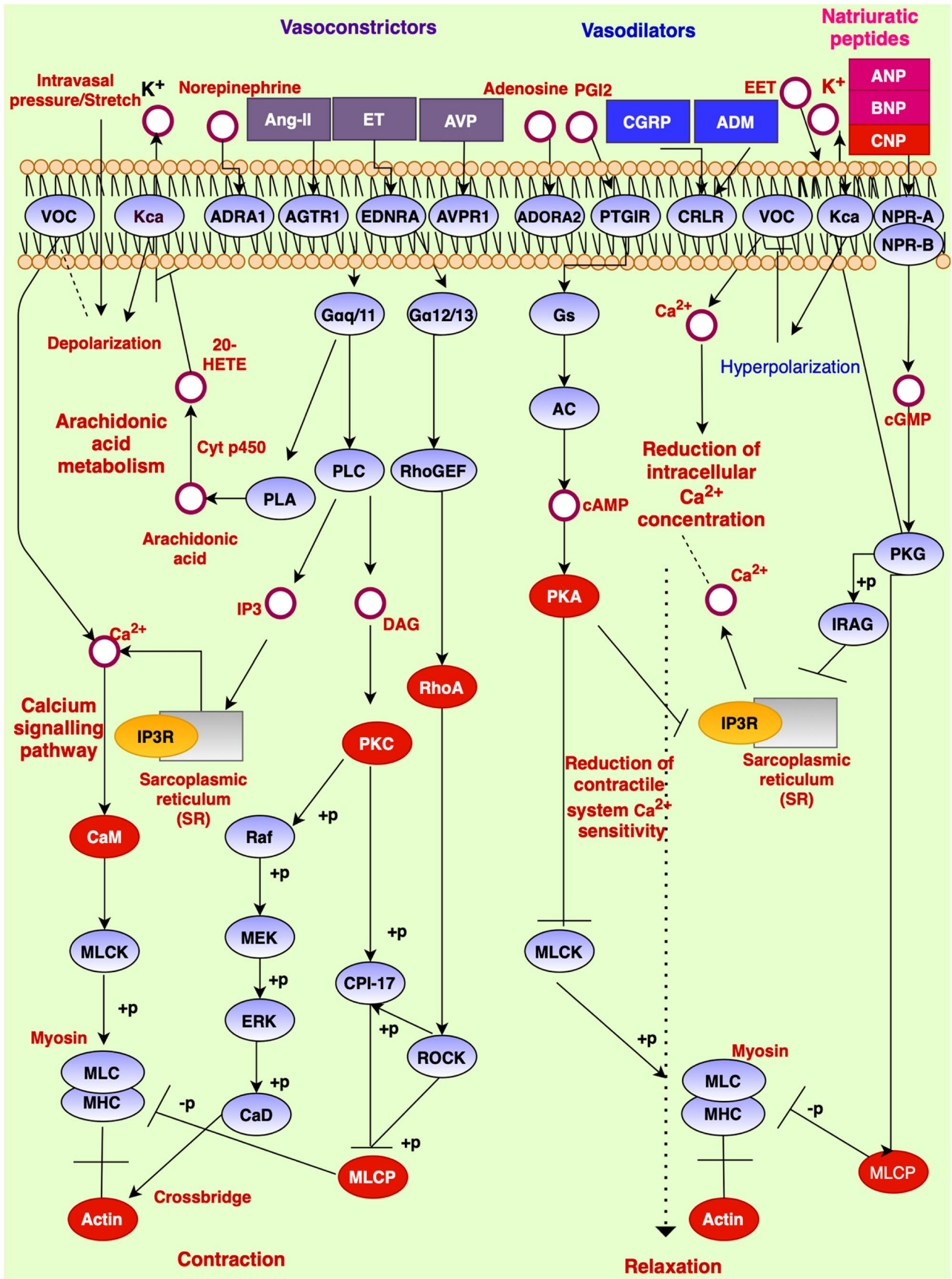
with other roles in the male and female reproductive systems. In porcine, SPP1 actively participates in sperm–zona interactions and is reported to be preventing polyspermy. Studies also reported that SPP1 is involved in adhesion of early germ cells to the basement membrane and epididymal maturation of the sperm (Hao et al. 2006).

