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# Metabolic utilization of intravenously injected iron from different iron sources in target tissues of broiler chickens

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ABSTRACT

No information is available regarding the utilization of iron (Fe) from different Fe sources at a target tissue level. To detect differences in Fe metabolic utilization among Fe sources, the effect of intravenously injected Fe on growth performance, hematological indices, tissue Fe concentrations and Fe-containing enzyme activities and gene expressions of Fe-containing enzymes or protein in broilers was investigated. On d 22 post-hatching, a total of 432 male chickens were randomly allotted to 1 of 9 treatments in a completely randomized design. Chickens were injected with either a 0.9% (wt/vol) NaCl solution (control) or a 0.9% NaCl solution supplemented with Fe sulphate or 1 of 3 organic Fe sources. The 3 organic Fe sources were Fe chelates with weak (Fe-MetW), moderate (Fe-ProtM) or extremely strong (Fe-ProtES) chelation strength. The 2 Fe dosages were calculated according to the Fe absorbabilities of 10% and 20% every 2 d for a duration of 20 d. Iron injection did not affect ($P > 0.05$) ADFI, ADG or FCR during either 1 to 10 d or 11 to 20 d after injections. Hematocrit and Fe concentrations in the liver and kidney on d 10 after Fe injections, and Fe concentrations in the liver or pancreas and ferritin heavy-chain (FTH1) protein expression level in the liver or spleen on d 20 after Fe injections increased ($P \leq 0.05$) as injected Fe dosages increased. When the injected Fe level was high at 20% Fe absorbability, the chickens injected with Fe-ProtES had lower ($P < 0.001$) liver or kidney Fe concentrations and spleen FTH1 protein levels than those injected with Fe-MetW or Fe-ProtM on d 20 after injections. And they had lower ($P < 0.05$) liver cytochrome C oxidase mRNA levels on d 20 after injections than those injected with Fe-MetW or Fe sulphate. The results from this study indicate that intravenously injected Fe from Fe-ProtES was the least utilizable and functioned in the sensitive target tissue less effectively than Fe from Fe sulfate, Fe-MetW or Fe-ProtM.

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## 1. Introduction

Iron (Fe) is an essential trace element required in numerous important biological processes of animals (Hansen et al., 2009; Ganz and Nemeth, 2011). Rapidly growing chicks have a high demand for Fe (Ma et al., 2016), so Fe additives are routinely supplemented into diets for optimal growth. Traditionally, Fe is added to diets in the form of inorganic salts which have many disadvantages, such as low bioavailability, high hydroscopicity and oxidation (Ma et al., 2014). In recent years, organic Fe sources have been developed as alternatives to traditional inorganic Fe sources. However, reported results on bioavailabilities of organic Fe sources are inconsistent (Cao et al., 1996; Yu et al., 2000; Shinde et al., 2011; Luiggi et al., 2014). Previous studies from our laboratory indicated that the bioavailabilities of organic Fe sources for broilers were closely correlated with their chelation strengths (quotient of formation [$Q_f$ ] values) between Fe and their ligands (Ma et al., 2014; Zhang et al., 2016). The Fe proteinate with

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moderate chelation strength is more available than iron sulfate in enhancing hemoglobin (Hb) and total body Hb Fe of broilers fed a casein–dextrose diet (Ma et al., 2014). Relative to Fe sulfate (assigned 100%), the bioavailabilities of organic Fe sources with weak, moderate and extremely strong chelation strength for broilers fed a conventional maize–soybean meal basal diet were 129%, 164% and 174%, respectively, therefore, organic Fe sources with greater $Q_f$ values showed higher Fe bioavailabilities (Zhang et al., 2016). However, it is not clear whether the differences in bioavailabilities of Fe from different sources were due to the differences in Fe absorption or in Fe metabolic utilization, or in both aspects because the method of Fe administration in the above studies was dietary supplementation. Recent studies from our laboratory have further indicated that organic Fe sources with greater $Q_f$ values showed higher Fe absorption in the small intestine of broilers (Li et al., 2017; Zhang et al., 2017; Lu et al., 2018). However, different absorptions of organic Fe sources in the small intestine of broilers could not fully explain the differences in their bioavailabilities, and thus, part of them might be associated with the different metabolic utilization of Fe from organic Fe sources at a target tissue level. However, no studies on this aspect have been reported before.

Direct injection of Fe sources into a vein might be an effective method to study the Fe metabolic utilization at a target tissue level by bypassing intestinal absorption (Davidsson et al., 1989; Li et al., 2008; Shen et al., 2013). Previous studies from our laboratory demonstrated that an intravenously injected organic manganese (Mn) or zinc (Zn) source with strong chelation strength had the lowest Mn or Zn utilization in the target tissues of broilers (Li et al., 2008; Shen et al., 2013). We hypothesized that the intravenously injected organic Fe source with extremely strong chelation strength would have the lowest Fe utilization in the target tissues of broilers. Therefore, the objective of the present study was to investigate the effect of intravenously injected Fe from different Fe sources on growth performance, hematological parameters, tissue Fe concentrations and Fe-containing enzyme activities and gene expressions of Fe-containing enzymes or protein to detect the differences in metabolic utilization of Fe from different Fe sources in the target tissues of broilers.

## 2. Materials and methods

### 2.1. Animal ethics

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

### 2.2. Experimental design and treatments

A completely randomized design was adopted in this experiment. The 9 treatments included a 0.9% (wt/vol) NaCl injection solution without Fe (the control), and the 0.9% saline solution supplemented with either Fe sulphate ($FeSO_4 \cdot 7H_2O$, reagent grade; 19.5% Fe by analysis) or 1 of 3 organic Fe sources, at 2 injected Fe dosages (see the details below). The 3 organic Fe sources used in the current study were the same as those used in our previous studies (Zhang et al., 2017; Lu et al., 2018), and they included Fe methionine with weak chelation strength (Fe-MetW, feed grade; 14.7% Fe and $Q_f$ = 1.37 by analysis), Fe proteinate with moderate chelation strength (Fe-ProtM, feed grade; 14.2% Fe and $Q_f$ = 43.6 by analysis), and Fe proteinate with extremely strong chelation strength (Fe-ProtES, feed grade; 10.2% Fe and $Q_f$ = 8,590 by analysis).

It was assumed that the amount of Fe injected should be close to the normal amount of Fe absorbed when chickens were fed a diet containing the optimal Fe. Therefore, the injected dosage of Fe was calculated using the following equation:

$$\text{Fe injected (mg/bird)} = \text{Fe absorbability (\%)} \times \text{Average daily feed intake (kg/d)} \times \text{Dietary supplemental Fe level (40 mg/kg)} \times 2\ \text{(d)}.$$

It has been reported that dietary Fe absorption in animals ranges from about 5% to 30% (Ni et al., 1995; Liu et al., 2019), so the values of 10% and 20% were used. The average daily feed intake was adjusted every 7 d based on the feed intakes from 22 to 42 d of age according to published guidelines (Yang, 2003). An inclusion of 40 mg/kg of Fe in a corn–soybean meal basal diet is a normally added Fe level as determined by a previous study from our laboratory (Ma et al., 2016). The “2 (d)” in the equation represents a single injection interval for every 2 d. The injected Fe concentrations in the saline solution supplemented with either Fe sulphate or 1 of 3 organic Fe sources were 2.08 mg of Fe/mL (10% Fe absorbability solution) and 4.16 mg of Fe/mL (20% Fe absorbability solution) from 22 to 28 d of age, and 2.75 mg of Fe/mL (10% Fe absorbability solution) and 5.50 mg of Fe/mL (20% Fe absorbability solution) from 29 to 35 d of age, and 3.17 mg of Fe/mL (10% Fe absorbability solution) and 6.34 mg of Fe/mL (20% Fe absorbability solution) from 36 to 42 d of age.

