# Changes in the Urine Proteome of Rats After Zinc Gluconate Gavage

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## Abstract

Zinc is an essential element for maintaining normal physiological functions in organisms. In this study, rats were administered 82 mg/kg · d of zinc gluconate (equivalent to a zinc dose of 11.7 mg/kg · d) via gavage for 4 days, and the urine proteome was comparatively analyzed before and after short-term zinc gluconate gavage. Many differential proteins have been reported to be associated with zinc, such as Mucin-2 (MUC-2) (14-fold higher before gavage than after, p=0.005), Transthyretin (3.9-fold higher after gavage than before, p=0.0004), and others. The biological processes (e.g., regulation of apoptotic process, immune system process, etc.), molecular functions (e.g., calcium ion binding, copper ion binding, signaling receptor activity, etc.), and KEGG pathways (e.g., complement and coagulation cascades, PI3K-Akt signaling pathway, etc.) enriched by differential proteins demonstrated relevance to zinc. This study explored the holistic effects of zinc on the organism from the perspective of urine proteomics, contributing to a deeper understanding of the biological functions of zinc and broadening the application potential of urine proteomics.

## Full Text

## Preamble

### Changes in the Urinary Proteome of Rats Following Intragastric Administration of Zinc Gluconate

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## Abstract

Zinc is an essential element for maintaining normal physiological functions in living organisms. In this study, rats were administered zinc gluconate at 82 mg/kg · d (equivalent to 11.7 mg/kg · d of elemental zinc) via intragastric gavage for 4 days. The urinary proteome was comparatively analyzed before and after short-term zinc gluconate administration. Many differential proteins have been reported to be associated with zinc, such as mucin-2 (MUC-2) (14-fold higher before vs. after gavage, $p = 0.005$) and transthyretin (3.9-fold higher after vs. before gavage, $p = 0.0004$). Enriched biological processes (e.g., regulation of apoptotic process, immune system process), molecular functions (e.g., calcium ion binding, copper ion binding, signaling receptor activity), and KEGG pathways (e.g., complement and coagulation cascades, PI3K-Akt signaling pathway) demonstrated correlations with zinc. This study explores the holistic effects of zinc on the organism from a urinary proteomics perspective, contributing to a deeper understanding of zinc' s biological functions and expanding the potential applications of urinary proteomics.

**Keywords:** zinc; urine; proteome; zinc gluconate; nutrients; mineral elements

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Zinc plays a vital role in various physiological processes. As a structural component and catalytic/regulatory cofactor for numerous enzymes and transcription factors, zinc participates in cellular DNA synthesis, protein synthesis, proliferation, maturation, death, immune responses, and antioxidant defense [1,2], while also serving as a regulatory and signal transduction element in intercellular and intracellular communication [3]. Zinc deficiency leads to various health problems, including growth retardation, immunodeficiency, hypogonadism, and neuronal and sensory dysfunction [4]. Abnormal zinc homeostasis is also associated with chronic diseases such as cancer, diabetes, depression, Wilson' s disease, and Alzheimer' s disease [5]. Zinc homeostasis in the body is regulated by zinc transporters and metallothioneins.

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 alterations in the body, making it a source of next-generation biomarkers [6]. Urinary proteins contain rich information that can reflect subtle changes in different systems and organs throughout the body. Our laboratory previously reported that the urinary proteome can systematically and comprehensively reflect the effects of magnesium L-threonate intake on the organism, with potential to provide clues for clinical nutrition research and practice [7].

Although the physiological functions of zinc have been extensively studied, no

research to date has investigated the holistic effects of zinc on the body from a urinary proteomics perspective. This study selected zinc gluconate as the supplement. Zinc gluconate is an organic zinc supplement with minimal gastric mucosal irritation, high bioavailability, good solubility, and wide application in health products, pharmaceuticals, and food. This study aims to investigate changes in the urinary proteome of rats after zinc gluconate intake, hoping to deepen understanding of zinc's physiological functions, provide new perspectives and clues for nutrition research, and contribute to more scientific guidance on human health and dietary regulation of trace elements.

### 2.1.1 Experimental Consumables

5 mL sterile syringes (BD), 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 reversed-phase peptide fractionation kit (Thermo Fisher Scientific, USA), iRT (indexed retention time, BioGnosis, UK).

