



Comparison of urinary proteome in the first two days after mating in male rats

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ABSTRACT

Objective: To explore whether differences between male rats on the next day of mating and on the day of mating can be reflected by the urine proteome.

Methods: Urine samples were collected from male Sprague-Dawley rats on the day of mating and the next day of mating. Urine samples were analysed by the label-free quantitative proteomics technique of high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS). Differential proteins of the urine proteome were analysed for protein function and biological pathways.

Results: 54 differential proteins were identified by comparing the urine proteome of rats on the next day of mating with that on the day of mating, and nearly two-thirds of the differential proteins were related to spermatogenesis.

Conclusions: The urine proteome has the potential to reflect spermatogenesis without interfering with it.

1. Introduction

Spermatogenesis is the process by which spermatogonial cells undergo proliferation and differentiation to form mature sperm, which involves chromosome fold reduction and cell deformation. There are three stages of mammalian spermatogenesis. First, the spermatogonium undergoes a series of mitosis to form a primary spermatocyte. Secondly, the primary spermatocytes produce haploid round sperm cells through meiosis. Finally, the round sperm cells are deformed into sperm with flagella. Paracrine, autocrine, and endocrine pathways all contribute to the regulation of this process, and the large number of structural elements and chemical factors involved in regulation make the network connecting various cellular activities during spermatogenesis unimaginably complex.¹ Urine is produced by the filtration of blood through the kidneys to eliminate metabolic waste, which is not controlled by the homeostatic regulation mechanism of the internal environment, and can be more sensitive to the various small changes produced by the body.² It has been shown that the urine metabolome can be used to differentiate normozoospermic infertile men from fertile individuals.³ However, no studies have been conducted to monitor spermatogenesis by urine proteome. After a mating of male rats, mature sperm is consumed to stimulate spermatogenesis in the testis. In this study, by collecting urine samples from rats on the day of mating and the next day of mating, we compared the urinary proteome and tried to explore whether spermatogenesis could be reflected in the urinary proteome.

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1.1. Experimental material

1.1.1. Experimental animal

Five 10-week-old male Sprague-Dawley rats and five 10-week-old female Sprague-Dawley rats were purchased from Beijing Weitonglihua Experimental Animal Biotechnology Company Limited. All rats were raised in a standard environment (room temperature 22 ± 1 °C, humidity 65%–70 %). All the rats were fed in the new environment for three days before the experiment began. All experimental operations were reviewed and approved by the Ethics Committee of the College of Life Sciences, Beijing Normal University with the approval number CLS-AWEC-B-2022-003.

1.2. Experimental method

1.2.1. Rats mating

Each of the five rats was cohoused with a female rat at 16:00. At 7:00 the next day, female rats were checked for vaginal plugs, and those who detected vaginal plugs were considered to be mating between male and female.

1.2.2. Urine sample collection

On the mating day, each of the five rats was placed into a single metabolic cage from 20:00 to 8:00 the next day and urine was collected through the metabolic cage. Urine was collected separately and temporarily stored in the -80 °C refrigerator as urine samples on the mating day. After urine collection, male rats were fed separately. On the next day after mating, each of the five rats was placed into a single metabolic cage from 20:00 to 8:00 the next day and urine was collected through the metabolic cage. Urine was collected separately and temporarily stored in the -80 °C refrigerator as urine samples on the next day after mating.

1.2.3. Urine sample treatment

Urine protein extraction: Rat urine samples were removed from the refrigerator at -80 °C and thawed at 4 °C. Rat urine samples were centrifuged at 4 °C, $12,000\times g$ for 30 min and the supernatants were transferred to new Eppendorf (EP) tubes. Triple volumes of precooled absolute ethanol was added, and the samples were homogeneously mixed and precipitated overnight at -20 °C. The mixture precipitated overnight was centrifuged at 4 °C, $12,000\times g$ for 30 min, the supernatant was discarded, and the ethanol was volatilized and dried. The precipitated protein was dissolved in lysis buffer (8 mol/L urea, 2 mol/L thiourea, 25 mmol/L dithiothreitol, 50 mmol/L Tris) and centrifuged at 4 °C at $12,000\times g$ for 30 min, and the supernatant was placed in a new EP tube. The protein concentration was measured by Bradford method.