### 2.3. Animals and diets

During 1 to 21 d of age, a total of 500 one-d-old Arbor Acres commercial male broilers (Huadu Broiler Breeding Corp., Beijing, China) were fed the same Fe-unsupplemented corn-soybean meal basal diet with all nutrients (except Fe) meeting or exceeding the requirements (NRC, 1994; Feeding standard of chicken, 2004, Table 1) of starting broilers to enhance their sensitivity to Fe injection. At 22 d of age, 432 birds were selected according to BW and randomly allotted to 1 of 9 treatments (8 replicate cages of 6 birds per cage) according to above experimental treatments. All injected solutions for all treatments contained the same concentration of methionine or lysine. All birds were fed on the same Fe-unsupplemented corn-soybean meal basal diet with all nutrients, except Fe, meeting or exceeding the requirements for growing broilers (NRC, 1994; Feeding standard of chicken, 2004, Table 1).

Birds were housed in electrically heated, thermostatically controlled stainless steel cages coated with plastic and equipped with plastic feeders and waterers. They were handled in accordance with the Arbor Acres Broiler Management Guide (Aviagen, 2009) for lighting and feeding, and allowed ad libitum access to tap water containing no detectable Fe. Each individual bird was injected with 0.5 mL of either the saline without Fe or with Fe addition through the vein of the wing every other day for 20 d. Feed intake and BW were recorded per cage on d 10 or 20 after injections to calculate ADFI, ADG and FCR during d 1 to 10 or d 10 to 20 after injections.

### 2.4. Sample collections and preparations

On d 10 and 20 after injections, 2 birds from each cage were selected according to the average BW of birds within the cage after fasting for 12 h. Blood samples were taken from each bird via heart puncture with stainless-steel needles equipped with heparinized blood-collection tubes. One part of the blood samples was stored at 4 °C for the analyses of hemoglobin (Hb) concentration and

**Table 1**
Ingredients and chemical composition of the basal diet for broilers, as-fed basis.

| Item | Day 1 to 21 | Day 22 to 42 |
|---|---|---|
| Ingredients, g/kg | | |
| Ground maize | 540.0 | 596.2 |
| Soybean meal | 374.0 | 320.3 |
| Soybean oil | 47.3 | 50.0 |
| Dicalcium phosphate[1] | 18.0 | 18.6 |
| Calcium carbonate[1] | 11.3 | 8.7 |
| Sodium chloride[1] | 3.0 | 3.0 |
| DL-Met | 3.2 | 1.4 |
| Premix[2, 3] | 3.2 | 2.0 |
| Chemical composition, g/kg | | |
| ME, MJ/kg | 12.61 | 12.98 |
| CP[4] | 217.0 | 203.0 |
| Lys | 11.2 | 10.0 |
| Met | 6.1 | 4.2 |
| Met + Cys | 9.0 | 7.2 |
| Ca[4] | 9.9 | 9.2 |
| Non-phytate P | 4.5 | 4.0 |
| Fe[4], mg/kg | 75.1 | 74.0 |

[1] Reagent grade.
[2] Provided miligrams per kilogram of diet from 1 to 21 d: retinyl acetate, 4.5; cholecalciferol, 0.11; DL-α-tocopheryl acetate, 16; menadione, 3; thiamin, 3; riboflavin, 9.6 pyridoxine, 3; vitamin $B_{12}$, 0.018; pantothenic acid, 15; niacin, 39; folic acid, 1.5; biotin, 0.18; choline, 700; Cu ($CuSO_4 \cdot 5H_2O$), 8; Mn ($MnSO_4 \cdot H_2O$), 110; Zn ($ZnSO_4 \cdot 7H_2O$), 60; I (KI), 0.35; Se ($Na_2SeO_3$), 0.15.
[3] Provided miligrams per kilogram of diet from 22 to 42 d: retinyl acetate, 3; cholecalciferol, 0.075; DL-α-tocopheryl acetate, 10.7; menadione, 2; thiamin, 2; riboflavin, 6.4; pyridoxine, 2; vitamin $B_{12}$, 0.012; pantothenic acid, 10; niacin,26; folic acid, 1; biotin, 0.1; choline, 500; Cu ($CuSO_4 \cdot 5H_2O$), 8; Mn ($MnSO_4 \cdot H_2O$), 80; Zn ($ZnSO_4 \cdot 7H_2O$), 40; I (KI), 0.35; Se ($Na_2SeO_3$), 0.15.
[4] Values determined by analysis. Each value based on triplicate determinations.

hematocrit (Hct), and the other was centrifuged at 3,000 × *g* for 10 min at 4 °C to isolate plasma, and then stored at −20 °C until analyses of plasma iron (PI) and total iron binding capacity (TIBC). The birds were subsequently killed by cervical dislocation; and their liver, kidney and heart samples were taken; A subsample was frozen at −20 °C for the analyses of Fe content, and succinate dehydrogenase (SDH), catalase (CAT) or cytochrome C oxidase (COX) activities, and another subsample on d 20 after infections was frozen in liquid N for the assays of CAT, SDH, COX or ferritin heavy chain (FTH1) mRNA and protein levels. The pancreas was also collected and frozen (−20 °C) for Fe concentration determinations. The spleen and right femur marrow samples on d 20 after injections were collected immediately and frozen in liquid nitrogen for the analyses of FTH mRNA and protein levels. The left tibia was excised and frozen in an individual heat-sealed polyethylene bag for Fe content analysis. Tibia bones were ashed in a muffle furnace at 550 °C as described by Ma et al. (2016). Samples of 2 individual chicks from each cage were pooled before analysis, and thus 8 replicate samples were obtained for each treatment.

### *2.5. Sample analyses*

#### *2.5.1. Iron concentrations*

Iron concentrations in the diets, water, and tissues were determined by inductively coupled plasma emission spectroscope (Thermal Jarrell Ash) after wet digestion with $HNO_3$ and $HClO_4$ as described by Li et al. (2017). Validation of the mineral analysis was conducted with the use of bovine liver powder (GBW (E) 080,193; National Institute of Standards and Technology) as a standard reference material.

#### *2.5.2. Blood indices*

The Hb concentration and Hct in the whole blood were analyzed by an automatic hematology analyzer (ABX Pentra DF 120; HORIBA Medical Inc., Montpellier, France). The PI and TIBC were determined by using the colorimetric method, and transferrin saturation (TS) in the plasma was calculated as PI/TIBC × 100 (Huebers et al., 1987).

#### *2.5.3. Enzyme activities*

The liver, heart and kidney samples were homogenized with 10% (wt/vol) ice-cold physiologic saline to obtain the homogenates for determination of enzyme activities. The SDH, CAT and COX activities were determined as described by Liao et al. (2017).

#### *2.5.4. mRNA levels*

The primer sequences for *Sdh*, *Cat*, *Cox*, *Fth1*, *β-actin* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) genes are shown in Table 2. The RNA isolation, reverse transcription and real-time PCR were performed as described previously (Ma et al., 2016). The abundances of *Cat*, *Sdh*, *Cox*, and *Fth1* mRNA were expressed as ratios of the target gene mRNA to the geometrical mean of β-actin and *GAPDH* mRNA (Liao et al., 2017).

#### *2.5.5. Western blotting*

The liver, heart, spleen or femur marrow samples were homogenized in ice-cold RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Then they were centrifuged for 4 min (12,000 × *g*, 4 °C), and the supernatants were subjected to western-blot analysis (Zhang et al., 2017). The GAPDH protein was used to normalize the expression levels of SDH, CAT, COX or FTH protein (Qin et al., 2017).

