

The characteristics of proteome and metabolome associated with contrasting sperm motility in goat seminal plasma

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Abstract

Sperm motility is an index tightly associated with male fertility. A close relationship between seminal plasma and sperm motility has been confirmed. This study was to assess the protein and metabolite profiles of seminal plasma obtained from adult goats with high or low sperm motility using the proteomic and metabolomic strategies. In total, 2098 proteins were found. 449 differentially expressed proteins (DEPs) were identified, and 175 DEPs were enriched in the high motility group. The obtained DEPs primarily exist in cytoplasm and extra-cellular portion. The Gene Ontology enrichment analysis demonstrated the main functional roles of these DEPs in regulating biological process, metabolic process of organic substances, cellular-metabolic process, primary-metabolic process, metabolic process of nitrogen compounds, etc. Additionally, the Kyoto-Encyclopedia of Genes and Genomes (KEGG) analysis revealed that these DEPs were primarily involved in phosphatidylinositol signaling system, salivary secretion, proteasome, apoptosis, mitophagy-animal, etc. Aided by the parallel reaction monitoring technology, the abundance changing pattern of 19 selected DEPs was consistent with that of the corresponding proteins obtained by TMT. A total of 4603 metabolites were identified in seminal plasma. 1857 differential metabolites were found between the high motility group and the low motility group, and 999 metabolites were up-regulated in the high motility group. The KEGG analysis demonstrated the primary involvement of the differential metabolites in metabolic and synthetic activities. In conclusion, we first established the proteome and metabolome databank of goat seminal plasma, detecting some proteins and metabolites which may affect sperm motility. This study will be valuable for understanding mechanisms leading to poor sperm motility.

Introduction

Artificial insemination (AI) can be definitely thought as one of the earliest and the most extensively utilized assisted reproductive technologies in animal breeding and production ¹. In brief, AI means that male sperm are introduced into female reproductive tract aided by specific tools. At present, AI has been recognized as an efficient and important approach for extending valuable genes in a stock population and improving their production traits ^{1,2}. As compared to natural mating, AI largely enhance the reproductive potential of an excellent male animal. Even after its death, using some long-term storage methods of its semen, we can still obtain its offspring ². Furthermore, AI can be combined with the estrus synchronization technique to realize the out-of-season breeding and improve the accuracy of expecting dates of parturition. Moreover, this technology can avoid disease transmission, since males used for AI generally experience some strict disease examinations ³.

Currently, AI has been extensively applied in the dairy industry ⁴. In general, the site where semen is deposited greatly influences the fertilization results of oocytes with sperm. Moreover, the number of motile sperm is also tightly associated with the conception rate. In comparison with fresh or cooled semen, more motile sperm are required when frozen-thawed semen is used for AI ⁵. It's known that the features of sheep and goat reproductive tract are different from that of cow, which prevents lots of sperm

entering uterus and makes sperm walk a long way to arrive at the oviduct and fertilize with oocytes. Currently, the fertility of AI is especially unsatisfied with the use of frozen-thawed sperm². So, it is becoming important to select those males that can produce semen with a high quality.

Healthy sperm motility is widely recognized as a core factor which can influence male fertility. Those defectively mobile or immobile sperm are usually unfruitful or sterilized except that some assisted reproductive techniques are used⁶. Some AI centers are currently using wave motion of semen as a main index to choose ejaculates for AI in sheep⁷. Furthermore, mass motility has been confirmed to be closely related to fertility in sheep⁸. However, the root causes leading to poor sperm motility are complicated and have not been determined until now.

Seminal plasma is an important part of semen, which is mainly composed of secretions derived from testicular, epididymis, and secondary sex gland. In seminal plasma, the components generally consist of proteins, ions, and metabolites like nucleosides, lipids, monosaccharides, amino acids, minerals, electrolytes, and also steroid hormones^{9,10}. Previous studies have proved that seminal plasma contributes a significant role in regulation of sperm motility and fertilization¹¹⁻¹⁵. Seminal plasma provides metabolic support, and it has a complicated and not well-understood effect on the physiological role of sperm. Additionally, seminal plasma also has certain impacts on the quality of chilled¹⁶ or frozen-thawed sperm¹⁷.

Some studies investigated the effects of proteins presented in seminal plasma on semen quality. In sheep, some investigators have attempted to study the proteome of seminal plasma¹⁸⁻²¹. In accordance with the report of Richard et al., variations in freezing resistance of sheep sperm is linked with origin and composition of seminal plasma. It was found that freezing with seminal plasma derived from sheep with high resistance to cryoinjury enhanced the post-thaw quality of sperm¹⁸. Furthermore, several protein markers potentially associated with sperm freezing resistance in sheep seminal plasma were identified by the same group. The proteins strongly linked with sperm freezing resistance, including 26S proteasome-complex, acylamino-acid releasing enzyme, alpha-mannosidase class 2C, heat-shock protein 90, tripeptidyl peptidase-2, TCP-1 complex, sorbitol-dehydrogenase, and transitional-endoplasmic reticulum ATPase, were detected. On the contrary, cystatin, zinc 2-alpha-glycoprotein, angiogenin 2-like protein, cartilage-acid protein-1, cathepsin-B, and ribonuclease-4 isoform-1 had negative effects on the resistance of sperm to cryoinjury¹⁹. In pig, the seminal plasma proteome and the associations between seminal plasma proteins and semen features have been established. Particularly, sperm motility had a strongly positive correlation with lactadherin¹³. In human, the analysis related to the seminal plasma proteome reflects reduced mitochondrial production, acrosome disruption, and DNA fragmentation, with several post-genomic functions associated to these alterations¹¹. Similarly, in rabbit, major seminal plasma-derived proteins contribute to the prevention of lipid peroxide radical damage and oxidative stress, membrane stability, sperm membrane transport and temperature control. Additionally, sperm motility had positive correlations with growth factor beta-nerve and cysteine rich secreted protein-1. However, a significantly negative association of sperm motility with galectin-1 existed¹⁰.

Since metabolites were end products of metabolic pathways, which also play important role in sperm physiology, such as motility, energy metabolism, and metabolic activity regulations²². Although certain seminal plasma components have beneficial effects on sperm cryotolerance, whereas others may have negative effects^{23,24}. In bull, some metabolites in seminal plasma, including 2 oxoglutaric-acid or fructose, can be used to evaluate their fertility as possible biomarkers²⁵. Hamamah et al. (1993) studied fertile and infertile male seminal plasma using ¹H nuclear-magnetic resonance spectra (NMR), finding important variations in concentrations of glyceryl phosphorylcholine-citrate and lactate among azoospermic and oligo-asthenozoospermic patients²⁶. The metabolite profile of rhesus macaque sperm was also investigated in order to establish the relationship among metabolism and energy source²⁷. Additionally, a metabolomics strategy in mouse sperm was used to evaluate the relationship between glycolytic-substrates and energy generation, which is critical for sperm motility²⁸. Furthermore, using both the NMR and the gas chromatography mass spectrometry (GC-MS), a total of 96 metabolites and more than 10 biological pathways have been detected in human sperm²⁹. More recently, Velho et al. (2018) identified 63 metabolites in bull seminal plasma, including 21 amino acids from bull with distinct field-fertility ratings, demonstrating the different metabolite patterns between the low-fertility and high-fertility bulls³⁰.

Currently, the application of AI in the goat industry is not popular as compared to the other stocks, such as cow or sheep. Moreover, the effect of seminal plasma on sperm motility in goats until now has not been clarified. Different from the other farm animals including sheep, goat seminal plasma contains phospholipase A, which is released from the bulbourethral gland. This protein can coagulate egg-yolk and hydrolyze the lecithin to fatty acids and spermicidal lyso-lecithins³¹. Therefore, during cryopreservation of goat semen, the effects of seminal plasma must be considered. In this study, we attempted to examine the variation of proteome and metabolome in goat seminal plasma associated with sperm motility using the high throughput technologies. This study will enrich our omics information related to goat semen. Furthermore, some obtained specific proteins or metabolites may be used as biomarkers for assessing the quality of goat semen and predicting of male fertility.

Results

Sperm quality assessment

In this study, twenty goats were equally separated into two groups. As shown in Fig.1., one group had a high motility (78.85%±2.44%). The other one had a low motility (62.16%±2.27%). The difference between these two groups was significant ($P<0.01$). However, no differences were found among these two groups with regards to the plasma membrane and acrosome integrity ($P>0.05$).

