Changes in the Urinary Proteome of Rats After Intragastric Administration of Polysaccharide Iron Complex

Authors: Shen Ziyun, Yang Minhui, Haitong Wang, Gao Youhe, Gao Youhe

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Abstract

Iron is an essential trace element for maintaining normal physiological functions in organisms, and no studies have investigated the overall impact of iron on the body from the perspective of urine proteomics. In this study, rats were administered polysaccharide iron complex via gavage (28 mg/kg · d of iron element, equivalent to the dose for preventing anemia in adults) for 4 days. Using two analytical methods—self before-after comparison and group comparison—we comparatively analyzed the urine proteome of rats before and after short-term gavage of polysaccharide iron complex. Many differential proteins have been reported to be associated with iron, including 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (7.7-fold higher before gavage than after, p=0.0039), p38 (14.5-fold higher after gavage than before, p=0.003), etc.; in before-after comparisons of individual rats, hepcidin was concurrently upregulated in 4 rats. The biological processes enriched by differential proteins include response to carbohydrate metabolic process, response to iron ion, regulation of apoptotic process, hematopoietic progenitor cell differentiation, etc.; molecular functions (such as complement binding, hemoglobin binding, etc.) and KEGG pathways (such as complement and coagulation cascades, cholesterol metabolism, malaria, etc.) also showed relevance to iron. This study, from the perspective of urine proteomics, contributes to a deeper understanding of the biological functions of iron and provides a new research perspective for the prevention, diagnosis, treatment, and monitoring of diseases related to iron metabolism disorders.

Full Text

Changes in the Urine Proteome of Rats After Intragastric Administration of Polysaccharide-Iron Complex

Ziyun Shen, Minhui Yang, Haitong Wang, Youhe Gao*

Gene Engineering Drug and Biotechnology Beijing Key Laboratory, College of Life Sciences, Beijing Normal University, Beijing 100875, China

Abstract

Iron is an essential trace element required for maintaining normal physiological functions in organisms. However, no studies have investigated the systemic effects of iron from the perspective of the urine proteome. In this study, rats were administered polysaccharide-iron complex (28 mg/kg·d iron element, equivalent to the dose used for anemia prevention in adults) via gavage for 4 days. Using both self-comparison (before vs. after) and group comparison approaches, we analyzed changes in the urine proteome before and after short-term administration. Many differential proteins were reported to be iron-related, including 2',3'-cyclic nucleotide 3' -phosphodiesterase (CNPase) (7.7-fold higher before vs. after gavage, p=0.0039) and p38 (14.5-fold higher after vs. before gavage, p=0.003). In individual rat comparisons, hepcidin was upregulated simultaneously in 4 rats. Enriched biological processes among differential proteins included carbohydrate metabolic processes, response to iron ions, regulation of apoptotic processes, and hematopoietic progenitor cell differentiation. Molecular functions (e.g., complement binding, hemoglobin binding) and KEGG pathways (e.g., complement and coagulation cascades, cholesterol metabolism, malaria) also showed iron-related associations. This study provides new insights into the biological functions of iron from a urine proteomics perspective and offers a novel research perspective for the prevention, diagnosis, treatment, and monitoring of iron metabolism disorders.

Keywords: Iron; Urine; Proteome; Polysaccharide-iron complex; Nutrients; Mineral elements

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Corresponding author: Youhe Gao (born 1964.06), male, professor, doctoral supervisor. Research focus: urine proteomics and urine biomarkers. Tel: 010-58804382; E-mail: gaoyouhe@bnu.edu.cn.

Introduction

Trace elements play indispensable roles in various physiological processes in organisms. In recent years, with in-depth research on the functions of trace elements, scientists have gradually recognized that trace element homeostasis is also associated with the pathogenesis of multiple diseases.

Iron is one of the essential trace elements required for maintaining normal physiological functions in organisms, participating in numerous vital biological processes such as oxygen transport, cellular respiration, and DNA synthesis. Iron metabolism disorders can lead to biochemical imbalance in the body, triggering a series of health problems [**?**].

Since urine is not part of the internal environment and lacks homeostatic mechanisms compared to plasma, it can accumulate early changes in physiological status and more sensitively reflect changes in the body, making it a source of next-generation biomarkers [**?**]. Urinary proteins contain rich information that can reflect subtle changes in different systems and organs of the body.

Our laboratory previously reported that the urine proteome can systematically and comprehensively reflect the effects of magnesium L-threonate intake on the body and has the potential to provide clues for clinical nutrition research and practice [**?**]. However, to date, no studies have investigated the effects of iron on the body from the perspective of the urine proteome.

This study selected polysaccharide-iron complex as an iron supplement, which can rapidly increase blood iron levels and elevate hemoglobin. It causes mild irritation to the gastrointestinal mucosa, has minimal adverse reactions, allows continuous administration, and has high absorption rates, making it suitable for preventing and treating iron deficiency anemia. This study aimed to investigate changes in the urine proteome of rats after short-term intake of polysaccharide-iron complex to further understand the biological functions and systemic effects of iron in organisms, providing a new research perspective for nutrition studies.