### *2.6. Statistical analyses*

Data were analyzed using SAS software (version 9.2; SAS Inst. Inc., Cary, NC, USA). To test the effect of the injected Fe, a single degree of freedom contrast was used to compare all injected Fe treatments with the control. Data excluding the control were further analyzed by two-way ANOVA with a model that included

**Table 2**
Primer sequences for real-time PCR amplification.

| Gene | GenBank ID | Primer sequences | Length, pb |
|---|---|---|---|
| *Cat* | NM001031215.2 | R: 5′-ACGCTGGGATGATGTTCTGG-3′<br>F: 5′-TTGCTGGAGAATCTGGGTC-3′ | 186 |
| *Sdh* | NM001080875 | R: 5′-CCTTCAAATGAGTCTGAGGGTT-3′<br>F: 5′- TACAAATCCATCGAGCCTTAC-3′ | 111 |
| *Cox* | MF102289.1 | R: 5′-GCACTCATAGAGTCCGTCCA-3′<br>F: 5′-GCAGGTGTTTCCTCCAT-3′ | 155 |
| *Fth1* | NM-205086.1 | R: 5′-GGTTGCGGTCGGTAAGT-3′<br>F: 5′-CGCCAGAACTACCACCAGG-3′ | 86 |
| *β-actin* | NM_205518.1 | R: 5′-TTCAGAGCCACATCATCCC-3′<br>F: 5′-CGGTACCAATTACTGGTGTTAGATG-3′ | 163 |
| *GAPDH* | NM_204305.1 | R: 5′-GCCTTCATTCACATCTATCACTGG-3′<br>F: 5′-CTTTGGCATTGTGGAGGGTC-3′ | 128 |

*Cat* = catalase; *Sdh* = succinate dehydrogenase; *Cox* = cytochrome C oxidase; *Fth1* = ferritin heavy chain 1; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase.

the main effects of the injected Fe source, injected Fe concentration and their interaction using the general linear model procedure of SAS. The replicate cage served as the experimental unit. Differences among the means were tested by the LSD test. $P \leqslant 0.05$ was considered to be statistically significant.

## 3. Results

### 3.1. Growth performance

Compared with the control, Fe injection had no effect ($P > 0.05$) on the ADG, ADFI or FCR during either 1 to 10 d or 11 to 20 d after injections (Table 3). Neither injected Fe source, Fe concentration, nor their interaction influenced ($P > 0.05$) the ADG, ADFI or FCR of broilers during either d 1 to 10 or d 11 to 20 after injections.

### 3.2. Hematological indices

Compared with the control, Fe injection increased ($P = 0.02$) Hct on d 10 after injections but did not affect ($P > 0.05$) all of the other blood parameters on d 10 or 20 after injections (Table 4). Injected Fe source, injected Fe concentration and their interaction did not influence ($P > 0.05$) TIBC and TS on both d 10 and 20 after injections, and Hb concentration on d 10 after injections. The PI on d 10 after injections was not affected ($P > 0.05$) by injected Fe source and injected Fe concentration, but was affected ($P = 0.04$) by their interaction. When the injected Fe level was high, the chickens injected with Fe-ProtES or Fe-MetW had higher ($P < 0.05$) PI than those injected with $FeSO_4 \cdot 7H_2O$ on d 10 after injections. The Hct on d 10 after injections, and Hb concentration and Hct on d 20 after injections were not affected ($P > 0.05$) by injected Fe source and the interaction between the injected Fe source and Fe level, but were increased ($P < 0.05$) by injected Fe level. The PI on d 20 after injections was not influenced ($P > 0.05$) by injected Fe level and the interaction between the injected Fe source and Fe level, but was affected ($P = 0.02$) by injected Fe source. The chickens injected with Fe-ProtES had lower ($P = 0.002$) PI than those injected with $FeSO_4 \cdot 7H_2O$ on d 20 after injections, but no differences ($P > 0.05$) were detected among the three organic Fe sources or among $FeSO_4 \cdot 7H_2O$, Fe-MetW and Fe-ProtM.

### 3.3. Iron concentrations

Compared with the control, Fe injection increased ($P < 0.01$) Fe concentrations in the liver and kidney on d 10 after injections, and Fe concentrations in the tibia bone ash on d 20 after injections (Table 5). Injected Fe source affected ($P < 0.01$) liver Fe concentrations on both d 10 and 20 after injections. The chickens injected with Fe-MetW or Fe-ProtM had higher ($P < 0.05$) liver Fe concentrations than those injected with $FeSO_4 \cdot 7H_2O$ or Fe-ProtES on d 10 after injections. Injected Fe level affected ($P < 0.01$) Fe concentrations in the liver, kidney and tibia bone ash on d 10 after injections, and Fe concentrations in the liver, pancreas and kidney on d 20 after injections, and the values of these parameters increased as injected Fe level increased ($P < 0.01$). The interaction between the injected Fe source and Fe level only affected ($P < 0.05$) Fe concentrations in the liver and kidney on d 20 after injections. When the injected Fe level was low, the chickens injected with Fe-MetW had higher ($P < 0.05$) liver Fe concentrations than those injected with $FeSO_4 \cdot 7H_2O$ or Fe-ProtES on d 20 after injections. However, when the injected Fe level was high, the chickens injected with $FeSO_4 \cdot 7H_2O$, Fe-MetW or Fe-ProtM had higher ($P < 0.01$) liver Fe concentrations than those injected with Fe-ProtES, and the chickens injected with Fe-MetW or Fe-ProtM had higher ($P < 0.01$) kidney Fe concentrations than those injected with Fe-ProtES on d 20 after injections.

**Table 3**
Effect of intravenously injected iron (Fe) on growth performance of broilers.

| Injected Fe | | Days after intravenous injections | | | | | |
|---|---|---|---|---|---|---|---|
| | | Day 1 to 10 (22 to 31 d of age) | | | Day 11 to 20 (32 to 41 d of age) | | |
| Source | Level[1] | ADFI, g/d | ADG, g/d | FCR, g/g | ADFI, g/d | ADG, g/d | FCR g/g |
| Control | 0[2] | 122 | 74.2 | 1.65 | 147 | 77.6 | 1.90 |
| $FeSO_4 \cdot 7H_2O$ | L[2] | 123 | 74.0 | 1.67 | 153 | 79.2 | 1.98 |
| | H[2] | 126 | 73.1 | 1.72 | 154 | 80.6 | 1.92 |
| Fe-MetW | L[2] | 124 | 74.7 | 1.66 | 146 | 77.8 | 1.88 |
| | H[2] | 121 | 73.6 | 1.64 | 149 | 74.9 | 2.01 |
| Fe-ProtM | L[2] | 122 | 71.8 | 1.71 | 152 | 81.2 | 1.88 |
| | H[2] | 123 | 76.3 | 1.62 | 150 | 73.5 | 2.09 |
| Fe-ProtES | L[2] | 121 | 72.8 | 1.66 | 153 | 83.7 | 1.83 |
| | H[2] | 119 | 72.3 | 1.65 | 152 | 83.5 | 1.83 |
| SEM | | 2.38 | 2.38 | 0.04 | 3.31 | 2.84 | 0.07 |
| Injected Fe source | $FeSO_4 \cdot 7H_2O$[3] | 124 | 73.6 | 1.69 | 154 | 79.9 | 1.95 |
| | Fe-MetW[3] | 122 | 74.1 | 1.65 | 148 | 76.3 | 1.95 |
| | Fe-ProtM[3] | 123 | 74.1 | 1.67 | 151 | 77.3 | 1.98 |
| | Fe-ProtES[3] | 120 | 72.6 | 1.65 | 152 | 83.6 | 1.83 |
| SEM | | 1.71 | 1.06 | 0.03 | 2.13 | 1.98 | 0.05 |
| Injected Fe level | L[4] | 122 | 73.8 | 1.67 | 151 | 80.4 | 1.89 |
| | H[4] | 122 | 73.3 | 1.66 | 151 | 78.1 | 1.96 |
| SEM | | 1.21 | 0.75 | 0.02 | 1.50 | 1.40 | 0.03 |
| *P*-value | | | | | | | |
| Fe source | | 0.33 | 0.70 | 0.61 | 0.22 | 0.06 | 0.13 |
| Fe level | | 0.91 | 0.63 | 0.62 | 0.99 | 0.24 | 0.15 |
| Fe source × Fe level | | 0.73 | 0.21 | 0.25 | 0.79 | 0.40 | 0.20 |

