RESEARCH ARTICLE

Expression Levels of Sperm Genes Associated with Energy Production Pathways Regulate Fertility Status of Bulls

Laxman Ramya[1,2], Divakar Swathi[1], Santhanahalli Siddalingappa Archana[1], Arunachalam Arangasamy[1], B. Krishnan Binsila[1], Balaganur Krishnappa[1], Sellappan Selvaraju[1*]

Abstract

Sperm biomolecules impact the reproductive success of a bull in many ways from fertilization to the health of the offspring. The energy produced within the sperm enables them to remain motile and allows them to participate in fertilization events. In this study, we deciphered the genes associated with energy production and their influence on the fertility rate. Post-thaw buffalo semen samples (n=21) were analysed for sperm kinematics and field fertility for selecting high (n=5) and low (n=5) fertile samples. Sperm total RNAs were extracted using a double lysis method followed by column-based extraction and sequenced using the Illumina platform. The expression levels of the genes involved in energy-regulating pathways were analyzed. Among these genes, 14.6% were significantly ($p<0.05$) differentially expressed between high and low fertile sperm. The expression levels of up-regulated genes such as *JAK3*, *DDX5*, *PRKCZ*, *CHD4*, *CHD5,* and *ADCY3* had a strong positive correlation ($r>0.5$) with progressive motility and velocities. The up-regulated genes were involved in chemokine signaling (*ADCY2* and *JAK2*, $p=0.0001$), calcium signaling (*AGTR1* and *SLC25A4*,$p=0.0002$) and oocyte meiosis (*ADCY2* and *ADCY3*, $p=0.049$). The increase in the expression of ATP-producing genes substantiate the improved progressive motility (64.51±5.79 vs 33.07±3.34 %), curvilinear velocity (70.58±4.32 vs 50.15±4.92 µm/sec), straight-line velocity (39.84±3.16 vs 26.02±2.52 µm/sec) and average path velocity (49.46±3.59 vs 32.99±3.45 µm/sec) in high fertile bulls compared to low fertile bulls. The study suggests that the expression level of differentially expressed genes associated with energy-regulating pathways influences the fertilizing ability of sperm.

Keywords: Bull fertility, Energy-production, Fertilization, Sperm kinematics, Sperm transcriptome

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Introduction

Buffalo is an economically important livestock resource in Asia contributing to 49% of the milk yield (Vohra *et al.,* 2021). Buffaloes have been considered poor breeders with low reproductive efficiency (Warriach *et al.,* 2015). Research is currently exploring various avenues to identify the causes behind conception rate variances, including factors related to gender as well as management. Although male and female contribute to the success or failure of conception, male assumes greater importance in artificial breeding as the semen from a bull has been used for impregnating thousands of cows during artificial insemination. Hence, selecting a high fertile (HF) bull becomes paramount. The fertilizing ability of the sperm determines the fertility status of the bull.

Fertilization is a multi-faceted process and the sperm takes a precarious journey through the female reproductive tract to reach the oocyte and fertilize. Sperm attain fertilization capability through a sequence of physiological and biochemical transformations including capacitation, acrosome reaction, etc. All these transformations of sperm are highly energy-demanding (Ickowicz *et al.,* 2012). For example, for successful capacitation, molecules such as Ca^{2+} HCO_3^-, serum albumin, etc., are essential. The influx of HCO_3^- inside the sperm increases the intracellular pH and adenylate cyclase, which in turn elevates the cAMP levels to provide energy for capacitation and fertilization. Hence, the sperm must possess the required energy to overcome the barriers of the female reproductive tract and power their journey to swim and reach the oocyte for fertilization. The outer dense fibers (ODFs), fibrous sheath (FS), and mitochondrial sheath (MS) wound around the axoneme, which provides energy for

[1]Reproductive Physiology Laboratory, Animal Physiology Division, ICAR-National Institute of Animal Nutrition and Physiology, Adugodi, Bengaluru-560030, India

[2]Department of Biotechnology, Jain Deemed -to be University, Bengaluru-560001, India.

