Metabolomic Analysis and Identification of Sperm Freezability Biomarkers in Boar Seminal Plasma

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Research

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Abstract

**Background:** In the freezing process of boar sperm, there are obvious differences of freezability between individuals. Studies suggest that specific freezability markers might be useful in good (GFE) and poor freezability ejaculates (PFE) selection prior to cryopreservation. Some potential markers of boar sperm freezability have been found from spermatozoa, little attention has been paid to seminal plasma. The seminal plasma is composed of secretions from testis, epididymis, and accessory sex glands, and the exposure of spermatozoa to small molecules such as metabolites can affect the sperm functions. However, details and significance of the seminal plasma metabolome related to boar sperm freezability are unknown. Therefore, the main aim of this study was to explore the difference in the metabolic level of seminal plasma between boars with differential freezability, and to explore the biomarkers of semen freezing tolerance.

**Results:** A total of 953 metabolites were identified in boar semen plasma by UHPLC-qTOF-MS analysis, and 50 metabolites show significant change between GFE group and PFE group. Further, twelve metabolites were subjected to metabolic target analysis and three metabolites (D-Aspartic acid, N-Acetyl-L-glutamate (NAG), and Inosine) show differences.

**Conclusions:** There is significant difference on metabolome of seminal plasma between GFE and PFE individuals. The D-Aspartic acid, NAG, and Inosine in seminal plasma may be potential markers for assessing sperm cryopreservation resistance in boars.

Background

Artificial insemination has been widely used in pig production worldwide. However, frozen–thawed boar semen account for less than 1% of the semen for insemination [1]. On one hand, boar spermatozoa in generally presents low freezability because of their high cold shock sensitivity[2, 3]. On the other hand, the quality of frozen-thawed boar semen shows strong variability associated with differences in freezability between individuals[4]. Therefore, it is meaningful to distinguish high and low freezability individuals before cryopreservation procedures, and choose the high freezability individuals for cryopreservation to improve the efficiency of artificial insemination utilizing post-thawed sperm. To solve this issue, researchers are engaged in a lot of work to distinguish good (GFE) and poor freezability ejaculates (PFE) [5–7].

Previous researches on the boar ejaculate freezability biomarkers mainly focused on proteomics. Numbers of proteins from sperm or seminal plasma, such as heat-shock protein 90 (HSP90AA1)[8], acrosin-binding protein (ACRPB)[9], triosephosphate isomerase (TPI)[9], and fibronectin 1 (FN1) [10] have been reported as markers for predicting boar ejaculate freezability[11]. In addition, a study demonstrated that genomic differences existed between good and poor freezers in the sequences of polymorphism restriction fragments of 16 candidate genetic markers[6]. Other freezability markers include patterns of
sperm motile subpopulations in extended semen\[12\], specific kinetic parameters evaluated at the cooling step\[13\], and acrosin activity \[14, 15\].

Sperm freezability is a complex phenotype, and it cannot be accurately predicted just based on conventional parameters \[13, 16\]. Current knowledge implies that the seminal plasma is much more than a nutrient medium. The seminal plasma is composed of secretions from testis, epididymis, and accessory sex glands. Seminal plasma contains a variety of substances, such as proteins, ions, and metabolites including amino acids, lipids, nucleosides, minerals, electrolytes, and steroid hormones\[17, 18\]. As metabolites are the final products of metabolism, the changes in the composition and content of which can reflect the state of sperm and individual metabolic timely\[19\]. Recent studies report that metabolites involve in sperm energy production, motility, protection, pH control and regulation of metabolic activity \[20\]. Furthermore, metabolites in seminal plasma may affect downstream and complementary changes in gene/protein expression\[21\]. Thus, we hypothesized that some particular metabolites in seminal plasma could be considered as markers of sperm freezability.

