

Effects of ferrous carbamoyl glycine on iron state and absorption in an iron-deficient rat model

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Abstract An iron-deficient rat model was established and used to determine the effects of different iron sources on iron metabolism and absorption. Iron-deficient rats were assigned to one of three treatment groups, and their diet was supplemented with deionized water (control), Fe-CGly, or FeSO₄ for 8 days via intragastric administration. Blood samples were obtained for analysis of iron-related properties, and the small intestine and liver were removed for quantitative reverse transcription PCR of genes related to iron metabolism. The serum total iron-binding capacity (TIBC) levels of rats in Fe-CGly and FeSO₄ supplementation groups was lower ($P < 0.05$) than that of the rats in the control group. The rats in Fe-CGly group exhibited higher ($P < 0.05$) plasma Fe and ferritin levels and lower ($P < 0.05$) TIBC levels compared with the rats in FeSO₄

groups. The relative expression of liver hepcidin increased ($P < 0.05$) by tenfold and 80-fold in the Fe-CGly and FeSO₄ groups, respectively, whereas divalent metal transporter 1, duodenal cytochrome *b*, and ferroportin 1 expression decreased ($P < 0.05$) in the duodenum in both Fe-CGly and FeSO₄ group. A comparison between Fe-CGly and FeSO₄ group showed that iron regulatory protein 1 (*IRP1*) and iron regulatory protein (*IRP2*) expressions were reduced ($P < 0.05$) in rats administered FeSO₄ than in rats administered with Fe-Cgly. These results indicate that Fe-CGly rapidly improves the blood iron status and that IRP1 and IRP2 may play an important role in the intestinal absorption of Fe-CGly.

Keywords Ferrous carbamoyl glycine · Hepcidin · IRP1/IRP2 · SD rats · FeSO₄

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Introduction

Iron (Fe) is a vital mineral that is essential for aerobic metabolism in many organisms; it contributes to various physiological functions such as oxygen transport and energy metabolism (Oliveira et al. 2014). The major biological function of iron requires its incorporation into proteins and enzymes such as hemoglobin and myoglobin. The relationship between dietary supplementation of iron and various pathological states in the body like iron deficiency and iron deficiency anemia (IDA) has been extensively investigated (Peto 2010). Iron deficiency and iron deficiency anemia (IDA) are micronutrient malnutrition conditions widespread in humans, livestock, and micro-livestock (such as rabbit and poultry) (Stoltzfus 2001). In contrast, excessive iron may lead to microbial infections or cause tissue damage. The biological

concentration of iron must therefore be closely monitored and regulated (Oliveira et al. 2014). Iron homeostasis in body tissues is balanced by two regulatory systems: One system functions systemically and relies on the hormone hepcidin and the iron exporter ferroportin, and the second system predominantly controls cellular iron metabolism through iron regulatory proteins that bind iron-responsive elements in regulated messenger RNAs (mRNAs) (Hentze et al. 2010).

Iron additives are important for the diet of both humans and livestock. To date, three stages of development of iron additives, corresponding to three generations of products, have been noted. The first generation consisted of inorganic iron sources such as ferrous chloride, ferrous sulfate, and ferrous carbonate. These inorganic sources have very poor biological availability and complex with other nutrients in the gut. The second generation consisted of simple organic iron sources such as ferrous fumarate and ferrous citrate; however, the biological activity of these compounds is low, and they may have adverse effects on other feed components (Wang et al. 2010). Therefore, researchers working with micro-livestock sought to develop safe and efficient forage additives that would overcome the shortcomings of the aforementioned iron additives.

One of such compound is iron carbamoyl glycine chelate (Fe-CGly), which has greater absorbability than other Fe sources (Wu et al. 2013; Wan et al. 2015). With the attendant benefits of increased absorption rate, better growth of animals, improved immunity, and increased compatibility with the environment, iron–amino acid chelates have recently received increased attention in animal nutrition (Hertrampf and Olivares 2004). Chelates of mineral are more bioavailable and efficiently absorbed from gut than those provided by inorganic salts (Vegarud et al. 2000). In addition, mineral chelates can potentially reduce mineral excretion and environmental contamination (Singh et al. 2015).

The improved bioavailability of iron–amino acid chelates has been attributed to highly efficient absorption, but the mechanism underlying the efficient absorption is unclear. This study therefore seeks to investigate the effects of Fe-CGly on the iron state and absorption in an iron-deficient rat model.

