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Changes in Post-translational Modifications of the Urinary Proteome Following Short-term Administration of Magnesium, Calcium, Zinc, and Iron Supplements in Rats

Authors: Su Yan, Shen Ziyun, Youhe Gao, Youhe Gao

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Abstract

Magnesium, calcium, zinc, and iron are essential mineral elements indispensable for maintaining mammalian physiological functions. This study investigated the changes in post-translational modifications (PTMs) of the urinary proteome in SD rats of different weeks of age following short-term gavage administration of magnesium L-threonate (MgT), calcium gluconate, zinc gluconate, and polysaccharide iron complex. The results revealed that urinary proteins in all mineral supplement groups exhibited PTMs changes, with the polysaccharide iron complex group showing more pronounced modification changes. Additionally, significant differences in response to the same mineral were observed among rats of different weeks of age. The differentially modified proteins were involved in a wide range of functions, with some proteins such as serum transferrin and $ $2-microglobulin being closely associated with mineral metabolism. This study explored the functional changes in rat urinary proteins following short-term mineral supplementation from the perspective of PTMs, providing a novel perspective for research on the physiological functions of mineral elements.

Full Text

Preamble

Alterations in Urinary Proteome Post-Translational Modifications Following Short-Term Intake of Specific Magnesium, Calcium, Zinc, and Iron Supplements in Rats

Yan Su[1], Ziyun Shen[1], Youhe Gao[1]*
[1]Gene Engineering Drug and Biotechnology Beijing Key Laboratory, College of Life Sciences, Beijing Normal University, Beijing 100875, China

Abstract

Magnesium, calcium, zinc, and iron are essential mineral elements indispensable for maintaining physiological functions in mammals. This study investigated changes in post-translational modifications (PTMs) of the urinary proteome in Sprague-Dawley (SD) rats of different ages following short-term oral administration of magnesium L-threonate (MgT), calcium gluconate, zinc gluconate, and polysaccharide-iron complex. The results revealed PTM alterations in urinary proteins across all mineral supplement groups, with the most pronounced changes observed in the polysaccharide-iron complex group. Notably, rats of different ages exhibited distinct responses to the same mineral supplement. The differentially modified proteins were involved in a wide range of biological functions, and several of them—such as serotransferrin and beta-2-microglobulin—are closely associated with mineral metabolism. This study explores functional changes in urinary proteins from a PTM perspective following short-term mineral supplementation in rats, providing a novel viewpoint for investigating the physiological functions of mineral elements.

Keywords: mineral elements; magnesium L-threonate (MgT); calcium gluconate; zinc gluconate; polysaccharide-iron complex; urinary proteome; post-translational modifications (PTMs)

Introduction

Magnesium, calcium, zinc, and iron are essential mineral elements that maintain mammalian physiological functions. Magnesium ions serve as crucial cofactors for over 300 enzymes, participating in energy metabolism, DNA repair, and cell signaling, particularly by regulating ATPase activity to influence cellular energy supply. Magnesium deficiency can lead to metabolic disorders and neurological dysfunction [1]. Calcium is not only a primary component of bones and teeth but also acts as a second messenger critical for neurotransmission, muscle contraction, and hormone secretion [2]. Zinc functions as a transcription factor activator and antioxidant enzyme component, maintaining cellular homeostasis by regulating immune responses and promoting protein synthesis; zinc deficiency can cause growth retardation and immune dysfunction [3]. Iron, as a core component of hemoglobin and cytochromes, is fundamental for oxygen transport and redox reactions, and its metabolic imbalance is closely associated with anemia and oxidative stress [4]. In recent years, mineral supplements have gained increasing attention for their potential in preventing and treating metabolic diseases. Previous studies have shown that aging can affect mineral absorption rates, tissue distribution, and biological effects [5].

Urine, which is not regulated by homeostatic mechanisms, can more directly reflect real-time physiological and pathological changes in the body. Urinary proteomics, as a non-invasive, continuously sampleable technique rich in low-molecular-weight proteins and peptides, provides an important tool for studying physiological and pathological states [6]. In particular, protein

post-translational modifications (PTMs), such as phosphorylation, acetylation, and glycosylation, are extensively involved in cell signal transduction and metabolic regulation, offering a novel perspective for elucidating the molecular mechanisms of mineral metabolism [7].

This study selected four clinically common mineral supplements: magnesium L-threonate (MgT), calcium gluconate, zinc gluconate, and polysaccharide-iron complex. MgT is a novel magnesium supplement with high bioavailability that can elevate brain magnesium levels, improve neural plasticity, and effectively cross the blood-brain barrier to exert neuroprotective effects [8]. Calcium gluconate, as an organic calcium salt, causes less gastrointestinal irritation than inorganic calcium and is widely used for calcium supplementation due to its good water solubility and absorption rate [9]. Zinc gluconate demonstrates good intestinal tolerance and immunomodulatory effects in clinical trials, rapidly releasing zinc ions to exert short-term effects [10]. Polysaccharide-iron complex is a gentle iron supplement with minimal gastrointestinal side effects, high bioavailability, and good tolerability, making it suitable for short-term intervention studies [11].

This study aimed to investigate the effects of short-term oral administration of these four mineral supplements on PTMs in the urinary proteome of SD rats of different ages using high-throughput proteomics technology. By systematically analyzing PTM changes in rat urine, we explored the impact of short-term magnesium, calcium, zinc, and iron intake on protein modifications, providing new clues for understanding the physiological functions of mineral elements and offering a fresh perspective for nutrition research.

