



# Dosage Effect of Zinc Glycine Chelate on Zinc Metabolism and Gene Expression of Zinc Transporter in Intestinal Segments on Rat

Danping Huang<sup>1</sup> · Qiaoling Hu<sup>1</sup> · Shenglin Fang<sup>1</sup> · Jie Feng<sup>1</sup>

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**Abstract** Zinc plays an essential role in various fundamental biological processes. The focus of this research was to investigate the dosage effect of zinc glycine chelate (Zn-Gly) on zinc metabolism and the gene expression of zinc transporters in intestinal segments. A total of 30 4-week-old SD rats were randomized into five treatment groups. The basal diets for each group were supplemented with gradient levels of Zn (0, 30, 60, 90, and 180 mg/kg) from Zn-Gly. After 1-week experiment, the results showed that serum and hepatic zinc concentration were elevated linearly with supplemental Zn levels from 0 to 180 mg Zn/kg. Serum Cu-Zn SOD activities resulted in a significant ( $P < 0.01$ ) quadratic response and reached the peak when fed 60 mg Zn/kg. There were linear responses to the addition of Zn-Gly from 0 to 180 mg Zn/kg on Cu-Zn SOD and AKP activities in the liver. In the duodenum, MT1 mRNA was upregulated with the increasing dietary Zn-Gly levels and reached the peak of 180 mg Zn/kg ( $P < 0.05$ ). Zip4 mRNA expression was downregulated with the increasing zinc levels ( $P < 0.05$ ) in both duodenum and jejunum. In the jejunum, Zip5 mRNA expression in 60 mg Zn/kg was higher compared with other groups ( $P < 0.05$ ). ZnT1 mRNA in duodenum was numerically increased with the rising levels of zinc content and was significantly higher ( $P < 0.05$ ) with 180 mg Zn/kg. In the duodenum, adding 60 or 90 mg Zn/kg increased PepT1 expression, but in the jejunum, 60 mg Zn/kg did not differ from 0 added Zn. In summary, there is a dose-

dependent effect of dietary Zn-Gly on serum and hepatic zinc levels and the activities of Cu-Zn SOD and AKP on rats. Dietary Zn-Gly has a certain effect on MT1, Zip4, Zip5, and ZnT1 expression, which expressed differently in intestinal segments with different levels of Zn-Gly load. Besides, Zn-Gly also could regulate PepT1 expression in intestinal segments.

**Keywords** Zinc glycine chelate · Dosage effect · Intestinal segments · Zinc transporters · Rat

## Introduction

Zinc is an essential trace element, which involves in more than 300 enzymatic reactions as a catalytic agent and regulates diverse cell processes such as DNA synthesis, gene expression, and oxidative stress [1].

Zinc in raw material is hardly to meet the demand of zinc for animals; zinc sulfate as an inorganic zinc source is commonly used for zinc sources in animal production [2]. However, inorganic zinc may have lower absorption efficiency and reduce the stabilities of vitamins and other nutrition [3]. In recent decades, organic chelated trace minerals, especially amino acid chelated trace minerals, have attracted considerable attention due to its better absorption and utilization [4]. It was found that supplementation of Zn-Met could improve the performance of animals including piglets [5] and broilers [6, 7]. High-dosage of Zn-Met could increase the zinc content in liver and tibia compared with other low-dosage groups [8]. Wang et al. proved that an appropriate dosage of Zn-Gly could improve the capacity of tissue deposition of zinc in piglet [9]. Feng et al. and Ma et al. also found that Zn-Gly could enhance growth performance and improved intestinal morphology in broilers [10, 11].

✉ Jie Feng  
fengj@zju.edu.cn

<sup>1</sup> Key Laboratory of Molecular Animal Nutrition, Ministry of Education, College of Animal Science, Zhejiang University, Hangzhou, People's Republic of China 310058

**Table 1** Composition of the experimental diet

Content	%	Content	%
Glucose	640	L-cystine	3
Casein	200	Choline chloride (50 %)	1.2
Corn oil	50	Ethoxyquin powder (60–66 %)	0.1
Cellulose	50	Glucose mixture of zinc glycine chelate	10.7
AIN 93G-Mx <sup>a</sup>	35	AIN 93G-VX <sup>b</sup>	10