In bull sperm, the oxidative phosphorylation pathway is predominant for energy production and high glycolytic activity may reduce intracellular pH and hinder capacitation (review: Storey 2008). Both the pathways can be used by the sperm to produce energy (Krzyszosiak et al. 1999; Magdanz et al. 2019) but different species may utilize various energy sources depending on the condition. In humans, the glycolytic pathway has been the predominant source for the generation of ATP to support the energy requirement for progressive motility and capacitation (Hereng et al. 2011). The aberrant expression of sperm-specific glycolytic enzymes is associated with immature and asthenozoospermic samples (Liu et al. 2019). The predominant glycolytic pathway in poor quality semen tends to indicate that the sperm were under stress and may hamper capacitation. Lysine is shown to increase the sperm concentrations (Nizza et al. 2010); its degradation of which in the poor quality semen may have a negative effect.

Quantitative proteomics analysis revealed that seven proteins (SPINK2, ACAT1, FAHD2A, PPP1CB, LPCAT2B, NEDD8 and NIN) were significantly upregulated and 33 proteins (YBX2, PACRG, VCP, ATP5F1D, SGTA, PGP, SORD, etc.) were significantly downregulated. In the present study, one of the significant abundant proteins, SPINK2, was validated with western blotting. SPINK2 protein is highly essential during different stages of spermatogenesis. The SPINK2 knockout male mice are infertile due to disrupted testicular integrity and increased germ cell apoptosis (Lee et al. 2011). SPINK2 plays a critical role to neutralize protease during the morphogenesis of sperm and formation of acrosomal structure (Jalkanen et al. 2006). In the present study also, the percentage of sperm with acrosome intactness was significantly higher in neat and frozen-thawed samples of good quality semen. Heterologous and homologous mutations of SPINK2 in humans cause oligozoospermia and azoospermia, respectively (Kherraf et al. 2017). In chicken, the expression of SPINK2 in seminal plasma was positively correlated with fertility (Thelie et al. 2019). SPINK2 also acts as a regulatory protein that blocks the action of a serine protease and prevents spontaneous capacitation and acrosome reaction (Ou et al. 2012). These studies along with the present study suggest that SPINK2 protein might regulate buffalo sperm quality by maintaining acrosome integrity.

Another abundant protein validated in the present study, NEDD8, has been reported to be essential for mitosis, cell cycle progression and morphogenesis (Tateishi et al.





**Fig. 6** Smooth muscle contraction pathway is enriched in buffalo sperm proteins. Proteins such as NPPC (CNP), RHOA and MLCP present in the sperm may regulate smooth muscle (uterus and oviduct) contraction in order to reach the sperm to the site of the oocyte. (The observed proteins in the sperm are highlighted in the red oval box; the pathways are re-created based on the KEGG database, Kanehisa Laboratories)

2001). The functional role of NEDD8 has not been reported in sperm. However, the NEDD8-related protein, Cullin-associated neddylation/NEDD8-dissociated protein1 (CAND1), is associated with low sperm motility in humans (Amaral et al. 2014). Through the process of neddylation, it modifies non-cullin protein to regulate protein activity, stability and localization (Yu et al. 2019). The positive association between the expression levels of NEDD8 and sperm motility in this study suggests that NEDD8 regulates buffalo bull fertility through promoting sperm progressive motility, which was significantly higher in neat and frozen-thawed samples of good quality semen. However, the exact role of NEDD8 in buffalo sperm function needs to be elucidated. The other abundantly expressed proteins in good quality sperm namely, ACAT1, PPP1CB, lysophosphatidylcholine acyltransferase 2B-like and NIN were reported to regulate various sperm functions importantly, fatty acid metabolism (Wakazono et al. 1995), sperm capacitation (Rotman et al. 2010), lipid metabolism (Moessinger et al. 2011) and locomotion (Bouckson-Castaing et al. 1996), respectively.

YBX2, an RNA binding protein, was significantly downregulated in good quality buffalo sperm. YBX2 showed stage-specific expression during spermatogenesis, i.e., highly expressed from pachytene spermatocyte to the elongating spermatid phase in adult mice (Kleene 2016). Due to the termination of RNA synthesis during mid-spermiogenesis, YBX2 regulates the stabilization, storage and transient suppression of mRNA (PRM1, PRM2, SMCP) translation as these proteins are essential during the final phases of spermatogenesis (Hecht 1998; Giorgini et al. 2001). However, the occurrence of YBX2 protein in matured sperm has not been reported in any species. In our study, YBX2 was upregulated in the poor quality semen indicating that genes, such as *PRM1* and *PRM2*, required for the chromatin condensation may be translationally repressed during spermiogenesis. This may result in the improper translation of protamine transcripts and lead to the defects in the chromatin distribution as observed in the poor quality semen samples of the present study.

