Comparison of Urinary Proteome in Male Rats During the First Two Days After Mating

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Abstract

Objective: To compare changes in the urine proteome of male rats during the first two days following mating behavior.

Methods: Urine samples were collected from Sprague-Dawley rats on the day of mating and the day after mating, and analyzed using label-free quantitative proteomics technology with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Differential proteins in the urine proteome were screened (FC>1.5 or <0.67, P<0.050) for protein function and biological pathway analysis.

Results: Comparison of the urine proteome between the day after mating and the day of mating in rats identified 43 differential proteins. Retrieval of differential proteins and related literature reports through the UniProt database and PubMed database revealed that nearly two-thirds of the differential proteins were associated with spermatogenesis.

Conclusion: The urine proteome changed on the day after mating compared to the day of mating in rats, and the known functions of some altered proteins were related to spermatogenesis.

Full Text

Comparison of Urinary Proteome in the First Two Days after Mating in Male Rats

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Objective: To compare changes in the urinary proteome of male rats during the first two days following mating behavior.

Methods: Urine samples were collected from Sprague-Dawley rats on the day of mating and the day after mating. Samples were analyzed using label-free quantitative proteomics via high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). Differential proteins in the urinary proteome were screened (fold change > 1.5 or < 0.67, $P < 0.05$) and subjected to protein function and biological pathway analysis.

Results: Comparison of urinary proteomes between the day after mating and the day of mating identified 43 differential proteins. Database searches of Uniprot and Pubmed revealed that nearly two-thirds of these differential proteins were associated with spermatogenesis.

Conclusions: The urinary proteome exhibited changes the day after mating compared to the day of mating, with known functions of some altered proteins related to spermatogenesis.

Keywords: urine proteome; mating; spermatogenesis

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Abstract

Objective: To compare urinary proteome changes in male rats during the first two days after mating behavior.

Methods: Urine samples from Sprague-Dawley rats were collected on the day of mating and the subsequent day, then analyzed using label-free quantitative proteomics by high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). Differential proteins (fold change > 1.5 or < 0.67, $P < 0.05$) were screened and analyzed for protein function and biological pathways.

Results: Forty-three differential proteins were identified when comparing the urinary proteome of the day after mating with that of the mating day. Database searches of Uniprot and Pubmed revealed that nearly two-thirds of these differential proteins were associated with spermatogenesis.

Conclusions: The urinary proteome changed significantly the day after mating compared to the mating day, with some altered proteins having known functions related to spermatogenesis.

Keywords: urine proteome; mating; spermatogenesis

1. Introduction

Spermatogenesis is the process by which spermatogonia undergo proliferation and differentiation to form mature sperm, involving chromosome ploidy reduction and cellular morphological transformation. Mammalian spermatogenesis comprises three stages: first, spermatogonia undergo a series of mitotic divisions to form primary spermatocytes; second, primary spermatocytes undergo meiosis to produce haploid round spermatids; and third, round spermatids undergo morphological transformation to become flagellated sperm. Paracrine, autocrine, and endocrine pathways all contribute to the regulation of this process, and the numerous structural components and chemical factors involved create an extraordinarily complex network connecting various cellular activities during spermatogenesis [1]. Urine is produced by blood filtration through the kidneys to eliminate metabolic waste. Unlike blood, it is not subject to homeostatic regulatory mechanisms, allowing it to more sensitively retain various subtle changes produced by the body [2]. Previous studies have shown that urinary metabolomics can be used to differentiate between normozoospermic infertile men and fertile men [3]. However, no studies have yet monitored spermatogenesis through urinary proteomics. After a single mating event in male rats, mature sperm are depleted, stimulating spermatogenesis in the testes. This study collected urine samples from rats after mating and on the mating day to conduct a comparative proteomic analysis, exploring whether the spermatogenesis process can be reflected in the urinary proteome.

2. Materials and Methods

2.1 Experimental Animals

Five 10-week-old male Sprague-Dawley rats and five 10-week-old female Sprague-Dawley rats were purchased from Beijing Huaweitong Lihua Experimental Animal Biotechnology Co., Ltd. All rats were housed in a standard environment (room temperature (22 $\pm$ 1)°C, humidity 65%-70%). Experiments began after three days of acclimation to the new environment. All experimental procedures followed the review and approval of the Animal 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 Mating 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 indicated successful mating.

2.2.2 Urine Sample Collection Urine was collected from male rats from 20:00 on the mating day to 8:00 the next morning and stored temporarily at -80°C as the mating day urine sample. After urine collection, male rats were housed individually. From 20:00 to 8:00 on the second day, urine was again collected and stored at -80°C as the day-after-mating urine sample.