Fe-MetW = Fe-Met with a weak chelation strength ($Q_f = 1.37$); Fe-ProtM = Fe proteinate with moderate chelation strength ($Q_f = 43.6$); Fe-ProtES = Fe proteinate with extremely strong chelation strength ($Q_f = 8.59 \times 10^3$).
[1] L represents the low injected Fe levels of 1.04, 1.38 and 1.58 mg (10% Fe absorbability solution) for each bird during d 22 to 28, d 29 to 35 and d 36 to 42, respectively; H represents the high injected Fe levels of 2.08, 2.76 and 3.16 mg (20% Fe absorbability solution) for each bird during d 22 to 28, d 29 to 35 and d 36 to 42, respectively.
[2] Data represent the means of 8 replicate cages (5 to 6 birds per cage during d 1to 10 and 3 to 4 birds per cage during d 11 to 20; $n = 8$).
[3] Data represent the means of 16 replicate cages (5 to 6 birds per cage during d 1 to 10 and 3 to 4 birds per cage during d 11 to 20; $n = 16$).
[4] Data represent the means of 32 replicate cages (5 to 6 birds per cage during d 1 to 10 and 3 to 4 birds per cage during d 11 to 20; $n = 32$).

**Table 4**
Effect of intravenously injected iron (Fe) on blood parameters of broilers.

| Injected Fe source | Injected Fe level[1] | Day 10 after intravenous injections (31 d of age) | | | | | Day 20 after intravenous injections (41 d of age) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | PI, μg/mL | TIBC, μg/mL | TS, % | Hb, g/L | Hct, % | PI, μg/mL | TIBC, μg/mL | TS, % | Hb, g/L | Hct, % |
| Control | 0[2] | 0.456 | 2.38 | 19.8 | 76.3 | 30.4* | 0.548 | 2.84 | 20.2 | 79.9 | 31.3 |
| $FeSO_4 \cdot 7H_2O$ | L[2] | 0.682[ab] | 2.66 | 25.5 | 79.4 | 31.0 | 0.789 | 2.77 | 29.1 | 78.0 | 31.2 |
| | H[2] | 0.504[b] | 2.43 | 22.4 | 83.0 | 32.9 | 0.756 | 2.49 | 32.0 | 81.0 | 32.2 |
| Fe-MetW | L[2] | 0.495[b] | 2.47 | 20.2 | 82.1 | 31.7 | 0.577 | 2.37 | 24.6 | 76.3 | 30.2 |
| | H[2] | 0.765[a] | 2.48 | 33.4 | 83.6 | 32.6 | 0.756 | 2.91 | 28.1 | 82.0 | 32.8 |
| Fe-ProtM | L[2] | 0.595[ab] | 2.08 | 31.8 | 81.6 | 31.7 | 0.697 | 2.66 | 26.7 | 78.8 | 31.6 |
| | H[2] | 0.680[ab] | 2.41 | 29.6 | 85.7 | 33.4 | 0.678 | 2.69 | 25.5 | 78.0 | 31.4 |
| Fe-protES | L[2] | 0.562[ab] | 2.56 | 23.1 | 80.3 | 30.9 | 0.587 | 2.70 | 22.1 | 80.0 | 32.4 |
| | H[2] | 0.753[a] | 2.56 | 32.4 | 81.9 | 32.3 | 0.585 | 2.48 | 22.8 | 82.1 | 33.0 |
| SEM | | 0.079 | 0.19 | 4.21 | 2.14 | 0.62 | 0.055 | 0.19 | 2.82 | 1.61 | 0.63 |
| Injected Fe source | $FeSO_4 \cdot 7H_2O$[3] | 0.593 | 2.54 | 23.9 | 81.2 | 31.9 | 0.773[a] | 2.63 | 30.5 | 79.5 | 31.7 |
| | Fe-MetW[3] | 0.630 | 2.47 | 26.8 | 82.9 | 32.1 | 0.671[ab] | 2.63 | 26.3 | 79.2 | 31.6 |
| | Fe-ProtM[3] | 0.638 | 2.24 | 30.7 | 83.7 | 32.6 | 0.688[ab] | 2.67 | 26.1 | 78.4 | 31.5 |
| | Fe-protES[3] | 0.658 | 2.56 | 27.7 | 81.1 | 31.6 | 0.587[b] | 2.58 | 22.5 | 81.1 | 32.7 |
| SEM | | 0.055 | 0.14 | 3.10 | 1.44 | 0.43 | 0.040 | 0.14 | 2.02 | 1.14 | 0.42 |
| Injected Fe level | L[4] | 0.583 | 2.44 | 25.1 | 80.9 | 31.3[b] | 0.663 | 2.63 | 25.6 | 78.3[b] | 31.4[b] |
| | H[4] | 0.676 | 2.44 | 29.4 | 83.6 | 32.8[a] | 0.696 | 2.64 | 27.1 | 80.7[a] | 32.4[a] |
| SEM | | 0.039 | 0.10 | 2.22 | 1.019 | 0.33 | 0.028 | 0.09 | 1.53 | 0.81 | 0.31 |
| *P*-value | | | | | | | | | | | |
| Fe source | | 0.88 | 0.39 | 0.52 | 0.51 | 0.53 | 0.02 | 0.98 | 0.06 | 0.43 | 0.13 |
| Fe level | | 0.11 | 0.83 | 0.19 | 0.07 | 0.002 | 0.42 | 0.91 | 0.47 | 0.04 | 0.02 |
| Fe source × Fe level | | 0.04 | 0.59 | 0.20 | 0.88 | 0.89 | 0.19 | 0.13 | 0.85 | 0.26 | 0.12 |

PI = pasma Fe; TIBC = total Fe-binding capacity; TS = transferrin saturation; Hb = hemoglobin; Hct = hematocrit; Fe-MetW = Fe-Met with a weak chelation strength ($Q_f = 1.37$); Fe-ProtM = Fe proteinate with moderate chelation strength ($Q_f = 43.6$); Fe-ProtES = Fe proteinate with extremely strong chelation strength ($Q_f = 8.59 \times 10^3$).
[a, b] Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).
*Different ($P < 0.02$) from all Fe supplemental groups.
[1] L represents the low injected Fe levels of 1.04, 1.38 and 1.58 mg (10% Fe absorbability solution) for each bird during d 22 to 28, d 29 to 35 and d 36 to 42, respectively; H represents the high injected Fe levels of 2.08, 2.76 and 3.16 mg (20% Fe absorbability solution) for each bird during d 22 to 28, d 29 to 35 and d 36 to 42, respectively.
[2] Data represent the means of 8 replicate cages (2 birds per cage; $n = 8$).
[3] Data represent the means of 16 replicate cages (2 birds per cage; $n = 16$).
[4] Data represent the means of 32 replicate cages (2 birds per cage; $n = 32$).

