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ORIGINAL ARTICLE

# High-throughput proteomic characterization of seminal plasma from bulls with contrasting semen quality

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**Abstract**

Seminal plasma proteins are the major extrinsic factors that can modulate the sperm quality and functions. The present study was carried out to compare the proteomic profiles of seminal plasma from breeding bulls producing good and poor quality semen in an effort to understand the possible proteins associated with semen quality. A total of 910 and 715 proteins were detected in the seminal plasma of poor and good quality semen producing bulls, respectively. A total of 705 proteins were common to both the groups, in which 380 proteins were upregulated and 89 proteins were downregulated in the seminal plasma of poor quality semen, while 236 proteins were co-expressed. The proteins negatively influencing sperm functions such as CCL2, UQCRC2, and SAA1 were among the top ten upregulated proteins in the seminal plasma of poor quality semen. Proteins having a positive role in sperm functions (NGF, EEF1A2, COL1A2, IZUMO4, PRSS1, COL1A1, WFDC2) were among the top ten downregulated proteins in the seminal plasma of poor quality semen. The upregulation of oxidation–reduction process-related proteins, histone proteins (HIST3H2A, H2AFJ, H2AFZ, H2AFX, HIST2H2AB, H2AFV, HIST1H2AC, HIST2H2AC, LOC104975684, LOC524236, LOC614970, LOC529277), and ubiquinol–cytochrome-c reductase proteins (UQCRB, UQCRFS1, UQCRQ, UQCRC1, UQCRC2) indicate deranged oxidation–reduction equilibrium, chromatin condensation and spermatogenesis in poor quality semen producing bulls. The expression of proteins essential for motile cilium (CCDC114, CFAP206, TEKT4), chromatin integrity (PRM2), gamete fusion (IZUMO4, EQTN), hyperactivation, tyrosine phosphorylation, and capacitation [PI3K–Akt signalling pathway-related proteins (COL1A1, COL2A1, COL1A2, SPP1, PDGFA, NGF)] were down regulated in poor quality semen producing bulls.

**Keywords** Seminal plasma · Semen quality · Breeding bulls · Sperm functions · Proteomics

## Introduction

As reproduction holds importance for the propagation of every species, optimal reproductive efficiency of dairy animals is essential for the sustainable dairying. In artificial breeding, ensuring the male fertility is indispensable, because semen from one bull is used for insemination of several thousands of cows (Foote 2010; Ugur et al. 2019). In general, breeding bulls are selected based on their ability to qualify the breeding soundness evaluation (BSE) procedures that involve assessment of selected physical, physiological, behavioural and andrological characteristics including preliminary semen quality assessment. However, a significant proportion of bulls that qualified the breeding soundness evaluation produced poor quality semen that are not fit for cryopreservation and are later culled based on poor fertility outcomes. In a study by Khatun et al (2013), it was reported that out of 414 Holstein Friesian crossbred bulls reserved for breeding purpose, only 25.64% of bulls produced quality semen that could be successfully cryopreserved for use in artificial breeding. Furthermore, it was also reported that a significant proportion of ejaculates produced by the breeding bulls were of poor quality leading to high ejaculate rejection

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rates (Aslam et al. 2014; Vijetha et al. 2014) that ultimately affects the breeding success and genetic improvement. The molecular background of poor semen quality in the bulls selected after vigorous screening, however, is not known.

The semen quality and fertilizing capacity of sperm depends on numerous factors, which includes the factors intrinsic to sperm, such as DNA, RNA, Protein and Metabolites (Rodriguez-Martinez 2006; Aslam et al. 2014, 2019; Saraf et al. 2020; Prakash et al. 2021; DasGupta et al. 2021). Besides these internal factors, the extrinsic factors from the milieu, where the sperm bathe and swim during its voyage, such as the secretions from the epididymis and accessory sex gland, can alter sperm functional attributes (Moura and Memili 2018). Seminal plasma is the amalgamated secretion from the testes, epididymis and accessory sex glands (seminal vesicle, prostate and bulbourethral gland). Seminal plasma proteins are the major extrinsic factors that can modulate the timely display of sperm maturational changes, such as capacitation, acrosome reaction, thereby affecting sperm–oocyte interaction, fertilization and early embryonic development. In addition, these proteins can offer antimicrobial activity and protection from sperm membrane damage and oxidative stress (Aslam et al. 2014; Viana et al. 2018). Despite the prime importance of seminal plasma proteins, they have not been studied extensively.

The proteomic strategy has emerged as an unswerving dependable tool in the past decade in andrological research (Wright et al. 2012). Besides seminal plasma proteins (Aslam et al. 2014; Viana et al. 2018), the intrinsic sperm proteins (Aslam et al. 2018, 2019; Ramesha et al. 2020), proteins of sperm membrane (Roncoletta et al. 2006), epididymal fluid (Moura et al. 2006), accessory sex gland fluid (Moura et al. 2007), spermatogenic and Sertoli cells (Tripathi et al. 2014; Tomer et al. 2021) in bovine were also studied earlier. Previously, 2D gel electrophoresis with immunostaining was used; however, due to limitations in the sole gel-based methods, mass spectrometry emerged as a reliable technique (Kumar et al. 2012). A majority of the earlier studies were primarily aimed to identify potential fertility associated sperm proteins; however, information on seminal proteins in relation to semen quality is very limited. In spite of the fact that seminal proteins are important for sperm quality and functions, the possible seminal plasma proteins controlling the semen quality were barely addressed. Filling this lacuna will help us to understand the molecular background of poor semen quality in bulls and to identify the intervention points for modulation of the semen quality in poor semen producing bulls. We hypothesised that semen quality is controlled by the seminal plasma and the seminal plasma proteome differ between bulls producing good and poor quality semen. Therefore, in the present study, we used high-throughput LC–MS/MS approach to identify the specific/differentially expressed proteins and pathways in seminal plasma from poor quality semen producing bulls as compared to good quality semen producing bulls.

