Changes in the urinary proteome during rat pregnancy

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Abstract

Objective: Pregnancy is a process during which the body' s physiological state undergoes profound changes. The normal course of pregnancy is crucial for ensuring proper fetal development and maintaining maternal health. We attempted to explore whether changes in the urinary proteome can reflect the process of pregnancy.

Methods: We collected urine samples from pregnant rats on gestational days 0, 2, 12, 14, 16, 18, and 20. Urinary proteomes at different time points were analyzed via LC-MS high-throughput mass spectrometry, and differential proteins obtained by comparison with gestational day 0 were subjected to biological pathway analysis.

Results: Changes in the urinary proteome on gestational day 2 were associated with pathways related to embryo implantation and placental differentiation. In late pregnancy (E16 and E18), urine was enriched for pathways related to organ development, primarily those associated with lung development. Interestingly, according to previous reports, the time point at which implantation-related pathways were identified in urine coincided precisely with the implantation window in rats; rat lung development occurs mainly during late pregnancy (E11-E21), and we found that lung development-related pathways in urine also aligned with this period. Furthermore, from late pregnancy to parturition, processes related to the coagulation system were found to become progressively more significant, consistent with the previously reported trend of continuously enhanced coagulation function during pregnancy.

Conclusion: (1) The urinary proteome can reflect the normal course of pregnancy; (2) Urine may serve as an effective strategy for monitoring the process of pregnancy.

Full Text

Urine Proteome Changes During Pregnancy in Rats

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Abstract

Objective: Pregnancy involves tremendous physiological changes, and normal gestation is essential for ensuring healthy fetal development and maternal well-being. This study explored whether urinary proteome changes could reflect the progression of pregnancy.

Methods: Urine samples were collected from pregnant rats on gestation days 0, 2, 12, 14, 16, 18, and 20. Urinary proteome profiles were analyzed using high-throughput liquid chromatography-tandem mass spectrometry (LC-MS/MS), and differentially expressed proteins at each time point were compared to day 0 for biological pathway analysis.

Results: On gestation day 2, urinary proteins were enriched in pathways related to embryo implantation and placental differentiation. During late pregnancy (E16 and E18), pathways associated with organ development, particularly lung development, were enriched. Notably, the appearance of implantation-related pathways in urine coincided precisely with the reported implantation window in rats. Similarly, rat lung development occurs primarily during late pregnancy (E11-E21), and our findings of lung development-related pathways aligned with this period. Furthermore, coagulation system-related processes became increasingly prominent from late pregnancy through delivery, consistent with previously reported trends of enhanced coagulation function during gestation.

Conclusion: (1) The urinary proteome can reflect normal pregnancy progression; (2) Urine may serve as an effective strategy for monitoring pregnancy.

Keywords: Pregnancy process; Embryonic development; Urinary proteome

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Introduction

Pregnancy is a physiological process involving substantial metabolic and hormonal adaptations, and abnormalities at any stage can lead to adverse outcomes [1]. Globally, approximately 200 million pregnancies occur annually, accompanied by 300,000 maternal deaths related to pregnancy and childbirth and 7 million perinatal fetal deaths [2]. Ensuring healthy pregnancy and deepening our understanding of gestational processes are crucial for normal fetal development

and minimizing maternal complications. Currently, ultrasound examination is the primary clinical method for monitoring fetal development [3]. Previous studies have reported numerous pregnancy-related metabolites and metabolic pathways in the blood metabolome during gestation [4]. Urine specimens are easily obtainable, lack homeostatic mechanisms that might mask early changes, and can accumulate early disease-related alterations [5]. Notably, urine can reflect central nervous system disorders, with relevant proteins crossing the blood-brain barrier and becoming enriched in urine, as demonstrated in Parkinson' s disease [6] and autism [7]. Urine also plays an important role in early tumor detection, with biomarkers identified for gliomas [8] and reflecting changes from minimal tumor cell burdens [9]. Given the sensitivity of urinary changes, we sought to explore through animal experiments whether the urinary proteome could reflect maternal changes and embryonic growth and development processes.