Identification of proteomic information

A total of 308933 spectra were obtained after the TMT analysis and searching by Maxquant (v1.5.2.8) in the present study. The number of the matched spectra was 36946. Furthermore, we detected 15724

peptides (14861 specific peptides) among these spectra, and 2299 proteins including 2098 quantified proteins. The basic information on the protein profile of goat seminal plasma, such as protein accession, protein definition, gene name, *P* value, etc., was revealed in Supplementary Figure S1.

Identification of DEPs

In total, 449 DEPs, with 1.5 folds shift and *P*-value of less than 0.05, were identified in goat seminal plasma with high or low motility. The detailed information associated with the identified DEPs, such as protein accession, protein definition, regulated type, *P*-value, gene name, etc, was shown in Supplementary Table S2. In comparison with the low motility group, 175 proteins were up-regulated in the high motility group, such as beta-galactosidase, ATP-citrate synthase, 3-phosphoinositide-dependent protein kinase 1-like, trafficking protein particle compound subunit 13 isoform X2, kinesin-like protein, a protein phosphatase inhibitor 2, etc. On the other hand, the abundance of 274 proteins significantly reduced in the high motility group, such as putative adenylate kinase 7, calpain-7-like protein, transmembrane protein 190, fibrous sheath interacting protein 2, angiotensin-converting enzyme, casein kinase II subunit beta, sperm acrosome-associated protein 5, etc.

Functional classification of DEPs

The results of the GO annotation analysis were presented in Fig.2. Regarding the classification of biological process, the acquired DEPs were significantly enriched in regulating biological processes, organic substance metabolic procedure, cellular metabolic procedure, primary metabolic procedure, nitrogen compound metabolic procedure, cell component organization, localization establishment, etc. Additionally, the acquired DEPs were primarily located in the intracellular region, intracellular organelle, membrane-bound organelle, endomembrane system, organelle lumen, non-membrane-bound organelle, etc., with regard to the GO analysis of cellular component. As concern molecular function, the DEPs were primarily involved in protein binding, hydrolase activities, ion binding, organic cyclic complex binding, transferrase activity, heterocyclic complex binding, etc.

Moreover, the COG/KOG functional classification statistics related to the acquired DEPs were presented in Fig.3. 67 DEPs were found to be involved in post-translational modification, protein turnover, and chaperone. 55 DEPs may have potentially functional roles in the pathways of signal transduction. There were 34 DEPs engaged in cytoskeleton. Additionally, some DEPs were found to function in metabolic activities, including energy manufacturing and adaptation (20), amino acids carrying with metabolism (18), carbohydrates carrying with metabolism (22), lipids carrying with metabolism (11), etc. Interestingly, three proteins, including myosin XVB, dynein light-chain Tctex-type-1, and dynein light-chain roadblock-type-2, were found to be associated with cell motility.

Subsequently, the results of the KEGG study were presented in Fig.4., including the top 20 substantially enriched pathways. The main pathways were revealed, including the phosphatidylinositol signaling system, salivary secretion, proteasome, apoptosis, mitophagy-animal, NOD-like receptor signaling pathway, etc. Furthermore, some DEPs were detected to be potentially involved in disease or infection,

such as tuberculosis, Kaposi sarcoma-associated herpesvirus infection, Huntington disease, staphylococcus aureus infection, etc.

PRM validation

In this study, in order to confirm the accuracy of the findings acquired in relation to seminal plasma proteome, 19 DEPs were selected for the PRM analysis, including phosphoglycerate mutase, ras-related protein Rab-11B, ATP-citrate synthase, peroxiredoxin, spermadhesin-1, T complex protein-1 sub-unit alpha, programmed cell death protein 5, testis-tissue sperm binding protein Li 69n, ubiquitin-like modifier-activating enzyme 1, zonadhesin, superoxide dismutase, sperm equatorial segment protein 1, thioredoxin reductase 2 (mitochondrial), acrosin-binding protein, zona pellucida binding protein, heat shock protein family-E (Hsp10) member-1, peroxiredoxin-1, aquaporin 7, and izumo sperm-egg fusion protein 4. The pattern of the fold changes in these proteins between these two groups, as shown in Table 1, was consistent with the results obtained by the TMT method.

Identification of metabolomic data

The metabolomic analysis of goat seminal plasma was performed using a non-targeted metabolomics strategy, including the positive and negative modes. In the present study, a total of 4603 metabolites have been quantified in seminal plasma derived from the two groups with high or low motility, including amino acids, peptides, fatty acids, lipids, sugars, nucleic acid, organic acids, and other metabolites. The detailed information associated with these identified metabolites, including index, mass, retention time (RT), compounds, formula, etc, was presented in Supplementary Table S3.

Determination of differential metabolites

Based on the Variable Importance in Projection (VIP), fold-change (FC), and *P*-value, the differential metabolites between these two groups were identified. The detailed information related to these identified metabolites were presented in Supplementary Table S4. Totally, 1857 differential metabolites were identified between the high motility group and the low motility group. Among these metabolites, 999 metabolites were up-regulated in the high motility group. However, 858 metabolites were significantly enriched in the low motility group. The numbers of various compounds presented in goat seminal plasma were shown in Fig.5.

Bioinformatics analysis of differential metabolites

The cluster analysis data of the acquired differential metabolites were shown in Fig.6. As shown in Fig. 6A, the cluster patterns between these two groups with high or low motility were opposite. The metabolites enriched in the high motility group were less abundant in the group with low motility instead. The correlation analysis results of differential metabolites were demonstrated in Fig.6B. Here, the Pearson correlation analysis method has been used to evaluate the correlation of differential metabolites. The top 50 differential metabolites with the largest VIP value were displayed.

In accordance to the VIP-value, the top 20 differential metabolites with largest VIP value were presented in Fig.6C. Among these metabolites, D-Ornithine (MW7114), ostruthin (MW2125), TRIPTOPHENOLIDE (MW2959), Ornithine (MW8535), Citrulline (MW6929), Glu-Phe (MW7553), Cocaine (MW1036), and 4-formyl Indole (MW6060) were enriched in the low motility group. However, 2-Methylguanosine (MW215), Glycerophosphorylcholine (MW7620), (3S)-3,6-Diaminohexanoate (MW5511), Deoxyadenosine (MW7155), Crotonobetaine (MW5523), Saccharopine (MW9069), Adenosine (MW6298), N,N-Dimethylguanosine (MW8389), L-Threonine (MW7963), 2,3-Bis (4 hydroxyphenyl) 1,2-propanediol (MW245), 7-Methylguanosine (MW6213), and L-Prolinamide (MW7951) were more abundant in the high motility group.

In addition, based on the fold change between these two groups, the most enriched differential metabolites were shown in Fig.6D. Thioetheramide-PC (MW9485), Adenosine (MW6298), N,N-Dimethylguanosine (MW8389), Isocitric acid (MW1661), Deoxyadenosine (MW7155), Glycocholic Acid (MW7625), Crotonobetaine (MW5523), 1-O (cis-9-Octadecenyl) 2-O-acetyl-sn glycerol 3-phosphocholine (MW5607), Acetylcarnitine (MW6280), and 17 α -Hydroxypregnenolone (MW5744) were significantly enriched in high motility group. On the other hand, N⁶-[(Indol-3-yl)acetyl]-L-lysine (MW8382), Pyroglutamic acid (MW2427), L-Proline (MW7953), Thr Leu Arg (MW9506), Dimethylglycine (MW1228), Arg Glu Ser Leu Glu (MW6447), 2-Keto-glutaramic acid (MW206), Glu-Phe (MW7553), Cocaine (MW1036), and Citrulline (MW6929) were more abundant in the low motility group.

Functional annotation of differential metabolites

In this study, the KEGG annotation was used to identify all potential pathways which the acquired differential metabolites may be involved in. As shown in Fig.7., most metabolites were mainly engaged on metabolic and synthetic activities, such as biosynthesis of secondary metabolites, lysine-biosynthesis, alpha linolenic-acid metabolism, porphyrin with chlorophyll metabolism, fatty acids metabolism, peptidoglycan biosynthesis, lipopolysaccharide biosynthesis, etc. The detailed information related to the KEGG annotation were included in the Supplementary Table S5.

Discussion

It has been reported that sperm motility is tightly associated with the results of AI⁶⁻⁸. Furthermore, seminal plasma plays a critical role in sperm motility regulation⁹⁻¹³. Mechanisms which lead to poor sperm motility, however, are still not clear and need to be elucidated. Therefore, the relationship between seminal plasma components and sperm motility needs to be explored. In the current study, we explored the effects of the protein and metabolite components included in seminal plasma on goat sperm motility using the proteomic and metabolic methods. The high-throughput technology can act as an efficient method to classify and identify proteins and metabolites for the prediction of their potential roles in complicated biological systems^{25,32}. We first established the proteome and metabolome datasets of goat seminal plasma, and then analyze potential functions and pathways in which the detected DEPs

and differential metabolites may be involved. These outcomes may be helpful for further understanding of mechanisms leading to poor sperm motility.

