Changes of urinary proteome in rats after intragastric administration of zinc gluconate

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Abstract: Zinc is an essential element for maintaining normal physiological function in living organisms. In this study, the urine proteome of rats before and after short-term intragastric administration of 82 mg/kg/d zinc gluconate (equivalent to 11.7 mg/kg/d zinc) was compared and analyzed. Many differential proteins have been reported to be zinc-related, such as mucin-2 (MUC-2) (14 times before compared with after gavage, p = 0.005) and transthyretin (3.9 times after gavage compared with before gavage, p = 0.0004). Biological processes enriched in differential proteins (e.g., regulation of apoptosis process, immune system process, etc.), molecular functions (e.g., calcium binding, copper binding, signaling receptor activity, etc.), KEGG pathways (e.g., complement and coagulation cascades, PI3K-Akt signaling pathway, etc.) showed correlation with zinc. In this study, we explore the overall effect of zinc on the body from the perspective of urine proteomics, which is helpful to deeply understand the biological function of zinc and broaden the application potential of urine proteomics.

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

1 Introduction

Zinc plays a crucial role in various physiological processes. Zinc is a structural component and a catalytic and regulatory cofactor of many enzymes and transcription factors, and is involved in DNA synthesis, protein synthesis, proliferation, maturation, death, immune response, antioxidant defense, and other life processes in cells[1,2]. Zinc also acts as a regulatory and signal transduction element in intercellular and intracellular communication[3].

Zinc deficiency can lead to a variety of health problems, including growth retardation, immunodeficiency, hypogonadism, and neuronal and sensory dysfunctions[4]. Abnormalities in zinc homeostasis have also been linked to the development of chronic diseases such as cancer, diabetes, depression, Wilson's disease, and Alzheimer's disease[5]. Zinc homeostasis is regulated by zinc transporter proteins and metallothioneins.

Since urine is not part of the internal environment, in contrast to plasma, there is no mechanism for homeostasis, and it is able to accumulate early changes in the physiological state of the organism, reflecting more sensitively the changes in the organism, and is a source of next-generation biomarkers[6]. The proteins in urine contain a wealth of information that can reflect small changes produced in different systems and organs of the organism. Our laboratory has previously reported that the urine proteome is able to reflect the effects of magnesium threonate intake on the body in a more systematic and comprehensive manner, and has the potential to provide clues for clinical nutrition research and practice[7].

Although the physiological functions of zinc have been extensively studied, to date, there

have been no studies exploring the overall effect of zinc on the organism from the perspective of the urinary proteome. In this study, zinc gluconate was chosen as the supplement for the study population. Zinc gluconate is an organic zinc supplement with low gastric mucosal irritation, easy absorption in the body, and high absorption and solubility, which is widely used in health care products, pharmaceuticals and food.

The aim of this study was to investigate the changes in the urinary proteome of rats after zinc gluconate intake, in the hope of deepening the understanding of the physiological functions of zinc, providing new perspectives and new clues for nutritional studies, and contributing to a more scientific guidance for human health and dietary regulation of trace elements.

2 Materials and Methods

2.1 Experimental materials

2.1.1 Experimental consumables

5 ml sterile syringe (BD), gavage needle (16-gauge, 80 mm, curved needle), 1.5 ml/2 ml centrifuge tube (Axygen, USA), 50 ml/15 ml centrifuge tube (Corning, USA), 96-well cell culture plate (Corning, USA), 10 kD filter (Pall, USA), Oasis HLB solid phase extraction column (Waters, USA), 1ml/200ul/20ul pipette tips (Axygen, USA), BCA kit (Thermo Fisher Scientific, USA), high pH reverse peptide separation kit (Thermo Fisher Scientific, USA), iRT (indexed retention time, BioGnosis, UK).

2.1.2 Experimental apparatus

Rat metabolic cages (Beijing Jiayuan Xingye Science and Technology Co., Ltd.), frozen 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 enzyme labeling instrument (BMG Labtech, Germany), oscillator (Thermo Fisher Scientific, USA), TS100 constant temperature mixer (Hangzhou Ruicheng Instruments Co. BMG Labtech), electronic balance (METTLER TOLEDO, Switzerland), -80 □ ultralow-temperature freezing refrigerator (Thermo Fisher Scientific, USA), EASY-nLC1200 Ultra High Performance Liquid Chromatography system (Thermo Fisher Scientific, USA), and Orbitrap Fusion Lumos Tribird Mass Spectrometer (Thermo Fisher Scientific, USA) were used.