Therefore, the aims of this study were to compare metabolome of seminal plasma between GFE and PFE, and to identify potential metabolites as biomarkers of freezability. We used an Ultra-high Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (UHPLC-qTOF-MS) based metabolomics approach to obtain the metabolic profile of seminal plasma from boars with good and poor sperm freezability. Furthermore, the potential metabolites were confirmed by targeted metabolomics analysis. The findings in the present study will provide a new perspective for boar sperm freezability predicting.

**Materials And Methods**

**Sample collection and preparation of seminal plasma**

The boars (n=15) were chosen based on production records during 2-years. All boars were Landrace raised under the same management conditions and received the same nutrition, and they were stalled in commercial herds. Semen was collected using the gloved hand method. The semen collection rhythm was twice a week and one single ejaculate per boar was used in this study.

After collection, the spermatozoa-rich fraction of each ejaculate (80–100 mL) was filtered through gauze and subsequently divided into two aliquots of equal volume. The first one was used for seminal plasma separation from spermatozoa through centrifugation at 500×g and 4 °C for 30 min. Seminal plasma preparations were then examined using phase microscopy to ensure no spermatozoa remained. Clean seminal plasma samples were then stored in liquid nitrogen. The other spermatozoa-rich fraction aliquot was diluted in Androhep Plus (Minitube, Germany) at 2×10^8, and then used to cryopreserve.

**Cryopreservation and thawing of sperm samples**
Fifteen ejaculates (> $2 \times 10^8$ spermatozoa/mL; > 75% motility) were chosen to cryopreserve. Firstly, the semen samples were stored at 17 °C to cool, then the semen was centrifuged at 500×g for 10 min. Soft sperm pellets were subsequently diluted to $2 \times 10^9$ spermatozoa/mL in Androstar® Cryo Plus (Minitube, Germany) containing 20% egg yolk. Then the spermatozoa were cooled slowly to 5°C for 5 h and subsequently diluted to $1 \times 10^9$ spermatozoa/mL with freezing medium containing 6% glycerol (Sigma–Aldrich, MO) at 5°C. Afterward, sperm samples were packed in 0.5 mL labeled plastic straws (Minitube, Germany). The straws were then transferred to a programmable freezer CryoMed 7457 (Thermo Fisher, MA). The cooling ramp was as follows: Wait at 4°C→2°C /min to 2°C→Hold for 1 min at 2°C→35°C /min to -30°C→Hold for 1 min at -30°C→35°C /min to -150°C→ Hold for 4 min at -150°C. The straws were finally plunged into liquid nitrogen and stored before use.

**Assessment of sperm quality**

Sperm motility parameters obtained were those described by Yeste et al[22]. Sperm motility assessment was carried out utilizing a commercial computer assisted sperm analysis (CASA) system (CASAS-QH-III, Tsinghua Tongfang Co., Ltd.). After evaluating three replicates per sample (a minimum of 1000 spermatozoa was counted per replicate), the corresponding mean standard error of the mean (SEM) was calculated.

**Sample classification into GFEs and PFEs**

To classify seminal plasma samples into two groups (GFEs vs. PFEs), spermatozoa were cryopreserved and thawed and sperm quality assessments were carried out at three different points: pre-freeze, refrigerated semen at 17 °C and frozen–thawed spermatozoa at 30 min post thawing. To distinguish seminal plasma samples between two groups of good (GFE) and poor (PFE) freezability, Boar sperm were characterized by the reduced of sperm motility.