**Table 5**
Effect of intravenously injected iron (Fe) on tissue Fe concentrations of broilers.

| Injected Fe source | Injected Fe level[1] | Day 10 after intravenous injections (31 d of age) | | | | Day 20 after intravenous injections (41 d of age) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Liver Fe, μg/g fresh tissue | Pancreas Fe, μg/g fresh tissue | Kidney Fe, μg/g fresh tissue | Tibia bone ash Fe, μg/g fresh tissue | Liver Fe, μg/g fresh tissue | Pancreas Fe, μg/g fresh tissue | Kidney Fe, μg/g fresh tissue | Tibia bone ash Fe, μg/g fresh tissue |
| Control | 0[2] | 73.6* | 63.8 | 39.1* | 542 | 115* | 14.3* | 47.6 | 623* |
| $FeSO_4 \cdot 7H_2O$ | L[2] | 88.5 | 69.5 | 46.2 | 569 | 175[c] | 23.3 | 44.9[cd] | 700 |
| | H[2] | 204 | 69.7 | 56.2 | 663 | 346[a] | 29.9 | 57.0[ab] | 748 |
| Fe-MetW | L[2] | 113 | 61.2 | 49.8 | 547 | 224[b] | 17.4 | 44.1[d] | 718 |
| | H[2] | 242 | 58.9 | 56.7 | 632 | 364[a] | 27.5 | 64.1[a] | 782 |
| Fe-ProtM | L[2] | 131 | 63.1 | 46.3 | 558 | 195[bc] | 17.8 | 46.0[c] | 690 |
| | H[2] | 227 | 68.2 | 48.0 | 626 | 361[a] | 28.3 | 62.1[a] | 736 |
| Fe-ProtES | L[2] | 106 | 53.8 | 41.8 | 581 | 171[c] | 20.9 | 51.7[bc] | 689 |
| | H[2] | 177 | 66.3 | 57.0 | 611 | 237[b] | 22.4 | 53.7[b] | 676 |
| SEM | | 2.55 | 11.3 | 1.39 | 24.9 | 10.1 | 1.21 | 1.28 | 21.1 |
| Injected Fe source | $FeSO_4 \cdot 7H_2O$[3] | 146[b] | 69.6 | 51.2 | 616 | 260 | 26.6 | 50.7 | 726 |
| | Fe-MetW[3] | 177[a] | 60.1 | 53.3 | 590 | 289 | 22.5 | 54.1 | 752 |
| | Fe-ProtM[3] | 179[a] | 65.6 | 47.1 | 592 | 278 | 23.1 | 54.0 | 713 |
| | Fe-ProtES[3] | 141[b] | 60.0 | 49.4 | 596 | 204 | 21.6 | 52.7 | 683 |
| SEM | | 11.2 | 7.9 | 1.24 | 21.4 | 12.1 | 1.23 | 1.65 | 15.4 |
| Injected Fe level | L[4] | 110[b] | 61.9 | 46.0[b] | 563[b] | 192 | 19.9[b] | 46.7 | 699 |
| | H[4] | 213[a] | 65.8 | 54.7[a] | 632[a] | 329 | 27.2[a] | 59.4 | 736 |
| SEM | | 5.66 | 6.27 | 1.64 | 12.9 | 6.81 | 6.27 | 1.24 | 11.7 |
| *P*-value | | | | | | | | | |
| Fe source | | 0.005 | 0.86 | 0.48 | 0.81 | <0.0001 | 0.18 | 0.66 | 0.12 |
| Fe level | | <0.0001 | 0.68 | 0.004 | 0.002 | <0.0001 | <0.0001 | <0.0001 | 0.07 |
| Fe source × Fe level | | 0.13 | 0.95 | 0.41 | 0.72 | 0.02 | 0.24 | 0.007 | 0.52 |

Fe-MetW = Fe-Met with a weak chelation strength ($Q_f = 1.37$); Fe-ProtM = Fe proteinate with moderate chelation strength ($Q_f = 43.6$); Fe-ProtES = Fe proteinate with extremely strong chelation strength ($Q_f = 8.59 \times 10^3$).
[a, b, c, d] Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).
*Different ($P < 0.01$) from all Fe supplemental groups.
[1] L represents the low injected Fe levels of 1.04, 1.38 and 1.58 mg (10% Fe absorbability solution) for each bird during d 22 to 28, d 29 to 35 and d 36 to 42, respectively; H represents the high injected Fe levels of 2.08, 2.76 and 3.16 mg (20% Fe absorbability solution) for each bird during d 22 to 28, d 29 to 35 and d 36 to 42, respectively.
[2] Data represent the means of 8 replicate cages (2 birds per cage; $n = 8$).
[3] Data represent the means of 16 replicate cages (2 birds per cage; $n = 16$).
[4] Data represent the means of 32 replicate cages (2 birds per cage; $n = 32$).

### 3.4. Enzyme activities

Compared with the control, Fe injection increased ($P < 0.05$) liver, heart and kidney CAT activities on d 20 after injections. Injected Fe source, injected Fe level and their interaction had no effect ($P > 0.05$) on the CAT and SDH activities in the heart and kidney on d 10 or 20 after Fe injections, and CAT and SDH activities in the liver on d 20 after Fe injections. Liver CAT activity was not affected ($P > 0.05$) by injected Fe source and the interaction between injected Fe source and injected Fe level, but was influenced ($P = 0.05$) by injected Fe level. Liver SDH activity on d 10 after injections and heart COX activity on d 10 or 20 after Fe injections were not influenced ($P > 0.05$) by injected Fe source and the interaction between injected Fe source and injected Fe level, but were affected ($P < 0.05$) by injected Fe level, respectively. All of the above detailed data are not shown because these parameters were not influenced by either injected Fe source or the interaction between injected Fe source and injected Fe level.

### 3.5. mRNA levels

Injected Fe source, injected Fe level and their interaction did not affect ($P > 0.05$) the mRNA levels of liver *Cat*, *Sdh* and *Fth1*, heart *Sdh*, and spleen and Femur marrow *Fth1* on d 20 after Fe injections (Table 6). Injected Fe source and injected Fe level had no effect ($P > 0.05$) on the liver *Cox* mRNA levels, but their interaction affected them ($P = 0.005$). When the injected Fe level was high, the birds injected with Fe-MetW had greater ($P < 0.01$) liver *Cox* mRNA levels than those injected with Fe-ProtM and Fe-ProtES, and the birds injected with $FeSO_4 \cdot 7H_2O$ had greater ($P = 0.02$) liver *Cox* mRNA levels than those injected with Fe-ProtES. The heart *Cat* and *Cox* mRNA levels on d 20 after injections were not affected ($P > 0.05$) by injected Fe source and the interaction between injected Fe source and injected Fe level, but decreased ($P \leq 0.05$) as injected Fe level increased.

### 3.6. Protein expression levels

Compared with the control, Fe injection increased ($P < 0.05$) FTH1 protein expression levels in the liver and spleen on d 20 after injections (Table 7). Injected Fe source, Fe level and their interaction did not affect ($P > 0.05$) the CAT and COX protein expression levels in the liver and heart, and SDH protein expression levels in the liver on d 20 after Fe injections. The FTH1 protein expression in the liver was affected ($P < 0.05$) by injected Fe source and Fe level, but was not affected ($P = 0.15$) by their interaction. The chickens injected with Fe-MetW had greater ($P < 0.05$) FTH1 protein expression levels in the liver than those injected with $FeSO_4 \cdot 7H_2O$ or Fe-ProtES, and there were no differences ($P > 0.05$) between Fe-ProtM and one of the other Fe sources, or $FeSO_4 \cdot 7H_2O$ and Fe-ProtES. The SDH protein expression levels in the heart were not influenced ($P > 0.05$) by injected Fe source and the interaction between injected Fe source and Fe level, but increased ($P = 0.02$) with increasing injected Fe level. Injected Fe source, injected Fe level and their interaction affected ($P < 0.05$) FTH1 protein expression levels in the spleen. As the injected Fe level was high, the birds injected with Fe-MetW or Fe-ProtM had greater ($P < 0.05$) FTH1 protein expression in the spleen than those injected with $FeSO_4 \cdot 7H_2O$ or Fe-ProtES, and there were no differences ($P > 0.05$) between Fe-MetW and Fe-ProtM, or $FeSO_4 \cdot 7H_2O$ and Fe-ProtES. The FTH1 protein expression levels in femur marrow were not affected ($P > 0.37$) by injected Fe level and the interaction between injected Fe source and Fe level, but were affected ($P = 0.005$) by injected Fe source. The birds injected with $FeSO_4 \cdot 7H_2O$ had greater ($P < 0.05$) FTH1 protein expression in femur marrow than those injected with Fe-ProtM or Fe-ProtES, and the birds injected with Fe-MetW had greater ($P = 0.04$) FTH1 protein expression in femur marrow than those injected with Fe-ProtES, but no differences ($P > 0.05$) were detected between $FeSO_4 \cdot 7H_2O$ and Fe-MetW, or Fe-ProtM and Fe-ProtES.