2.2.3 Urine Sample Processing Urinary Protein Extraction: Frozen rat urine samples were thawed at 4°C, then centrifuged at 12,000 × g for 30 min at 4°C. Two milliliters of supernatant were transferred to 2 mL tubes (500 L per tube), and three volumes of pre-cooled absolute ethanol were added. Samples were gently mixed by inversion and precipitated overnight at -20°C. The overnight mixture was centrifuged at 12,000 × g for 30 min at 4°C, the supernatant was discarded, and ethanol was allowed to evaporate to dryness. Protein pellets were resuspended in lysis buffer (containing 8 mol/L urea, 2 mol/L thiourea, 25 mmol/L dithiothreitol, 50 mmol/L Tris), centrifuged at 12,000 × g for 30 min at 4°C, and the supernatant was transferred to new 1.5 mL tubes to obtain urinary proteins. Protein concentration was determined using the Bradford method.

Urinary Protein Digestion: One hundred micrograms of urinary protein sample were transferred to a 1.5 mL tube, and 25 mmol/L NH_4HCO_3 solution was added to a total volume of 200 L. Twenty millimolar dithiothreitol solution (DTT, Sigma) was added, vortexed, heated in a metal bath at 97°C for 10 min, and cooled to room temperature. Fifty millimolar iodoacetamide (IAA, Sigma) was added, vortexed, and reacted at room temperature in the dark for 40 min. Ten-kilodalton ultrafiltration tubes (Pall, Port Washington, NY, USA) were prepared by adding 200 L UA solution (8 mol/L urea, 0.1 mol/L Tris-HCl, pH 8.5) to the membrane and centrifuging at 14,000 × g for 5 min at 18°C; the filtrate was discarded and the wash repeated. IAA-treated urinary protein samples were added to the membrane and centrifuged at 14,000 × g for 30 min at 18°C; the filtrate was discarded, leaving urinary proteins on the membrane. The membrane was washed with 200 L UA solution at 14,000 × g for 30 min at 18°C, repeated twice, then washed with 25 mmol/L NH_4HCO_3 solution at 14,000 × g for 30 min at 18°C, repeated twice. Trypsin (Trypsin Gold, Promega, Fitchburg, WI, USA) was added at a 1:50 enzyme-to-protein ratio and digested at 37°C for 15 h. After digestion, the filtrate was collected by centrifugation at 13,000 × g for 30 min at 4°C as the peptide mixture. Peptides were desalted using HLB solid-phase extraction columns (Waters, Milford, MA), lyophilized using a vacuum dryer, and stored at -20°C.

2.2.4 LC-MS/MS Tandem Mass Spectrometry Analysis Lyophilized peptide mixtures were dissolved in 0.1% formic acid, quantified using a BCA kit, and diluted to 0.5 g/ L. Six microliters of each sample were pooled and separated using a high pH reverse-phase peptide fractionation kit (Thermo Fisher Scientific). Ten fractions were collected by centrifugation, lyophilized, and reconstituted in 0.1% formic acid. iRT reagent (Biognosys, Switzerland) was

added to the ten fractions and all individual samples at a 10:1 sample-to-iRT volume ratio to calibrate extracted peptide peak retention times.

The ten fractions were separated using an EASY-nLC1200 chromatography system (Thermo Fisher Scientific, USA), and the separated peptides were analyzed using 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 for library construction using Swiss-iRT and Uniprot-mouse databases (version 2.0, Thermo Scientific). Based on the library results, 39 variable windows were established for DIA methods for individual samples. One microgram of peptides from individual samples was separated using the EASY-nLC1200 system and analyzed by the Orbitrap Fusion Lumos Tribrid mass spectrometer in Data Independent Acquisition (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 imported into Spectronaut Pulsar (Biognosys AG, Switzerland) software for analysis. Peptide abundance was calculated by summing the peak areas of fragment ions in MS2. Protein abundance was calculated by summing the abundances of constituent peptides.

2.2.6 Data Analysis Each sample underwent three technical replicates, and average values were used for statistical analysis. This study performed a before-and-after comparison between mating day and day-after-mating samples to screen for differential proteins. Screening criteria were: intergroup fold change (FC) ≥ 1.5 or ≤ 0.67, and two-tailed unpaired t-test P-value < 0.05. Identified differential proteins were analyzed using 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.

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3. Results

3.2.1 Differential Proteins

Comparing urinary proteins between the day after mating and the mating day, differential proteins were screened using criteria of FC ≥ 1.5 or ≤ 0.67 and two-tailed unpaired t-test $P < 0.05$. The results showed that 43 differential proteins could be identified between the day after mating and the mating day. First, the STRING database was used to analyze protein-protein interactions among the identified differential proteins, with results shown in [Figure 1: see original paper]. Next, differential proteins were sorted by FC magnitude and retrieved through Uniprot, with results presented in .

3.2.2 Functional Analysis of Differential Proteins

Literature searches of the 43 identified differential proteins in the PubMed database revealed that 26 proteins or other members of their families have been reported to be associated with spermatogenesis.