Corresponding Author: Sellappan Selvaraju; Reproductive Physiology Laboratory, Animal Physiology Division, ICAR-National Institute of Animal Nutrition and Physiology, Adugodi, Bengaluru-560030, India e-mail: selvarajuars@gmail.com

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the sperm to move (Dcunha *et al.,* 2020). The dynein ATPases hydrolyse ATP to produce the whipping of flagellum and thus the sperm motility depends on the amount of available ATP. Further, decreased levels of ATP are associated with hyperactivation and an increase in the curvilinear velocity and flagellar beat cross frequency (Sansegundo *et al.,* 2022). Thus it becomes evident that sperm bioenergetics may shed light on understanding the fertility status.

Sperm are transcriptionally and translationally silent but are packed with the necessary biomolecules required for the different facets of fertilization (Parthipan *et al.,* 2017; Selvaraju *et al.,* 2021). In recent years, there has been a growing trend in investigating the genetic markers linked to fertility and semen quality. Bovine sperm has 5000-7000 transcripts contributing to the fertilization process (Selvaraju *et al.,* 2017). Transcripts present in the sperm are though indicative of remnants of spermatogenesis, some of them are essential for further downstream fertilization and embryonic developmental processes (Jodar *et al.,* 2013). Since sperm are metabolically active, the majority of these transcripts are expected to be associated with the energy-associated pathways. Hence assessing the relative expression levels of genes associated with energy production can help understand if such genes can be used as diagnostic markers for fertility. The present study was aimed to identify the genes influencing energy production pathways in sperm and their association with sperm kinematics, for establishing a panel of energy-regulating genes in predicting the fertility status of the bulls.

Materials and Methods

Semen Collection

All the experiments were carried out as per the approval of the Institute Animal Ethics Committee (vide: NIANP/IAEC/1/2020/11). All methods were carried out following with relevant guidelines and regulations. Frozen semen samples from Murrah buffalo bulls (n=21) were purchased from the ICAR - Central Institute for Research on Buffaloes (CIRB), Haryana, India and stored in liquid nitrogen until further analyses. The frozen-thawed semen samples were washed with a 50% density gradient solution (Bovipure Nidacon, Sweden) at 300 g for 15 min at room temperature. The sperm pellet was collected and resuspended in phosphate-buffered saline (1X PBS) for RNA extraction.

Assessment of Sperm Functional Attributes

Sperm kinematics such as progressive motility (PM), straight-line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), and the mucous penetration (MP) ability were assessed by CASA (Computer Assisted Semen Analyzer, Sperm Class Analyzer, version 6.4, Microptic, Spain) as described earlier (Selvaraju *et al.,* 2021). The fertility data based on verified pregnancy were obtained from at least 500 inseminations for each of these buffalo bulls.

Grouping of Bulls

The bulls were categorized into two groups, high fertile (HF, n=5) and low fertile (LF, n=5) through a ranking analysis conducted within their respective groups. This ranking process involved evaluating each bull's attributes in comparison to the herd average. For each of the attribute tested, a bull receives 1 point for each attributes exceeding the herd average value. Based on the summed points, the bulls were classified into HF and LF.

Further, the bulls were also classified as good and poor quality semen producers based on the individual sperm kinematic attributes. Based on the herd average value, the top five bulls having higher value were considered as good and otherwise as bad quality semen producers. The bulls of each group were compared using a student t-test and identified to be significantly (p<0.05) varying between the groups.

