Organic iron absorption by in situ ligated jejunal and ileal loops of broilers¹

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ABSTRACT: The objective of this study was to determine the effect of organic and inorganic Fe sources on the Fe absorption and gene expression of Fe and amino acid transporters in the ligated jejunal and ileal segments of broilers. The in situ ligated jejunal and ileal loops from Fe-deficient broiler chicks (28-d-old) were perfused with Fe solutions containing 0, 3.58, or 7.16 mM Fe from one of the following Fe sources: Fe sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), the mixtures of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ with either Met or Gly, Fe-Gly chelate, or three Fe-amino acid or protein chelates with weak, moderate or extremely strong chelation strengths (Fe-Met W, Fe-Pro M, or Fe-Pro ES), respectively, for up to 30 min. Iron absorption was increased ($P < 0.0001$) as the perfused Fe concentrations increased, and no differences ($P > 0.07$) were detected in the Fe absorption between the jejunum and ileum. Regardless of intestinal segments, Fe absorption was higher ($P < 0.006$) for Fe-Pro ES and Fe-Pro M than for $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and for Fe-Pro ES than for Fe-Met W. Glycine but not Met supplementation increased ($P < 0.03$) the absorption of Fe as FeSO_4 . Regardless of Fe source, Fe addition inhibited ($P < 0.05$) the mRNA expressions of divalent metal

transporter 1 (**DMT1**) in the jejunum and ileum, but enhanced ($P < 0.05$) the mRNA expressions of L-type amino transporter 1 (**LAT1**) and B⁰-type amino acid transporter 1 (**B⁰AT1**) in the jejunum and ileum. No differences ($P > 0.05$) among different Fe sources were observed in the mRNA expression levels of Fe and amino acid transporters in both the jejunum and the ileum. The mRNA expression levels of *DMT1*, ferroportin 1, *B⁰AT1*, or y⁺LAT1 were higher ($P < 0.0001$), but those of excitatory amino acid transporter 3, *LAT1*, or y⁺L-type amino transporter 2 were lower ($P < 0.04$) in the jejunum than in the ileum. The supplementation of inorganic or organic Fe had no effect ($P > 0.14$) on the protein expression levels of DMT1 and FPN1 in the jejunum and ileum. The above results indicate that organic Fe sources with stronger chelation strengths showed higher Fe absorption in the jejunum and ileum of broiler chicks. Glycine was more effective in facilitating Fe absorption than Met as a ligand. The mRNA expressions of Fe and amino acid transporters in the jejunum were different from those in the ileum. The DMT1, LAT1, and B⁰AT1 might be involved in the Fe absorption in the jejunum or ileum of broilers.

Key words: amino acid transporter, broiler, divalent metal transporter, ferroportin 1, gene expression, organic iron absorption

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INTRODUCTION

Iron is essential for animals, and involved in DNA synthesis, respiration, and key metabolic reactions (Dunn et al., 2007; Ganz and Nemeth, 2011). Iron additives are usually used in broiler diets to prevent Fe deficiencies or support optimal growth. However, the bioavailabilities of Fe sources vary greatly due to their chemical traits (Lai et al., 2012; Zhang et al., 2016a). It has been demonstrated that the bioavailabilities of organic Fe sources for broilers are closely related to their chelation strengths [quotient of formation (Q_f) values] between Fe and their ligands, and the organic Fe sources with greater Q_f had higher bioavailabilities (Ma et al., 2014; Zhang et al., 2016a). A recent study (Li et al., 2017) from our laboratory indicated that when the perfused Fe concentration was as low as 0.45 mM, no differences among inorganic and organic Fe sources were detected in the Fe absorption of ligated duodenal, jejunal or ileal loops of broilers. It was also found that that organic Fe sources with greater Q_f values showed higher Fe absorption in the duodenum of broilers at higher concentrations (equal to or higher than 3.58 mM) of perfused Fe (Li et al., 2017; Zhang et al., 2017). However, Fe absorption as organic Fe sources in the jejunum and ileum at the above higher concentrations of perfused Fe and its mechanisms remain unclear.

The Fe absorption mechanism of inorganic Fe has been well investigated in both in vitro and in vivo studies with CaCo-2 cells (Zoller et al., 2002) and rodents (Abboud and Haile, 2000; Frazer et al., 2003). In the process of Fe^{2+} transport, the divalent metal transporter 1 (DMT1), which is expressed on the apical transmembrane, actively transports reduced dietary Fe into intestinal enterocytes (Canonne-Hergaux et al., 2001). Subsequently, when body requires Fe, the cytosolic Fe is transported via ferroportin 1 (FPN1) which is expressed on the basolateral membrane, and is the sole identified Fe transporter from the intracellular to bloodstream (Abboud and Haile, 2000; McKie et al., 2000). Zhang et al. (2017) indicated that regardless of Fe source, Fe perfusion inhibited the mRNA expression of DMT1, but enhanced the mRNA expression of FPN1 in the duodenum of broilers, suggesting that DMT1 and FPN1 might be involved in duodenal Fe absorption of broilers. Several types of amino acid transporters, excitatory amino acid transporter 3 (EAAT3), L-type amino transporter 1 (LAT1), B⁰-type amino acid transporter 1 (B⁰AT1), y⁺L-type amino transporter 1 (y⁺LAT1) and y⁺L-type amino transporter