Materials and Methods

2.1.1 Experimental Consumables

5 mL sterile syringes (BD Company), gavage needles (16 gauge, 80 mm, curved), 1.5 mL/2 mL centrifuge tubes (Axygen, USA), 50 mL/15 mL centrifuge tubes (Corning, USA), 96-well cell culture plates (Corning, USA), 10 kDa filters (Pall, USA), Oasis HLB solid-phase extraction columns (Waters, USA), 1 mL/200 L/20 L pipette tips (Axygen, USA), BCA assay kit (Thermo Fisher Scientific, USA), high pH reverse-phase peptide separation kit (Thermo Fisher Scientific, USA), iRT (indexed retention time, BioGnosis, UK).

2.1.2 Experimental Instruments

Rat metabolic cages (Beijing Jiayuan Xingye Technology Co., Ltd.), refrigerated high-speed centrifuge (Thermo Fisher Scientific, USA), vacuum concentrator (Thermo Fisher Scientific, USA), DK-S22 electric thermostatic water bath (Shanghai Jinghong Experimental Equipment Co., Ltd.), full-wavelength multifunctional microplate reader (BMG Labtech, Germany), oscillator (Thermo Fisher Scientific, USA), TS100 thermomixer (Hangzhou Ruicheng Instrument Co., Ltd.), electronic balance (METTLER TOLEDO, Switzerland), -80°C ultra-low temperature freezer (Thermo Fisher Scientific, USA), EASY-nLC1200 ultra-high performance liquid chromatography (Thermo Fisher Scientific, USA), Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, USA).

2.1.3 Experimental Reagents

Polysaccharide-iron complex capsules (National Drug Approval Number H20030033) were produced by Shanghai Pharmaceuticals Qingdao Guofeng Pharmaceutical Co., Ltd. Additional reagents included: Trypsin Gold (Promega, USA), dithiothreitol DTT (Sigma, Germany), iodoacetamide IAA (Sigma, Germany), ammonium bicarbonate NH4HCO3 (Sigma, Germany), urea (Sigma, Germany), purified water (Wahaha, China), mass spectrometry-grade methanol (Thermo Fisher Scientific, USA), mass spectrometry-grade acetonitrile (Thermo Fisher Scientific, USA), mass spectrometry-grade pure water (Thermo Fisher Scientific, USA), Tris-Base (Promega, USA), thiourea (Sigma, Germany).

2.1.4 Analysis Software

Proteome Discoverer (Version 2.1, Thermo Fisher Scientific, USA), Spectronaut Pulsar (Biognosys, UK), Ingenuity Pathway Analysis (Qiagen, Germany), R studio (Version 1.2.5001), Xftp 7, Xshell 7.

2.2.1 Animal Model Establishment

This study used 17-week-old rats to minimize the impact of growth and development during the gavage period. Five healthy male SD (Sprague Dawley) rats aged 9 weeks ($250\pm20g$) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. There humidity 65%-70%) for 8 weeks. When their body weight reached 500-600 g, experiments began. All experimental procedures followed the review and approval of the Ethics Committee of the College of Life Sciences, Beijing Normal University.

Tolerable Upper Intake Level (UL) refers to the average maximum daily intake level of a nutrient that is unlikely to pose any risk of adverse health effects to almost all individuals in a specific life stage and gender group. Recommended Nutrient Intakes (RNI) refers to the intake level that meets the needs of 97-98% of individuals in a specific age, gender, and physiological condition group.

According to the Chinese Dietary Guidelines, the daily RNI for iron is 20 mg/d, and the UL is 42 mg/d [**?**]. Following the anemia prevention dose indicated in the polysaccharide-iron complex instructions, each capsule contains 150 mg iron element, with adults taking 1-2 capsules daily (150-300 mg iron/d). This dose converts to approximately 14-28 mg/kg · d for rats based on body surface area and weight. In this study, the gavage dose for rats was 28 mg/kg · d, equivalent to the adult anemia prevention dose. Three grams of polysaccharide-iron complex (approximately 1.4 g iron) were dissolved in 500 mL sterile water to prepare the gavage solution. Each rat received 5 mL of polysaccharide-iron solution daily via gavage once per day for 4 consecutive days. The first day of gavage was designated as Fe-D1, and so on. Sampling time points were set before and after gavage for self-control comparison. Samples collected the day before gavage served as the control group, designated as Fe-D0 (sample numbers

51-55). Samples collected on the 4th day of gavage served as the experimental group, designated as Fe-D4 (sample numbers 61-65).

[Figure 1: see original paper] Research methodology and technical route

2.2.2 Urine Sample Collection

On the day before gavage (D0) and 4 days after gavage (D4), each rat was placed individually in a metabolic cage at the same time, with food and water withheld for 12 hours. Urine was collected overnight and stored at -80°C.

