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# Different Zinc Sources Have Diverse Impacts on Gene Expression of Zinc Absorption Related Transporters in Intestinal Porcine Epithelial Cells

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**Abstract** This study was conducted to investigate the effects of zinc sources on gene expression of zinc-related transporters in intestinal porcine epithelial cells (IPEC-1). IPEC-1 cells were treated with zinc glycine chelate (Zn-Gly), zinc methionine (Zn-Met), and zinc sulfate ($ZnSO_4$), respectively, for measurement of cell viability. Then, the relative expression of zinc-related transporters in IPEC-1 in response to different zinc sources (50 μmol/L zinc) was measured. Zinc transporter SLC39A4 (ZIP4) expression was selectively silenced to assess the function of ZIP4 in inorganic and organic zinc absorption. The result showed that Zn-Gly and Zn-Met had lower cell damage compared with $ZnSO_4$ on the same zinc levels. Different zinc sources improved the expression of metallothionein1 (MT1) and zinc transporter SLC30A1 (ZnT1) messenger RNA (mRNA) compared with the control ($P < 0.05$), while ZIP4 decreased ($P < 0.05$) in response to zinc addition. MT1 and ZnT1 mRNA expressions in Zn-Gly and Zn-Met were higher than those in $ZnSO_4$, and ZIP4 mRNA expression in Zn-Met was the lowest among three kinds of zinc sources ($P < 0.05$). Expression of divalent metal transporter 1 (DMT1) mRNA in control was significantly higher ($P < 0.05$) than added different zinc sources groups. Silencing of ZIP4 significantly decreased MT1 mRNA expression in $ZnSO_4$ and Zn-Gly treatments, reduced zinc absorption rate, and increased DMT1 mRNA expression in $ZnSO_4$ compared with negative control. In summary, different zinc sources could improve zinc status on IPEC-1 cells and organic zinc had lower cell damage compared with $ZnSO_4$. Moreover, Zn-Gly and Zn-Met are more efficient on zinc absorption according to the expression of various zinc-related transporters MT1, ZIP4, ZnT1, and DMT1. ZIP4 played a direct role in inorganic zinc uptake, and the absorption of zinc in Zn-Gly depends on ZIP4 partly, while absorption of Zn-Met is less dependent on ZIP4.

**Keywords** Zinc glycine chelate · Zinc methionine · Zinc sulfate · Zinc transporters · RNA interference

## Introduction

Zinc plays critical roles in various biochemical processes and functions, as intracellular/intercellular signaling component and structural/catalytic center of diverse metalloenzymes [1–3]. Zinc homeostasis mainly relies on the subcellular, cellular, tissue, and the whole body by active transport, which depends on zinc uptake, intracellular storage, and excretion [4, 5]. Small intestine, as a major site of zinc absorption, alters body zinc status by changing zinc absorption efficiency by largely unknown molecular adaptation mechanisms. To date, a number of proteins involved in intestinal zinc metabolism have been identified [6, 7], such as MT1, DMT1, ZIP4, and ZnT1. MT1 acts as a main zinc-binding protein in cell cytosol. And, DMT1 is involved in divalent metal uptake in enterocytes, including zinc and iron. ZIP4 belongs to ZIP family (solute-linked carrier SCL39), which mainly transports zinc ions from outside into cytoplasm. Conversely, ZnT1 belongs to ZnT family (solute-linked carrier SLC30), which mainly decreases intracellular zinc levels.

Many research studies have confirmed that zinc amino acid chelates have better bioavailability than inorganic zinc in animals [8–12]. However, few data are available regarding the absorption characteristics of zinc amino acid chelate in small

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intestine. Therefore, the purposes of current study were to compare the effects of Zn-Gly, Zn-Met, and $ZnSO_4$ on expression of zinc-related transporters and detect the function of ZIP4 on organic zinc absorption in IPEC-1 cells.