## Materials and methods

The study was conducted at Theriogenology lab, Southern Regional Station of ICAR–National Dairy Research Institute, Bengaluru, Karnataka 560030, India. Prior approval of the Institutional Animal Ethics Committee was obtained for the experimental procedures (Approval No: 1904/GO/ReBi/L/16/CPCSEA). Semen production characteristics of Holstein Friesian breeding bulls ($n = 50$) maintained at Nandini sperm station, Bangalore, Karnataka were evaluated over the period of over 1 year. All the experimental bulls (age 4–6 years; large tall type) were maintained under uniform managemental conditions including housing, feeding (Daily ration of 3 kg concentrate, 2–4 kg hay, 100 g mineral mixture, ad libitum green fodder and fresh water) and health care measures as per the Minimum Standard Protocol for breeding bull management (MSP, Govt of India), and were routinely used for artificial breeding. The number of ejaculates rejected from subsequent processing and cryopreservation, owing to poor initial semen quality, were recorded. Only those ejaculates fulfilling standard requirements ($>600$ million spermatozoa/mL; $>70\%$ progressive motility and $<20\%$ sperm abnormalities) were considered as fit for cryopreservation and artificial breeding. Other ejaculates were rejected from subsequent cryopreservation and use in artificial breeding. The ejaculate rejection rate was calculated based on the following formula.

$$\text{Ejaculate Rejection Rate (ERR) (\%)} = \left[(\text{Total number of ejaculate rejected}/\text{Total number of ejaculate collected}) \times 100\right].$$

A total of 12 bulls having contrasting ejaculate rejection rates [Six bulls with very high ERR ($>60\%$) and the remaining six bulls with very low ERR ($<5\%$)] were selected for this study. Ejaculates were collected from the bulls using artificial vagina as per the standard procedure and the seminal plasma separated from freshly ejaculated semen of these 12 bulls were used for proteomic profiling.

## Sample preparation

25 µg protein from each sample was reduced with 5 mM TCEP [tris (2-carboxyethyl) phosphine] and further alkylated with 50 mM iodoacetamide and then digested with Trypsin (1:50, Trypsin/lysate ratio) for 16 h at 37 °C. Digests were cleaned using a C18 silica cartridge to eliminate the salt and dried using a speed vac. The dried pellet was resuspended in buffer A (5% acetonitrile, 0.1% formic acid).

## Mass spectrometric analysis of peptide mixtures

Experiments were performed on an Ultimate 3000 RSLC-nano system coupled with a Thermo QE Plus. 1 µg was loaded on C18 column 50 cm, 3.0 µm Easy-spray column (Thermo Fisher Scientific). Peptides were eluted with a 0–40% gradient of buffer B (80% acetonitrile, 0.1% formic acid) at a flow rate of 300 nl/min) and injected for MS analysis. LC gradients were run for 100 min. MS1 spectra were acquired in the Orbitrap at 70 k resolution. Dynamic exclusion was employed for 10 s excluding all charge states for a given precursor. MS2 spectra were acquired at 17,500 resolutions.

## Data processing

All samples were processed and RAW files generated were analysed with Proteome Discoverer (v2.4) against the Uniprot reference proteome database as provided. For Sequest and Amanda search, the precursor and fragment mass tolerances were set at 10 ppm and 0.5 Da, respectively. The protease used to generate peptides, i.e., enzyme specificity was set for trypsin/P (cleavage at the C terminus of "K/R: unless followed by "P") along with maximum missed cleavages value of two. Carbamidomethyl on cysteine as fixed modification and oxidation of methionine and N-terminal acetylation were considered as variable modifications for database search. Both peptide spectrum match and protein false discovery rate were set to 0.01 FDR.

## Differential analysis

The abundance values of each sample were used for differential statistical analysis. Protein Abundance values were filtered on the basis of valid values. Filtered values were Log2 transformed followed by Z-score standardization. Student T-Test is used as the numbers of groups according to the study and statistical significance was considered for *P* values less than or equal to 0.05. Significance is calculated using Benjamini Hochberg FDR (cutoff = 0.05).

The seminal plasma of good and poor quality semen producing bulls were considered as control and treatment groups, respectively. Differentially expressed proteins were identified by calculating fold change of expression values (log base2) with respect to control samples. Differentially expressed proteins include upregulated (> onefold) and downregulated proteins (< − onefold). Annotation of proteins were carried out by online bioinformatic resources based on the existing information about Bos taurus genes. Gene ontology (GO) analysis and pathway analysis of differentially expressed transcripts were carried out using DAVID Bioinformatics Resources 6.8 (Laboratory of Human Retrovirology and Immunoinformatics, USA) based on Huang et al. (2009) protocols. Interactions between proteins possessing functions and pathways related to spermatogenesis and sperm function were analyzed using Cluego app (v2.5.3, Integrative Cancer Immunology, Jerome Galon) via Cytoscape bio informatics software platform [3.7.1, U.S. National Institute of General Medical Sciences (NIGMS), USA].

# Results

In the present study, using mass spectrometry, 920 proteins were detected in the seminal plasma of Holstein Friesian dairy bulls. Among which, 780 proteins were characterized based on UniProt database and the remaining 140 proteins remain uncharacterized. The gene ontology analysis of these proteins revealed their involvement in 44 biological processes, 62 cellular components and 58 molecular functions. Top ten biological processes, cellular components and molecular functions, and pathway enrichment of proteins are given in additional file 1.

## Differences in protein expression between the seminal plasma of good and poor quality semen producing bulls

A total of 910 and 715 proteins were detected in the seminal plasma of poor and good quality semen producing bulls, respectively. A total of 705 proteins were detected in both the groups, whereas 205 and 10 proteins were found exclusively in poor and good quality semen producing bulls, respectively. The differentially expressed proteins between the good and poor quality semen producing bulls were identified by calculating the log2 of abundance ratio (fold change) and the cutoff as $\pm 1$ ($\leq 0.5$ abundance ratio considered as downregulated and $\geq 2$ abundance ratio considered as upregulated). Among the 705 detected proteins in seminal plasma of both groups, 380 proteins were upregulated (> 1 fold change) and 89 proteins were downregulated (< -1 fold change) in poor semen producing bull seminal plasma as compared to good quality semen producing bull seminal plasma, while 236 proteins were neutrally expressed (between 1 and -1 fold change) in both the groups. The top ten abundantly upregulated and ten downregulated proteins in the seminal plasma of poor quality bulls compared to

