# Kinetics Absorption Characteristics of Ferrous Glycinate in SD Rats and Its Impact on the Relevant Transport Protein

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**Abstract** Ferrous glycinate (Fe-Gly) maintains high bioavailability in animals, but its exact absorption mechanism is still unknown. Here, we studied on the absorption kinetics of ferrous glycinate and its impact on the relevant transport protein in Sprague-Dawley (SD) rats. A total of 72 SD rats (male, BW 100±6.25 g) were randomly allotted to three treatments. These treatments were perfused with 1 mL of normal saline, ferrous sulfate ($FeSO_4$), and ferrous glycinate (71.35 mg/L as iron) separately. Four rats were selected from each treatment for collection of blood from the tails at certain times (15, 30, 45, 60, 75, 90, 120, 240, and 360 min) after gavage. Moreover, other six rats selected from each treatment were slaughtered for sampling after gavage at 2, 4, and 6 h to evaluate the expression of intestinal transport protein. Pharmacokinetic parameters of iron were determined by one-compartmental analysis. Compared with $FeSO_4$, the peak plasma concentration of iron ($C_{max}$) is higher in the rats given gavage with Fe-Gly ($P<0.05$). Four hours after gavage with Fe-Gly, the expression of divalent metal transporter 1 (DMT1) in the duodenum is significantly decreased ($P<0.05$), but the expression of ferroportin 1 (Fpn1) is significantly increased ($P<0.05$). This study indicates that Fe-Gly as iron sources can be absorbed more and utilized faster than $FeSO_4$, and they had different effects on the expression of intestinal transport protein.

**Keywords** Ferrous glycinate · Absorption kinetics · Transport protein

## Introduction

Iron is an essential trace element for animal growth and health, which is involved in numerous vital biologic processes, including oxygen transport, DNA biosynthesis, energy metabolism, and oxidative phosphorylation [1, 2]. For lack of a controlled excretion mechanism, iron homeostasis in the body is mainly regulated by its absorption and transport through duodenum epithelia [3]. However, iron deficiency is a prevalent nutritional problem for humans and animals [4]. It has a long history of supplementation iron to diets to prevent and treat iron deficiency in animals [5–7], but different iron sources varied in their bioavailability. It was reported that metal chelated with amino acid or protein has good bioavailability in animals [8–10]. Studies have showed that ferrous glycinate (Fe-Gly) was more effective in animal production compared with ferrous sulfate ($FeSO_4$) [11, 12], and the fact that Fe-Gly could be easily absorbed and maintained high bioavailability in humans in spite of the presence of iron absorption inhibitory factors such as phytic acid [13, 14] also made it an effective iron reinforcing agent.

However, the data as to the absorption process of Fe-Gly and studies about the mechanism of its high bioavailability is limited. Therefore, the main objective of the present research was to study on the kinetics absorption characteristics of Fe-Gly and its impact on the relevant intestinal transport proteins in Sprague-Dawley (SD) rats.

## Materials and Methods

### Animals and Experimental Design

The experiment was conducted in the Laboratory Animal Center of Zhejiang University. Seventy-two SD rats (male,

**Table 1** Composition and nutrient levels of basal diets

| Ingredients | Percentage |
|---|---|
| Corn starch | 39.75 |
| Casein lactic | 20.00 |
| Granular sugar | 10.00 |
| Dextrin | 13.20 |
| Solka Floc-40 | 5.00 |
| Mineral mix | 3.50 |
| Vitamin mix | 1.00 |
| L-cystine | 0.30 |
| Choline bitartrate | 0.25 |
| Soy oil | 7.00 |

Supplied mineral mix contained (based on total diet) calcium, 0.52 %; phosphorus, 0.20 %; potassium, 0.38 %; sodium, 0.11 %; magnesium, 0.05 %; iron, 52.02 ppm; zinc, 36.76 ppm; manganese, 11.33 ppm; copper, 6.73 ppm; cobalt, 0.02 ppm; and iodine, 0.21 ppm. Supplied vitamin mix contained (based on total diet) vitamin A, 4.00 IU/g; vitamin $D_3$, 1.00 IU/g; alpha-tocopherol, 75.00 IU/kg; thiamine, 5.00 ppm; riboflavin, 6.0 0 ppm; niacin, 30.00 ppm; pantothenic acid, 15.00 ppm; choline, 1,000.00 ppm; pyridoxine, 6.00 ppm; folic acid, 2.00 ppm; biotin, 0.20 ppm; vitamin $B_{12}$, 25.00 μg/kg; and vitamin K, 0.90 ppm

After 3 days of pre-feeding, all rats were fasted for 12 h before the experiment. Then, these treatments were perfused with 1 mL of normal saline, $FeSO_4$, and Fe-Gly (71.35 mg/L as iron), respectively. All the animal studies were approved by the Animal Ethics Committee of Zhejiang University.