Sperm RNA Extraction, Quality Control and Transcriptome Sequencing

Buffalo sperm RNA was isolated using the combined lysis followed by a kit-based method (Parthipan *et al.,* 2015; Selvaraju *et al.,* 2023). In brief, 40 x 10^6 sperm per sample were lysed by double lysis method followed by total RNA extraction using a silica membrane-based column (PureLink RNA mini kit, Invitrogen, USA). The extracted total RNA was subjected to DNase treatment (TURBO DNA-free kit, Ambion, USA) to remove the traces of genomic DNA. The absence of genomic DNA and RNA contaminants from other cell was confirmed by the absence of the cell-specific markers using RT-qPCR (Table 1). The total RNA concentration was measured fluorometrically (Qubit 4.0, Invitrogen, USA) and purity was measured spectro-photometrically by calculating the ratio of 260/280 and 260/230 nm (NanoDrop, ND-1000, Thermo Scientific, USA). The RNA integrity was estimated using an Agilent TapeStation (Agilent Technologies, USA). The sperm total RNA was devoid of intact 18S and 28S rRNA peaks with RIN values below 2.5, the library was prepared from total sperm RNA (100 ng) using a random primer strategy without poly A selection using the NEB Next Ultra II Directional RNA library kit (New England Biolabs, USA). The libraries were sequenced (150PE) using Illumina Hiseq X and the Fastq files were initially subjected to quality check and subsequently to the downstream data analysis.

Bioinformatic Data Analysis

The generated fastq files were processed to remove the adapters using Trimmomatic and the processed reads were mapped against the water buffalo genome (UOA_WB_1; GCF_003121395.1, NCBI database) using STAR aligner. The mapping QC was done using Qualimap. Genes involved in energy associated processes were listed from the UniProt KB database using the keyword "energy production". Gene abundance was calculated using StringTie for all the genes associated with energy production. The expression

Table 1: List of primers used for confirming the absence of genomic DNA and other cell RNA contaminants

Sl. No.	Gene ID	Primer sequence 5' to 3'	Product size (bp)	NCBI accession number	Contaminant
1	*PRM1*	F -ATGGCCAGATACCAATGCT	224	OL955514	gDNA
		R -GTGGCATGTTCAAGATGTGG			
2	*GPX4*	F -AATGTGGCCTCGCAATGAGG	164	XM_025292793.2	gDNA
		R -CCAGCGGCGAACTCTTTGAT			
3	*PTPRC*	F -TTCAGAAGGACGCATGCTGT	137	XM_044942636.2	Leukocytes
		R -GGTGGGGTAGAGTTTCCTGC			
4	*CKIT*	F -GAATAGCTGGCATCAGGGTG	224	XM_006058312.2	Germ cells
		R-CCAGATCCACATTCTCTCCATC			
5	*CDH1*	F - CTGCATTCCTGGCTTTGGTG	171	XM_006047636.2	Somatic cells
		R - GTAAGCACGCCATCTGTGTG			

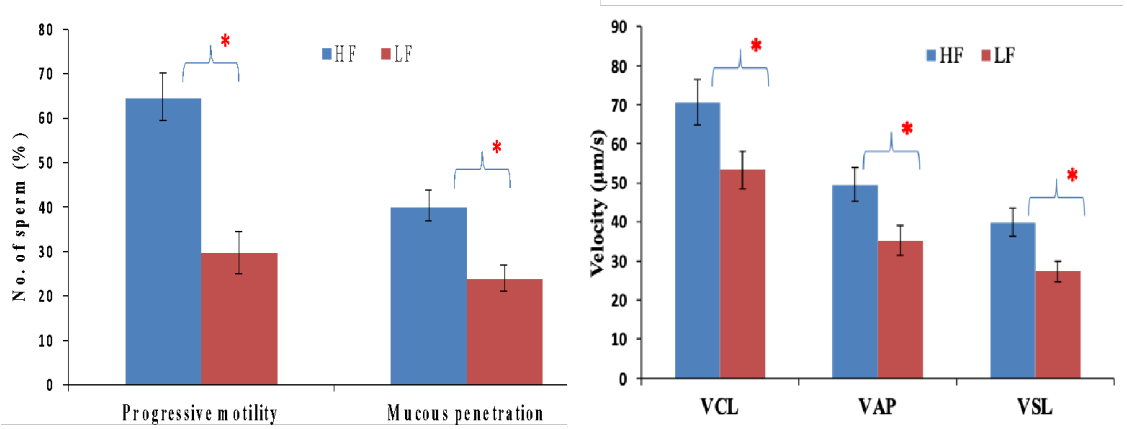

Fig. 1. Grouping of bulls based on sperm kinematics. Bulls were grouped into high fertile (HF, n=5) and low fertile LF, n=5) based on the sperm functional parameter ranking analysis.