## 4. Discussion

The results from the present study indicated that based on the liver Fe concentration on d 10 or 20 after Fe injections, and PI and femur marrow FTH1 protein expression level on d 20 after Fe injections, the intravenously injected organic Fe source with extremely strong chelation strength had the lowest Fe utilization in the target tissues of broilers, which has supported our hypothesis. Our results provided scientific experimental bases for developing and applying the organic Fe sources with appropriate chelation strengths and high metabolic utilization of Fe in broiler production.

The intrinsically labeled radioisotope method is a good way to verify mineral metabolism and utilization in animals. In the present study, we did not adopt this method because none of the commercial organic Fe products used had been manufactured with intrinsic radiotracers or stable isotopes of Fe. The intravenous injection method was considered an effective method to detect differences in metabolic utilization of minerals in the sensitive target tissues of animals (Zhou et al., 1994; Li et al., 2008; Shen et al., 2013). Therefore, the intravenous injection technique was used in the current study to detect differences in the metabolic utilization of Fe from different Fe sources in the sensitive target tissues of broilers. As an evaluation marker of utilization, growth observation is generally unresponsive for many trace elements (Luo et al., 2007; Lu et al., 2007; Huang et al., 2009). In the present study, injected Fe source did not influence growth performance of birds, which is in line with our previous studies (Ma et al., 2014; Zhang et al., 2016), indicating that growth performance might be affected by factors other than Fe source, and was not sensitive for assessment of the metabolic utilization of Fe sources for broilers.

Hematologic indices are commonly used to assess iron status for chicks. In the present study, Hb concentration on d 20 after Fe injections and Hct on d 10 or 20 after Fe injections increased as the injected Fe dosage increased. These results were similar to those of Zhang et al. (2016), who found that Hb and Hct could be positively affected by dietary Fe level in broilers. Blood Hb concentration and Hct have been considered as responsive criteria to assess the bioavailabilities of Fe (Spears et al., 1992; Aoyagi and Baker, 1995; Ettle et al., 2008; Ma et al., 2014). Ma et al. (2014) reported that blood Hb concentration was a sensitive index in reflecting differences in bioavailability among different Fe sources. However, Zhang et al. (2016) demonstrated that Hb, Hct and PI lack the sufficient sensitivity required to detect differences of bioavailabilities among Fe sources. The disparity might be mainly due to the different diets used in the above 2 studies (purified diet in the study of Ma et al. (2014) vs. corn–soybean meal diet in the study of Zhang et al. (2016)). The PI represents the Fe concentration that binds to transferrin. The results from the present study indicated that PI, but not Hb and Hct, was a sensitive indicator to detect the differences in metabolic utilisation of injected Fe among Fe sources. The Fe from injected Fe-ProtES was less utilizable for PI accumulation in broilers on d 20 after injection than that from injected $FeSO_4 \cdot 7H_2O$. The different methods of Fe administration (present intravenously injection vs. dietary supplementation in the study of Zhang et al. (2016)) might partially explain the inconsistency.

Previous studies in broilers demonstrated that Fe concentrations in the liver and kidney, especially in the liver, increased as dietary Fe concentration increased (Ma et al., 2014, 2016; Zhang et al., 2016). Our present study indicated that liver and kidney Fe

**Table 6**
Effect of intravenously injected iron (Fe) on mRNA levels of Fe-containing enzymes and protein in the tissues of broilers on d 20 after intravenous injections.

| Injected Fe source | Injected Fe level[1] | Liver | | | | Heart | | | Spleen | Femur marrow |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *Cat*, RQ[2] | *Sdh*, RQ[2] | *Cox*, RQ[2] | *Fth1*, RQ[2] | *Cat*, RQ[2] | *Sdh*, RQ[2] | Cox, RQ[2] | *Fth1*, RQ[2] | *Fth1*, RQ[2] |
| Control | 0[3] | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| $FeSO_4 \cdot 7H_2O$ | L[3] | 0.93 | 1.04 | $0.95^{bc}$ | 0.98 | 1.18 | 1.06 | 1.02 | 0.97 | 0.96 |
| | H[3] | 1.18 | 1.02 | $1.02^{ab}$ | 0.86 | 0.97 | 0.91 | 0.92 | 0.98 | 1.13 |
| Fe-MetW | L[3] | 0.89 | 0.99 | $0.91^{bc}$ | 0.84 | 0.91 | 1.01 | 0.88 | 0.96 | 1.18 |
| | H[3] | 0.93 | 1.01 | $1.10^{a}$ | 0.90 | 0.87 | 0.96 | 0.85 | 0.93 | 0.95 |
| Fe-ProtM | L[3] | 1.03 | 1.03 | $0.96^{bc}$ | 0.83 | 1.09 | 1.04 | 0.98 | 0.94 | 0.86 |
| | H[3] | 1.09 | 1.06 | $0.94^{bc}$ | 0.99 | 0.94 | 1.04 | 0.71 | 0.97 | 0.98 |
| Fe-ProtES | L[3] | 1.11 | 1.03 | $0.96^{bc}$ | 0.87 | 1.10 | 1.16 | 0.93 | 0.87 | 0.98 |
| | H[3] | 1.06 | 1.06 | $0.88^{c}$ | 0.94 | 0.78 | 1.10 | 0.76 | 1.03 | 0.91 |
| SEM | | 0.10 | 0.04 | 0.04 | 0.08 | 0.13 | 0.06 | 0.06 | 0.07 | 0.09 |
| Injected Fe source | $FeSO_4 \cdot 7H_2O$[4] | 1.06 | 1.03 | 0.98 | 0.92 | 1.07 | 0.99 | 0.97 | 0.97 | 1.04 |
| | Fe-MetW[4] | 0.91 | 1.00 | 1.01 | 0.87 | 0.89 | 0.99 | 0.86 | 0.95 | 1.06 |
| | Fe-ProtM[4] | 1.06 | 1.05 | 0.95 | 0.91 | 1.02 | 1.04 | 0.84 | 0.95 | 0.92 |
| | Fe-ProtES[4] | 1.08 | 1.04 | 0.92 | 0.91 | 0.94 | 1.13 | 0.85 | 0.95 | 0.95 |
| SEM | | 0.08 | 0.03 | 0.03 | 0.05 | 0.09 | 0.05 | 0.04 | 0.05 | 0.07 |
| Injected Fe level | L[5] | 0.99 | 1.02 | 0.95 | 0.88 | $1.07^{a}$ | 1.07 | $0.95^{a}$ | 0.94 | 0.99 |
| | H[5] | 1.06 | 1.04 | 0.98 | 0.92 | $0.89^{b}$ | 1.00 | $0.81^{b}$ | 0.98 | 0.99 |
| SEM | 0.05 | 0.02 | 0.02 | 0.04 | 0.06 | 0.03 | 0.03 | 0.04 | 0.06 | |
| *P*-value | | | | | | | | | | |
| Fe source | | 0.34 | 0.62 | 0.11 | 0.91 | 0.50 | 0.10 | 0.15 | 0.98 | 0.67 |
| Fe level | | 0.35 | 0.61 | 0.18 | 0.42 | 0.05 | 0.15 | 0.002 | 0.40 | 0.98 |
| Fe source × Fe level | | 0.60 | 0.88 | 0.005 | 0.42 | 0.74 | 0.70 | 0.25 | 0.60 | 0.45 |