## Materials and Methods

### Cell Culture

IPEC-1 cells were derived from the un-suckled newborn piglets [13]. The cells were cultured in DMEM/F12 medium containing 10 % FBS, 2 mmol/L L-glutamine, insulin (5 mg/L), transferrin (5 mg/L), selenium (5 μg/L), epidermal growth factor (5 μg/L), and penicillin-streptomycin (Sigma) at 37 °C in a 5 % $CO_2$ atmosphere. IPEC-1 cells from passages 30–50 were used in present study.

### Cell Viability Assay

IPEC-1 cells were seeded in 96-well cell culture plates at a density of $10^4$. When approximate 80 % confluence was reached, cells were treated with DMEM containing Zn-Gly, Zn-Met, and $ZnSO_4$, respectively, with final zinc concentrations of 0, 50, 100, 150, and 200 μmol/L. After incubation for 6, 12, and 24 h, cell viability was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Following the treatments (Zn-Gly, Zn-Met, and $ZnSO_4$), 20 μL MTT (5 mg/mL) was added per well and then incubated for 4 h at 37 °C. After removal of the medium, formazan was dissolved by 150 μL DMSO and absorbance was measured at a wavelength of 570 nm by an enzyme-linked immunosorbent assay reader (Bio-Rad). Cell viability was calculated as previously described [14].

### MT1, DMT1, ZIP4, and ZnT1 mRNA Expressions in IPEC-1 Cells

IPEC-1 cells ($10^5$) were seeded in six-well plates and then cultured with 50 μmol/L zinc concentration of Zn-Gly, Zn-Met, and $ZnSO_4$, respectively, for 6 h, and 0 μmol/L zinc concentration was set as control. Total RNA was isolated from IPEC-1 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and 0.5 μg RNA was reverse-transcribed into cDNA in a final volume of 10 μL reaction by PrimeScript ™ RT reagent kit (Takara, Tokyo, Japan). Then, the reaction was incubated at 30 °C for 10 min, 42 °C for 30 min, and 70 °C for 15 min. Real-time PCR was performed by an iQ™ 5 Real-Time PCR Detection System (Bio-Rad Inc., Hercules, CA, USA). The following protocol was used: 1 min at 95 °C, and then for 40 cycles of amplification (10 s at 95 °C and 25 s at 63 °C). Primer sequences for MT1, DMT1, ZIP4, ZnT1, and β-actin were designed with Primer Express 2.0 (Table 1). Results were normalized to β-actin and the relative gene expression level was determined by $2^{-\Delta\Delta Ct}$ method [15].

### ZIP4 siRNA Transfection

The ZIP4 small Interfering RNA (siRNA) duplex used in this study was as follows: ZIP4-s, 5′-CUCAG UACUUCGUGGACUUTT-3′, and ZIP4-a, 5′-AAGUCCACGAAGUACUGAGTT-3′. And, the negative control siRNA duplex (s, 5′-UUC UCC GAA CGU GUC ACG UTT-3′; a, 5′-ACG UGA CAC GUU CGG AGA ATT-3′) was used to verify the selective effect of siRNA. IPEC-1 cells were seeded in six-well plates and transfected with siRNA, oligonucleotides 1 to 4, and negative control (Table 2). The siRNA transfections (100 pmol per well in six-well plates) were incubated with Lipofectamine™2000 Reagent (Invitrogen, Carlsbad, CA, USA) following protocols provided by manufacturers. Total RNA was isolated at 24 and 48 h, and ZIP4 messenger RNA (mRNA) expression was analyzed by real-time PCR as described above. ZIP4 expression was normalized to β-actin.

### Transfection with ZIP4 siRNA on Cell Viability

Control, negative control, and ZIP4-siRNA-transfected cells ($10^5$ cells/mL) were seeded in 96-well plates. The control contains 500 μL DMEM + 1.5 mL DMEM/F12. The negative control contains negative siRNA. After 24- and 48-h incubation, the cells were harvested for cell viability by MTT using the same protocol as described above.