<sup>a</sup> Composition of mineral mixture as follows: calcium carbonate 357.0 %, chromium potassium sulfate 0.2750 %, potassium dihydrogen phosphate 196.0 %, boric acid 0.0815 %, sodium 74.00 %, fluoride 0.0635 %, potassium citrate 70.78 %, nickel carbonate 0.0318 %, potassium 46.60 %, lithium chloride 0.0174 %, hydroxide 24.00 %, selenium sodium 0.0103 %, ferric citrate 6.060 %, potassium iodate 0.0100 %, sodium 1.450 %, ammonium 0.0080 %, copper sulfate 0.670 %, ammonium vanadate 0.0066 %, manganese carbonate 0.630 %, glucose 222.3059 %

<sup>b</sup> Composition of vitamin mixture as follows: VE (500 IU/g) 15.000 %, VB6 0.700 %, nicotine 3.000 %, VB2 0.600 %, VB12(0.1 %) 2.500 %, VB1 0.600 %, Biotin (0.1 %) 2.000 %, folate 0.200 %, calcium pantothenate 1.600 %, VK 0.075 %, VD3(100, 000 IU/g) 1.000 %, glucose 971.925 %, VA(500,000 IU/g) 0.800 %

Bioavailability can be defined as the proportion of the administered substance capable of being absorbed and available for use or storage, which always correlated with absorption efficiency [12]. It is well known that zinc ions are mainly absorbed in small intestine by two families of zinc transporters, which include the ZnT family and Zip family. The ZnT protein is responsible for reducing the intracellular zinc through zinc efflux, while the Zip proteins tend to promote zinc transport into cytoplasm [13]. Yue et al. showed that in 2 and 6 h after gavage on rats, Zn-Gly was more effective in improving body zinc status than ZnSO<sub>4</sub>, but ZnSO<sub>4</sub> did more efficiently on the regulation of zinc transporters in small intestine [14]. However, whether or not there is a Zn-Gly dosage effect on zinc metabolism and the gene expression of zinc

transporters in different intestinal segments are not clear. Thus, current study was designed to evaluate the dosage effect of Zn-Gly on zinc metabolism and gene expression of zinc-related transporters in intestinal segments on rats.

## Materials and Methods

### Animals and Diets

A total of 30 4-weeks -old SD rats weighing  $91.02 \pm 7.55$  g were randomly allotted to five treatments with six replicates. Dietary treatments were designed with five gradients of Zn-Gly, which contain 0, 30, 60, 90, and 180 mg Zn/kg. The basal diet was based on the AIN-93G required for rodents (Table 1).

During the experiment, the rats were housed in stainless steel cages separately and given ad libitum access to feed and deionized water. Room temperature was maintained at 23–25 °C and humidity was 40–60 %. All experiments were according to the rules of the Animal Care and Use Committee of Zhejiang University (Hangzhou, China).

### Treatments and Sample Collection

After 2 days of pre-feeding (containing 30 mg Zn/kg), rats were given diets with five different levels of Zn-Gly for a week. Blood samples received from eyeball artery were isolated by centrifuging at 3000×g for 10 min at 4 °C and then stored at –80 °C until analysis. All rats were killed by means of cervical dislocation. Liver were cut out immediately and snap-frozen in liquid nitrogen, followed by storing at –80°C until analysis. Proximal segment about 3–5 cm of duodenum and jejunum was cut out, and mucosa was scraped. The steps are as follows: Firstly, a section of intestine (3–5 cm, proximal segment) was excised and cut open the segments with the

**Table 2** Choosing primers for qPCR

Gene	GenBank reference	Sequence forward and reverse primers	Length (bp)
GAPDH	NM_017008	F:5-GGTGGACCTCATGGCCTACAT-3 R:5-GCCTCTCTTGTCTCAGTATCCT-3	88
ZnT1	NM_022853.1	F:5-CACGCTAGTGGCTAACACCA-3 R:5-AGGAAAACACGGGTTACAC-3	295
Zip4	NM_001077669.1	F:5-ATGAGCTGCCTCACGAACT-3 R:5-CTGCTAGAGCCACGTAGAGG-3	129
Zip5	NM_001108728.1	F:5-GGAACTGGAGCTGAGTGTGGATGT-3 R:5-GCAGAAGCCTGTGGAAGAGGTCTA-3	136
MT1	BC058442.1	F:5-GCCTTCTTGTTCGCTTACACC-3 R:5-CTTCTTGCAGGAGGTGCATT-3	139
PepT1	D50664	F:5'-GGGAGAACGGAATCAGATTCGTCAG-3 R:5'- TTGCTGGCGCTGTGACTGGTG-3'	100