Materials and methods

Animals and treatments

Thirty male Sprague–Dawley (SD) rats were used to determine the effects of Fe-CGly on iron absorption and regulation. These SD rats were fed for 14 days pre-

experimental period with an iron-deficient diet in order to stimulate a ‘nutritional anemia status’ (Blood hemoglobin level (<120 g/L) indicated the nutritional anemia status) (Borel et al. 1991). The Hb levels of normal or anemic rats are presented in Table 1. The iron-deficient rat model was built after the pre-experimental period, and then the anemic rats (Hb level were about 95 g/L) were subsequently used to determine the effects of Fe-CGly on iron absorption and regulation. In total, 30 rats with an average weight of 116 ± 2.35 g were allotted on a weight equalization basis to one of the three treatment groups (10 rats per treatment): control, Fe-CGly, and FeSO₄ groups. The experimental treatment consisted of daily administration of 1 mL of deionized water (control), 1.88 mmol Fe-CGly, or 1.88 mmol FeSO₄, respectively, for a period of 8 days by gavage. The offered Fe quantity in Fe-Cgly or FeSO₄ group was enough for the recovery of Hb level (Weber et al. 2010).

All rats were fed with basal diet having a previously established composition (Wu et al. 2012; Zhou et al. 2012). The basal diet contained 397.5 g/kg corn, 200 g/kg casein, 132 g/kg maltodextrin, 100 g/kg sucrose, 70 g/kg corn oil, 50 g/kg fibers (cellulose), 30 g/kg canola, 35 g/kg mineral mixture (with no iron), 10 g/kg vitamin mixture, 3 g/kg DL-methionine, 100 g/kg sodium chloride, and 2 g/kg choline bitartrate. The detected trace element content was 987.9 mg/kg for Al, 36.2 mg/kg for Zn, and 24.1 mg/kg for Fe.

The animals were housed individually in cages (26.0 cm × 19.0 cm) and fed a ration prepared according to the recommendations of the American Institute of Nutrition (1993) for adult rats (Lien et al. 2001). The rats were maintained under a controlled environment at $22 \text{ }^\circ\text{C} \pm 2 \text{ }^\circ\text{C}$ and relative air humidity of $60 \% \pm 10 \%$ under a 12-h daylight/dark cycle (dark from 0800 to 2000 h), with water and food available ad libitum throughout the experiment (Wu et al. 2012; Zhou et al. 2012).

Fe-CGly (C₆H₁₀N₄O₆Fe) was obtained from Guangzhou Tanke Bio-Tech Co. Ltd (Guangzhou, China; >99.99 % purity, 24 % iron content). All of the other reagents used in this study were of analytical grade or better quality. This study was performed in accordance with the Chinese guidelines for animal welfare and approved by the Animal Care and Use Committee of the Institute of Subtropical Agriculture of the Chinese Academy of Sciences.

Table 1 Hb levels of normal or anemic rats in the present study

	Normal SD rat	Anemic SD rat
Hb (g/L)	148.45 ± 12.32	95.19 ± 5.85

Sample collection

At 0800 h on day 8, all animals were deprived of basal diet and killed by exsanguination (anaesthetized before exsanguination). Blood samples (1.5 mL per rat) were collected and centrifuged to obtain plasma, which was stored at -20°C until analysis. The small intestine and liver were excised, and the duodenum, jejunum, and ileum were separated, thoroughly flushed with sterile saline, immediately frozen in liquid nitrogen, and then stored at -80°C for mRNA analysis (Wu et al. 2015).

Fe analysis

The samples (liver, kidney, and spleen) were digested by the microwave digestion system MK-II (Shanghai Shin Co., Shanghai, China) using a Milli Q microwave digester (Millipore Corp., Bedford, MA, USA). Fe levels were determined by inductively coupled plasma mass spectrometry (ICP-MS) (ELAN6000, PerkinElmer SCIEX, USA) with the same operating parameters as previously described (Wu et al. 2012). All vessels used in this experiment were treated with 10 % nitric acid for over 24 h.