Urine digestion of urine protein: 100 µg urine protein sample was taken into 1.5 mL EP tube, and 25 mmol/L NH_4HCO_3 solution was added to make the total volume 200 µL. 20 mM Dithiothreitol solution (DTT, Sigma, prepared in 25 mmol/L NH_4HCO_3 solution) was added and mixed. The metal bath was heated at 97 °C for 10 min, and cooled to room temperature. 50 mM Iodoacetamide (IAA, Sigma, prepared in 25 mmol/L NH_4HCO_3 solution) was added, mixed and reacted at room temperature for 40 min away from light. 200 µL UA solution (8 mol/L urea, 0.1 mol/L Tris-HCl, pH 8.5) was added to the 10 kDa ultrafiltration tube (Pall, Port Washington, NY, USA), centrifuged at 18 °C, $14,000\times g$ for 5 min and repeated once; freshly treated samples were added and centrifuged at 18 °C, $14,000\times g$ for 30 min. The urine protein was on the filter membrane. 200 µL UA solution was added, centrifuged at 18 °C, $14,000\times g$ for 30 min and repeated twice. 25 mmol/L NH_4HCO_3 solution was added, centrifuged at 18 °C, $14,000\times g$ for 30 min and repeated twice. Trypsin Gold (Promega, Fitchburg, WI, USA) was added at the ratio of 1:50 for enzyme digestion, and the water bath at 37 °C for 15 h. After digestion, the peptides were collected by centrifugation at 4 °C at $13,000\times g$ for 30 min, desalted by HLB solid phase extraction column (Waters, Milford, MA). The peptides were lyophilized with a vacuum dryer, and stored at -20 °C.

1.2.4. LC-MS/MS analysis

The digested samples were reconstituted with 0.1 % formic acid, and peptides were quantified with BCA peptide quantification kit. The peptide concentration was then diluted with 0.1 % formic acid to 0.5 µg/µL. Mixed peptide samples were prepared from 6 µL of each sample and separated by high pH reversed phase peptide separation kit (Thermo Fisher Scientific, Waltham, MA, USA). Ten fractions were collected by centrifugation, lyophilized with a vacuum dryer and reconstituted with 0.1 % formic acid. iRT reagent (Biognosys, Switzerland) was added to each fraction and each digested sample with a volume ratio of sample: iRT of 10:1 to calibrate the retention times of the extracted peptide peaks.

For analysis, 1 µg of peptide from each fraction and each digested sample was loaded onto a trap column and separated on a reverse-phase C18 column (50 µm \times 150 mm, 2 µm) using the EASY-nLC1200 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA).⁴ The elution for the analytical column lasted 90 min with a gradient of 4%–90 % mobile phase B (gradient: mobile phase A: 0.1 % formic acid, mobile phase B: 80 % acetonitrile; flow rate 0.3 µL/min). Peptides were analysed with an Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

To generate the spectrum library, Ten fractions were subjected to mass spectrometry in data-dependent acquisition (DDA) mode, and 10 raw files were generated. Mass spectrometry data were collected in high sensitivity mode. A complete mass spectrometric scan was obtained in the 350–1200 m/z range with a resolution set at 60,000. 10 raw files were imported into Proteome Discoverer software for library construction using Swiss-iRT and Uniprot-Rat databases (version 2.0, Thermo Scientific). 39 variable window Data Independent Acquisition (DIA) methods for DIA mode of each digested sample were set up according to the results of database construction.

Each digested sample was analysed using Data Independent Acquisition (DIA) mode. DIA mode was performed using the DIA method with 39 windows. After every 7 samples, a single DIA analysis of the pooled peptides was performed as a quality control.