2.2.3 Urine Sample Processing

Two milliliters of urine sample were thawed and centrifuged at 4°C, 12,000×g for 30 minutes to remove cell debris. The supernatant was collected, and 40 L of 1 M dithiothreitol (DTT, Sigma) stock solution was added to achieve a working concentration of 20 mM DTT. After mixing, the sample was heated in a metal bath at 37°C for 60 minutes. After cooling to room temperature, 100 L of iodoacetamide (IAA, Sigma) stock solution was added to achieve the working concentration of IAA, mixed well, and reacted in the dark at room temperature for 45 minutes.

After the reaction, the sample was transferred to a new centrifuge tube, mixed thoroughly with three volumes of pre-cooled absolute ethanol, and placed at -20°C for 24 hours to precipitate proteins. After precipitation, the sample was centrifuged at 4°C, 10,000×g for 30 minutes, the supernatant was discarded, and the protein precipitate was dried. The protein precipitate was resuspended in 200 L of 20 mM Tris solution. After centrifugation, the supernatant was retained, and protein concentration was determined using the Bradford method.

Using the filter-aided sample preparation (FASP) method, the urinary protein extract was added to the membrane of a 10 kDa ultrafiltration tube (Pall, Port Washington, NY, USA), washed three times with 20 mM Tris solution, and re-suspended in 30 mM Tris solution. Trypsin (Trypsin Gold, Mass Spec Grade, Promega, Fitchburg, WI, USA) was added to each sample at a ratio of urinary protein:trypsin = 50:1 for digestion, followed by incubation at 37°C for 16 hours. The filtrate after enzymatic digestion was the peptide mixture. The collected peptide mixture was desalted using Oasis HLB solid-phase extraction columns, vacuum-dried, and stored at -80°C. The lyophilized peptide powder was resuspended in 30 L of 0.1% formic acid water, and peptide concentration was determined using a BCA assay kit. The peptide concentration was diluted to 0.5 g/ L, and 4 L from each sample was taken as a mix.

2.2.4 LC-MS/MS Tandem Mass Spectrometry Analysis

All identification samples were spiked with diluted 100-fold iRT standard solution at a sample:iRT volume ratio of 20:1 to unify retention time. Data-independent acquisition (DIA) was performed on all samples, with each sample

repeated 3 times. One mix sample was inserted every 10 injections as a quality control. One microgram of sample was separated using EASY-nLC1200 liquid chromatography (elution time: 90 min, gradient: mobile phase A: 0.1% formic acid, mobile phase B: 80% acetonitrile). The eluted peptides were analyzed by Orbitrap Fusion Lumos Tribrid mass spectrometer to generate corresponding raw files.

2.2.5 Data Processing and Analysis

Raw files collected in DIA mode were imported into Spectronaut software for analysis. High-confidence protein standards were peptide q value < 0.01. Peak area quantification was applied to quantify proteins based on the peak area of all fragment ions of secondary peptides. Automatic normalization was performed. Proteins containing two or more specific peptides were retained. Missing values were replaced with 0. The content of different proteins identified in each sample was calculated. Samples from rats before gavage were compared with samples after 4 days of gavage to screen for differential proteins.

Unsupervised hierarchical clustering analysis (HCA), principal component analysis (PCA), and OPLS-DA analysis were performed using the Wukong platform (https://omicsolution.org/wkomics/main/). Functional enrichment analysis of differential proteins was conducted using the DAVID database (https://david.ncifcrf.gov/) to obtain results in three aspects: biological process, cellular localization, and molecular function. Differential proteins and related pathways were searched based on the Pubmed database (https://pubmed.ncbi.nlm.nih.gov/). Protein-protein interaction network analysis was performed using the STRING database (https://cn.string-db.org/).

2.2.6 Random Grouping Analysis

When using omics technology to study disease biomarkers, differences between disease and control groups are typically screened. Due to the large omics data and limited sample size, differences between the two groups may be randomly generated. Therefore, we used a random grouping statistical analysis strategy, which is suitable for clinical omics disease biomarker studies with limited sample size, to determine whether differences between groups were randomly generated [**?**].

The 10 samples from before (n=5) and after gavage (n=5) were randomly divided into two groups. Among all random combinations, the average number of differential proteins was calculated for all random combinations under the same screening conditions.

2.2.7 Analysis of Differential Proteins and Functional Annotations Using Pubmed Database

Differential proteins and functional annotations were searched and analyzed in Pubmed. The specific search criteria were simultaneous inclusion of keywords

and iron in the title or abstract, for example, "iron[Title/Abstract] AND heme [Title/Abstract]" . These articles were then read and screened one by one to analyze the association of differential proteins and enriched molecular functions, biological processes, and pathways with iron.

Results

3.1 Characteristics of Rats After Gavage with Polysaccharide-Iron Complex

During the experiment, we observed the drinking, feeding, body weight, and hair characteristics of rats after gavage with polysaccharide-iron complex. We found that body weight remained relatively stable before and after gavage, with normal drinking, feeding, and activity. After gavage, the rats' feces were black, and their hair was relatively messy, possibly due to excessive iron intake.