### 2.1.2 Experimental Instruments

Rat metabolic cages (Beijing Jiayuan Xingye Technology), refrigerated high-speed centrifuge (Thermo Fisher Scientific, USA), vacuum concentrator (Thermo Fisher Scientific, USA), DK-S22 electric thermostatic water bath (Shanghai Jinghong Experimental Equipment), full-wavelength multifunctional microplate reader (BMG Labtech, Germany), shaker (Thermo Fisher Scientific, USA), TS100 thermomixer (Hangzhou Ruicheng Instrument), 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

Zinc gluconate was purchased from Shanghai Yuanye Bio-Technology (CAS No. 4468-02-4, molecular formula $C_{12}H_{22}ZnO_{14}$, purity $ 98*{4}HCO{3}$ (Sigma, Germany), urea (Sigma, Germany), purified water (Wahaha, China), MS-grade methanol (Thermo Fisher Scientific, USA), MS-grade acetonitrile (Thermo Fisher Scientific, USA), MS-grade pure water (Thermo Fisher Scientific, USA), Tris-Base (Promega, USA), and 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 gavage. Five healthy male SD (Sprague Dawley) rats aged 9 weeks (250 ± 20 g) were purchased from Beijing Vital River Laboratory Animal Technology. Rats were housed under standard conditions (room temperature (22 ± 2)°C, humidity 65%-70%) for 8 weeks until reaching 500-600 g before beginning experiments. All procedures followed the review and approval of the Ethics Committee of the College of Life Sciences, Beijing Normal University.

The tolerable upper intake level (UL) refers to the average maximum daily nutrient intake level that is unlikely to cause any adverse effects or health risks for almost all individuals in a specific physiological stage and gender group. The recommended nutrient intake (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 Dietary Guidelines for Chinese Residents, the UL for zinc is 40 mg/d [8]. Converting this human UL to rat dosage based on body surface area and weight yields approximately 3.6 mg/kg·d of elemental zinc, equivalent to 25.3 mg/kg·d of zinc gluconate.

In this study, rats received 11.7 mg/kg·d of elemental zinc (82 mg/kg·d of zinc gluconate), which is 3 times the UL. To prepare the gavage solution, 4.2 g of zinc gluconate was dissolved in 500 mL sterile water. After one week of normal feeding, each rat received 5 mL of zinc gluconate solution daily via gavage once per day for 4 consecutive days to establish a high-zinc model. The first gavage day was designated Zn-D1, and so on. Sampling time points were set before and after gavage for self-controlled comparison. Samples collected before gavage served as the control group (Zn-D0, sample numbers 36-40), while samples collected on day 4 of gavage served as the experimental group (Zn-D4, sample numbers 46-50).

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

### 2.2.2 Urine Sample Collection

On the day before mineral supplementation (D0) and 4 days after supplementation (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 12,000 × g for 30 minutes at 4°C to remove cell debris. The supernatant was mixed with 40 L of 1 M DTT stock solution (Sigma) to achieve a final DTT concentration of 20 mM, incubated in a metal bath at 37°C for 60 minutes, then cooled to room temperature. One hundred microliters of iodoacetamide (IAA, Sigma) stock solution were added to reach the working concentration, mixed, and reacted in

the dark at room temperature for 45 minutes. After the reaction, samples were transferred to new centrifuge tubes, mixed with three volumes of pre-cooled absolute ethanol, and precipitated at -20°C for 24 hours. Following precipitation, samples were centrifuged at 10,000 × g for 30 minutes at 4°C, the supernatant was discarded, and protein pellets were dried. Dried pellets were 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, urinary protein extracts were loaded onto 10 kDa ultrafiltration tubes (Pall, Port Washington, NY, USA), washed three times with 20 mM Tris solution, and resuspended in 30 mM Tris solution. Trypsin (Trypsin Gold, Mass Spec Grade, Promega, Fitchburg, WI, USA) was added at a 50:1 protein-to-enzyme ratio for digestion at 37°C for 16 hours. The filtrate containing the 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, and peptide concentration was determined using a BCA assay kit. Peptide concentration was adjusted to 0.5  g/ L, and 4  L from each sample was pooled as a mix.

### 2.2.4 LC-MS/MS Tandem Mass Spectrometry Analysis

All identification samples were spiked with iRT standard solution (diluted 100-fold) at a 20:1 sample-to-iRT ratio to unify retention time. Data-independent acquisition (DIA) was performed on all samples in triplicate, with one mix sample inserted every 10 runs as 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). Eluted peptides were analyzed by Orbitrap Fusion Lumos Tribrid mass spectrometer to generate raw files.

### 2.2.5 Data Processing and Analysis

Raw files acquired in DIA mode were imported into Spectronaut software for analysis. High-confidence proteins were defined as those with peptide q-value $< 0.01$. Protein quantification was performed using peak area quantification of all fragment ion peaks from secondary peptides, with automatic normalization. Proteins containing two or more specific peptides were retained, missing values were replaced with 0, and protein abundances were calculated for each sample. Samples collected before mineral supplementation were compared with those collected 4 days after supplementation 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 for biological processes, cellular localization, and molecular functions. Differential proteins and related pathways

were searched using 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/).