*Cat* = catalase; *Sdh* = succinate dehydrogenase; *Cox* = cytochrome C oxidase; *Fth1* = ferritin heavy chain 1; RQ = relative quantities; Fe-MetW = Fe-Met with a weak chelation strength ($Q_f$ = 1.37); Fe-ProtM = Fe proteinate with moderate chelation strength ($Q_f$ = 43.6); Fe-ProtES = Fe proteinate with extremely strong chelation strength ($Q_f = 8.59 \times 10^3$).
[a,b,c] Mean values within a column with unlike superscript letters were significantly different ($P \leq 0.05$).
[1] L represents the low injected Fe levels of 1.04, 1.38 and 1.58 mg (10% Fe absorbability solution) for each bird during d 22 to 28, d 29 to 35 and d 36 to 42, respectively; H represents the high injected Fe levels of 2.08, 2.76 and 3.16 mg (20% Fe absorbability solution) for each bird during d 22 to 28, d 29 to 35 and d 36 to 42, respectively.
[2] The mRNA levels were calculated as the RQ of the target gene mRNA to the geometric mean of β-actin mRNA and *GAPDH* mRNA.
[3] Data represent the means of 8 replicate cages (2 birds per cage; *n* = 8).
[4] Data represent the means of 16 replicate cages (2 birds per cage; *n* = 16).
[5] Data represent the means of 32 replicate cages (2 birds per cage; *n* = 32).

concentrations increased as the injected Fe dosage increased, which was similar to the previous findings (Ma et al., 2014, 2016; Zhang et al., 2016), suggesting that the injected Fe from different Fe sources could be mobilized and deposited in the liver and kidney of chickens. Target tissue accumulations of trace minerals have been considered to be sensitive criteria for assessment of their bioavailabilities (Baker and Ammerman, 1995; Cao et al., 1996, 2002). In the present study, liver and kidney Fe concentrations on d 20 after Fe injections were sensitive enough to detect the differences in the tissue utilization of injected Fe among Fe sources. Based on these sensitive criteria, the Fe from the injected Fe-ProtES was less utilizable for liver and kidney tissues of broilers than that from the injected Fe-MetW, Fe-ProtM or $FeSO_4 \cdot 7H_2O$. These results are in agreement with those of previous studies (Li et al., 2008; Shen et al., 2013), which showed that the injected organic Mn or Zn source with strong chelation strength was the least favorable for tissue Mn or Zn utilization by broilers.

Iron is vital for the functions of numerous iron-containing enzymes, such as CAT, SDH and COX. Research with broilers and pigs has demonstrated that the CAT activities in the liver increased as dietary Fe levels increased (Ma et al., 2016; Feng et al., 2009). Brandsch et al. (2002) also reported that CAT activity in rat liver increased by feeding high iron diets. Similarly, the results from the present study indicate that CAT activity in the liver on d 10 after injections increased as the injected Fe dosages increased, suggesting that injected Fe can be utilized in the synthesis of CAT in the liver of chickens. Feng et al. (2009) found that SDH activity in the liver of pigs first increased, and then decreased when the dietary supplemental Fe levels increased from 0 to 120 mg/kg. The same trends were observed in a study on broilers (Ma et al., 2016). The results from the current study showed that SDH activity in the liver on d 10 after injections reduced as the injected Fe dosages increased, implying that high Fe addition might downregulate the synthesis of SDH in the liver. Our previous results indicated that the COX activity in heart increased quadratically as dietary Fe level increased, and it was sufficiently sensitive to evaluate Fe status and Fe requirements for broilers fed a maize-soybean-meal diet from 22 to 42 d of age (Liao et al., 2017). de Deungria et al. (2000) observed that COX activity in the brain of rats increased as supplemental Fe increased. In the present study, COX activity in the heart on d 10 or 20 after injections increased as the injected Fe dosages increased, which is consistent with the previous results. Additionally, in our present study, no differences were found in the CAT, SDH or COX activities in the liver, heart and kidney among Fe sources, indicating that these enzyme activities in the tissues lack enough sensitivity to detect the differences in tissue utilization of injected Fe from different Fe sources in broilers.

The gene expression of Fe-containing enzymes might be another type of new and more sensitive biomarker to reflect the iron status in the body of chickens (Ma et al., 2016; Liao et al., 2017). In the present study, *Cat* and *Cox* mRNA levels in the heart on d 20 after injections decreased as the injected Fe dosages increased, indicating that higher Fe injection might repress the gene transcription of these enzymes. The same trend was observed for *Cat* mRNA in the heart of broilers at 7 d of age when more Fe was added to the basal diet (Zhang et al., 2016). Ma et al. (2016) and Liao et al. (2017) also found that *Cox* mRNA in heart of broilers increased as dietary added Fe concentration increased from 0 to 60 mg/kg, and then began to decrease as dietary added Fe concentration was equal to or higher than 80 mg/kg. These above phenomena might be due to the Fe homoeostatic control mechanisms in the body, but the exact mechanism is still unknown. Our previous study showed that

**Table 7**
Effect of intravenously injected iron (Fe) on protein expression levels of Fe-containing enzymes and protein in the tissues of broilers on d 20 after intravenous injections.

| Injected Fe source | Injected Fe level[1] | Liver | | | | Heart | | | Spleen | Femur marrow |
|---|---|---|---|---|---|---|---|---|---|---|
| | | CAT, RQ[2] | SDH, RQ[2] | COX, RQ[2] | FTH1, RQ[2] | CAT, RQ[2] | SDH, RQ[2] | COX, RQ[2] | FTH1, RQ[2] | FTH1, RQ[2] |
| Control | 0[3] | 1.37 | 1.02 | 0.84 | 0.07* | 0.68 | 1.14 | 0.49 | 0.31* | 0.71 |
| $FeSO_4 \cdot 7H_2O$ | L[3] | 1.26 | 1.02 | 1.00 | 0.43 | 0.85 | 1.14 | 0.39 | $0.63^{bc}$ | 1.11 |
| | H[3] | 1.34 | 0.99 | 0.89 | 0.92 | 0.87 | 1.13 | 0.43 | $0.69^{b}$ | 0.86 |
| Fe-MetW | L[3] | 1.47 | 1.03 | 1.03 | 0.54 | 0.87 | 1.03 | 0.47 | $0.66^{bc}$ | 0.85 |
| | H[3] | 1.42 | 0.96 | 0.89 | 1.15 | 0.93 | 1.17 | 0.47 | $1.07^{a}$ | 0.84 |
| Fe-ProtM | L[3] | 1.44 | 1.04 | 0.79 | 0.55 | 0.88 | 1.02 | 0.52 | $0.48^{bc}$ | 0.69 |
| | H[3] | 1.39 | 1.02 | 1.02 | 1.03 | 0.78 | 1.25 | 0.49 | $1.14^{a}$ | 0.71 |
| Fe-ProtES | L[3] | 1.30 | 0.86 | 1.05 | 0.54 | 0.85 | 1.09 | 0.44 | $0.39^{c}$ | 0.62 |
| | H[3] | 1.31 | 0.95 | 0.97 | 0.82 | 0.84 | 1.16 | 0.40 | $0.74^{b}$ | 0.60 |
| SEM | | 0.07 | 0.04 | 0.07 | 0.03 | 0.04 | 0.03 | 0.02 | 0.03 | 0.03 |
| Injected Fe source | $FeSO_4 \cdot 7H_2O$[4] | 1.30 | 1.00 | 0.94 | $0.67^{b}$ | 0.86 | 1.13 | 0.41 | 0.66 | $0.98^{a}$ |
| | Fe-MetW[4] | 1.45 | 0.99 | 0.96 | $0.84^{a}$ | 0.90 | 1.09 | 0.47 | 0.86 | $0.85^{ab}$ |
| | Fe-ProtM[4] | 1.41 | 1.03 | 0.91 | $0.79^{ab}$ | 0.83 | 1.14 | 0.51 | 0.81 | $0.70^{bc}$ |
| | Fe-ProtES[4] | 1.31 | 0.91 | 1.01 | $0.68^{b}$ | 0.85 | 1.13 | 0.42 | 0.57 | $0.61^{c}$ |
| SEM | | 0.06 | 0.05 | 0.07 | 0.05 | 0.03 | 0.03 | 0.02 | 0.05 | 0.05 |
| Injected Fe level | L[5] | 1.37 | 0.98 | 0.97 | $0.51^{b}$ | 0.86 | $1.07^{b}$ | 0.46 | 0.54 | 0.82 |
| | H[5] | 1.36 | 0.98 | 0.94 | $0.98^{a}$ | 0.86 | $1.18^{a}$ | 0.45 | 0.91 | 0.76 |
| SEM | 0.05 | 0.03 | 0.04 | 0.02 | 0.03 | 0.02 | 0.03 | 0.04 | 0.05 | |
| *P*-value | | | | | | | | | | |
| Fe source | | 0.50 | 0.44 | 0.78 | 0.047 | 0.71 | 0.92 | 0.65 | 0.01 | 0.005 |
| Fe level | | 0.95 | 0.92 | 0.73 | <0.0001 | 0.89 | 0.02 | 0.87 | <0.0001 | 0.38 |
| Fe source × Fe level | | 0.95 | 0.75 | 0.23 | 0.15 | 0.68 | 0.27 | 0.96 | 0.02 | 0.54 |

