Comparison of Urinary Proteome in Male Rats Pre- and Post-Mating

Authors: Wang Haitong, Zhao Chenyang, Gao Youhe, Gao Youhe

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Abstract

Objective: To investigate whether the differences between male rats on the mating day versus pre-mating, and on the day after mating versus the mating day can be reflected through the urinary proteome. Methods: Urine samples were collected from Sprague-Dawley male rats pre-mating, on the mating day, and on the day after mating, and were analyzed using label-free quantitative proteomics technology by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Differential proteins in the urinary proteome were screened for protein functional analysis; differential post-translational modifications in the urinary proteome were screened for analysis. Results: Comparison of urinary proteomes between the mating day and pre-mating in rats identified 9 differential proteins, among which some were associated with sperm-egg binding and catecholamine neurotransmitter metabolism. Comparison of urinary proteomes between the day after mating and the mating day identified 54 differential proteins, nearly two-thirds of which were associated with spermatogenesis. Comparison of post-translational modifications in urinary proteomes between the mating day and pre-mating identified 45 differential modifications, among which some modifications were on proteins associated with the androgen receptor signaling pathway. Comparison of post-translational modifications in urinary proteomes between the day after mating and the mating day identified 53 differential modifications, among which some modifications were on proteins associated with the androgen receptor signaling pathway. Conclusion: The urinary proteome has the potential to reflect the stimulation of mating behavior on the organism and the process of spermatogenesis. Compared to studies investigating spermatogenesis through semen collection, the urinary proteome can monitor the process of spermatogenesis without interfering with it.

Full Text

Preamble

Comparison of Urine Proteome in Male Rats Before and After Mating

Haitong Wang[1], Chenyang Zhao[2], Youhe Gao[1]*

[1]Beijing Key Laboratory of Genetic Engineering Drug and Biotechnology, College of Life Sciences, Beijing Normal University, Beijing 100875, China

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Abstract

Objective: To investigate whether differences between the mating day and pre-mating, as well as between the day after mating and the mating day, can be reflected in the urine proteome.

Methods: Urine samples were collected from Sprague-Dawley male rats before mating, on the mating day, and on the day after mating. Identification was performed using label-free quantitative proteomics technology based on high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). Differential proteins in the urine proteome were screened for protein functional analysis, and differential post-translational modifications in the urine proteome were screened for analysis.

Results: Comparison of the urine proteome between the mating day and pre-mating identified 9 differential proteins, some of which were related to sperm-egg binding and catecholamine neurotransmitter metabolism. Comparison between the day after mating and the mating day identified 54 differential proteins, with nearly two-thirds related to spermatogenesis. Comparison of post-translational modifications in the urine proteome between the mating day and pre-mating revealed 45 differential modifications, with some modified proteins associated with the androgen receptor signaling pathway. Comparison between the day after mating and the mating day revealed 53 differential modifications, with some modified proteins also associated with the androgen receptor signaling pathway.

Conclusion: The urine proteome has the potential to reflect both the stimulation of the body by mating behavior and the process of spermatogenesis. Compared with studies on spermatogenesis that require semen collection, the urine proteome can monitor spermatogenesis without interfering with the process.

