# Research Note: Metabolic utilization of iron from different iron sources in primary cultured hepatocytes of broiler embryos

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**ABSTRACT** The present study was carried out to evaluate the effects of iron (**Fe**) sources and levels on the Fe concentration and expressions of iron-containing enzymes or protein in primary cultured hepatocytes of broiler embryos. The hepatocytes were incubated with 0, 0.25 and 0.50 mmol/L added Fe from either Fe sulfate, or 1 of 3 organic Fe chelates with weak (Fe-Met W), moderate (Fe-Pro M), or extremely strong (Fe-Pro ES) chelation strengths for 24 h. The results showed that all supplemental Fe treatments had higher ($P < 0.05$) Fe concentration, succinate dehydrogenase (***SDH***), *CAT* and ferritin heavy chain 1 (***FTH1***) mRNA levels than those in the control group. The hepatocytes incubated with Fe-Prot ES had lower ($P < 0.009$) Fe concentration than those incubated with Fe sulfate, Fe-Met W or Fe-Prot M. The *SDH* mRNA level was lower ($P < 0.05$) in Fe sulfate and Fe-Prot ES groups than in Fe-Prot M group. In conclusion, the Fe from Fe-Prot ES was less utilizable than Fe from Fe sulfate, Fe-Met W or Fe-Pro M in primary cultured hepatocytes of broiler embryos.

**Key words:** chick embryo hepatocyte, iron source, iron-containing enzyme, ferritin

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## INTRODUCTION

Iron (**Fe**) is an essential trace element that has a variety of vital functions in physiological and biochemical process of humans and animals. Previous studies have shown that dietary Fe supplementation could positively affect growth performance, antioxidation and immune functions of chickens (Vahl and van, 1987; Xie et al., 2019).

Traditionally, Fe is often added to diets of broilers as the inorganic Fe sulfate. However, the bioavailabilities of the inorganic Fe are low due to its high hydroscopicity and oxidation, and high excretion. To overcome these limitations, researchers have been trying to develop and use of organic Fe sources (Shinde et al., 2011; Luiggi et al., 2014). Previous studies demonstrated that the bioavailabilities of organic Fe sources for broiler chickens are closely related to their chelation strengths (defined as the quotient of formation [$\mathbf{Q_f}$]) between Fe and their ligands, and organic Fe sources with stronger chelation strength showed higher bioavailabilities (Zhang et al., 2017). 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. Liver is the reliable site for storing Fe and plays an important role in the regulation of Fe homeostasis in the body. Accordingly, primary cultured broiler hepatocytes might be an ideal in vitro model for exploring the differences in metabolic utilization of Fe from different Fe sources.

In fact, Fe functions in the body as a metal cofactor for a variety of enzymes, such as succinate dehydrogenase (**SDH**), catalase (**CAT**), and cytochrome c oxidase (**COX**). The SDH, CAT, and COX play a central role in cellular respiration and energy metabolism. Ferritin heavy chain 1 (**FTH1**) is a 21-kDa subunit of the ferritin complex and plays a vital role in proper $Fe^{2+}$ management required for cell growth and function. Our recent study has indicated that hepatic *COX* mRNA level and splenic FTH1 protein level were sensitive enough for detecting differences in tissue utilization of injected Fe from different Fe sources (Lu et al., 2022). Therefore, we hypothesized that the expression of iron-containing enzyme or protein might be sensitive criteria



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to detect the differences of metabolic utilization for different Fe sources in primary cultured hepatocytes of broiler embryos.

The present study was conducted to assess the effects of Fe sources and levels on the Fe concentration and expressions of iron-containing enzymes or protein, and to further explore differences in the metabolic utilization of different forms of Fe sources in primary cultured hepatocytes of broiler embryos.

## MATERIALS AND METHODS

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 guidelines. Ethical approval on animal survival was given by the animal ethics committee of IAS-CAAS.

### Preparations of Primary Cultured Hepatocytes of Chick Embryos

Hepatocytes were isolated from the liver tissue of 14-day-old chicken embryos as described previously (Zhou and Zhang, 2005). Chicken embryonic primary hepatocytes were cultured in L-15 medium (Invitrogen) containing fetal bovine serum (**FBS**), 10 $\mu$g/mL of transferrin, 10 $\mu$g/mL of vitamin C, $10^{-6}$ mol/L dexamethasone, $10^{-6}$ mol/L insulin, and 100 U/mL penicillin/streptomycin. After about 72 h of incubation, these cells were used for the following experiment.