2.1 Experimental Animals and Model Establishment

Data for the mineral supplement intervention groups and control groups in this study were derived from previously published peer-reviewed articles and preprint platform results from our laboratory [12][13][14][15]. Healthy male SD rats (250$±20g)werepurchasedfromBeijingVitalRiverLaboratoryAnimalTechnologyCo., Ltd.Allratswereacclim humidity 65%-70%, 12-hour light-dark cycle) before experiments began. All experimental procedures were reviewed and approved by the Animal Ethics Committee of the College of Life Sciences at Beijing Normal University (approval number: CLS-AWEC-B-2022-003).

According to the Chinese Dietary Guidelines, the tolerable upper intake levels (UL) for calcium, zinc, and iron are 2000 mg/d, 40 mg/d, and 42 mg/d, respectively. Based on interspecies dose conversion using body surface area and weight, these correspond to rat intake levels of approximately 180 mg/kg · d for calcium, 3.6 mg/kg · d for zinc, and 37.8 mg/kg · d for iron, equivalent to doses of approximately 2000 mg/kg · d for calcium gluconate, 25.3 mg/kg · d for zinc gluconate, and about 28 mg/kg · d for polysaccharide-iron complex (calculated as elemental iron). Referencing Slutsky et al., the minimum effective dose of MgT for enhancing memory function in rats is 604 mg/kg · d (containing 50

mg/kg・d elemental magnesium), and MgT is considered the most suitable form of magnesium for oral administration, with a recommended dose of 100 mg/kg・d elemental magnesium [16][17]. Based on these criteria, the daily gavage doses for each group were established as shown in Table 1 .

All supplements were dissolved in sterile water to prepare gavage solutions: zinc gluconate group (4.2 g in 500 mL water), calcium gluconate group (16.125 g in 500 mL water), polysaccharide-iron complex group (3 g in 500 mL water), and MgT group (24.16 g in 200 mL water).

For the young group experiments, twelve 6-week-old rats were randomly divided into four groups (n=3), with each group receiving one mineral supplement (MgT, calcium gluconate, zinc gluconate, or polysaccharide-iron complex). For the adult group experiments, five rats were sequentially treated with MgT, calcium gluconate, zinc gluconate, and polysaccharide-iron complex at 14, 17, 18, and 19 weeks of age, respectively, with washout periods between treatments. During the experiments, rats were gavaged once daily for four consecutive days. Urine samples were collected the day before gavage (as control) and on day 4 of gavage (as experimental group) for self-controlled comparison. All urine samples were stored at -80°C.

2.2 Urine Sample Processing

Two milliliters of urine sample were thawed and centrifuged at 12,000×g for 30 minutes at 4°C. The supernatant was collected, and 40 L of 1M dithiothreitol (DTT, Sigma) stock solution was added to achieve a final DTT concentration of 20 mM. After thorough mixing, the solution was incubated in a metal bath at 37°C for 60 minutes, then cooled to room temperature. Subsequently, 100 L of iodoacetamide (IAA, Sigma) stock solution was added to reach the working concentration of IAM, mixed thoroughly, and reacted in the dark at room temperature for 45 minutes. After the reaction, samples were transferred to new centrifuge tubes, mixed with 3 volumes of pre-cooled absolute ethanol, and stored at -20°C for 24 hours to precipitate proteins. The precipitated samples were centrifuged at 10,000×g for 30 minutes at 4°C, the supernatant was discarded, and the protein pellet was dried before resuspension in 200 L of 20 mM Tris solution. After another 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 kD ultrafiltration tubes (Pall, Port Washington, NY, United States) and washed three times with 20 mM Tris solution. Proteins were resuspended in 30 mM Tris solution, and trypsin (Trypsin Gold, Promega, Fitchburg, WI, United States) was added at a protein-to-enzyme ratio of 50:1 for enzymatic digestion at 37°C for 16 hours. The digested filtrate containing the peptide mixture was collected, desalted using Oasis HLB solid-phase extraction columns, vacuum-dried, and stored at -80°C. The dried 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 to create a mixed sample.

2.3 LC-MS/MS Tandem Mass Spectrometry Analysis

Peptides were resuspended in 0.1% formic acid to a concentration of 0.5 g/ L and separated using a Thermo Easy-nLC 1200 nano-liquid chromatography system with a 90-minute gradient elution program. The mobile phase consisted of phase A (0.1% formic acid) and phase B (80% acetonitrile). Separated peptides were then analyzed using an Orbitrap Fusion Lumos Tribrid mass spectrometer in data-independent acquisition (DIA) mode.

2.4 Open-pFind Non-restrictive Modification Search

pFind Studio software (version 3.2.1, Institute of Computing Technology, Chinese Academy of Sciences) was used for unrestricted modification searching of each sample's raw mass spectrometry data using default parameters. The search database was the Rattus norvegicus protein database downloaded from UniProt (https://www.uniprot.org) with a version date of September 2024. Instrument type was set to HCD-FTMS, enzyme to trypsin, enzyme specificity to full, and maximum missed cleavage sites to 2. Mass error tolerances for both precursor and fragment ions were set to $±$20 ppm, and search mode was set to open search. The false discovery rate (FDR) threshold at the peptide level was set to 1%.