GAPDH glyceraldehyde-3-phosphate dehydrogenase, ZnT1 zinc transporter SLC30A1, Zip4 zinc transporter SLC39A4, Zip5 zinc transporter SLC39A5, MT1 metallothionein1, PepT1 peptide transporter 1

**Table 3** Dose-dependent effect of glycine chelated zinc on the zinc level in serum and liver of rat

Item	Zn (mg/kg, supplemented as Zn-Gly)					SEM		
	0	30	60	90	180	Zn-Gly <sup>a</sup>		
						Linear	Quadratic	
Serum								
Zn (mg/L)	0.17	0.54	0.64	1.82	2.75	0.19	0.001	0.001
Liver								
Zn (mg/kg)	32.25	36.31	42.41	56.00	61.98	2.37	0.001	0.253

Values are presented as means: *n* = 6 for per treatment

SEM standard error of the mean

<sup>a</sup> Linear and quadratic effects of increasing dietary Zn supplementation (0–180 mg/kg) as Zn-Gly

scissors. Then, flush the intestinal segments with cold 9 g/L NaCl to remove the digesta. The mucosa was gently scraped with razor blade and put into 1.5-mL Eppendorf tubes.

**Determination of Mineral Concentration**

0.5~1.0 g liver sample was weighed for mineral analysis. Briefly, liver sample was charred on the electric furnace and then 500 °C dry ashed for 8 h in muffle furnace. After cooling, the sample was dissolved with hydrochloric acid and then made a constant volume. Contents of Zn were analyzed with flame atomic absorption spectrophotometry (AA-6300, Shimadzu, Tokyo, Japan). The serum zinc content was measured by direct dilution method; the specific steps are as follows: 5 mL of 10 % nitric acid was dropped into 500 µL serum, and then diluted with deionized–distilled water [15]. The zinc concentrations in serum and liver were measured by flame atomic absorption spectrophotometry, and ultraviolet spectrophotometry was used to determine the alkaline phosphatase (AKP). AKP activity was determined according to the modified method described by Gonzalez et al. [16]. One

hundred microliters of crude enzyme solutions as a sample mixed with 2.0 mL of substrate (p-nitrophenyl phosphate in glycine–NaOH buffer pH 9 for AKP) was incubated at 37 °C for 30 min. Then, after adding 2.9 mL of 0.1 N NaOH, the absorbance was measured by spectrophotometry at 405 nm and the activity calculated by the amount of p-nitrophenyl released. Cu-Zn SOD (copper zinc superoxide dismutase) activities were measured with an SOD detection kit (Nanjing Jiancheng Bioengineering Institute, China). The principle is according to Wang and Chen in which its ability inhibit superoxide anion generated by xanthine and xanthine oxidase reaction system [17]. The OD values were measured at 550 nm, and the activity was expressed as U/mL (serum) and U/mg (liver).

**Quantitative Real-Time PCR**

In order to extract total RNA, intestinal mucosa removed from small intestine sections was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). DNase was added for treating the total RNA to avoid any DNA pollution.

**Table 4** Impact of different doses glycine chelated zinc on the Cu-ZnSOD enzyme/Alkaline phosphatase activity in serum and liver of rat