Methods

2.1 Experimental Animals

Ten pregnant Wistar rats on gestation day 0 were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All animals were maintained on standard laboratory chow at 25°C ± 2°C with a 12-hour light/dark cycle. This study was approved by the Ethics and Animal Welfare Committee of Beijing Normal University (CLS-EAW-2020-022). Rats were housed two per cage during early pregnancy and individually during late pregnancy.

2.2 Sample Preparation

Urine samples were collected on gestation days 0, 2, 12, 14, 16, 18, and 20. Pregnant rats were placed in metabolic cages for 12 hours to obtain approximately 10 mL of urine, which was centrifuged at 14,000 × g for 30 minutes at 4°C and stored at -80°C. For processing, 4–6 mL of each sample was centrifuged at 14,000 × g for 30 minutes at 4°C, followed by addition of at least three volumes of ethanol and overnight incubation at -20°C. The precipitate was resuspended in 100 µL of lysis buffer (8 mol/L urea, 2 mol/L thiourea, 50 mmol/L Tris, 25 mmol/L dithiothreitol), and protein concentration was quantified using a BCA assay kit.

For digestion, 100 µg of protein was digested with trypsin, and urinary proteins were treated with FASTAP enzyme [4]. Protein samples were placed on 10 kDa filter membranes (Pall, Port Washington, NY, USA), washed twice with UA solution, reduced with 20 mmol/L dithiothreitol at 37°C for 1 hour, and alkylated with 50 mmol/L iodoacetamide (IAA, Sigma) in the dark for 45 minutes. After multiple washes with YA and NH4HCO3, trypsin was added at an enzyme-to-protein ratio of 1:50, and digestion proceeded at 37°C for 15 hours. Digested peptides were desalted using HLB columns (Waters, Milford, MA, USA) and lyophilized in a vacuum freeze dryer.

2.3 Sample Fractionation

Digested peptides were diluted to 0.5 µg/µL with 1% FA. All samples (1 µg each) were pooled into a mix sample and loaded onto a high-pH reversed-phase fractionation column (84,868, Thermo Fisher Scientific). Peptides were eluted with different acetonitrile concentrations (5, 7.5, 10, 12.5, 15, 17.5, 20, and 50%) to obtain 10 gradient fractions, which were then lyophilized and reconstituted in 20 µL of 1% FA.

2.4 LC-MS/MS Analysis

To calibrate retention time for peak extraction, iRT peptides were added to each sample at a 10:1 peptide-to-iRT volume ratio. For each sample, 1 µg was loaded onto a trap column (75 µm × 2 cm, 3 µm, C18, 100 Å) at 300 µL/min and separated on an analytical column (75 µm × 250 mm, 2 µm, C18, 100 Å) with a 90-minute gradient of 4–35% mobile phase B using an Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

The 10 fractions were analyzed using data-dependent acquisition (DDA) for database searching, with full MS scans acquired at 350–1500 m/z and resolution set to 120,000. MS/MS scans were performed in Orbitrap mode at 30,000 resolution. Individual samples were analyzed using data-independent acquisition (DIA) with 36 variable isolation windows, full scan resolution of 60,000 (m/z range 350–1400), and DIA scan resolution of 30,000. All samples were pooled into a mix for quality control analysis.

2.5 DIA Data Analysis

Raw DDA data from fractionated samples were imported into Proteome Discoverer (version 2.1; Thermo Scientific) for database searching, following the detailed procedures described by Weijing et al. [10]. Raw DIA data from all samples were imported into Spectronaut. Protein analysis was performed using the implemented IDPicker algorithm to generate protein groups [11], and all results were filtered at a Q-value < 0.01 (corresponding to 1% FDR). Peptide intensity was calculated by summing the peak areas of corresponding fragment ions in MS2, and protein intensity was determined by summing the respective peptide intensities.

2.6 Data Analysis

Results were exported from Spectronaut. Filtering criteria included a miscleavage rate $< 1\%$ and requirement of at least two unique peptides for protein identification. QC samples were used to evaluate mass spectrometry stability. Proteins with QC CV < 0.3 were retained, and missing values were imputed using the sequential-KNN method (https://www.omicsolution.org/wkomics/main/) for each sample.

One-way ANOVA was used for inter-group comparisons, with each gestational

time point compared to day 0 to identify differential proteins. Differential protein criteria: proteins identified by at least two peptides, fold change (FC) > 1.5 or < 0.67, and t-test P-value < 0.05. All results are expressed as mean ± standard deviation.

