Original paper

Estimation of the Iron Absorption from Ferrous Glycinate-loaded Solid Lipid Nanoparticles by Rat Everted Intestinal Sac Model

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Solid lipid nanoparticle (SLN) could be adopted as a potential nanovehicle for improving iron absorption. The rat everted intestinal sac model was employed to investigate the absorption of ferrous glycinate SLNs, to evaluate the resistance of the SLNs against phytic acid and zinc, and to estimate the effect of the SLN particle sizes on iron absorption. The results showed that the iron absorption was obviously enhanced after ferrous glycinate incorporation into SLNs. The inhibitory effects of phytic acid and zinc on iron absorption could be partly prevented by SLNs, and meanwhile the iron absorption could be improved. Furthermore, the results indicated that the relationship between ferrous glycinate SLNs and iron absorption was size-dependent, and the absorption increased with decreasing of SLN particle sizes. The results suggested that the SLNs could alter the absorption pathways of iron besides simple carriers. SLN could be an effective carrier to improve the absorption of nonheme iron.

Keywords: Ferrous glycinate solid lipid nanoparticle, iron absorption, everted intestinal sac model, phytic acid, particle size

Introduction

Iron deficiency is one of the most prevalent malnutrition in developing countries, and nutritional anemia induced by the mineral deficiency affects roughly a sixth of the world's population (Lopez *et al.*, 2016). The major causes of the problem are the inadequate intake of food iron and low iron bioavailability in diets (Abbaspour *et al.*, 2014). To enhance the supply of absorbable iron-rich food and to increase the bioavailability of dietary iron are presently efficient and economical strategies employed to overcome iron deficiency in developing countries (Collings *et al.*, 2013; Pasricha *et al.*, 2013). Many developed techniques, such as solid lipid nanoparticles (SLNs), liposomes, multiple emulsions, have been used to improve the bioavailability of nonheme iron (Hosny *et al.*, 2015; Jimenez-Alvarado *et al.*, 2009; Yuan *et al.*, 2013). Iron-loaded SLNs have been considered to be a potential and effective iron supplement to treat iron deficiency (Zariwala *et al.*, 2013).

SLNs, as a kind of colloidal delivery systems, usually formed from lipid and/or lipid-like substances, have been widely investigated and utilized in many fields, such as drug, cosmetic and food. SLNs have been used to deliver conventional oral iron salts. Iron SLNs, compared with the usual nonheme iron and/or free iron salts, were higher bioavailable and more stable (Hosny *et al.*, 2015; Zariwala *et al.*, 2013). Iron-loaded SLNs could eliminate the major drawbacks of conventionally used iron tablet, such as constipation, allergic reactions (Hosny *et al.*, 2015).

Microdisperse systems have been considered as effective carriers of food ingredients (Cheng *et al.*, 2014; Tan *et al.*, 2014). It is well-known that the absorption of common iron salts could be severely blocked by external factors, such as phytic acid, divalent metal ions (Andrews *et al.*, 2014; Olivares *et al.*, 2012). Large quantities of phytic acid are contained in cereal-based and legume-based diets, which have been evidenced to exert a negative effect on iron absorption in humans. Iron uptake even from porridges

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made of low-extraction flours was still very low due to phytic acid inhibitory effect. Certain divalent metal ions, such as Zn^{2+} , are another important inhibitory factor of iron absorption because of the antagonistic interaction between metal ions. Meanwhile, the bioavailability, bioactivity and distribution of core materials were remarkably influenced by SLN physicochemical properties (Wang *et al.*, 2005). For instance, particle size plays a critical role for the absorption of particulate matter delivered through the oral route (Kulkarni and Feng, 2013).

Rat everted intestinal sac technique has been extensively adopted to predict the intestinal absorption of a variety of drugs and food ingredients in humans (Tharabenjasin *et al.*, 2014). The *in vitro* model shows promise as a useful screening tool for estimating the absorption of active ingredients. The role of SLNs in iron absorption could be established using the model.