We observed downregulation of HSP proteins (HSPA8, HSP90, HSP70) in good quality sperm. HSP proteins are induced to express during stimuli and help to withstand the stress (Baena et al. 2018) and improve sperm survival through membrane repair and protecting mechanism (Moein-Vaziri et al. 2014; Weber et al. 2020).

The downregulation of HSP proteins in good quality sperm suggests that these animals are less prone to stress conditions, whereas subfertile/poor population of sperm is susceptible to stress and manifested as upregulation of these proteins for sperm survival and repair mechanisms (Erata et al. 2008). Similar to the present study, other proteins such as FH (Martinez-Heredia et al. 2008), ELSPBP1 (D'Amours et al. 2012), ARMC3 (D'Amours et al. 2019) and NCO2A (Qin et al. 2014) downregulated in good quality sperm were earlier reported to be associated with decreased sperm quality.

Taken together, in the upregulated proteins of good quality semen, the biological processes such as metabolic activity, mitochondrial membrane organization, cilia development and function, fusion of sperm to egg plasma membrane (EQTN, IZUMO1 and LYZL6;  $p = 0.02$ ) and adenylate kinase activity (AK1 and AK3;  $p = 0.014$ ) were enriched along with the pathways associated with oxidative phosphorylation and calcium-dependent events including PKA activation (PRKAR2A and PRKAR1B;  $p = 0.019$ ) and post-translational protein phosphorylation (ALB, SPP1 and QSOX1;  $p = 0.03$ ). These enriched functions are indicative of the presence of metabolically active, acrosome intact sperm essential for traveling through the female reproductive tract and can undergo acrosome reaction at a suitable microenvironment. In addition, a significantly higher percentage of progressive forward motile and acrosome intact sperm observed in the neat and frozen-thawed good quality semen samples suggests that these attributes are likely associated with the differentially expressed proteins of good quality semen.

No single test has been developed and also no single protein can be used for assessing semen quality and bull fertility. Since the fertilization is a multifaceted process, many proteins regulate the sperm function and earlier study from our lab also indicates that the ratio of different proteins influences sperm fertility (Somashekar et al. 2017). In-depth studies on these differentially expressed proteins may reveal their regulatory role in sperm fertilization competence.

## Conclusion

The sperm proteome profile deciphered the molecular functions of sperm during fertilization and early embryonic development. The proteins regulating membrane integrity were enriched in the good quality semen, whereas the amino acid degradation pathways were enriched in the poor quality semen samples. These differentially expressed proteins will be useful for predicting the presence of functionally competent sperm in the ejaculate for reproductive success.

**Table 3** Enriched (top 10) biological processes and pathways associated with sperm proteins uniquely present in good and poor quality semen samples