2.5 Bioinformatics Analysis of Protein Post-Translational Modifications

Following unrestricted modification searching, modification identification results (PROTEIN files) were obtained for each sample. A Python script (pFind_{{{protein}}*{{contrast}}}*{script}) was obtained from GitHub (https://github.com/daheitu/scripts_{{{for}}*{{pFind3}}}*{protocol}.io) to integrate modification information across different samples. Differentially modified proteins were identified by comparing experimental and control groups using the following criteria: fold change (FC) $\geq$ 2.0 or $\leq$ 0.5, and two-tailed paired t-test P-value $<$ 0.01. Differentially modified proteins were annotated and functionally queried using the UniProt database, and relevant literature was searched via PubMed (https://pubmed.ncbi.nlm.nih.gov) to further analyze the potential functions of these modifications.

3.1.1 Urinary Proteome PTM Analysis of MgT 9-Week-Old Group

Comparison of post-translational modifications between the experimental group after 4 days of gavage and the pre-gavage control group revealed one differential

modification in the 9-week-old group, affecting one protein type. Details are listed in Table 2 .

Table 2 Differentially selected modifications in the MgT 9-week-old group (FC$ $2.0 or $ $0.5, P<0.01)

Uniprot ID	Peptide	Modification	Control group	Experimental group	change	P value
P12346	EGVCPEGSIDSAPVK	4,Carbamidomethyl[C];	5.06E-03			

Literature searches for the identified modified proteins were conducted in the PubMed database. P12346 corresponds to Serotransferrin (FC=2.9, P=5.06E-03), which is primarily responsible for iron transport and cellular uptake. Transferrin saturation (TSAT) is an internationally recognized biomarker of iron nutritional status; TSAT decreases during iron deficiency with compensatory increased transferrin synthesis, while TSAT increases during iron overload. Currently, no studies have documented a relationship between serotransferrin and magnesium status [18].

3.1.2 Urinary Proteome PTM Analysis of MgT 14-Week-Old Group

Comparison between the experimental group after 4 days of gavage and the pre-gavage control group identified 29 differential modifications in the 14-week-old group, affecting 19 protein types. Details are listed in Table 3 .

Table 3 Differentially selected modifications in the MgT 14-week-old group (FC$ $2.0 or $ $0.5, P<0.01)

Uniprot ID	Peptide	Modification	Control group	Experimental group	change	P value
P36373	SQSFPHPLYEKPSLMR	1,Acetyl[S];	[illegible]33E-03			
Q9JHB9	SGSGCSLLQEVIR	5,Carbamidomethyl-DTT[C];	4.68E-03			
P02780	SGSGCSLLQEVIR	5,Carbamidomethyl-DTT[C];	4.68E-03			
P02761	QHIDVLENSLGFK	4,Asp->Ser[D];	4.81E-03			
Q9JHB9	ASGSGCSILDEVIR	0,Amidine[AnyN-term];	8.64E-03			
P02780	ASGSGCSILDEVIR	0,Amidine[AnyN-term];	8.64E-03			
P81827	DEICAWGVGMTTR	2,Glu->His[E];	9.01E-03			
P83121	DEICAWGVGMTTR	2,Glu->His[E];	9.01E-03			

Uniprot ID	Peptide	Modification	Control group	Experimental group	change	P value
P81828	DEICAWVVTTR	2,Glu->His[E];	9.01E-03			
P07647	VSQSFQDPLYIPVFMR	2,O-Ethyl-N,N-diMePhospho[S];	2.66E-03			
P81827	DEICAWVDVITR	4,Delta_H(4)C(3)O(1)[C];	6.13E-03			
P83121	DEICAWVDVITR	4,Delta_H(4)C(3)O(1)[C];	6.13E-03			
P81828	DEICAWVDVITR	4,Delta_H(4)C(3)O(1)[C];	6.13E-03			
P02781	ELEEFDAPPEAVEANLK	0,CH-12A[AnyN-term];	3.78E-03			
P07861	SESQMDITDINAPKPK	5,Oxidation[M];	6.89E-04			
P07151	KIPNIEMSDLSFSK	7,Oxidation[M];	6.65E-03			
P20059	GECQSEGVLFFQGNR	3,Carbamidomethyl[C];	3.04E-03			
P15083	CPVLVGTPQALVQEGYTER	2,Pro->Tyr[P];	3.07E-03			
P83121	TVEHGSIIVGGTNCC	13,Gly[N](Dicarbamidomethyl[C	3.82E-03			
P81828	TVEHGSIIVGGTNCC	13,Gly[N](Dicarbamidomethyl[C	3.82E-03			
P20767	QVTHEGNTVEK	2,Val->Ser[V];	4.64E-03			
P10960	TNSSFVQHLVDAHVK	2,HexNAc[N];	9.84E-03			
P36373	LLEDEPFASQHTC	0,SQHTC_{13C}(6)[AnyN-term];	7.76E-03			
P36374	LLEDEPFASQHTC	0,SQHTC_{13C}(6)[AnyN-term];	7.76E-03			
P81828	CTSFDSTGCGHVGR	10,Carbamidomethyl[C];	9.73E-03			
P13432	Q9R0T4	APDPTPLSNPPTQLHSTEQSDNQNKDAQVN-TLEVHVCDCEGTVNNCMK	0,Phenylisocyanate_{2H}(5)[AnyN-term]; 3.20E-03			
Q9R0T4	LSDNQNKDQVTTLEVHVDCEGTVNNCMK	18,Carbamidomethyl[C];20,Carbamidomethyl[C];27,Carbamidomethyl[C];28,Oxidation[M]	6.51E-03			
P07314	QPLSSMCPSHVDKDG	0,Gln->pyro-Glu[AnyN-termQ];6,Oxidation[M];7,Carbamidomethyl[C];	9.26E-03			

Literature searches revealed that some identified proteins have documented relationships with magnesium concentration changes. P07861 corresponds to Neprilysin (FC=0.4, P=6.89E-04), whose activity may be affected by magnesium concentration. Specifically, low magnesium concentrations (0.0 and 0.4 mM) cause a 50% decrease in Neprilysin activity without altering protein levels [19], suggesting that magnesium changes may regulate Neprilysin function through activity modulation. P07151 corresponds to Beta-2-microglobulin (FC=0.5, P=6.65E-03), which research has identified as an important independent factor for elevated serum concentration, with levels negatively correlated with serum magnesium concentration [20].