Compared with the usual iron supplement (ferrous sulfate), ferrous glycinate is more stable, higher bioavailable and safer (Jeppsen and Borzelleca, 1999; Marchetti *et al.*, 2000). The iron amino acid chelate has been widely used to combat iron deficiency in many countries. The iron chelate had been encapsulated in SLNs to prevent the iron salt from being dissociated in acid environment. The purposes of the present study were to research whether the well-known inhibitors of iron absorption (i.e. phytic acid and zinc) would affect the transport of ferrous glycinate SLNs, and to estimate the influences of particle sizes on the iron SLNs uptake.

Materials and Methods

(1) Materials Stearic acid, soybean lecithin, polyvinyl alcohol (PVA) and Tween 80 were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Phytic acid and ZnCl_2 were obtained from Sigma-Aldrich Co., LLC (Shanghai, China). All of the other chemicals were of reagent grade and purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Ferrous glycinate was synthesized according to CN Patent ZL200410065260.3.

(2) Preparation of Ferrous Glycinate SLNs Ferrous glycinate SLNs were prepared by double emulsion solvent evaporation method described previously, with some modifications (Zariwala *et al.*, 2013). Briefly, the organic phase was prepared by dissolving stearic acid (100 mg) and soybean lecithin (25 mg) in a 10-mL dichloromethane and pre-warming to 60°C in a water bath. Aqueous emulsifier phase (3 mL) with or without the addition of ferrous glycinate (20 mg) was heated to equivalent temperature. The organic phase and the aqueous phase were homogenized using ultrasonication (VCX400, Sonics & Material, USA) in cycle of sonication for 1 s and standby for 1 s (3 min, 300 W, 60°C), and then the primary emulsion was obtained and poured into 20 mL PVA (1%, w/v) solution, the mixture system was homogenized again (300 W, 60°C) to form a w/o/w emulsion. The resultant double emulsion was rotary evaporated, cooled in an ice bath for 15 min. At last, the residual organic solvent was evacuated by nitrogen gas.

(3) Procedures to Purify Ferrous Glycinate SLNs and to Control SLN Particle Sizes The purification and particle size control of ferrous glycinate SLNs was processed simultaneously. Firstly, SLNs were separated according to the particle size by Sephadex G-100 column (20 cm × 1 cm id), and the samples were eluted using 0.9% (w/v) NaCl solution, and the eluate was collected by test tubes per minute. Secondly, SLNs were extruded to adjust particle sizes using an ultrafiltration cell (Amicon stirred cell 8010, Millipore, USA) with a polycarbonate membrane (1.0, 0.6, 0.4, 0.2, and 0.1 µm pores). Samples were extruded through a membrane using N_2 gas, and extruded solution was collected. The obtained solution could be further extruded to reduce the particle size using a membrane with next smaller pore size. At last, the first step was repeated to eliminate free iron. Particle size and size distribution were measured by dynamic light scattering with a ZEN3600 Zetasizer nano instrument (Malvern Instrument, Worcs, UK).

(4) Everted Intestinal Sac Experiments

a) Animals One hundred and eight rats (Sprague-Dawley, 7-week-old male, 200 – 250 g) were purchased from Shanghai Laboratory Animal Research Center (Chinese Academy of Sciences, Shanghai, China). The rats were housed four to each cage at controlled conditions (50% air humidity, 25°C, 12-h light cycle). The animals were acclimatized for 1 week and were fed on tap water and pellet food (Shanghai Laboratory Animal Research Center, Chinese Academy of Sciences, Shanghai, China). All animal testing was conducted according to national standard “Laboratory Animal-Requirements of Environment and Housing Facilities” (GB 14925-2001) of China. The care of laboratory animal and the animal experimental operation was in keeping with “Administration Rule of Laboratory Animal” of China. This research was approved by the Ethics Committee on Animal Experiments of Yangtze University, China.

b) Perfusion Buffer The perfusion buffer was PBS buffer solution (50 mM) with the pH adjusted to 7.4 using NaOH. The suspensions of ferrous glycinate SLNs were dispersed at predetermine iron concentration in the presence of predetermine amounts of phytic acid or ZnCl_2 .