2 (y⁺LAT2), were demonstrated to participate in amino acid transport in the intestine (Duelli et al., 2000; Amara and Fontana, 2002; Sperandeo et al., 2005; Jiang et al., 2015), but it is still unknown whether the above-mentioned Fe and amino acid transporters participate in the absorption of Fe as organic Fe sources (such as Fe amino acid chelates) in the jejunum and ileum of broilers.

We hypothesized that the organic Fe sources with greater Q_f values might have higher Fe absorption, and the above-mentioned Fe and amino acid transporters might be involved in the absorption of Fe as organic Fe sources in the jejunal and ileal segments of broilers. Therefore, the objective of the present study was to determine the effect of organic and inorganic Fe sources on Fe absorption and gene expressions of Fe and amino acid transporters in in situ ligated jejunal and ileal segments of broilers to test the above hypothesis.

MATERIALS AND METHODS

Experimental Design and Treatments

A completely randomized design involving $7 \times 2 \times 2$ (Fe sources $\times$ perfused Fe concentrations $\times$ small intestinal segments) factorial arrangement of treatments was adopted in the present study. The seven different Fe sources included inorganic Fe sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, reagent grade; 19.5 % Fe by analysis), Fe-Gly chelate (Fe-Gly, feed grade, 12.85% Fe by analysis), the mixture of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ with either Met or Gly at a 1: 2 molar ratio of Fe to Met or Gly (Fe + Met or Fe + Gly), Fe-Met with weak chelation strength (Fe-Met W, feed grade, 14.7% Fe, and $Q_f = 1.37$ by analysis), Fe proteinate with moderate chelation strength (Fe-Prot M, feed grade, 14.2 % Fe, and $Q_f = 43.6$ by analysis), and Fe proteinate with extremely strong chelation strength (Fe-Prot ES, feed grade, 10.2% Fe, and $Q_f = 8,590$ by analysis). The Fe-Gly, Fe-Met W, Fe-Prot M, and Fe-Prot ES used in the present study are commercially available, and their Fe concentrations and Q_f values are all from previous studies in our laboratory (Zhang et al., 2016a, 2017). The two perfused Fe concentrations were 3.58 and 7.16 mM Fe, or two small intestinal segments were jejunal and ileal loops, respectively. Additionally, the two control treatments (jejunal and ileal loops) with Fe-free solution were designed to exclude the effect of endogenous Fe for Fe absorption calculation and also as the 0 Fe source for gene expressions of transporters. Therefore, there were a total of 30 treatments. The jejunum or ileum of each bird was used as one replicate of the ligated loops of the

corresponding small intestinal segments, and there were a total of 10 jejunal or ileal replicate loops ($n = 10$) per treatment.

Birds, Diets, and Preparations and Fe Perfusions of Intestinal Loops

All experimental procedures were approved by the Animal Management Committee (in charge of animal welfare issue) of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (IAS-CAAS, Beijing, China) and performed in accordance with the guidelines. Ethical approval on animal survival was given by the animal ethics committee of IAS-CAAS.

Two hundred 1-d-old Arbor Acres commercial male chicks (Huadu Broiler Breeding Corp., Beijing, PR China) were housed in electrically heated and thermostatically controlled cages. The birds were allowed ad libitum access to a Fe-sufficient corn-soybean diet (146 mg Fe/kg diet by analysis, as-fed basis) and tap water with no detectable Fe for the first 21 d. Then, the birds were fed a Fe-deficient casein-dextrose basal diet (4.94 mg Fe/kg diet by analysis, as-fed basis) for the next following 7 d to deplete Fe stores in the body (liver Fe concentration was 41.2 ng/g, fresh basis). Diets used in this experiment as well as broiler feeding and management were the same as those used in a previous study from our laboratory (Zhang et al., 2017). At 28 d of age, after an overnight fast, 150 birds were weighed, selected and used to prepare the in situ ligated jejunal or ileal loops of 30 treatments of 10 replicates each. Each bird provided one replicate jejunal segment and one replicate ileal segment. Preparations and Fe perfusions of jejunal or ileal loops followed the same procedures as described previously in our laboratory (Zhang et al., 2016b, 2017).