3.2 Urinary Protein Identification and Unsupervised Clustering Analysis

A total of 1803 proteins were identified in urine samples before gavage (D0) and on the 4th day of gavage (D4) (meeting unique peptides > 1, FDR $< 1\%$). Unsupervised hierarchical clustering analysis (HCA) and principal component analysis (PCA) were performed on total proteins. The results are shown in Figures 2 and 3. HCA and PCA results showed that significant changes occurred in the urine proteome of rats after polysaccharide-iron complex intake, which may reflect the body' s rapid response to exogenous iron. However, the sample points were relatively dispersed, indicating certain individual differences.

[Figure 2: see original paper] Hierarchical clustering analysis (HCA) of total proteins in urine samples before and on the 4th day of gavage with polysaccharide-iron complex

[Figure 3: see original paper] Principal component analysis (PCA) of total proteins in urine samples before and on the 4th day of gavage with polysaccharide-iron complex

3.3.1 Differential Protein Analysis

Missing values were replaced with 0, and group comparison was performed between samples before gavage and samples on the 4th day of gavage, screening out 157 differential proteins. The screening criteria were: P value < 0.05 in T-test analysis, Fold change (FC) > 1.5 or < 0.67. See supplementary table for details.

Among them, 52 differential proteins had P values < 0.01, showing very significant changes before and after gavage, as shown in Table 1 . PubMed database was used to analyze the protein functions and literature retrieval of the 52 differential proteins. Literature showing the correlation between differential proteins

and iron is listed in the table.

Proteins downregulated in rat urine after polysaccharide-iron intake included 2' ,3' -cyclic-nucleotide 3' -phosphodiesterase (CNPase), S100 calcium binding protein A7-like 2 (S100A7l2), tissue inhibitor of metalloproteinases 1 (TIMP-1), and integral membrane protein 2B (Itm2b).

CNPase is a myelin marker with FC of 0.13. S100A7 is a protein that can induce immunomodulatory activity. In the brains of offspring from pregnant rats fed iron-deficient diets, expression of CNPase and S100 calcium binding protein decreased, indicating that iron availability affects oligodendrocyte development [**?**]. Compared with control rats, TIMP-1 was overexpressed in rats taking the iron chelator deferiprone.

The FC of proto-oncogene c-Crk adapter molecule (p38) was 14.53. p38 protein expression was upregulated in iron-overloaded bone marrow mesenchymal stem cells (BMSC) [**?**]. The FC of NPC intracellular cholesterol transporter 1 was 4.38. Studies have shown that iron overload increases intracellular cholesterol [**?**]. The FC of carbonic anhydrase (CA) was 3.8. Studies in experimental animals showed that elevated oxidative stress in red blood cells leads to formation of autoantibodies against carbonic anhydrase and anemia [**?**]; carbonic anhydrase may have interfering effects on iron metabolism [**?**].

Table 1 Significantly changed differential proteins in comparison between Fe-D0 and Fe-D4 groups (P value < 0.01, FC > 1.5 or < 0.67)

Protein Accessions	Genes	Related to Iron
P13233	S100a7l2	[6]
D3Z9U8	Timp1	
P30120	Itm2b	
Q5XIE8	Lilra5	
A0A0G2JTC1	Selenop	
P25236	Wfdc2	
Q8CHN3	Sirpa	
P97710	Ncam1	
P10354	Xpnpep2	
Q501W2	Amy1a	
D3ZM39	P07314	
F1LUV9	Q568Z6	
C0JPT7	Q6TUD4	
P50430	A0A0G2K3G0	
A0A0H2UI19	D3ZUM4	
Q99MA2	A0A096P6L8	
Q6P9V1	P51635	
E9PSQ1	P10247	
P85971	G3V8X5	
...	...	...

3.3.2 Random Grouping Results

To determine the likelihood that differential proteins identified by group comparison were randomly generated, we performed random grouping validation on the total proteins identified in the 10 samples from both groups. Using the same differential protein screening criteria (FC $\geq$ 1.5 or $\leq$ 0.67, P $<$ 0.01), 126 random groupings yielded an average of 10.82 differential proteins, with a randomly identified protein ratio of 21.15%, indicating that at least 79.85% of differential proteins were not generated by randomness. Random grouping test results are shown in Table 2 . The probability that our screened 52 differential proteins (FC $\geq$ 1.5 or $\leq$ 0.67, P $<$ 0.01) were randomly generated is very low, demonstrating that these differential proteins are indeed associated with short-term intake of polysaccharide-iron complex supplements.

Table 2 Random grouping results for Fe-D0 and Fe-D4 groups using screening criteria of FC $\geq$ 1.5 or $\leq$ 0.67, P $<$ 0.01

Total number of proteins	Average number of differential proteins with false random combinations	Screening criteria	Ratio (average numbers of proteins with false random combinations/number of correctly identified differential proteins)
126	10.82	FC $\geq$ 1.5 or $\leq$ 0.67, P $<$ 0.01	21.15%

We also performed random grouping validation using screening criteria of FC $\geq$ 1.5 or $\leq$ 0.67, P $<$ 0.05 on the total proteins identified in the 10 samples from both groups. After 126 random groupings, the average number of differential proteins was 55, with a randomly identified protein ratio of 35.08%, indicating that at least 65% of differential proteins were not generated by randomness. The probability that our screened 157 differential proteins (FC $\geq$ 1.5 or $\leq$ 0.67, P $<$ 0.05) were randomly generated is relatively low.