| Sl. no.                    | Process and pathways  | Enrichment FDR | Genes                          |
|----------------------------|---|----------------|--------------------------------|
| Good quality semen samples |   |                |                                |
| Biological process         |   |                |                                |
| 1                          | Mesenchyme migration  | 1.24E−07       | ACTA2, ACTA1, ACTC1, ACTG2     |
| 2                          | Organelle organization  | 5.91E−05       | MTA2, RHOA, TIMM44, DNAL1,     |
| 3                          | Tissue migration  | 0.001          | RHOA, ATP5F1A, ACTA2, MACF1    |
| 4                          | Skeletal muscle satellite cell migration                                    | 0.001          | RHOA, RHOC                     |
| 5                          | Mesenchyme morphogenesis  | 0.001          | ACTA2, ACTA1, ACTC1, ACTG2     |
| 6                          | Cristae formation   | 0.010          | APOO, ATP5F1D, ATP5F1A         |
| 7                          | Cytoskeleton organization   | 0.011          | RHOA, DNAL1, MACF1, RHOC       |
| 8                          | Movement of cell or subcellular component                                   | 0.012          | RHOA, DYNLRB1, RHOC, DYNLRB2   |
| 9                          | Muscle contraction  | 0.018          | RHOA, ACTA2, ACTC1, ENO1       |
| 10                         | Inner mitochondrial membrane organization                                   | 0.018          | APOO, ATP5F1D, ATP5F1A         |
| Pathways                   |   |                |                                |
| 1                          | Fluid shear stress and atherosclerosis                                      | 0.011          | GSTM3, HSP90AA1, RHOA, HSP90B1 |
| 2                          | Oxidative phosphorylation   | 0.023          | NDUFA8, ATP5F1A, ATP5F1D       |
| 3                          | Carbon metabolism   | 0.023          | ENO1, HIBCH, IDH3A             |
| 4                          | Vascular smooth muscle contraction  | 0.023          | RHOA, ACTA2, ACTG2             |
| 5                          | Estrogen signaling pathway  | 0.023          | HSP90AA1, SHC1, HSP90B1        |
| 6                          | Parkinson's disease   | 0.023          | NDUFA8, ATP5F1A, ATP5F1D       |
| 7                          | Protein processing in endoplasmic reticulum                                 | 0.027          | PDIA3, HSP90AA1, HSP90B1       |
| 8                          | Metabolic pathways  | 0.029          | ENO1, HIBCH, MMAB, IDH3A       |
| 9                          | Biosynthesis of amino acids   | 0.033          | ENO1, IDH3A                    |
| 10                         | Thermogenesis   | 0.039          | NDUFA8, ATP5F1A, ATP5F1D       |
| Poor quality semen samples |   |                |                                |
| Biological process         |   |                |                                |
| 1                          | Glucose metabolic process   | 0.0001         | GALM, FBP1, ACADM, PDHA1       |
| 2                          | Organic acid metabolic process  | 0.0001         | HADHA, OGDH, ACADM, SRR        |
| 3                          | Pyruvate metabolic process  | 0.0001         | SRR, PDHB, PDHA1, PDHA2        |
| 4                          | Tricarboxylic acid cycle  | 0.0001         | OGDH, PDHA1, PDHB, PDHA2       |
| 5                          | Citrate metabolic process   | 0.0001         | OGDH, PDHA1, PDHB, PDHA2       |
| 6                          | Protein localization to nuclear body  | 0.0001         | CCT7, CCT6A, CCT8              |
| 7                          | Positive regulation of protein localization to chromosome, telomeric region | 0.0001         | CCT7, CCT6A, CCT8              |
| 8                          | Positive regulation of establishment of protein localization to telomere    | 0.0001         | CCT7, CCT6A, CCT8              |
| 9                          | Protein localization to Cajal body  | 0.0001         | CCT7, CCT6A, CCT8              |
| 10                         | Regulation of protein localization to Cajal body                            | 0.0001         | CCT7, CCT6A, CCT8              |
| Pathways                   |   |                |                                |
| 1                          | Glycolysis/gluconeogenesis  | 6.92E−06       | GALM, FBP1, PDHA1, PDHA2, PDHB |
| 2                          | Valine, leucine, and isoleucine degradation                                 | 3.80E−05       | HSD17B10, HADHA, HADH, ACADM   |
| 3                          | Glucagon signaling pathway  | 0.0004         | FBP1, PDHA1, PDHA2, PDHB       |
| 4                          | Fatty acid degradation  | 0.0005         | HADHA, HADH, ACADM             |
| 5                          | Fatty acid metabolism   | 0.0008         | HADHA, HADH, ACADM             |
| 6                          | Tryptophan metabolism   | 0.008          | HADHA, HADH                    |
| 7                          | Lysine degradation  | 0.014          | HADHA, HADH                    |
| 8                          | Central carbon metabolism in cancer   | 0.0001         | FGFR2, PDHA1, PDHA2, PDHB      |
| 9                          | PPAR signaling pathway  | 0.0001         | DBI, FABP3, ACADM, UBC         |
| 10                         | Pyruvate metabolism   | 0.0004         | PDHA1, PDHA2, PDHB             |