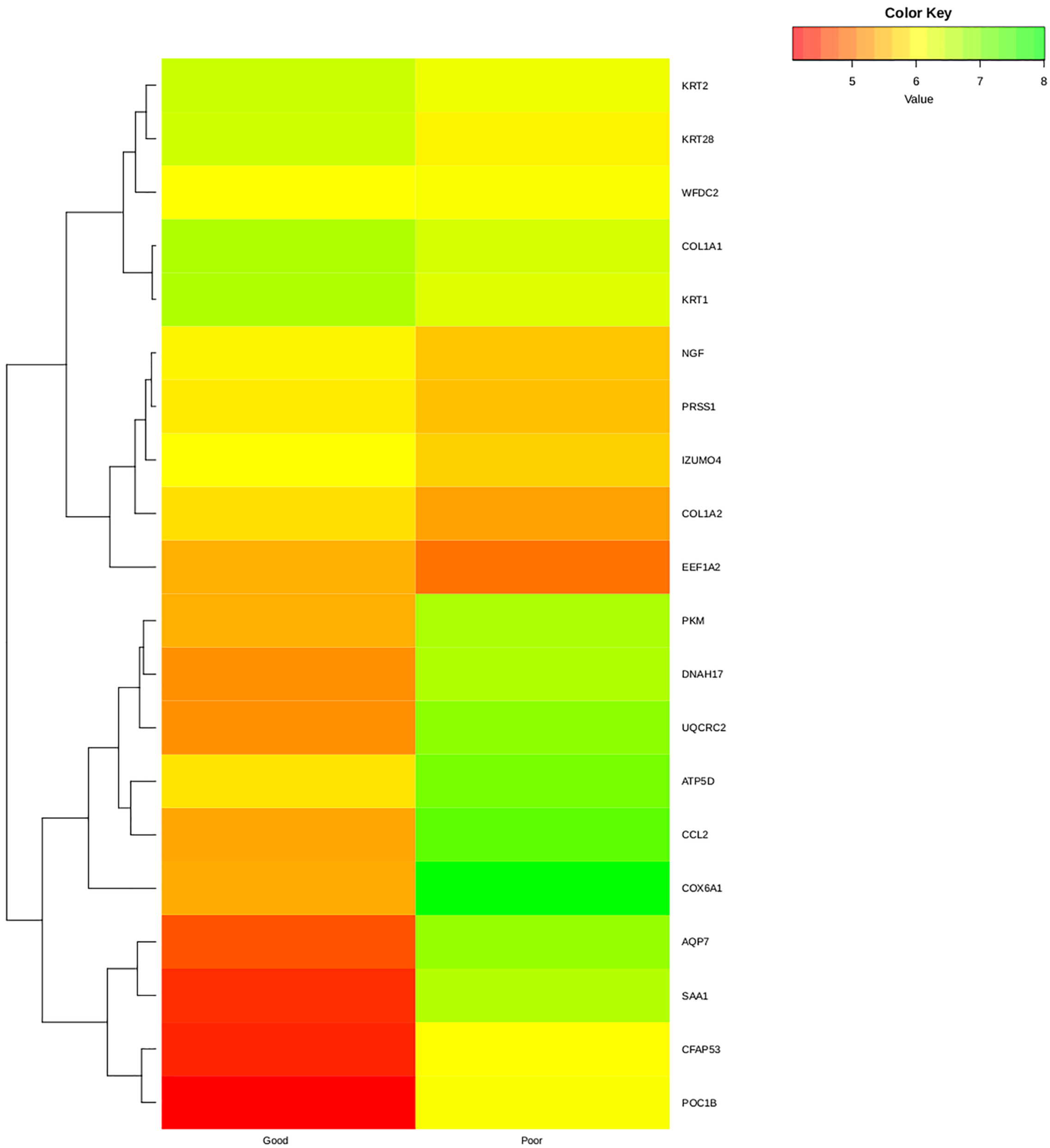


**Fig. 1** Heat map of top 20 differentially expressed seminal plasma proteins in poor quality semen producing bulls

good quality semen producing bulls are shown in Fig. 1 and Table 1.

## Functional annotation of differentially expressed proteins in seminal plasma of poor quality semen producing bulls

The functional classification of upregulated proteins in the seminal plasma of poor quality semen producing bulls revealed that they were involved in the gene ontology terms, such as 49 biological processes, 44 cellular components, and 37 molecular functions. Top ten among each of these categories are shown in Fig. 2. Biological processes include oxidation–reduction process (GO:0055114), chromatin silencing (GO:0006342), glycolytic process (GO:0006096), spermatogenesis (GO:0007283), sperm capacitation (GO:0048240) and sperm motility (GO:0030317). The list of upregulated proteins involved in the important biological process are shown in Table 2.

The functional classification of downregulated proteins in the seminal plasma of poor quality semen producing bulls revealed that they were involved in the gene ontology terms, such as 12 biological processes, 11 cellular components, and 8 molecular functions (shown in

**Table 1** Top 20 abundantly differentially expressed seminal plasma proteins in poor quality bulls compared to good quality semen producing bulls

| Accession | Description | Gene name | Abundance ratio |
|---|---|---|---|
| *10 abundantly upregulated proteins* | | | |
| P13182 | Cytochrome c oxidase subunit 6A1, mitochondrial | COX6A1 | 6.64 |
| Q32L74 | Aquaporin 7 | AQP7 | 5.99 |
| A5PKH9 | POC1 centriolar protein B | POC1B | 5.70 |
| P05630 | ATP synthase subunit delta, mitochondrial | ATP5D | 5.06 |
| P28291 | C–C motif chemokine 2 | CCL2 | 4.89 |
| Q861V4 | Ubiquinol–cytrochrome-c reductase (Subunit II) | UQCRC2 | 4.60 |
| F1N7G5 | Cilia and flagella associated protein 53 | CFAP53 | 4.58 |
| B3IVN4 | M1-type pyruvate kinase | PKM | 4.57 |
| P35541 | Serum amyloid A protein | SAA1 | 4.55 |
| E1BLB4 | Dynein Axonemal Heavy Chain 17 | DNAH17 | 4.49 |
| *10 abundantly downregulated proteins* | | | |
| P13600 | Beta-nerve growth factor | NGF | – 2.87 |
| Q32PH8 | Elongation factor 1-alpha 2 | EEF1A2 | – 2.70 |
| P02465 | Collagen alpha-2(I) chain | COL1A2 | – 2.61 |
| Q148H6 | Keratin, type I cytoskeletal 28 | KRT28 | – 2.53 |
| G3N0V2 | Keratin 1 | KRT1 | – 2.40 |
| A6QPE2 | Izumo Sperm-Egg Fusion Protein 4 | IZUMO4 | – 2.19 |
| G3MZ71 | Keratin 2 | KRT2 | – 2.01 |
| P00760 | Serine protease 1 | PRSS1 | – 1.97 |
| P02453 | Collagen alpha-1(I) chain | COL1A1 | – 1.91 |
| Q3T0Z0 | WAP four-disulfide core domain 2 | WFDC2 | – 1.56 |