1.2.5. Quantitative analysis of label- DIA

Data were collected as previously.⁵ Specifically, the raw file of each digested sample collected in DIA mode was imported into Spectronaut Pulsar (Biognosys AG, Switzerland) for analysis. The abundances of peptide were calculated by summing the peak areas of the respective fragment ions in MS₂. Mass tolerance: ± 40 ppm. Peptide Confidence Level set to 95 %. Global normalization. Protein intensities were summed from their respective peptide abundances to calculate protein abundances.

1.2.6. Data analysis

The LC-MS/MS analysis technique was repeated 3 times for each sample, and the PG.Quantity average value for each identified protein was taken for statistical analysis. Five rats were used as biological replicates. Five urine samples on the mating day group were compared with five urine samples on the next after mating day group. The identified proteins were compared to screen for differential proteins. The differential protein screening conditions were as follows: PG.Quantity fold change (FC) ≥ 1.5 or ≤ 0.67 between groups

Table 1

Urine differential proteins between the next day of mating and the day after mating.

Uniprot ID	Protein names	Fold change	Trend	P value	Related to implantation
Q8R431	Monoglyceride lipase	13.75	↑	1.18E-02	7
P0C0A1	Vacuolar protein-sorting-associated protein 25	8.48	↑	3.99E-02	8
Q63531	Ribosomal protein S6 kinase alpha-1	7.8	↑	4.51E-02	9 10
Q63060	Glycerol kinase	4.87	↑	9.32E-03	11
P63259	Actin, cytoplasmic 2	4.5	↑	3.53E-02	12 13 14
P63095	Guanine nucleotide-binding protein G(s) subunit alpha isoforms short	3.75	↑	2.19E-02	15 16
Q62753	Syntaxin-binding protein 2	3.63	↑	0.044062	17
P04897	Guanine nucleotide-binding protein G(i) subunit alpha-2	3.6	↑	4.16E-02	18 19
Q2QDE7	Deoxyribonuclease-1-like 1	3.09	↑	0.037949	20
Q6PCU2	V-type proton ATPase subunit E 1	3.07	↑	7.32E-03	21 22
Q9Z0W7	Chloride intracellular channel protein 4	2.92	↑	3.59E-02	23
P06866	Haptoglobin	2.73	↑	3.80E-02	24 25
P61983	14-3-3 protein gamma	2.65	↑	0.019226	26
P04692	Tropomyosin alpha-1 chain	2.41	↑	0.01404	27
P03994	Hyaluronan and proteoglycan link protein 1	2.35	↑	0.032237	28
Q6MG61	Chloride intracellular channel protein 1	2.34	↑	2.44E-02	23
Q6P9T8	Tubulin beta-4B chain	2.33	↑	4.48E-02	29 30
Q497B0	Omega-amidase NIT2	2.24	↑	3.07E-02	
Q99PW3	Sialidase-1	2.23	↑	0.035028	
Q811X6	Lambda-crystallin homolog	2.18	↑	0.046418	
Q3KRD8	Eukaryotic translation initiation factor 6	2.12	↑	0.027884	31
Q4KM73	UMP-CMP kinase	2.04	↑	0.024033	
Q811M5	Complement component C6	2.04	↑	2.98E-02	
Q9EPB1	Dipeptidyl peptidase 2	2.04	↑	0.00621	32 33
P50399	Rab GDP dissociation inhibitor beta	2.03	↑	3.24E-02	34
Q9Z173	Adhesion G protein-coupled receptor L3	2.01	↑	0.033569	
P51635	Aldo-keto reductase family 1 member A1	1.94	↑	5.39E-03	35 36
Q6DGG1	Protein ABHD14B	1.94	↑	1.46E-02	
Q5XHZ9	Pachytene checkpoint protein 2 homolog	1.9	↑	9.31E-03	37 38
Q5XFX0	Transgelin-2	1.86	↑	4.20E-02	39 40
Q32KJ6	N-acetylgalactosamine-6-sulfatase	1.85	↑	1.68E-02	
P51607	N-acylglucosamine 2-epimerase	1.84	↑	4.69E-02	
Q5FVH2	Phospholipase D3	1.82	↑	0.015003	41 42
P08689	Sex hormone-binding globulin	1.80	↑	0.006293	43 44 45
P32755	4-hydroxyphenylpyruvate dioxygenase	1.78	↑	0.026115	
Q6TMA8	Angiopietin-related protein 4	1.78	↑	0.040734	
Q920A6	Retinoid-inducible serine carboxypeptidase	1.74	↑	1.97E-03	
Q5M843	2-oxoglutarate and iron-dependent oxygenase domain-containing protein 3	1.65	↑	0.049558	
Q5M7T9	Threonine synthase-like 2	1.64	↑	4.00E-03	
Q568Z6	IST1 homolog	1.62	↑	0.049048	
Q66H12	Alpha-N-acetylgalactosaminidase	1.61	↑	0.037511	
Q6TUD4	Protein YIPF3	1.61	↑	0.041193	
P63102	14-3-3 protein zeta/delta	1.6	↑	4.71E-02	46 47
P35704	Peroxisome oxidoreductin-2	1.58	↑	3.22E-02	48 49
Q6VBQ5	Myeloid-associated differentiation marker	1.58	↑	0.044019	
B0BND0	Ectonucleotide pyrophosphatase/phosphodiesterase family member 6	1.57	↑	4.42E-02	
Q5U2P2	Immunoglobulin superfamily member 11	1.57	↑	7.04E-03	
P10111	Peptidyl-prolyl cis-trans isomerase A	1.56	↑	3.94E-02	50
P19804	Nucleoside diphosphate kinase B	1.54	↑	3.45E-02	51 52
P17046	Lysosome-associated membrane glycoprotein 2	1.52	↑	0.018791	53 54
P47820	Angiotensin-converting enzyme	1.52	↑	0.001539	55 56
Q642A7	Protein FAM151A	1.51	↑	1.65E-02	
Q6AYR9	Tetraspanin-1	0.60	↓	0.003997	57
P02625	Parvalbumin alpha	0.4	↓	1.37E-02	58