Monoglyceride lipase is highly expressed in the testis and directly participates in regulating human testicular physiology, including spermatogenesis and Leydig cell function, as part of the endocannabinoid system [4]. Vacuolar protein-sorting-associated protein 33B mutation in Caenorhabditis elegans causes infertility with arrested spermatocytes, indicating that this protein participates in the formation of sperm-specific organelles [5]. 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 cavity formation [6]. This protein also regulates Sertoli cell blood-testis barrier dynamics through Akt1/2, thereby regulating F-actin organization and adhesion function at cell interfaces to promote preleptotene spermatocyte transit across the blood-testis barrier during rat spermatogenesis [7]. Glutathione S-transferase plays an important role in spermatogenesis and normal sperm function [8], and null genotype of Glutathione S-transferase theta-1 is associated with spermatogenic failure and may contribute to susceptibility to spermatogenic dysfunction and male infertility in Chinese populations [9]. Glycerol kinase 2, which shares high homology with Glycerol kinase, is crucial for proper crescent-like mitochondrial arrangement to form the mitochondrial sheath during mouse spermatogenesis, and knockout of this gene causes disorganized mitochondrial sheaths in sperm flagella [10]. Actin is involved in various aspects of spermatogenesis, with active remodeling of the actin cytoskeleton occurring during sperm cell shaping and differentiation [11,12]. However, the molecular mechanisms by which actin cytoskeleton organization responds to spermatogenesis in germinal epithelial cells remain largely unexplored [13].

Prostasomes, enzyme-containing vesicles secreted by the prostate, fuse with sperm at neutral or slightly acidic pH, transferring certain molecules to sperm. While dipeptidyl peptidase IV enzyme activity is not inherently present in sperm, this enzyme is transferred from prostasomes to sperm, conferring new membrane-bound enzymatic activity and altering surface catalytic activity [14]. Guinea pig sperm contain Dipeptidyl peptidase II, which is restricted to a compartment within the sperm acrosome [15]. During spermatogenesis, spermatogonia undergo mitosis and meiosis to form bundles of spermatids connected by cytoplasmic bridges, which individualize to form single spermatids. The individualization process involves formation of a cytoskeletal protein and membrane complex around the spermatid nucleus called the individualization complex, which divides the shared membrane into individual membranes surrounding each spermatid. The 95F myosin in Unconventional myosin is a component of the individualization complex, participating in membrane reorganization during individualization, and its function is essential for this process; partial loss-of-function mutations cause male infertility [16].

In ram reproductive organs, Guanine nucleotide-binding protein G(s) subunit alpha is expressed in a tissue-specific and age-dependent manner, with high expression in the epididymis, suggesting it may affect epididymal luminal fluid composition and thus the microenvironment for sperm maturation, playing an important role in spermatogenesis and development of the testis and epididymis in the ram reproductive system [17]. Rat Sertoli cells lack Guanine nucleotide-binding protein G(o) subunit alpha, while this protein is highly expressed in pachytene spermatocytes, suggesting it may function at this stage of spermatogenesis [18]. Guanine nucleotide-binding protein G(i) subunit alpha-1, alpha-2, alpha-3, and Guanine nucleotide-binding protein G(o) subunit alpha have been detected in mouse spermatocytes and spermatids. As spermatocytes develop into spermatids, Guanine nucleotide-binding protein G(o) subunit alpha levels decrease [19]. The association of Guanine nucleotide-binding protein G(i) subunit with the developing acrosome suggests a 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 [19,20].

V-type proton ATPases play an important role in rabbit sperm capacitation [21]. Rat round spermatids regulate intracellular pH through HCO_3^--dependent transport systems and a putative proton-conducting pathway, V-type proton ATPases. These pH regulatory mechanisms appear specifically designed to withstand acid challenges [22]. Chloride intracellular channel proteins exist in bovine epididymal sperm. Chloride intracellular channel protein 1, 4, and 5 are all present in sperm and occupy different intracellular locations. They can all bind to PP1$ $2in sperm, and given that PP1$ $2is a key enzyme regulating sperm motility, Chloride intracellular channel pr binding proteins [23].

The N-myc downstream-regulated gene (NDRG) family consists of four members: NDRG-1, NDRG-2, NDRG-3, and NDRG-4. Ndrg3 is a critical gene causing homozygous lethality in early embryonic development and regulates male meiosis in mice. NDRG3 expression is specifically enhanced in germ cells and peaks in pachytene spermatocytes. In Ndrg3-deficient germ cells, ERK activation is attenuated, and double-strand break repair and synaptonemal complex formation during meiosis are impaired [24]. Haptoglobin is an iron transport protein expressed in Sertoli cells, Leydig cells, and germ cells of rat testis, 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 [25]. Sertoli cells play a critical role in spermatogenesis, expressing receptors for the major hormonal regulators follicle-stimulating hormone (FSH) and testosterone (T). After FSH stimulation 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 [26].