### MT1 and DMT1 Expression and Zinc Absorption Rate

Negative control and ZIP4 siRNA were transfected following the same protocol described above. After 24-h incubation, cells were treated with 50 μmol/L Zn-Gly, Zn-Met, and $ZnSO_4$ for another 6 h, respectively. Then, cells were collected for measurement of gene expression and intracellular zinc absorption rate. MT1 and DMT1 expressions were determined as described above. Intracellular zinc absorption rate was tested as previously described [16]. IPEC-1 cells were washed twice with cold phosphate-buffered saline (PBS), continuously washed with 1 mmol/L EDTA and PBS, and then digested with nitric acid and diluted with H2O to 10 mL when measured. Intracellular zinc concentration was tested by inductively coupled plasma mass spectrometry (ICP-MS).

### Statistical Analysis

Data were analyzed by SPSS (version 19.0) using one-way ANOVA. Differences between means were determined by a Tukey's honestly significance difference test. Gene expression and zinc concentration in transfected cells were conducted by

**Table 1** Primer sequences for quantitative real-time PCR

| Item | No. in Genbank | Primer sequences (5′ to 3′) | Length |
|---|---|---|---|
| β-actin | XM_003124280.3 | CCTGCGGCATCCACGAAAC<br>TGTCGGCGATGCCTGGGTA | 123 |
| MT1[a] | NM_001001266.2 | CTGTGCCTGAAGTCTGGGGAA<br>CACAGAAAAAGGGATGTAGCATG | 115 |
| DMT1[b] | NM_001128440.1 | GCTCTCATACCCATCCTCACGTTT<br>GGACGTAAACCACGACGAAGTACA | 142 |
| Zip4[c] | XM_001925360.3 | CAGGGTCATCTGGGAAAGGAAGC<br>CCGGCACTCAGGCACATCGTG | 101 |
| ZnT1[d] | NM_001139470.1 | AAAATGTGAAGACCCGACATCGTA<br>AGGTTGAATGGTGGTAGCGTGAA | 94 |

[a] Metallothionein1

[b] Divalent metal transporter 1

[c] Zinc transporter SLC39A4

[d] Zinc transporter SLC30A1

independent-samples *t* test. Data were given as mean value ± standard deviation and $P < 0.05$ was declared to be statistically significant.

## Results

### Cell Viability Assay

MTT assay of different zinc sources showed that IPEC-1 cell viability decreased significantly ($P < 0.05$) when high doses of zinc were added at 6, 12, or 24 h (Fig. 1). Zn-Gly and Zn-Met had lower cell damage compared with $ZnSO_4$ on the same levels. Zinc concentration of 50 μmol/L and 6-h cell incubation were selected as suitable conditions for the following experiments.

### MT1, DMT1, ZIP4, and ZnT1 mRNA Expressions

Figure 2 shows that different zinc sources improved the expression of MT1 and ZnT1 mRNA compared with the control ($P < 0.05$), while ZIP4 decreased ($P < 0.05$) in response to zinc addition. MT1 and ZnT1 mRNA expressions in Zn-Gly and Zn-Met were higher ($P < 0.05$) than those in $ZnSO_4$, and ZIP4 mRNA expression in Zn-Met was the lowest among three kinds of zinc sources ($P < 0.05$). Expression of DMT1 mRNA in different zinc sources groups was significantly downregulated ($P < 0.05$) compared with control.

### Cell Viability Assay in ZIP4 siRNA Cells

IPEC1 transfected with four siRNA oligonucleotides resulted in higher inhibition of ZIP4 mRNA levels in 24 and 48 h ($P < 0.05$), and a 64.60 % ($P < 0.05$) and 78.43 % reduction were showed in siRNA 1 (Fig. 3). Moreover, transfection with ZIP4-siRNA1 in IPEC-1 cells had no significant effect on cell viability in 24 or 48 h ($P > 0.05$) in Fig. 3. So ZIP4 siRNA1 and 24 h were selected for the following studies.