*indicates the level of significance ($p<0.05$).

differences of these genes between the HF and LF bulls were compared using DESeq2.

The differentially expressed genes were further annotated for the Gene Ontology using UniProt KB. Gene enrichment analyses were carried out using ShinyGo (Ge *et al.,* 2020) and Cytoscape plugins.

Statistical Analysis

The significance between the two different groups was calculated using the student's t-test. The significance of the enrichment terms represented as false discovery rate (FDR) corrected p-values were calculated using the hypergeometric test and the corrections were made using the Benjamini and Hochberg (FDR) corrections. The association between the up-regulated genes and the sperm kinematic parameters was assessed using the Pearson correlation coefficient. The values of sperm functional parameters were presented as mean ± SEM. Significance was set at $p<0.05$.

Results and Discussion

Grouping of Bulls

Sperm progressive motility (64.51±5.79 vs 29.81±4.81 %), VCL (70.58±4.32 vs 53.52±3.89 µm/sec), VAP (49.46±3.59 vs 35.16±2.65 µm/sec), VSL (39.84±3.16 vs 27.31±1.81 µm/sec) and MP (39.92±4.02 vs 23.96±2.96 %) were significantly higher in the HF bulls compared to the LF bulls (Fig. 1). The significant difference in the motility and the velocity parameters between the HF and LF bulls indicates that sperm kinematics may be an important component defining the success of the fertilization process.

Mapping of the Reads to the Buffalo Genome

An average of 63.5% of reads were mapped to the genome, of which 18.6% were mapped to the exonic, 37.6% to the intronic and 43.7% to the intergenic regions (Fig. 2). The mapping percentage denotes the high-quality data for the downstream processing. The majority of the sperm transcript reads were mapped to the intergenic, intronic followed by the exonic regions similar to the reported earlier (Ramya *et al.,* 2021). The percentage of exonic reads was significantly higher in the HF bulls compared to LF bulls (HF, 32.58±12.73 vs LF, 4.61±0.87), however the intronic (HF, 30.44±6.33 vs LF, 44.77±0.47) and intergenic (HF, 36.682±7.01 vs LF, 50.592±0.97) reads were higher in LF bulls compared to the HF bulls. The higher percentage of the exonic reads in the HF bulls is indicative of the presence of functional transcripts in the sperm.

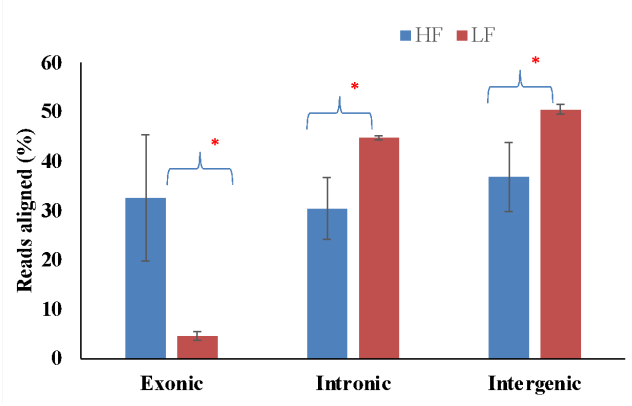

Fig. 2: Mapping summary of HF and LF bulls. *indicates the level of significance (p<0.05).