**Table 4** List of proteins significantly differentially expressed and their functions in good quality semen samples. The functions were reported from published literature and UniprotKB

| Sl. no.                       | Protein ID  | Fold difference | p value  | Function   |
|-------------------------------|---|-----------------|----------|--|
| <b>Upregulated proteins</b>   |   |                 |          |  |
| 1                             | Serine protease inhibitor Kazal-type 2-like (SPINK2)                                | 10.34           | 1.18E-03 | SPINK2 is required for maintaining normal spermatogenesis. Regulates serine protease-mediated apoptosis in male germ cells (Lee et al. 2011)   |
| 2                             | Acetyl-CoA acetyltransferase, mitochondrial (ACAT1)                                 | 3.15            | 1.00E-02 | Inhibitory action on acrosin isoform proteins (Thélie et al. 2019)<br>Catalyzes the last step of the mitochondrial beta-oxidation pathway, an aerobic process breaking down fatty acids into acetyl-CoA (Wakazono et al. 1995; <a href="http://www.uniprot.org/uniprot/P24752">www.uniprot.org/uniprot/P24752</a> )    |
| 3                             | Fumarylacetoacetate hydrolase domain-containing protein 2-like isoform X1 (FAHD2A)  | 3.14            | 2.00E-02 | Hydrolase activity and metal ion binding ( <a href="http://www.uniprot.org/uniprot/Q96GK7">www.uniprot.org/uniprot/Q96GK7</a> )  |
| 4                             | Serine/threonine-protein phosphatase PPI-beta catalytic subunit isoform X1 (PPP1CB) | 3.09            | 4.00E-02 | Role in sperm capacitation (Rotman et al. 2010)  |
| 5                             | Lysophosphatidylcholine acyltransferase 2B-like (LPCAT2)                            | 2.26            | 2.00E-02 | Lipid metabolism (Moessinger et al. 2011)  |
| 6                             | Neddylin (NEDD8)  | 2.06            | 4.00E-02 | Regulate protein activity, stability and localization (Yu et al. 2019)<br>Mitosis, cell cycle progression and morphogenesis (Tateishi et al. 2001)   |
| 7                             | Ninein isoform X2 (NIN)   | 1.43            | 4.00E-02 | Locomotion, phagocytosis and cilia movement (Bouckson-Castaing et al. 1996)<br>Role in microtubule nucleation, by recruiting the gamma-tubulin ring complex to the centrosome (Stillwell et al. 2004)  |
| <b>Downregulated proteins</b> |   |                 |          |  |
| 1                             | Predicted: Parkin coregulated gene protein homolog (PACRG)                          | 13.43           | 3.22E-04 | Role in sperm differentiation (spermiogenesis) (Lorenzetti et al. 2004)  |
| 2                             | Predicted: Y-box-binding protein 2 (YBX2)   | 25.86           | 6.41E-04 | YBX2 is essential for spermatid differentiation (Kleene 2016)  |
| 3                             | Predicted: proteasome subunit alpha type-7 isoform X1 (PSMA7)                       | 2.14            | 1.11E-03 | Regulates proteasome degradation (Liu et al., 2006)  |
| 4                             | Predicted: sorbitol dehydrogenase isoform X2 (SORD)                                 | 3.83            | 3.58E-03 | Produces energy through the glycolytic pathway (Cao et al. 2009)   |
| 5                             | Predicted: heat shock 70 kDa protein 6-like (HSPA6)                                 | 3.04            | 3.90E-03 | Associated with heat tolerance (Baena et al. 2018)<br>Sperm-ZP interaction and fertilization (Naaby-Hansen and Herr. 2010)   |
| 6                             | Predicted: transitional endoplasmic reticulum ATPase-like (VCP)                     | 8.54            | 8.60E-03 | ATP binding and hydrolase activity ( <a href="https://www.uniprot.org/uniprot/A0A0V1HY68">https://www.uniprot.org/uniprot/A0A0V1HY68</a> )   |
| 7                             | Predicted: low-quality protein: nuclear receptor coactivator 2 (NCOA2)              | 2.27            | 9.28E-03 | Association with male infertility<br>Androgen deprivation induces NCoA2, which in turn mediates activation of PI3K signaling and promotes PCA metastasis (Qin et al. 2014)   |
| 8                             | Predicted: pyruvate dehydrogenase E1 component subunit beta, mitochondrial (PDHB)   | 2.92            | 9.47E-03 | Conversion of pyruvate to acetyl-CoA and CO <sub>2</sub> and thereby links the glycolytic pathway to the tricarboxylic cycle.<br>Sperm capacitation (Rezende et al. 2018)  |
| 9                             | Predicted: propionyl-CoA carboxylase beta chain, mitochondrial (PCCB)               | 2.81            | 0.01     | Gluconeogenesis (Crisà et al. 2010)<br>Mitochondrial enzyme involved in the catabolism of odd chain fatty acids (Jiang et al. 2005)  |
| 10                            | Predicted: proteasome subunit alpha type-2 (PSMA2)                                  | 2.81            | 0.01     | Proteasome complex involved in the proteolytic degradation of most intracellular proteins ( <a href="https://www.uniprot.org/uniprot/P25787">https://www.uniprot.org/uniprot/P25787</a> )<br>Proteasomes remain associated with the sperm head following ZP-induced acrosomal exocytosis (Zimmerman and Sutovsky 2009) |
| 11                            | Predicted: heat shock 70 kDa protein 1B-like (HSPA1B)                               | 2.62            | 0.01     | Involved in sperm protection (Weber et al. 2020)   |