Keywords: Urinary proteome; Mating; Fertilization; Spermatogenesis

1 Introduction

Male mating behavior is an innate behavior controlled by neural circuits in the brain that can be performed without prior experience. These neural circuits regulate male sexual drive and reward through dopamine release [59]. Spermatogenesis is the process by which spermatogonia proliferate and differentiate into mature sperm, involving chromosome ploidy reduction and cellular morphological transformation. Paracrine, autocrine, and endocrine pathways all contribute to the regulation of this process, and the numerous structural elements and chemical factors involved create an incredibly complex network connecting various cellular activities during spermatogenesis [1]. Urine is produced by blood filtration through the kidneys to excrete metabolic waste. Unconstrained by homeostatic regulatory mechanisms, urine can more sensitively retain various subtle changes produced by the body [2]. Previous studies have shown that the urinary metabolome can be used to distinguish between normozoospermic infertile men and fertile men [3]. However, no studies have yet monitored mating behavior and spermatogenesis through the urine proteome. A single mating event in male rats not only stimulates relevant neural circuits in the brain but also consumes mature sperm, thereby stimulating spermatogenesis in the testes. In this study, we collected urine samples from rats before mating, on the mating day, and on the day after mating to conduct a comparative proteomic analysis, attempting to explore whether mating behavior and spermatogenesis processes can be reflected in the urine proteome.

2 Materials and Methods

2.1 Animals and Ethics

Ten-week-old male Sprague-Dawley rats (n=5) and ten-week-old female Sprague-Dawley rats (n=5) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All rats were housed in a standard environment (temperature (22$±$1)°C, humidity 65%-70%). The rats were acclimated to the new environment for three days before the experiment. All experimental procedures were reviewed and approved by the Animal Welfare and Ethics Committee of the College of Life Sciences, Beijing Normal University (approval number: CLS-AWEC-B-2022-003).

2.2 Experimental Procedures

2.2.1 Rat Co-housing Male and female rats were co-housed at a 1:1 ratio at 16:00. The following day at 7:00, female rats were examined for vaginal plugs. The presence of a vaginal plug was considered evidence of successful mating.

2.2.2 Urine Sample Collection Urine was collected from male rats from 20:00 to 8:00 the next day. After urine collection, male rats were co-housed with female rats. On the mating day, urine was collected from 20:00 to 8:00 the next day and temporarily stored at -80°C as the mating day urine sample. After

collection, male rats were housed individually. Urine was continuously collected from 20:00 to 8:00 on the second day and temporarily stored at -80°C as the day-after-mating urine sample.

2.2.3 Urine Sample Processing Urine Protein Extraction: Rat urine samples were removed from the -80°C freezer and thawed at 4°C. The samples were centrifuged at 4°C, 12,000×g for 30 min. Two mL of supernatant was taken, and each 500 L aliquot was placed in a 2 mL centrifuge tube. Three volumes of pre-chilled absolute ethanol were added, gently mixed by inversion, and proteins were precipitated overnight at -20°C. The overnight mixture was centrifuged at 4°C, 12,000×g for 30 min, the supernatant was discarded, and ethanol was allowed to evaporate to dryness. The protein pellet was resuspended in lysis buffer (containing 8 mol/L urea, 2 mol/L thiourea, 25 mmol/L dithiothreitol, 50 mmol/L Tris). The sample was centrifuged at 4°C, 12,000×g for 30 min, and the supernatant was transferred to a new 1.5 mL tube to obtain urinary proteins. Protein concentration was determined using the Bradford method.

Urinary Protein Digestion: One hundred g of urinary protein sample was placed in a 1.5 mL centrifuge tube, and 25 mmol/L NH4HCO3 solution was added to a total volume of 200 L. Twenty mM dithiothreitol solution (DTT, Sigma) was added, vortexed, and heated in a metal bath at 97°C for 10 min, then cooled to room temperature. Fifty mM iodoacetamide (IAA, Sigma) was added, vortexed, and reacted in the dark at room temperature for 40 min. A 10 kDa ultrafiltration tube (Pall, Port Washington, NY, USA) was used. Two hundred L of UA solution (8 mol/L urea, 0.1 mol/L Tris-HCl, pH 8.5) was added to the filter membrane and centrifuged at 18°C, 14,000×g for 5 min. The lower filtrate was discarded, and the wash was repeated once. The iodoacetamide-treated urinary protein sample was added to the filter membrane and centrifuged at 18°C, 14,000×g for 30 min. The lower filtrate was discarded, leaving the urinary proteins on the filter membrane. Two hundred L of UA solution was added to wash the urinary proteins and centrifuged at 18°C, 14,000×g for 30 min, repeated twice. Twenty-five mmol/L NH4HCO3 solution was added to wash the urinary proteins and centrifuged at 18°C, 14,000×g for 30 min, repeated twice. Trypsin (Trypsin Gold, Promega, Fitchburg, WI, USA) was added at a 1:50 enzyme-to-protein ratio for digestion, and the sample was incubated in a water bath at 37°C for 15 h. After digestion, the filtrate was collected by centrifugation at 4°C, 13,000×g for 30 min. This filtrate was the peptide mixture. The peptide mixture was desalted using an HLB solid-phase extraction column (Waters, Milford, MA), lyophilized using a vacuum dryer, and stored at -20°C.