Besides, the PRM, as a new-developed MS technology, was used to verify the results of the TMT method in the present study. In previous studies, this technology has been used for the quantification and detection of specific proteins among biological samples^{32,34}. It can calculate dozens of proteins simultaneously with greater quantity sensitivity and assurance as compared to those conventional methods of protein verification including western blot or immunofluorescence approach³³. Based on the acquired results, a high consistency between the PRM results and the TMT results further confirmed that the proteomic data acquired in this study were accurate and believable. In addition, the percentages of sperm with intact plasma membrane among these samples used were similar, implying that the proteins and metabolites identified in seminal plasma cannot be derived from leakage of sperm during the treatment.

In accordance with our study, the abundance of 445 proteins was significantly different between the high motility group and the low motility group, and these proteins may be related to the alteration of sperm motility. The GO results showed that the DEPs primarily function in metabolic activities, such as the metabolism of organic substances, the cellular metabolic procedure, the main metabolic procedure, and the metabolic procedure of nitrogen compounds. It is known that sperm motility is highly linked with cellular metabolism. The normal moving capability of sperm requires the support of ATP. Some DEPs related to ATP production were found in this study. For example, ATP-citrate synthase was more abundant in the high motility group than the low motility group. ATP-citrate synthase is involved in ATP synthesis. In rat, reduced sperm motility and concentration have been found to be induced by a reduction in the activity of ATP-citrate synthase³⁵. Moreover, a decrease in the level of cellular ATP and an increase in oxidative stress were also observed³⁵. Additionally, phosphoglycerate kinase 2 (PGK2), an isozyme that catalyzes the first step in the ATP-generating glycolytic pathway, was also up-regulated within the high motility group. This protein is also known for its important roles in sperm motility and male fertility³⁶. Similarly, 6-phosphogluconate dehydrogenase was involved in the pentose phosphate pathway and engaged on ATP production, consequently supporting motility of sperm³⁷.

84 DEPs were found to be associated with response to stress. Mature sperm are highly sensitive to environmental stimuli. [Sperm lysozyme-like protein 1, as an intra-acrosomal oolemmal-binding sperm protein](#), was significantly down-regulated in the high motility group. This protein has been found to be involved in binding of sperm to egg plasma membrane during fertilization³⁸. Also, we found the abundance of heat shock protein family E increased instead in the low motility group. In human, heat shock proteins, which are highly expressed in the testis, is correlated with male fertility³⁹. Furthermore, in bull, the level of sperm heat shock protein reduced significantly after the freezing and thawing process, which possibly linked with reduced sperm motility, plasma membrane integrity and acrosome integrity⁴⁰.

Regarding molecular function, 23 DEPs were found to function in regulation of oxidoreductase activity. Peroxiredoxin, an important antioxidant in mammalian semen, was less abundant in the low motility group. In human, the sperm suspension supplementation obtained from asthenozoospermic men efficiently enhanced sperm motility and DNA integrity through minimizing reactive oxygen levels ⁴¹. Superoxide dismutase 1 plays a pivotal role in antioxidation by scavenging superoxide anions. Some studies have demonstrated that superoxide dismutase 1 is tightly associated with sperm quality, including sperm motility ⁴²⁻⁴⁴. But, in this study, superoxide dismutase 1 was found to be more abundant in the low motility group. The reason is not known, and we preliminarily hypothesize that this phenomenon may be related to a compensatory increase in goat seminal plasma with low motility.

The COG/KOG functional classification was also used to assess the potentially functional roles of the DEPs. 34 DEPs were found to be engaged on skeleton structure and function, including 11 DEPs up-regulated in the high motility group. A previous study has confirmed the position of kinesin-like proteins in sperm flagella, so kinesin-like proteins may play vital roles in intra-flagellar transport and flagellar formation during spermatogenesis ⁴⁵. The other down-regulated DEPs, such as actin-like protein 7B, tektin-5, dynein, tubulin, etc, are well known to be involved in construction of skeleton. However, the role of kinesin-like proteins in seminal plasma still need to be elucidated. Furthermore, three proteins, including myosin XVB, dynein light chain Tctex-type 1, and dynein light chain roadblock-type 2, were found to directly influence cell motility in this study.

To the best of our knowledge, this study is the first to conduct a comprehensive assessment of small ruminant seminal plasma metabolome, including amino acids, fatty acids, peptides, sugars, nucleosides, organic and inorganic compounds. Moreover, an association of specific seminal plasma metabolites with goat sperm motility were determined. Metabolites are derived from metabolic reactions and presented in many biochemical pathways ⁴⁶. Moreover, metabolites have potentiality to act as biomarkers for assessment of male fertility ⁴⁷⁻⁵¹. Sperm are suspended in seminal plasma that shows certain qualitative and quantitative variation in its biochemical composition ⁵². Currently, roles of seminal plasma are still not well known. However, exposure of sperm to some metabolite-like components during storage of sperm, such as sugars, citric acid, amino acids, can influence sperm fertility ⁵³. The removal of seminal plasma is generally suggested for goat sperm preservation, due to the toxic interaction between seminal plasma and egg yolk or milk which is the main protectant in traditional extenders ^{31,53}. Additionally, the interaction of metabolites with other molecules in the uterine environment also affects fertilization, implantation, fetal and placental developments ⁵⁴. Furthermore, some metabolites in seminal plasma, such as amino acids, peptides, sugars, fatty acids, steroids and nucleosides, are involved in some important physiological activities, influencing energy production, motility, pH control, membrane protection and metabolic activity of sperm ⁵⁵⁻⁵⁸.

As presently evaluated by the untargeted metabolomics analysis, the major known metabolites in goat seminal plasma were defined as peptides, followed by amino acids, enzymes, carbohydrates, fatty acids, and nucleosides. We identified 321 peptides and 44 amino acids in goat seminal plasma. By contrast, in

bull, 21 metabolites were classified as amino acids, peptides, and their analogues in seminal plasma³⁰. Similarly, other researchers have detected 20⁵⁹ to 23 amino acids⁶⁰ in bull seminal plasma using the GC-MS method. In addition, a large number of amino acids were found in goat epididymal fluid⁵⁸. Besides involvement of composing proteins⁶¹, amino acids have been reported to be extensively engaged on sperm biology, including protection and regulation of metabolic activity⁵⁸. Moreover, amino acids can protect ram sperm during cryopreservation by reducing injury caused by lipid peroxidation and free radicals⁶².

49 carbohydrates were identified in seminal plasma. Carbohydrates are essential for sperm function because these molecules are the critical components involved in energy production pathways⁵⁷. Glycolysis is used by mammalian sperm to obtain energy. During this process, the glycolysable carbohydrates included in seminal plasma, such as fructose, are required for ATP production, leading to increased respiratory activity to support optimum sperm motility and survival^{63,64}. According to a previous investigation, fructose was one of the most predominant metabolites in bull seminal plasma³⁰. However, owing to the different analyzing method used, it cannot determine the real concentration of fructose in goat seminal plasma in this study. But, the concentration of fructose in the high motility group was significantly higher than that in the low motility group. The finding was similar to that reported in bull. In that study, fructose was more enriched in the high fertility group as compared to the low fertility group³⁰. Fructose is the primary energy source for sperm and the major carbohydrate in seminal plasma of mammals⁶⁴⁻⁶⁶. Currently, fructose has been found in seminal plasma of several species, including bull³⁰, buffalo⁶⁷, goat^{67,68}, ram⁶⁹, boar⁷⁰, human⁷¹, and rabbit⁷². As revealed by this study, fructose is extensively involved in fundamental pathways of energy production for goat sperm. In addition, Yousef et al. suggested that a reduction in fructose concentration observed in seminal plasma of rabbits intoxicated with aluminum chloride may be one of factors leading to reduced sperm motility⁷². Therefore, it may be concluded here that less fructose concentration in goat seminal plasma reduces the energy supply to sperm, negatively affecting their motility.

In addition, citric acid, with a high fold change between these two groups, was more abundant in the high motility group. Similar to our report, in bull, a significant enrichment of citric acid in seminal plasma were found in the high fertility bull³⁰, implying that citric acid may be act as a potential biomarker for assessment of sperm motility. Citric acid is also presented in semen of other species, such as boar⁷³, human⁷¹, and rabbit⁷⁴. Citric acid is reported to be involved in pH regulation in boar semen. Furthermore, it can act as a chelator for zinc, magnesium, and calcium⁷³. In human, the concentration of zinc, magnesium and calcium in seminal plasma and their chelation influence sperm metabolism, consequently affecting sperm transport, acrosome reaction, and fertilization⁷⁵. In addition, based on a recent study, citric acid in seminal plasma was found to be associated with bull fertility by potentially affecting sperm capacitation and acrosome reaction⁹.