and paired T-test was $P < 0.05$.⁶ The differential proteins were analysed by Uniprot website (<https://www.uniprot.org/>) and the relevant literature was searched in Pubmed database (<https://pubmed.ncbi.nlm.nih.gov>) to analyze the function of the differential proteins.

2. Experimental results and analysis

2.1. Comparison of urine proteome between the day after mating and the day after mating

2.1.1. Differential protein

All of the samples were identified 925 proteins. The urine protein of the next day of mating was compared with that of mating day. The screening conditions for differential protein were $FC \geq 1.5$ or ≤ 0.67 , and the paired T-test was $P < 0.05$. The results showed that 54 differential proteins could be identified on the day after mating compared with the day of mating. The differential proteins were arranged in the order of FC from largest to smallest and retrieved by Uniprot. The results were shown in Table 1.

2.1.2. Differential protein function analysis

The 54 identified differential proteins were searched through PubMed database, and 26 of them or other members of their family were reported to be associated with spermatogenesis. The function of these proteins is shown below :

Monoglyceride lipase is highly expressed in the testes and is directly involved in regulating human testicular physiology as part of the endocannabinoid system, including spermatogenesis and the function of stromal cells.⁷

Mutations in Vacuolar protein-sorting associated protein 33 B from the same family as Vacuolar protein-sorting associated protein 25 cause sterility in *C. elegans*, Spermatocyte with division arrest indicates that this protein is involved in the formation of sperm-specific organelles.⁸

Ribosomal protein S6 kinase alpha-1 substrate Ribosomal protein S6 is critical for spermatogenesis, and knockdown of this protein in Chinese bream resulted in spermatogenesis defects, including germ cell loss, mature sperm retention, and cavity formation.⁹ The protein can also regulate the blood-testicular barrier of supporting cells through Akt1/2, and then regulate the organization of F-actin, regulate the adhesion function of the intercellular interface, and promote the transport of blood-testicular barrier of spermatocytes in the pre-leptotene stage during rat spermatogenesis.¹⁰