2.1.3 Experimental reagents

Zinc gluconate (Zinc gluconate) was purchased from Shanghai Yuanye Biotechnology Co., Ltd, CAS No. 4468-02-4, molecular formula C12H22ZnO14, purity over 98%. In addition, Trypsin Golden (Promega, USA), dithiothreitol (DTT) (Sigma, Germany), iodoacetamide (IAA) (Sigma, Germany), ammonium bicarbonate NH4HCO3 (Sigma, Germany), urea (Sigma, Germany), and purified water (Wahaha, China) were used, Methanol for mass spectrometry (Thermo Fisher Scientific, USA), Acetonitrile for mass spectrometry (Thermo Fisher Scientific, USA), Purified water for mass spectrometry (Thermo Fisher Scientific, USA), Tris-Base (Promega, USA), Thiourea (Sigma, Germany) and other reagents.

2.1.4 Analysis software

Proteome Discoverer (Version2.1, Thermo Fisher Scientific, USA), Spectronaut Pulsar (Biognosys, UK), Ingenuity Pathway Analysis (Qiagen, Germany); R studio (Version1.2.5001); Xftp 7; Xshell 7.

2.2 Experimental Methods

2.2.1 Animal modeling

The present study was conducted using 17-week-old rats to minimize the effects of growth and development during gavage. Five healthy SD (Sprague Dawley) 9-week-old male rats (250±20 g) were purchased from Beijing Viton Lihua Laboratory Animal Technology Co. The rats were kept in a standard environment (room temperature (22±2)□, humidity 65%-70%) for 8 weeks and weighed 500-600 g. The experiments were started, and all experimental operations followed the review and approval of the Ethics Committee of the School of Life Sciences, Beijing Normal University.

Dietary nutrient tolerable upper intake levels (UL, tolerable upper intake levels): the average daily maximum intake of a nutrient for a population of a certain physiological stage and gender, which has no side effects or risks to the health of almost all individuals. Recommended nutrient intakes (RNI): intake levels that meet 97-98% of the individual needs of a particular age, gender, or physiological group.

According to the Dietary Guidelines for Chinese Residents, the UL for zinc is 40 mg/d[8]. The UL for humans was converted to a dose for rats based on body surface area and body weight equal to approximately 3.6 mg/kg-d, i.e., 25.3 mg/kg-d of zinc gluconate. In the present study, the dose of zinc in rats was 11.7 mg/kg-d by gavage, and that of zinc gluconate was 82 mg/kg-d, which was three times the UL. 4.2 g of zinc gluconate was dissolved in 500 ml of sterile water and configured as a gavage solution. After one week of normal feeding, each rat was gavaged with 5 ml of zinc gluconate solution once a day for 4 consecutive days to establish a high zinc model. The first day of gavage was recorded as Zn-D1, and so on. Sampling time points were set before and after gavage for their own before and after control. The samples collected on the day before gavage were the control group, recorded as Zn-D0, and the samples were numbered 36-40, and the samples collected on the 4th day of gavage were the experimental group, recorded as Zn-D4, and the samples were numbered 46-50.

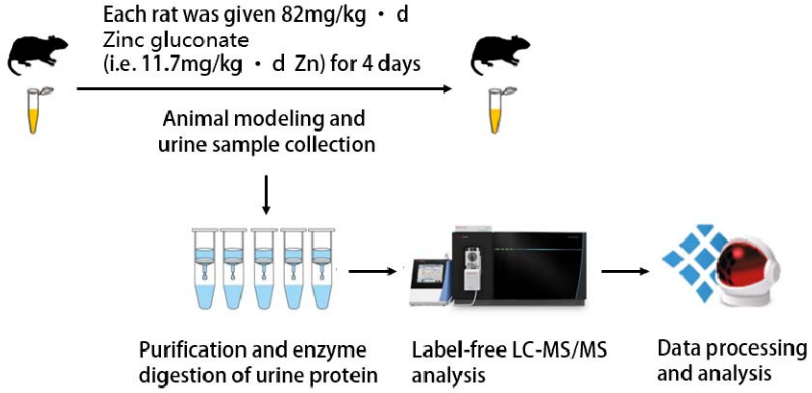

Fig. 1 Research methodology and technical route

2.2.2 Urine sample collection

One day before the start of gavage mineral supplementation (D0) and 4 days after gavage mineral supplementation (D4), each rat was individually placed in a metabolic cage at the same time, fasted and dehydrated for 12 h. Urine was collected overnight, and the urine samples were collected and placed in the refrigerator at -80°C for temporary storage.