### MT1 and DMT1 mRNA Expression and Zinc Absorption Rate in ZIP4 siRNA Cells

As shown in Fig.4, there were 16.94 % ($P < 0.05$) and 33.62 % ($P < 0.05$) reduction in MT1 mRNA

**Table 2** Primer sequences for ZIP4 siRNA

| siRNA | | Primer sequences (5′ to 3′) |
|---|---|---|
| ZIP4 siRNA-663 | Sense | CUC AGU ACU UCG UGG ACU UTT |
| | Antisense | AAG UCC ACG AAG UAC UGA GTT |
| ZIP4 siRNA-711 | Sense | CCA ACA UCA CAC UGG CUG ATT |
| | Antisense | UCA GCC AGU GUG AUG UUG GTT |
| ZIP4 siRNA-1583 | Sense | GUG CAC AAC UUC GCU GAU GTT |
| | Antisense | CAU CAG CGA AGU UGU GCA CTT |
| ZIP4 siRNA-1978 | Sense | GCU GUC UCU GUA UGA GGA UTT |
| | Antisense | AUC CUC AUA CAG AGA CAG CTT |
| Negative control -siRNA | Sense | UUC UCC GAA CGU GUC ACG UTT |
| | Antisense | ACG UGA CAC GUU CGG AGA ATT |

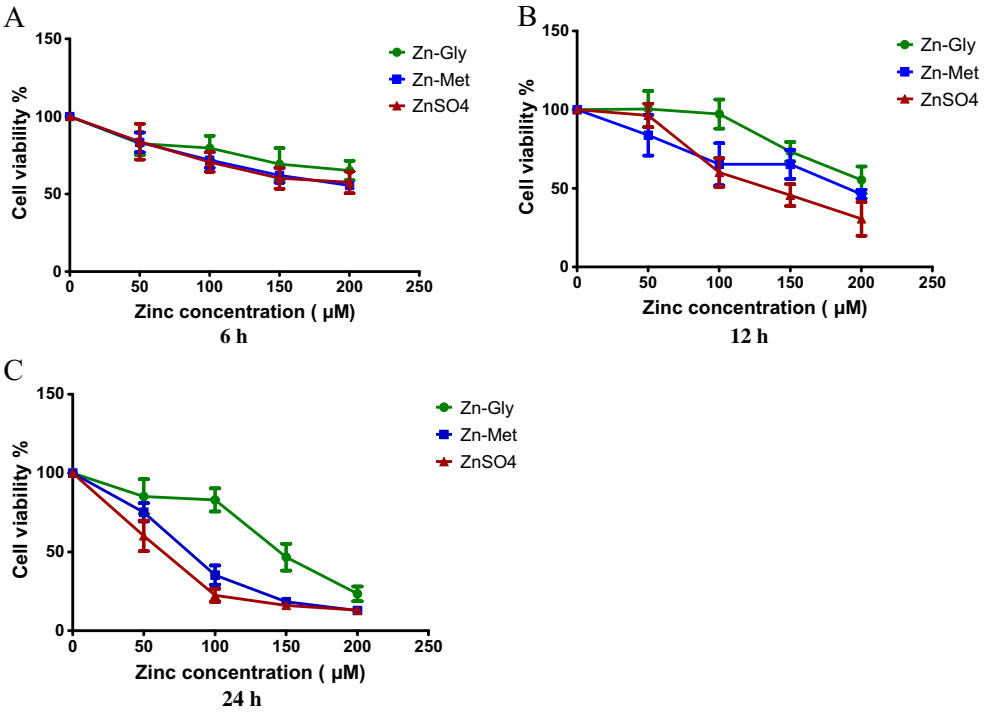


**Fig. 1** Viability of IPEC-1 cells following 6-, 12-, and 24-h incubation with different zinc sources. Values are means ± SD ($n = 6$)

expression in ZnSO4 and Zn-Gly when silencing ZIP4 mRNA expression. However, DMT1 mRNA expression of ZIP4-siRNA cells in Zn-Gly and Zn-Met were significantly decreased by 28.95 % ($P < 0.05$) and 26.44 % ($P < 0.05$), respectively. Silencing of ZIP4 caused a 73.33 % ($P < 0.05$) decrease of zinc