In conclusion, this study first established the proteomic and metabolomic databanks of goat seminal plasma. Based on this, the DEPs and differential metabolites that may be involved in regulation of sperm motility were determined. There were 175 up-regulated and 274 down-regulated DEPs in high motility group. The identified DEPs were primarily engaged on some essential sperm functions and pathways, such as control of biological processes, metabolic processes, organization of cellular components, phosphatidylinositol signaling system, salivary secretion, proteasome, apoptosis, etc. A total of 1857 differential metabolites were identified between the high motility group and the low motility group, and 999 metabolites were up-regulated in the high motility group. Furthermore, most differential metabolites were mainly involved in some metabolic and synthetic activities. Therefore, the proteins and metabolites acquired in the present study may be helpful for us to further understand mechanisms leading to poor sperm motility. In addition, the identified DEPs or metabolites can also act as biomarkers to assess goat semen quality, and may be used for directing AI.

Materials And Methods

Ethics statement

The ethical committee of Yunnan Animal Science and Veterinary Institute (Kunming city of Yunnan province, China) has approved all experiments including animal usage in this study (201909006). In addition, during the whole experiment, the authors strictly complied with Regulations on the Administration of Laboratory Animals (Order-No.2 of the State Science and Technology Commission of the people's Republic of China, 1988) and Regulations on the Administration of Experimental Animals of Yunnan Province (the Standing Committee of Yunnan Provincial People's Congress 2007.10). We confirmed that all authors complied with the ARRIVE guidelines.

Chemicals and reagents

Unless otherwise mentioned, the chemicals, reagents, and kits have been purchased from Sigma-Chemical Company (St. Louis, Mo, United States). The Andromed extender was purchased from Minitüb GmbH (Hauptstrasse 41, 84184 Tiefenbach, Germany).

Animals and management

In this study, the semen used was collected from a newly developed breed-*Yunshang black goats*. To collect semen, 20 bucks (2–3 years old) were used during September of 2019 (their reproductive season). Routine anthelmintic handling and vaccination against rabies and tetanus were conducted. The bucks were raised under the standardized conditions of feeding, lodging and light. The daily diet consisted of 29.5% maize, 23% soybean, 1.5% calcium monophosphate, 1% premis, 0.5% sodium-bicarbonate, 0.5% NaCl, 19% broad bean-bran, 10% alfalfa-Grass, and 15% corn-silage. The bucks had free access to salt and drink.

Semen collection, dilution, and motility assessment

In this study, semen was collected using artificial vagina and directly transported to the laboratory within 10 minutes. Two successive ejaculates of one buck obtained over a 10 min period were pooled for its semen quality analysis. Instantly after collecting, we counted volume of semen and observed semen color. Mass motility was first assessed by observing the wave motion pattern of fresh undiluted semen^{76, 77}. However, the assessment of mass motility is subjectively carried out on the basis of the experience and knowledge of the technicians, so it is only a rough assessment. Concentrations of sperm were analyzed using Nucleo-Counter® SP 100™ (Chemo-Metic AS, Allerød, Denmark). Following the initially assessment, quality of the used ejaculates satisfied with the criteria in the experiments were as follows: mass motility: ≥ 3.0 ; sperm concentration: $\geq 2500 \times 10^6$ sperm/mL; normal morphology: $\geq 75\%$.

After the above mass motility assessment, the motility of sperm was analyzed using a computer-assisted sperm CASA system installed with the Sperm Class Analyzer (SCA) software (SCA Evolution; Microptic, Barcelona, Spain). A specific program in this software is designed for the evaluation of goat sperm. The detailed parameter setting for this program was as follows: Calibration name, 10 \times ; Calibration value ($\mu\text{m}/\text{pixel}$), 0.475323; Capture method, Ph-; Grid distance (μm), 100; Analysis timeout, 15; Box size, 152; Frame rate (fps), 25; number of images, 25; Resolution, Low; Style, automatic; Minimum Area, $3\mu\text{m}^2$; Maximum Area, $70\mu\text{m}^2$; Drifting ($\mu\text{m}/\text{s}$), 0; Static ($\mu\text{m}/\text{s}$) < 10 ; slow-medium velocity ($\mu\text{m}/\text{s}$), 45; Rapid velocity ($\mu\text{m}/\text{s}$), 75; progressive motility (STR $>$), 80; connectivity (pixels), 12; VAP points (pixels), 5; VCL/VAP, VCL.

When the motility was examined, the collected semen samples were diluted using the Andromed extender to a final concentration of 20×10^6 sperm/mL. 10 μl drop of sperm solution was placed on a slide and covered using a cover slip (18mm \times 18mm). Initially, the heated plate (38°C) with a magnification of 100 \times have been installed on a phase-contrast microscope (Nikon, ECLIPSE-E200, Japan), and the progressive motility (PM, %) values were analyzed. Ten fields per drop including a total of 500 sperm has been recorded for every sample. Based on the obtained sperm motility values, the used bucks were separated into two groups with a higher ($\geq 75\%$) or lower motility ($\leq 65\%$).

When performing the proteomic analysis of goat seminal plasma, there are 5 bucks with higher or lower motility in each group. However, when performing the metabolomic analysis of seminal plasma, there are 10 bucks with higher or lower motility in each group. The semen from these bucks used were analyzed separately and not pooled during this whole experiment.

Sperm plasma membrane and acrosome assessment

The hypo-osmotic swelling test (HOST) has been used to test the integrity of sperm plasma membrane as described in a previous study⁷⁸. In brief, 20 μL of semen was incubated in 200 μL of the hypo-osmotic solution (9 g/l fructose and 4.9 g/l sodium citrate, 100 mOsm/kg) at 37 °C for 60 minutes. Then, 10 μL of solution was mounted on a microscope slide and covered using a cover slip. A total of 200 sperm were assessed in each time. Sperm with visible coiling tails were counted under the phase contrast microscope with a magnification of 400 \times for each sample.

FITC-PSA staining together with flow cytometry was used to assess the acrosome status of goat sperm ⁷⁷. In brief, semen was diluted using the TALP buffer to a fixed concentration of 10×10^6 sperm/mL. Then, 200 μ L of the above sample was stained using 50 μ L propidium-iodide (PI) (50 μ g/mL) and 0.5 μ L FITC PSA (2mg/mL), followed by incubation in a dark and humid environment for 15 minutes at 37°C. Finally, the percentages of FITC-PSA and PI stained sperm were analyzed by flow cytometry. The concentration of alive sperm with intact acrosome and plasma membrane were identified as PI and FITC-PSA negative.

A FacStar-plus flow cytometer (FAC SCalibur, Becton-Dickinson and Co., Franklin Lakes, NJ, USA) was used to perform the flow cytometry analysis. The green fluorescence emitted from FITC-PSA were detected on the FL1 photodetector (530/30BP-filter). The red fluorescence generated from PI was observed on the FL2 photodetector (670LP-filter). The Ar ion blue laser was used to excite those fluorochromes (488 nm). The fluorescence information was shown in the logarithmic mode using the Cell-Quest Pro-3.1 program (BD-Biosciences). According to the guideline of International Society for the Advancement of Cytometry (ISAC), the data was obtained from 100,000 events for further study using the Cell-Quest program (Becton Dickinson).

Seminal plasma exaction and purification

The seminal plasma exaction process was defined in a previous report ⁷⁹. In brief, following semen collection, seminal plasma was extracted separately from sperm cells via centrifugation at $10,000 \times g$ for 10 minutes in a microfuge at 4°C. Then, the supernatants were gently collected and centrifuged again at the same condition. The collected seminal plasma was further filtered via a 0.22 μ m Millipore filter (Millipore). The seminal plasma samples were preserved at -80 °C for the proteomics and metabolomic analysis.

Protein extraction and trypsin digestion

The protein extraction process has been described in a previous study ³². In brief, before the extraction of total proteins in seminal plasma, all samples were initially sonicated for three times using ice, applying the highly intensity ultrasonic-processor (Scientz) in the lysis buffer (8M urea, 1% protease inhibitor cocktail). The supernatants were collected after centrifugation at 12,000 g at 4 °C for 10 minutes, and the protein concentrations were measured using the BCA kit as instructed by the manufacturer.