2.2.4 LC-MS/MS Analysis The lyophilized peptide mixture was dissolved in 0.1% formic acid. Peptide concentration was quantified using a BCA assay kit and diluted to 0.5 g/ L. Six L of each sample was mixed, and separation was performed using a high pH reverse-phase peptide fractionation kit (Thermo Fisher Scientific). Ten fractions of eluate were collected by centrifugation, lyophilized

using a vacuum dryer, and reconstituted in 0.1% formic acid. The ten fractions and all individual samples were spiked with iRT reagent (Biognosys, Switzerland) at a sample:iRT volume ratio of 10:1 to calibrate the retention time of extracted peptide peaks.

The ten fractions were separated using an EASY-nLC 1200 chromatography system (Thermo Fisher Scientific, USA). The separated peptides were analyzed by an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, USA) in Data Dependent Acquisition (DDA) mode to generate ten raw files. These were imported into Proteome Discoverer software (version 2.0, Thermo Scientific) for library construction using Swiss-iRT and Uniprot-Rat databases. Based on the library construction results, a DIA method with 39 variable windows was established for individual sample Data Independent Acquisition (DIA) mode. One g of peptides from individual samples was separated using an EASY-nLC 1200 chromatography system (Thermo Fisher Scientific, USA). The separated peptides were analyzed by an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, USA) in DIA mode using the newly established DIA method to generate raw files.

2.2.5 Label-free DIA Quantitative Analysis Individual sample raw files collected in DIA mode were analyzed using Spectronaut Pulsar (Biognosys AG, Switzerland) software. Peptide abundance was calculated by summing the peak areas of each fragment ion in MS2. Protein abundance was calculated by summing the abundance of its constituent peptides. Each sample was analyzed with three technical replicates.

2.2.6 Open-pFind Unrestricted Modification Search pFind Studio software (version 3.2.1, Institute of Computing Technology, Chinese Academy of Sciences) was used for unrestricted modification searching of three technical replicates per sample using default parameter settings. The database was the Rattus norvegicus database downloaded from UniProt (updated to September 2024). Instrument type was HCD-FTMS, trypsin with full enzyme specificity, and a maximum of two missed cleavage sites. Precursor mass tolerance was $±$20 ppm, fragment mass tolerance was $±$20 ppm, and open search was selected. The filtering criterion was a false discovery rate (FDR) <1% at the peptide level.

2.2.7 Protein Data Analysis For each sample, three technical replicates were averaged for statistical analysis. This experiment performed before-and-after comparisons. The mating day was compared with pre-mating to screen for differential proteins. Differential protein screening criteria were: fold change (FC) between groups $ $1.5 or $ $0.67, and P-value <0.05 from two-tailed paired t-test analysis. The day after mating was compared with the mating day to screen for differential proteins using the same criteria. Identified differential proteins were analyzed through the Uniprot website

(https://www.uniprot.org/) and relevant literature was searched in the Pubmed database (https://pubmed.ncbi.nlm.nih.gov) for functional analysis.