Determination of blood iron properties

Blood hemoglobin (Hb), red blood cell (RBC) number, and mean corpuscular volume (MCV) were estimated using an automatic hematology analyzer (Cell-Dyn 4000, Abbott Laboratories, Abbott Park, IL, USA). An automated biochemistry analyzer (Synchron CX Pro; Beckman Coulter, Fullerton, CA, USA) was used to determine the total iron-binding capacity (TIBC) of the samples. Commercial kits for these indexes were purchased from Beijing Chemclin Biotech Co., Ltd. (Beijing, China) and used according to the manufacturer's instructions. The ELISA commercial kit used for plasma ferritin determination was also purchased from Beijing Chemclin Biotech Co., Ltd. (Beijing, China).

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was isolated from small intestine and liver samples using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was reverse-transcribed into cDNA using the SuperScript First-Strand Synthesis System kit (Life Technologies),

Quantitative PCR analyses were performed on an ABI 7900HT system. Quantitative PCR amplification (Applied Biosystems, Foster City, CA, USA) was performed in a total volume of 10 μL , including cDNA, SYBR Green mix, ROX reference dye, and specific primers. The PCR primer

pairs that were used to amplify hepcidin, divalent metal transporter (*DMT1*), ferroportin 1 (*Fpn1*), duodenal cytochrome b (*Dcytb*), iron regulatory protein 1 (*IRP1*), iron regulatory protein 2 (*IRP2*), and β -actin cDNA fragments are listed in Table 2. The qRT-PCR conditions were as follows: 30 s of denaturation at 94°C followed by 30 cycles of 30 s of annealing at 60°C , and 30 s of extension at 72°C . Relative quantification of gene amplification by qRT-PCR was performed using cycle threshold (Ct) values. The comparative Ct value method was employed to quantitate expression levels for genes relative to those for β -actin.

Western blot

Frozen samples were powdered under liquid nitrogen, and lysed in RIPA buffer (150 mM NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris-HCl at pH 7.4), plus a protease inhibitor cocktail (Roche, Shanghai, China) and phosphatase inhibitors (Thermo Scientific, Bremen, Germany). Soluble proteins were subjected to SDS-PAGE, and transferred to PVDF membranes (Millipore, Billerica, MA), blocked with 5 % nonfat milk in TBS-0.05 % Tween-20 for 1 h and incubated overnight with iron regulatory protein 1 (IPR 1; 1:500), iron regulatory protein 2 (IPR 2; 1:500), and β -actin (1:1000) (Santa Cruz, CA, USA) antibodies followed by horseradish peroxidase-linked secondary antibodies (Santa Cruz, CA, USA). The immunostaining signals were detected with enhanced chemiluminescence kit (Applygen Technologies Inc., Beijing, China). The density of the specific protein bands of interest was measured using AlphaMager 2200 (Alpha Innotech Corporation, CA, USA) software and normalized by that of β -actin.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) in a completely randomized design (CRD) and were analyzed using SPSS 13.0 (SPSS, USA). Data are presented as the mean \pm SEM. Differences between significant means were separated using Tukey's test. Significant differences were considered at $P < 0.05$.

Results

Growth and iron-associated phenotype of treated and control rats

Administration with Fe-CGly and FeSO_4 showed no significant effect ($P > 0.05$) on final body weight and weight gain of rats (Table 3). We then examined the blood iron-

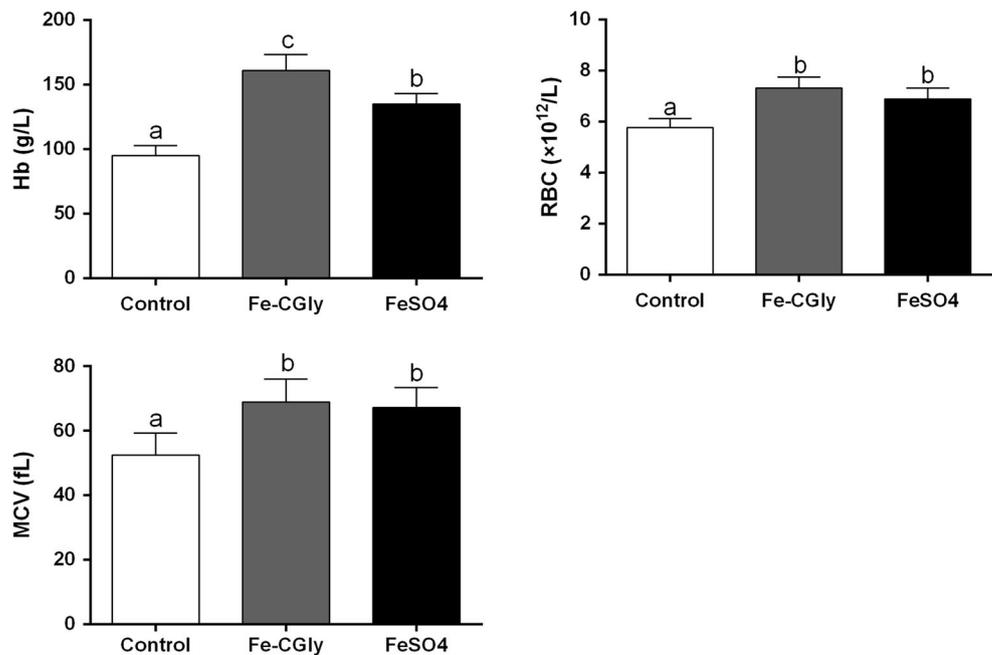
Table 2 Sequences of primers for hepcidin, DMT1, Fpn1, Dcytb, IRP1, IRP2, and β -actin