The protein mixture was reduced by 5mM dithiothreitol at 56 °C for 30 minutes and alkylated using 11 mM iodoacetamide for 15 minutes at room temperature in darkness for absorption. The urea concentration in the protein samples were diluted to less than 2 M applying 100 mM triethylammonium bicarbonate. After the above treatments, trypsin was applied for the first digestion overnight at a trypsin to protein mass ratio (1: 50), and then for the second digestion for 4 hours at a trypsin to protein mass ratio (1: 100).

TMT labeling, HPLC fractionation, and LC-MS/MS analysis

The peptides were desalinated through the Strata X-C18 SPE column (Phenomenex) and vacuum dried, following digestion with trypsin. Peptides were reassembled into 0.5M triethylammonium bicarbonate and operated for the 10-PLEX TMT package according to the instructions of manufacture for the TMT/iTRAQ-kit. In short, one unit of the TMT/iTRAQ mixture was thawed and reassembled into 24 μ l acetonitrile (defined as the volume of mixture needed to mark of 100 μ g proteins). The peptide solutions were incubated for 2 hours at room temperature, pooled, desalted, and dried through vacuum centrifugation.

Using an Agilent-300 Extend C18 column (5 μ m particles, 4.6 mm ID, 250 mm length), the samples were fractionated into various fractions through the high-pH reverse phase HPLC. In brief, peptides were initially separated in 10 mM ammonium-bicarbonate (pH-10) for 80 min into 80 fractions with gradient of 2% to 60% acetonitrile. Later, the peptides were combined into 9 fractions and dried by vacuum centrifugation.

The tryptic peptides were dissolved in the solvent-A (0.1 % formic-acid, 2 % acetonitrile), and straightly loaded into a home-made reversed phase analytical column (20 cm length, 100 μ m i.d.). The gradient was comprised of an increasing from 6% to 22% solvent-B (0.1% formic acid in 90% acetonitrile) during 38 minutes, 22% to 32% in 14 minutes, and an increase to 80% for 4 minutes, then maintaining at 80% for the last 4 minutes. All processes were operated at stable flow rate of 450 nL/min using the EASY-nLC 1200 UPLC system.

The peptides were subjected to the NSI sources in Q-ExactiveTM HF X (Thermo), followed by the tandem-mass spectrometry (MS/MS) together online with the UPLC. The applied electrospray tension was 2.0 kV. The m/z assay size for a complete scan was 350 to 1600, and the Orbitrap detected the whole peptides at resolution of 120,000. The peptides have been chosen for MS/MS with the NCE setting at 28. The fragments were identified at a resolution of 30,000 in the Orbitrap. A data dependent process that exchanged from one MS-scan to 20 MS/MS dynamic exclusion scans using 30.0 s. Automatic gain-control (AGC) was fixed at 1E5. The first set mass was fixed at 100 m/z.

Bioinformatics analysis of proteomic data

The MS/MS data was analyzed applying the explore engine Max-Quant (v-1.5.2.8). In *Capra aegagrus hircus* database concatenated using the reversed decoy database, the tandem mass spectra were detected. Trypsin/P has been determined as the cleavage enzyme which allows up to 2 lacking cleavages. First, the mass tolerance of precursor ions was fixed at 20 ppm in first check and 5 ppm was in second check. The mass tolerance for fragments ions were fixed at 0.02 Da. Carbamido-methyl on Cys was clarified for a set direction, and changeable directions were specified for oxidation on Met and acetylation on the protein N-term. FDR was changed to < 1 % and a lowest score was fixed to > 40 for the modified peptides. The minimum length of a peptide was fixed at 7. The TMT 10-PLEX was chosen for the quantification process. In MaxQuant, the parameters were fixed as the default values. The *P* values were calculated using the t-test of the two-sample two-tailed Student. Proteins with fold change of > 1.50 and *P* value <0.05 were identified as up-regulated DEPs between the high motility group and the low

motility group. However, the proteins with fold change of <0.667 and P value < 0.05 were identified as the down-regulated proteins.

The Gene Ontology (GO) annotation was performed based on the UniProt GOA database (<http://www.ebi.ac.uk/GOA/>). At first, the IDs of identified proteins were transformed to UniProt-IDs, mapping to GO IDs with the protein IDs. Unless some identified proteins can be annotated by the UniProt-GOA database, the InterProScan will be applied to annotate protein's GO functionality using the protein sequence alignment procedure. Based on the UniProt-GOA database, the DEPs were classified into three types: biological procedure, cell compartment, and molecular functions. For each type, two tailed Fisher's exact-test was used to test the enrichment of the DEPs against all identified proteins. The GO with a modified P value < 0.05 was considered significantly. In addition, the information related to subcellular localization of the obtained DEPs was inferred with Wolfpsort (<http://www.genscript.com/psort/wolfpsort.html>).

The KEGG online service tools KAAS (<http://www.genome.jp/kaas-bin/kaas>) was used to identify pathways that the obtained DEPs are enriched. Firstly, KAAS was applied to annotate the KEGG database description of the identified proteins. Then, the annotation results were mapped on the KEGG pathway database applying the KEGG online service tools KEGG mapping. Based on the KEGG database, the two tailed Fisher's exact-test was used to detect the enriched channels to test the enrichment of DEPs against the entire detected proteins. The pathway was considered significantly with a corrected P value <0.05 . According to the KEGG website, these pathways were specified into hierarchical groups.

The analysis of protein domain was conducted applying the InterPro domain database (<http://www.ebi.ac.uk/interpro/>). For the identified proteins in each category, the InterPro (resources that allows functional evaluation of protein sequencing by identifying proteins in various groups and estimating the existence of domains and major locations) database have been scanned, and two tailed Fisher's exact-test has been applied to analyze the enrichment of DEPs against those identified proteins. Proteins domain with a corrected P value < 0.05 was thought significant.

Parallel reaction monitoring (PRM) validation

The seminal plasma separation and total protein extraction were the same as the above procedure. The digested peptides were submitted to the PRM analysis. PRM is a newly developed approach to verify proteins using the quadrupole-Orbitrap mass spectrometer^{38,80}. In brief, the tryptic peptides were mixed in the solvent A and eluted in a reversed phase analytical column using the gradient solvent B (6 %-25 % over 40 minutes, 25 %-35 % over 12 minutes, 80 % over 4 minutes, and 80 % over the last 4 minutes) at a rate of 500 nL/min. The peptides measured (1.5 mg per sample) were analyzed with an online Q ExactiveTM plus the Orbitrap mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) coupled with the UPLC. The applied electrospray tension was 2.2 kV. The full MS scans (400-960 m/z) were obtained at a resolution of 70,000 using AGC of 3E6 and a highest injection time (MIT) of 50 ms. A data independent protocol (one MS scan followed by 20 MS/MS scans) was used for the MS/MS scans with

the following parameters: resolution, 17,500; NEC, 27; AGC, 1E5; MIT, 120 ms; insulation window, 1.6 m/z. The PRM data was analyzed using the Skyline 3.6. The results were quantified for every peptide, and the DEPs detected were screened and compared with the MS data derived from the TMT.

Seminal plasma metabolite exaction

In brief, the collected seminal plasma samples were first warmed on ice. The samples were vortexed for 30 seconds to ensure complete mixing. Then, 3 volumes of ice-cold methanol were added to 1 volume of seminal plasma, followed by vortexing for 3 minutes. The mixture was further centrifuged at 12,000 g for 10 minutes at 4 °C. The supernatant was collected and centrifuged at 12,000 g for 5 minutes at 4 °C again. After filtration through a 0.22 µm filter membrane, the supernatants were transferred into the injection bottles. Finally, the samples were preserved at -80 °C prior to the LC-MS/MS analysis. In addition, the pooled QC samples were simultaneously prepared by mixing 10 µL of each exacted mixture.

HPLC

The exacted seminal plasma samples were analyzed using the LC-ESI-MS/MS system (UPLC, Shim-pack UFLC SHIMADZU CBM A system, <https://www.shimadzu.com/>; MS, QTRAP 6500+ System, <https://sciex.com/>). The analytical parameters were set as following: UPLC: column, Waters ACQUITY UPLC HSS T3 C18 (1.8 µm, 2.1 mm×100 mm); the column temperature, 35 °C; the flow rate, 0.3 mL/min; the injection volume, 1 µL; the solvent system, water (0.01 % methanolic acid): acetonitrile; the gradient program of positive ion, 95:5 (V/V) at 0 minutes, 79:21 (V/V) at 3.0 minutes, 50:50 (V/V) at 5.0 minutes, 30:70 (V/V) at 9.0 minutes, 5:95 (V/V) at 10.0 minutes, and 95:5 (V/V) at 14.0 minutes; the gradient program of negative ion, 95:5 (V/V) at 0 minutes, 79:21 (V/V) at 3.0 minutes, 50:50 (V/V) at 5.0 minutes, 30:70 (V/V) at 9.0 minutes, 5:95 (V/V) at 10.0 minutes, and 95:5 (V/V) at 14.0 minutes.