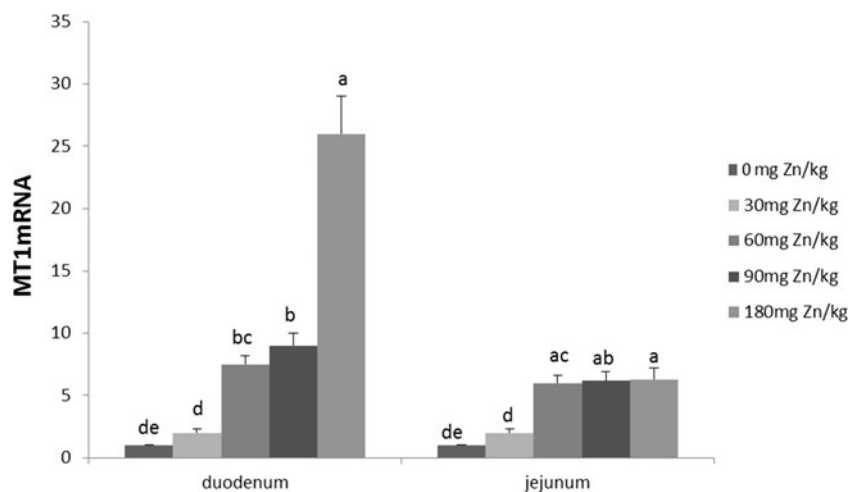
Item	Zn (mg/kg, supplemented as Zn-Gly)					SEM		
	0	30	60	90	180	Zn-Gly <sup>a</sup>		
						Linear	Quadratic	
Serum								
Cu-ZnSOD (U/mL)	196.47	251.03	345.71	213.49	237.27	13.82	0.564	0.003
AKP (U/mL)	57.28	59.25	79.95	65.57	69.65	2.84	0.103	0.167
Liver								
Cu-ZnSOD (U/mg)	94.12	111.85	169.20	165.59	204.33	9.02	0.001	0.680
AKP (U/mg)	57.27	59.38	68.53	68.22	71.67	1.81	0.003	0.614

Values are presented as means: *n* = 6 for per treatment

SEM standard error of the mean

<sup>a</sup> Linear and quadratic effects of increasing dietary Zn supplementation (0–180 mg/kg) as Zn-Gly

**Fig. 1** Effect of different doses of glycine chelated zinc on MT1 mRNA level in rat duodenum and jejunum. Relative mRNA expression of MT1 was detected by quantitative RT-PCR. Results are presented as mean  $\pm$  SE ( $n = 6$ /treatment), and the different noted lowercase letters are statistically significant ( $P < 0.05$ )



Transcription system which was used for the cDNA template synthesis consists of 2  $\mu$ g RNA, Oligo-dT 1  $\mu$ L, 5 $\times$  buffer 4  $\mu$ L, dNTP 2  $\mu$ L, and M-mlevtrans 1  $\mu$ L. These materials mentioned above were firstly incubated for 10 min at 25  $^{\circ}$ C and secondly incubated for 1 h at 42  $^{\circ}$ C. Primers for MT1, Zip4, Zip5, ZnT1, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) designed with Primer Express 2.0 (Table 2) were synthesized by Invitrogen (Invitrogen Ltd., Shanghai, China). Using 1  $\mu$ L cDNA, 10  $\mu$ L SYBR (2 $\times$ ), 0.8  $\mu$ L of each primer, 0.4  $\mu$ L RoxDye II, and 7  $\mu$ L RNase-free water in a final volume of 20  $\mu$ L, SYBR<sup>®</sup> Premix Ex-Taq TM Kit (Takara, Otsu, Japan) was applied for quantitative RT-PCR and it was used by an iQTM 5 real-time PCR detection system (Bio-Rad Inc., Hercules, CA, USA). The reaction protocol comprised as follows: an initial denaturation at 95  $^{\circ}$ C for 30 s, followed by 40 cycles for 5 s, and then 60  $^{\circ}$ C for 30 s. Melting curves and PCR efficiency were checked to make sure that the amplification of the single PCR product is consistent [18]. Target genes were

normalized to GAPDH, and the relative gene expression level was calculated by  $2^{-\Delta\Delta C_t}$  method.

### Statistical Analysis

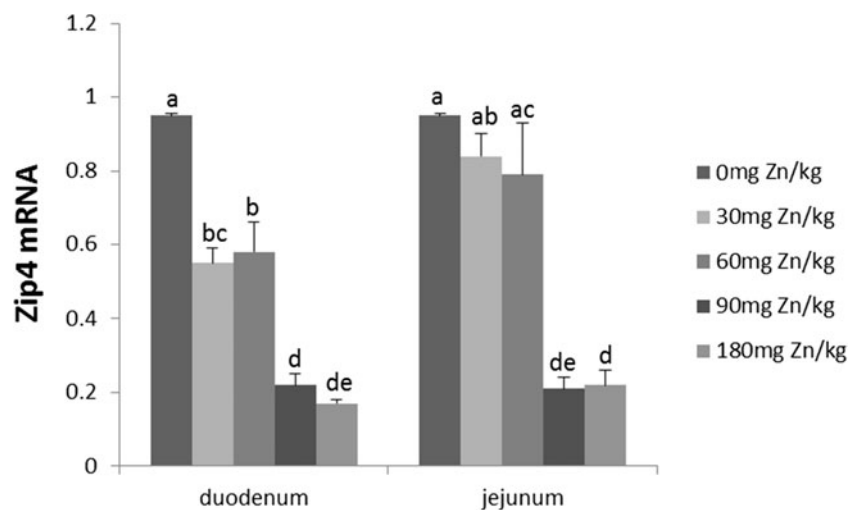
One-way ANOVA analysis following LSD post hoc test was used for statistical analyses by SPSS (13.0). A significant level of 0.05 was used for evaluating the difference among treatments. The planned single degree of freedom tests included the linear and quadratic dose effects of Zn-Gly.