2.2.3 Urine sample processing

Two milliliters of urine sample was removed, thawed, and centrifuged at 4 °C and 12,000×g for 30 minutes. The supernatant was removed, and 1 M dithiothreitol (DTT, Sigma) storage solution (40 µl) was added to reach the working concentration of DTT (20 mM). The solution was mixed well and then heated in a metal bath at 37 °C for 60 minutes and allowed to cool to room temperature.

Then, iodoacetamide (IAA, Sigma) storage solution (100 µl) was added to reach the working concentration of IAM, mixed well and then reacted for 45 minutes at room temperature protected from light. At the end of the reaction, the samples were transferred to new centrifuge tubes, mixed thoroughly with three times the volume of precooled anhydrous ethanol, and placed in a freezer at -20 °C for 24 h to precipitate the proteins.

At the end of precipitation, the sample was centrifuged at 4 °C for 30 minutes at 10,000×g, the supernatant was discarded, the protein precipitate was dried, and 200 µl of 20 mM Tris solution was added to the protein precipitate to reconstitute it. After centrifugation, the supernatant was retained, and the protein concentration was determined by the Bradford method.

Using the filter-assisted sample preparation (FASP) method, urinary protein extracts were added to the filter membrane of a 10-kD ultrafiltration tube (Pall, Port Washington, NY, USA) and washed three times with 20 mM Tris solution. The protein was resolubilized by the addition of 30 mM Tris solution, and the protein was added in a proportional manner (urinary protein:trypsin = 50:1) to each sample. Trypsin (Trypsin Gold, Mass Spec Grade, Promega, Fitchburg, WI, USA) was used to digest proteins at 37 °C for 16 h.

The digested filtrate was the peptide mixture. The collected peptide mixture was desalted by an Oasis HLB solid phase extraction column, dried under vacuum, and stored at -80 °C. The peptide mixture was then extracted with a 0.1% peptide mixture. The lyophilized peptide powder was redissolved by adding 30 µL of 0.1% formic acid water, and then the peptide concentration was determined by using the BCA kit. The peptide concentration was diluted to 0.5 µg/µL, and 4 µL of each sample was removed as the mixed sample.

2.2.4 LC-MS/MS analysis

All identification samples were added to a 100-fold dilution of iRT standard solution at a ratio of 20:1 sample:iRT, and the retention times were standardized. Data-independent acquisition (DIA) was performed on all samples, and each sample measurement was repeated 3 times, with 1-mix samples inserted after every 10 runs as a quality control. The 1-µg samples were 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 entered into the Orbitrap Fusion Lumos Tribird mass spectrometer for analysis, and the corresponding raw files of the samples were generated.2.2.5 Data processing and analysis

The raw files collected in DIA mode were imported into Spectronaut software for analysis, and the highly reliable protein standard was peptide q value<0.01. The peak area quantification method was applied to quantify the protein by applying the peak area of all fragmented ion peaks of secondary peptides, and the automatic normalization was processed.

Proteins containing two or more specific peptides were retained, and missing values were replaced with 0. The amount of different proteins identified in each sample was calculated, and samples from rats before gavage of mineral supplements were compared with samples from rats 4 days after gavage of mineral supplements to screen for differential proteins.

Unsupervised cluster 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 performed using the DAVID database (https://david.ncifcrf.gov/) to obtain results in 3 areas: biological process, cellular localization and molecular function. Differential proteins and related pathways were searched based on Pubmed database (https://pubmed.ncbi.nlm.nih.gov/). Protein interaction network analysis was performed using the STRING database (https://cn.string-db.org/).

3 Results and Discussion

3.1 Differential protein analysis

The missing values were replaced with 0. 112 differential proteins were screened by comparing the pre-gavage samples of rats with the samples on day 4 of gavage. Differential proteins were screened under the following conditions: p-value < 0.05 by t-test analysis, and Fold change (FC) > 1.5 or < 0.67. Sequence numbers of 5 differential proteins were not queried in Uniprot, probably because the entries had been deleted or merged.

Using the Uniprot database, Protein Accessions was entered into the search box to download the name function, GO analysis results of the proteins. Using PubMed database to analyze the protein function of differential proteins and literature search, the relationship between differential proteins and zinc was analyzed in detail one by one. The specific method is as follows: enter the protein name of the differential protein together with zinc into the Pubmed search box, and the search range is title/abstract, for example, "zinc [Title/Abstract] AND Protein [Title/Abstract]". The literature was then read to confirm the relationship between the differential proteins and zinc. The 107 differential proteins and related literature are listed in Table 1.