3.3.3 Biological Pathway Analysis

The 157 differential proteins (P value $<$ 0.05, FC $>$ 1.5 or $<$ 0.67) were imported into the DAVID database, enriching 53 biological processes (BP), as shown in Table 3 .

Multiple biological pathways were reported to be related to iron' s biological functions, such as complement activation, glucose transmembrane transport,

response to estrogen, establishment of endothelial barrier, negative regulation of apoptotic process, response to iron ion, carbohydrate metabolic process, zymogen activation, cellular response to interleukin-6, sodium ion transport, cell-matrix adhesion, and hematopoietic progenitor cell differentiation.

According to literature, intravenous iron formulations induce complement activation in vivo [**?**]. Iron metabolism disorders affect aging [**?**]. Systemic and cellular iron and glucose metabolic pathways are interconnected [**?**]. Elevated estrogen levels are associated with increased systemic available iron [**?**]. Estrogen administration upregulates transferrin [**?**]. Long-term dexamethasone administration reduces liver iron concentration in rats [**?**]. Intracellular iron chelation enhances endothelial barrier function [**?**]. Iron induces reactive oxygen species (ROS) production and apoptosis [**?**]. Lower serum iron levels are significantly associated with higher serum IL-6 levels, and IL-6 promotes ferroptosis in bronchial epithelial cells by inducing ROS-dependent lipid peroxidation and disrupting iron homeostasis [**?**, **?**]. Iron accumulation in bronchial epithelial cells depends on sodium transport [**?**]. L-ascorbic acid can promote iron absorption [**?**]. Host antimicrobial mechanisms can reduce iron availability to pathogens, and there are multiple ferritins that affect innate immune responses [**?**]. Ferritin has inhibitory effects on the in vitro growth of human hematopoietic progenitor cells and in vitro proliferation of T lymphocytes [**?**]. Iron overload inhibits endochondral ossification [**?**]. Iron regulates L-cystine uptake and downstream GSH production in two types of mammalian cells [**?**].

Due to space limitations, other biological processes and their related literature with iron are shown in the table.

Table 3 Biological process (BP) enrichment analysis of differential proteins between Fe-D0 and Fe-D4 groups (P value < 0.05, FC > 1.5 or < 0.67) (P value < 0.05, sorted by P value from smallest to largest)

Biological Process (BP)	Count	P-Value	Related to Iron
complement activation	···	···	[34,35]
aging	···	···	[32]
glucose transmembrane transport	···	···	[33]
complement activation, classical pathway	···	···	[34,35]
response to estrogen	···	···	[34,35]
...	...	...	...

3.3.4 Molecular Function and KEGG Pathway Analysis

The 157 differential proteins (P value < 0.05, FC > 1.5 or < 0.67) were imported into the DAVID database, enriching 23 molecular functions, as shown in Table 4 .

Table 4 Molecular function (MF) enrichment analysis of differential proteins between Fe-D0 and Fe-D4 groups (P value < 0.05, FC > 1.5 or < 0.67) (P value

< 0.05, sorted by P value from smallest to largest)

Molecular Function (MF)	Count	P-Value
macromolecular complex binding	···	2.00E-07
integrin binding	···	1.90E-05
calcium ion binding	···	2.90E-05
protein homodimerization activity	···	5.30E-05
...	...	...

The 157 differential proteins (P value < 0.05, FC > 1.5 or < 0.67) were imported into the DAVID database, enriching 10 KEGG pathways including lysosome, complement and coagulation cascades, focal adhesion, glycosaminoglycan degradation, regulation of actin cytoskeleton, amoebiasis, malaria, leukocyte transendothelial migration, systemic lupus erythematosus, and metabolic pathways (Table 5). Lysosomes are major regulators of iron metabolism [**?**]. Intravenous iron formulations induce complement activation in vivo [**?**]. High intracellular iron oxide nanoparticle concentrations affect cytoskeleton and focal adhesion kinase-mediated signaling [**?**]. Iron administration greatly increases susceptibility to amoebiasis in iron-deficient herders [**?**]. Iron is a cofactor for Plasmodium falciparum development [**?**]. Iron sucrose and iron gluconate significantly inhibit polymorphonuclear leukocyte (PMN) transendothelial migration [**?**]. Many studies have demonstrated iron' s important role in immune response, and increasing evidence suggests that iron homeostasis may be abnormal under chronic inflammatory conditions in systemic lupus erythematosus [**?**].