CAT = catalase; SDH = succinate dehydrogenase; COX = cytochrome C oxidase; FTH1 = ferritin heavy chain 1; RQ = relative quantities; Fe-MetW = Fe-Met with a weak chelation strength ($Q_f$ = 1.37); Fe-ProtM = Fe proteinate with moderate chelation strength ($Q_f$ = 43.6); Fe-ProtES = Fe proteinate with extremely strong chelation strength ($Q_f = 8.59 \times 10^3$).
$^{a,b}$ Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).
*Different ($P < 0.05$) from all Fe supplemental groups.
[1] L represents the low injected Fe levels of 1.04, 1.38 and 1.58 mg (10% Fe absorbability solution) for each bird during d 22 to 28, d 29 to 35 and d 36 to 42, respectively; H represents the high injected Fe levels of 2.08, 2.76 and 3.16 mg (20% Fe absorbability solution) for each bird during d 22 to 28, d 29 to 35 and d 36 to 42, respectively.
[2] The protein levels were calculated as the RQ of the target gene protein to the glyceraldehyde-3-phosphate dehydrogenase protein.
[3] Data represent the means of 8 replicate cages (2 birds per cage; $n$ = 8).
[4] Data represent the means of 16 replicate cages (2 birds per cage; $n$ = 16).
[5] Data represent the means of 32 replicate cages (2 birds per cage; $n$ = 32).

liver *Cox* mRNA level was affected by dietary Fe concentration, and was a new and sensitive criterion for assessing the Fe requirements of broilers (Ma et al., 2016). The present study indicated that injected Fe concentrations had no effect on the liver *Cox* mRNA levels on d 20 after Fe injections, but the interaction of injected Fe source and Fe concentration influenced them. When the injected Fe concentration was high, the birds injected with Fe-MetW had greater liver *Cox* mRNA levels than those injected with Fe-ProtM and Fe-ProtES, and the birds injected with $FeSO_4 \cdot 7H_2O$ had greater liver *Cox* mRNA levels than those injected with Fe-ProtES. Therefore, under higher Fe injection, the liver *Cox* mRNA level was sensitive enough to detect the differences in the tissue utilization of injected Fe among Fe sources, and injected Fe from organic Fe source with extremely strong chelation strength was the least utilizable for liver *Cox* mRNA expression of broilers. These results are similar to those of Li et al. (2008), who found that based on the heart manganese-containing superoxide dismutase mRNA level of broilers, the injected Mn amino acid chelate with strong chelation strength was the least favorable for tissue Mn utilization by broilers. Zhang et al. (2016) reported that based on *Sdh* mRNA expression levels in the liver and kidney of broilers on d 21, the relative bioavailabilities of organic Fe sources with greater chelation strength showed higher Fe bioavailabilities. In the current study, as injected Fe concentration increased, the SDH protein expression level in the heart of broilers increased, but its mRNA level in the liver and heart did not change, suggesting that Fe might regulate this enzyme expression primarily at a translational level. The above disparity might have mainly resulted from the different approaches of Fe administration (present intravenous injection vs dietary supplementation in the study of Zhang et al. (2016)). In addition, the difference in ages of broilers between the 2 studies (the present study: d 31 and 41 vs d 21 in the study of Zhang et al. (2016)) might partially explain the inconsistency.

Ferritin is a ubiquitous intracellular Fe storage protein. Although the genomes of many species contain multiple copies of heavy-chain and light-chain sequences, the chicken genome contains only a single copy of the heavy-chain gene (Stevens et al., 1987). Ferritin has been found in many tissues, and is deposited mainly in the spleen, liver, and bone marrow (Matsuno et al., 1985; Oshtrakh et al., 2006). The expression of ferritin is tightly controlled by the intracellular Fe concentration (Muckenthaler and Hentze, 1997). When the Fe level is low, the expression of ferritin is suppressed to avoid intracellular Fe sequestration. The opposite scenario takes place as Fe is abundant. Iron modulates ferritin synthesis post-transcriptionally in animals (Hentze and Kühn, 1996; Eisenstein et al., 1997). Han et al. (2000) reported that dietary supplemental Fe increased the protein expression of FTH1 in the brain of rats. The present study demonstrated that FTH1 protein expression in the liver and spleen increased as the injected Fe dosages increased, which is in line with the results of Han et al. (2000). To our knowledge, no information is available regarding the effect of supplemental Fe as different Fe sources on the FTH1 protein expression in the tissues of chickens. In the current study, FTH1 protein expression levels in the spleen of broilers on d 20 after injections were sensitive enough to detect differences in the tissue utilization of injected Fe in broilers among Fe sources. Based on the criterion, intravenously injected Fe from Fe-ProtES was the least utilizable Fe source. This might be due to its extremely strong chelation strength of the bonds between Fe and ligands, which retarded Fe from the organic Fe source being mobilized for

metabolic utilization in the target tissues of broilers. The results from the present study and our previous studies (Zhang et al., 2016, 2017; Lu et al., 2018) suggest that more organic Fe from Fe-ProtES could better resist interference from the low pH in the stomach and complex factors in the gut and directly reach the intestinal brush border, where it is hydrolyzed and absorbed as an ion, resulting in higher Fe bioavailability. The results from the present study also indicate that there are differences not only in the absorption of Fe in the small intestine, but also in the metabolic utilization of Fe from organic Fe sources with different chelation strengths in the target tissues of broilers. Obviously and surely, in practice with dietary supplemental Fe, we should choose organic Fe with a strong chelation strength because it has the highest bioavailability reflecting both the Fe absorption in the gut and metabolic Fe utilization in the target tissues of broilers. However, further studies will be needed to address the relative bioavailability, absorption and metabolic utilization of Fe from the most strongly chelated EDTA Na–Fe in broilers.

## 5. Conclusions

The results from the current study indicated that liver and kidney Fe concentrations, and liver *Cox* mRNA levels and spleen FTH1 protein expression levels were sensitive enough for detecting differences in tissue utilization of injected Fe from different Fe sources in broilers. Based on the above biomarkers, intravenously injected Fe from the organic Fe source with extremely strong chelation strength was the least utilizable Fe source and functioned in the sensitive target tissue less effectively than Fe from Fe sulfate or the other 2 organic Fe sources with weak or moderate chelation strength. These findings might provide a further insight into the metabolic utilization mechanism of Fe in the target tissues of chickens.

## Author contributions

**Lin Lu**: Conceptualization, Methodology, Funding acquisition, Writing – original draft preparation. **Xueyu Dong**: Investigation, Data curation. **Xuelian Ma**: Investigation. **Liyang Zhang**: Resources, Project administration. **Sufen Li**: Resources, Software. **Xugang Luo**: Supervision, Funding acquisition, Writing – review & editing. **Xiudong Liao**: Validation, Writing – review & editing.

## Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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