Sample Collections and Preparations

The Fe absorption rate was highest at 30 min after perfusion (Zhang et al., 2016b, 2017; Li et al., 2017), therefore, 2 mL of perfused solutions in the ligated jejunal and ileal loops were harvested with syringes at 30 min after perfusion, and then all chicks were killed by cervical dislocation. The harvested solutions were centrifuged at $3000 \times g$ for 10 min, then the supernatants were collected and divided into two parts, one part was stored at 4 °C for analyzing phenol red concentrations to correct the changes in Fe concentrations resulting from water absorption or intestinal secretion since phenol red acts as a non-absorbable marker in the luminal

medium (Zhang et al., 2016b, 2017), and the other part was frozen at -20 °C until Fe concentrations were analyzed. After the perfused solutions were collected, the ligated jejunal and ileal loops of the control group and groups treated with 3.58 mM Fe from each Fe source were excised, flushed with ice-cold saline solution and slit lengthwise. Then, the mucosa was scraped with an ice-cold microscope slide, immediately frozen in liquid nitrogen, and stored at -80 °C until mRNA and protein expression analyses of the transporters.

Determinations of Fe and Phenol Red Contents

Iron contents in the perfusion solutions of the loops were determined by coupled plasma emission spectroscopy (Thermal Jarrell Ash, Waltham, MA) after wet digestion with HNO_3 and HClO_4 (Li et al., 2017). The bovine liver powder was used as the standard reference to validate the mineral analysis [GBW (E) 080193, National Institute of Standards and Technology, Beijing]. The concentrations of phenol red in perfusion solutions were assayed by a method as previously described (Zhang et al., 2016b, 2017; Li et al., 2017). Final volumes of solutions and Fe absorption velocities were calculated according to the equations shown in a previous study (Zhang et al., 2017).

Analyses of mRNA Expression Levels by Real-Time Quantitative PCR

Total RNA in the intestinal mucosa was extracted using TRIzol reagent (catalog number 15596018, Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The concentrations of total RNA were estimated by measuring ultraviolet light absorbance at 260 nm (NanoDrop 2000, Waltham, MA). The reverse transcriptions of mRNA were conducted using the PrimeScript RT reagent Kit with gDNA Eraser (Catalog number RR047A, Takara Bio Inc., Otsu, Japan) following the manufacturer's proposals. Real-time quantitative PCR was performed in triplicate on an ABI 7900HT system (Life technologies) using the SYBR Green PCR Master Mix (Catalog number 4367659, Life Technologies) according to optimized PCR protocols (Zhang et al., 2017). The primers of *DMT1*, *FPN1*, *EAAT3*, *LAT1*, *B⁰AT1*, *y⁺LAT1*, *y⁺LAT2*, *β -actin* (housekeeping gene) and *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* gene (housekeeping gene) were chosen with Primer Express Software (Applied Biosystems Inc., Foster, CA), and their information was given in Table 1. The

Table 1. Primer sequences for real-time PCR amplification

Gene ¹	GenBank ID ²	Primer sequences ³	Position and length, bp
<i>β-Actin</i>	NM_205518.1	F: 5'-CGGTACCAATTACTGGTGTAGATG-3' R: 5'-GCCTTCATTACATCTATCACTGG-3'	1,518 to 1,680, and 163
<i>GAPDH</i>	NM_204305.1	F: 5'-CTTTGGCATTGTGGAGGGTC-3' R: 5'-ACGCTGGGATGATGTTCTGG-3'	552 to 679, and 128
<i>DMT1</i>	NM_001128102.1	F: 5'-AGCCGTTACCACTTATTTTCG-3' R: 5'-GGTCCAAATAGGCGATGCTC-3'	223 to 352, and 130
<i>FPN1</i>	XM_015289163.1	F: 5'-GGAGACTGGGTGGACAAGAAGTC-3' R: 5'-GATGCATTCTGAACAACCAAGGA-3'	558 to 625, and 68
<i>EAAT3</i>	XM_424930.5	F: 5'-TGCTGCTTTGGATTCCAGTGT-3' R: 5'-AGCATGACTGTAGTGCAGAAGTAATATAT-3'	916 to 990, and 75
<i>LAT1</i>	NM_001030579.2	F: 5'-TGGCCTTGTACAGTGGTCTT-3' R: 5'-GCTTCGGACTTCAGCATCTG-3'	849 to 1,044, and 196
<i>B⁰AT1</i>	XM_419056.5	F: 5'-TGCGTAGGGTTTTGTGTTGG-3' R: 5'-AACTCCAGACTCCCACACTG-3'	252 to 438, and 187
<i>y⁺LAT1</i>	XM_015282844.1	F: 5'-CAGAAAACCTCAGAGCTCCCTTT-3' R: 5'-TGAGTACAGAGCCAGCGCAAT-3'	936 to 1,006, and 71
<i>y⁺LAT2</i>	XM_001231336.4	R: 5'-GCCCTGTACAGTAAATCAGACAAGA-3' R: 5'-TTCAGTTGCATTGTGTTTTGGTT-3'	494 to 575, and 82

¹*GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *DMT1* = divalent metal transporter 1; *FPN1* = ferroportin 1; *EAAT3* = excitatory amino acid transporter 3; *LAT1* = L-type amino transporter 1; *B⁰AT1* = B⁰-type amino acid transporter 1; *y⁺LAT1* = y⁺-L-type amino transporter 1; *y⁺LAT2* = y⁺-L-type amino transporter 2.