## Results

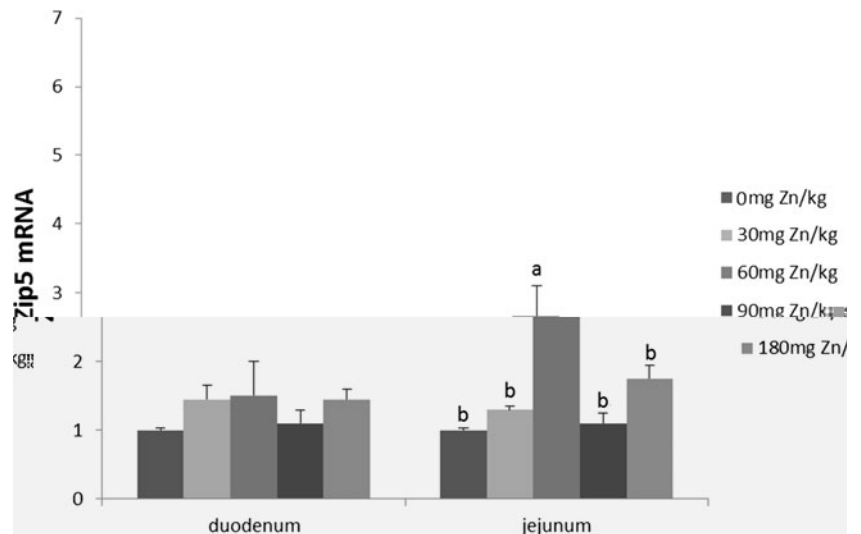
### Zinc Concentration in Serum and Liver

Table 3 displayed the dosage effect of Zn-Gly on zinc status of serum and liver. Serum and hepatic zinc concentration were elevated linearly with supplemental Zn levels from 0 to

**Fig. 2** Dose effect of glycine chelated zinc on Zip4 mRNA level in rat duodenum and jejunum. Relative mRNA expression of Zip4 was detected by quantitative RT-PCR. Results are presented as mean  $\pm$  SE ( $n = 6$ /treatment), and the different noted lowercase letters are statistically significant ( $P < 0.05$ )



**Fig. 3** Effect of different doses of glycine chelated zinc on Zip5 mRNA level in rat duodenum and jejunum. Relative mRNA expression of Zip5 was detected by quantitative RT-PCR. Results are presented as mean ± SE (*n* = 6/treatment), and the different noted lowercase letters are statistically significant (*P* < 0.05)



180 mg Zn/kg (serum, *P* = 0.001; liver, *P* = 0.001). Moreover, zinc concentration in serum showed a significant (*P* < 0.01) quadratic regression. Serum Zn was significantly higher (*P* < 0.05) when 90 and 180 mg Zn/kg was fed, and hepatic Zn was increasingly higher from 60 to 180 mg Zn/kg in the diet.