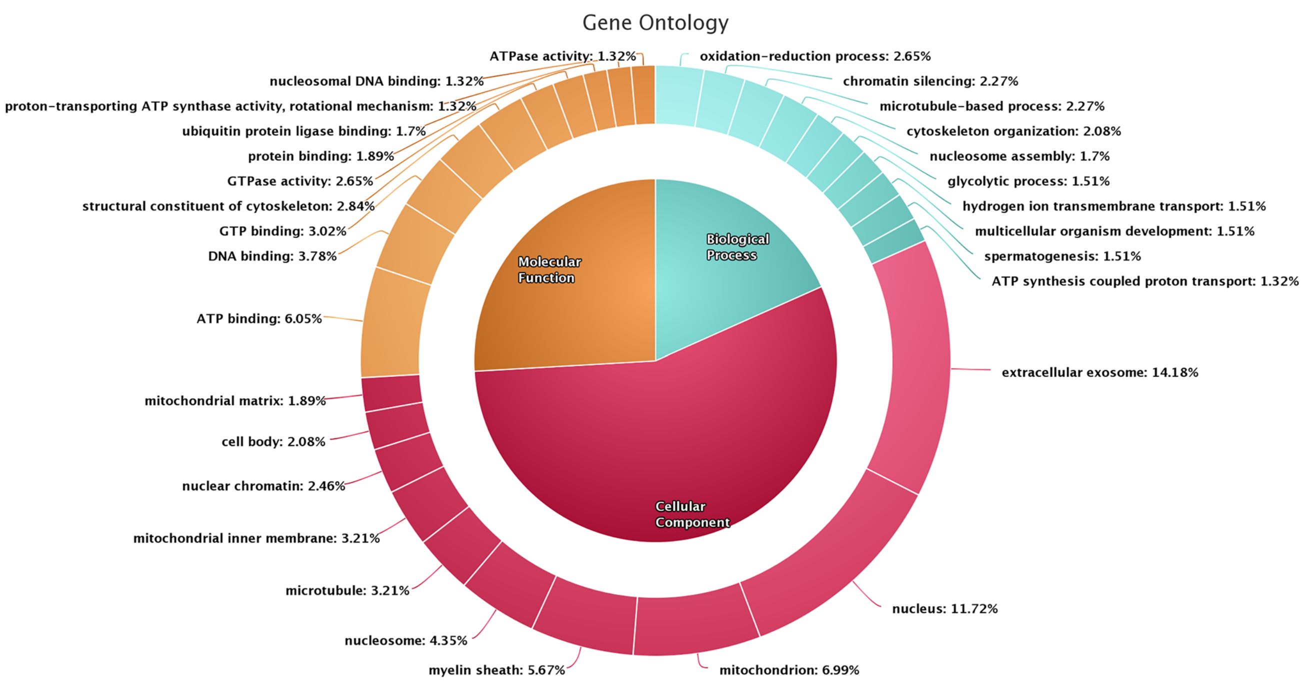


**Fig. 2** Functional annotation of upregulated proteins in the seminal plasma of poor quality semen producing bulls based on the gene ontology terms

**Table 2** List of upregulated proteins and their important enrichments in biological process

| Biological process | Count | Gene symbol |
|---|---|---|
| Oxidation–reduction process | 14 | NDUFA6, GPX4, NDUFB4, NDUFA2, NDUFB1, SDHA, CYB5R1, LDHA, SCCPDH, ALDH2, GAPDHS, UQCRFS1, CDO1, LDHAL6B |
| Chromatin silencing | 12 | LOC104975684, HIST3H2A, H2AFJ, H2AFZ, LOC524236, H2AFX, HIST2H2AB, LOC614970, H2AFV, LOC529277, HIST1H2AC, HIST2H2AC |
| Glycolytic process | 8 | TPI1, PGAM2, GAPDHS, ALDOC, ENO1, PGK2, ENO2, ENO3 |
| Spermatogenesis | 8 | SPATA32, ODF2, ODF3, H2AFX, HSPA2, CYLC2, CYLC1, SPATA19 |
| ATP synthesis coupled proton transport | 7 | ATP5B, ATP5E, ATP5D, ATP5A1, ATP5C1, ATP5H, ATP5F1 |
| Sperm capacitation | 4 | BSP5, PRKACA, DLD, ROPN1 |
| Sperm motility | 4 | LDHC, TEKT3, AKAP4, ROPN1 |