SD rats were reared in a clean standard room. Their diet (Table 1) was formulated according to the international standards of experimental animals, AIG-93G (purchased from Slack Experimental Animals LLC, Shanghai). The room temperature was maintained at about 23~25 °C, and the relative humidity was 40~60 %. Twelve hours of alternating lighting was used for the SD rats. All rats were housed in a stainless steel cage and given deionized water to avoid uptaking extra iron except from the diet.

## Sample Collection

After gavage, four rats were selected from each treatment for collection of blood from the tails at certain times (15, 30, 45, 60, 75, 90, 120, 240, and 360 min). Serum was separated by centrifugation at 3,000×*g* for 10 min at 4 °C and stored at −80 °C until kinetics absorption study.

Moreover, other six rats selected from each treatment were slaughtered for sampling after gavage at 2, 4, and 6 h respectively. The rats were sacrificed by cervical dislocation, and about 3 cm of the duodenum just proximal to the pyloric sphincter was removed, washed with normal saline, packed with sterile and RNase-free silver paper, and after, rapidly frozen in liquid nitrogen and stored at −80 °C for later analyses of divalent metal transporter 1 (DMT1) and ferroportin 1 (Fpn1).

## Pharmacokinetic Analysis

Concentration of iron in the serum was analyzed with an atomic absorption spectrophotometer (AA6501, Shimadzu Ltd., Kyoto, Japan) [15]. Plasma concentration-time plots according to the data of serum iron in different time points were made. Pharmacokinetic parameters of iron were calculated using PKSlover 1.0 [16] by one-compartmental analysis. The involved pharmacokinetic parameters are listed as follows: $t_{1/2}$, terminal half-life; V/F, apparent volume of distribution; CL/F, clearance; $C_{max}$, the observed peak plasma concentration; $T_{max}$, observed time to achieve $C_{max}$; AUC, the area under the plasma concentration-time curve; and MRT, the mean residence time.

## Quantification Real-time PCR Analysis

Expression of duodenum-specific transcripts was analyzed by RT-PCR [17, 18]. Primers for DMT1, Fpn1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed with Primer Express 2.0, Beacon designer, and GenBank (Sangon Biotech, Table 2). Total RNA from mouse duodenum was extracted by Trizol Reagent kits (Jiancheng Bioengineering Institute, Nanjing, China) [19]. RNA was then reverse transcribed into cDNA with the M-MLV Reverse Transcriptase RNase H- (Promega). Real-time PCR was conducted using the iQ™5 real-time multiplexing system (Bio-Rad Inc., Hercules, CA, USA). The flourescence quantitative PCR mixtures contained 1 μL of cDNA, 12.5 μL 2× SYBR green PCR master mix, 1 μL each of the forward and reverse

**Table 2** Choosing primers for quantitative PCR

| Item | No. in GenBank | Sequences | Length | Annealing temperature |
|---|---|---|---|---|
| GAPDH | NM_017008 | 5′-GGTGGACCTCATGGCCTACAT-3′<br>5′-GCCTCTCTCTTGCTCTCAGTATCCT-3′ | 88 | 61 |
| DMT1 | AY336075 | 5′-GTGGTGGCTGGGGCTTCATTG-3′<br>5′-GGGTCTTGGCCTGTCCGTTCA-3′ | 88 | 63 |
| Fpn1 | AF394785 | 5′-CTGGTGGAACTCTACGGAAACG-3′<br>5′-GGGACGTCTGGGCCACTTTAA-3′ | 139 | 61 |

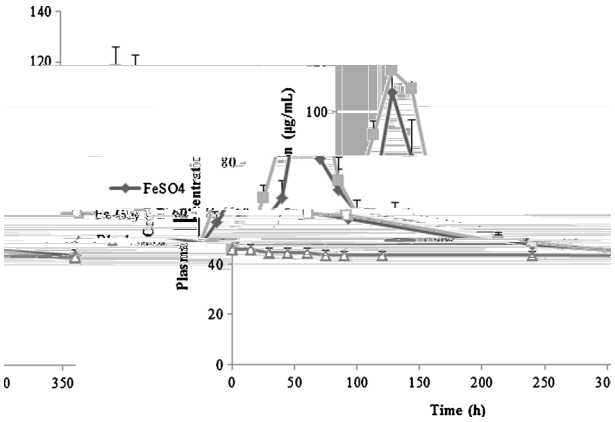


**Fig. 1** Mean plasma iron concentration versus time after gavage with different iron sources to SD rats. *Error bars* represent standard deviation of the mean ($n$=4)

primers, and 9.5 μL of RNase-free deionized water in a final volume of 25 μL. This standard PCR protocol was carried out at 15 s at 95 °C and 30 s at 60 °C for 40 cycles, and then thermal denaturing step was done in order to generate the dissociation curves to verify amplification specificity. Results were normalized to GAPDH. The relative quantification was performed using Relative Expression Software Tool-384 (Bio-Rad Inc., Hercules, CA, USA) which is based on the calculation of $2^{-\Delta\Delta Ct}$ value.