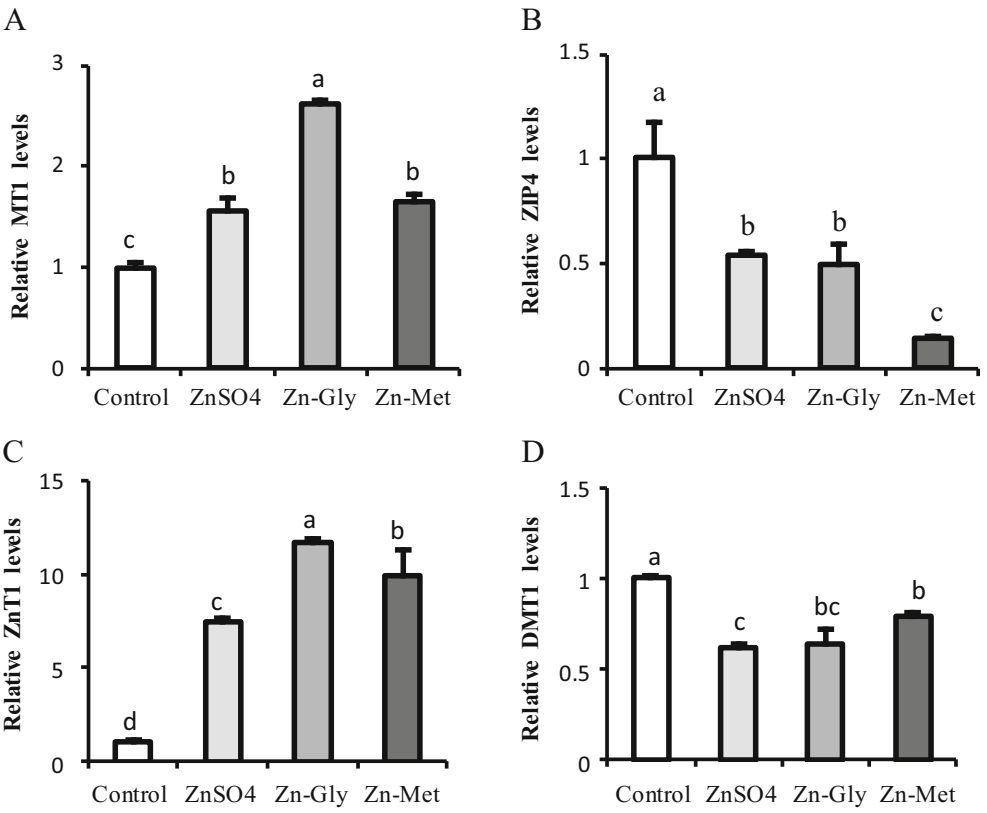


**Fig. 2** Relative mRNA levels of zinc-related transporters in IPEC-1 cells after 6-h incubation with 50 μmol/L $ZnSO_4$, Zn-Gly, and Zn-Met, respectively. Values are means ± SD ($n = 3$). Labeled means without a common letter differ, $P < 0.05$. MT1, metallothionein1; ZIP4, zinc transporter SLC39A4; ZnT1, zinc transporter SLC30A1; DMT1, divalent metal transporter 1