Table 1 Differential proteins in the comparative analysis of the Zn-D0 and Zn-D4 groups (p-value < 0.05, FC > 1.5 or < 0.67)

Uniprot Accessions	Gene Names	FC	P	Related to zinc
Q9QZK9	Dnase2b Dlad	0.0506	5E-04	[10,9]
Q62635	Muc2	0.071	0.005	[12,11]
Q6VPP3	Clca4 Clca6 Prp3	0.4802	0.041	
P00507	Got2 Maat	0.6139	0.03	[13]
F1LQU8	Prss30	1.5103	0.045	
P10247	Cd74	1.5134	0.022	[14]
G3V928	Lrp1	1.5187	0.05	
Q9WUC4	Atox1 Rah1	1.5274	0.007	[15]
P32038	Cfd Adn Df	1.5277	0.032	
P20611	Acp2	1.551	0.04	[16]
Q4QQW8	Plbd2 RDCR-0918-3	1.5535	0.014	
D3ZUD8	Tm9sf3	1.5539	0.009	
A0A0G2KA90	Dsc1	1.572	0.044	
Q5XI77	Anxa11 rCG_39189	1.5788	0.049	[17]
Q8K4G9	Nphs2	1.5907	0.003	
B5DF65	Blvrb	1.5917	0.036	[18]
Q66HT1	Aldob	1.5929	0.032	

P61972	Nutf2 Ntf2	1.5941	0.022	
Q05030	Pdgfrb Pdgfr Pdgfr1	1.5978	0.014	
A0A0H2UHY8	Aspa	1.6	0.048	[19]
B2RZB5	Chmp2a	1.6007	0.022	
A0A0H2UHL7	Il1r2	1.6023	0.018	[20]
P62815	Atp6v1b2 Atp6b2 Vat2	1.627	0.048	[21]
O08557	Ddah1 Ddah	1.627	0.04	[22]
G3V7W1	Pdcd6 Alg2	1.6292	0.032	
A0A0G2KAJ7	Col12a1	1.633	0.038	
A0A0G2JSK5	Itgb1	1.6443	0.034	
Q641Z7	Smpdl3a Asml3a	1.6488	0.039	
F1M4J1	LOC100294508	1.6561	0.043	
P07897	Acan Agc Agc1	1.6661	0.029	
G3V6T7	Pdia4	1.6706	0.005	[23]
Q01460	Ctbs Ctb	1.672	0.019	[24]
P97710	Sirpa Bit Mfr Ptpns1 Shps1 Sirp	1.6735	0.044	
P29975	Aqp1 Chip28	1.6756	0.019	[25]
Q5PPG2	Lgmn	1.6794	0.029	
Q5BJU0	Rras2	1.6818	0.021	[26]
D3ZUR5	Slurp2 RGD1308195	1.6849	0.03	
D3ZUM4	Glb1	1.6871	0.022	
Q80WF4	Tmem132a Gbp Hspa5bp1	1.6875	0.015	
Q9QYU4	Crym	1.6945	0.028	
P13852	Prnp Prn Prp	1.7001	0.008	[27]
D3ZAN2	Cdhr2	1.7042	0.028	[28]
D4A4S5	Folr2	1.7067	0.024	[29]
A2IBE0	Car14 Ca14 rCG_52058	1.7073	0.024	[30]
Q641Z8	Pef1	1.7094	0.012	
D3ZV56	Rnf150	1.7113	0.037	[31]
A0A0G2JXZ9	Ptprj	1.7196	0.04	[32]
P20760	Igg-2a	1.7246	0.017	[33]
G3V6A6	Chmp1b2	1.7278	0.016	
Q9QZK8	Dnase2 Dnase2a Dnl2	1.7285	0.02	[10,9]
P27590	Umod	1.7326	0.009	[34]
A0A0H2UI19	F12	1.7398	0.037	[35]
F1LZJ4	Hyi RGD1561416	1.7494	0.039	
Q5I0D5	Lhpp	1.7629	0.017	
Q63772	Gas6	1.7648	0.036	
Q9R066	Cxadr Car	1.7656	0.015	
G3V8M6	Folr1	1.771	0.047	[29]
D3ZET1	Nectin4 Pvrl4	1.7813	0.024	
A0A0G2JYC4	LOC100361907 Cfh LOC100364175	1.782	0.028	[37,36]
D3ZCG9	Itga3 ITGA3	1.7927	0.036	
Q642B4	Clec14a	1.7944	0.028	