The present study was performed to evaluate the effects of supplemental Fe sources and levels on Fe concentration and the expressions of key iron-containing enzymes and protein in the primary cultured hepatocytes. Our pre-experiment results demonstrated that the hepatocytes incubated with 0.25 or 0.50 mmol/L added Fe at 24 h had the higher Fe concentration, CAT and COX activities than other treatment groups (data not shown). Therefore, in the present study, the cells were treated with 0 (**control**), 0.25 or 0.50 mmol/L added Fe as the Fe sulfate (**$FeSO_4$•$7H_2O$**), Fe-Met with weak chelation strength (**Fe-Met W**; $Q_f = 1.37$), Fe proteinate with moderate chelation strength (**Fe-Prot M**; $Q_f = 43.6$), or Fe proteinate with extremely strong chelation strength (**Fe-Prot ES**; $Q_f = 8{,}590$) for 24 h. The Fe-Met W, Fe-Prot M, and Fe-Prot ES used in the present study were the same as that used in our previous study (Zhang et al., 2017).

### Determinations of Fe Concentration, SDH, COX, CAT Activities

The Fe concentration in hepatocytes was analysed according to the instructions in the commercial kits (SeicnCell, CA). The activities of SDH, COX, and CAT in hepatocytes were determined using the detection kits of SDH, COX, or CAT (Nanjing Jiancheng Bioengineering Institute, China), respectively.

### Total RNA Isolation and Real-Time PCR

Total RNA was isolated from hepatocytes using TRIzol reagent (Life Technologies, CA) according to the manufacturer's instruction, and then treated with RNase-free DNase and reverse-transcribed to cDNA using the PrimeScript RT Master Mix kit (Takara, Kyoto, Japan). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

### Statistical Analyses

Data from the present study were analyzed by SAS statistical software (version 9.2, SAS Institute Inc., NC). All data were analyzed using single degree of freedom contrast to compare all supplemental Fe treatments with the control. Data excluding the control were further analyzed as a 4 × 2 (Fe sources × Fe levels) factorial arrangement of treatments by two-way ANOVA with a model including the main effects of Fe sources, Fe levels, and their interaction. Each replicate served as the experimental unit. If the variances were significant, differences among means were tested by the least significant difference method, and the statistical significance was set at $P < 0.05$.

## RESULTS AND DISCUSSION

The liver is the center for the regulation of Fe homeostasis in the body, and hepatic Fe concentration was considered to be a sensitive criterion for assessment of Fe bioavailability (Lu et al., 2022). Furugouri reported that hepatic concentrations of total Fe, non-hemin Fe, hemosiderin Fe and ferritin Fe increased linearly with increasing levels of Fe in pigs (Furugouri, 1972). In our present study, there was no difference in cell viability among the different treatment groups (data no shown) and all supplemental Fe treatments had higher Fe concentration in primary cultured hepatocytes of broiler embryos than those in the control group (Table 1). Furthermore, the hepatocytes incubated with FeSO4•7H2O, Fe-Met W, and Fe-Prot M had higher Fe concentration than those incubated with Fe-Prot ES group (Table 1). These results are in agreement with the previous study (Lu et al., 2022), which showed that the injected organic Fe source with strong chelation strength was the least favorable for tissue Fe utilization by broilers.

Iron is a vital element required for the function of numerous enzymes (Liao et al. 2017). Iron-containing enzymes, such as SDH, COX, and CAT, play an important role in cellular respiration and energy metabolism. Our previous study showed that SDH, COX, and CAT activities in liver increased as dietary supplemental Fe level increased and hepatic SDH, COX and CAT activities were new and sensitive criteria to evaluate the dietary Fe requirements of broilers (Ma et al., 2016). Our

**Table 1.** Effects of added iron (Fe) sources and levels on the Fe concentration and iron-containing enzyme activities in hepatocytes of broiler embryos.