**Related Enzyme Activities in Serum and Liver**

The addition of Zn-Gly also affected Cu-Zn SOD and AKP activities in serum and liver (Table 4). Serum Cu-Zn SOD activities resulted in a significant (*P* < 0.01) quadratic response and was significantly highest (*P* < 0.05) when 60 mg Zn/kg was fed. Compared with other treatments, the highest activities of serum AKP was also observed in 60 mg Zn/kg (*P* < 0.05). In addition, there were linear responses to the addition of Zn-Gly from 0 to 180 mg Zn/kg on Cu-Zn SOD

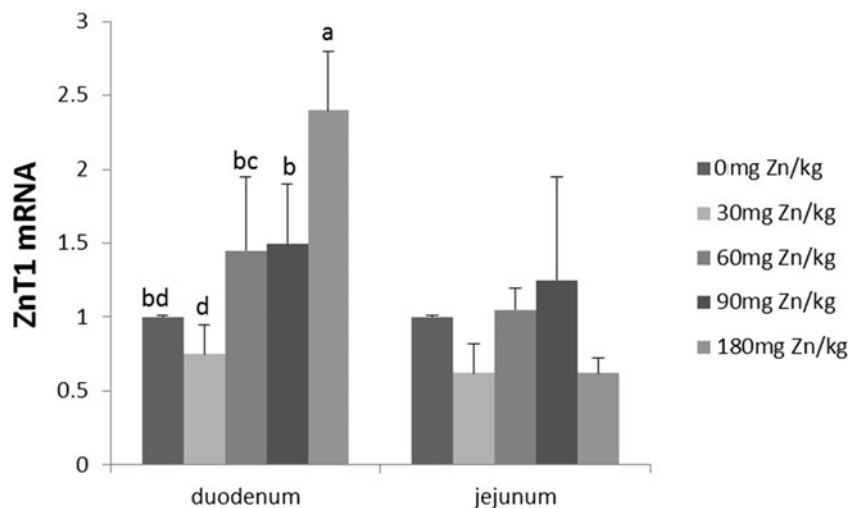
and AKP activities in the liver (Cu-Zn SOD, *P* = 0.001; AKP, *P* = 0.003), with the greatest Cu-Zn SOD activities when 60 and 180 mg Zn/kg was fed and 60, 90, or 180 mg Zn/kg significantly (*P* < 0.05) increased AKP activities.

**Effect of Diverse Doses of Zn-Gly on MT1, ZnT1, Zip4, Zip5, PepT1 Gene Expression**

*Relative mRNA Expression of MT1*

The effects of different levels of Zn-Gly on the expression of MT1 mRNA in intestinal segments are presented in Fig. 1. MT1 mRNA elevated with the increasing dietary Zn levels and reached the peak in 180 mg Zn/kg group (*P* < 0.05) in duodenum. MT1 mRNA in diets with 60, 90, and 180 mg Zn/kg was higher (*P* < 0.05) than 0 and 30 mg Zn/kg in both duodenum and jejunum.

**Fig. 4** Dose effect of glycine chelated zinc on ZnT1 mRNA level in rat duodenum and jejunum. Relative mRNA expression of ZnT1 was detected by quantitative RT-PCR. Results are presented as mean ± SE (*n* = 6/treatment), and the different noted lowercase letters are statistically significant (*P* < 0.05)



#### *Relative mRNA Expression of Zip4*

Figure 2 showed the mRNA expression of Zip4 in duodenum and jejunum. Zip4 mRNA expressions were downregulated with increasing zinc levels in both duodenum and jejunum. Zip4 mRNA in diet with 90 and 180 mg Zn/kg was significantly lower ( $P < 0.05$ ) than 0, 30, and 60 mg Zn/kg in both duodenum and jejunum.

#### *Relative mRNA Expression of Zip5*

The expression of Zip5 mRNA in duodenum and jejunum is shown in Fig. 3. In jejunum, Zip5 mRNA expression in 60 mg Zn/kg was significantly higher ( $P < 0.05$ ) than all other treatments. However, no significant differences were observed among all treatments in duodenum ( $P > 0.05$ ).

#### *Relative mRNA Expression of ZnT1*

The effects of different concentrations of Zn-Gly on the expression of ZnT1 were shown in Fig. 4. ZnT1 mRNA in duodenum was numerically upregulated with the rising levels of zinc content and was significantly higher ( $P < 0.05$ ) when 180 mg Zn/kg was fed. ZnT1 mRNA expression in jejunum had no significant differences ( $P > 0.05$ ) between all groups.

#### *Relative mRNA Expression of PepT1*

Figure 5 displayed the effects of different levels of Zn-Gly on mRNA expression of PepT1. Adding 60 and 90 mg Zn/kg had the highest levels of PepT1 mRNA expression

especially in 180 mg Zn/kg. It may result from the fact that the dietary zinc permeating through the duodenum first present a higher concentration, which indicates that duodenum is possibly the major site of intestinal zinc absorption [30].