Genes	Primer	5'-3'	Products (bp)
Hepcidin	Forward	GCTGCCTGTCTCCTGCTTCT	160
	Reverse	TGGTGTCTCGCTTCCTTCG	
DMT1	Forward	CTACTTGGGTTGGCAGTGTTG	102
	Reverse	CCGCTGGTATCTTCGCTCA	
Fpn1	Forward	TCGTAGCAGGAGAAAACAGG	145
	Reverse	GAAATGAAACCACAGCCAAT	
Dcytb	Forward	TCTGGACTCCTCTTTGG	107
	Reverse	TCTGGTGGGAATGAATGGT	
IRP1	Forward	TGGGGTGTAGGTGGTATTGA	155
	Reverse	TGTCGGAGGTGCTTGTA	
IRP2	Forward	AAGCCGAAACTCAGGAACA	193
	Reverse	AGGAGGGATGACTGCCACA	
β -Actin	Forward	TCGTGCGTGACATTAAGAG	134
	Reverse	ATTGCCGATAGTGATGACCT	

Table 3 Effects of intragastric administration of ferrous carbamoyl glycine chelate on the growth of anemic SD rats

	Initial body weight (g)	Final body weight (g)	Gain (g)
Control group	116.1 \pm 7.4	126.1 \pm 8.6	10.0 \pm 2.4
Fe-CGly	115.8 \pm 7.0	126.2 \pm 8.3	10.4 \pm 1.8
FeSO ₄	117.7 \pm 6.9	127.9 \pm 7.9	10.2 \pm 2.5

Data are expressed as mean \pm SEM, $n = 8$. Values in a row with different superscript differ ($P < 0.05$)

Fig. 1 Effects of intragastric administration of ferrous carbamoyl glycine chelate on iron-associated properties of the blood of anemic SD rats. Data are expressed as mean \pm SEM, $n = 8$. Values with different superscript differ ($P < 0.05$)

associated properties (Fig. 1). Furthermore, administration of Fe-CGly increased ($P < 0.05$) Hb levels (3.97 %) to a greater extent than FeSO₄ did. Control group had the lowest ($P > 0.05$) Hb levels. Administration of Fe-CGly or FeSO₄ resulted in increased RBC and MCV levels when

compared with the control group. However, similar values of RBC and MCV were obtained for rats fed with diets supplemented with Fe-CGly and FeSO₄. When compared with the control group, rats administered Fe-CGly exhibited higher ($P < 0.05$) serum Fe (7.66 %) and ferritin

levels (4.79 %) and lower ($P < 0.05$) TIBC levels (12.54 %). A comparison between the two iron sources showed higher ($P < 0.05$) serum iron (3.73, ferritin (3.07 %) levels, and lower TIBC levels (2.49 %) in rats administered Fe-CGly than in those administered FeSO₄ (Fig. 2). Thus, Fe-CGly increased serum iron and ferritin concentration in anemic rats and decreased TIBC in more effective way than FeSO₄ in anemic rats.