²ID = identity.

³F = forward; R = reverse.

expression of target genes was normalized using the geometric mean of *β-actin* and *GAPDH* with the 2^{-ΔΔCT} method as described previously (Livak and Schmittgen, 2001; Sun et al., 2017).

Tissue Preparations and Western Blottings

The intestinal mucosa samples (40 mg) were crushed and homogenized in 0.4 mL of ice-cold RIPA lysis buffer (catalog number P0013B; Beyotime, Haimen, China) with protease inhibitors (catalog number 4693159001, Roche, Penzberg, Germany). Then, the homogenates were centrifuged at 12,000 × *g* for 4 min at 4 °C. After that, the supernatants were subjected to western-blot analysis (Zhang et al., 2017). The GAPDH protein was used to normalize the expression level of the target protein (Qin et al., 2017).

Statistical Analyses

All data were analyzed using the GLM procedure (version 9.2; SAS Institute Inc., Cary, NC). The analysis of Fe absorption velocities was performed using three-way ANOVA, and the model included the effects of Fe source, Fe concentration, intestinal segment, and their interaction of two or three factors. The mRNA expression levels of each transporter in different intestinal

segments were determined simultaneously, thus the data for the mRNA expression levels of transporters were analyzed using two-way ANOVA, and the model included the effects of Fe source, intestinal segment, and their interaction. In addition, the protein expression levels of each Fe transporter in different intestinal segments were not determined simultaneously, thus the analysis of DMT1 and FPN1 protein expression levels was subjected to one-way ANOVA. If the variances were significant, differences among means were tested with the LSD method, and statistical significance was detected at *P* ≤ 0.05. Each ligated intestinal segment loop was used as the experimental unit.

RESULTS

Iron Absorption in the Ligated Intestinal Loops of Chicks

Iron source and concentration affected (*P* < 0.001) Fe absorption in the ligated jejunal and ileal loops of chicks, but no interaction (*P* > 0.71) of either 2 or 3 factors was observed (Table 2). Iron absorption was higher (*P* < 0.03) for Fe-Prot M and Fe-Prot ES than for FeSO₄•7H₂O, for Fe-Pro ES than for Fe-Met W, and for Fe + Gly and Fe-Gly than for

Table 2. Effects of Fe source and concentration on the Fe absorption in the ligated jejunum and ileum of broilers

Fe source ¹	Fe absorption (nmol·min ⁻¹ ·cm ⁻¹)			
	3.58 mM		7.16 mM	
	Jejunum ²	Ileum ²	Jejunum ²	Ileum ²
FeSO ₄ ·7H ₂ O	11.7	12.4	16.9	16.5
Fe + Met	13.7	13.9	17.3	17.9
Fe + Gly	13.8	14.2	18.9	19.1
Fe-Gly	12.4	14.9	19.1	19.5
Fe-Met W	13.0	13.6	18.4	19.1
Fe-Prot M	14.0	15.7	17.9	20.8
Fe-Prot ES	15.7	17.3	20.8	21.1
Pooled SEM	0.51			
Fe source ³				
FeSO ₄ ·7H ₂ O	14.4 ^c			
Fe + Met		15.7 ^{bc}		
Fe + Gly	16.5 ^b			
Fe-Gly	16.5 ^b			
Fe-Met W		16.0 ^{bc}		
Fe-Prot M		17.1 ^{ab}		
Fe-Prot ES	18.7 ^a			
Pooled SEM	0.66			
Fe concentration ⁴				
3.58 mM	14.0 ^b			
7.16 mM	18.8 ^a			
Pooled SEM	0.36			
Intestinal segment ⁵				
Jejunum	16.0			
Ileum	16.9			
Pooled SEM	0.36			
<i>P</i> -value ^d				
Fe source	0.001			
Fe concentration	<0.0001			
Intestinal segment	0.08			
Source × concentration	0.97			
Source × segment	0.93			
Concentration × segment	0.65			
Source × concentration × segment	0.98			

^{a-c}Within a column lacking a common superscript differ ($P < 0.03$).

^dProbability of main effect.

¹Fe + Gly = the mixture of FeSO₄·7H₂O with Gly; Fe + Met = the mixture of FeSO₄·7H₂O with Met; Fe-Gly = Fe-Gly chelate; Fe-Met W = Fe-Met with a weak chelation strength [quotient of formation (Q_f) = 1.37]; Fe-Prot M = Fe proteinate with moderate chelation strength (Q_f = 43.6); Fe-Prot ES = Fe proteinate with extremely strong chelation strength (Q_f = 8,590).

²Data represent the means of 10 replicates ($n = 10$).

³Data represent the means of 40 replicates ($n = 40$).

⁴Data represent the means of 140 replicates ($n = 140$).

⁵Data represent the means of 140 replicates ($n = 140$).