Transferrin receptor protein was identified in the proteome of mouse neonatal

testis, indicating its involvement in meiosis. Transferrin receptor protein is essential for progression of spermatocyte meiosis, particularly for DNA double-strand break repair and chromosome synapsis [27]. Transferrin receptor protein is found only in human spermatocytes and early spermatids. In patients with spermatogenic diseases, Transferrin is always present in Sertoli cells, while Transferrin receptor protein is only detected when spermatocytes are present. In human seminiferous tubules, Sertoli cells are devoted to Transferrin production and storage, while spermatocytes and early spermatids utilize Transferrin [28].

Many processes in spermatogenesis depend on cytoskeletal dynamics, organelle movement, and particularly microtubule regulation. Data from transgenic mouse models indicate 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, β-tubulin 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 construct microtubules in the sperm flagellum [30].

The Rab GDP dissociation inhibitor family includes Rab GDP dissociation inhibitor alpha, which participates in actin cytoskeleton organization and regulates cell morphology and motility; its expression is decreased in asthenozoospermic patients [31]. The ability to metabolize specific steroids increases during testicular development, such as elevated expression of Aldo-keto reductase family 1 member C3 during domestic cat testis development [32]. Aldo-Keto Reductase mRNA levels are higher in silkworm testis than in other tissues, playing an important role in silkworm spermatogenesis [33].

Pachytene checkpoint protein 2 homolog, according to Uniprot database searches, is expressed in male germ cell nuclei and participates in biological processes including sperm 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 structural development during meiosis, mediating the non-crossover pathway in early meiotic recombination and efficiently completing homologous chromosome synapsis by affecting both crossover and non-crossover pathways, being essential for effective synapsis of sex chromosomes. Mouse Pachytene checkpoint protein 2 is required for recombination and normal higher-order chromosome structure during meiosis, playing a potential role in non-crossover repair of double-strand breaks. Male mice with homozygous mutations in this gene show complete chromosome synapsis in spermatocytes, but cells die in pachytene due to defective double-strand break repair during recombination, resulting in testicular tissue lacking post-meiotic cells [34,35].

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 [35].

Acute heat stress impairs 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 testis to resist heat-induced damage [36]. Transgelin gene expression levels are lower in testicular tissue of older animals [37].

Adhesion G protein-coupled receptor A3 is a known marker of spermatogonial stem cells; 55% of mice with this gene knocked out are infertile from puberty despite normal spermatogenesis and epididymal sperm numbers [38]. 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 [39]. Mature spermatids must be released from attached Sertoli cells, and proteins identified as participating in adhesion between Sertoli cells and mature spermatids include 14-3-3 protein zeta/delta, which appears only in tubule segment lysates during spermiation. However, its exact role in spermatogenesis and how it interacts with or affects other signal transduction pathways in the testis remain unknown [40].

Peroxiredoxin-2 has antioxidant properties and may participate in maintaining the oxidative balance of the mouse spermatogenesis environment [41]. Peroxiredoxin-2 can also maintain normal development of neonatal rat germ cells [42]. Peptidyl-prolyl cis-trans isomerase A is upregulated in mouse testis treated with environmental estrogens, which reduce sperm count and cause male infertility, though the molecular mechanisms of this effect remain unclear [43].

Nucleoside diphosphate kinase B is distributed in the manchette microtubule structure of sperm (a transient microtubule structure in elongating 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 basal pole of elongating spermatid nuclei. Nucleoside diphosphate kinase isoforms may have specific functions in the phosphotransfer network of human spermatogenesis and flagellar motility [44]. Nucleoside diphosphate kinase plays a key role in spermatogenesis by increasing levels of the antioxidant enzyme glutathione peroxidase 5 to eliminate reactive oxygen species [45].

Combined treatment with injectable testosterone undecanoate and oral levonorgestrel enhances Parvalbumin alpha expression to inhibit 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 [46].

4. Discussion

Despite potential interference in experimental results, this study preliminarily demonstrates that even with a small sample size, there are significant differences between the urinary proteome on the 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 correlate with spermatogenesis in database searches, our results suggest these proteins may still be associated with spermatogenesis and could serve as target proteins for further investigation. This study demonstrates the potential of urinary proteomics for studying the spermatogenesis process, providing a urinary proteomic approach for exploring pathogenic mechanisms, target discovery, and new diagnostic methods for men with abnormal spermatogenesis. Future experiments could consider expanding the animal sample size or collecting clinical samples for further research. Additionally, this study reflects the sensitivity of the urinary proteome, opening new avenues for urinary biomarker exploration.

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Note: Figure translations are in progress. See original paper for figures.