Effect of iron supplementation on iron levels in tissues (liver, kidney, and spleen)

As shown in Fig. 3, following administration with Fe-CGly, iron levels in the liver (15 %) and spleen (20.12 %) increased significantly ($P < 0.05$). A comparison between Fe-CGly and FeSO₄ group showed similar iron concentration in liver and spleen samples. After iron administration, the iron content of the kidneys did not change significantly in either Fe-CGly or FeSO₄ groups ($P > 0.05$).

Expression of genes associated with iron homeostasis

Highest ($P < 0.05$) gene expression of hepcidin in the liver was obtained with FeSO₄ group, followed by Fe-CGly, while the lowest ($P < 0.05$) expression was noticed with control group (Fig. 4). Subsequently, the expression of genes involved in iron uptake in the duodenum was also determined (Fig. 5). *DMT1*, *Fpn1*, and *Dcytb* mRNA expression decreased ($P < 0.05$) in rats receiving either Fe-CGly or FeSO₄ relative to that in the control group

($P < 0.05$) with no significant differences between Fe-CGly and FeSO₄ group. Finally, the gene expressions of *IRP1* and *IRP2*, which encode global iron regulators, were determined in the duodenum (Fig. 6a). A comparison between Fe-CGly and FeSO₄ group showed that *IRP1* and *IRP2* expressions were reduced in rats administered FeSO₄ than in rats administered with Fe-CGly. In rats that received Fe-CGly, *IRP1* expression increased significantly compared with that in the FeSO₄ and control group ($P < 0.05$). The protein expression of *IRP1* and *IRP2* were also determined in the duodenum (Fig. 6b). In rats that received Fe-CGly, the protein expression of *IRP1* and *IRP2* increased significantly compared with that in the FeSO₄ and control group ($P < 0.05$).

Discussion

Iron plays an important role in many metabolic processes of the body. The present study confirmed that administration with either Fe-CGly or FeSO₄ showed no effect on the growth performance of rats. The fact that the blood Hb concentration of rats were restored when administered either of the 2 iron sources (Fe-CGly or FeSO₄) in this study confirmed that iron administration generally promoted improved blood Hb concentration. However, the increased plasma Fe level, ferritin, and faster increase in blood Hb levels recorded in rats administered Fe-CGly than their counterparts administered FeSO₄ implied a higher bioavailability of iron in rats administered iron ferritin than FeSO₄ group. Improved bioavailability of iron

Fig. 2 Effects of intragastric administration of ferrous carbamoyl glycine chelate on plasma iron, TIBC, and ferritin concentrations in anemic SD rats. Data are expressed as mean \pm SEM, $n = 8$. Values with different superscript differ ($P < 0.05$)

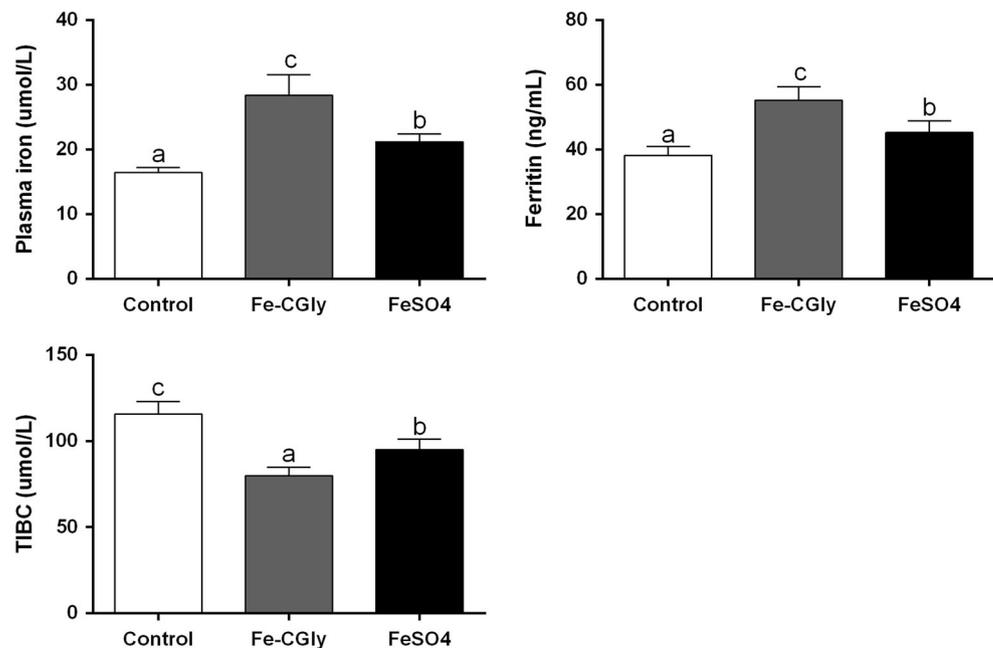


Fig. 3 Effects of intragastric administration of ferrous carbamoyl glycine chelate on tissue Fe in the livers, kidneys, and spleens of anemic rats. Data are expressed as mean \pm SEM, $n = 8$. Values with different superscript differ ($P < 0.05$)