Differential Gene Expression and Enrichment Analysis

Differential gene expression analysis revealed that 55 (14.6%) out of the 377 genes were significantly ($p < 0.05$) differentially expressed. Among these 46 and 2 genes were significantly up-regulated and down-regulated, respectively, in the HF bulls (Fig. 3). Genes involved in the energy production were majorly up-regulated in the HF bulls indicating their significance in the fertilization and successful pregnancy outcomes. Genes including *EGFR*, *JAK3*, and *TSSK6* were significantly up-regulated in the HF bulls. When the male and the female gametes interact, the sperm tend to stimulate the production and secretion of cytokines by the cumulus cells and these secretory products facilitate the sperm capacitation. *JAK3* involved in the JAK/STAT pathway is reported to be triggered by numerous cytokines, that are aiding in sperm capacitation process (Lachance and Leclerc, 2011).

Epidermal growth factor receptors (EGFRs) are tyrosine kinase receptors activated by peptic ligands inducing the formation of homo/hetero dimers (Jaldety *et al.,* 2012). EGFR signaling produces the required amount of reactive oxygen species (ROS) for capacitation and acrosome reaction (Kowsar *et al.,* 2021). Further, EGFR transactivation activates G protein-coupled receptor signaling, which in turn generates a Ca^{2+} signal and a transient rise in the intracellular calcium levels, resulting in capacitation, actin polymerization and acrosome reaction (Breitbart and Etkovitz, 2011). Adenylate cyclases (ADCs) are responsible for generating cAMP and are involved in olfactory signal transduction. Sperm with $Adcy3^{-/-}$ phenotype had reduced motility in mice (Tong *et al.,* 2016).

The up-regulated genes were involved in biological processes such as protein phosphorylation (*DYRK2, PI4K2B,* and *CDK17, FDR=6.2E-19*), stress response (*HSP90AA1*, *TRPV4*, and *IL2*, 7.53E-09), cellular respiration (*ND4*, *ND1*, and *COX3*, 7.5E-09), protein tyrosine kinase signaling (*FGFR2*, *PTK2B*, and *EPHB2*, 4.31E-07), osmotic stress response (*TRPV4*, *PTK2B*, and *EGFR*, 1.5E-02), ROS response (*PTK2B*, *ND6*, and *ND5*, 5.77E-03) and olfactory behaviour (*CHD7* and *ADCY3*, 3.54E-03) (Fig. 4).

Protein phosphorylation of the flagellar proteins helps the sperm acquire hyperactivated motility, which is crucial for the sperm to penetrate the cumulus and bind with the zona pellucida (Naz and Rajesh, 2004). An increase in protein tyrosine phosphorylation is a hallmark of capacitation and hyperactivated motility in human sperm. Protein tyrosine kinase signaling is associated with different functions of fertilization such as spermatogenesis, epididymal maturation,