## Western Blotting Analysis

The proteins were extracted by T-PER Tissue Extraction Reagent (Pierce, USA) from rat intestinal tissues, and then proteins were quantitated by BCA Quantification Kit (Beyotime Institute of Biotechnology, Shanghai, China). Tissue lysates (total protein of 60 μg) were separated by 10 % SDS-PAGE and transferred to a PVAF membrane. Membranes were blocked with 5 % nonfat milk in T-TBS buffer at room temperature for 1 h. Thereafter, the membranes were immunoblotted with primary antibodies (Santa Cruz

**Table 3** Pharmacokinetic parameters after gavage with different iron sources to the rats

| Pharmacokinetic parameters | Unit | $FeSO_4$ | Fe-Gly |
|---|---|---|---|
| $t_{1/2}$ | h | 0.493 | 0.426 |
| V/F | L | 4.24 | 2.86 |
| CL/F | L/h | 5.68 | 4.42 |
| $C_{max}$ | mg/L | 35.54 | 52.76[a] |
| $T_{max}$ | h | 0.73 | 0.63 |
| AUC | mg/(L h) | 70.25 | 89.27 |
| MRT | h | 1.458 | 1.261 |

The values are the mean

$t_{1/2}$ terminal half-life, *V/F* apparent volume of distribution, *CL/F* clearance, $C_{ma}$ the observed peak plasma concentration, $T_{ma}$ observed time to achieve $C_{max}$, *AUC* the area under the plasma concentration-time curve, *MRT* the mean residence time

[a] Significantly different from $FeSO_4$

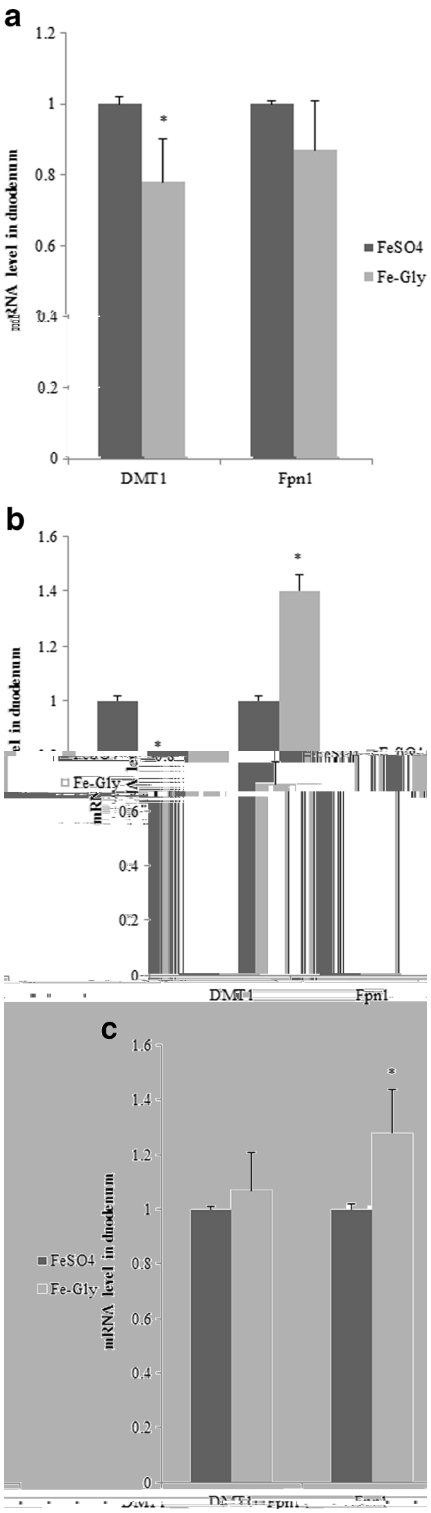


**Fig. 2** The mRNA expression of DMT1 and Fpn1 in the duodenum. **a**–**c** Representations of 2, 4, and 6 h after gavage, respectively. Values are presented as mean±SD ($n$=6). *$P$<0.05, significant difference of mean values between the two groups

Biotechnology, Inc., USA) overnight at 4 °C, followed by incubation with goat anti-mouse IgG (Pierce, USA) for 1 h at room temperature. Blots were visualized by SuperSignal® West Dura Extended Duration Substrate (Pierce, USA), and the protein expression levels were quantified by densitometric scan.