FeSO₄·7H₂O. However, there were no differences ($P > 0.08$) between Fe-Prot ES and Fe-Prot M or Fe-Met W and FeSO₄·7H₂O, or among Fe + Met, Fe + Gly, Fe-Gly, Fe-Met W, and Fe-Prot M. Iron absorption increased ($P < 0.001$) with increasing Fe concentration, and no differences ($P > 0.07$) in Fe absorption were detected between the jejunum and ileum.

mRNA Expression Levels of Fe and Amino Acid Transporters in the Ligated Intestinal Loops

The *DMT1*, *LAT1*, and *B⁰AT1* mRNA levels were affected ($P < 0.02$) by Fe source and intestinal segment, but not influenced by their interaction ($P > 0.22$) (Table 3). The *DMT1* mRNA levels were lower ($P < 0.005$), but *LAT1* and *B⁰AT1* mRNA

Table 3. Effects of Fe source and intestinal segment on the mRNA expressions of Fe and amino acid transporters in the ligated jejunal and ileal loops of broilers¹

Intestinal segment	Fe source ²	<i>DMT1</i> RQ ³	<i>FPN1</i> RQ ³	<i>EAAT3</i> RQ ³	<i>LAT1</i> RQ ³	<i>B⁰AT1</i> RQ ³	<i>y⁺LAT1</i> RQ ³	<i>y⁺LAT2</i> RQ ³
Jejunum	Control	0.24	0.47	3.24	0.93	1.39	2.08	0.65
	FeSO ₄ •7H ₂ O	0.13	0.48	2.95	0.84	2.79	4.40	0.68
	Fe + Met	0.13	0.44	2.84	1.61	4.24	4.26	0.70
	Fe + Gly	0.13	0.34	2.59	1.22	3.90	4.61	0.71
	Fe-Gly	0.13	0.36	2.78	0.97	3.75	4.15	0.74
	Fe-Met W	0.12	0.37	2.35	0.96	2.89	3.86	0.74
	Fe-Prot M	0.11	0.35	2.16	1.10	4.77	4.05	0.66
	Fe-Prot ES	0.14	0.43	2.43	1.13	3.15	5.62	0.81
Ileum	Control	0.061	0.09	2.05	0.78	0.28	0.70	1.62
	FeSO ₄ •7H ₂ O	0.033	0.11	3.30	2.52	0.97	1.90	1.95
	Fe + Met	0.031	0.13	3.74	2.31	0.72	1.33	2.44
	Fe + Gly	0.031	0.12	2.90	3.04	0.80	1.30	1.97
	Fe-Gly	0.033	0.14	3.41	2.17	0.87	1.37	1.92
	Fe-Met W	0.031	0.14	2.96	3.14	0.93	1.11	1.82
	Fe-Prot M	0.029	0.12	2.70	2.20	0.95	1.17	1.98
	Fe-Prot ES	0.032	0.12	3.31	2.93	0.84	0.96	2.01
Pooled SEM		0.016	0.051	0.39	0.37	0.65	0.69	0.24
Fe source ⁴								
	Control	0.15 ^a	0.28	2.65	0.86 ^b	0.84 ^b	1.39	1.14
	FeSO ₄ •7H ₂ O	0.079 ^b	0.30	3.13	1.68 ^a	1.88 ^a	3.15	1.32
	Fe + Met	0.082 ^b	0.29	3.29	1.96 ^a	2.48 ^a	2.80	1.57
	Fe + Gly	0.078 ^b	0.23	2.75	2.13 ^a	2.35 ^a	2.96	1.34
	Fe-Gly	0.082 ^b	0.25	3.10	1.57 ^a	2.31 ^a	2.76	1.33
	Fe-Met W	0.077 ^b	0.26	2.66	2.05 ^a	1.91 ^a	2.49	1.28
	Fe-Prot M	0.069 ^b	0.24	2.43	1.65 ^a	2.86 ^a	2.61	1.32
	Fe-Prot ES	0.088 ^b	0.28	2.87	2.03 ^a	2.00 ^a	3.29	1.41
	Pooled SEM	0.011	0.034	0.29	0.27	0.42	0.48	0.16
Intestinal segment ⁵								
	Jejunum	0.14 ^a	0.41 ^a	2.67 ^b	1.10 ^b	3.36 ^a	4.13 ^a	0.71 ^b
	Ileum	0.034 ^b	0.12 ^b	3.05 ^a	2.39 ^a	0.80 ^b	1.23 ^b	1.96 ^a
	Pooled SEM	0.005	0.015	0.13	0.12	0.19	0.24	0.077
<i>P</i> -value ^c								
	Fe source	0.0041	0.97	0.26	0.010	0.0002	0.14	0.61
	Intestinal segment	<0.0001	<0.0001	0.039	<0.0001	<0.0001	<0.0001	<0.0001
	Fe source × intestinal segment	0.97	0.094	0.21	0.23	0.31	0.074	0.74

^{a,b}Within a column in a group lacking a common superscript differ ($P < 0.05$).^cProbability values of main effect.¹Data represent the means of 10 replicates ($n = 10$).