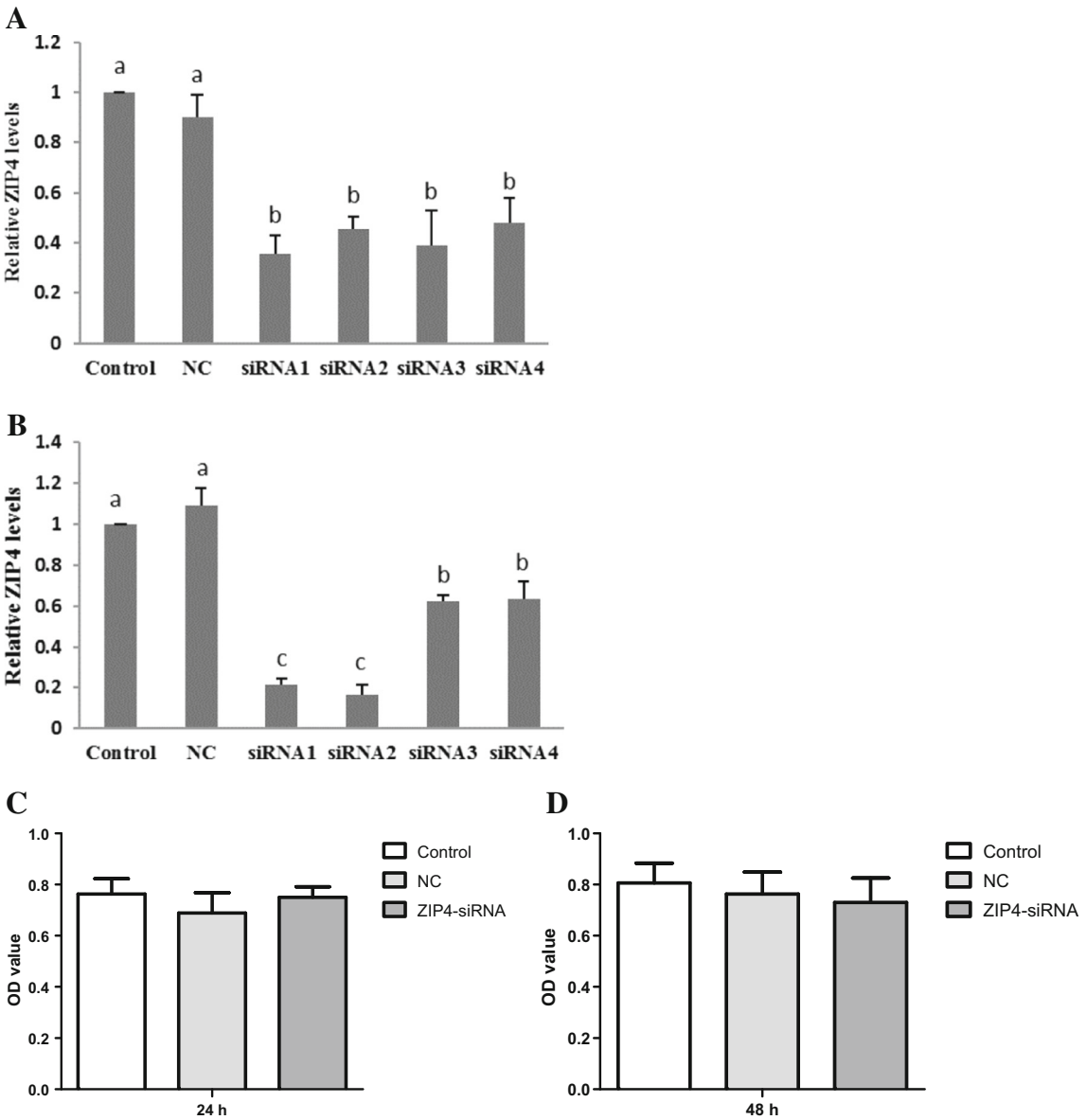


**Fig. 3** ZIP4 siRNA transfections. Four oligonucleotides of ZIP4 siRNA were transfected to inhibit ZIP4 mRNA expression in IPEC-1 cells. Relative ZIP4 levels were determined in 24 h (**a**) and 48 h (**b**). ZIP4 siRNA1 on cell proliferation in 24 h (**c**) and 48 h (**d**). Values are means ± SD ($n$ = 3). Labeled means without a common letter differ, $P < 0.05$

absorption rate in $ZnSO_4$ compared with negative control. However, zinc absorption rate in Zn-Gly and Zn-Met did not differ between negative control and ZIP4 siRNA groups (Fig.5).