Glycerol kinase 2, which has a high degree of homology with Glycerol kinase kinase, is crucial for the correct arrangement of crescent mitochondria to form mitochondrial sheath during mouse spermatogenesis. Knockout of this gene will lead to the disorder of mitochondrial sheath of sperm flagella.¹¹

Actin is involved in various aspects of spermatogenesis, and with spermatogenesis, the Actin cytoskeleton exhibits active remodeling during this process, participating in the shaping and differentiation of sperm cells.^{12,13} However, the molecular mechanisms by which Actin cytoskeletal tissue responds to spermatogenesis in spermatogenic epithelial cells remain largely unexplored.¹⁴

Guanine nucleotide-binding protein G(s) subunit alpha is expressed in a tissue-specific and age-dependent manner in the reproductive organs of the ram. The protein is expressed at high levels in the epididymis, suggesting that it may influence the composition of the epididymal lumen fluid, thus, it affects the microenvironment of sperm maturation and may play an important role in spermatogenesis and the development of the testis and epididymis in the ram reproductive system.¹⁵ Guanine nucleotide-binding protein G(o) subunit alpha, from the same family as Guanine nucleotide-binding protein G(s) subunit alpha, The high level of expression in rat spermatocytes during pachytene stage suggests that it may play a role in this stage of spermatogenesis.¹⁶

Syntaxin binding protein 2 in sertoli cells regulates spermatogonial stem cell maintenance through directly interacting with connexin 43 in the testes of neonatal mice. And interactions between Sertoli and germ cells are essential for spermatogenesis and male fertility.¹⁷

Guanine nucleotide-binding protein G(i) subunit alpha-1, Guanine nucleotide-binding protein G(i) subunit alpha-2, Guanine nucleotide-binding protein G(i) subunit alpha-3 and Guanine nucleotide-binding protein G(o) subunit alpha were detected in mouse spermatocytes and sperm cells. When spermatocytes develop into sperm cells, Guanine nucleotide-binding protein G(o) subunit alpha levels are reduced.¹⁸ Guanine nucleotide-binding protein G(i) subunit is associated with the developing acrosome, which may play a role in acrosome biogenesis. The Guanine nucleotide-binding protein G(i) subunit is found in the acrosomal region of mammalian sperm and is part of a complex required for signal transduction to induce acrosomal exocytosis.^{18,19}

Deoxyribonuclease-1 is associated with the regulation of apoptosis, and seminal plasma levels of this protein, are important for correct spermatogenesis. Levels of deoxyribonuclease-1 are progressively decreased in varicocele when compared to adolescents without varicocele. Deoxyribonuclease-1 levels are positively correlated with sperm concentration and morphology. Moreover, deoxyribonuclease-1 is capable of discriminating a varicocele that causes alterations to semen quality from one that does not.²⁰

V-type proton ATPases play an important role in the capacitation process of rabbit sperm.²¹ Rat round sperm cells regulate intracellular pH through V-type proton ATPases, HCO₃⁻ entry pathways, Na⁺/HCO₃⁻ dependent transport systems, and postulated proton conduction pathways. These pH adjustments appear to be specifically designed to withstand the acid challenge.²²

There are Chloride intracellular channel protein in bovine epididymis sperm. Chloride intracellular channel protein 1, Chloride intracellular channel protein 4 and Chloride intracellular channel protein 5, are present in the sperm, and occupy different positions in the cell. Both are able to bind to PP1 γ 2 in sperm, and since PP1 γ 2 is a key enzyme regulating sperm motility, the PP1 γ 2-binding protein Chloride intracellular channel protein may play an important role in sperm function.²³