3.2.1 Urinary Proteome PTM Analysis of Calcium Gluconate 9-Week-Old Group

Comparison between the experimental group after 4 days of gavage and the pre-gavage control group identified one differential modification in the 9-week-old group, affecting one protein type. Details are listed in Table 6 .

Table 4 Differentially selected modifications in the calcium gluconate 9-week-old group (FC$ $2.0 or $ $0.5, P<0.01)

Uniprot ID	Peptide	Modification	Control group	Experimental group	change	P value
P24090	DCTGQEVTDPAK	2,Trioxidation[C];	2.49E-03			

Literature searches identified P24090 as Alpha-2-HS-glycoprotein (FC=0, P=2.49E-03). Studies have shown that calcification load is negatively correlated with serum Alpha-2-HS-glycoprotein levels. This protein can regulate calcium solubility and bioavailability by forming colloidal calcium-protein particles that circulate in the blood, helping to maintain calcium homeostasis [21].

3.2.2 Urinary Proteome PTM Analysis of Calcium Gluconate 16-Week-Old Group

Comparison between the experimental group after 4 days of gavage and the pre-gavage control group identified 11 differential modifications in the 16-week-old group, affecting 10 protein types. Details are listed in Table 7 .

Table 5 Differentially selected modifications in the calcium gluconate 16-week-old group (FC$ $2.0 or $ $0.5, P<0.01)

Uniprot ID	Peptide	Modification	Control group	Experimental group	change	P value
P27590	AHWSD[illegible]	4,Hex(1)NeuGc(1)[S];	[illegible]E-03			
P81827	LQCFR[illegible]	1,Acetyl[Protein N-term];15,Carbamidomethyl[C];	[illegible]E-03			
P07647	VGSICLA[illegible]SEMK	5,Carbamidomethyl[C];	[illegible]E-03			
P36373	TDLML[illegible]LEGGK	6,Cys->SecNEM[C];	[illegible].58E-03			
Q64230	AIIEHEI[illegible]GFFHEQSR	5,His->Glu[H];	[illegible]E-03			
P14841	QEADAS[illegible]VQRN	[illegible]Any N-term;	1.71E-03			
Q6IE52	PEPCGS[illegible]PETKR	0,[illegible][Any N-term];	[illegible]E-04			

Uniprot ID	Peptide	Modification	Control group	Experimental group	change	P value
P00786	CGSCWTFSTTGALESAVAK	7,Phe->Cys[F];	8.64E-03			
P22283	CSFQWEFCNK	1,Carbamidomethyl[C];8,Carbamidomethyl[C];	8.64E-03			
P15950	LVNCPFEEQHTEQLK	4,Dichlorination[C];	3.88E-03			
P36373	TDLMLCACELEGGK	6,Cys->SecNEM[C];	9.96E-03			

Literature searches revealed that some proteins show correlations with calcium concentration changes. P27590 corresponds to Uromodulin (FC=0, P=4.18E-03), a secreted protein primarily expressed in the thick ascending limb of Henle's loop that participates in urine concentration and calcium metabolism, regulating systemic calcium balance. In calcium metabolic regulation, Uromodulin can significantly stimulate the expression level of transient receptor potential vanilloid receptor 5 (TRPV5) on the apical membrane of renal tubular epithelial cells by inhibiting endocytosis, thereby enhancing calcium reabsorption efficiency. This regulatory mechanism represents an important protective mechanism against urinary stone formation [22]. Q6IE52 corresponds to Murinoglobulin-2 (FC=2.6, P=8.44E-04). Studies on its homolog $ $2-microglobulin show that calcium binding at physiological pH causes conformational changes, protein precipitation into amorphous forms, and subsequent transformation into amyloid aggregates. These aggregates exist as microscopic particles that do not progress to larger amyloid deposits. However, when renal function is impaired, particularly during dialysis, $ $2-microglobulin concentrations may temporarily increase, generating large aggregates that deposit in bone joints and convert to amyloid proteins during dialysis-related amyloidosis [23].

3.3.1 Urinary Proteome PTM Analysis of Zinc Gluconate 9-Week-Old Group

Comparison between the experimental group after 4 days of gavage and the pre-gavage control group identified six differential modifications in the 9-week-old group, affecting six protein types. Details are listed in Table 4.