| Item | Added Fe levels (mmoL/L) | Fe concentration ($\mu$moL/mg protein) | SDH activity (U/mg protein) | CAT activity (U/mg protein) | COX activity (mU/mg protein) |
|---|---|---|---|---|---|
| Control group[1] | 0 | 0.44* | 10.6 | 15.4 | 14.9 |
| $FeSO_4 \bullet 7H_2O$[1] | 0.25 | 1.05 | 12.28 | 17.88 | 21.00 |
| | 0.50 | 1.73 | 9.81 | 15.98 | 17.65 |
| Fe-Met W[1] | 0.25 | 1.07 | 10.88 | 16.07 | 16.80 |
| | 0.50 | 1.82 | 9.06 | 15.56 | 14.88 |
| Fe-Prot M[1] | 0.25 | 0.93 | 10.23 | 15.32 | 17.72 |
| | 0.50 | 2.02 | 10.12 | 16.15 | 17.36 |
| Fe-Prot ES[1] | 0.25 | 0.63 | 9.77 | 15.92 | 17.90 |
| | 0.50 | 1.38 | 10.44 | 15.72 | 20.40 |
| Pooled SE | | 0.13 | 1.42 | 0.80 | 1.56 |
| Fe sources[2] | $FeSO_4 \bullet 7H_2O$ | 1.39a | 11.04 | 16.93 | 19.33 |
| | Fe-Met W | 1.45a | 9.97 | 15.81 | 15.84 |
| | Fe-Prot M | 1.47a | 10.18 | 15.73 | 17.54 |
| | Fe-Prot ES | 1.00b | 10.10 | 15.82 | 19.15 |
| Pooled SE | | 0.09 | 1.01 | 0.56 | 1.06 |
| Added Fe levels[3] | 0.25 | 0.92b | 10.79 | 16.30 | 18.35 |
| | 0.50 | 1.74a | 9.86 | 15.85 | 17.57 |
| Pooled SE | | 0.07 | 0.71 | 0.40 | 1.56 |
| *P*-value | | | | | |
| Fe sources | | 0.004 | 0.87 | 0.39 | 0.10 |
| Add Fe levels | | <0.001 | 0.38 | 0.45 | 0.47 |
| Fe sources × levels | | 0.41 | 0.70 | 0.40 | 0.28 |

Abbreviations: CAT, catalase; COX, cytochrome c oxidase; Fe-Met W, Fe-Met with a weak chelation strength ($Q_f = 1.37$); Fe-Prot M, Fe proteinate with moderate chelation strength ($Q_f = 43.6$); Fe-Prot ES, Fe proteinate with extremely strong chelation strength ($Q_f = 8.59\times10^3$); SDH, succinate dehydrogenase.

*Different from all supplemental Fe treatments by single degree of freedom contrast analysis ($P < 0.001$).

[a,b]Differences among means were tested by the least significant difference method and means with different lowercase letters within the same column differ ($P < 0.009$).

[1]Data represent the means of 6 replicates (n = 6).

[2]Data represent the means of 12 replicates (n = 12).

[3]Data represent the means of 24 replicates (n = 24).

**Table 2.** Effects of added iron (Fe) sources and levels on the mRNA expression levels of iron-containing enzymes and ferritin in hepatocytes of broiler embryos.

| Item | Added Fe levels(mmoL/L) | *SDH* (RQ) | *CAT* (RQ) | *COX1* (RQ) | *COX7A2L* (RQ) | *FTH1* (RQ) |
|---|---|---|---|---|---|---|
| Control group[1] | 0 | 1.01* | 1.06* | 1.00 | 1.01 | 1.00* |
| $FeSO_4 \bullet 7H_2O$[1] | 0.25 | 1.08 | 1.33 | 1.17 | 1.12 | 1.21 |
| | 0.50 | 1.08 | 0.98 | 1.04 | 1.18 | 1.39 |
| Fe-Met W[1] | 0.25 | 1.12 | 1.33 | 1.05 | 1.18 | 1.08 |
| | 0.50 | 1.32 | 1.36 | 1.11 | 1.42 | 1.35 |
| Fe-Prot M[1] | 0.25 | 1.27 | 1.36 | 1.10 | 1.26 | 1.34 |
| | 0.50 | 1.22 | 1.29 | 1.22 | 1.39 | 1.40 |
| Fe-Prot ES[1] | 0.25 | 1.14 | 1.38 | 1.06 | 1.11 | 1.15 |
| | 0.50 | 1.06 | 1.20 | 1.07 | 1.21 | 1.40 |
| Pooled SE | | 0.06 | 0.08 | 0.07 | 0.11 | 0.10 |
| Fe sources[2] | $FeSO_4 \bullet 7H_2O$ | 1.08c | 1.15 | 1.10 | 1.15 | 1.30 |
| | Fe-Met W | 1.22ab | 1.35 | 1.08 | 1.30 | 1.21 |
| | Fe-Prot M | 1.25a | 1.33 | 1.16 | 1.33 | 1.37 |
| | Fe-Prot ES | 1.10bc | 1.29 | 1.07 | 1.16 | 1.28 |
| Pooled SE | | 0.05 | 0.06 | 0.05 | 0.08 | 0.07 |
| Added Fe levels[3] | 0.25 | 1.15 | 1.35a | 1.09 | 1.17 | 1.20b |
| | 0.50 | 1.17 | 1.21b | 1.11 | 1.30 | 1.39a |
| Pooled SE | | 0.03 | 0.05 | 0.04 | 0.05 | 0.05 |
| *P*-value | | | | | | |
| Fe sources | | 0.03 | 0.15 | 0.59 | 0.23 | 0.51 |
| Added Fe levels | | 0.70 | 0.03 | 0.82 | 0.10 | 0.01 |
| Fe sources × levels | | 0.22 | 0.17 | 0.38 | 0.81 | 0.74 |