Haptoglobin, an iron transporter expressed in Sertoli, Leyden, and germ cells of the rat testis but not in the epididymis, may play an important role in iron metabolism in the testis. Testis Haptoglobin mRNA levels steadily increase during maturation after birth,

indicating its involvement in spermatogenesis.²⁴ Sertoli cells play a key role in spermatogenesis and express receptors for follicle-stimulating hormone (FSH) and testosterone (T), the major hormonal regulators of spermatogenesis. FSH stimulation of Sertoli cells in pigs resulted in an increase in inhibin- α , inhibin- β , plaquoglobulin, haptoglobulin, D-3-phosphoglycerate dehydrogenase, and sodium/potassium transport atpase in Sertoli extracellular vesicles.²⁵

Sloss of 14-3-3 protein gamma leads to decreased desmosome formation and a decrease in cell-cell adhesion in vitro, and in the mouse testis in vivo, leading to defects in testis organization and spermatogenesis.²⁶

The stable F-actin cones of spermatogenesis contain Tropomyosin 1 and failure of F-actin cone formation is associated with failure of Tropomyosin 1 to accumulate at the cone initiation sites.²⁷

In the male poultry *Anas platyrhynchos* Hyaluronan and proteoglycan link protein 2 gene, has been identified as candidate genes regulating reproductive capacity.²⁸

Many processes in spermatogenesis depend on the dynamic changes of the cytoskeleton, the movement of organelles, and especially the regulation of microtubules. Data from transgenic mouse models suggest that the coordination of microtubule dynamics is critical for male fertility.²⁹ During spermatogenesis, a structure called "nuage" appears and disappears as spermatogenic cells differentiate. Nuage can be divided into four types: Irregularly Shaped Perinuclear Granule (ISPG), Intermitintermittal Cement (IMC), and Satellite Body (SB), Chromatoid Body (CB). ISPG, IMC and SB were observed in pachytene spermatocytes, while CB was observed in round sperms. In rat round sperm cells, Tubulin beta is translated from mRNA stored in CB, and assembled with Tubulin alpha outside CB to form a structural unit of microtubules: $\alpha\beta$ -heterodimer, to construct microtubules in the sperm fella.³⁰

In *Drosophila melanogaster* Eukaryotic translation initiation factor 4E-5 is essential for male fertility. Eukaryotic translation initiation factor 4E-5 protein localizes to the distal ends of elongated spermatid cysts, and Eukaryotic translation initiation factor 4E-5 mutants exhibit defects during post-meiotic stages, including a mild defect in spermatid cyst polarization. Eukaryotic translation initiation factor 4E-5 mutants also have a fully penetrant defect in individualization, resulting in failure to produce mature sperm.³¹

Human testicular peritubular cells transport sperm and contribute to the ecological niche and immune monitoring of spermatogonial stem cells. Secretome analysis revealed replicative senescence of human testicular peritubular cells with elevated Dipeptidyl peptidase 4 levels, which may play a role in spermatogenesis, and testicular Dipeptidyl peptidase 4 may further represent a possible drug target.³² Dipeptidyl peptidase 4 inhibitors are a new class of anti diabetic compounds that affect spermatogenesis during the clinical course, leading to a dramatic deterioration of semen quality in patients.³³

Rab GDP dissociation inhibitor beta and family member Rab GDP dissociation inhibitor alpha are involved in the organization of actin cytoskeleton and also regulate cell morphology and cell motility. The expression of this protein is decreased in patients with asthenospermia.³⁴

Increased ability to metabolize specific steroids during testicular development, such as increased expression of Aldo-keto reductase family 1 member C3, the same family member as Aldo-keto reductase family 1 member A1, during testicular development in domestic cats.³⁵ The level of Aldo-Keto Reductase mRNA in silkworm testis is higher than that in other tissues, which plays an important role in the spermatogenesis of silkworm.³⁶