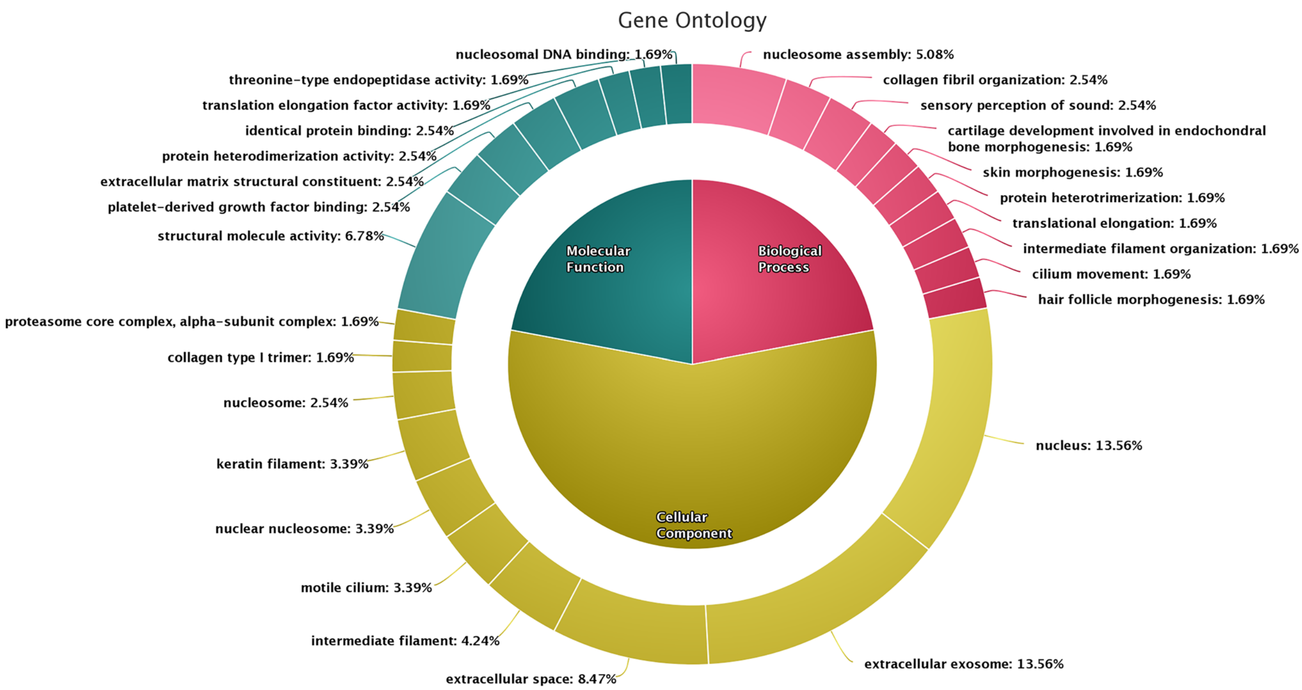


**Fig. 3** Functional annotation of downregulated proteins in the seminal plasma of poor quality semen producing bulls based on the gene ontology terms

Fig. 3). Biological processes include nucleosome assembly (GO:0006334), collagen fibril organization (GO:0030199), protein heterotrimerization (GO:0070208), intermediate filament organization (GO:0045109), and translational elongation (GO:0006414). The interaction between the downregulated proteins involved in different functions are shown in Fig. 4.

## Pathway enrichment of differentially expressed proteins in poor quality bull seminal plasma

The pathway enrichment analysis of upregulated proteins in poor quality bull seminal plasma revealed 35 pathways; the major pathways include metabolic pathways (bta01100), oxidative phosphorylation (bta00190), and glycolysis/gluconeogenesis (bta00010). The top 10 upregulated and downregulated pathways in the seminal plasma of poor quality bulls are shown in Fig. 5. The list of proteins involved in important upregulated and downregulated pathways are shown in Table 3. The pathway enrichment analysis of downregulated proteins in poor quality bull seminal plasma revealed the 11 pathways including PI3K–Akt signalling pathway (bta04151), protein digestion and absorption (bta04974), focal adhesion (bta04510) and ECM–receptor interaction (bta04512). The involvement of downregulated proteins in PI3K–Akt signalling

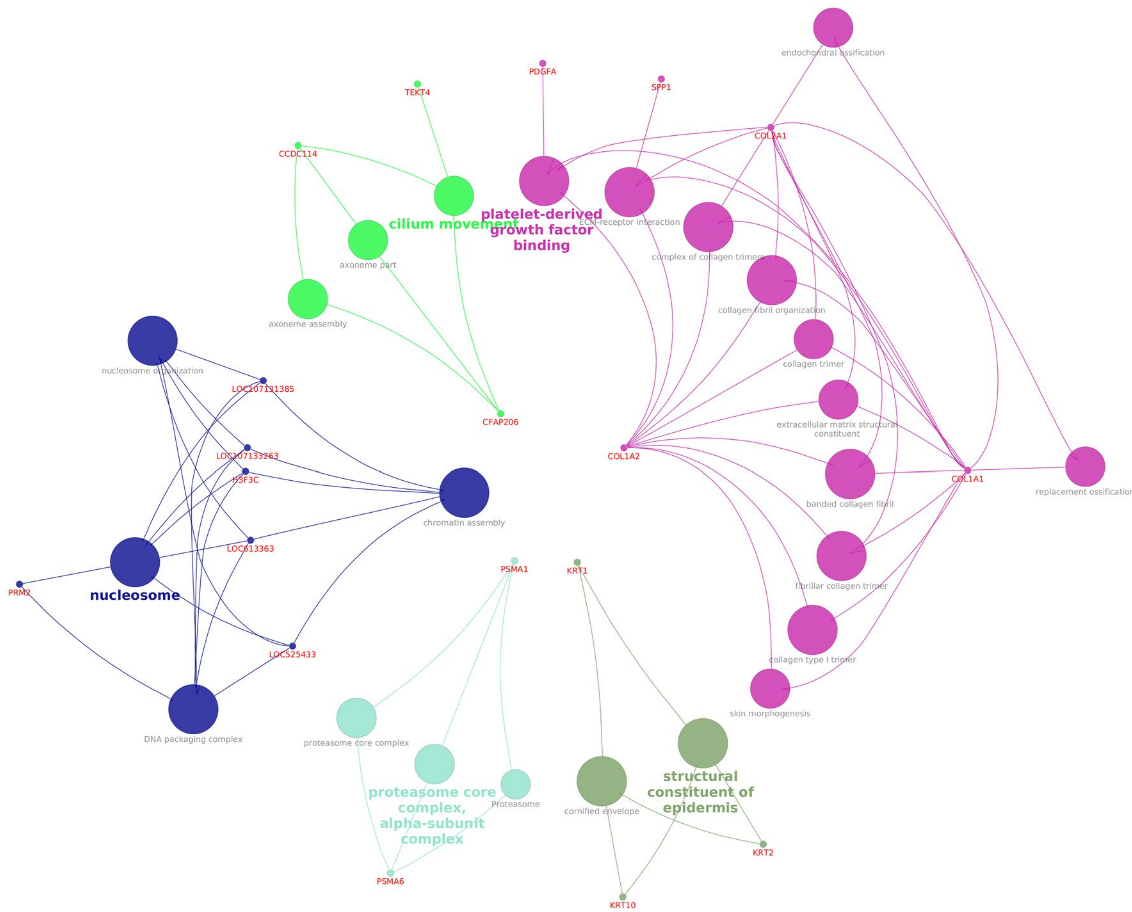


**Fig. 4** Interaction between the downregulated proteins involved in different functions

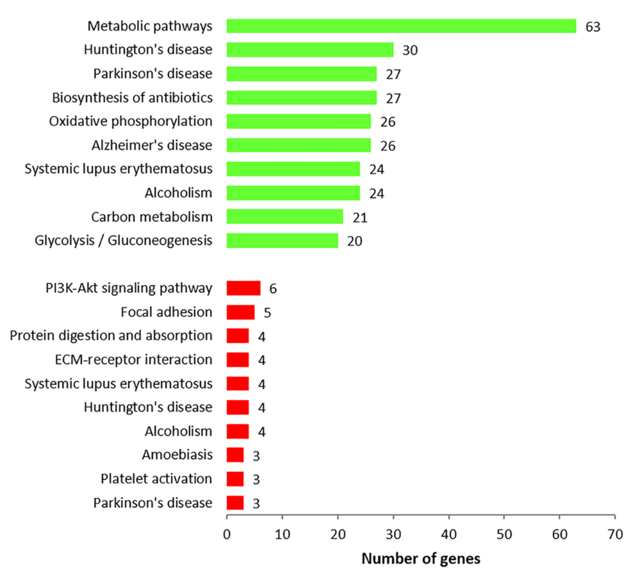


**Fig. 5** 20 highly enriched differentially expressed pathways in seminal plasma of poor quality semen producing bulls

pathway (COL1A1, COL2A1, COL1A2, SPP1, PDGFA, NGF) were located using KEGG mapper (Fig. 6).