c) Everted Intestinal Sac Experiment Procedure The everted intestinal sac studies were performed as previously described (Salphati *et al.*, 2001). Briefly, the rats were fasted overnight (free access to tap water) before the everted intestinal sac experiments. Animals were anesthetized using an i.p. injection of thiobutabarbital sodium (150 mg/kg). The intestinal segments of interest were identified (duodenum, starting 2 cm below the pylorus) and isolated. Subsequently, an overdose of anesthesia was used to sacrifice the rats. Intestinal segments were rinsed with cold Tyrode's solution (4°C) and immediately placed in oxygenated (O_2/CO_2 , 95:5, v/v) Tyrode's solution. Then, the intestinal segments were gently everted over using a glass rod. One end of the segment was tied with a silk thread forming a sac, while the other end was attached to a sampler. After the blank solution (1 mL) was

introducing into the everted sac (serosal side), a 10-cm-long everted intestinal sac was prepared. An appropriate amount of aerated Tyrode's solution was introduced in the everted intestinal sac.

The sacs were placed in 250 mL triangular flasks with 100 mL perfusion buffer solution, respectively. These flasks were incubated in an oscillating water bath (100 cycles/min) at 37°C. Aliquots of 0.1 mL were sampled from sacs at 0, 30, 60, 90, and 120 min followed by immediate replenishment of the same volume of aerated Tyrode's solution, respectively. The everted intestinal sacs were incubated for 120 min in effect experiments of phytic acid, zinc ion and particle size. After incubation, the transport was stopped by washing the inserts three times with ice-cold Tyrode's solution. Experiments were independently repeated three times.

These intestinal segments were opened by a longitudinal incision. The lengths and width of opened segments were measured, and the areas were calculated. These samples were determined using atomic absorption spectrophotometry (AAS, Zeenit 700P, Analytik Jena AG, Germany) at 248.3 nm.

(5) *Data Analysis and Statistical Evaluation* The cumulative absorption of intestinal segments per unit area (Q) was calculated based on the area of intestinal segment and the concentrations of iron as determined by AAS. Q was calculated using the following equations:

$$Q = \frac{\sum_{i=1}^{n-1} C_i \times 0.1 + C_n \times V}{A} \quad \text{.....Eq. 1}$$

Where C_i is the iron concentration of the i time point sample; C_n is the iron concentration of the n time point sample; A is the area of intestinal segment (cm^2); Q is the cumulative absorption of intestinal segments per unit area.

Statistical analysis of the data obtained was performed according to Tukey's test with the Matlab (Version 7.11.0.584(R2010b), The MathWorks, Inc., Natick, Massachusetts, USA). P values of ≤ 0.05 were considered to imply significant difference. Variance within treatment groups was expressed as standard deviation (S.D.).

Results

(1) *Effect of Incubation Time on Q from Ferrous Glycinate SLNs* Cumulative absorption of intestinal segments per unit area ferrous glycinate SLNs with increasing incubation time at four levels, 30, 60, 90, and 120 min was evaluated. As shown in Fig. 1, the Q values of ferrous glycinate SLNs were time-dependent in the period from 30 to 120 min, which was in agreement with that of ferrous glycinate, and the values tended to increase with increasing incubation time. The Q values of ferrous glycinate were significantly different ($P < 0.05$) at different concentration. Iron absorption had not become saturated during 10 – 100 $\mu\text{mol/L}$ of ferrous glycinate, implying that the uptake mechanism of the chelated iron in duodenum may be passive diffusion (Zhang *et al.*, 2013). However, the Q values of ferrous glycinate SLNs were

Fig. 1. Effects of incubation time on cumulative absorption of intestinal segments per unit area (Q). Ferrous glycinate SLNs and ferrous glycinate are expressed in solid line series and dashed line series, respectively. The results of ferrous glycinate SLNs and ferrous glycinate were compared at the same time. The data marked the same letter represented no significant difference; the data marked the different letter represented significant difference. The values in the legend were iron concentrations. Values are means $\pm$ S.D., $n = 3$.

significantly higher ($P < 0.05$) than that of ferrous glycinate. Compared to ferrous glycinate, the relative Q of ferrous glycinate SLNs 10, 50 and 100 $\mu\text{mol/L}$ and incubation for 120 min were 1.78-fold, 1.33-fold and 1.31-fold, respectively. The obviously higher Q values of ferrous glycinate SLNs could be ascribed to the properties of SLNs, which plays an important role in the transcytosis of nanoparticles. The results indicated that the iron from ferrous glycinate SLNs could be more efficiently transported.

