Proteomic Analysis of Capacitated and Non-Capacitated Spermatozoa of Yanbian Yellow Cattle

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Research

Keywords: Yanbian yellow cattle, Capacitated sperm, Proteomics, Liquid chromatography-mass spectrometry, Signal transduction pathway

Posted Date: December 15th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-125470/v1>

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Abstract

Background: Sperm capacitation is a process which occurs prior to fertilization, and is essential for producing high-quality living embryos. The main purpose of this study was to explore the difference of proteomics between capacitated and non-capacitated sperm of Yanbian yellow cattle. Bioinformatic analyses of LC-MS/MS data included GO enrichment, KEGG pathway enrichment, and protein-protein interaction (PPI) analysis.

Results: The results revealed 23 specific proteins in the capacitated group and 345 in the non-capacitated group. Compared with non-capacitated sperm, capacitated sperm exhibited 89 upregulated proteins and 509 downregulated proteins. Western blotting was used to confirm our proteomics data. The expression level of PSMD1 in the capacitated sperm group was significantly lower than that in the non-capacitated sperm group, and the expression level of HSPA5 was significantly higher than in the non-capacitated sperm group.

Conclusions: Our results revealed that many proteins were differentially expressed between capacitated and non-capacitated sperm, particularly those involved in the proteasome signaling and protein transport signaling pathways. This work enhances our understanding of molecular processes involved in sperm viability in Yanbian yellow cattle, and provides a framework for future studies.

Background

Fresh sperm from mammals cannot be used for fertilization. Before fertilization, sperm must undergo a capacitating process, which is important for the production of high-quality living embryos [1]. Capacitation is an important prerequisite for fertilization [2], involving a series of biochemical and physiological changes. Unlike somatic cells, sperm are highly specialized cells with a very small amount of cytoplasm and organelles. Mature sperm lose the potential of gene expression, which makes the transcription and translation process completely silent. Sperm capacitation is protein-dependent, and post-translational modification of proteins plays a key role in the capacitation mechanism [3]. The dynamic changes of proteins are crucial to better understand the underlying mechanisms of sperm capacitation. Because mature sperm are transcriptionally inactive, protein phosphorylation is a valuable means of controlling their physiology [4, 5]. The phosphorylation of many proteins is triggered by ubiquitin proteasome system. The proteasome is a giant protein complex that degrades unwanted or damaged proteins by breaking peptide bonds. Proteasomes have been shown to exist in mammalian sperm and play an important role in fertilization [6, 7]. In humans, mice, wild pigs, and bulls [8], the mechanism of sperm capacitation has been studied using proteomics, and some proteins and tyrosine phosphorylated proteins specific to sperm capacitation have been found.

At present, quantitative proteomics has been widely used in the quantitative study of protein abundance in different functional states of sperm. However, there are few studies on proteomics during sperm capacitation. Recent advances in two-dimensional electrophoresis (2-de) for protein separation and mass spectrometry (MS) for peptide sequencing have facilitated the identification of proteins, leading to a rapid expansion in sperm proteomics research. In this study, we used LC-MS combined with Labelfree method to reveal the proteome differences before and after sperm capacitation in Yanbian yellow cattle. Yanbian yellow cattle are used for both labor and meat in the Yanbian Korean Autonomous Prefecture in Jilin Province, and are

considered a protected national resource. Mass spectrometry is a comprehensive technique used in proteomics to elucidate protein levels and their composition in cells [9]. We used quantitative proteomics to study the differences in proteins of sperm in capacitated and non-capacitated states in Yanbian yellow cattle. This study will add to the theoretical foundation needed for improving the breeding of Yanbian yellow cattle.