## Discussion

Seminal plasma encompasses RNA, proteins, lipids, ions and other components dissolved or encapsulated in exosomes or extracellular vesicles (Vojtech et al. 2014). Seminal plasma proteins play an important role in sperm phenotypic characteristics, function and fertilizing potential. Therefore, analysing the seminal proteins would give a broad picture of their involvement in semen quality; however, the seminal plasma proteins remain underexplored. This study reports the proteomic profile of seminal plasma in Holstein Friesian bulls and the alterations in seminal plasma proteome in bulls producing poor quality semen as compared to good quality semen producing bulls.

We found that the negative influencers of sperm function and fertility such as C–C motif chemokine 2 (CCL2), Ubiquinol–cytrochrome-c reductase, Subunit II (UQCRC2) and Serum amyloid A (SAA1) were among the top 10

**Table 3** List of proteins involved in important upregulated and downregulated pathways

| Pathways | Count | Gene symbol |
|---|---|---|
| *Upregulated pathways* | | |
| Metabolic pathways (bta01100) | 63 | ACADVL, ACAA2, ATP5C1, ENO1, ENO2, COX6A1, ENO3, IL4I1, ALDH2, LOC538702, UQCRFS1, DLAT, ACADM, GLUL, PDHX, TPI1, PGAM2, COX6B2, SDHA, ATP5F1, COX6B1, HADHB, PKM, NDUFS6, GAPDHS, UQCRC1, ALDOC, NDUFS1, UQCRC2, LAP3, DLD, FH, UQCRB, NDUFB4, AK1, ATP5A1, NDUFB1, ATP5H, PDHB, COX5A, GK2, ATP5B, NFS1, LDHA, ATP5E, ATP5D, PGK2, NDUFV2, ACSS1, PDHA2, NDUFA7, PDHA1, NDUFA6, GK, PKLR, MDH2, NDUFA2, UQCRQ, NT5C1B, ACO2, CDO1, PFKM, LDHAL6B |
| Oxidative phosphorylation (bta00190) | 26 | UQCRB, NDUFB4, ATP5A1, NDUFB1, ATP5C1, COX7A2, ATP5H, COX6A1, COX5A, ATP5B, ATP5E, ATP5D, UQCRFS1, NDUFV2, NDUFA7, NDUFA6, NDUFA2, COX6B2, ATP5F1, SDHA, COX6B1, NDUFS6, UQCRQ, UQCRC1, NDUFS1, UQCRC2 |
| Glycolysis/Gluconeogenesis (bta00010) | 20 | PDHA2, PDHA1, TPI1, PKLR, PGAM2, PDHB, ENO1, ENO2, ENO3, LDHA, PKM, ALDH2, GAPDHS, ALDOC, PGK2, DLAT, ACSS1, PFKM, DLD, LDHAL6B |
| *Downregulated pathways* | | |
| PI3K–Akt signalling pathway (bta04151) | 6 | COL1A1, COL2A1, COL1A2, SPP1, PDGFA, NGF |
| Focal adhesion (bta04510) | 5 | COL1A1, COL2A1, COL1A2, SPP1, PDGFA |
| Protein digestion and absorption (bta04974) | 4 | COL1A1, PRSS1, COL2A1, COL1A2 |
| ECM–receptor interaction (bta04512) | 4 | COL1A1, COL2A1, COL1A2, SPP1 |