**Ultra-high Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (UHPLC-qTOF-MS) data acquisition**

Analysis data was acquired using a UHPLC-high definition quadrupole time-of-flight MS instrument (UHPLC-qTOF SYNAPT G1 HD-MS system, Waters) equipped with TripleTOF 6600 (Q-TOF, AB Sciex). A binary solvent method consisting of eluent A (25mM NH4Ac and 25mM NH4OH in water pH=9.75) and acetonitrile (B) was carried with elution gradient as follows: 0 min, 95% B; 0.5 min, 95% B; 7 min, 65% B; 8 min, 40% B; 9 min, 40% B; 9.1 min, 95% B; 12 min, 95% B, delivered at 0.5mL min-1. The Triple TOF mass spectrometer was used for its ability to acquire MS/MS spectra on an information-dependent basis (IDA) during an LC/MS experiment. In this mode, the acquisition software (Analyst TF 1.7, AB Sciex) continuously evaluates the full scan survey MS data as it collects and triggers the acquisition of MS/MS spectra depending on preselected criteria. In each cycle, 12 precursor ions whose intensity greater than
100 were chosen for fragmentation at collision energy (CE) of 30 V (15 MS/MS events with product ion accumulation time of 50 msec each). ESI source conditions were set as following: Ion source gas 1 as 60 Psi, Ion source gas 2 as 60 Psi, Curtain gas as 35 Psi, source temperature 600°C, Ion Spray Voltage Floating (ISVF) 5000 V or -4000 V in positive or negative modes, respectively.

**Multivariate data (MVD) analysis**

UHPLC-qTOF-MS data was analyzed using SIMCA 13 software (Umetrics, Umea, Sweden) and interactive XCMS (version 3.2). Before exporting the data to SIMCA for visualization and biomarker selection, the LC-MS raw data was firstly processed (noise elimination, peak picking, alignment and retention time correction) with MarkerLynxTM software (version 4.1, Waters Corporation, Milford, MA, USA). The following parameters were used for data processing: retention time (Rt) range of 2.5-11 min, mass range of 100-1000 Da, mass tolerance of 0.02 Da, Rt window of 0.2 min. The data matrix obtained from MarkerLynxTM processing was then exported into SIMCA 13 for PCA and OPLS-DA analyses. The data were Pareto-scaled and no transformation was used. For the XCMS analyses, the MassLynxTM raw data (.raw) were converted to NetCDF format using the DataBridge application in MassLynxTM (Waters, MA, USA). The converted data (NetCDF format) were then use XCMS for processing, statistical analysis, visualization and biomarker identification as described by [23]. The parameters were as follows: feature detection set as centWave method, minimum peak width = 5, maximum peak width = 20, retention time correction set as Obiwarp method, Profstep = 1, alignment set as m/z width = 0.015, min fraction = 0.5, bw = 5, and statistics set as statistical test = unpaired parametric t-test (Welch t-test), paired t-test and posthoc analysis with the threshold p-value = 0.01 and fold-change = 1.5.

**Relative distribution and statistical analysis**

Total intensity values (integrated area under the peak) from MarkerLynxTM XS software (Waters Corporation, Manchester, UK) pre-processed data matrixes were used for univariate statistical analyses. SPSS software (IBM SPSS Statistics for Windows, Version 22. Armonk, NY: IBM Corp.) was used for such descriptive statistics. Here, Univariate Analysis of Variance (ANOVA) was performed as 2-tailed complete randomized blocks, and used to compare the nontreated with the different time points of treated cells. ANOVA was followed by the Bonferroni post hoc test where differences between the means were considered significant at p < 0.05, and indicated in the Box-and-Whiskers plots.

**Targeted metabolomics analysis**

Targeted metabolomics analysis was performed using QTRAP 5500 (AB SCIEX), The target metabolomics metabolite extraction method is as same as UHPLC-qTOF-MS data acquisition. We perform absolute quantification of candidate differential metabolites based on standard products, and the standard products are purchased from Yuanye Biological Technology Co., Ltd.
Results

Classification of boar ejaculates in GFE and PFE groups

Ten Landrace boars in the same age were chosen in the study. The semen collected from the selected boars showed similar sperm parameters. And fresh sperm with motility more than 75% was processed for freezing. The sperm motility of fresh sperm, 17°C, and thawed were analyzed (Figure 1A, B). The difference in the sperm freezability of these boars was evaluated based on the ratio of thawed motility to fresh motility (the relative sperm motility) (Figure 1C, D). Five GFE and five PFE semen were chosen, as description in Figure 1C.