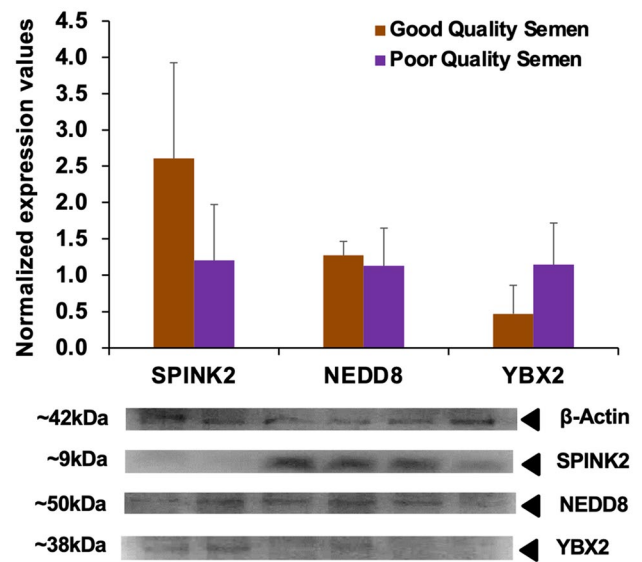
Table 4 (continued)

| Sl. no. | Protein ID   | Fold difference | p value | Function  |
|---------|--|-----------------|---------|---|
| 12      | Predicted: heat shock 70 kDa protein 1-like isoform X1 (HSPA1L)                                | 2.4             | 0.01    | Binding of spermatozoa to the zona pellucida (Matsumoto and Fujimoto. 1990)<br>Spermatogenesis and male fertility (Liu et al. 2019)                       |
| 13      | Predicted: ATP synthase subunit delta, mitochondrial (ATP5F1D)                                 | 4.91            | 0.02    | Produces ATP through proton gradient across the membrane by electron transport complexes of the respiratory chain (Oláhová et al. 2018)                   |
| 14      | Predicted: uncharacterized protein C2orf57 homolog   | 3.97            | 0.02    | No well-defined function reported ( <a href="https://www.uniprot.org/uniprot/A0A1S2ZJL5">https://www.uniprot.org/uniprot/A0A1S2ZJL5</a> )                 |
| 15      | Predicted: vesicle-associated membrane protein-associated protein A isoform X1                 | 1.8             | 0.02    | Transfer cholesterol from the ER to organelles (Amini-Bavil-Olyaei et al. 2013)   |
| 16      | Predicted: armadillo repeat-containing protein 4 isoform X3 (ARMC3)                            | 1.96            | 0.02    | Protein identified in cryopreserved ram spermatozoa (Pini et al. 2018)<br>Abundant in high fertile but immotile bovine spermatozoa (D'Amours et al. 2019) |
| 17      | Predicted: protein FAM71B  | 3.07            | 0.02    | Isolated from human sperm nuclei (de Mateo et al. 2011)<br>May be involved in RNA biogenesis (van Koningsbruggen et al. 2007)                             |
| 18      | Predicted: hydroxysteroid dehydrogenase-like protein 2 (HSDL2)                                 | 2.54            | 0.02    | Located in cell peroxisome in human HSDL2 and involved in fatty acid metabolism (Kowalik et al. 2009)   |
| 19      | Predicted: fibrous sheath-interacting protein 2-like (Fspip2l)                                 | 1.83            | 0.02    | Associated with the sperm fibrous sheath ( <a href="http://www.ncbi.nlm.nih.gov/gene/401024">www.ncbi.nlm.nih.gov/gene/401024</a> )                       |
| 20      | Predicted: acyl-coenzyme A thioesterase 13 (ACOT13)  | 2.69            | 0.03    | Necessary for fibrous sheath assembly and for the overall axonemal and flagellar biogenesis (Martinez et al. 2018)  |
| 21      | Predicted: phosphoglycolate phosphatase (PGP)  | 3.89            | 0.03    | Regulate intracellular levels of acyl-CoAs, free fatty acids and coenzyme A (Cao et al. 2009)   |
| 22      | Predicted: aldose 1-epimerase isoform X1 (GALM)  | 3.54            | 0.03    | Glucose, lipid and energy metabolism ( <a href="https://www.uniprot.org/uniprot/Q2T9S4">https://www.uniprot.org/uniprot/Q2T9S4</a> )                      |
| 23      | Predicted: CKLF-like MARVEL transmembrane domain-containing protein 2-like (Cmtm2b)            | 2.21            | 0.03    | Carbohydrate metabolic process ( <a href="https://www.uniprot.org/uniprot/A0A1S3CWF4">https://www.uniprot.org/uniprot/A0A1S3CWF4</a> )                    |