Methods

Culture medium

The capacitation medium was modified BO solution, consisted of 110.0 mmol/L NaCl, 6.02 mmol/L KCl, 1.75 mmol/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.02 mmol/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 31.0 mmol/L NaHCO_3 , 0.83 mmol/L $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 8.2 mmol/L sodium pyruvate, 6 mmol/L HEPES, 10000 IU/100 mL penicillin, 5 mg/100 mL streptomycin, 10 mol/L caffeine, 5 mg/mL BSA, 20 $\mu\text{g}/\text{mL}$ heparin (pH = 8.0). The non-capacitation medium was modified Tyrode's bicarbonate buffered medium, consisted of 100 mmol/L NaCl, 3.1 mmol/L KCl, 25.0 mmol/L NaHCO_3 , 0.3 mmol/L NaH_2PO_4 , 0.4 mmol/L EDTA, 21.6 mmol/L sodium lactate, 2.0 mmol/L CaCl_2 , 0.4 mmol/L MgCl_2 , 10 mmol/L HEPES, 1.0 mmol/L pyruvate, 6 mg/mL BSA).

The antibodies used were anti-phosphotyrosine antibody, mouse monoclonal antibody (P1869, Sigma, 1:2000); anti-mouse IgG (Fc specific)-peroxidase, antibody produced in goat (A0168, Sigma, 1:2000); rabbit anti-GRP78/HSPA5 antibody (NBP1-06274, NOVUS, 1:2000); rabbit anti-proteasome 19S Subunit S1 antibody (PA1-973, Thermo Fisher, 1:1000); actin (AA128, Beyotime, 1:1000); anti-rabbit IgG (whole molecule)–peroxidase antibody produced in goat (A0545, Sigma, 1:1000).

Semen collection and preparation

Three healthy and disease-free fresh semen from Yanbian yellow cattle aged 3–5 years were collected. The semen were washed with phosphate buffer saline (PBS) three times, sperm concentration was adjusted to 5×10^7 cells/mL, and centrifuged at $800 \times g$ for 5 min to remove the seminal plasma. Then resuspended in non-capacitated medium (for non-capacitated treatment) and the capacitated medium (for capacitated treatment) respectively, both groups were incubated in 5% CO_2 incubator with maximum saturation humidity of 95% and a temperature of 38.5°C for 1 h.

Capacitation status detection

The kinetic parameters of capacitated and non-capacitated sperm were measured by Computer Assisted Sperm Analysis (CASA) systems.

The tyrosine phosphorylation levels of bovine sperm protein were measured using western blotting. Sperm proteins were extracted from both the capacitated and non-capacitated groups, some sperm proteins concentrations were determined by the BCA kit, then SDS-PAGE and western blot analysis were performed.

Sperm acrosome morphological fluorescence labeling staining

According to the instructions in the peanut agglutinin fluorescence labeling (PNA-FITC) staining kit (GENMED SCIENTIFICS INC.U.S.A), the sperm death/viability and acrosomal integrity were analyzed using fluorescence microscopy and flow cytometry.

Filter Aided Proteome Preparation (FASP) enzymolysis

In each group, 100 µg of protein solution was made up to 100 µL with 25 mM ammonium bicarbonate. DTT (1 M) was added to the protein solution, which was then incubated at 57°C for 1 h. Next, 10 µL of 1M iodoacetamide was added; the sample was mixed and incubated at room temperature for 40 min. Reductive alkylation proteins were filtered using a 10 K ultrafiltration tube at 12,000 rpm, and discarded the solution at the bottom of the collection tube. Proteins bound to the membrane were washed four times with ammonium bicarbonate dissolution buffer. Trypsin formulated in dissolution buffer was added, and the proteins were digested overnight at 37°C. Enzymatically digested peptides were collected and concentrated by centrifugation, and dried.

ZipTip desalting

The dried polypeptides were concentrated by centrifugation, desalted on a ZipTip C18 column, dried, and prepared for mass spectrometry analysis. For desalting, peptides were reconstituted in 0.1% formic acid (FA). The desalting column was activated with 100% acetonitrile and equilibrated with 0.1% FA. Add the redissolved sample into the desalting column to make the sample flow slowly through the desalting column. Peptides trapped on the desalting column were washed with 0.1% FA solution and then eluted with a solution of 0.1% FA and 80% acetonitrile. The peptides were dried using a speed vacuum concentrator.