Q9QYL8	Lypla2	1.8004	0.02	
A0A0G2K7Y0	Cr1l Cd46	1.8118	0.024	[38]
B0BMY7	Twf2 LOC684352	1.824	0.014	
D3ZXY4	Aldh8a1 LOC683474	1.825	0.04	[39]
Q5U362	Anxa4	1.8369	0.01	[40,17]
A0A0A0MXX5	Bdh2	1.8479	0.034	
B2GUV5	Atp6v1g1 rCG_55259	1.8597	0.044	[21]
G3V6D9	Nherf2 Slc9a3r2	1.8675	0.028	[41]
Q99MH3	Hamp Hepc	1.8785	0.019	[42]
G3V843	F2	1.8956	2E-04	[43]
P84039	Enpp5	1.9142	0.034	
Q6AYS7	Acy1a Acy1	1.9179	0.022	[44]
G3V647	Pdxk	1.9439	0.001	[46,45]
P38438	Tgfbr2	1.9797	0.024	[47]
P14668	Anxa5 Anx5	2.0246	0.025	[40]
A0A0G2JYP3	Rbm12b	2.0448	0.047	
Q75NI5	Cdh15 rCG_51596	2.052	0.036	[28]
P85971	Pgls	2.0661	0.03	
A0A0G2K676	Chia	2.0755	0.041	
P23606	Tgm1	2.1635	0.02	
F1LPT7	Chmp4c	2.1714	0.027	
A0A0G2K3Y5	Csf1r	2.2031	0.002	[48]
G3V9J1	Mug2	2.2639	0.011	
D3ZGN2	Cpne5	2.2899	2E-04	
C0JPT7	Flna	2.3252	3E-05	[49]
F1M7X4	Erbb4	2.4418	0.019	
P34900	Sdc2 Hspg1 Synd2	2.4541	0.043	
Q4QQV8	Chmp5	2.455	0.03	
F1M957	Vwf	2.5633	0.007	[50]
P81155	Vdac2	2.5944	0.036	[51]
P62749	Hpcal1	2.6987	0.048	
D4A1G1	Acyp2	2.794	0.006	
G3V7L8	Atp6v1e1	3.3257	0.002	[53,52]
P39069	Ak1	3.3922	0.015	[54]
P55280	Cdh6 Kcad	3.6041	0.029	[28]
Q07008	Notch1	3.7666	0.045	[55,52]
Q64361	Lxn	3.855	0.021	
P08721	Spp1 2b7 Spp-1	3.9115	0.038	[56]
P02767	Ttr Tt	3.9473	4E-04	[57]
D3ZCA0	Plpbp PROSC Prosc	4.5092	0.006	[46,45]
G3V8P3	Celsr2	6.5915	0.046	[28]
P46844	Blvra Blvr	6.8089	0.041	[58]
P23928	Cryab	7.7838	0.026	[60,59]
D3ZGP2	Edar	∞	0.045	[61]

D4A6P1	Sez6l2	∞	0.049	[63,62]
Q5U2U2	Crkl	∞	0.034	

According to Uniprot analysis, many differential proteins have the function of binding zinc ions, including acid sphingomyelinase-like phosphodiesterase 3a, membrane-bound carbonic anhydrase 14, ectonucleotide pyrophosphatase/phosphodiesterase family member 5 (E-NPP), and Biliverdin reductase A (BVR A). Hepcidin (FC=1.9, p=0.019) are involved in biological processes including the response to zinc ions. Zinc may be involved in the regulation of hepcidin production[42] .

Zinc is a structural component of many proteins (e.g., a variety of enzymes and transcription factors) and is a regulatory cofactor for a number of differential proteins that modulate protein activity. For example, dimethylarginine dimethylaminohydrolase 1 (DDAH-1) is inhibited by zinc ions. DDAH-1 is downregulated in the hippocampus of zinc-deficient rats[22]. The

There were 20 differential proteins that met $p<0.01$, most of which were reported to be associated with zinc.

Deoxyribonuclease-2-β had an FC of 0.05 and a p-value of 0.0005. Deoxyribonuclease II is activated by intracellular acidification that occurs during apoptosis, and zinc inhibits intracellular acidification associated with apoptosis, thereby inhibiting deoxyribonuclease II[10]. Mucin-2 (MUC-2) had an FC of 0.07 and a p-value of 0.005. Increased abundance of MUC2 was found in the jejunum of the offspring of mothers on a high-zinc diet[12].