## 3.1 Differential Protein Analysis

After replacing missing values with 0 and comparing pre-gavage samples with day 4 post-gavage samples, 112 differential proteins were identified. Screening criteria were: t-test p-value $< 0.05$ and fold change (FC) $> 1.5$ or $< 0.67$. Five differential protein accession numbers could not be found in UniProt, possibly due to deleted or merged entries.

Using the UniProt database, protein accession numbers were entered into the search field to download protein names, functions, and GO analysis results. PubMed was used for functional analysis and literature retrieval of differential proteins, with detailed analysis of each protein' s relationship with zinc. The search method involved entering the protein name together with "zinc" in the PubMed search field, limited to title/abstract (e.g., "zinc[Title/Abstract] AND Protein[Title/Abstract]" ). Literature was then reviewed to confirm the relationship between differential proteins and zinc. The 107 differential proteins and related references are listed in Table 1 .

According to UniProt analysis, many differential proteins possess zinc ion binding functions, including acid sphingomyelinase-like phosphodiesterase 3a, membrane-bound carbonic anhydrase 14, ectonucleotide pyrophosphatase/phosphodiesterase family member 5 (E-NPP 5), and biliverdin reductase A. Hepcidin (FC = 1.9, p = 0.019) participates in biological processes including response to zinc ion, and zinc may be involved in regulating hepcidin production [42].

Zinc serves as a structural component of many proteins (such as various enzymes and transcription factors) and as a regulatory cofactor for some differential proteins, modulating protein activity. For example, dimethylarginine dimethylaminohydrolase 1 (DDAH-1) is inhibited by zinc ions, and DDAH-1 is downregulated in the hippocampus of zinc-deficient rats [22].

Twenty differential proteins met the criterion of $p < 0.01$, most of which have been reported to be zinc-related. Deoxyribonuclease-2-$\beta$ showed an FC of 0.05 and p-value of 0.0005. Deoxyribonuclease II is activated by intracellular acidification occurring during apoptosis, and zinc inhibits apoptosis-associated intracellular acidification, thereby suppressing deoxyribonuclease II [10]. Mucin-2 (MUC-2) showed an FC of 0.07 and p-value of 0.005. Offspring of high-zinc diet mothers showed increased MUC2 abundance in the jejunum [12].

Major prion protein (PrP) (FC = 1.7, p = 0.008) may be involved in neuronal zinc homeostasis [27]. Transthyretin (FC = 3.9, p = 0.0004) has a reciprocal relationship with zinc [57].

Pyridoxal phosphate homeostasis protein showed an FC of 4.5 and p-value of

0.006. Pyridoxal kinase showed an FC of 1.9 and p-value of 0.001. At physiological concentrations, zinc stimulates pyridoxal kinase activity, promoting pyridoxal phosphate formation [46].

Three differential proteins—seizure related 6 homolog like 2, ectodysplasin-A receptor, and Crk-like protein—showed infinite FC, meaning they were undetectable in pre-gavage samples but detected in post-gavage samples. According to literature, zinc is associated with neuronal injury and death after seizures [62,63]. The gene edar is inflammation-related and shows specific responses to zinc oxide nanoparticles (ZnO NPs) [61]. Due to space limitations, only a few examples are listed; differential proteins and related references are detailed in Table 1.

## 3.2 Biological Pathway Analysis

GO analysis of the 112 differential proteins (p-value $< 0.05$, FC $> 1.5$ or $< 0.67$) using the DAVID database enriched 83 biological processes (BP) (p-value $< 0.05$), as shown in Table 2 .

Zinc is essential for normal reproductive system function. Enriched biological processes include androgen catabolic process, positive regulation of estradiol secretion, and response to steroid hormone. Zinc is critical for androgen expression [64], and estradiol affects zinc homeostasis in ovarian follicles by controlling ZnT9 expression [65].

Adequate zinc intake reduces cardiovascular disease risk, and zinc plays a key role in maintaining vascular health by improving circulation and reducing arterial inflammation [66]. Enriched biological processes include blood coagulation, heart development, aorta morphogenesis, cardiac muscle cell proliferation, regulation of angiogenesis, vascular endothelial growth factor receptor-2 signaling pathway, and heart looping.

Zinc participates in brain neurotransmission, contributing to memory, learning, and cognitive function [1]. Enriched biological processes include synaptic vesicle lumen acidification, positive regulation of neuroblast proliferation, cerebrospinal fluid secretion, regulation of Schwann cell migration, and neural crest formation.