Table 5 KEGG pathway enrichment analysis of differential proteins between Fe-D0 and Fe-D4 groups (P value < 0.05, FC > 1.5 or < 0.67) (P value < 0.05, sorted by P value from smallest to largest)

KEGG Pathway	Count	P-Value	Related to Iron
Lysosome	···	4.80E-05	[63]
Complement and coagulation cascades	···	2.00E-04	[33]
Focal adhesion	···	7.90E-04	[64]
Glycosaminoglycan degradation	···	9.20E-04	···
...	...	...	...

3.4.1 Differential Protein Screening in Individual Rats

The urine proteome can sensitively reflect changes in body status but is also influenced to some extent by genetic factors [**?**], age [**?**], gender [**?**, **?**], ethnicity [**?**], region [**?**], exercise [**?**, **?**], dietary habits, mental state, circadian rhythm, medication [**?**, **?**], and other environmental factors, showing inter- and intra-individual variability [**?**, **?**]. Animal models are easy to control variables and can reduce changes in human urine samples due to irrelevant variables. However,

even different individuals of the same species have some differences. Therefore, this study used a self-control analysis method to reduce the impact of individual differences and help identify potentially important information.

The specific analysis method for self-comparison was as follows: missing values were replaced with 0, and three technical replicates of each rat's pre-gavage sample (D0) were compared with three replicates of the day 4 post-gavage sample (D4) using two-tailed paired comparison. Differential protein screening criteria were: P value < 0.05 in T-test analysis, Fold change (FC) > 1.5 or < 0.67.

Screening results were as follows: Rat #1 had 194 differential proteins, Rat #2 had 368, Rat #3 had 520, Rat #4 had 230, and Rat #5 had 148.

3.4.2 Shared Biological Processes, Molecular Functions, and Pathway Analysis

Functional annotation of differential proteins from the five rats was performed separately using the DAVID database with screening criteria of $p < 0.05$. Venn diagram analysis was used to analyze the overlap of biological processes, molecular functions, and pathways among the 5 rats.

Rat #1 enriched 126 biological processes; Rat #2 enriched 163; Rat #3 enriched 212; Rat #4 enriched 167; Rat #5 enriched 77. Three biological processes were shared among all 5 rats (100% of the experimental group), including carbohydrate metabolic process, aging, and cell-matrix adhesion. Literature shows that iron metabolism disorders are associated with aging [**?**] and glucose metabolic pathways are interconnected [**?**], and ferroptosis is associated with multiple signaling pathways including cell adhesion [**?**, **?**].

Sixteen biological processes were shared among 4 rats (80% of the experimental group). These biological processes and literature related to iron are shown in the table. Additionally, biological processes such as response to iron ion and regulation of heme-dependent eIF2α phosphorylation were shared among 3 rats (60% of the experimental group).

Table 6 Shared biological processes (BP) among 4 or 5 rats (DAVID database GO analysis)

Biological Process (BP)	Related to Iron
carbohydrate metabolic process	[33]
aging	[32]
cell-matrix adhesion	[50,57]
negative regulation of cysteine-type endopeptidase activity	···
negative regulation of endopeptidase activity	···
positive regulation of fibroblast proliferation	···
lipid metabolic process	···
cell adhesion mediated by integrin	···
response to estrogen	[34,35]

Biological Process (BP)	Related to Iron
glomerular filtration	···
phagocytosis, engulfment	···
positive regulation of cell migration	···
positive regulation of ERK1 and ERK2 cascade	···
cellular response to lipopolysaccharide	···
positive regulation of protein kinase B signaling	···
zymogen activation	···
regulation of systemic arterial blood pressure	···
complement activation, classical pathway	···
glutathione metabolic process	[50,57]

Rat #1 enriched 35 molecular functions; Rat #2 enriched 67; Rat #3 enriched 75; Rat #4 enriched 61; Rat #5 enriched 31. Three molecular functions were shared among all 5 rats (100%), including protein binding, macromolecular complex binding, and calcium ion binding. Eleven molecular functions were shared among 4 rats (80%), including hemoglobin beta binding. Iron is an essential component for hemoglobin synthesis in the body and is crucial for oxygen transport and cellular respiration.

Table 7 Shared molecular functions (MF) among 4 or 5 rats (DAVID database GO analysis)

Molecular Function (MF)
protein binding
macromolecular complex binding
calcium ion binding
proton-transporting ATPase activity, rotational mechanism
hydrolase activity
protease binding
cysteine-type endopeptidase inhibitor activity
serine-type endopeptidase inhibitor activity
phosphatidylserine binding
hemoglobin beta binding
endopeptidase inhibitor activity
peptidase activity
receptor binding
serine-type endopeptidase activity

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed using the DAVID website. Rat #1 enriched 30 KEGG pathways; Rat #2 enriched 41; Rat #3 enriched 49; Rat #4 enriched 35; Rat #5 enriched 10. Two KEGG pathways were shared among all 5 rats (100%):

lysosome and phagosome. Five KEGG pathways were shared among 4 rats (80%): malaria, endocytosis, African trypanosomiasis, Staphylococcus aureus infection, and sphingolipid metabolism. Literature indicating the association of these pathways with iron is listed in the table.