Metabolomic analysis based on UHPLC-qTOF-MS technology

A total of 953 metabolites were identified after UHPLC-qTOF-MS analysis of these seminal plasma samples, regardless of groups. Metabolites were identified and categorized according to their major chemical classes, including carboxylic acids and derivatives, organooxygen compounds, amino acids, peptides, and alogues, fatty amides, fatty acyls, benzene and substituted derivatives, purine nucleotides, pyrimidine nucleotides, glycosyl compounds, fatty acids and conjugates (Figure 2). A total of 534 (POS, 298 & NEG, 236) features could be mapped to current databases. According to the classification of metabolites, it was found that in the POS mode, the main metabolites were organic acids and their derivatives which contains 68 metabolites, accounting for 24% of all metabolites detected. The remaining metabolites were carboxylic acids and their derivatives (10%), nucleosides, nucleotides, and alogues ranked third (8%), followed by the organic oxygen compounds, organoheterocyclic compounds, lipids and lipid-like molecules, benzene and its substituted derivatives, benzenoids, and the other 14 metabolites (Figure 2A). In the NEG mode, 87 kinds of organic oxygen compounds metabolites was detected, accounting for 37% of all metabolites detected, followed by nucleosides, nucleotides, and alogues (10%), carboxylic acids and their derivatives (10%) ranked third, followed by organoheterocyclic compounds (8%), lipids and lipid-like molecules (8%), fatty acyls (4%), and 12 others metabolites (Figure 2B).

Identification of potential freezability biomarkers

To identify potential biomarkers in seminal plasma associated with the sperm freezability, OPLS-DA was applied to the classification of GFE group and PFE group (Figure 3). There was a clear separation between two groups in both positive (Figure 3A) and negative ion mode (Figure 3B) in OPLS-DA score plot. Besides, according to the permutation results (Figure 3C, D), the $R^2_Y$ value of the OPLS-DA model is close to 1 ($R^2_Y=0.87, 0.84$), which can better reflect the real situation and without over-fitting. Based on the analysis of OPLS-DA method, the calculated VIP and $P$ value is shown in Figure 4. According to the OPLS-DA and volcano plot, metabolites with VIP score greater than 1 and $P$-value less than 0.05 were identified and considered as candidate freezability markers. And the cluster analysis of each candidate
metabolite is shown in the Figure 5A, B. Finally, A total of 50 metabolites show significant difference between GFE group and PFE group (Table 1, 2).

Pathway analysis of metabolites

We analyzed these metabolites by KEGG pathways analysis of the differential metabolites pathway (Table 3). These differential metabolites were mainly enriched in amino acids biosynthetic metabolic pathways such as alanine, aspartic acid, glutamic acid, arginine, proline, cysteine, and methionine biosynthetic metabolic pathways, some of the metabolites are enriched in purine metabolism, pyrimidine metabolism, terpenoid backbone biosynthesis, aminoacyl tRNA biosynthesis and other metabolic pathways (Figure 6A, B).

Confirmation of freezability biomarkers by targeted metabolic analysis

Among the 50 candidate metabolites obtained from the above analysis, 3-Methylhistidine ($P = 0.036$), Phenethyl Caffeinate ($P = 0.049$), S-Adenosyl-L-homocysteine ($P = 0.049$), D-Aspartic acid ($P = 0.031$), L-Methionine ($P = 0.018$), DL-2-Aminoadipic acid ($P = 0.014$), L-Glutamine ($P = 0.009$), N-Acetyl-L-glutamate (NAG) ($P = 0.009$), Cytidine ($P = 0.023$), Inosine ($P = 0.012$), Quercetin ($P = 0.034$), and Norethindrone Acetate ($P = 0.024$) were chosen and further verified by targeted metabolism. The result reveals that D-Aspartic acid, NAG, and Inosine show significant difference between GFE and PFE ($P < 0.05$) (Figure 7).