Zinc is involved in the production and regulation of immune system cells and aids wound healing [67]. Enriched biological processes include regulation of cytokine production involved in inflammatory response, inflammatory response, immune system process, wound healing, and regulation of phagocytosis.

Zinc protects cells from oxidative damage by free radicals and reduces oxidative stress [1]. Enriched biological processes include positive regulation of reactive oxygen species metabolic process and cellular response to hydrogen peroxide.

Zinc is important for regulating gene expression, DNA metabolism, chromatin structure, cell proliferation, maturation, and apoptosis [68]. Enriched biological processes include regulation of apoptotic process, cellular response to growth

factor stimulus, regulation of cell death, apoptotic process, regulation of cell proliferation, apoptotic DNA fragmentation, regulation of cysteine-type endopeptidase activity involved in apoptotic process, and regulation of gene expression.

As a structural component of many proteins (such as various enzymes and transcription factors), zinc participates in carbohydrate, protein, and lipid metabolism, aiding nutrient absorption [1]. Enriched biological processes include response to organic substance, cellular chloride ion homeostasis, cellular sodium ion homeostasis, response to vitamin D, aspartate metabolic process, and water homeostasis.

## 3.3 Molecular Function and KEGG Pathway Analysis

GO analysis of the 112 differential proteins (p-value $< 0.05$, FC $> 1.5$ or $< 0.67$) using the DAVID database enriched 24 molecular functions (MF) (p-value $< 0.05$), as shown in Table 3 .

Enriched molecular functions include cuprous ion binding, cupric ion binding, and copper ion binding. Complex interactions exist among iron, copper, and zinc [70,69]. Excessive zinc intake can cause copper deficiency, leading to reduced iron absorption and ultimately anemia [71].

Eighteen differential proteins were enriched for calcium ion binding, four for cadherin binding, and three for calcium-dependent phospholipid binding. There appears to be an interaction signal between zinc and calcium ions [5], and zinc homeostasis may be closely related to intracellular calcium signaling [72].

Zinc ions participate in extracellular signal recognition, signal transduction, and second messenger metabolism [67]. Six differential proteins were enriched for signaling receptor activity. Other enriched molecular functions include protease binding, receptor binding, ATPase activity, protein binding, and integrin binding.

Additionally, enriched biological processes included response to calcium ion, positive regulation of ERK1 and ERK2 cascade, regulation of protein kinase B signaling, cellular phosphate ion homeostasis, regulation of kinase activity, regulation of protein tyrosine kinase activity, Notch signaling pathway, enzyme-linked receptor protein signaling pathway, peptidyl-tyrosine phosphorylation, and positive regulation of protein phosphorylation.

GO analysis of the 112 differential proteins (p-value $< 0.05$, FC $> 1.5$ or $< 0.67$) using the DAVID database enriched 10 KEGG pathways (p-value $< 0.05$), as shown in Table 4 .

Enriched KEGG pathways include complement and coagulation cascades, human papillomavirus (HPV) infection, ECM-receptor interaction, regulation of actin cytoskeleton, hematopoietic cell lineage, metabolic pathways, and PI3K-Akt signaling pathway.

Chronic zinc deficiency significantly impairs cell-mediated immunity, antibody responses and affinity, complement system, and phagocytic activity [73]. High dietary zinc is negatively associated with high-risk HPV (hrHPV) infection [74]. In neuronal cells, zinc deficiency induces oxidative stress and alters normal cytoskeletal structure and dynamics [75]. Zinc released from lysosomes is essential for ERK and PI3K/Akt activation [67]. Excessive zinc intake can cause copper deficiency, leading to reduced iron absorption and ultimately anemia [71].

These results demonstrate that short-term zinc gluconate supplementation affects the organism, and the rat urinary proteome can reveal changes in zinc-related proteins and biological functions, indicating that urinary proteomics can comprehensively and systematically reflect holistic changes in the body. This study provides clues for understanding zinc' s metabolic processes, mechanisms of action, and biological functions in vivo from a urinary proteomics perspective, while offering new research perspectives and methodological insights for future nutrition studies.

This study has several limitations. Zinc gluconate was used as the supplement, but the analysis focused primarily on the correlation between differential proteins and biological functions with zinc, without addressing the effects of gluconate ions on the body. On one hand, zinc' s effects on the organism are more pronounced and easier to observe; on the other hand, research on the effects of gluconate ions is limited. Due to resource constraints, this study used only one concentration of zinc gluconate in 5 rats. Future studies should consider adding different supplement forms, concentration gradients, larger sample sizes, and expanded research subjects. We hope future researchers can build upon our methods and findings to further supplement and facilitate the translation and application of experimental results for the benefit of human health.

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