Table 6 Differentially selected modifications in the zinc gluconate 9-week-old group (FC$ $2.0 or $ $0.5, P<0.01)

Uniprot ID	Peptide	Modification	Control group	Experimental group	change	P value
Q63041	CGNKVAEEVRALVQK	0,Ethylphosphate[AnyN-term];	9.85E-03			
P81827	QTYPDELSAWVVVPSHR	0,Succinyl_V{2H}(4)[AnyN-term];	8.16E-03			

Uniprot ID	Peptide	Modification	Control group	Experimental group	change	P value
P83121	QTYPDEIICSAWWVV	0,Succinyl_V{13}C(4)[AnyN-term];	8.16E-03			
P81828	QTYPDEIICSAWWVV	0,Succinyl_V{13}C(4)[AnyN-term];	8.16E-03			
P02761	VFMQHIDVLENSLGF	0,Cytopiloyne[AnyN-term];	9.85E-03			
P12346	DCTGNFCLFR	2,Carbamidomethyl[C];7,Carbamidomethyl[C];	2.31E-03			

Literature searches revealed that some proteins show correlations with zinc concentration changes. P12346 corresponds to Serotransferrin (FC=0.4, P=2.31E-03), an important zinc-binding protein in plasma that is closely related to dietary zinc intake and zinc homeostasis, participating in the regulation of zinc absorption, utilization, transport, and tissue distribution [24].

3.3.2 Urinary Proteome PTM Analysis of Zinc Gluconate 17-Week-Old Group

Comparison between the experimental group after 4 days of gavage and the pre-gavage control group identified 14 differential modifications in the 17-week-old group, affecting 11 protein types. Details are listed in Table 5.

Table 7 Differentially selected modifications in the zinc gluconate 17-week-old group (FC$ $2.0 or $ $0.5, P<0.01)

Uniprot ID	Peptide	Modification	Control group	Experimental group	change	P value
P81827	PDEICAWVMYVTTR	5,BMP-piperidinol[C];	4.46E-04			
P83121	PDEICAWVMYVTTR	5,BMP-piperidinol[C];	4.46E-04			
P81828	PDEICAWVMYVTTR	5,BMP-piperidinol[C];	4.46E-04			
Q6IRK9	GLLVDTVGPR	0,Ethylphosphate[AnyN-term];	4.64E-03			
P27590	AHWSDHCHLVSTHEK	4,dHex(1)Hex(2)[S];	4.09E-03			
P01835	DGVLDSVTDQDSK	5,Carboxy[D];	6.99E-03			
P01836	DGVLDSVTDQDSK	5,Carboxy[D];	6.99E-03			
Q64319	STNPASKGSDV	0,GSD_z-1_H_V{...}[AnyN-term];	5.88E-03			
P02761	LNGDWFSIVVASNK	5,Delta_H(4)O(2)[W];	5.38E-03			

Uniprot ID	Peptide	Modification	Control group	Experimental group	change	P value
P02761	LCEAHG[illegible]	1,Xle->Cys[L];	9.49E-03			
P02761	GNLDVA[illegible]	7,Delta_H(4)C(3)O(1)[K];	[illegible]			
Q9QZ76	EGDLAG[illegible]QEVLIS[illegible]	5,Ala->Tyr[A];	[illegible]E-03			
P04764	AMQEFM[illegible]GASS[illegible]	0,GIST-Quat_{2H}(3)[AnyN-term];	[illegible]E-03			
P02761	KLCEAH[illegible]	0,Succinyl[AnyN-term];	6.30E-03			

Literature searches revealed that some proteins show correlations with zinc concentration changes. Q64319 corresponds to Amino acid transporter heavy chain SLC3A1 (FC=0.4, P=3.88E-03). SLC3A1, SLC30, and SLC39 belong to different subfamilies of the solute carrier (SLC) family, with distinct yet potentially interconnected functions. The SLC30 family primarily transports zinc out of cells or sequesters it in specific cytoplasmic regions when intracellular zinc levels rise, maintaining physiological zinc concentrations [25]. The SLC39 family is mainly responsible for zinc uptake into cells, maintaining dynamic zinc balance [26]. While SLC3A1 is known primarily for amino acid transport and no direct link to zinc has been established, its potential association with zinc regulation warrants investigation given the complex interactions among SLC superfamily members in cellular metabolic networks and possible indirect connections between amino acid metabolism and zinc homeostasis. P01836 corresponds to Ig kappa chain C region, A allele (FC=0.3, P=6.99E-03). Zinc is an essential trace element for normal immune function, regulating immune cell activity and function. Both zinc deficiency and excess can affect immune responses, including antibody production and function [27].

3.4.1 Urinary Proteome PTM Analysis of Polysaccharide-Iron Complex 9-Week-Old Group

Comparison between the experimental group after 4 days of gavage and the pre-gavage control group identified 38 differential modifications in the 9-week-old group, affecting 31 protein types. Details are listed in Table 8 .

Table 8 Differentially selected modifications in the polysaccharide-iron complex 9-week-old group (FC$ $2.0 or $ $0.5, P<0.01)

Uniprot ID	Peptide	Modification	Control group	Experimental group	change	P value
P81827	ECIGTT[illegible]	2,Carbamidomethyl[C];	5.40E-0[illegible]			