ESI-QTRAP-MS/MS

The LIT and triple quadrupole scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (QTRAP) (QTRAP 6500+ LC-MS/MS System) installed with an ESI Turbo Ion-Spray interface and controlled by the Analyst 1.6.3 software (Sciex). The QTRAP was operated in both positive and negative ion modes. The parameters of the ESI source operation were set as the following: the source temperature: 500 °C; the ion spray voltage (IS): 5500 V (positive) and -4500 V (negative); the ion source gas I (GSI): 55.0 psi; the gas II (GSII): 60.0 psi; the curtain gas (CUR): 25.0 psi; the collision gas (CAD): high. The instrument tuning and mass calibration were performed in 10 and 100 µmol/L polypropylene glycol solutions using the QQQ and LIT modes, respectively. A specific set of MRM transitions were monitored during each period based on the metabolites eluted within this period.

Metabolomics data analysis

The original data files acquired by the LC-MS analysis were first converted into mzML format using the ProteoWizard software. Peak extraction, alignment, and retention time correction were performed by the

XCMS program. The “SVR” method was used to correct the peak area. The peaks were filtered in accordance with a deletion rate > 50% in each group of samples. After the above treatments, the identified metabolite information was obtained by searching the laboratory’s self-built database, the public database (Metlin), and metDNA. Finally, the statistical analysis was carried out by the R program. The statistical analysis includes the univariate analysis and the multivariate analysis. The univariate statistical analysis was performed using Student’s t-test and variance multiple analysis. The multivariate statistical analysis was carried out using these approaches including principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA).

Statistical analysis

The data associated with motility, plasma membrane and acrosome integrity of goat sperm were statistically analyzed by T-test using the JMP10.0 software (SAS Institute Inc., Cary, NC, USA). Data normality and homogeneity of variances were verified using the Shapiro–Wilk normality tests and Levene’s tests, respectively. The data were presented as means \pm SEM. It was thought that the data with value of $P < 0.05$ or $P < 0.01$ was statistically significant.

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Declarations

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Author Contribution

B.J and J.L: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Writing - original draft, Writing - review & editing. **C.L and S.M:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - review & editing. **Y.F:** Conceptualization, Formal analysis, Methodology, Review & editing. **Q.W and G.Q:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing - review & editing.

Competing interests

The authors declare no competing interests.

Tables

Table 1. Validation of DEPs using parallel reaction monitoring (PRM) analysis

Protein accession	Protein name	Protein gene	G/B Ratio (PRM)*	G/B value (PRM)	P	G/B Ratio (TMT)*
A452EPH7	Phosphoglycerate mutase	BPGM	3.54	1.84E-02		1.64
I1W1N4	Ras-related protein Rab-11B	RAB11B	4.28	5.70E-03		1.73
A452FSK9	ATP-citrate synthase	ACLY	4.40	3.88E-02		1.52
A452E5B4	Peroxiredoxin	PRDX5	2.88	3.19E-02		1.53
A452DLM2	Spermadhesin-1	LOC102175091	2.84	8.60E-02		1.75
A452EVX4	T-complex protein 1 subunit alpha	TCP1	2.57	3.16E-02		1.59
A452DSU1	Programmed cell death protein 5	PDCD5	1.86	2.08E-01		1.53
A452G6T9	Testis tissue sperm-binding protein Li 69n	PSMD14	3.72	6.53E-03		1.65
A452FJ29	Ubiquitin-like modifier-activating enzyme 1	UBA1	2.78	1.23E-02		1.50
A452EC76	Zonadhesin	ZAN	0.01	3.27E-03		0.35
A452E294	Superoxide dismutase	SOD2	0.24	1.00E-03		0.41
A452EFP3	Sperm equatorial segment protein 1	SPESP1	0.05	8.31E-05		0.51
A452DL42	Thioredoxin reductase 2, mitochondrial	TXNRD2	0.24	1.48E-02		0.57
A452E876	Acrosin-binding protein	ACRBP	0.19	2.95E-02		0.41
A452DWD6	Zona pellucida binding protein	ZPBP	0.23	7.26E-03		0.38
A452E0C3	Heat shock protein family E (Hsp10) member 1	HSPE1	0.60	7.50E-02		0.66
A452E1R9	Peroxiredoxin 1	PRDX1	0.39	1.60E-01		0.61
A452DNC1	Aquaporin 7	AQP7	0.22	4.31E-06		0.60
A452EAI1	Izumo sperm-egg fusion protein 4	IZUMO4	0.08	6.55E-03		0.44

Figures

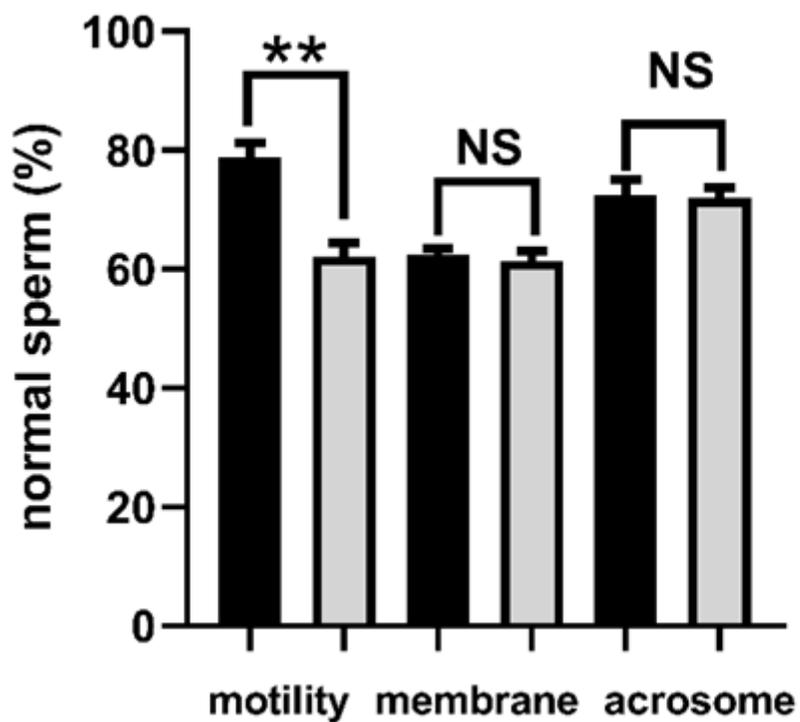


Figure 1

The motility, plasma membrane and acrosome integrity of goat sperm. The black column represented the high motility group, and the grey column represented the low motility group. All data were expressed as means \pm SEM. “**” represents a significant difference ($P < 0.01$). “NS” represents no difference ($P > 0.05$).

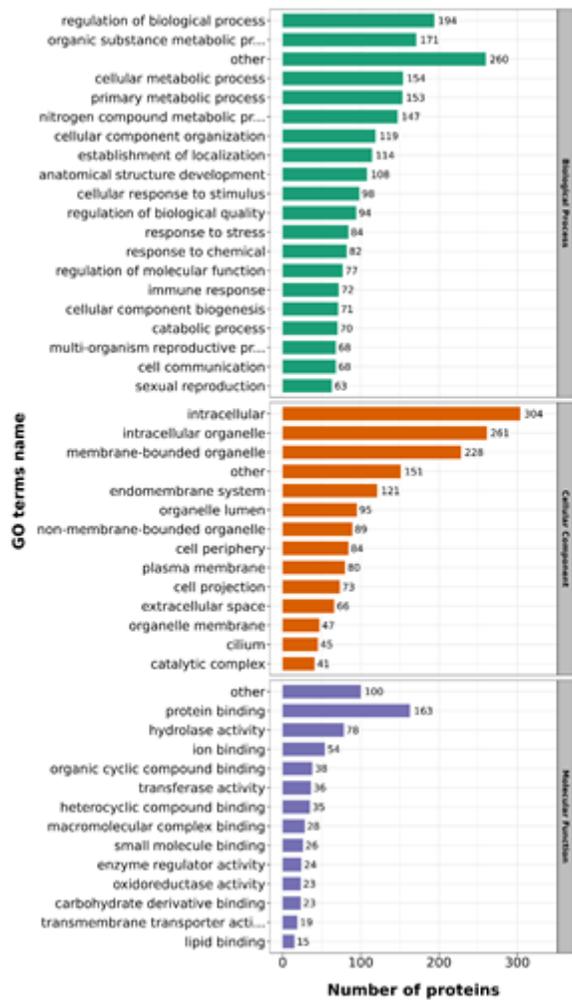


Figure 2

Statistical distribution chart of the identified DEPs based on each GO category. The DEPs were classified into biological process, cellular compartment and molecular function. The bar represents the number of DEPs.