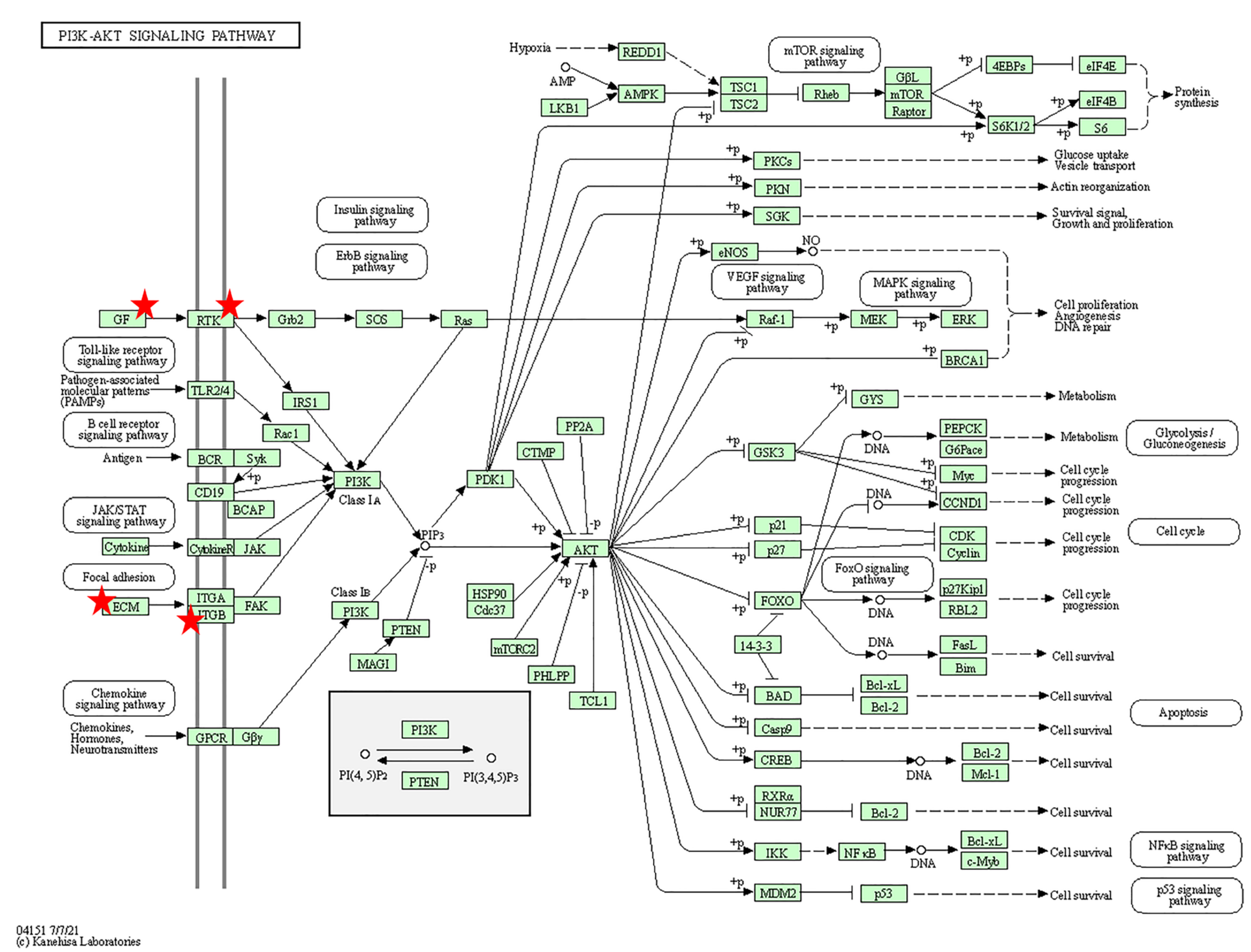


**Fig. 6** PI3K–Akt signalling pathway with downregulated proteins in the seminal plasma of poor quality semen producing bulls

upregulated proteins in the seminal plasma of poor quality semen producing bulls. It has been reported that higher levels of CCL2 was associated with subfertility by causing damage to Leydig cells and hypogonadism (Jiang et al. 2020). Therefore, the upregulation of CCL2 in poor quality semen producing bulls in our study may indicate altered Leydig cell functions in those bulls. Earlier study Park et al. (2012) identified that UQCRC2 was highly expressed in poor fertility bulls and nutilin-3a mediated decrease in male fertility happens through UQCRC2 (Shukla et al. 2013). The acute phase protein, SAA1, is released upon inflammatory stimulus; therefore, the upregulation of this protein in poor quality semen producing bulls might be related to the subclinical inflammatory changes in the reproductive tract (Ye and Sun 2015). Other proteins include Cytochrome c oxidase subunit 6A1, mitochondrial (COX6A1—protective role during abundant ROS production; Rahman et al. 2016), Aquaporin 7 (AQP7—positively correlated with sire conception rate; Kasimanickam et al. 2017), POC1 centriolar protein B (POC1B—sperm centriole quality biomarker; Turner et al. 2021), Cilia and flagella associated protein 53 (CFAP53—vital for sperm flagella biogenesis; Wu et al. 2021) and Dynein Axonemal Heavy Chain 17 (DNAH17—maintains sperm motility; Whitfield et al. 2019).

The proteins having a positive role in sperm functions (NGF, EEF1A2, COL1A2, IZUMO4, PRSS1, COL1A1, WFDC2) were among the top 10 downregulated proteins in the seminal plasma of poor quality semen producing bulls. Beta-nerve growth factor (NGF) was the highly downregulated protein in poor quality semen producing bulls, which is having a role in enhancing sperm motility (Li et al. 2010), viability (Sanchez-Rodriguez et al. 2019) and sire conception rate (Lima et al. 2020). Elongation factor 1-alpha 2 (EEF1A2) is involved in transcription regulation and protein synthesis in spermatogonia and spermatocytes (Kido and Lau 2008). Collagen alpha-1(I) chain (COL1A1) and Collagen alpha-2(I) chain (COL1A2) are involved in the detachment and movement of germ cells during spermatogenesis (He et al. 2005; Chen et al. 2012). Its high expression was reported in the spermatogenic cells of zebu bulls (Tomar et al. 2021). Izumo Sperm–Egg Fusion Protein 4 (IZUMO4) is expressed in the sperm head and is vital for gamete fusion; the diminished levels of this protein were reported after acrosome reaction. The addition of cryoprotective agent increased the IZUMO4 levels in sperm (Yoon et al. 2016). Serine protease 1 (PRSS1) is involved in the ZP reaction and block of polyspermy (Peng et al. 2012). WAP four-disulfide core domain protein 2 (WFDC2), also known as Human Epididymis Protein 4, is essential for sperm motility, maturation and fertilization (Kant et al. 2019). Overall, expression of all these proteins that have a vital role in sperm functions and fertility were downregulated in poor quality semen.

A majority of the upregulated proteins in poor quality semen producing bulls were involved in oxidation–reduction process (GO:0055114). Oxidation–reduction potential in sperm is a measure of equilibrium or balance between the oxidants and antioxidants. The oxidation–reduction process is correlated with poor quality semen (Agarwal et al. 2016). In accordance with that, oxidation–reduction process is upregulated in poor quality semen producing bulls in our study. The chromatin silencing process (GO:0006342) was upregulated in poor quality semen, in which many proteins are histone family proteins. Since the majority of histones should be replaced by protamines during the spermiogenesis to cause chromatin condensation, the upregulation of many histones (HIST3H2A, H2AFJ, H2AFZ, H2AFX, HIST2H2AB, H2AFV, HIST1H2AC, HIST2H2AC, LOC104975684, LOC524236, LOC614970, LOC529277) in poor quality semen indicate improper chromatin condensation in poor quality semen producing bulls. The sperm motility-related enolases (ENO1, ENO2, ENO3) were involved in glycolytic process (GO:0006096). The upregulated proteins vital for sperm motility such as outer dense fibre proteins (ODF2, ODF3), spermatogenesis-associated proteins (SPATA19, SPATA32), and cell cycle-related proteins such as cyclins (CYLC1, CYLC2) were involved spermatogenesis (GO:0007283) biological process (Lacroix et al. 2016). The seven upregulated proteins such as ATP5B, ATP5E, ATP5D, ATP5A1, ATP5C1, ATP5H, and ATP5F1 were involved in ATP synthesis coupled proton transport (GO:0015986), which are crucial for the production of energy for sperm motility (Aslam et al. 2018). Though, the majority of these are positively related to sperm function and semen quality, the upregulation of these proteins in the seminal plasma of poor quality semen producing bulls is quite intriguing. These functions should be studied further to affirm their connections with semen quality.