²Fe + Met = mixture of FeSO₄•7H₂O with Met; Fe + Gly = mixture of FeSO₄•7H₂O with Gly; Fe-Gly = Fe-Gly chelate; Fe-Met W = Fe-Met with a weak chelation strength [quotient of formation (Q_f) = 1.37]; Fe-Prot M = Fe proteinate with moderate chelation strength (Q_f = 43.6); Fe-Prot ES = Fe proteinate with extremely strong chelation strength (Q_f = 8,590); *DMT1* = divalent metal transporter 1; *FPN1* = ferroportin 1; *EAAT3* = excitatory amino acid transporter 3; *LAT1* = L-type amino transporter 1; *B⁰AT1* = B⁰-type amino acid transporter 1; *y⁺LAT1* = y⁺L-type amino transporter 1; *y⁺LAT2* = y⁺L-type amino transporter 2.

³The mRNA levels were calculated as the relative quantities (**RQ**) of the target gene mRNA to the geometric mean of β-actin mRNA and GAPDH mRNA using the 2^{-ΔΔCT} method.

⁴Data represent the means of 20 replicates ($n = 20$).⁵Data represent the means of 80 replicates ($n = 80$).

levels were higher ($P < 0.05$) in the ligated intestinal loops perfused with solutions containing different Fe sources than in those perfused with the Fe-free control solution, but there were no differences ($P > 0.06$) among the Fe treatment groups. Additionally, the *DMT1* or *B⁰AT1* mRNA levels were higher

($P < 0.0001$), and *LAT1* mRNA levels were lower ($P < 0.001$) in the jejunum than in the ileum.

The *FPN1* and *EAAT3* mRNA levels in the ligated intestinal loops were affected ($P < 0.0001$) by intestinal segment, but not influenced ($P > 0.09$) by Fe source and Fe source × intestinal segment

interaction (Table 3). The *FPN1* mRNA levels were higher ($P < 0.0001$), but the *EAAT3* mRNA levels were lower ($P < 0.04$) in the jejunum than in the ileum.

The intestinal segment affected ($P < 0.0001$) the y^+LAT1 and y^+LAT2 mRNA levels in the ligated intestinal loops, but Fe source and the interaction between Fe source and intestinal segment did not affect ($P > 0.07$) them (Table 3). The y^+LAT1 mRNA levels were greater ($P < 0.0001$), but the y^+LAT2 mRNA levels were lower ($P < 0.0001$) in the jejunum than in the ileum.

Protein Expression Levels of Fe Transporters in the Ligated Intestinal Loops

Iron source had no effect ($P > 0.50$) on the protein expression levels of DMT1 and FPN1 in the ligated jejunal and ileal loops of chicks (Table 4).

DISCUSSION

The results of this study showed that regardless of Fe source, Fe absorption in the jejunum and ileum increased as the perfused Fe concentration increased, which was consistent with our previous results on duodenal Fe absorption of broilers (Li et al., 2017; Zhang et al., 2017). A series of studies have demonstrated that organic Fe is more bioavailable than inorganic Fe in broilers (Shinde et al., 2011; Ma et al., 2014; Kwiecień et al., 2015; Zhang et al., 2016a), and the chelation strength might be an important factor which influences the

bioavailabilities of the organic trace elements (Li et al., 2004, 2005; Huang et al., 2009, 2013; Liu et al., 2012, 2013; Ma et al., 2014; Zhang et al., 2016a). Results from this study agree with our previous findings (Li et al., 2017; Zhang et al., 2017), indicating that organic Fe sources with greater Q_f values had higher Fe absorption in the duodenum of broilers, which might explain why the organic Fe sources with greater Q_f values has higher bioavailability (Zhang et al., 2016a). There are two hypotheses to interpret the difference of organic trace elements in the mechanisms of absorption and utilization (Ashmead, 1993). One hypothesis is that the organic mineral complex or chelate with optimal Q_f could resist the interference of the antinutritional factors from the digestive tract and directly reach the intestinal brush border, where it can be hydrolyzed and absorbed as ions into the blood, resulting in a higher bioavailability than the inorganic form (Cook et al., 1972). The other is that it could maintain its structural integrity in the digestive tract and arrive at absorptive sites in the small intestine as the original intact molecules, and then be absorbed and metabolized as such, rendering superior bioavailability to the inorganic source (Ashmead, 2001). However, until now, there has been no direct evidence to support either of these hypotheses. Additionally, in this study, Gly but not Met supplementations significantly increased the absorption of Fe as $FeSO_4 \cdot 7H_2O$, indicating that Gly was more effective in facilitating Fe absorption than Met as a ligand. However, a previous study

Table 4. Effect of Fe source on the protein expression levels of DMT1 and FPN1 in the ligated jejunum and ileum loops of broilers¹

Fe source ²	Jejunum		Ileum	
	DMT1, RQ ³	FPN1, RQ ³	DMT1, RQ ³	FPN1, RQ ³
Control	1.00	1.00	1.00	1.00
$FeSO_4 \cdot 7H_2O$	0.84	0.94	1.12	1.08
Fe + Met ³ F	1.03	1.03	0.61	1.39
$FeSO_4 \cdot 7H_2O$ + Met				
Fe + Gly ³ $FeSO_4 \cdot 7H_2O$ + Gly	0.82	1.00	0.80	1.26
Fe-Gly ³	0.89	0.97	1.05	0.86
Fe-Met W ³	0.85	0.92	0.83	1.13
Fe-Prot M ³	0.89	1.00	0.81	0.81
Fe-Prot ES ³	0.82	0.99	0.75	1.10
SEM	0.11	0.14	0.20	0.22
P-value ^a	0.84	1.00	0.67	0.51

^aProbability values of main effect.