2.2.8 Protein Post-translational Modification Data Analysis After Open-pFind unrestricted modification searching, PROTEIN files containing post-translational modifications for each sample were obtained. A Python script (pFind_{{{protein}}*{{contrast}}}*{script}) was downloaded from the GitHub platform (https://github.com/daheitu/scripts_{{{for}}*{{pFind3}}}*{protocol}.io) to summarize modification identification results across different samples, i.e., PROTEIN files [60]. The mating day was compared with pre-mating to screen for differential modifications. Screening criteria were: fold change (FC) between groups $ $1.5 or $ $0.67, and P-value <0.05 from two-tailed paired t-test analysis. The day after mating was compared with the mating day to screen for differential modifications using the same criteria. Proteins containing differential modifications were analyzed through Uniprot.

3 Results

3.1 Comparison of Urine Proteome Between Mating Day and Pre-mating

3.1.1 Differential Proteins The mating day urine proteome was compared with pre-mating to screen for differential proteins using the criteria: FC$ $1.5 or $ $0.67, and two-tailed paired t-test P<0.05. The results showed that 9 differential proteins could be identified between mating day and pre-mating. These differential proteins were sorted by FC value from largest to smallest and searched through Uniprot, with results shown in Table 1 .

3.1.2 Functional Analysis of Differential Proteins The 9 identified differential proteins were searched for their functions and biological processes through Uniprot. Some proteins were found to be related to fertilization and neurotransmitter metabolism.

Testis-expressed protein 101 functions in fertilization by controlling sperm binding to the zona pellucida and sperm migration to the oviduct, participating in biological processes such as sperm-zona pellucida binding, fertilization, and flagellated sperm motility.

Catechol O-methyltransferase catalyzes the O-methylation of catecholamine neurotransmitters and catechol hormones, thereby inactivating them, and shortens the biological half-life of certain neuroactive drugs (such as L-dopa, alpha-methyldopa, and isoproterenol). Catecholamine neurotransmitters are a class of neurotransmitters with important physiological functions in humans, mainly including norepinephrine, epinephrine, and dopamine. During mating, the brain releases dopamine, producing feelings of pleasure and satisfaction.

3.2 Comparison of Urine Proteome Between Day After Mating and Mating Day

3.2.1 Differential Proteins The day-after-mating urine proteome was compared with the mating day to screen for differential proteins using the criteria: FC$ $1.5 or $ $0.67, and two-tailed paired t-test P<0.05. The results showed that 54 differential proteins could be identified between the day after mating and the mating day. These differential proteins were sorted by FC value from largest to smallest and searched through Uniprot, with results shown in Table 2 .

3.2.2 Functional Analysis of Differential Proteins The 54 identified differential proteins were searched in the PubMed database, revealing that 33 proteins or other members of their families have been reported to be associated with spermatogenesis.

Monoglyceride lipase is highly expressed in the testis and, as part of the endocannabinoid system, directly participates in regulating human testicular physiology, including spermatogenesis and Leydig cell function [7].

Vacuolar protein-sorting-associated protein 25 belongs to the same family as Vacuolar protein-sorting-associated protein 33B. Mutations in the latter cause infertility in Caenorhabditis elegans with arrested spermatocytes, indicating involvement in the formation of sperm-specific organelles [8].

The substrate of Ribosomal protein S6 kinase alpha-1, Ribosomal protein S6, is essential for spermatogenesis. Knockdown of this protein in Chinese sturgeon causes spermatogenesis defects, including germ cell loss, retention of mature sperm, and lumen formation [9]. This protein also regulates Sertoli cell blood-testis barrier dynamics through Akt1/2, thereby regulating F-actin organization, adhesion function at intercellular interfaces, and promoting preleptotene spermatocyte transport across the blood-testis barrier during rat spermatogenesis [10].

Glycerol kinase 2, which is highly homologous to Glycerol kinase, is essential for the correct arrangement of crescent-like mitochondria to form the mitochondrial sheath during mouse spermatogenesis. Knockout of this gene causes disorganization of the mitochondrial sheath in sperm flagella [11].