Pachytene checkpoint protein 2 homolog was searched by Uniprot database. The results showed that this protein is expressed in the male reproductive nucleus, and is involved in biological processes such as sperm development, sperm formation, male meiosis, synaptic complex, meiosis recombination, double-strand break repair, and meiosis recombination checkpoint signaling pathway. This protein plays a key role in chromosome recombination and chromosome structure development during meiosis. In the early stage of meiosis recombination, it mediates the non-crossed pathway and also influences the crossed and non-crossed pathways to effectively complete homologous chromosome association, which is necessary for the effective sex chromosome synapsis. Mouse Pachytene checkpoint protein 2 is required for recombination and normal higher-order chromosome structure during meiosis. Pachytene checkpoint protein 2 plays a potential role in the non-crossed repair of double-strand breaks in meiosis. The spermatocytes of male mice with a complete mutation in this gene can fully associate with their chromosomes. However, in the process of chromosome recombination, the loss of this leads to the failure of repair of double-strand breaks, cell death at the pachytene stage, and the lack of post-meiotic cells in the testicular tissue.^{35,37} In males, Pachytene checkpoint protein 2 is required for sex chromosome syndesmosis and the formation of sex bodies (transcription-silencing subnuclear domains formed by the X and Y chromosomes).³⁸

Acute heat stress impels translation, protein folding and protein degradation processes in chicken testis, leading to apoptosis and interfering with spermatogenesis. After acute heat stress, Transgelin is upregulated in the testis to resist heat-induced damage.³⁹ The expression level of Transgelin gene was low in testicular tissue of aged animals.⁴⁰

Phospholipase D6 as a Golgi-localized protein of pachytene spermatocytes and developing spermatids and suggest that its subcompartment-specific distribution within the Golgi apparatus may be related to the specific functions of this organelle during spermatogenesis.⁴¹ Phospholipase D isozymes are involved in the spermatogenesis of the mouse testis.⁴²

Sex hormone-binding globulin expression in the dog testis is positively correlated with sperm concentration, total and progressive motility, plasma membrane integrity and acrosome integrity, and Sex hormone-binding globulin is negatively correlated with low sperm mitochondrial activity. In the epididymis, Sex hormone-binding globulin expression is only positively correlated with plasma membrane integrity. In addition, Sex hormone-binding globulin expression in the testes is associated with epididymis Sex hormone-binding globulin expression and morphologically normal cells. Sex hormone-binding globulin play key roles in spermatogenesis and sperm maturation, being essential for male reproductive success.⁴³ The cauda epididymidis is the rat major sperm storage region whose androgenic supply, essential for the sperm viability, is provided by the vasculature and is dependent upon testosterone diffusion through the stromal tissue to reach the epithelial cells. Stromal Sex hormone-binding globulin in the androgenic supply of the sperm storage region of the epididymis.⁴⁴ Sperm Sex hormone-binding globulin isoform levels correlate significantly with age and sperm motility and may influence sperm function in relation to male fertility.⁴⁵

The 14-3-3 protein plays a key regulatory role in both mitosis and meiosis. In mice, 14-3-3 protein epsilon is essential for normal sperm function and male fertility.⁴⁶ Mature spermatids need to be released from their attached sertoli cells. Identified proteins involved in the adhesion between sertoli cells and mature spermatids include 14-3-3 protein zeta/delta, which was only found in the tubular segment lysate during sperm release. But what exactly is its role in spermatogenesis, and how does it interact or influence other signal transduction pathways in the testes remain unknown.⁴⁷

Peroxiredoxin-2 has some antioxidant properties and may be involved in maintaining oxidative balance in the spermatogenic environment of mice.⁴⁸ Peroxiredoxin-2 also maintained the normal development of reproductive cells in newborn rats.⁴⁹

Peptidyl-prolyl cis-trans isomerase A is upregulated in the testes of mice treated with environmental estrogen, which reduces male sperm count and leads to male infertility. However, the molecular mechanism of its effect on male infertility remains unclear.⁵⁰