Uniprot ID	Peptide	Modification	Control group	Experimental group	change	P value
P05545	DQAEINTXSALFIDK	5,Xle->Cys[I];	9.85E-03			
P00758	CVEAHKIEDVTDIMKCAGEMDGGK	1,Didehydro[C];	6.73E-03			
P83121	STGFCYVGR	0,ICPL_{13C}(6)[AnyN-term];	9.85E-03			
P81828	SFDSTGFCHVGR	2,Phe->Ser[F];	9.68E-03			
P81828	TGFCHVGRQKCQTYPDEICAWVVVTTR	19,Carbamidomethyl[C];	5.40E-04			
Q64240	CIQFYGGCK	1,Carbamidomethyl[C];9,Carbamidomethyl[C];	9.85E-03			
P27590	CQLQSLGPMK	0,Pyro-carbamidomethyl[AnyN-termC];	8.16E-03			
P81828	SFDSTGFCHVGRQKCQTYPDEICAWVVVTTR	23,Carbamidomethyl[C];	3.44E-03			
P05545	IRDEELSCSV	8,Carbamidomethyl[C];	3.44E-03			
P02770	TPTLVEAAR	0,ASD_z+2_{ion}[AnyN-term];	7.50E-04			
Q0PMD2	CAGESFQVVVR	1,Carbamidomethyl[C];	#DIV/0!			
Q9R0T4	SITGPGADKPPVGVFSR	9,Lys->CamCys[K];	7.89E-03			
P20611	FPLGPCPR	6,Carbamidomethyl[C];	#DIV/0!			
P83121	PDEICAWVVVTTR	5,Cys->Ser[C];	2.06E-03			
P81828	PDEICAWVVVTTR	5,Cys->Ser[C];	#DIV/0!			
P81827	PDEICAWVVVTTR	5,Cys->Ser[C];	3.44E-03			
P07522	NLDPASVEPR	0,Ethylphosphate[AnyN-term];	#DIV/0!			
P10960	SLPCDICK	4,Carbamidomethyl[C];7,Carbamidomethyl[C];	3.88E-03			
P07314	QPLSSMCPSIIVDK	7,Carbamidomethyl[C];	#DIV/0!			
P07314	QPLSSMCPSIIVDKDGK	7,Carbamidomethyl[C];	6.66E-03			
P02780	QCFLDQTDK	2,Carbamidomethyl[C];	#DIV/0!			
P22283	DYIEQNIHAVR	0,Carbamyl[AnyN-term];	5.06E-03			
P13635	DCNKPSPDDDIQDR	2,Carbamidomethyl[C];	#DIV/0!			
P83121	PDEICAWVVVTTR	5,NEM[C];	5.87E-03			
P81828	PDEICAWVVVTTR	5,NEM[C];	#DIV/0!			
P81828	TSFDSTGFCHVGR	0,Succinyl_{2H}(4)[AnyN-term];	3.87E-03			
P81827	PDEICAWVVVTTR	5,NEM[C];	#DIV/0!			
Q63041	VLGTLACQTQEIR	7,Carbamidomethyl[C];	#DIV/0!			
P19218	STINVEEDNGVSSESR	0,Ethylphosphate[AnyN-term];	#DIV/0!			

Uniprot ID	Peptide	Modification	Control group	Experimental group	change	P value
P01835	PPSTEQLATGGFNSVQL	16,betaA[C];	#DIV/0!			
P07522	VVRVNLDPASVPRN	0,NBS[Any N-term];	#DIV/0!			
P07522	VNLDPASVPR	3,Xle->Cys[L];	#DIV/0!			
P22282	DNCPFEEIQTEQLKR	3,Iodoacetamide_{13C}(6)[C];	#DIV/0!			
P02780	PYVQDHFTEK	1,Pro->Cys[P];	#DIV/0!			
P14046	GMYESLPVVAVK	2,Oxidation[M];	#DIV/0!			
P21704	WLIPDSADTTATSTHCAY	16,Carbamidomethyl[C];	#DIV/0!			

Note: #DIV/0! indicates a "from none to present" change in differential modification.

Literature searches revealed that some proteins show correlations with iron concentration changes. P02770 corresponds to Albumin (FC=9.5, P=3.44E-03). Studies show that albumin levels in iron-deficiency anemia patients are significantly lower than in healthy controls, and albumin levels positively correlate with serum iron, transferrin saturation, and ferritin levels [28], indicating that iron concentration changes may affect albumin levels. Q9R0T4 corresponds to Cadherin-1 (FC=18, P=3.44E-03). Research has found that Cadherin-1 expression significantly decreases in iron-overloaded hepatocytes, leading to weakened cell-cell adhesion [29]. P20611 corresponds to Lysosomal acid phosphatase (FC=26.3, P=1.73E-04). In iron-overloaded hepatocytes, both lysosome number and lysosomal acid phosphatase activity increase [30].

Among proteins with "from none to present" differential modifications: P13635 corresponds to Ceruloplasmin (P=5.06E-03). When iron load increases, ceruloplasmin promotes iron loading onto transferrin by increasing oxidase activity, thereby accelerating iron clearance, and its activity may be dynamically adjusted according to dietary iron load [31]. Q63041 corresponds to Alpha-1-macroglobulin (P=5.87E-03), which can bind free heme with high affinity. During dietary iron excess or hemolysis-induced heme release, plasma concentration and urinary excretion of this protein increase significantly, suggesting its potential as a biomarker for iron nutritional overload or hemolytic dietary stress [32]. P21704 corresponds to Deoxyribonuclease-1 (P=9.85E-03), whose activity is directly related to dietary iron intake. High-iron diets cause iron overload, leading to increased reactive oxygen species and apoptosis, ultimately resulting in elevated DNase-1 activity [33].

3.4.2 Urinary Proteome PTM Analysis of Polysaccharide-Iron Complex 16-Week-Old Group

Comparison between the experimental group after 4 days of gavage and the pre-gavage control group identified 61 differential modifications in the 17-week-old group, affecting 34 protein types. Details are listed in Table 9 .