Discussion

Cryopreservation of sperm is important for preservation of the boar sperm. In general, 40%-50% of the sperm population can not survive after cryopreservation process, even when "optimized" cooling/thawing protocols are used\cite{16, 24, 25}. There is a considerable variability between and within ejaculates in their ability to withstand cryopreservation procedures. Mammalian seminal plasma is mainly formed by secretions of the epididymis and accessory sex glands\cite{26}. Seminal plasma contains large spectra of metabolites and the current concept states that seminal plasma can modulate sperm function. Previously, metabolites have been identified in the bull and boar seminal plasma and attempts were made for exploring the candidate biomarker of fertility\cite{27}. The current study aimed to find a specific metabolomics signature in the seminal plasma of high freezability boars. Our study is in fact the first to compare the seminal plasma metabolome of boars between GFE and PFE. This model gives a global view of the metabolites in boar seminal plasma with high freezability and low freezability. Moreover, we confirmed the candidate metabolite biomarkers utilizing targeted metabolome method. In general, the main compounds in boar seminal plasma in the present study were carboxylic acids and derivatives, organonitrogen compounds, amino acids, peptides, and their alogues, fatty acyls, purine nucleosides, pyrimidine nucleosides, fatty acids and their conjugates. The changed 50 metabolites enriched in amino acids biosynthetic metabolic pathways, purine metabolism, pyrimidine metabolism, terpenoid backbone
biosynthesis, aminoacyl tRNA biosynthesis and other metabolic pathways. The enriched metabolic pathways implied that the variant metabolic function of sperms between GFE and PFE. Based on functional analysis such as KEGG, we chose 12 metabolites which could be confirmed by targeted metabolome. Finally, the results confirm that D-Aspartic acid, NAG, and Inosine were lower in GFE group than PFE group.

It is interesting that the level of D-Aspartic acid is higher in GFE group than PFE group. Previous studies reported that D-aspartic acid occurs in human seminal plasma and the concentration of D-Aspartic acid was significantly reduced in oligoasthenoteratospermic individuals[28]. Then studies on Leydig cells and spermatogonia in vitro demonstrated a direct effect of D-Aspartic on the steroidogenic pathway and spermatogenesis. Therefore, D-Aspartic mainly functions as a modulator of spermatogenesis in mammalian[29]. Further, the D-Aspartic treatment can increase the motility of sperm[30]. However, attempts using D-Aspartic to improve the reproductive activity of animals of commercial interest have yielded mixed results. Of course, there might be low correlation between sperm motility and freezability. The higher concentration of D-Aspartic acid in seminal plasma might impede the sperm cryotolerance ability.

NAG is synthesized from acetyl-CoA and glutamate by N-acetyl glutamate kinase, which catalyzes the key regulatory step in the pathway to arginine biosynthesis. Moreover, in mammalian, NAG is an allosteric catalyst of carbamoylphosphate synthase-I (CPS-I) [31]. While CPS-I catalyzed synthesis of carbamoyl phosphate (CP) is necessary for mitochondria to convert ornithine to citrulline[32]. Therefore, the NAG is the essential co-factor of CPS1 in the urea cycle[33]. Mammalian NAG is found in the mitochondrial matrix of cells in the liver and intestines[34]. The lower NAG level might result from the low enzyme active of N-acetyl glutamate kinase. There is a study reported that the existence of ionotrophic glutamate receptors and glutamate transporters in sperm. It also indicates that glutamate receptors and transporters might have functions other than neurotransmission in sperm[35]. The different level of NAG between GFE and PFE group implies that the amino acid biosynthesis is related to the sperm freezability.

Inosine is the main substance in the pathway of uric acid metabolism. Inosine has good permeability to the cell membrane and can directly enter the cell, convert it into nucleotides, and then further become ATP to participate in metabolism[36]. Exogenous Inosine could accelerate differentiation of rat intestinal epithelial cells[37]. However, there are only few studies report the Inosine in seminal plasma[38]. It seems that Inosine level was significantly higher in the seminal plasma of oligozo- and azoospermic than normozoospermic men[36]. The Inosine in seminal plasma might activate pyruvate oxidases, increase the activity of coenzyme A, and stimulate metabolism in sperm. The negative correlation between Inosine level and sperm freezability indicate that the role of nucleosides metabolism in sperm cold shock sensitivity.