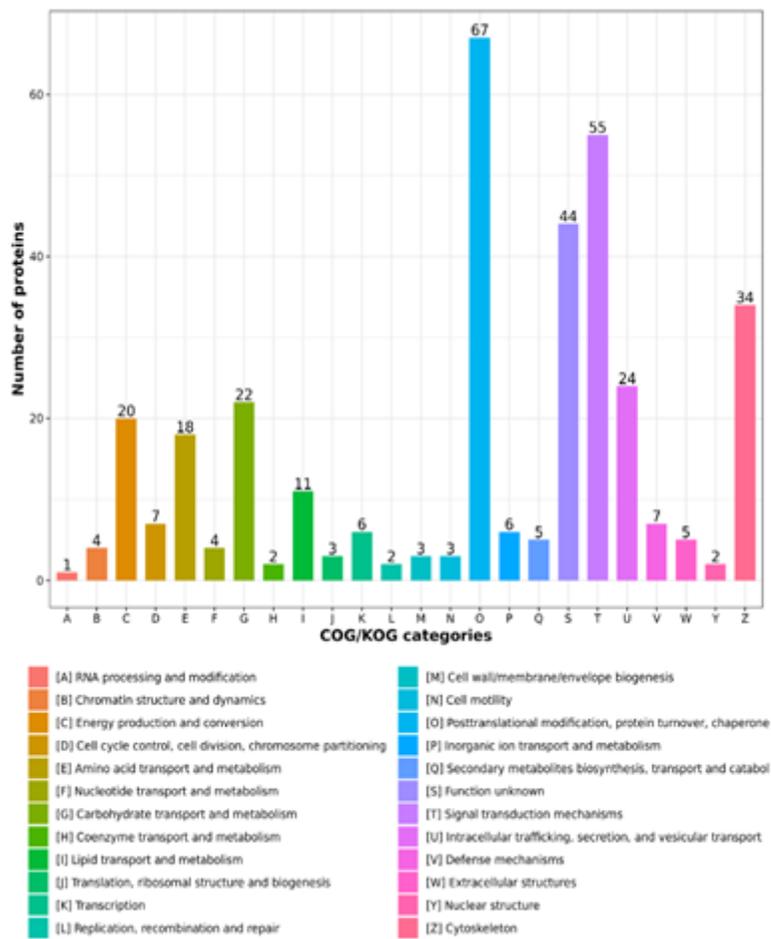


Figure 3

COG/KOG functional classification chart of the identified DEPs. The values on each bar represented the number of proteins that are involved in this function.

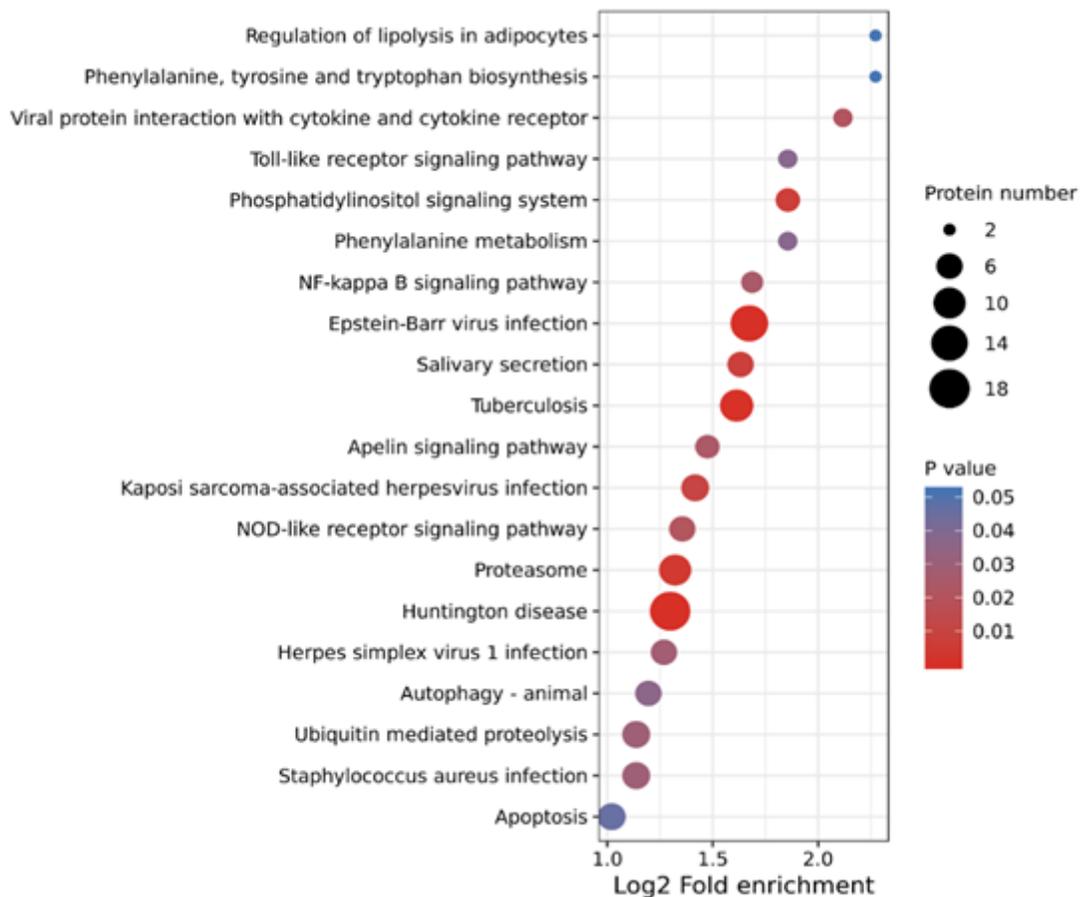


Figure 4

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEPs. The P value was calculated using a Fisher's exact test. The X-axis represents the Log2 transformed ratio of DEP compared to the identified proteins, and the Y-axis means KEGG pathway. The size of each bubble refers to the number of the DEPs. The color indicates the significance P value, and the size of circle indicates the number of DEPs in terms.

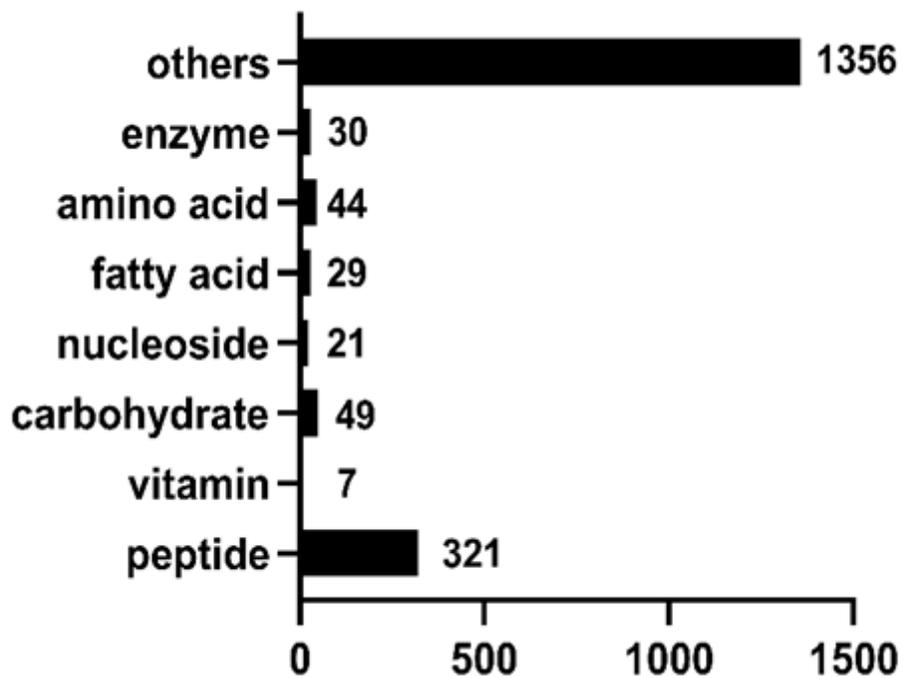


Figure 5

The classification of differential metabolites presented in goat seminal plasma between these two groups. The number on each column represents the number of this kind of metabolites.