¹Data represent the means of 10 replicates ($n = 10$).

²Fe + Met = mixture of $FeSO_4 \cdot 7H_2O$ with Met; Fe + Gly = mixture of $FeSO_4 \cdot 7H_2O$ with Gly; Fe-Gly = Fe-Gly chelate; Fe-Met W = Fe-Met with a weak complex strength [quotient of formation (Q_f) = 1.37]; Fe-Prot M = Fe proteinate with moderate chelation strength (Q_f = 43.6); Fe-Prot ES = Fe proteinate with extremely strong chelation strength (Q_f = 8,590).

³The protein levels were calculated as the RQ of the target gene protein to the glyceraldehyde-3-phosphate dehydrogenase protein, and the average expression level of protein in the control treatment was used as a calibrator; DMT1 = divalent metal transporter 1; FPN1 = ferroportin 1.

with broilers showed that Gly supplementation did not improve the Fe absorption as FeSO_4 in the small intestine (Jia et al., 2015). The disparity in these results might be due to the differences in perfused Fe concentrations (present 3.58 or 7.16 mM vs. previous 5.96 mM) and perfusion time (present 30 min vs. previous 120 min) used in the two studies.

The absorption mechanism of inorganic Fe source has been well studied. Inorganic Fe must be reduced first before it can be utilized. In this process, membrane-associated ferrireductase (duodenal cytochrome *b* reductase, **Dcytb**) is the only Fe-regulated ferric reductase in the duodenal mucosa, and that loss of Dcytb affects Fe absorption (McKie, 2008). After Fe^{3+} is reduced to Fe^{2+} , the latter is absorbed via DMT1 into the intestinal epithelium (Gunshin et al., 1997), and then is exported into the bloodstream by the basolateral Fe exporter FPN1 (Donovan et al., 2000; McKie et al., 2000) in conjunction with the ferroxidase hephaestin (Tako et al., 2010). The FPN1 is a sole identified Fe exporter in mammals and essential for Fe exporting from enterocytes to bloodstream (Donovan et al., 2005). However, how organic Fe sources such as the Fe amino acid chelates or Fe proteinates were transferred to the bloodstream, and the status of organic Fe in the process of Fe transport across the intestinal epithelial cell was still unclear. In the current study, the *DMT1* mRNA expression was higher in the jejunum than in the ileum, which was consistent with our previous findings on inorganic Fe absorption of broilers (Zhang et al., 2006b), indicating that the *DMT1* mRNA expression gradually reduced as the extension of the small intestine. In addition, our present results also demonstrated that regardless of Fe source, the addition of Fe decreased the mRNA expression of *DMT1* in the jejunum and ileum of broilers. Similarly, some studies with chicks, rats, bull calves, or pigs have also shown a depressive effect of high Fe supplementation on duodenal *DMT1* expression (Hansen et al., 2009, 2010; Zhang et al., 2017). Fe^{2+} is highly reactive, and can be easily oxidized to cause cell and tissue injury (Ganz and Nemeth, 2012), and excess Fe in the enterocytes might downregulate the *DMT1* mRNA expression to prevent toxic accumulation of Fe. Previous studies showed that the *FPN1* mRNA expression gradually decreased from duodenum to ileum (McKie et al., 2000; Zhang et al., 2016b), which is in line with the results from the present study showing that the *FPN1* mRNA expression in the jejunum was higher than that in the ileum. Zhang et al. (2017) found that the perfusion of Fe (3.58 mM) as organic

or inorganic Fe sources upregulated the mRNA expression of *FPN1* in the duodenum of broilers. However, in the current study, the supplementation of the same dose of Fe as organic or inorganic Fe sources did not influence the mRNA expression of *FPN1* in the jejunum and ileum. These results suggest that FPN1 might play a crucial role in the Fe absorption in the duodenum, but play a limited role in the jejunum and ileum. Additionally, in this study, no difference was detected in the *DMT1* and *FPN1* mRNA expression levels in the jejunum and ileum among the different Fe sources, which might result from the very short time (30 min) of Fe perfusion. Zhang et al. (2017) obtained similar results in a study on the duodenal *FPN1* expression regulated by Fe treatment.