In summary, for the first time we found that there are significant differences of metabolic profile between GFE and PFE individuals. Furthermore, some candidate metabolites were confirmed by the targeted metabolic analysis. Of course, it can be inferred that one indicator alone may not be able to accurately
evaluate, and multiple markers may be needed to predict sperm freezability. It will be meaningful to evaluate sperm freezability in combination with genome, proteome, metabolome, and epigenome data.

**Conclusions**

This study for the first time investigated the metabolome profile of boar seminal plasma with high and low freezability. Fifty metabolites show significant difference between GFE group and PFE group. The carboxylic acids and derivatives, amino acid, peptides, and alogues, organooxygen compounds, and fatty amides are the main component of these changed metabolites. Moreover, our results indicate that the D-Aspartic acid, NAG, and Inosine might be the potential markers associated with freezability. Further studies would be required to investigate the mechanism underlying the relationship between metabolites and sperm freezability.

**Declarations**

**Ethics approval and consent to participate**

All processes were performed according to guidelines for the ethical treatment of animals and were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University.

**Consent for publication**

The authors declare consent for publication.

**Availability of data and materials**

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**
Experiments were designed by X.G. Weng, and Z.H. Liu. Data were collected by Y.T. Zhang. Statistical analyses were performed by Y.T. Zhang. The manuscript was drafted by X.G. Weng and Y.T. Zhang with input from all authors.

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**Tables**

Table 1 Candidate differential metabolites screened in positive mode
| MS2 name                                          | MEAN GFE | MEAN PFE | VIP      | P- VALUE | FOLD CHANGE |
|--------------------------------------------------|----------|----------|----------|----------|-------------|
| 1,7-Dimethyluric acid                            | 0.0942   | 0.0379   | 2.5269   | 0.0083   | 2.4825      |
| 1-Myristoyl-sn-glycero-3-phosphocholine           | 0.0052   | 0.0137   | 2.1016   | 0.0359   | 0.3759      |
| 1-Oleoyl-sn-glycero-3-phosphocholine              | 0.0016   | 0.0028   | 1.7158   | 0.0272   | 0.5566      |
| 2-Butoxyethanol                                   | 0.0259   | 0.0122   | 1.9992   | 0.0496   | 2.1198      |
| 3.alpha.-Mannobiose                               | 4.1080   | 2.3077   | 1.8162   | 0.0008   | 1.7801      |
| 3-Hydroxyphenylacetic acid                        | 0.1205   | 0.0723   | 1.5893   | 0.0458   | 1.6674      |
| 3-Methylhistidine                                 | 1.1480   | 0.8328   | 1.1579   | 0.0356   | 1.3786      |
| Argininosuccinic acid                             | 0.0390   | 0.0179   | 2.3860   | 0.0262   | 2.1754      |
| Asp-Arg                                          | 0.0749   | 0.0378   | 2.0141   | 0.0178   | 1.9786      |
| Coumarin                                         | 0.0176   | 0.0084   | 2.0150   | 0.0016   | 2.1020      |
| Dacarbazine                                       | 0.0775   | 0.0428   | 1.6797   | 0.0476   | 1.8089      |
| Galactinol                                       | 0.5637   | 0.3181   | 1.6433   | 0.0019   | 1.7721      |
| Indole                                           | 0.0433   | 0.0262   | 1.5523   | 0.0021   | 1.6541      |
| Myristoleic acid                                 | 0.0162   | 0.0118   | 1.1880   | 0.0233   | 1.3761      |
| Nobiletin                                        | 0.0272   | 0.0164   | 1.7471   | 0.0006   | 1.6621      |
| norpropoxyphene                                   | 0.0028   | 0.0069   | 2.2637   | 0.0386   | 0.4030      |
| Phenethyl Caffeiate                               | 0.0219   | 0.0131   | 1.4684   | 0.0494   | 1.6794      |
| Primaquine                                        | 1.3228   | 1.0533   | 1.1033   | 0.0467   | 1.2558      |
| Pro-Asn                                          | 0.0040   | 0.0017   | 2.3885   | 0.0229   | 2.4268      |