2.7 Functional Analysis

Biological processes, cellular components, and molecular functions were analyzed using Gene Ontology (GO) through the DAVID website. Ingenuity Pathway Analysis (IPA) software (Qiagen, Mountain View, CA, USA) was used for biological pathway and disease/function analysis. GraphPad Prism 8.0.1 was used for graphing.

Results

3.1 Establishment of the Pregnant Rat Model

This study used ten female Wistar rats mated with three male rats. Maternal weight changes are shown in Figure 1, and litter sizes are presented in Table 1 (mean ± SD: 15.4 ± 1.854 pups per rat). No significant differences in litter size were observed among the pregnant rats, and all gave birth at 20–21 days, indicating consistent pregnancy progression. Five pregnant rats were selected for urinary proteome analysis on gestation days 0, 2, 12, 14, 16, 18, and 20, yielding 50 urine samples.

3.2 Changes in the Urinary Proteome of Pregnant Rats

We employed high-throughput LC-MS/MS to analyze urinary proteome changes across different gestational time points. Results for each pregnant rat at each time point are provided in Supplementary Table 1. Each gestational time point was compared to day 0. Based on screening criteria (FDR < 1.0%, at least two peptides per protein), 1,621 proteins were identified. After imputing missing values for the mix sample and filtering for QC CV < 0.3, 1,604 proteins remained. Missing value imputation using the Wukong platform (https://www.omicsolution.org/wkomics/main/) yielded 1,209 proteins for subsequent screening and analysis (Supplementary Table 2).

Comparisons with gestation day 0 identified 162, 175, 51, 69, 220, and 200 differential proteins on days 2, 12, 14, 16, 18, and 20, respectively (FC > 1.5 or < 0.67, P < 0.05). The Venn diagram of differential proteins at each time point (Figure 2) shows that days 2 and 20 had the most unique differential proteins, suggesting greater physiological differences during early pregnancy and near-term periods.

3.3 Biological Processes in Early Pregnancy

We investigated early pregnancy physiological changes through urinary proteome analysis. IPA analysis of day 2 differential proteins revealed several

key biological pathways (Figure 3): Wound Healing Signaling, Glucocorticoid Receptor Signaling, Methylthiopropionate Biosynthesis, Ascorbate Recycling (Cytosolic), Glutathione Redox Reactions II, Lactose Degradation III, Th1 and Th2 Activation Pathway, and Vitamin-C Transport.

Glucocorticoid Receptor Signaling represents a major intracellular signaling regulator that directly controls mouse embryo implantation and endometrial remodeling [16]. Notably, this process occurs during the rat implantation window [12], and its detection before implantation may reflect preparatory changes. However, excessive glucocorticoids may adversely affect trophoblast development and differentiation [17]. The Th1 and Th2 Activation Pathway is associated with normal pregnancy and complications, with Th1 (inflammatory) responses diminishing and Th2 responses enhancing during successful pregnancy [18]. Maternal Th2-type immunity appears beneficial, while Th1-type immunity may harm fetal development [18]. Vitamin-C Transport is crucial for placental weight and infant birth weight, influencing trophoblast differentiation and placental steroid hormone synthesis essential for fetal development and pregnancy maintenance [19]. These urinary proteome changes closely correlate with embryo implantation and trophoblast development, with timing consistent with the rat implantation period, demonstrating urine' s sensitivity in reflecting early pregnancy changes.

3.4 Biological Processes in Late Pregnancy

Embryonic growth and development differentially affect maternal physiology in late pregnancy. We explored these changes through urinary proteome analysis of gestation days 12, 14, 16, 18, and 20 (Figure 4A–4E). On day 12, enriched pathways included G 12/13 Signaling, Synaptogenesis Signaling, Hepatic Fibrosis/Hepatic Stellate Cell Activation, Rho Family GTPases Signaling, GP6 Signaling, and Airway Pathology in Chronic Obstructive Pulmonary Disease. RHOGDI Signaling on day 12 plays important roles in regulating trophoblast cell migration [20], while Hepatic Stellate Cell Activation is crucial for liver development and hematopoiesis. GP6 Signaling is associated with platelet hyperaggregation in miscarriage [21], and GP6 gene variants affect pregnancy loss [22].