Table 9 Differentially selected modifications in the polysaccharide-iron complex 17-week-old group (FC$ $2.0 or $ $0.5, P<0.01)

Uniprot ID	Peptide	Modification	Control group	Experimental group	change	P value
P07861	TWRRCANYVNGNMSNA	0,Unknown_{382}[AnyN-term];	3.82E-03			
P02761	MQHIDVLENSLGFKH	0,Acetyl_{2H}(3)[AnyN-term];	1.58E-03			
P02782	VVAETLLYCELECGMK	13,Carbamidomethyl[C];	8.58E-03			
P02780	GSGCSILDNVAR	0,[illegible][AnyN-term];	5.41E-03			
Q9JHB9	GSGCSILDNVAR	0,[illegible][AnyN-term];	5.41E-03			
P02761	VFMQHIDVLENSLGFK	0,Glycidamide[AnyN-term];	2.47E-03			
P22283	FSHDTYLSNIK	8,FormylS;	4.64E-03			
P02761	VFMQHIDVLENSLGFK	9,Xle->Ser[L];	2.89E-03			
P36373	VGSTCLASGWGSTK	0,Methylthio[AnyN-term];	2.06E-03			
P36375	VGSTCLASGWGSTK	0,Methylthio[AnyN-term];	2.06E-03			
P07861	QSNSLNYGGIGMVIGHELTHGFDDNGR	0,Gln->pyro-Glu[AnyN-termQ];	6.99E-03			
P02770	AADKDNCFATEGPNLVAR	0,Acetyl_{2H}(3)[AnyN-term];	7.25E-03			
P01835	SQRFPNAEEAEITK	0,Amidine[AnyN-term];	4.18E-03			
P07647	LVSQSFQHPDYIPVFMK	16,Oxidation[M];	6.05E-03			
P00689	VFVDNHLDNQR	0,Ethylphosphate[AnyN-term];	3.88E-03			
P02770	CVEDYLSAILNR	0,Formyl[AnyN-term];	2.84E-03			
P01835	ADYESHNLYTCEVVHK	11,Cys->Dha[C];	8.50E-03			
Q08420	MGLATSLAGPHSILGR	1,Oxidation[M];	8.15E-03			

Uniprot ID	Peptide	Modification	Control group	Experimental group	change	P value
P02770	RPCFSALTVDETYVPK	3,Cys->Dha[C];	4.46E-04			
P81827	ICQTYPDEICAWVVATTR	0,Ethylphosphate[AnyN-term];	4.64E-03			
P83121	ICQTYPDEICAWVVATTR	0,Ethylphosphate[AnyN-term];	4.64E-03			
P10960	NYVDQYSAEVAVQMMHEM	4,Asp->Cys[D];	4.81E-03			
Q08420	SAMLPPLDQRQITGLAVIFS	1,Hex(1)HexNAc(1)[S];	9.01E-03			
P81827	EICAWVVATTR	0,Acetyl[ProteinN-term];3,Carbamidomethyl[C];	3.88E-03			
P81828	EICAWVVATTR	0,Acetyl[ProteinN-term];3,Carbamidomethyl[C];	3.88E-03			
P83121	EICAWVVATTR	0,Acetyl[ProteinN-term];3,Carbamidomethyl[C];	3.88E-03			
P07522	VNLDPASXPPR	3,Xle->Cys[L];	6.15E-03			
P02761	MQHIDVLENSLGFK	0,Diisopropylphosphate[AnyN-term];	6.18E-03			
P85971	LVPFDHAESTYGLYR	2,Val->Gln[V];	1.88E-03			
P08649	EPFLSCCK	6,Carbamidomethyl[C];7,Carbamidomethyl[C];	5.65E-05			
P01835	DSTYSMSSTLSLTK	1,Cation_{Fe}{2}[III][D];	3.11E-03			
P01836	DSTYSMSSTLSLTK	1,Cation_{Fe}{2}[III][D];	3.11E-03			
P10252	ILEYFPNGK	7,Deamidated[N];	3.08E-03			
P02761	KLCEAHGNTC	0,NTC[AnyN-term;	4.81E-03			
Q6DGG1	QDPMGSSSFTQHLK	0,SBF[AnyN-term];	8.44E-04			
Q63041	FGQVLLVDEK	1,Phe->Arg[F];	4.97E-03			
P81828	LQCFRCTSFDSTGEGNSVGR	0,Formyl[AnyN-term];3,GGC;15,Carbamidomethyl[C];	3.38E-03			
O35217	KEPLTAYNPEEFQVHR	0,Phenylisocyanate_{2H}(5)[AnyN-term];	8.58E-03			
P01835	TYSMSSTLSLTK	0,Phosphogluconoylation[AnyN-term];	8.58E-03			
P01836	TYSMSSTLSLTK	0,Phosphogluconoylation[AnyN-term];	8.58E-03			
P07522	LAINWIDGESHR	0,ESP[AnyN-term];	4.64E-03			
P27590	TENGESSQAR	0,Iodoacetanilide[AnyN-term];	4.64E-03			