Table 8 Shared KEGG pathways among 4 or 5 rats (DAVID database GO analysis)

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway	Related to Iron
Lysosome	[63]
Phagosome	[95]
Malaria	[66]
Endocytosis	[103]
African trypanosomiasis	[97]
Staphylococcus aureus infection	[58]
Sphingolipid metabolism	[98]

3.4.3 Analysis of Differentially Expressed Proteins Commonly Upregulated or Downregulated in Multiple Rats

Differential proteins obtained from each rat' s before-after comparison were divided into upregulated and downregulated groups based on FC. Compared with pre-gavage samples (D0), post-gavage samples (D4) from the 5 rats identified 129, 309, 425, 148, and 69 upregulated differential proteins (FC $>$ 1.5, P $<$ 0.05), respectively. The 5 rats identified 65, 59, 95, 82, and 79 downregulated differential proteins (FC $<$ 0.67, P $<$ 0.05), respectively. Venn diagrams show the overlap of differential proteins identified before and after gavage among the 5 rats, as shown in Figures 4 [Figure 4: see original paper] and 5 [Figure 5: see original paper]. Differential protein names and overlap details are listed in supplementary tables.

[Figure 4: see original paper] Venn diagram of upregulated differential proteins (FC $>$ 1.5, P $<$ 0.05) from self-control comparison of 5 rats

[Figure 5: see original paper] Venn diagram of downregulated differential proteins (FC $<$ 0.67, P $<$ 0.05) from self-control comparison of 5 rats

Detailed search and analysis were performed for differential proteins that were commonly upregulated or downregulated in 4 or 5 rats, as shown in Table 9 .

S100 calcium binding protein A7 (S100 calcium binding protein A7 like 2, S100A7l2), prostatic steroid-binding protein C1, and cystatin-related protein (Cystatin-related protein 1) were commonly downregulated in all 5 rats. Expression of S100 calcium binding protein decreased in the brains of offspring from pregnant rats fed iron-deficient diets [**?**]. Prostatic epithelial cells synthesize hepcidin, and hepcidin synthesis and secretion are significantly increased

in prostate cancer cells and tissues [**?**]. Cystatin C is positively correlated with serum ferritin [**?**].

Seven proteins including spermine binding protein, cystatin-related protein 2, Cullin 1, decay accelerating factor 1, prostatic glandular kallikrein-6, and submandibular glandular kallikrein-9 were downregulated in 4 rats. Literature review revealed that multiple proteins (or their families) are associated with iron metabolism or ferritin, as detailed in Table 9.

Eleven proteins were upregulated in 4 rats, including hepcidin, which participates in maintaining iron homeostasis as a signaling molecule. Other proteins such as H-2 class II histocompatibility antigen gamma chain, neutral and basic amino acid transport protein rBAT, solute carrier family 22 member 12, acyl-CoA synthetase short-chain family member 3, glutamate-cysteine ligase catalytic subunit, and beta-galactosidase were also found to be associated with iron metabolism or ferritin.

Table 9 Differential proteins commonly upregulated or downregulated in 4 or 5 rats

Protein Accessions	Gene Names	Related to Iron
D3ZFC6	Itih4	[101,102]
F1M8K0	Cenpf	
E9PT83	Akr1a1	[24,25]
P51635	Alr	[103]
P10247	Slc3a1	[104]
Q64319	Nbat	[105]
Q3ZAV1	Slc22a12	
Q99MH3	Urat1	
A0A0G2K047	Hamp	[106]
P19468	Hepc	[104]
D3ZUM4	Acss3	
D3Z9U8	Gclc	
P02782	Glclc	
P22282	S100a7l2	[6]
G3V928	RGD1562234	
Q4YNX7	Psbpc1	[99]
B1WBY1	Scgb1d2	
P22283	Andpro	
A0A0G2K176	Crp1	[100]
A0A0G2QC50	Sbp	[107]
D4A263	Zg16b	
D4A6I7	Z4YNX7	
Q62894	Cd55	[108]
G3V6A0	Daf1	[109]
D3ZFC6	P36374	
F1LQT4	P07647	[110]

Protein Accessions	Gene Names	Related to Iron
Q5M891	Klk6	
D3ZWD6	Klk-8	
A0A088DKH8	Klk8	
A2IBE2	Klk9	
G3V7K5	Klk-9	
A0A0G2K227	Klks3	
Q6AYC4	···	
O70257	···	
D3ZAT0	···	
Q63768	···	
D4A076	···	

3.4.4 Functional Enrichment Analysis of Differentially Expressed Proteins Commonly Upregulated or Downregulated in Multiple Rats

Functional annotation was performed for differential proteins commonly upregulated or downregulated in 3, 4, or 5 rats. The enriched biological processes (Table 10), molecular functions (Figure 6 [Figure 6: see original paper]), and KEGG pathways (Table 11) were analyzed.

A total of 44 biological processes were enriched, and literature searches were conducted for their correlation with iron. Related literature is detailed in Table 10.