### Statistical Analysis

The data were analyzed by one-way ANOVA of SPSS 16.0. Multiple comparisons were made by least significant difference (LSD). Values were expressed as means ± standard deviation (SD). $P$ value < 0.05 was considered to be statistically significant.

## Results

As shown in Fig. 1, the plasma concentration-time profiles are very similar between two iron source treatments; both curves reached the peak in 50 min, but the Fe-Gly group maintained higher iron levels on each time point especially in the 0- to 100-min period compared with the $FeSO_4$ group. Absorption was rapid as indicated by the occurrence of mean peak plasma concentrations in less than 0.8 h within different iron source treatments (Table 3). There was no significant difference in the AUC values between the two iron sources, but the AUC of Fe-Gly was enhanced by 27.07 % compared with that of $FeSO_4$. The V/F values were 32.55 % lower and the CL/F values was 22.18 % lower in the Fe-Gly group. Compared with that of $FeSO_4$, the $C_{max}$ of Fe-Gly was increased ($P$<0.05), but the MRT values of Fe-Gly was 13.51 % lower than that of $FeSO_4$.

Different iron sources have different effects on the mRNA expression of transport proteins in the duodenum (Fig. 2). Compared with that in the $FeSO_4$ group, the mRNA level of DMT1 in the Fe-Gly group was lower in 2 and 4 h ($P$<0.05), but the difference vanished in 6 h. The Fpn1 mRNA of Fe-Gly had a slight decrease in 2 h after gavage, but higher Fpn1 mRNA was observed in the next 4 h compared with that of $FeSO_4$ ($P$<0.05).

The protein expressions of DMT1 and Fpn1 in the duodenum were shown in Fig. 3. After gavage, the expression of DMT1 in the Fe-Gly group was lower than that in the $FeSO_4$ group in 4 and 6 h ($P$<0.05). The expression of Fpn1 in the Fe-Gly group was enhanced in 4 h compared with that in the $FeSO_4$ ($P$<0.05), whereas the expression level of Fpn1 between the two groups is similar in 6 h ($P$>0.05).