LC-MS/MS analysis

Vacuum dried samples were reconstituted with 0.1% FA, and 1–2 µg of peptide was used for LC-MS. Peptide separation was performed using an EASY-nLC 1200 (Thermo Scientific, USA) equipped with a self-filled trap column (C18, 5 µm, 100 µm × 2 cm) and an analytical column (C18, 1.9 µm 75 µm × 20 cm) at a flow rate of 200 nL/min. Tandem mass spectrometry was performed on an Orbitrap Fusion Lumos (Thermo Scientific, USA) in data dependent scan (Data Dependent Acquisition, DDA) mode. The full scan resolution was 60,000 (FWHM), and the mass-to-charge ratio range was set to m/z 375–1600. In HCD Fragmentation mode, the collision energy was set to 30%.

Quantitative analysis of protein

This experiment used the Precursor Intensity to conduct Label Free quantitative analysis. The iBAQ data and LFQ data in the MaxQuant software were used for analysis.

GO enrichment analysis

Gene Ontology (GO) annotates genes and gene products in terms of their cellular component, biological process, and molecular function.

Enrichment analysis of KEGG pathway

KEGG (Kyoto Encyclopedia of Genes and Genomes) is the main database used to systematically analyze information about gene functions, genomes and protein groups. When the pathway enrichment analysis was

carried out, the different proteins were mapped with the pathways included in KEGG database. Furthermore, Fisher algorithm is used to calculate the probability of mapping differential proteins to different pathways, which is called pathway enrichment analysis. $P < 0.05$ indicating that the pathway was significantly enriched, which was affected by the experimental treatment.

Statistical analysis

All experiments were performed three times, and data were analyzed using Proteome Discoverer 1.4 and the Mascot server (Version 2.3, Matrix Science, London, UK). For western blot experiments, band density was determined using ImageJ software. All data were analyzed with ANOVA in using SPSS19. The Duncan test was used to compare the values and make multiple comparisons. When $P < 0.05$, the difference was considered to be significant ; and the test data were expressed as mean $\pm$ SD.

Results

Detection of capacitation status

The kinetic parameters of sperm before and after capacitation are shown in Table 1. VCL (curvilinear velocity, m/s) and ALH (amplitude of lateral head displacement, μm) increased significantly after sperm capacitation ($P < 0.05$), indicating that hyperactivation occurred during sperm capacitation.

The changes of protein tyrosine phosphorylation level are shown in Fig. 1. It can be seen from the figure that the protein tyrosine phosphorylation level of capacitated sperm is higher than that of non-capacitated sperm, indicating that protein tyrosine phosphorylation occurs during sperm capacitation.

Acrosome morphology test results

The results of fluorescence microscopy (Fig. 2) showed that the acrosome region of non-capacitated sperm showed bright green fluorescence, and the fluorescence of the capacitated sperm decreased slightly. Sperm in both treatment groups had strong acrosomal integrity, but a series of changes may occur in the acrosomal after capacitation, leading to a slight decrease in fluorescence intensity.

The results of flow cytometry (Fig. 3) showed that the number of living cells decreased, the number of dead cells increased, and the integrity of acrosome and membrane decreased, which indicated that the membrane and acrosome were damaged during sperm capacitation.

Experimental enzyme digestion efficiency and search results

The enzymatic cleavage efficiency for each sample was counted using the MaxQuant software. The enzymatic cleavage efficiency of all samples was greater than 95% (Table 2). Table 3 shows the types of capacitated and non-capacitated proteins that were identified.

Qualitative analysis of proteins

The results of the detection of proteins which differed between the capacitated and non-capacitated sperm groups are shown in Table 4. Among the samples in the capacitation treatment group, 23 specific proteins

were identified. The non-capacitance treatment group had 345 specific proteins. The analysis of protein similarities and differences between the two groups is shown in Fig. 4.

Protein Quantitative Analysis

Quantitative analysis was carried out for the protein whose normalized quantitative value was not 0 in all samples. In the inter group comparison, the normalized signal mean value of all samples in each group was calculated to calculate the inter group Ratio. The protein dataset was screened to determine differential proteins between groups using a cutoff of fold change ≥ 2 between groups (i.e., ratio ≥ 2 or ≤ 0.5) and $P < 0.05$. The proteins differentially expressed between the two groups are shown in Table 5. We identified 89 upregulated proteins and 509 downregulated proteins in the capacitation group compared to the non-capacitation group.