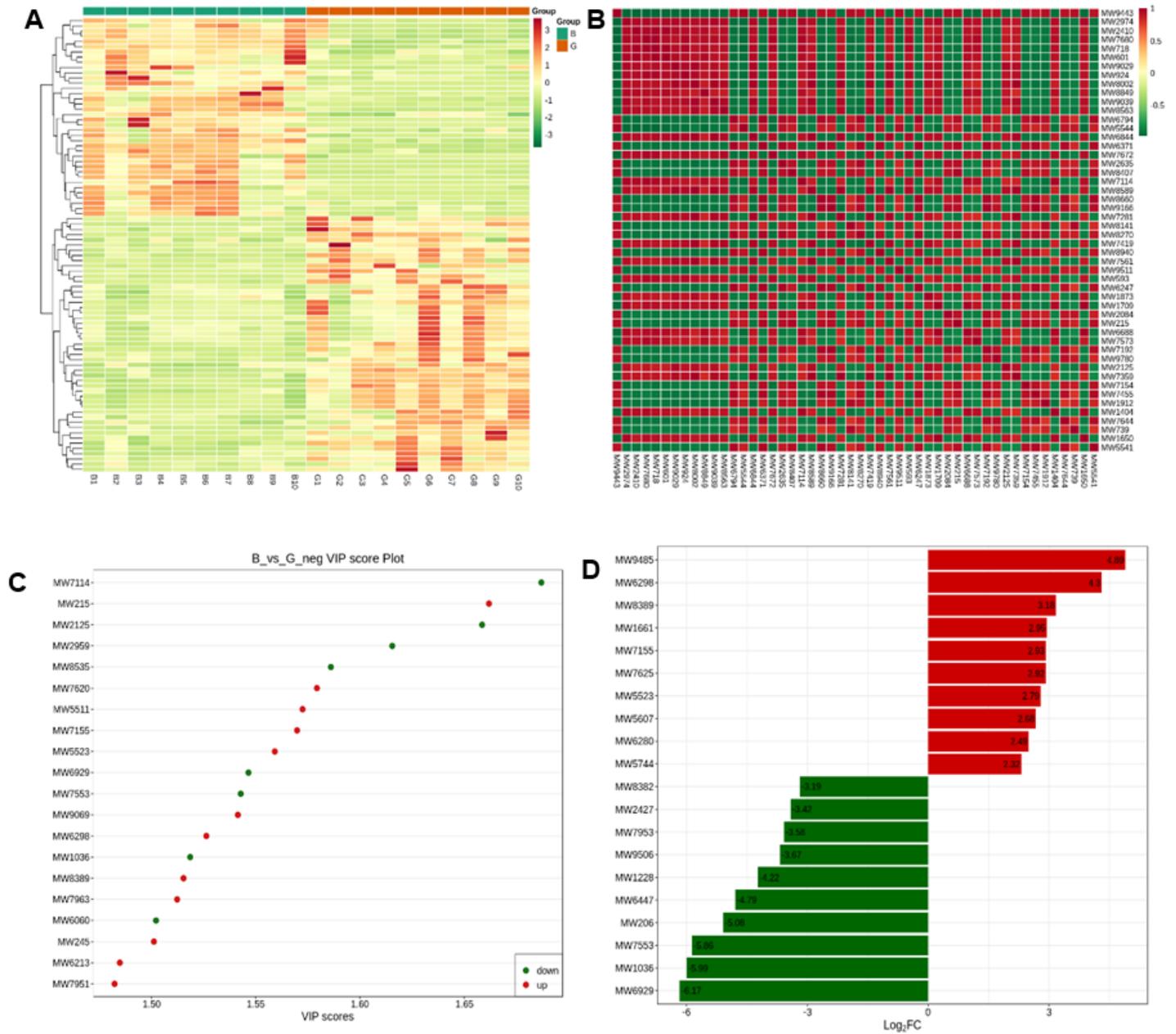


Figure 6

The bioinformatics analysis of differential metabolites between these two groups. The figure A represented the cluster heatmap of different metabolites. The metabolites with significant difference were normalized and clustered in this map. The X-axis represented the samples, and the Y-axis represented the differential metabolites. Red represented the highly expressed metabolites, and green represented the lowly expressed metabolites. The figure B represented the correlation results of differential metabolites analyzed by the Pearson correlation analysis method. Red color indicates strong positive correlation and green color indicates strong negative correlation. The figure C represented the top 20 differential metabolites with the largest VIP value. Abscissa represents the VIP value, and ordinate represents differential metabolite. Red represents up-regulated metabolites, and green represents down-regulated

metabolites. The figure D represented the top 20 differential metabolites with the largest FC value. Abscissa represents \log_2FC value, and ordinate represents differential metabolites. Red represents up-regulated metabolites, and green represents down-regulated metabolites.

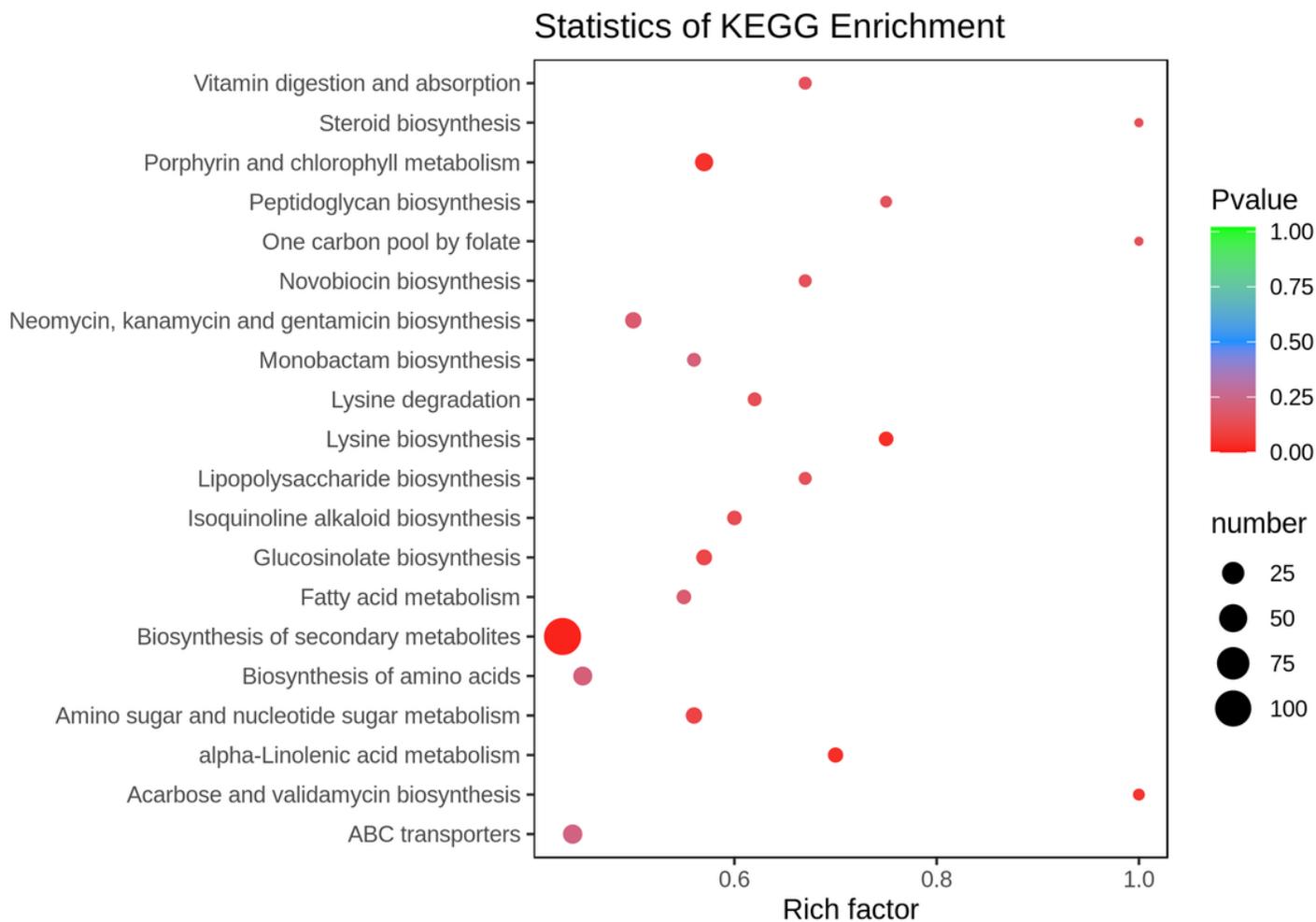


Figure 7

The KEGG enrichment map of differential metabolites. Abscissa represents Rich factor, and ordinate represents the pathway name. The color of the point represented the P value. Red indicates that the enrichment is more significant. The size of the points represents the number of metabolites involved in this pathway.

Supplementary Files

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