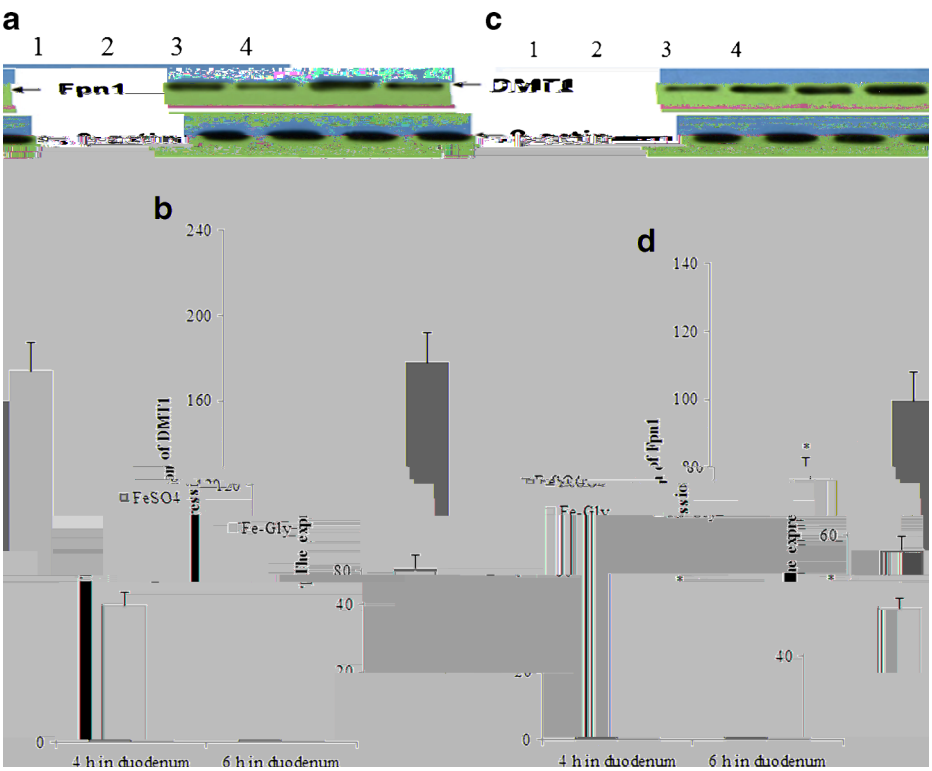


**Fig. 3** **a** Western blotting analysis of DMT1 expression in the duodenum: *lane 1*, DMT1 expression of the $FeSO_4$ group in the duodenum, 4 h after gavage; *lane 2*, DMT1 expression of the Fe-Gly group in the duodenum, 4 h after gavage; *lane 3*, DMT1 expression of the $FeSO_4$ group in the duodenum, 6 h after gavage; *lane 4*, DMT1 expression of the Fe-Gly group in the duodenum, 6 h after gavage. **b** The results of DMT1 expression by densitometric scan. **c** Western blotting analysis of Fpn1 expression in the duodenum: *lane 1*, Fpn1 expression of the $FeSO_4$ group in the duodenum, 4 h after gavage; *lane 2*, Fpn1 expression of the Fe-Gly group in the duodenum, 4 h after gavage; *lane 3*, Fpn1 expression of the $FeSO_4$ group in the duodenum, 6 h after gavage; *lane 4*, Fpn1 expression of the Fe-Gly group in the duodenum, 6 h after gavage. **d** The results of Fpn1 expression by densitometric scan

## Discussion

Many studies have shown that amino acid-chelated iron was better absorbed than inorganic iron such as ferrous sulfate [20–22]. In the present research, we established rat intragastric administration model to compare the intestinal absorption characteristics of ferrous sulfate and ferrous glycinate. The assay was liner in the concentration range of 0–140 μg/mL with the correlation coefficients $R^2 > 0.999$, and it was suitable for the later plasma pharmacokinetics study on iron [23–25]. The absorption curves of two iron sources were similar, but higher plasma iron levels were observed on each time point especially from the 0- to 100-min period in the Fe-Gly group compared with those in the $FeSO_4$ group. It can be inferred that ferrous glycinate indeed maintained better absorption efficiency than ferrous sulfate, which was confirmed by the pharmacokinetic parameters. The area under the plasma concentration-time curve (AUC) was used to evaluate the total absorption of drugs in the body, and the mean residence time (MRT) reflected the utilization of drugs in the body. The enhanced AUC values and decreased MRT values in the Fe-Gly group indicated that ferrous glycinate was better absorbed and utilized faster than ferrous sulfate. Thus, iron sources from ferrous glycinate exhibited better bioavailability compared with ferrous sulfate.

DMT1 and Fpn1 were very important intestinal membrane transport proteins for iron absorption. Distributed in the brush border of duodenal villi and crypts, DMT1 is responsible for transporting iron across the apical membrane into the enterocyte [26, 27]. As for Fpn1, which are abundantly expressed on the basolateral membrane of polarized enterocytes in the duodenum, is the only known iron exporter acting as a basolateral iron pump [28, 29]. An increased expression level of DMT1 and Fpn1 has been observed in patients with iron deficiency [30, 31], whereas the expression of DMT1 is decreased after high oral iron intakes [32]. Similar results were reported in various cell culture models [33, 34]. Duodenum is the main iron absorption site in the body [35], and the expression levels of DMT1 and Fpn1 in the duodenum have been shown to be regulated in response to iron status [3, 36]. Therefore, the paper focused on the expression of DMT1 and Fpn1 in the duodenum. In the present study, lower mRNA abundance of DMT1 was observed in the Fe-Gly group during 2 and 4 h, which was consistent with the protein expression results by Western blotting analysis. It indicated that Fe-Gly is easier to be absorbed, for the increased cell iron can reduce DMT1 levels [37]. There were no significant differences in Fpn1 mRNA level in 2 h after gavage, but Fpn1 mRNA level of Fe-Gly was increased in 4 and 6 h compared with that of $FeSO_4$. The protein expression of Fpn1 in Fe-Gly was significantly increased in 4 h, but the high expression levels were not sustained in 6 h after gavage. The Fpn1 protein expression was not consistent with the mRNA expression; a part of the reason may be the regulation of Fpn1 at a transcriptional level [26]. The results also revealed that ferrous glycinate is more effective in the transportation because of the relatively high Fpn1 levels. The reasons for the different effects of two iron sources on the relevant transporters warrant further study.

## Conclusions

In summary, the present study indicated that ferrous glycinate is more effective in absorption and utilization than ferrous sulfate in SD rats. The expression of iron-related transporters also differed between the $FeSO_4$ and Fe-Gly groups. As for the relationship of the absorption rate and different intestinal transporters' expression between two iron sources, it remains to be further researched.

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