Nucleoside diphosphate kinase B is distributed in the manchette microtubule structure of sperm (a transient microtubule structure in elongated sperm that plays an important role in nuclear enrichment and sperm tail formation). Nucleoside diphosphate kinase A is distributed instantaneously in the round sperm nucleus and asymmetrically in the cytoplasm of the elongated sperm nucleus base. Nucleoside diphosphate kinase subtypes may have specific functions in phosphate transfer networks involved in human spermatogenesis and flagellar motility.⁵¹ Nucleoside diphosphate kinase plays a key role in spermatogenesis by increasing the level of glutathione peroxidase 5 in mouse cells to eliminate reactive oxygen species.⁵²

Lamp2 (encoding lysosome-associated membrane protein-2) is a target of Fork head box J2. Fork head box J2 overexpression in the germ cells of mouse testes affects chaperone-mediated autophagy by upregulating LAMP2A, leading to spermatogenesis failure at the initiation of meiosis, thus resulting in male infertility.⁵³ Lysosome-associated membrane protein-1 is expressed in the later stages of spermatogenesis (acrosome stage), whereas Lysosome-associated membrane protein-2 is expressed throughout spermatogenesis.⁵⁴

Somatic angiotensin-converting enzyme was found to play an important role in spermatogonial stem cell self-renewal through the regulation of MAPK-dependent cell proliferation. Spermatogonial stem cell self-renewal is an indispensable part of spermatogenesis.⁵⁵ Similarly, testicular angiotensin-converting enzyme plays a key role in male fertility. ATP in sperm of mice lacking testicular angiotensin-converting enzyme activity is 9.4-fold lower than in normal mouse sperm. Angiotensin-converting enzyme inhibitors also reduce ATP production by 72 % in mouse sperm. Testicular angiotensin-converting enzyme inactivation severely affects oxidative metabolism, and sperm display lower levels of oxidative enzymes, leading to a reduced rate of mitochondrial respiration. Reduced sperm energy production in mice lacking testicular angiotensin-converting enzyme activity leads to defects in their physiological functions, including motility, acrosomal enzyme activity, and fertilization in vitro and in vivo.⁵⁶

The natural down-regulation of Tspan8 in sertoli cells during puberty is a prerequisite for establishing male fertility. The natural down-regulation of Tspan8 is prevented specifically in sertoli cells from puberty up to adulthood. Adult male rats showed around 98 % reduction in sperm count.⁵⁷

The combination of injection of testosterone undecanoate and oral levonorgestrel enhanced the expression of Parvalbumin alpha and inhibited spermatogenesis. Parvalbumin alpha protects testicular cells from apoptosis and promotes cell survival, and may be an early molecular target for hormone-induced spermatogenesis inhibition.⁵⁸

3. Discussion

Rats were self-controlled in the experiment, and the interval between two urine collection was only one day, so as far as possible, the interference of individual differences and their own growth and development could be excluded. Therefore, even with a small sample size, the results of this study could preliminarily show that there were significant differences in the urine proteome of rats on the second day after mating and on the mating day, and most of the differences were related to spermatogenesis. Although the correlation between the remaining differential proteins and spermatogenesis had not been found in the database, the results of this study suggested that these proteins may still be related to spermatogenesis and could be further studied as target proteins of spermatogenesis. This study demonstrated the potential of urine proteome in studying spermatogenesis, and provided a method of urine proteomics for exploring the pathogenesis of abnormal spermatogenesis in males, discovering targets and new diagnostic methods. And the results of this study indicated that the study of spermatogenesis by semen actually interfered with the spermatogenesis process at the same time as the collection of semen for the study, while the urine proteomic method in our study could be carried out without affecting spermatogenesis. Further experiments may consider expanding the sample size of experimental animals or collecting clinical samples for research. At the same time, it also reflected the sensitivity of urine proteome and opened up a new field of urine exploration.

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CRedit authorship contribution statement

Haitong Wang: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Chenyang Zhao:** Methodology, Conceptualization. **Youhe Gao:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors have no conflict of interest to disclose.

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