| 24      | Predicted: phosphoglycerate kinase 2   | 2.3             | 0.03    | Sperm plasma membrane protein that is essential for male fertility (Fujihara et al. 2018)   |
| 25      | Predicted: midkine isoform X2 (MDK)  | 2               | 0.04    | Essential for sperm motility and male fertility but is not required for the completion of spermatogenesis (Danshima et al. 2010)                          |
| 26      | Predicted: hypoxia upregulated protein 1 isoform X2 (HYOU1)                                    | 3.37            | 0.04    | Tumor growth and metastasis (Filippou et al. 2020)  |
| 27      | Predicted: hyaluronidase PH-20 (SPAMI)   | 1.76            | 0.04    | Cell growth and metastasis (Li et al. 2019)   |
| 28      | Predicted: heat shock protein HSP 90-alpha isoform X1 (HSP90AA1)                               | 2.09            | 0.04    | Penetrating the layer of cumulus cells by digesting hyaluronic acid and involved in sperm-egg adhesion (Gmachl et al. 1993)                               |
| 29      | Predicted: epididymal sperm-binding protein 1 (ELSPBP1)  | 2.4             | 0.04    | Upregulated in the normozoospermic sperm in human (Bansal et al. 2015)  |
| 30      | Predicted: small glutamine-rich tetrapeptide repeat-containing protein alpha isoform X1 (SGTA) | 4.41            | 0.05    | Detected only in dead bull spermatozoa (D'Amours et al. 2012)<br>Pathogenesis of polycystic ovary syndrome (Goodarzi et al. 2008)                         |
| 31      | Predicted: coiled-coil domain-containing protein 173 (Ccde42)                                  | 2.22            | 0.05    | Ccdc isoform such as Ccdc42 is required for spermatogenesis and the mutation disrupts sperm motility (Pasek et al. 2016)                                  |



**Table 4** (continued)

| Sl. no. | Protein ID   | Fold difference | p value | Function   |
|---------|--|-----------------|---------|--|
| 32      | Predicted: fumarate hydratase, mitochondrial (FH)    | 1.63            | 0.05    | Significantly higher expression in asthenozoospermic samples (Martínez et al. 2008)<br>Component of tricarboxylic acid (TCA) cycle |
| 33      | Predicted: heat shock cognate 71 kDa protein (HSPA8) | 2.28            | 0.05    | Protects sperm survival through membrane repair mechanism (Moein-Vaziri et al. 2014)   |



**Fig. 7** Validation of LC–MS/MS protein profiles using western blot. The expression of upregulated (SPINK2 and NEDD8) and downregulated (YBX2) sperm proteins was validated. The  $\beta$ -actin was used for normalization

### Compliance with ethical standards

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

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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**Author contributions** BBK, SS and NSKG: designed the experiment; ASS, RL and SD: performed experiments; RA: data interpretation; BBK, SS, ASS and NSKG: wrote the paper; RB: critically discussed the data; BBK, SS, NSKG and DTP: critically discussed the data, carried out data interpretation and prepared manuscript. All authors reviewed the manuscript.

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