Actin is involved in various aspects of spermatogenesis, with active remodeling of the actin cytoskeleton during this process, participating in sperm cell shaping and differentiation [12,13]. However, the molecular mechanisms by which actin cytoskeleton organization responds to spermatogenesis in seminiferous 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 ram reproductive organs. This protein is highly expressed in the epididymis, suggesting it may affect epididymal luminal fluid composition and thus the microenvironment for sperm maturation,

potentially playing an important role in spermatogenesis and testicular and epididymal development in the ram reproductive system [15]. Guanine nucleotide-binding protein G(o) subunit alpha, belonging to the same family, is highly expressed in rat pachytene spermatocytes, suggesting its potential role during this stage of spermatogenesis [16].

Syntaxin-binding protein 2 in Sertoli cells regulates spermatogonial stem cell maintenance by directly interacting with connexin 43 in neonatal mouse testes. Interactions between Sertoli cells 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 spermatids. As spermatocytes develop into spermatids, Guanine nucleotide-binding protein G(o) subunit alpha levels decrease [18]. The association of Guanine nucleotide-binding protein G(i) subunit with the developing acrosome suggests its role in acrosome biogenesis. Guanine nucleotide-binding protein G(i) subunit is present in the acrosomal region of mammalian sperm and is part of the complex required for signal transduction leading to acrosomal exocytosis [18,19].

Deoxyribonuclease-1 is associated with apoptosis regulation. The seminal plasma level of this protein is important for proper spermatogenesis. Compared with adolescents without varicocele, adolescents with varicocele have decreased Deoxyribonuclease-1 levels, which positively correlate with sperm concentration and morphology. Furthermore, Deoxyribonuclease-1 can distinguish varicocele that causes altered semen quality from varicocele that does not [20].

V-type proton ATPases play an important role in rabbit sperm capacitation [21]. Rat round spermatocytes regulate intracellular pH through V-type proton ATPases, HCO3–dependent transport systems, and putative proton-conducting pathways. These pH regulatory mechanisms appear specifically designed to withstand acid challenges [22].

Chloride intracellular channel proteins are present in bovine epididymal sperm. Chloride intracellular channel protein 1, Chloride intracellular channel protein 4, and Chloride intracellular channel protein 5 are all present in sperm and occupy different intracellular locations. They can all bind to PP1$ 2$in sperm. Given that PP1 2$is a key enzyme regulating sperm motility, Chloride intracellular channel protei
binding proteins, may play important roles in sperm function [23].

Haptoglobin is an iron transport protein expressed in Sertoli cells, Leydig cells, and germ cells in rat testes, but not in the epididymis, and may play an important role in testicular iron metabolism. Testicular Haptoglobin mRNA levels steadily increase during postnatal maturation, suggesting its involvement in spermatogenesis [24]. Sertoli cells play a critical role in spermatogenesis, expressing receptors for the major hormonal regulators of spermatogenesis, follicle-stimulating hormone (FSH) and testosterone (T). After FSH stimula-

tion of porcine Sertoli cells, inhibin-α, inhibin-β, plakoglobin, haptoglobin, D-3-phosphoglycerate dehydrogenase, and sodium/potassium-transporting ATPase increase in Sertoli cell extracellular vesicles [25].

In both in vitro and in vivo mouse experiments, deletion of 14-3-3 protein gamma leads to reduced desmosome formation and decreased cell-cell adhesion, resulting in testicular tissue and spermatogenesis defects [26].

During spermatogenesis, the stable F-actin cone contains Tropomyosin 1, and failure of F-actin cone formation is associated with failure of Tropomyosin 1 accumulation at cone initiation sites [27].