(2) *Effects of Phytic Acid and Zinc Ion on Q from Ferrous Glycinate SLNs* Usually, nonheme iron from the daily food is poorly absorbed because of the presence of absorption depressors. Phytic acid is a powerful inhibitor of nonheme iron absorption and it extensively exists in plant-based diets, such as cereals, legumes. As can be seen from Fig. 2, the Q values of ferrous glycinate SLNs and ferrous glycinate decreased with increasing of phytic acid concentrations. However, the Q value profiles were distinct between the two iron supplements. Compared with ferrous glycinate, however, iron uptake from ferrous glycinate SLNs was less suppressed by phytic acid. For instance, at the iron concentration of 100 $\mu\text{mol/L}$ and at phytic acid concentrations of 100, 200, and 500 $\mu\text{mol/L}$, the Q values were decreased by 8.9%, 14.5%, and 22.5% for ferrous glycinate SLNs and by 14.1%, 21.4% and 37.7% for ferrous glycinate, respectively. The results revealed that ferrous glycinate SLNs had 1.31-fold, 1.39-fold, 1.42-fold and 1.63-fold potency against ferrous glycinate at iron concentration of 100 $\mu\text{mol/L}$ and at phytic acid concentration of 0, 100, 200 and 500 $\mu\text{mol/L}$, respectively.

It is conceived as a critical nutrition issue that mineral elements, especially divalent metal ions, are commonly involved in the competitive inhibition in foods. For example, the interaction between iron and zinc could significantly reduce iron absorption.

Fig. 2. Effects of phytic acid on cumulative absorption of intestinal segments per unit area (Q). Ferrous glycinate SLNs and ferrous glycinate are expressed in solid line series and dashed line series, respectively. The results of ferrous glycinate SLNs and ferrous glycinate were compared at the same phytic acid concentration. The data marked the same letter represented no significant difference; the data marked the different letter represented significant difference. The values in the legend were iron concentrations. Values are means $\pm$ S.D., $n = 3$.

Fig. 3. Effects of $ZnCl_2$ on cumulative absorption of intestinal segments per unit area (Q). Ferrous glycinate SLNs and ferrous glycinate are expressed in solid line series and dashed line series, respectively. The results of ferrous glycinate SLNs and ferrous glycinate were compared at the same $ZnCl_2$ concentration. The data marked the same letter represented no significant difference; the data marked the different letter represented significant difference. The values in the legend were iron concentrations. Values are means $\pm$ S.D., $n = 3$.

The effects of zinc on Q values of ferrous glycinate SLNs were presented in Fig. 3. The Q values of ferrous glycinate SLNs decreased in response to increasing of zinc concentration. Compared with free ferrous glycinate, however, inhibitory effect of zinc on iron absorption was alleviated by the incorporation ferrous glycinate into SLNs. For instance, the absorption values at the iron concentration of 100 $\mu\text{mol/L}$ and at zinc concentration of 10, 50 and 200 $\mu\text{mol/L}$ were decreased by 6.7%, 13.6% and 21.7% for ferrous glycinate SLNs and by 7.1%, 15.9% and 27.3% for ferrous glycinate, respectively. The relative efficiency of ferrous glycinate SLNs were about 1.31-fold, 1.32-fold, 1.35-fold and 1.64-fold at iron concentration of 100 $\mu\text{mol/L}$ and at the zinc concentration of 0, 10, 50 and 200 $\mu\text{mol/L}$, respectively. The results suggested that the

Fig. 4. Effects of particle size of ferrous glycinate SLNs on cumulative absorption of intestinal segments per unit area (Q). The results of ferrous glycinate SLNs containing different amount iron were compared at the same particle size. The data marked the same letter represented no significant difference; the data marked the different letter represented significant difference. The values in the legend were iron concentrations. Values are means $\pm$ S.D., $n = 3$.

antagonism between the two mineral elements may be actively prevented by SLNs.