Among them, 15 biological processes overlapped with group analysis results, including zymogen activation, positive regulation of fibroblast proliferation, cell-matrix adhesion, aging, integrin-mediated cell adhesion, antimicrobial humoral immune response mediated by antimicrobial peptide, response to estrogen, acute-phase response, positive regulation of peptidyl-tyrosine phosphorylation, response to ethanol, negative regulation of apoptotic process, L-cystine transport, glycoside catabolic process, carbohydrate metabolic process, and metanephric proximal tubule development. Additionally, biological processes such as glutathione metabolic process, regulation of systemic arterial blood pressure, and regulation of heme-dependent eIF2α phosphorylation were enriched. Many biological processes are related to iron' s biological functions.

Table 10 Biological process (BP) enrichment analysis of proteins commonly upregulated or downregulated in 3 or more rats (DAVID database GO analysis)

Biological Process (BP)	Count	P-Value	Related to Iron
glutathione metabolic process	···	···	[50,57]

Biological Process (BP)	Count	P-Value	Related to Iron
regulation of systemic arterial blood pressure	···	···	[50,57]
zymogen activation	···	···	[100]
amino acid transmembrane transport	···	···	[111]
positive regulation of fibroblast proliferation	···	···	[112]
cell-matrix adhesion	···	···	[113]
aging	···	···	[50,57]
negative regulation of cysteine-type endopeptidase activity	···	···	···
···	···	···	···

Seventeen molecular functions were enriched, including hemoglobin binding. Seven molecular functions overlapped with group analysis results, including cysteine-type endopeptidase inhibitor activity, protease binding, macromolecular complex binding, receptor binding, calcium ion binding, integrin binding, and arylsulfatase activity.

Ten KEGG pathways were enriched, with 4 overlapping with group analysis results, including lysosome, glycosaminoglycan degradation, regulation of actin cytoskeleton, and malaria. Literature searches were conducted for the correlation of enriched KEGG pathways with iron, with related literature detailed in Table 11.

[Figure 6: see original paper] Molecular function (MF) enrichment analysis of proteins commonly upregulated or downregulated in 3 or more rats (DAVID database GO analysis)

Table 11 KEGG pathway enrichment analysis of proteins commonly upregulated or downregulated in 3 or more rats (DAVID database GO analysis)

KEGG Pathway	Count	P-Value	Related to Iron
Lysosome	···	···	[124]

KEGG Pathway	Count	P-Value	Related to Iron
Sphingolipid metabolism	···	···	[125]
Other glycan degradation	···	···	[126]
Glycosaminoglycan degradation	···	···	···
Regulation of actin cytoskeleton	···	···	···
Gap junction	···	···	···
Phagosome	···	···	···
African trypanosomiasis	···	···	···
Cholesterol metabolism	···	···	···
Malaria	···	···	···

Discussion

Iron overload is generally defined as excessive accumulation of iron in the body beyond the range required for normal metabolism. This condition can be caused by various factors, such as long-term iron supplementation, genetic diseases (e.g., hereditary hemochromatosis), and chronic inflammatory states. In recent years, the occurrence and negative effects of iron overload have attracted attention. Iron overload occurs worldwide, particularly in economically developed regions, seriously affecting human health and safety (especially in children). Iron overload affects lipid peroxidation and nutrient metabolism, and is closely related to the occurrence and development of cardiovascular diseases. The health impacts of iron overload on organisms are multifaceted, including but not limited to increased intracellular oxidative stress, tissue damage, and impaired organ function, which can lead to serious cardiovascular and nervous system diseases.

In this study, the dose of polysaccharide-iron complex administered to rats was 28 mg/kg · d (iron basis), equivalent to the adult anemia prevention dose. According to literature review, the concentration of polysaccharide-iron complex used in this study would require gavage for more than 4 weeks to establish an iron overload model [**?**]. This study administered polysaccharide-iron complex (28 mg/kg · d iron) to rats for 4 days to investigate the systemic effects of short-term polysaccharide-iron complex gavage. This study is expected to provide clues for the prevention, diagnosis, treatment, and monitoring of iron metabolism disorders (such as anemia caused by iron deficiency and cardiovascular diseases caused by iron overload), filling the gap in urine proteomics in the field of iron metabolism.

This study employed both self-comparison and group comparison analysis methods, providing more comprehensive and reliable data validation. The application of self-comparison methods reduced the impact of individual differences on experimental results, improved experimental stability and reproducibility, and is of great significance for result credibility. The results from the two analysis methods validated each other, demonstrating that the urine proteome can reflect the effects of short-term polysaccharide-iron complex intake on the body, making the results more credible.

The results indicate that after short-term polysaccharide-iron complex intake, the rat urine proteome can display changes in iron-related proteins and biological functions. Short-term supplementation with polysaccharide-iron complex affects the body, and the urine proteome can comprehensively and systematically reflect the body' s overall changes. This study provides clues for in-depth understanding of iron metabolism processes, mechanisms, and biological functions in organisms from a urine proteomics perspective, while offering new research perspectives and methodological insights for future related studies, which has potential significance for the prevention, diagnosis, treatment, and monitoring of iron metabolism disorders.

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