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

Many processes in spermatogenesis depend on cytoskeletal dynamics and organelle movement, particularly microtubule regulation. Data from transgenic mouse models demonstrate that coordination of microtubule dynamics is essential 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), Intermitochondrial Cement (IMC), Satellite Body (SB), and Chromatoid Body (CB). ISPG, IMC, and SB are observed in pachytene spermatocytes, while CB is observed in round spermatids. In rat round spermatids, Tubulin beta is translated from mRNA stored in CB and assembles with Tubulin alpha outside CB to form the structural unit of microtubules, the $\alpha\beta$-heterodimer, to build microtubules in sperm flagella [30].

In Drosophila, Eukaryotic translation initiation factor 4E-5 is essential for male fertility. Eukaryotic translation initiation factor 4E-5 localizes to the distal end of elongated spermatid cysts. Mutants of Eukaryotic translation initiation factor 4E-5 show defects in post-meiotic stages, including mild defects in spermatid cyst polarization. Eukaryotic translation initiation factor 4E-5 mutants also have fully penetrant defects in sperm individualization, resulting in failure to produce mature sperm [31].

Human testicular peritubular cells transport sperm and contribute to the spermatogonial stem cell niche and immune surveillance. Secretome analysis revealed that human testicular peritubular cells undergo replicative senescence with elevated Dipeptidyl peptidase 4 levels, which may play a role in spermatogenesis. Testicular Dipeptidyl peptidase 4 may further represent a potential drug target [32]. Dipeptidyl peptidase 4 inhibitors are a new class of anti-diabetic compounds that affect spermatogenesis during clinical use, causing rapid deterioration of semen quality in patients [33].

Rab GDP dissociation inhibitor beta belongs to the same family as Rab GDP dissociation inhibitor alpha, which participates in actin cytoskeleton organization and regulates cell morphology and motility. Expression of this protein is

decreased in asthenozoospermic patients [34].

Aldo-keto reductase family 1 member A1 belongs to a family whose members have increased ability to metabolize specific steroids during testicular development, such as increased expression of Aldo-keto reductase family 1 member C3 during domestic cat testicular development [35]. Aldo-Keto Reductase mRNA levels are higher in silkworm testes than in other tissues, playing an important role in silkworm spermatogenesis [36].

Pachytene checkpoint protein 2 homolog is expressed in male germ cell nuclei and participates in biological processes including spermatid development, spermiogenesis, male meiosis, synaptonemal complex, meiotic recombination, double-strand break repair, and meiotic recombination checkpoint signaling. It plays a critical role in chromosome recombination and chromosomal structure development during meiosis. It mediates the non-crossover pathway in early meiotic recombination and is required for efficient homologous chromosome synapsis by affecting both crossover and non-crossover pathways, essential for effective sex chromosome synapsis. Mouse Pachytene checkpoint protein 2 is required for recombination and normal higher-order chromosome structure during meiosis. It plays a potential role in non-crossover repair of double-strand breaks during meiosis. Male mice with homozygous mutations in this gene show complete chromosome synapsis in spermatocytes, but due to defects in double-strand break repair caused by the deletion, cells die in the pachytene stage, and testicular tissue lacks post-meiotic cells [37,38]. In humans, Pachytene checkpoint protein 2 is required for sex chromosome synapsis and sex body (the transcriptionally silent subnuclear domain formed by X and Y chromosomes) formation [38].

Acute heat stress impairs translation, protein folding, and protein degradation processes in chicken testes, leading to apoptosis and interfering with spermatogenesis. After acute heat stress, Transgelin is upregulated in testes to resist heat-induced damage [39]. Expression levels of Transgelin gene are lower in testicular tissues of older animals [40].

Phospholipase D6 is a Golgi-localized protein in pachytene spermatocytes and developing spermatids, and its specific distribution in the Golgi may be related to the specific functions of this organelle during spermatogenesis [41]. Phospholipase D isozymes are involved in spermatogenesis in mouse testes [42].