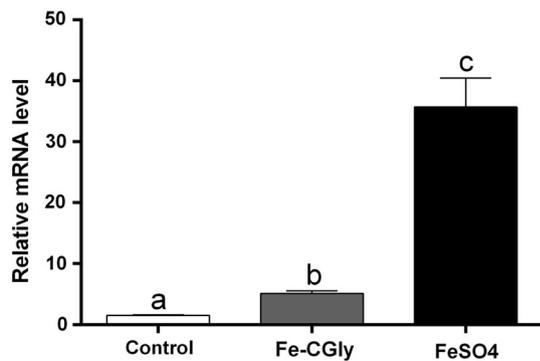
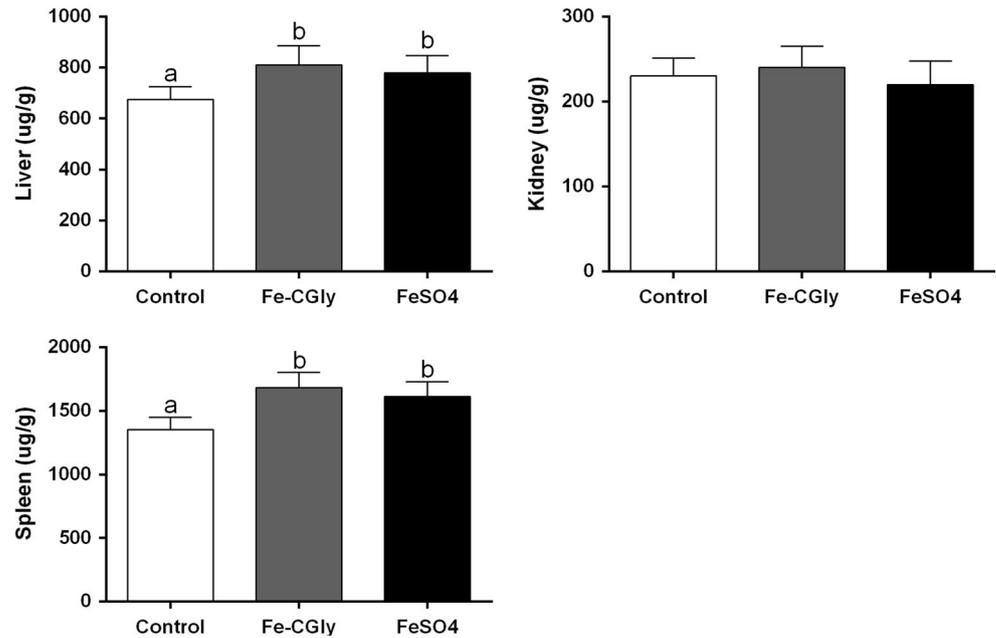


Fig. 4 Hepcidin mRNA expression levels following the administration of FeCGly or FeSO₄. Data are expressed as mean \pm SEM, $n = 8$. Values with different superscript differ ($P < 0.05$)

in Fe-CGly group could be attributed to the excellent chemical stability, high biological value, and high solubility of iron in the amino acid chelates (Ashmead 2001; Hertrampf and Olivares 2004). The trend observed in this

study agreed with previous findings which showed improved iron absorption in glycine-chelated iron than ferrous sulfate (Bovell-Benjamin et al. 2000).

Liver is a reliable response criterion of mineral status and relative bioavailability (Bailey et al. 2001). Increased liver iron concentration recorded with rats on Fe-CGly and FeSO₄ when compared to control group confirmed the assertion that iron administration directly influences their concentration in organs. The low liver and spleen iron concentration recorded for rats on control group could be attributed to deficient iron levels of the diet. As expected, Fe-CGly should improve the visceral iron concentration. However, administration of either Fe-CGly or FeSO₄ in the current study showed similar iron concentration in liver and spleen samples. This may be due to the greater catabolism of iron in liver and spleen of pigs in Fe-CGly group.