Excitatory amino transporter 3 is a Na^+ -dependent transporter with a high affinity for anionic amino acids, including Asp and Glu (Kanai and Hediger, 2004). A previous study on broilers demonstrated that quantities of *EAAT3* mRNA were greatest in the ileum, intermediate in the jejunum, and lowest in the duodenum (Gilbert et al., 2007). Our results are in an agreement with these findings. The LAT1 is an amino acid transporter, which preferentially transports large neutral amino acids with branched or aromatic side chains such as Leu, Ile, Val, Phe, Tyr, Try, Met, and His (Kanai et al., 1998). In this study, the *LAT1* mRNA levels in the ileum were greater than those in the jejunum. Similar results were reported by other scientists (Gilbert et al., 2007). The $\text{B}^0\text{AT1}$ transporter belongs to B^0 transport system, which mediates the Na^+ -driven uptake of a broad range of neutral amino acids into epithelial cells of small intestine and kidney proximal tubule (Bröer et al., 2004). The lack of $\text{B}^0\text{AT1}$ leads to the reduction of neutral amino acid absorption in the intestine and loss of neutral amino acids in the urine, which causes an overload of amino acids in the lumen of the intestine and reduced systemic amino acid availability (Dave et al., 2004). In this study, the *B⁰AT1* mRNA was mainly expressed in the jejunum, which is consistent with the results from a previous study on *Mus musculus* (Bröer et al., 2004). Among the members of the LAT family, $\text{y}^+\text{LAT1}$ and $\text{y}^+\text{LAT2}$, are two unique amino acid transporters of y^+L transport system because of their peculiar Na^+ dependence (Kanai et al., 2000). System y^+L was originally identified as a transport system that transports both neutral and basic amino acids in the erythrocyte plasma membrane (Devés et al., 1992). In the current study, the $\text{y}^+\text{LAT1}$ mRNA levels were greater in the jejunum than in the ileum, whereas

the y^+LAT2 mRNA levels were greater in the ileum as compared with the jejunum. These results were not in line with a previous study (Gilbert et al., 2007), which indicated that the mRNA expression levels of the two transporters were similar in the jejunum and ileum. The different ages (present day 28 vs. previous day 14) of the birds in the two studies might partially explain the inconsistency. The supplementation of Fe as organic or inorganic Fe sources did not affect the y^+LAT1 and y^+LAT2 mRNA expression levels in the jejunum and ileum, suggesting that the y^+LAT1 and y^+LAT2 might not participate in the absorption of Fe as organic Fe sources.

There have been some reports providing evidence to support the above-mentioned second assumption that complexed or chelated trace minerals might be absorbed in their intact form. Wapnir et al. (1983) demonstrated that the Zn–L–His complex could be absorbed in its intact form by perfused rat intestines in the same way as His. Lowe et al. (1994) reported that Zn as an amino acid chelate was transported across the intestinal enterocyte and into the circulation in an intact form in dogs. A previous study with Caco-2 cells revealed that Cu^{2+} was transported as a complex of Met into the epithelium, which was mediated by amino acid transporters (Gao et al., 2014). However, whether Fe as Fe amino acid chelates was absorbed as free ions or amino acid complexes is still not well understood. In this study, the addition of Fe as Fe amino acid chelates enhanced the mRNA expression of *LAT1*, and *B⁰AT1* in the small intestine, suggesting that these amino acid transporters might participate in the absorption of Fe as Fe amino acid chelates. However, no differences were found in the mRNA expression of amino acid transporters among organic or inorganic Fe treatment groups. Thus, it could not be elucidated why the Fe absorption of organic Fe sources with stronger chelation strengths was better than that of inorganic Fe source.

It was reported that the rats perfused with Fe–Gly chelate had lower DMT1 protein expression but higher FPN1 protein expression in the duodenum after 4-h perfusion (Zhuo et al., 2014). In this study, Fe treatment had no effect on the DMT1 and FPN1 protein expression levels in the jejunum and ileum of broilers, which is in an agreement with the results from our previous study (Zhang et al., 2017), indicating that the DMT1 and FPN1 protein levels in the duodenum were not sensitive parameters in reflecting the changes of Fe status of broilers. These results might be due to the very short time

(30 min) of Fe perfusion. It is suggested that the maximal abundance of protein expression might exhibit a few hours after the enhanced quantities of corresponding mRNA (Blalock et al., 1988; Frazer et al., 2003). Therefore, further studies are needed to investigate the DMT1 and FPN1 protein expressions in the broiler's small intestine subjected to a longer-time Fe perfusion.

In conclusion, the organic Fe sources with stronger chelation strengths had higher Fe absorption in the jejunum and ileum of broilers. As a ligand, Gly was more effective than Met in facilitating Fe absorption. The mRNA expressions of Fe and amino acid transporters changed along with the extension of the small intestine. The DMT1, LAT1, and *B⁰AT1* might participate in the absorption of Fe as Fe amino acid chelates in the jejunum or ileum. Further studies are needed to elucidate the exact mechanisms of organic Fe absorption mediated by amino acid transporters in the small intestine of chicks.

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