The major prion protein (PrP) (FC=1.7,p=0.008) may be involved in neuronal zinc homeostasis[27]. There is a correlation between transthyretin (FC=3.9,p=0.0004) and zinc[57].

Pyridoxal phosphate homeostasis protein (PPSP) has an FC of 4.5 and a p-value of 0.006. pyridoxal kinase (PK) has an FC of 1.9 and a p-value of 0.001. At physiological concentrations, zinc stimulates pyridoxal kinase activity and promotes pyridoxal phosphate formation[46].

The FC for three differential proteins, Seizure related 6 homolog like 2, Ectodysplasin-A receptor, and Crk-like protein, was ∞, i.e., this protein was not detected in the pre-gastric sample and was detected in the post-gastric sample. protein was not detected in the pre-gastric samples, while it was detected in the post-gastric samples. According to the literature, zinc is associated with neuronal damage and death after seizures[62,63]. The gene edar is associated with inflammation and specifically responds to zinc oxide nanoparticles (ZnO NPs)[61]. Due to space limitations, only a few examples are listed, and the differential proteins, as well as the relevant literature, are detailed in Table 1.

3.2 Biological pathway analysis

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

Zinc is essential for the proper functioning of the reproductive system. Differential proteins are enriched to biological processes including androgen catabolic processes, positive regulation of estradiol secretion, and response to steroid hormones. Zinc is essential for androgen expression[64] , estradiol affects zinc homeostasis in the follicle by controlling the expression of ZnT 9[65].

Adequate intake of zinc reduces the risk of cardiovascular disease, and zinc plays a key role in maintaining vascular health by improving blood circulation and reducing arterial

inflammation[66]. Differential proteins are enriched to biological processes including blood coagulation, heart development, aortic morphogenesis, cardiomyocyte proliferation, regulation of angiogenesis, vascular endothelial growth factor receptor-2 signaling pathway, and cardiac circulation.

Zinc is involved in neural signaling in the brain, aiding memory, learning, and cognitive functions[1]. Differential proteins are enriched to biological processes including acidification of synaptic vesicle lumen, 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 cells of the immune system and can help heal wounds[67]. Differential proteins are enriched to biological processes including the regulation of cytokine production involved in the inflammatory response, inflammatory responses, immune system processes, wound healing, and the regulation of phagocytosis.

Zinc protects cells from oxidative damage by free radicals and reduces oxidative stress[1]. Differential proteins are enriched to biological processes including positive regulation of reactive oxygen species metabolic processes and cellular response to hydrogen peroxide.

Zinc is important for regulating gene expression, DNA metabolism, chromatin structure, cell proliferation, maturation, and apoptosis[68]. Differential proteins are enriched to biological processes including regulation of the apoptotic process, cellular response to growth factor stimulation, 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.

Zinc is a structural component of many proteins (e.g., many enzymes and transcription factors) and is involved in the metabolism of carbohydrates, proteins, and lipids, aiding in nutrient absorption[1]. Biological processes that differential protein enrichment to include response to organic matter, cellular chloride homeostasis, cellular sodium homeostasis, response to vitamin D, aspartate metabolic processes, and water homeostasis.

Table 2 Biological processes (BP) to which differential proteins were enriched in the Zn-D0 and Zn-D4 groups (P-value < 0.05)

Term	Count	%	P-Value
collecting duct development	4	3.9	2.40E-06
blood coagulation	6	5.9	4.00E-05
cell adhesion	10	9.8	1.20E-04
cell migration	9	8.8	1.50E-04
positive regulation of ERK1 and ERK2 cascade	8	7.8	2.60E-04
negative regulation of apoptotic process	12	11.8	3.30E-04
positive regulation of protein kinase B signaling	7	6.9	4.80E-04
multicellular organism development	5	4.9	5.00E-04
Homophilic cell adhesion via plasma membrane adhesion molecules	6	5.9	1.10E-03
heart development	8	7.8	1.40E-03
cellular phosphate ion homeostasis	3	2.9	1.60E-03
positive regulation of cell-substrate adhesion	4	3.9	1.80E-03
cellular response to growth factor stimulus	5	4.9	2.00E-03
response to organic substance	6	5.9	2.40E-03
cellular chloride ion homeostasis	3	2.9	2.90E-03