Transporters which may be involved in zinc permeation across intestinal epithelial cells are Zip4, Zip5, and ZnT1 [31]. Zip4 is located on the apical membrane of enterocytes and takes up dietary zinc into enterocytes. Zip4 mRNA expression is responsive to dietary zinc, which exhibit upregulation under zinc deficiency and downregulation under increased zinc concentration at the mRNA level [32]. Results of this study showed that Zip4 mRNA induced by low-dose treatments expressed significantly higher than high-dose Zn groups both in the duodenum and jejunum. Dufner-Beattie et al. [32] reported that the expression of Zip4 mRNA and protein significantly ( $P < 0.05$ ) increased with Zn deficiency while added dietary Zn or oral gavage Zn could decreased Zip4 mRNA in mice. Current study showed that there were no significant differences on Zip4 mRNA expression when fed with the same dosage between the duodenum and jejunum ( $P > 0.05$ ). Tohru et al. indicated that the relative expression of Zip4 showed no difference in any segments of the small intestine. It maybe result from zinc levels regulating the stability of Zip4 mRNA in small intestine, while it did not have much effect on transcription of Zip4 [33].

Zip5 is localized at the basolateral membrane of enterocytes under the condition of zinc repletion [34]. When dietary zinc is replete, Zip5 was responsible for transporting Zn from the blood into enterocytes in order to excrete zinc to the intestinal lumen [35]. While in zinc deficiency, Zip5 protein was internalized and degraded in mice [32]. The current study showed that there were no significant differences between all groups in duodenum. Zinc status did not make an effort on the expression and translation of Zip5 but on post-translational level [33]. Dufner-Beattie et al., (2004) indicated that Zip5 mRNA levels are not altered in response to dietary zinc. Interestingly, in our research, Zip5 mRNA expression in 60 mg Zn/kg was significantly higher compared with all other groups in the jejunum, which needs further study.

ZnT1 is expressed on the basolateral membrane of enterocytes, and its mRNA expression is induced under increased zinc concentrations [36]. Current study found that the expression of ZnT1 mRNA was numerically increased and reached the highest expression in duodenum when adding 180 mg Zn/kg. McMahon and Cousins found that the expression levels of ZnT1 mRNA in small intestine are not affected by low zinc diet on rats [28]. ZnT1 mRNA was markedly greater in both small intestine and kidney when a supplemental zinc intake (180 mg Zn/kg) was provided [37]. McMahon and Cousins also reported that dietary zinc supplementation (180 mg Zn/kg) increased the level of ZnT1 mRNA and protein about 50 and 10 % compared with 5 mg Zn/kg groups in small intestine on rats. There was no significant difference

among the five treatments on the content of ZnT1 mRNA in the jejunum in current study. The different effects of Zn-Gly on ZnT1 mRNA expression between duodenum and jejunum may further prove that duodenum is the major site of small intestine on zinc absorption.

Intestinal peptide transporter (PepT1) was expressed in small intestine, mainly on apical membranes of epithelial cells. Some researchers indicated that metal amino acid chelates may be absorbed by other transporters like PepT1 [38, 39]. Few studies were carried out on the relationship between PepT1 and amino acid metal chelate. Liao et al. reported that PepT1 mRNA and protein expression was significantly higher in the ferrous bisglycinate (Fe-Gly) groups than FeSO<sub>4</sub>, which suggested that the transport of Fe-Gly was associated with PepT1 [40]. In present study, 60 mg Zn/kg enhanced PepT1 mRNA expression in duodenum. We did not find difference between 0 and 60 mg Zn/kg on PepT1 mRNA expression in jejunum, which may also indicate PepT1 to exert function mainly in duodenum. However, the concrete mechanism of Zn-Gly on the intestinal absorption is uncertain and worthy of further study.

## Conclusion

In summary, there is a dose-dependent effect of dietary Zn-Gly on serum and hepatic zinc concentration on rats, as well as the activities of Cu-Zn SOD and AKP. Dietary Zn-Gly has certain effects on MT1, Zip4, Zip5, and ZnT1 expression, which expressed differently in intestinal segments with different levels of Zn-Gly load. Our current study also showed that Zn-Gly could regulate the expression of PepT1. However, whether PepT1 is involved in the absorption of organic zinc needs further study.

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