(3) Effect of Particle Size on Q from Ferrous Glycinate SLNs

Particle size plays a critical roles in nanoscale drug delivery. Iron intake of ferrous glycinate SLNs at four particle sizes were measured (Fig. 4). As can be seen from Fig. 4, the Q values decreased with particle size increasing of ferrous glycinate SLNs. The iron absorption of SLNs with 100 and 200 nm diameters behaved more efficient than that with the particle scales of 500 and 1000 nm ($P < 0.05$). As an example, at the iron concentration of 100 $\mu\text{mol/L}$, compared to 100-nm-size SLNs, the iron uptake of SLNs with the sizes of 200, 500 and 1000 nm was decreased by 21.9%, 59.3% and 82.9%, respectively. The evident size-dependent characteristic maybe indicate that the recognition and internalization of ferrous glycinate SLNs could be regulated by the particle sizes.

Discussions

In the present study, the iron absorption from ferrous glycinate SLNs was clearly evaluated using everted intestinal sac model. The effects of common inhibitors and particle sizes on iron transport from ferrous glycinate SLNs were elucidated. The stability of ferrous glycinate is a very important factor for intestinal absorption. The iron was stable even with pasteurization, so it should be stable under the experimental condition (Ashmead, 2001).

Judging from the Q values (Fig. 1), ferrous glycinate SLNs could be absorbed more rapidly than ferrous glycinate. It may be owed to the possibility that the absorption pathways of ferrous glycinate could be changed via encapsulation in SLNs. Ferrous glycinate could be transported by the formation of part of the nonheme-iron pool, and the iron from ferrous glycinate could pass through small intestinal mucosa by passive diffusion (Sheehan, 1976). In contrast, ferrous glycinate SLNs could be taken up as

common SLNs, and the core material may be actively carried by SLNs (Zhang *et al.*, 2013). Nanoparticles could be acquired via the M-cells in the Peyer's patches and the isolated follicles of the gut-relevant lymphoid tissue besides the normal intestinal enterocytes (Florence, 1997). In addition, previous studies had reported that some macromolecules, such as peptides and proteins, and fat particles could be absorbed intact by endocytic pathway and/or intestinal epithelial cells. Similar studies have evidenced that nanoparticles could be absorbed in an intact form across the intestinal epithelium (Hamman *et al.*, 2005).

Phytic acid extensively exists in cereal-and/or legume-based dietary (Andrews *et al.*, 2014). The absorption of iron can be severely restrained because of the formation of insoluble chelate compound by the coordination between the organic acid and iron. In previous studies, the negative effect of phytic acid on iron absorption could be decreased by acid-salt washing method, phytase and genetic modification (Hurrell *et al.*, 1992; Petry *et al.*, 2013; Troesch *et al.*, 2009). Compared to that of ferrous sulfate, the absorption of ferrous glycinate was less suppressed by phytic acid, but the bioavailability of iron glycine was still seriously cut down because of the chelation between the organic acid and iron (Fox *et al.*, 1998). The Q value profiles (Fig. 2) illustrated that the iron absorption from ferrous glycinate SLNs was higher than that from ferrous glycinate, and the inhibition of phytic acid was reduced. The results indicated that SLNs could behave anti-inhibition ability in food vehicles. It could be likely attributed to the fact that the ferrous glycinate was incorporated into SLNs, and the iron was kept from contacting the organic acid and prohibiting the formation of insoluble iron chelates.