GO enrichment analysis

We next performed GO enrichment analysis to determine the functions of the genes differentially expressed between the capacitated and non-capacitated sperm. GO analysis is used to define the concepts related to gene functions and the way in which these functions are related. Analysis of cellular components for the 729 proteins indicated that nearly half of the differentially expressed proteins were located in “extracellular exosomes” and “extracellular spaces”. The biological process analysis showed that almost all of the processes had many proteins that were downregulated in the capacitated sperm. In terms of molecular function, “cadherin binding involved in adhesion between cells” was the strongest classification (Fig. 5 A-C).

KEGG pathway enrichment analysis

To determine the pathways associated with the differentially expressed proteins, we performed KEGG pathway enrichment analysis (Fig. 6). Some of the important pathways are shown in Table 6. The analysis showed that metabolic pathways were the most involved, and that proteins such as SGSH, ARSB, and BTBD are mainly involved in energy metabolism during sperm capacitation. In addition, we identified two other signaling pathways that appear to be important for sperm capacitation-proteasome signaling pathways and protein transport signaling pathways. PSMD1 and HSPA5 are essential proteins in both pathways respectively. The pathway map is shown in Fig. 7.

Protein-protein interaction (PPI) analysis

PPI analysis was performed for each of the differentially expressed proteins. In the PPI interaction map, the node size indicates the number of proteins interacting with each other, with larger nodes indicating more protein interactions. The color of node indicates upregulation (red) and downregulation (green), and the color intensity indicates the degree of modulation. The thickness of the edge indicates the reliability of the interaction, and the thicker the edge, the higher the reliability. The proteins involved in the proteasome and protein transport signaling pathways are shown in Fig. 8. These pathways are highly connected.

Western blot analysis

To validate the results obtained for differentially expressed proteins identified by quantitative proteomics, western blot analysis was used to examine the expression levels of the PSMD1 and HSPA5 proteins in capacitated and non-capacitated spermatozoa of Yanbian yellow cattle. As shown in Fig. 9 and Fig. 10, the expression levels of PSMD1 in the capacitated group was significantly lower than in non-capacitated group, and the expression level of HSPA5 in capacitated group was significantly higher than in the non-capacitated group. These results are consistent with our mass spectrometry results.

Discussion

After ejaculation, in order to acquire the ability to fertilize, sperm must undergo a maturation process called capacitation. It is generally believed that motility is one of the most important characteristics related to sperm fertilization, and it is an expression of sperm motility and structural integrity [10]. Hyperactivation is a highly asymmetric movement mode that occurs when the sperm is close to the oocyte. It is the guarantee for the sperm to complete the fertilization with the oocyte at the appropriate position and time. The increase of tyrosine phosphorylation (pY) is one of the most important processes in capacitation, protein phosphorylation in tyrosine residues has become a marker to define sperm capacitation [11]. This protein modification has been proposed to play a role in sperm motility in some species.

Changes of hyperactivation parameters before and after sperm capacitation

In most species, mature sperm remains immobile in the epididymis until released, and they quickly begin to swim. This process is called activation of motion. The capacitated sperm produces a nearly symmetrical flagellum rhythm, which is driven by a nearly linear trajectory. When sperm are overactive, the extent to which the flagellum bend is increased, usually on only one side of the flagellum. Sperm hyperactivation is essential for fertilization because translucency of the zona pellucida requires sperm hyperactivation. A large number of studies in various species have shown that hyperactive exercise is essential for in vivo fertilization because it helps sperm penetrate the zona pellucida [12].