In the present study, highest hepcidin expression was obtained with FeSO₄ group than Fe-CGly group. Hepcidin regulates the activity of ferroportin1 (Fpn1), an intestinal

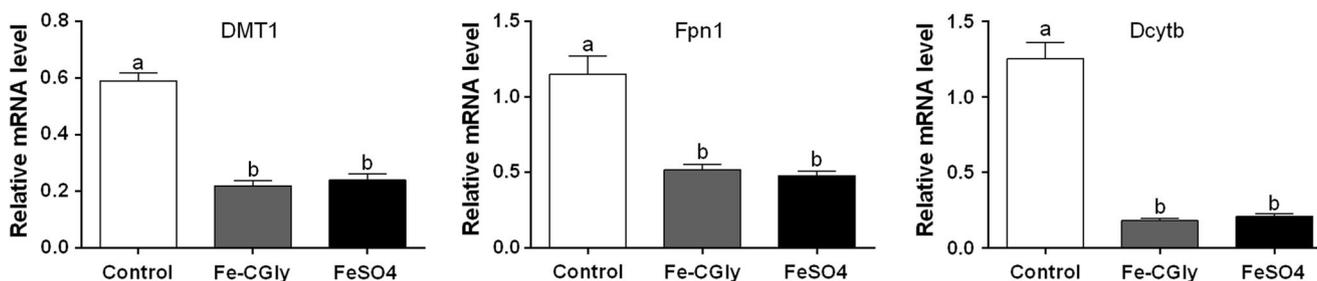
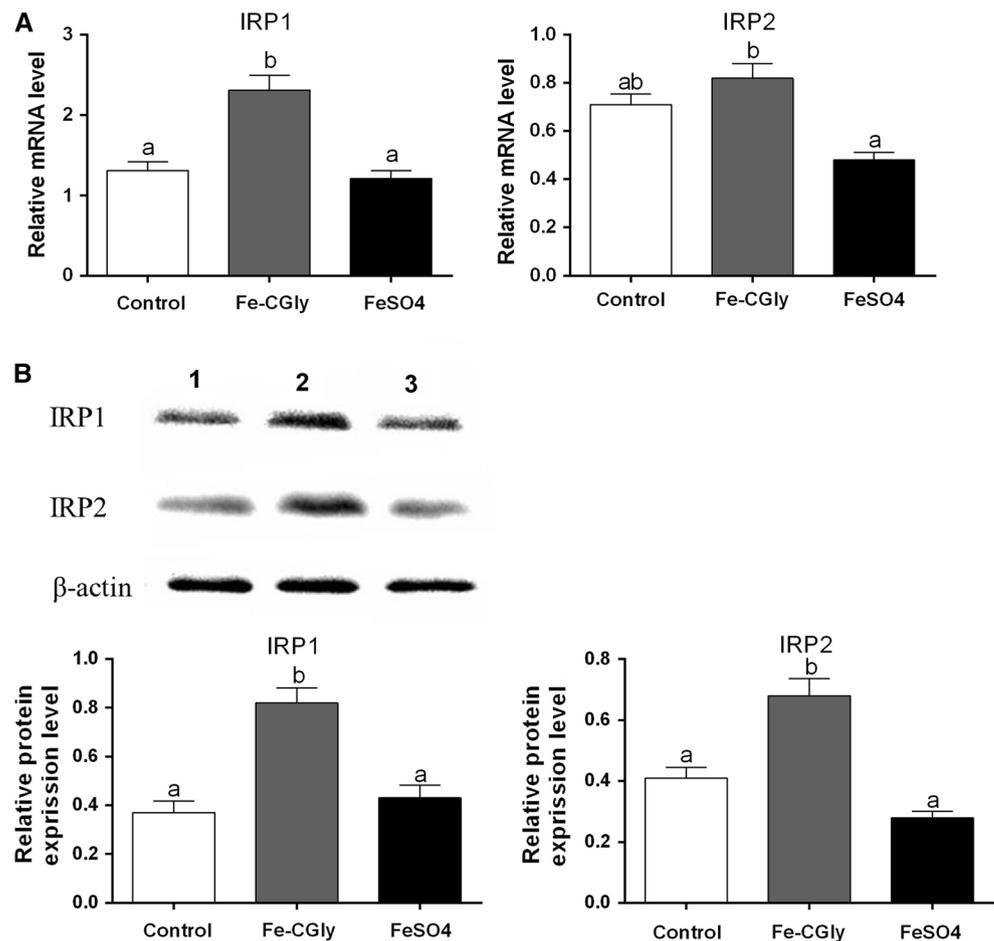