It has proved that the interaction between iron and zinc caused antagonistic responses (Kordas and Stoltzfus, 2004). It is a possible mechanism of the interplay between iron and zinc that these chemically similar ions compete a common absorptive pathway for some portions. The probable interaction sites were located on divalent metal transporter 1 (DMT1) and/or Zip14 protein (Espinoza *et al.*, 2012; Iyengar *et al.*, 2012). The results depicted in Fig. 3 revealed that the iron absorption from ferrous glycinate SLNs was still decreased. However, the iron absorption from ferrous glycinate SLNs was higher than that of ferrous glycinate. It could be supposed that SLNs had remitted the antagonism between the two metal elements. Furthermore, the absorption pathway of ferrous glycinate could be altered by encapsulating it in SLNs. The absorption of SLNs could be enhanced via the chylomicron-association pathway and/or the M cell uptake pathway (Qi *et al.*, 2012). Pereira *et al.* confirmed that iron-loaded nanoparticles could be uptaken by endocytosis (Pereira *et al.*, 2013). So it could be deduced that the iron from SLNs could be absorbed not only via the conventional absorption pathway of nonheme iron but also by direct delivery of SLNs. The different uptake pathway could avoid the competition between iron and zinc for transporters and decrease the antagonism.

It has been found that the absorption of SLNs exhibited clear size and surface characteristic-dependency of nanoparticles. The results (Fig. 4) showed that iron absorption from ferrous glycinate SLNs decreased with increasing of particle sizes. It could be ascribed to the fact that the smaller particles could penetrate cell membranes; particles with sizes of about 100 – 200 nm could be internalized by the receptor-mediate endocytosis; larger particles with sizes of about 500 – 1000 nm could be taken up via phagocytosis (Win and Feng, 2005). Rejman *et al.* presumed that the particles with sizes of about ≤ 200 nm could reach late endosomal-lysosomal compartments (Rejman *et al.*, 2004). In addition, it has been found that nanoparticles with about ≤ 100 nm could alter signaling process essential for basic cell functions, and nanoparticles should not be merely regarded as plain conveyors, but could also play a simulative role in mediating biological effects. These results of the present study were in keeping with that of literatures in which particle absorption increased with decreasing of particle sizes (Florence, 1997). The SLNs were adopted to the assays without submitting to an *in vitro* digestion (gastric) previously. It could partly influence the results. The effect of *in vitro* digestion on iron absorption of SLNs still needs to be clarified by further studies.

It should be pointed out that the advantages of this model are a relatively large surface area available for absorption and the presence of a mucus layer, and the potential disadvantage of everted intestinal sac model is the presence of the muscularis mucosa, which might evoke an underestimation of the transport of active ingredients with a tendency to bind with muscle cells. The living system is more complex than *in vitro* models, in an *in vitro* condition there is probability of less or no enzymatic action. *In vitro* and *in vivo* correlation has not been established between the everted intestinal sac and *in vivo* model (Alam *et al.*, 2012; Cardin and Mason, 1976; Gruden, 1977). However, frequently results from the everted intestinal sac model have been in agreement with *in vivo* findings. The everted intestinal sac model is an efficient tool for studying *in vitro* absorption mechanisms of active ingredients, intestinal metabolism of active ingredients, role of transporter in active ingredient absorption, and for investigating the role of intestinal enzymes during active ingredient transport through the intestine. The results of everted sac model experiments could be used as an effective indicator of the absorption of ferrous glycinate SLNs.

Conclusions

The iron transport from ferrous glycinate SLNs was assessed using rat everted intestinal sac model. The overall absorptive profile of ferrous glycinate could be increased by incorporation into SLNs. The inhibitory effects of phytic acid and zinc could be markedly reduced using SLNs encapsulating ferrous glycinate. Moreover, the absorption of ferrous glycinate SLNs was size-dependent, and the uptake of the SLNs was enhanced with

decreasing particle sizes. The absorption characteristics of ferrous glycinate SLNs could be attributed to the protection of lipid vesicles, and the absorption pathways of ferrous glycinate could be altered by SLNs. It could be concluded that SLNs may be an effective carrier of ferrous glycinate, and subsequently the absorption of iron could be improved.

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