Computer Assisted Sperm Analysis (CASA) system has been used to detect the percentage of hyperactivated sperm. VCL is a representative index of hyperactive motility, VCL and ALH are parameters greatly increased in hyperactive sperm, BCF, ALH and VCL are indicative parameters of sperm motility [13]. The results showed that the VCL, ALH and BCF of capacitated sperm were higher than those of non capacitated sperm, non-capacitated spermatozoa were in a relatively low beat-cross frequency (BCF) and did not fully perform its swing function, it is suggested that the state of non capacitated sperm is to reduce energy consumption, so as to prepare for the increase of sperm swing frequency and smooth penetration of zona pellucida after capacitation [13]. LIN (linearity) represents the linear motion pattern of sperm. The LIN of the capacitated sperm was smaller than that of the non capacitated sperm, because the hyperactivation of the sperm after capacitation was highly asymmetric, and the linearity of the sperm movement decreased. STR (straightness) is a parameter of sperm motility. The STR of capacitated sperm is larger than that of non capacitated sperm, and the motility of capacitated sperm is enhanced, which is conducive to sperm penetrating the zona pellucida. The results showed that the LIN of the capacitated sperm was smaller than that of the non

capacitated sperm, and the STR of the capacitated sperm was larger than that of the non capacitated sperm. Studies have shown that sperm with high VSL (Straight line velocity) has better sperm-oocyte binding ability [14], our results showed that the VSL of capacitated sperm was higher than that of non capacitated sperm.

Studies have shown that sperm without hyperactivation cannot cross the zona pellucida and bind to the oocyte [12]. Hyperactivation ability is regarded as an important index of male reproductive performance, and it is an indispensable link to ensure the successful fertilization of male animals [15]. It is known that the phosphorylation of sperm protein and the protein tyrosine phosphorylation downstream of the pathway are one of the maintenance conditions of sperm hyperactivity [16]. Therefore, the level of protein tyrosine phosphorylation was detected next.

Protein phosphorylation is a marker of capacitation

Post-translational modifications, such as protein phosphorylation or dephosphorylation, regulate sperm capacitation, hyperactivation, and acrosome reactions, which are essential for sperm to reach, bind, penetrate, and fuse with oocytes [17, 18]. In most species, immunocytochemistry has shown that tyrosine phosphorylated proteins are mainly located in the flagellum of capacitated spermatozoa [19]. During capacitation, the distribution of tyrosine phosphorylation proteins in the flagellum is related to sperm hyperactivation. The correlation between protein tyrosine phosphorylation and capacitation has not been fully established. However, some studies have shown that protein tyrosine phosphorylation is a marker of capacitation [20]. Arcelay and Visconti proposed that the increase of Tyr (tyrosine) phosphorylation level of sperm protein is usually considered as a marker of late capacitation [21, 22]. Zapata et al. evaluated sperm capacitation by measuring protein tyrosine phosphorylation levels, and detected a significant increase in total phosphotyrosine content [23].

In mice, the presence of tyrosine phosphorylated proteins is considered essential for acrosome reactivity and fertilization in vitro, therefore it is regarded as the end point of capacitation. However some researchers believe that the appearance of tyrosine phosphoprotein is not always the end point of capacitation [24]. No fertilization has been reported in the absence of tyrosine phosphorylation. In this experiment, the curvilinear velocity and tyrosine phosphorylation level of capacitated sperm were significantly higher than those of non-capacitated sperm, indicating that hyperactivation and protein tyrosine phosphorylation occurred during sperm capacitation. Some studies have found that tyrosine phosphorylation is a marker of capacitation [25], leading to sperm hyperactivation and rapid beating of flagella during asymmetric sperm movement. This result is consistent with this experiment. It has also been found that tyrosine phosphorylation proteins related to capacitation are associated with male fertility [26].

The role of the proteasome in sperm capacitation

Proteasomes are responsible for the breakdown of most proteins in cells. The active site of proteolysis is located in the 20S core particles (CP). The only ATP-hydrolyzed proteins in the proteasome are the six ATPases present in the regulatory granules [27, 28]. The main discovery with respect to proteasome regulation is that mammalian PKA phosphorylation stimulates the function of the 26S proteasome [29]. Proteasomes exist in sperm of many species and participate in a variety of sperm functions, including capacitation [30]. Several studies have shown that mammalian sperm have all of the components of the 26S proteasome.