Abbreviations: *CAT*, catalase; *COX 1*, cytochrome c oxidase subunit 1; *COX7A2L*, cytochrome c oxidase subunit 7A2 like; *FTH1*, ferritin heavy chain 1; Fe-Met W, Fe-Met with a weak chelation strength ($Q_f = 1.37$); Fe-Prot M, Fe proteinate with moderate chelation strength ($Q_f = 43.6$); Fe-Prot ES, Fe proteinate with extremely strong chelation strength ($Q_f = 8.59\times10^3$); RQ, relative quantity; *SDH*, succinate dehydrogenase.

*Different from all supplemental Fe treatments by single degree of freedom contrast analysis ($P < 0.05$).

[a,bc]Differences among means were tested by the least significant difference method and means with different lowercase letters within the same column differ ($P < 0.05$).

[1]Data represent the means of 6 replicates (n = 6).

[2]Data represent the means of 12 replicates (n = 12).

[3]Data represent the means of 24 replicates (n = 24).

current study showed that the Fe sources, added Fe levels and their interaction did not affect SDH, CAT, and COX activities in hepatocytes (Table 1), which is in line with the study of our previous and recent in vivo studies with broilers (Zhang et al., 2017; Lu et al., 2022). These results indicated that these enzyme activities lack enough sensitivity to detect differences in metabolic utilization of Fe from different Fe sources in primary cultured hepatocytes of broiler embryos.

Assays of the gene expressions of selected Fe-containing enzymes might provide sensitive functional biomarkers for assessing Fe bioavailability. Ma et al. and Zhang et al. reported that hepatic *SDH* mRNA expression level on d 21 increased linearly as dietary Fe concentration increased (Ma et al., 2016; Zhang et al., 2017). Interestingly, our present research showed that all supplemental Fe treatments had higher *SDH, CAT* and *FTH1* mRNA levels in primary cultured hepatocytes of broiler embryos than those in the control group (Table 2). And the added Fe levels affected the *CAT* and *FTH1* mRNA in primary cultured hepatocytes of broiler embryos. The *SDH* mRNA level was affected by Fe sources (Table 2). Additionally, in the present study, the *SDH* mRNA level in hepatocytes was lower in Fe-Prot ES group than in Fe-Prot M group (Table 2), indicating that Fe-Prot ES might have lower Fe utilization than Fe-Prot M in hepatocytes of broiler embryos. Our recent study also indicated that injected Fe from Fe-Pro ES was less utilizable than that from Fe sulfate, Fe-Met W or Fe-Pro M for hepatic *COX* mRNA expression of broilers (Lu et al., 2022). These results suggest that although the organic Fe source with greater chelation strength showed higher Fe absorption in small intestine of broilers, their Fe utilization in the liver was not as good as that of the Fe-Prot M and Fe-Met W. 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 liver of broilers. Our current findings might provide a further insight into the metabolic utilization of Fe from different iron sources in the target tissue of broilers.

In conclusion, the present study indicated that the Fe concentration and *SDH* mRNA level were sensitive criteria to detect differences in the metabolic utilization of Fe from different Fe sources in primary cultured hepatocytes of broiler embryos. And the Fe from Fe-Prot ES was less utilizable than Fe from Fe sulfate, Fe-Met W, or Fe-Prot M in primary cultured hepatocytes of broiler embryos.

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## DISCLOSURES

None of the authors have any conflicts of interest to declare.

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