Sex hormone-binding globulin functions as an androgen transport protein, with each dimer binding one steroid molecule, specifically targeting 5-dihydrotestosterone, testosterone, and 17-estradiol. It regulates plasma metabolic clearance rate of steroid hormones by controlling their plasma concentration and participates in biological processes of primary spermatocyte growth. Canine testicular Sex hormone-binding globulin expression positively correlates with sperm concentration, total and progressive motility, plasma membrane integrity, and acrosome integrity, and negatively correlates with low sperm mitochondrial activity. In the epididymis, Sex hormone-binding globulin expression only positively correlates with sperm plasma membrane

integrity. Expression of Sex hormone-binding globulin in testes and epididymis is associated with morphologically normal cells. Sex hormone-binding globulin plays a critical role in spermatogenesis and sperm maturation, essential for male reproductive success [43]. The cauda epididymis is the main storage area for rat sperm, and its androgen supply is essential for sperm survival, provided by the vasculature and dependent on testosterone diffusion through stromal tissue to epithelial cells. Stromal Sex hormone-binding globulin plays a role in androgen supply to the sperm storage region of the epididymis [44]. Sperm Sex hormone-binding globulin isoform levels significantly correlate with age and sperm motility and may affect sperm functions related to male fertility [45].

14-3-3 protein plays key regulatory roles 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. Proteins identified to be involved in adhesion between Sertoli cells and mature spermatids include 14-3-3 protein zeta/delta, which appears only in tubule segment lysates during sperm release. However, its exact role in spermatogenesis and how it interacts with or affects other signal transduction pathways in the testis remain unknown [47].

Peroxiredoxin-2 has antioxidant properties and may be involved in maintaining oxidative balance in the mouse spermatogenesis environment [48]. Peroxiredoxin-2 can also maintain normal development of neonatal rat germ cells [49].

Peptidyl-prolyl cis-trans isomerase A is upregulated in mouse testes treated with environmental estrogens, which reduce sperm count and cause male infertility. However, the molecular mechanisms of its effect on male infertility remain unclear [50].

Nucleoside diphosphate kinase B is distributed in the manchette microtubule structure of sperm (a transient microtubule structure in elongated spermatids that plays an important role in nuclear condensation and sperm tail formation). Nucleoside diphosphate kinase A is transiently distributed in round spermatid nuclei and asymmetrically in the cytoplasm at the nuclear base of elongated spermatids. Nucleoside diphosphate kinase isoforms may have specific functions in the phosphate transfer network of human spermatogenesis and flagellar motility [51]. Nucleoside diphosphate kinase plays a critical role in spermatogenesis by increasing levels of the intracellular antioxidant enzyme glutathione peroxidase 5 to eliminate reactive oxygen species in mice [52].

The gene encoding Lysosome-associated membrane protein-2, LAMP2, is a target of Fork head box J2. Overexpression of Fork head box J2 in mouse testicular germ cells affects chaperone-mediated autophagy by upregulating lysosome-associated membrane protein 2A, leading to spermatogenesis failure at the onset of meiosis and resulting in male infertility [53].

Lysosome-associated membrane protein-1 is expressed in later stages of spermatogenesis (acrosomal phase), while Lysosome-associated membrane protein-2

is expressed throughout the entire spermatogenesis process [54].

Somatic angiotensin-converting enzyme plays an important role in spermatogonial stem cell self-renewal by regulating 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. Sperm from mice lacking testicular angiotensin-converting enzyme activity have 9.4-fold lower ATP than normal mouse sperm. ACE inhibitors can also reduce ATP production in mouse sperm by 72%. Inactivation of tACE severely affects oxidative metabolism, with sperm showing lower levels of oxidative enzymes, leading to reduced mitochondrial respiration rates. Reduced energy production in sperm from mice lacking testicular angiotensin-converting enzyme activity leads to physiological functional defects, including motility, acrosomal enzyme activity, and in vivo and in vitro fertilization capacity [56].