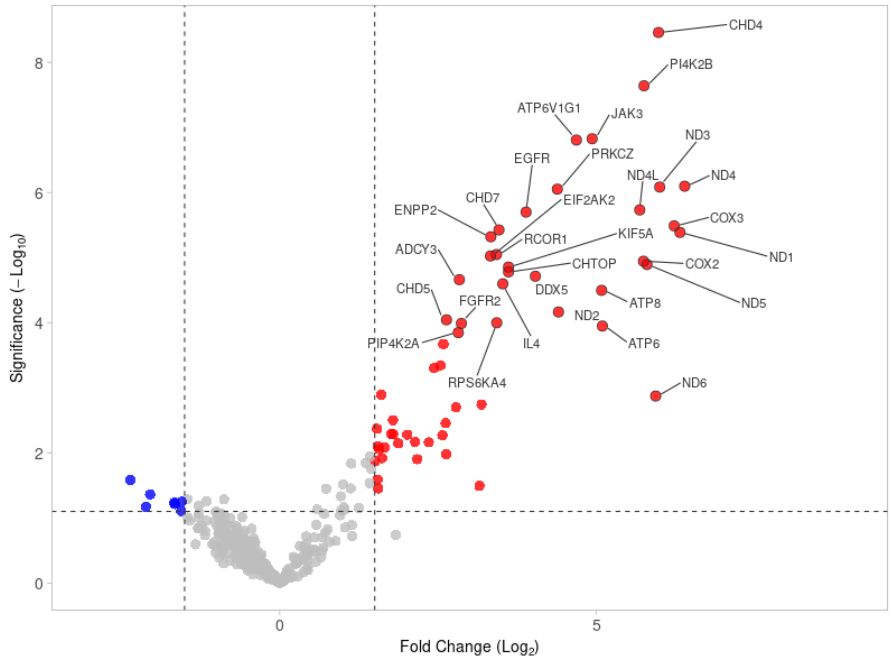

Fig. 3: Differential gene expression between HF and LF bulls. Genes including *PI4K2B*, *JAK3* and *TSSK6* were significantly up-regulated, *PTEN* and *IDE* were significantly down- regulated in the HF bulls.

capacitation, acrosomal exocytosis, membrane interaction and fusion (Ijiri *et al.,* 2012).

Osmotic stress influences the sperm functional attributes and male fertility (Lavanya *et al.,* 2021). Sperm are exposed to the inevitable stress conditions during their journey in the female reproductive tract. ROS-induced oxidative stress affects male fertility (Mannucci *et al.,* 2022). In the HF bulls up-regulation of the stress-responsive genes such as *HSP90AA1, TRVP4,* and *PTK2B* helps the sperm to counteract such stress and achieve successful fertilization. *HSP90AA1* is a molecular chaperone providing resistance against oxidative stress (Casas *et al.,* 2010). Ion channels were reported to trigger hyperactivated motility and thus control the fertilizing ability of the sperm. *TRPV4* is a non-selective cationic channel involved in the temperature, osmolality and Ca^{2+} influx. Osmolality and shear stress activate *TRPV4* ion channels (Nowicka-Bauer and Szymczak-Cendlak, 2021). The expression and localization differ between high and low-motile sperm indicating *TRPV4* is an important factor regulating sperm motility (Kumar *et al.,* 2016). Detection of external cues is crucial for the sperm to locate the oocyte (Flegel *et al.,* 2016). Expression of genes involved in olfactory behaviour helps the sperm to sense the signals sent by the oocyte and direct their path towards the oocyte for establishing fertilization.

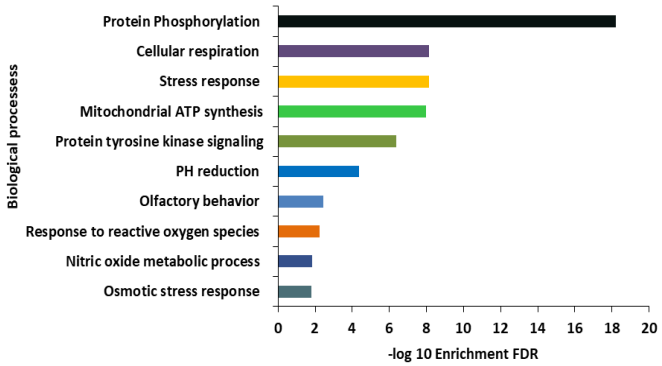

Fig. 4: Biological processes of the up-regulated genes.

The pathway enrichment analysis identified the processes such as oxidative phosphorylation (ATP6, ATP8, and COX3, 1.20E-19), chemokine signaling (ADCY2, ADCY3, and PRKCZ, 2.09E-04), PI3K-Akt signaling (EGFR, FGFR2, and HSP90AA1, 6.43E-04), calcium signaling (SLC25A4, PTK2B, and FGFR2, 6.43E-04) and phospholipase D signaling (ADCY2, ADCY3 and EGFR, 4.90E-3) (Fig. 5).

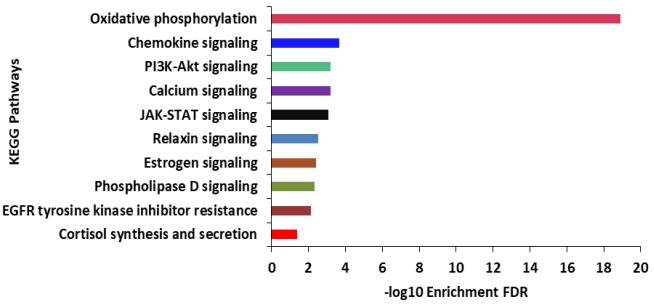

Fig. 5: Pathways of the up-regulated genes in HF bulls.