Fig. 5 *DMT1*, *Fpn1*, and *Dcytb* mRNA expression levels in the duodenum following the administration of FeCGly or FeSO₄. Data are expressed as mean \pm SEM, $n = 8$. Values with different superscript differ ($P < 0.05$)

Fig. 6 *IRP1* and *IRP2* expression levels (**a** mRNA level, **b** protein level) in the duodenum following the administration of FeCGly or FeSO₄. ¹control group, ²Fe-Cgly group, ³FeSO₄ group. Data are expressed as mean \pm SEM, $n = 8$. Values with different superscript differ ($P < 0.05$)



and macrophage iron exporter, by inducing its internalization and degradation (Ross et al. 2012). Hepcidin is a peptide hormone secreted by the liver in response to iron loading and inflammation (Nemeth et al. 2004), and its expression is regulated in response to the iron content in the body (De Domenico et al. 2007). Body iron homeostasis is maintained by the hepcidin-Fpn1 regulatory axis and the cellular IRE/IRP (iron regulatory element, iron regulatory protein) system for regulating the expression of iron absorption- and transport-related proteins (Lash and Saleem 1995; Hentze et al. 2010; Theil 2011). The blood iron concentration is a direct reflection of the iron content in the body. So, compared with FeSO₄ group, the suppression of hepcidin expression in Fe-Cgly group may be due to the higher blood iron concentration.

When the body is in a state of iron deficiency, the rate of intestinal absorption of iron is accelerated. DMT1 is the only known membrane transporter for the intestinal absorption of non-heme iron, primarily at the duodenum brush border (Andrews 1999). The dietary levels of iron influence DMT1 expression and distribution. During iron deficiency, DMT1 mRNA levels increase at the rat duodenal crypt-villus junction, and DMT1 protein is mainly

distributed in the cell membranes on the free surface of intestinal microvilli. Under normal iron levels, DMT1 is mainly distributed in the cytoplasm (Frazer et al. 2007). In a subtype of DMT1 with the mRNA 3'-UTR containing an iron response element (IRE), DMT1 expression is regulated by iron levels via the IRE/IRP system (Schneider and Leibold 2003). In IRP system mainly including IRP1 and IRP2, IRP1 emerged as a key regulator of erythropoiesis and iron absorption by controlling hypoxia inducible factor 2 α (HIF2 α) mRNA translation, while IRP2 appears to dominate the control of iron uptake and heme biosynthesis in erythroid progenitor cells by regulating the expression of transferrin receptor 1 (TfR1) and 5-aminolevulinic acid synthase 2 (ALAS2) mRNAs, respectively (Wilkinson and Pantopoulos 2014).

The results of the present study revealed that hepcidin expression levels in the rat liver were elevated after iron supplementation, indicating that hepcidin secretion into the bloodstream was also elevated, thereby decreasing Fpn1 expression in the duodenal mucosa. The iron content in the liver and spleen of rats fed an iron-supplemented diet showed marked improvement with both supplements. Compared to the control group, SD anemic rats that

received both Fe-Cgly and FeSO₄ showed decreased duodenal expression of *DMT1*, *Dcytb*, and *Fpn1*, indicating that the capacity for intestinal absorption of Fe²⁺ decreased significantly. Additionally, the gene and protein levels of IRP1/IRP2 were higher in the Fe-Cgly group than in the control and FeSO₄ groups. Thus, we thought that IRP1/2 may play an important role in the intestinal absorption of Fe-Cgly.

Conclusion

Our results indicated that compared to the inorganic FeSO₄, Fe-Cgly can rapidly improve the blood iron status of anemic rats in vivo. Furthermore, Fe-Cgly and FeSO₄ may have similar roles in the body, and IRP1/IRP2 may play an important role in the absorption process of Fe-Cgly.

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Compliance with ethical standards

Conflict of interest None of the authors has any conflict of interest to declare.

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