Uniprot ID	Peptide	Modification	Control group	Experimental group	change	P value
P14046	YMVLVPSQLYTETPEK	6,Pro->Asn[P];	8.58E-03			
Q03626	YMVLVPSQLYTETPEK	6,Pro->Asn[P];	8.58E-03			
Q6IE52	YMVLVPSQLYTETPEK	6,Pro->Asn[P];	8.58E-03			
P02761	GETFQLMVLYGR	0,Any N-term;	3.88E-03			
Q63041	QQNSHGLTSSTQDTAVALQ	11,Thr->Asn[T];	4.81E-03			
P81827	CAWVVVTDER	1,Delta_S(-1)Se(1)[C];	8.77E-03			
P81828	CAWVVVTDER	1,Delta_S(-1)Se(1)[C];	8.77E-03			
P83121	CAWVVVTDER	1,Delta_S(-1)Se(1)[C];	8.77E-03			
Q08420	DQPQITGSMLFR	1,SulfanilicAcid_{13C}(6)[D];	6.99E-03			
P16391	EDLKTWTAADMAAQITR	4,BHAc[K];	5.37E-03			
P07522	DLSKEVASLIRGGSPK	4,LRGG+dimethyl[K];	2.89E-03			
Q9QX79	DGYMLTLQR	4,Oxidation[M];	6.99E-03			
P14841	PQEADASEEGVQR	3,Cation_Li[E];	4.46E-04			
P36373	VGSTCLASGWGSTK	5,Delta_S(-1)Se(1)[C];	#DIV/0!			
P36375	VGSTCLASGWGSTK	5,Delta_S(-1)Se(1)[C];	3.88E-03			
P13432	PDPNGQSIPIVCTLIPSNVQP	0,Acetyl[AnyN-term];	#DIV/0!			
P01835	DSTYSMSSTLSLTK	0,Formyl[AnyN-term];6,Oxidation[M];	8.58E-03			
P01836	DSTYSMSSTLSLTK	0,Formyl[AnyN-term];6,Oxidation[M];	8.58E-03			
Q9ES87	SLQTPRPLQQLFHPS	0,Delta_H(2)C(2)[AnyN-term];	2.52E-03			

Literature searches revealed that some proteins show correlations with iron concentration changes. The 17-week-old group shared several iron-related differentially modified proteins with the 9-week-old group, such as Albumin and Alpha-1-macroglobulin. Additionally, P02782 corresponds to Prostatic steroid-binding protein C1 (FC=0.1, P=8.58E-03), which is associated with hepcidin synthesis and secretion in prostate cancer cells. Hepcidin is a key iron regulatory protein whose expression and secretion are significantly increased in prostate cancer cells and tissues [34]. PSBP C1 may influence iron metabolism by reg-

ulating hepcidin expression. P00689 corresponds to Pancreatic alpha-amylase (FC=0.5, P=3.88E-03). Studies have found that iron regulatory proteins are significantly correlated with pancreatic alpha-amylase levels, and iron metabolism regulation may affect pancreatic cell physiological functions, thereby influencing alpha-amylase synthesis and secretion [35].

Notably, both 9-week-old and 17-week-old groups shared a common differential modification: Pro-epidermal growth factor (Pro-EGF) modification "3,Xle->Cys[L]" on the "VNLDPASVPPR" sequence, with FC values of #DIV/0! (indicating "from none to present" change) and 2.8, respectively. The epidermal growth factor receptor (EGFR) plays a key role in iron metabolism by regulating transferrin receptor 1 distribution [36]. As a member of the EGF family, Pro-EGF expression and function may be regulated by EGFR signaling pathways, thereby indirectly affecting iron metabolism.

Discussion

This study explored changes in urinary proteome PTMs following short-term gavage of four common mineral supplements—magnesium L-threonate, calcium gluconate, zinc gluconate, and polysaccharide-iron complex—in rats. Changes in protein concentration and post-translational modifications provide complementary information, characterizing the effects of short-term, low-dose interventions from two different dimensions.

At the modification level, all mineral groups exhibited differential modification sites, with polysaccharide-iron complex gavage inducing the most numerous PTM changes, suggesting that iron metabolism may have a strong influence on protein modification regulation. These differentially modified proteins have diverse functions, encompassing metal ion binding, immune modulation, oxidative stress response, and other aspects. These results not only align with known biological knowledge of mineral-protein interactions but also suggest that urinary PTM analysis can serve as a novel tool for revealing mineral physiological functions.

Age factors also demonstrated distinct effects in this study, with the same mineral inducing markedly different PTM changes in rats of different ages. This is consistent with known phenomena of age-related changes in absorption efficiency, enzyme activity, and hormonal environments, further emphasizing the necessity of considering age factors in nutritional interventions [5].

However, it should be noted that functional annotations for most identified PTMs in mineral metabolism remain incomplete in the current field, which objectively limits the depth of analysis of specific modification events in this study. This indicates that future research should employ targeted modification proteomics combined with functional experiments to clarify the effects of key modification sites on mineral homeostasis and related physiological functions. Additionally, this study has certain limitations. While we focused on analyzing the correlation between differentially modified proteins and magnesium, cal-

cium, zinc, and iron metabolism, and although the selected supplements are widely used compounds in clinical and basic research with relatively small non-specific effects from carrier components, other components such as threonate, gluconate, and polysaccharide carriers may still have potential effects on the body. Moreover, the relatively limited sample size may affect the generalizability and statistical power of the results. However, as an exploratory study, its primary purpose is to preliminarily characterize the features and trends of mineral-protein modification interactions and provide clues and directions for subsequent in-depth mechanistic investigations. Future studies should consider expanding sample sizes and establishing appropriate carrier control groups to exclude potential effects of non-mineral components. Furthermore, combining targeted validation, functional experiments, and multi-omics integration strategies will help uncover key modification events regulated by minerals and their physiological and pathological significance, providing new theoretical foundations for precision nutrition interventions.

In summary, this study preliminarily explored changes in the rat urinary proteome following short-term supplementation with magnesium L-threonate, calcium gluconate, zinc gluconate, and polysaccharide-iron complex from a PTM perspective. We identified differentially modified proteins consistent with previously studied mineral metabolism-related proteins, providing clues for understanding the metabolic processes and biological functions of magnesium, calcium, zinc, and iron in rats and offering a new window for nutrition research.

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