positive regulation of protein localization to plasma membrane	4	3.9	3.10E-03
positive regulation of cell migration	7	6.9	3.30E-03
synaptic vesicle lumen acidification	3	2.9	3.60E-03
aorta morphogenesis	3	2.9	4.10E-03
positive regulation of kinase activity	4	3.9	5.30E-03
phagocytosis	4	3.9	5.70E-03
cell-substrate adhesion	3	2.9	5.90E-03
cell-cell adhesion mediated by cadherin	3	2.9	7.00E-03
response to xenobiotic stimulus	9	8.8	7.10E-03
neuron migration	5	4.9	8.00E-03
negative regulation of cell proliferation	8	7.8	8.40E-03
cellular sodium ion homeostasis	3	2.9	9.30E-03
positive regulation of fibroblast proliferation	4	3.9	9.30E-03
negative regulation of cytokine production involved in inflammatory response	3	2.9	9.90E-03
positive regulation of protein tyrosine kinase activity	3	2.9	9.90E-03
negative regulation of collateral sprouting of intact axon in response to injury	2	2	1.00E-02
Cellular response to folic acid	2	2	1.00E-02
inflammatory response	7	6.9	1.10E-02
response to calcium ion	4	3.9	1.20E-02
cardiac muscle cell proliferation	3	2.9	1.20E-02
response to vitamin D	3	2.9	1.30E-02
positive regulation of angiogenesis	5	4.9	1.30E-02
regulation of actin cytoskeleton organization	4	3.9	1.40E-02
negative regulation of cell death	4	3.9	1.40E-02
positive regulation of reactive oxygen species metabolic process	3	2.9	1.50E-02
immune system process	3	2.9	1.60E-02
positive regulation of neuroblast proliferation	3	2.9	2.00E-02
folic acid transport	2	2	2.00E-02
negative regulation of cell projection organization	2	2	2.00E-02
heterophilic cell-cell adhesion via plasma membrane cell adhesion molecules	3	2.9	2.10E-02
positive regulation of epithelial cell migration	3	2.9	2.20E-02
positive regulation of peptidyl-tyrosine phosphorylation	4	3.9	2.30E-02
Notch signaling pathway	4	3.9	2.30E-02
cellular response to hydrogen peroxide	4	3.9	2.40E-02
folic acid import into cell	2	2	2.50E-02
positive regulation of protein monoubiquitination	2	2	2.50E-02
protein localization to nuclear pore	2	2	2.50E-02
cerebrospinal fluid secretion	2	2	2.50E-02
androgen catabolic process	2	2	2.50E-02
positive regulation of chemokine production	3	2.9	2.70E-02
dendrite morphogenesis	3	2.9	2.80E-02

muscle organ development	3	2.9	2.90E-02
chitin catabolic process	2	2	3.00E-02
response to Thyroglobulin triiodothyronine	2	2	3.00E-02
vascular endothelial growth factor receptor-2 signaling pathway	2	2	3.00E-02
apoptotic process	7	6.9	3.10E-02
outflow tract morphogenesis	3	2.9	3.30E-02
enzyme linked receptor protein signaling pathway	2	2	3.50E-02
positive regulation of estradiol secretion	2	2	3.50E-02
urate biosynthetic process	2	2	3.50E-02
positive regulation of Schwann cell migration	2	2	3.50E-02
positive regulation of cell proliferation	8	7.8	3.70E-02
heart looping	3	2.9	3.80E-02
positive regulation of gene expression	8	7.8	3.90E-02
response to steroid hormone	3	2.9	3.90E-02
COPII vesicle coating	2	2	4.00E-02
positive regulation of cytokine-mediated signaling pathway	2	2	4.00E-02
aspartate metabolic process	2	2	4.00E-02
wound healing	4	3.9	4.00E-02
animal organ development	3	2.9	4.00E-02
negative regulation of cysteine-type endopeptidase activity involved in apoptotic process	3	2.9	4.10E-02
peptidyl-tyrosine phosphorylation	3	2.9	4.40E-02
neural crest formation	2	2	4.40E-02
water homeostasis	2	2	4.40E-02
negative regulation of biomineral tissue development	2	2	4.40E-02
positive regulation of phagocytosis	3	2.9	4.50E-02
positive regulation of protein phosphorylation	5	4.9	4.60E-02
apoptotic DNA fragmentation	2	2	4.90E-02

3.3 Molecular function and KEGG pathway analysis

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

The molecular functions enriched to include cuprous ion binding, cupric ion binding, and copper ion binding. There are complex interactions between iron, copper and zinc[70,69]. Excessive zinc intake leads to copper deficiency, which leads to decreased iron absorption and ultimately anemia[71].