The movement of the sperm towards the oocyte appears to be dependent on external regulatory factors such as the chemokines secreted by the oocyte. Chemokine receptors belong to the G protein-coupled receptors (Caballero-Campo *et al.,* 2014) and chemokine signaling pathways help the sperm reach the oocyte, fertilize the egg and achieve a successful pregnancy. PI3K pathway regulates capacitation-induced sperm motility and acrosome reaction. PI3K-AKT pathway is crucial for progesterone-mediated tyrosine phosphorylation (Sagare-Patil *et al.,* 2013). Calcium signalling mechanisms are fundamental for successful fertilization as calcium is a crucial ion for sperm motility, capacitation and acrosome reaction (Finkelstein *et al.,* 2020). Phospholipase D signaling is critical for sperm hyperactivation (Itach *et al.,* 2007) and for early embryo developmental processes (Lalonde *et al.,* 2006).

The up-regulated genes were localised in the respirasome, mitochondrial inner membrane, membrane protein complex, and transcription export complex and regulate molecular

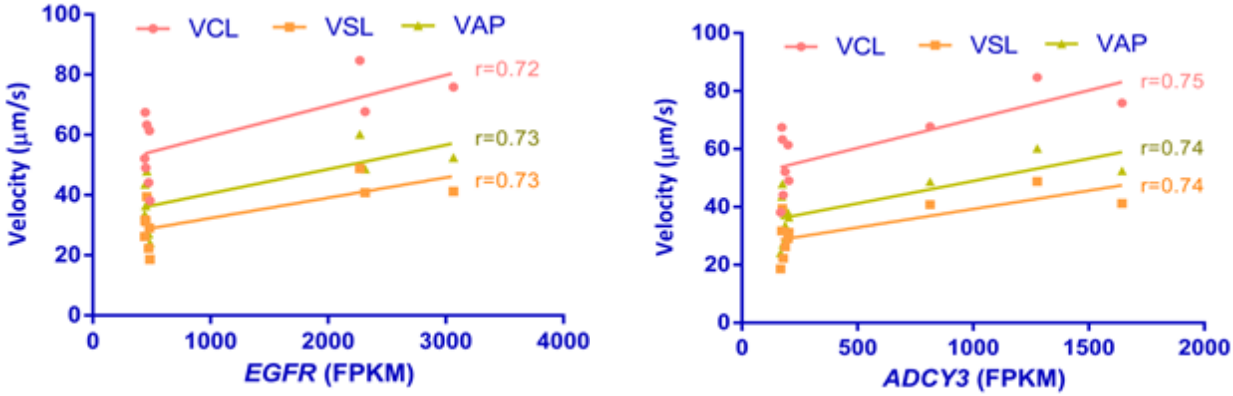

Fig. 6: Correlation analysis between up-regulated genes and sperm kinematic parameters. *EGFR* is associated with acrosome reaction and *ADCY3* is associated with sperm motility were significantly positively correlated with velocity parameters.

functions such as ATP binding, phosphorylation, osmosensor, ion channel and adenylate cyclase activities.

Correlation Findings

Pearson correlation analysis identified that the up-regulated genes such as *JAK3*, *DDX5*, *PRKCZ*, *CHD4*, *CHD5*, *ADCY3*, *TMBIM6*, *EGFR* and *RFK* had strong positive correlation (r>0.6) with all the sperm kinematic parameters such as VCL, VSL, PM and VAP (Fig. 6).

Conclusion

The genes associated with the energy production pathways can influence sperm motility, capacitation, hyperactivation and acrosome reaction. The expression levels of *JAK3*, *DDX5*, *PRKCZ*, *CHD4*, *CHD5,* and *ADCY3* can be used for predicting sperm kinematics and fertility status of the bull. These genes could be used as a panel for diagnosing the fertility status of the bulls.

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