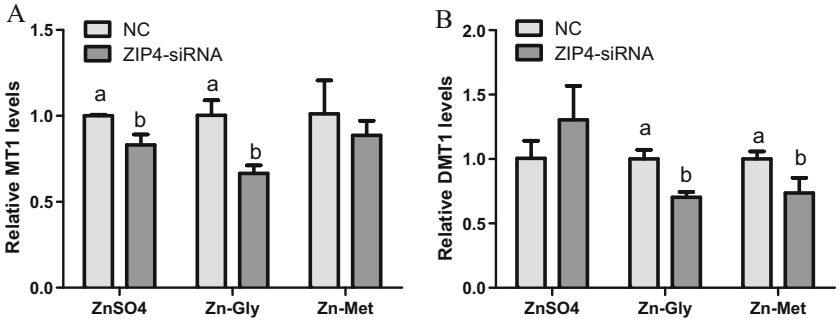


**Fig. 4** Different zinc sources on MT1 and DMT1 mRNA expression in IPEC-1 cells transfected with ZIP4 siRNA. Values are means ± SD ($n$ = 3). Labeled means without a common letter differ, $P < 0.05$

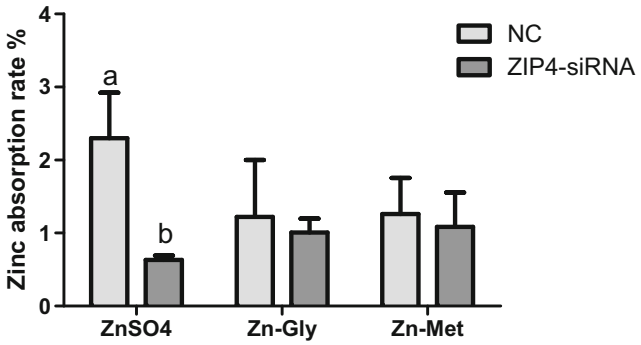


**Fig. 5** Different zinc sources on zinc absorption rate in IPEC-1 cells transfected with ZIP4 siRNA. Values are means ± SD ($n$ = 3). Labeled means without a common letter differ, $P < 0.05$

## Discussion

High doses of zinc exceeding nutritional needs may possibly pose a risk of toxic effects, especially for intestinal cells that are first to be exposed to dietary zinc in vitro [17]. The current study found that cell viability was obviously decreased when the added zinc concentration was greater than 100 μmol/L with the increased incubation time. This was similar to previous findings in IPEC-J2 in vitro [18]. The present study also showed that Zn-Gly and Zn-Met had lower cell damage than $ZnSO_4$ at the same level, which demonstrated that organic zinc could effectively protect enterocytes from high amount zinc ion damage. It may be a result of the good water solubility of $ZnSO_4$, and the reduction of gastrointestinal factor impact in vitro could instantly generate a large number of zinc ions causing toxic effects on cells.

Metallothionein1 (MT1) which plays a role for post-translational regulation is directly related to changes in intracellular zinc levels, which has frequently been used to estimate shifts in cellular zinc content [19, 20]. In vivo and in vitro findings have indicated that MT1 expressions were regulated by zinc [5, 21], and high zinc supply could induce an increase in cellular MT1 expressions [22, 23]. Our study showed that expressions of MT1 mRNA with different zinc sources were significantly higher than control, especially in Zn-Gly added treatment, which means that different zinc sources improved intracellular zinc level. Lodemann et al. (2015) reported that the expression of MT was significantly increased at 200 μmol/L $ZnSO_4$ than 0 added Zn in IPEC-J2 and Caco-2 cells [24]. Masaki et al. (2007) showed that Zn-Gly could significantly increase MT mRNA and protein on HaCaT cells [25]. This suggested that organic zinc might provide more available zinc than inorganic zinc sources [26]. Having a cyclic structure made organic zinc structure stable so that zinc could be used by transcription factor (MTF-1) more easily and regulated MT gene expression more effectively [27].