A recent study described in detail the role of the proteasome degradation A kinase anchored fibrosheath protein AKAP3 (A kinase-anchored protein 3) in bovine sperm capacitation, this is related to the ability of the bull to acquire energy and acrosome exocytosis [31]. Bovine sperm shows activity of all three major proteasome core enzymes [32]. The acrosomal proteasome may play a dual role during the sperm-zona pellucida interaction, the first is the mediator of acrosome exocytosis, followed by protease, which promotes the local degradation of zona pellucida matrix, leading to the formation of fertilization suture [33]. The degradation of soluble zona pellucida proteins by bovine sperm proteasome has not been reported, but it has been confirmed in humans [34] and Japanese quail [35] [36]. In humans, sperm proteasomes are involved in acrosomal exocytosis and the duration of Ca^{2+} influx before [37]. Sperm treatment with proteasome inhibitors blocks the ability and alters the pattern of protein phosphorylation without affecting sperm motility or ZP binding ability [38]. Furthermore, TK and PKA inhibitors decreased the activity of proteasome during capacitation. PSMD1 is a subunit of proteasome 19S regulatory granules and acts as a docking site for ADRM1, ADRM1 is another proteasome subunit that absorbs ubiquitinated substrates for proteolysis. PSMD1 is sumoylated by SUMO E3 enzyme PIASy. Ryu et al. found that sumoylation of a key lysine residue adjacent to the ADRM1 binding domain in PSMD1 regulates the association between ADRM1 and PSMD1. Moreover, the sumoylation-dependent interaction between PSMD1 and ADRM1 may alter the composition and function of the proteasome [39]. These findings confirm the mechanism by which the SUMO family of ubiquitin-like proteins regulates ubiquitin-mediated protein degradation. Our results showed that the expression level of PSMD1 in the capacitated sperm group (H) of three Yanbian yellow cattle was significantly lower than that in the non-capacitated sperm group (F), indicating that ubiquitin mediated protein degradation may be involved in the capacitation process of bull sperm. Some reports suggest that ubiquitin proteasome system may play a role in sperm capacitation, acrosomal reaction, and/or sperm-oocyte fusion [40]. Pizarr et al. verified the existence of proteasomes in the sperms of several mammals, and Sanche et al. demonstrated their importance in the process of in vitro fertilization and acrosomal exocytosis in bovine [41]. All this evidence indicate that sperm proteasome plays an active role in the capacitation.

The role of chaperones in sperm capacitation

Chaperones are a group of proteins characterized by the ability to recognize the exposed hydrophobic surfaces of newly synthesized or partially folded proteins, and help them achieve their functional conformations. Thus, chaperone proteins prevent incorrect reactions that would otherwise lead to misfolding and aggregation of the protein. Prototypes chaperones were initially identified by increased expression after cells exposure to environmental stresses such as heat shock, and are therefore referred to as “heat shock proteins” (HSPs) or “cell stress response proteins”. It is becoming increasingly apparent that these proteins play important roles in a variety of essential cellular processes that aid in protein folding, intracellular transport, membrane transport, and protein degradation. Therefore, these proteins are highly conserved during evolution, and are widely expressed in almost all eukaryotic and prokaryotic cells. Several somatic and germ cell-specific molecular chaperones have been identified in male germ line, and appear to be essential for cell cycle progression in the initial stage of spermatogenesis [42]. Heat shock proteins are ubiquitous molecules in cells and acting as molecular chaperones under stress conditions, including carcinogenesis [43]. HSPA5 (GRP78) is involved in two signaling pathways: thyroid hormone synthesis and protein transport. Lachance et al. reported two separate chaperone proteins, HSP60 and GRP78, that are both expressed by oviduct epithelial cells (OECs) and regulated the potential for sperm motility, protein tyrosine phosphorylation, and intracellular

calcium levels during capacitation. Our results showed that the expression level of HSPA5 in the capacitated sperm group (H) was significantly higher than that in the non-capacitated sperm group, suggesting that HSPA5 participates in the process of bull sperm capacitation. HSPA5 and HSPD1 proteins have been shown to bind to human sperm [44], and since chaperones are present in seminal plasma they may be adsorbed to the cell surface only after ejaculation and semen liquefaction [45]. Data from Lobo et al. also showed that GRP78 was phosphorylated at serine, threonine, and tyrosine residues, indicating that sperm GRP78 phosphorylation is converted during sperm maturation [46]. The heat-shock protein HSPA5 may be involved in capacitation.