Natural down-regulation of Tspan8 in pubertal Sertoli cells is a prerequisite for establishing male fertility. Specifically preventing this natural down-regulation of Tspan8 in Sertoli cells from puberty to adulthood reduces sperm count by approximately 98% in adult male rats [57].

Combined treatment with injectable testosterone undecanoate and oral levonorgestrel enhances Parvalbumin alpha expression and inhibits spermatogenesis. Parvalbumin alpha can protect testicular cells from apoptosis and promote cell survival, and may be an early molecular target for hormone-induced spermatogenesis inhibition [58].

3.3 Comparison of Post-translational Modifications in Urine Proteome Between Mating Day and Pre-mating

Post-translational modifications in the urine proteome between mating day and pre-mating were compared using screening criteria: FC$ $1.5 or $ $0.67, and two-tailed paired t-test P<0.05. The results showed that 45 differential modifications could be identified between mating day and pre-mating, sorted by FC value from largest to smallest as shown in Table 3 . Except for P02625, the proteins containing these differential modifications showed no differences between mating day and pre-mating in rats. GO analysis in Uniprot revealed that some proteins with differential modifications (P02780, Q9JHB9, and P02782) are related to steroid binding and androgen receptor signaling pathway; Q03626 is related to embryo implantation; P98158 is related to male gonad development; P05371 is related to spermatogenesis; P48199 is related to response to testosterone. Additionally, some proteins are related to neuron development regulation and synaptic transmission. Detailed GO analysis results are provided in Supplementary File 1.

3.4 Comparison of Post-translational Modifications in Urine Proteome Between Day After Mating and Mating Day

Post-translational modifications in the urine proteome between the day after mating and mating day were compared using screening criteria: FC$ $1.5 or $ $0.67, and two-tailed paired t-test P<0.05. The results showed that 53 differential modifications could be identified between the day after mating and mating day, sorted by FC value from largest to smallest as shown in Table 4 . Except for P02625 and Q642A7, the proteins containing these differential modifications showed no differences between the day after mating and mating day in rats. GO analysis in Uniprot revealed that some proteins with differential modifications (P48199) are related to response to estradiol and testosterone; P02780 and Q9JHB9 are related to androgen receptor signaling pathway; P12346 is related to cellular response to follicle-stimulating hormone stimulus; P13635 is related to female pregnancy; P98158 is related to male gonad development. Additionally, a few proteins are related to neuron development regulation. Detailed GO analysis results are provided in Supplementary File 2.

4 Discussion

This experiment used rat self-control with only a one-day interval between the two urine collections, minimizing interference from individual differences and self-growth and development on the results. Therefore, despite the small sample size, our results can preliminarily indicate that differences exist in the urine proteome between mating day and pre-mating in rats, and these differences are related to fertilization and euphoric response. The urine proteome shows significant differences between mating day and the day after mating, with most differential proteins related to spermatogenesis. Although the remaining differential proteins have not yet been found to be associated with spermatogenesis in databases, our results suggest that these proteins may still be related to spermatogenesis and can be further studied as target proteins for spermatogenesis. Compared with the urine proteome itself, changes in protein post-translational modifications are more significant, providing a new window for developing more accurate and sensitive urinary detection methods for body status monitoring and early screening of various diseases. In-depth exploration of urine proteome post-translational modifications has important scientific significance and clinical prospects.

This study demonstrates the potential of urine proteomics in investigating mating behavior and spermatogenesis processes, providing a urinary proteomics approach for exploring pathogenic mechanisms, target discovery, and new diagnostic methods for male infertility caused by abnormal spermatogenesis. Our results indicate that semen-based spermatogenesis studies actually interfere with the spermatogenesis process during semen collection, whereas the urinary proteomics method used in this study can be performed without affecting spermatogenesis. Future experiments could consider expanding the sample size of experimental animals or collecting clinical samples for research. This also reflects

the sensitivity of the urinary proteome, opening new avenues for urine-based exploration.

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