Huang et al. (2013) and Takahashi et al. (2012) indicated that a protective mechanism appears together with the downregulation of zinc transporters such as ZIP4, the upregulation of efflux transporters as ZnT1, and the zinc-binding protein MT when cells are exposed to high zinc concentrations [28, 29]. ZIP4 was responsible for zinc uptake in apical membrane, which exhibits upregulation under zinc deficiency and downregulation under increased zinc concentration at the mRNA levels [30, 31]. In the current study, ZIP4 mRNA expression in zinc addition was significantly downregulated compared with control. This was similar to previous findings, which reported repressed abundance of ZIP4 mRNA with varying zinc supply in mice and cultured cells [5, 31, 32]. ZnT1 is located on the basolateral membrane of enterocytes, and its mRNA expression is induced under increased zinc concentrations [33]. The present study found that ZnT1 mRNA expression in organic zinc was elevated ($P < 0.05$) than that in inorganic zinc especially with the highest levels observed in Zn-Gly. Lodemann et al. (2015) found that ZnT1 mRNA showed an upregulation with zinc addition and supported an effort of the cell to mediate Zn efflux that could protect cells from higher zinc toxicity. The downregulation of ZIP4 mRNA expression and the upregulation of ZnT1 mRNA expression with zinc addition demonstrated the improvement of intracellular zinc status, and MT1 as a heavy-metal-binding protein determines the tissue distribution of endogenous zinc, which can be induced similarly with ZnT1 by increasing the zinc concentration [34]. DMT1, as a common uptake pathway for many divalent metals, also played a role in zinc ion influx [35]. The current study showed that the mRNA expressions of DMT1 were declined in either inorganic or organic zinc addition compared with control. Shen et al. (2008) indicated that the cooperated upregulation of DMT1 and ZIP4 mRNA expression would contribute to enhance zinc absorption in Caco-2 cells [22]. It may be a result of the protective feedback mechanism to maintain intracellular zinc homeostasis [36]. The present results on ZIP4, ZnT1, and DMT1 expression were in accordance with the results of MT1, which indicated that zinc uptake was reduced by inhibition of ZIP4 and DMT1 expression and promotes the expression of ZnT1 to strengthen the efflux of zinc until intracellular zinc was satisfied; the results also implied the effect of organic zinc is more obviously.

To further study organic zinc absorption in the small intestine, ZIP4 mRNA expression was silenced by siRNA in this study. ZIP4 played a direct role in inorganic zinc uptake [7, 37]. It was found that MT1 mRNA expression showed a greater decrease in ZIP4 siRNA cells ($P < 0.05$ for $ZnSO_4$ and Zn-Gly), suggesting the participation of ZIP4 in $ZnSO_4$ and Zn-Gly metabolism. As the result of ZIP4 mRNA expression being silenced, zinc absorption rate in $ZnSO_4$ was significantly decreased and the mRNA expression of DMT1 in $ZnSO_4$ was increased. Martin (2014) also reported that DMT1 appears to play a minor role in zinc homeostasis [38]. This was similar to the findings by Geiser et al., who reported rapidly

decreased total zinc in small intestine from ZIP4 intestine knockout mice [30]. However, DMT1 mRNA expression in Zn-Gly and Zn-Met was significantly lower than that in $ZnSO_4$ treatment, and zinc absorption rate in Zn-Gly and Zn-Met did not differ between ZIP4-siRNA cells and negative control. These results proved that ZIP4 played a direct role in inorganic zinc uptake and also indicated that the absorption of zinc in Zn-Gly depends on ZIP4 partly, while zinc in Zn-Met may have other absorption pathway in small intestine, which needs further study.

## Conclusions

In summary, different zinc sources could improve zinc status on IPEC-1 cells and organic zinc had lower cell damage compared with $ZnSO_4$. Moreover, Zn-Gly and Zn-Met are more efficient on zinc absorption according to the expression of various zinc-related transporters like MT1, ZIP4, ZnT1, and DMT1. ZIP4 played a direct role in inorganic zinc uptake, and the absorption of zinc in Zn-Gly depends on ZIP4 partly, while absorption of Zn-Met is less dependent on ZIP4.

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