On day 14, enriched pathways included Airway Pathology in Chronic Obstructive Pulmonary Disease, Complement System, LXR/RXR Activation, Atherosclerosis Signaling, Prostanoid Biosynthesis, Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F, and Coagulation System. The Complement System is essential for placental development and protects normal pregnancy progression [23]. Maternal IL-17A induces fetal cerebral cortical abnormalities [24], and the presence of such development-related factors in urine during normal pregnancy suggests potential for detecting developmental abnormalities. Coagulation-related pathways first appeared on day 14.

On day 16, enriched biological processes included cellular response to platelet-derived growth factor stimulus, cell migration, positive regulation of defense response to virus by host, epidermal growth factor receptor signaling pathway, ubiquitin homeostasis, negative regulation of cholesterol efflux, and blood coagulation. PDGF-BB and PDGF-AA affect myocardial development [25], while epidermal growth factor receptor signaling is a key regulator of fetal lung surfactant synthesis and placental vascular development [26]. Negative regulation of cholesterol efflux reflects physiologically increasing maternal cholesterol levels during pregnancy, which may initiate early atherosclerotic changes [27].

On day 18, major pathways included Airway Pathology in Chronic Obstructive Pulmonary Disease, Glucocorticoid Receptor Signaling, WNT/ -catenin Signaling, Complement System, Th2 Pathway, and PCP (Planar Cell Polarity) Pathway. These pathways relate to organ growth and development. Glucocorticoid Receptor Signaling is critical in lung embryogenesis [28], while WNT/ -catenin signaling plays key roles in lung development, injury, and repair [30]. The PCP pathway regulates coordinated cell behaviors during morphogenesis, including lung cilia orientation [31] and kidney development [32]. Rat lung development occurs primarily in late pregnancy (E11–E21) and matures fully only after birth [33]. Our identification of multiple lung development-related pathways on day 18 suggests significant fetal lung development at this stage, consistent with reported developmental timelines.

On day 20, enriched pathways included Extrinsic Prothrombin Activation Pathway, Thymine Degradation, GP6 Signaling, Coagulation System, Role of Tissue Factor in Cancer, Complement System, IGF-1 Signaling, Factors Promoting Cardiogenesis in Vertebrates, and Eicosanoid Signaling. The GP6 pathway at this stage primarily relates to coagulation, as platelet activation via GP6-collagen interactions is essential for thrombus formation [34]. Maternal IGF-1 deficiency causes placental dysfunction [35], while prostanoid biosynthesis is associated with human labor [36]. Coagulation-related pathways, including Extrinsic Prothrombin Activation and Coagulation System, showed significant changes. Fibrinogen concentration and platelet count increase during pregnancy as a physiological response to prevent excessive bleeding at delivery [37]. Progressive gestational enhancement of coagulation function, increased thrombin generation, and fibrinolysis create a hypercoagulable state that maintains placental function and prevents hemorrhage during delivery [38]. Our results show gradually increasing prominence of procoagulant processes from day 12 to day 20, with the greatest enrichment on day 20, preparing for parturition.

Our findings demonstrate that the urinary proteome reflects pregnancy changes across different stages: implantation and trophoblast differentiation pathways on day 2; coagulation pathways appearing on day 14; organ development processes (pancreas, lung, kidney) on days 16 and 18; lung development pathways prominently on day 18; and coagulation function pathways on day 20. The temporal consistency between enriched biological pathways and corresponding gestational processes—implantation within the implantation window, lung de-

velopment in late pregnancy, and progressively enhanced coagulation—demonstrates that the urinary proteome sensitively reflects distinct pregnancy stages and contains information about normal embryonic development.

Although based on animal experiments, these results suggest the need for larger clinical studies to explore whether maternal urinary proteome can reflect human pregnancy progression. The normal pregnancy urinary proteome could serve as a clinical database for effective monitoring of gestational abnormalities.

In summary, urine enriches pregnancy-related biological processes at different gestational times, proving that the urinary proteome can reflect normal pregnancy progression. Clinically, urine has potential as an effective strategy for monitoring pregnancy.

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