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

Zinc ions are involved in extracellular signal recognition, signal transduction and second messenger metabolism[67]. The 6 differential proteins are enriched to the molecular function of

signaling receptor activity. The molecular functions enriched to also include protease binding, receptor binding, ATPase activity, protein binding, and integrin binding.

Meanwhile, the biological processes that differential proteins are enriched to include response to calcium ions, positive regulation of ERK1 and ERK2 cascades, 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.

Table 3 Molecular functions (MF) enriched to differential proteins in Zn-D0 and Zn-D4 groups (P value < 0.05)

Term	Count	%	P-Value
calcium ion binding	18	17.6	1.60E-07
integrin binding	7	6.9	1.50E-04
identical protein binding	23	22.5	4.50E-04
protease binding	6	5.9	8.50E-04
cuprous ion binding	3	2.9	1.20E-03
signaling receptor activity	6	5.9	1.30E-03
receptor binding	8	7.8	4.70E-03
cadherin binding	4	3.9	5.70E-03
proton-transporting ATPase activity, rotational mechanism	3	2.9	1.00E-02
protein binding	18	17.6	1.00E-02
deoxyribonuclease II activity	2	2	1.10E-02
folic acid receptor activity	2	2	1.10E-02
extracellular matrix binding	3	2.9	1.30E-02
methotrexate binding	2	2	1.60E-02
ATPase binding	4	3.9	1.90E-02
protein homodimerization activity	10	9.8	1.90E-02
chitinase activity	2	2	2.10E-02
cupric ion binding	2	2	2.10E-02
transmembrane receptor protein tyrosine kinase activity	3	2.9	3.00E-02
calcium-dependent phospholipid binding	3	2.9	3.40E-02
phosphatase activity	3	2.9	3.90E-02
thyroid hormone binding	2	2	4.20E-02
chitin binding	2	2	4.20E-02
copper ion binding	3	2.9	4.70E-02

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

KEGG pathways enriched to include complement and coagulation cascades, Human papillomavirus (HPV) infection, ECM-receptor interactions, regulation of the actin cytoskeleton, hematopoietic cell profiles, metabolic pathways, and the PI3K-Akt signaling pathway.

Chronic zinc deficiency leads to a marked reduction in cell-mediated immunity, antibody response and antibody 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 the normal structure and dynamics of the cytoskeleton[75]. Zinc released from lysosomes is essential for ERK and PI3K / Akt activation[67]. Excessive zinc intake leads to copper deficiency, resulting in decreased iron absorption and ultimately anemia[71].

Table 4 KEGG pathways enriched to differential proteins in Zn-D0 and Zn-D4 groups (P value <0.05)

Term	Count	%	P-Value
Complement and coagulation cascades	5	4.9	3.40E-03
Human papillomavirus infection	9	8.8	3.50E-03
Focal adhesion	6	5.9	1.50E-02
Collecting duct acid secretion	3	2.9	1.60E-02
ECM-receptor interaction	4	3.9	2.50E-02
Regulation of actin cytoskeleton	6	5.9	2.60E-02
Hematopoietic cell lineage	4	3.9	2.70E-02
Metabolic pathways	19	18.6	3.20E-02
PI3K-Akt signaling pathway	7	6.9	3.90E-02
Endocytosis	6	5.9	4.50E-02

4 Perspective

The results illustrate that short-term zinc gluconate supplementation affects the organism, and the urine proteome of rats can show changes in zinc-related proteins and biological functions, and also suggests that the urine proteome can comprehensively and systematically reflect the overall changes in the organism. The present study provides clues for an in-depth understanding of the metabolic process, mechanism of action, and biological function of zinc in organisms from the perspective of urine proteomics, as well as new research perspectives and methodological insights for future nutritional studies.

However, the supplement used in this study was zinc gluconate, but the correlation of differential proteins and biological functions with zinc was analyzed, not the effect of gluconate ions on the organism. On the one hand, the effect of zinc on the organism is more significant and easier to observe; on the other hand, there are fewer studies on the effect of gluconate ion on the organism. Due to limited resources, only one concentration of zinc gluconate was used in this study to gavage five rats, and subsequent studies may consider adding different supplement forms, adding different concentration gradients, increasing the number of samples, and expanding the study population. We expect that subsequent researchers will utilize the methodology and results of this study to make further additions to help translate and apply the experimental results for the benefit of human health.

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