Table 2 Candidate differential metabolites screened in negative mode
| MS2 name                                         | MEAN GFE | MEAN PFE | VIP     | P-VALUE | FOLD CHANGE |
|-------------------------------------------------|----------|----------|---------|---------|-------------|
| Tridecanoic acid (Tridecylic acid)              | 0.2783   | 0.6348   | 2.1164  | 0.0153  | 0.4384      |
| Tosyllysine Chloromethyl Ketone                 | 0.0120   | 0.0199   | 1.6392  | 0.0006  | 0.6027      |
| Tetrahydrocorticosterone                        | 0.0206   | 0.0426   | 1.4649  | 0.0138  | 0.4821      |
| Saccharin                                       | 0.1809   | 0.5057   | 2.4826  | 0.0422  | 0.3577      |
| Quercetin                                       | 0.5422   | 0.2871   | 1.6347  | 0.0343  | 1.8883      |
| Pyrethrosin                                     | 0.0056   | 0.0154   | 2.0506  | 0.0298  | 0.3597      |
| Phenol                                          | 0.0525   | 0.0866   | 1.4885  | 0.0362  | 0.6054      |
| Norethindrone Acetate                           | 0.1871   | 0.3038   | 1.4178  | 0.0241  | 0.6158      |
| Nomilin                                         | 4.0172   | 5.8818   | 1.3541  | 0.0054  | 0.6830      |
| Nname,Clofibric Acid                            | 0.0258   | 0.0524   | 1.8773  | 0.0131  | 0.4927      |
| N-Acetyl-L-glutamate                            | 0.0198   | 0.0346   | 1.5573  | 0.0320  | 0.5740      |
| N-Acetylglucosamine 1-phosphate                 | 0.1879   | 0.1014   | 1.5174  | 0.0417  | 1.8531      |
| Myristoleic acid                                | 0.3421   | 0.7712   | 1.8389  | 0.0427  | 0.4436      |
| L-Methionine                                    | 0.1183   | 0.2343   | 1.7950  | 0.0182  | 0.5052      |
| L-Glutamine                                     | 0.0513   | 0.1409   | 2.2264  | 0.0088  | 0.3640      |
| L-Galactono-1,4-lactone                         | 0.0082   | 0.0202   | 1.9233  | 0.0276  | 0.4045      |
| Inosine                                         | 7.1245   | 22.2369  | 2.6884  | 0.0123  | 0.3204      |
| Hydroxyphenyllactic acid                        | 0.0556   | 0.1204   | 1.9547  | 0.0010  | 0.4615      |
| Geranyl diphosphate                             | 0.0421   | 0.0768   | 1.7488  | 0.0044  | 0.5485      |
| Ellipticine                                     | 0.1565   | 0.2469   | 1.3680  | 0.0177  | 0.6337      |
| DL-2-Aminoadipic acid                           | 0.2106   | 0.2768   | 1.1342  | 0.0140  | 0.7611      |
| D-Aspartic acid                                 | 0.0685   | 0.0959   | 1.1970  | 0.0310  | 0.7142      |
| Cytidine                                        | 0.1239   | 0.0715   | 1.5283  | 0.0234  | 1.7324      |
| Caprylic acid                                   | 0.5436   | 1.4953   | 2.2286  | 0.0132  | 0.3635      |
| Acadesine (Drug)                                | 0.0146   | 0.0336   | 1.7311  | 0.0400  | 0.4358      |
| 5'-O-methylthymidine                            | 0.0151   | 0.0303   | 2.0358  | 0.0498  | 0.4992      |
| 3-Hydroxydodecanoic acid                        | 0.0328   | 0.0745   | 1.7901  | 0.0298  | 0.4403      |
| Metabolite             | Value1 | Value2 | Value3 | Value4 | Value5 |
|-----------------------|--------|--------|--------|--------|--------|
| 3-Guanidinopropanoate | 0.2673 | 0.1166 | 1.9724 | 0.0098 | 2.2931 |
| 2-Thiocytidine        | 0.5193 | 0.9476 | 1.5898 | 0.0415 | 0.5480 |
| 1-Methyladenosine     | 0.0051 | 0.0090 | 1.7015 | 0.0122 | 0.5626 |