Conclusion

In this study, we identified many differentially expressed proteins between capacitated and non-capacitated Yanbian yellow cattle sperm. The changes in PSMD1 and HSPA5 levels, which are associated with the proteasome signaling and protein transport signaling pathways. PSMD1 as a proteasome subunit regulates ubiquitin-mediated protein degradation, which may play a role in sperm capacitance in Yanbian yellow cattle. HSPA5, as a heat shock protein involved in protein transport pathway, may regulate sperm capacitation in Yanbian yellow cattle. These results provide a foundation for understanding the molecular processes driving sperm capacitation in these cattle, and for improving the reproductive capacity of Yanbian yellow cattle. However, this study is not deep enough, and further research is needed.

Abbreviations

GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; PPI: Protein-protein interaction; PBS: Phosphate buffer saline; CASA: Computer Assisted Sperm Analysis; VCL: Curvilinear velocity, m/s; ALH: Amplitude of lateral head displacement; BCF: Beat-cross frequency; LIN: Linearity; STR: Straightness; VSL: Straight line velocity; PY: Phosphorylation; HSPs: Heat shock proteins

Declarations

Acknowledgements

Thank you for the samples provided by Benfu cattle farm in Yanji city.

Funding

This study was supported by the Key Agricultural Technology Research Project of Jilin Provincial Science and Technology Department [20190301037NY].

Availability of data and materials

The data and computing programs used in this manuscript are available from the corresponding author on request.

Authors' contributions

YJ conceived and designed the experiment. CX and YJ wrote the manuscript. YQL and YCX detected the capacitated state. CX, YQL and MMC performed data analysis. ZCL, XLQ and YYZ collected the samples. CX and ZCL performed the rest of the experiment.

Ethics approval and consent to participate

None of the authors have any conflict of interest to declare. Procedures of animal tissue sampling were followed under the guideline from the research ethical committee of Animal Center for Biomedical Experimentation at YanBian University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Kinetic parameters of Yanbian yellow cattle sperm before and after capacitation

A

Different treatments	Kinetic parameters					
	VCL	ALH	VSL	LIN	BCF	STR
	μm/s	μm	μm/s	%	Hz	%
Non-Capacitated	154.40±3.38 ^b	5.68±0.23 ^b	86.77±2.63	52.10±2.38	23.47±2.34	67.76±2.85
Capacitated	196.80±4.37 ^a	6.69±0.09 ^a	92.47±2.97	46.05±3.40	26.06±3.31	74.32±3.33

B

Different treatments	Kinetic parameters					
	VCL	ALH	VSL	LIN	BCF	STR
	μm/s	μm	μm/s	%	Hz	%
Non-Capacitated	164.43±3.79 ^b	5.80±0.13 ^b	87.10±3.52	53.32±3.02	23.72±3.50	68.43±4.22
Capacitated	200.03±3.09 ^a	6.86±0.18 ^a	93.03±2.90	46.67±4.44	26.26±3.14	76.57±3.14

C

Different treatments	Kinetic parameters					
	VCL	ALH	VSL	LIN	BCF	STR
	$\mu\text{m/s}$	μm	$\mu\text{m/s}$	$\%$	Hz	$\%$
Non-Capacitated	157.20 $\pm$ 3.12 ^b	5.80 $\pm$ 0.28 ^b	87.22 $\pm$ 2.47	51.96 $\pm$ 2.53	23.24 $\pm$ 2.77	66.77 $\pm$ 2.99
Capacitated	199.37 $\pm$ 2.05 ^a	6.96 $\pm$ 0.15 ^a	93.28 $\pm$ 4.00	46.01 $\pm$ 3.51	25.47 $\pm$ 2.92	74.28 $\pm$ 3.86

Note: A, B and C are the serial Numbers of the three yanbian yellow cattle.