A majority of upregulated proteins (63 proteins) in poor quality semen producing bulls are involved in the metabolic pathways (bta01100), which is critical for every cell including spermatozoa. Metabolic pathway is crucial for germ cell development, sperm functions and fertilization (Piomboni et al. 2012). Metabolic pathways provide energy for spermatozoa by generating ATP by oxidative phosphorylation (bta00190) and glycolysis (bta00010). Proteins involved in both of these pathways were upregulated in poor quality semen producing bulls. This finding in our study is contrasting; however, the functions of majority of the genes involved in these pathways are not known and there is a possibility that those genes may negatively regulate metabolic pathways. A total of 26 and 20 upregulated proteins in poor quality semen producing bulls were involved in oxidative phosphorylation (bta00190) and glycolysis/gluconeogenesis (bta00010), respectively. Oxidative phosphorylation generates ATP in mitochondria, while glycolysis generates ATP in head and principal piece (du Plessis et al. 2015). Though, oxidative phosphorylation is having positive roles in semen quality, many upregulated proteins involved in this pathway were ubiquinol–cytochrome-c reductase proteins (UQCRB, UQCRFS1, UQCRQ, UQCRC1, and UQCRC2), which are involved in tumorigenesis and reported to negatively influence semen quality (Park et al. 2012; Han et al. 2019). The ubiquinol–cytochrome C reductase core protein II is reported to be involved in the degradation of p53, which is vital for proper spermatogenesis (Han et al. 2019). Therefore, upregulation of ubiquinol–cytochrome-c reductase proteins in poor quality semen producing bulls might impair spermatogenesis and result in poor semen quality.

A good number of downregulated proteins in poor quality semen were involved in nucleosome assembly (GO:0006334) biological process. Nucleosome is a subunit of chromatin and it encompasses DNA turns wrapped around histones. Proper nucleosome assembly is vital for the chromatin integrity (Dutta et al. 2001). Downregulation of proteins involved in nucleosome assembly (LOC107133263, LOC525433, H3F3C, LOC613363, LOC107131385) in poor quality semen indicate compromised chromatin integrity in sperm. Two downregulated proteins (CCDC114, CFAP206) were involved in cilium movement (GO:0003341). The deficiency of CCDC114 (coiled-coil domain containing protein 114) resulted in the absence of outer dynein arms (Inaba and Mizuno 2016) and the loss of CFAP206 in cilium hampered sperm motility (Beckers et al. 2020). Downregulation of these two proteins in our study indicate the ciliopathy in poor quality semen producing bulls. In the context of cellular component, downregulated proteins were majorly (16 proteins) related to nucleus (GO:0005634) including important sperm function-related proteins, such as PRM2 (Protamine 2-vital for chromatin integrity), IZUMO4 and EQTN (Equatorin—essential for gamete fusion; Ito et al. 2018). Four proteins were involved in motile cilium (GO:0031514) including TEKT4 (Tektin4), which is crucial for coordinated sperm flagellar beating and its absence caused asthenozoospermia (Roy et al. 2007). Since, histones should be replaced by the protamines during spermatogenesis, the downregulation of protamine (PRM2) and the upregulation of histones (HIST3H2A, H2AFJ, H2AFZ, H2AFX, HIST2H2AB, H2AFV, HIST1H2AC, HIST2H2AC, LOC104975684, LOC524236, LOC614970, LOC529277) ascertain the improper chromatin compaction in poor quality semen producing bulls.

PI3K–Akt signalling pathway (bta04151) was the major pathway enriched with downregulated proteins (COL1A1, COL2A1, COL1A2, SPP1, PDGFA, NGF) in poor quality semen producing bulls as compared to good quality semen producing bulls. These genes were mapped into KEGG pathway, in which COL1A1, COL2A1, and COL1A2 were mapped in both extracellular matrix (ECM) and integrin beta (IGFB). The PDGFA, NGF and SPP1 are mapped in receptor tyrosine kinase (RTK), growth factors (GF) and integrin beta (IGFB), respectively. As all these genes are involved in upstream process of PI3K–Akt signalling pathway, the down regulation of these genes could affect the subsequent downstream of that pathway. PI3K–Akt pathway (bta04151) is involved in the regulation of hyperactivation, tyrosine phosphorylation, and capacitation (O'Flaherty et al. 2006). COL1A1 and COL1A2 are vital for self-renewal of spermatogonia and movement of germ cells during spermatogenesis, respectively (Chen et al. 2012). PDGFA (platelet-derived growth factor-A) influences epididymal development and its function (Basciani et al. 2004). SPP1 (Secretary phosphoprotein 1), also known as Osteopontin (OPN), is having important roles in fertilization, implantation and placentation (Johnson et al. 2003). SPP1 improves embryo development by decreasing apoptosis (Hao et al. 2008). Nearly the same set of proteins involved PI3K–Akt signalling pathway (bta04151) were also involved in Focal adhesion (bta04510), Protein digestion and absorption (bta04974) and ECM–receptor interaction (bta04512). These pathways are important for the sperm attachment and interaction with oocyte or oviductal epithelium. Downregulation of proteins and pathways essential for sperm functions, in our study, indicate the altered molecular mechanisms in poor quality semen producing bulls.

## Conclusion

Collectively, the upregulation of oxidation–reduction process-related proteins, histone proteins (HIST3H2A, H2AFJ, H2AFZ, H2AFX, HIST2H2AB, H2AFV, HIST1H2AC, HIST2H2AC, LOC104975684, LOC524236, LOC614970, LOC529277), and ubiquinol–cytochrome-c reductase proteins (UQCRB, UQCRFS1, UQCRQ, UQCRC1, UQCRC2) indicate deranged oxidation–reduction equilibrium, chromatin condensation and spermatogenesis in poor quality semen producing bulls. Downregulation of proteins essential for motile cilium (CCDC114, CFAP206, TEKT4), chromatin integrity (PRM2), gamete fusion (IZUMO4, EQTN), hyperactivation, tyrosine phosphorylation, and capacitation [PI3K–Akt signalling pathway-related proteins (COL1A1, COL2A1, COL1A2, SPP1, PDGFA, NGF)] indicate perturbed molecular mechanisms related to sperm functions in poor quality semen producing bulls.

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**Data availability** The datasets analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

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

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