Table 3 KEGG pathways analysis of the differential metabolites pathway
| Pathway                                                      | model |
|--------------------------------------------------------------|-------|
| Metabolic pathways - Sus scrofa (pig)                        | POS   |
| Biosynthesis of amino acids - Sus scrofa (pig)               | POS   |
| Caffeine metabolism - Sus scrofa (pig)                       | POS   |
| Galactose metabolism - Sus scrofa (pig)                      | POS   |
| Histidine metabolism - Sus scrofa (pig)                      | POS   |
| Phenylalanine metabolism - Sus scrofa (pig)                  | POS   |
| Phenylalanine, tyrosine and tryptophan biosynthesis - Sus scrofa (pig) | POS   |
| Tryptophan metabolism - Sus scrofa (pig)                     | POS   |
| 2-Oxocarboxylic acid metabolism - Sus scrofa (pig)          | NEG   |
| ABC transporters - Sus scrofa (pig)                          | NEG   |
| Aminoacyl-tRNA biosynthesis - Sus scrofa (pig)               | NEG   |
| AMPK signaling pathway - Sus scrofa (pig)                    | NEG   |
| Antifolate resistance - Sus scrofa (pig)                     | NEG   |
| Ascorbate and aldarate metabolism - Sus scrofa (pig)        | NEG   |
| Biosynthesis of amino acids - Sus scrofa (pig)               | NEG   |
| Central carbon metabolism in cancer - Sus scrofa (pig)      | NEG   |
| D-Glutamine and D-glutamate metabolism - Sus scrofa (pig)   | NEG   |
| Fatty acid biosynthesis - Sus scrofa (pig)                   | NEG   |
| GABAergic synapse - Sus scrofa (pig)                         | NEG   |
| Glutamatergic synapse - Sus scrofa (pig)                     | NEG   |
| Glyoxylate and dicarboxylate metabolism - Sus scrofa (pig)  | NEG   |
| Lysine degradation - Sus scrofa (pig)                        | NEG   |
| Metabolic pathways - Sus scrofa (pig)                        | NEG   |
| Mineral absorption - Sus scrofa (pig)                        | NEG   |
| Nitrogen metabolism - Sus scrofa (pig)                       | NEG   |
| Proximal tubule bicarbonate reclamation - Sus scrofa (pig)  | NEG   |
| Purine metabolism - Sus scrofa (pig)                         | NEG   |
| Pyrimidine metabolism - Sus scrofa (pig)                     | NEG   |
Figure 1

Screening semen for differential freezability.

Figure 2
Metabolites and chemical class of seminal plasma. A, B was detected in positive and negative ions mode respectively.

Figure 3

Metabolomics profile of GFE and PFE group. OPLS-DA scores plots based on the data from in positive (A) v.s. in negative mode (B). Permutation test of OPLS-DA model for group GFE v.s. PFE in positive mode (C) and negative mode (D).
**Figure 4**

VIP value and volcano maps of metabolites. VIP scores of metabolites in GFE and PFE (TOP15) obtained in positive (A) and negative mode (B). Heat map with red or green boxes on the right indicates high and low abundance ratio, respectively, of the corresponding metabolite in GFE and PFE. Volcano maps of metabolites in the positive (C) and negative (D) mode.
Figure 5

Heat map of metabolites with different content between GFE and PFE groups. A, B was analyzed in positive and negative ions mode respectively.
Figure 6

Metabolic pathway analysis of changed metabolites identified in boar seminal plasma. A, B was analyzed in positive and negative ions mode respectively.
Figure 7

Content of metabolites confirmed by targeted metabolic analysis.