Table 2. Statistics of enzyme cutting efficiency

Sample name	Total number of peptide digestion sites	The number of peptides with a missing cut site of 2	Proportion of missing cut site	Enzyme digestion efficiency
Non-Capacitated	19606	459	2.34%	97.66%
Capacitated	12437	320	2.57%	97.43%

Table 3. Statistical table of the number of searching proteins

Different treatments	Number of protein identification species
Non-Capacitated	2535
Capacitated	1951

Table 4. Qualitative differential protein statistics

Specific detection sample	Number of protein species
Capacitated	23
Non-Capacitated	345

Table 5. Statistical table of differential proteins

Comparison between groups	Up-regulate species	Down-regulated species	Total
Capacitated and Non-Capacitated	89	509	598

Table 6. The KEGG pathway analysis of differentially expressed proteins

Pathway Name	Protein Counts	Gene Name
Metabolic pathways	85	SGSH, ARSB, BTB, CNDP2, ATP6AP1, PGD...
lysosomal	46	SGSH, ARSB, GM2A, LGMN, ATP6AP1, HEXB...
Oxidative phosphorylation	13	COX7A1, NDUFA9, ATP6AP1, COX7C, ATP6V1H, ATP6V1D...
proteasome	10	PSMB7, PSMD1, PSMD12, PSME1, PSMD11, PSMC3...
Thyroid hormone synthesis	7	HSP90B1, GPX5, GPX3, GNAS, HSPA5, PDIA4, LRP2
Protein transport	4	IMMP1L, SPCS3, SRPRA, HSPA5

Figures

Figure 1

Phosphorylation levels of protein tyrosine in bovine spermatozoa of capacited and noncapacited Yanbian yellow cattle. The left figure shows the immunoblot result of the phosphorylation level of sperm protein tyrosine, and the right figure shows the relative expression levels.

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Figure 2

PNA-FITC staining observed on capacitated and noncapacitated sperm of Yanbian yellow cattle. (A) The acrosomal region of non-capacitated sperm shows bright green fluorescence; (B) The fluorescence of capacitated sperm decreased slightly.

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PNA-FITC flow cytometry results of capacitated and noncapacitated sperm of Yanbian yellow cattle. (A) Flow cytometry image; (B) Histogram.

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Figure 4

Analysis of protein similarities and differences between the two groups

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Figure 5

GO analysis of differentially expressed proteins. (A) Histogram showing the number of proteins involved in cell components; (B) Histogram showing the number of proteins involved in biological processes; (C) Histogram showing the number of proteins involved in molecular function.

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Figure 6

Pathway analysis data of enabled and non-capacited sperms show the sample dotplot

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Figure 7

Dstatistics of regulated enrichment by KEGG pathway analysis. (a) KEGG diagram of proteasome signaling pathway; (b) KEGG diagrams of protein transport signaling pathways. The red box represents upregulation. The green box represents the down-regulated protein.

Figure 8

PPI interaction diagram The size of nodal protein represents the number of proteins interacting with it; The color of nodal protein indicates up-down, up-down to red and down to green; The darker the color is, the greater the modulation degree is. The thickness of the interaction relation line (edge) between node proteins

indicates the trust degree of the interaction (the trust degree of the interaction relation is rated by the STRING database). The thicker the edge is, the higher the trust degree is.

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Figure 9

Western blot analysis of PSMD1 protein The result of western blotting is shown on the left, and the relative protein expression is shown on the right.

Figure 9

Western blot analysis of PSMD1 protein The result of western blotting is shown on the left, and the relative protein expression is shown on the right.

Figure 10

Western blot analysis of HSPA5 protein The result of western blotting is shown on the left, and the relative protein expression is shown on the right.

Figure 10

Western blot analysis of HSPA5 protein The result of western blotting is shown on the left, and the relative protein expression is shown on the right.