In vitro iron absorption of α -lactalbumin hydrolysate-iron and β -lactoglobulin hydrolysate-iron complexes

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ABSTRACT

To study the feasibility of promoting iron absorption by peptides derived from α -lactalbumin and β -lactoglobulin, the present work examined the transport of iron across Caco-2 monolayer cell as in vitro model. Caco-2 cells were seeded in bicameral chambers with α -lactalbumin hydrolysate-Fe (α -LAH-Fe) complex and β -lactoglobulin hydrolysate-Fe (β -LGH-Fe) complex, α -LAH and iron mixture, β -LGH and iron mixture, FeSO_4 and ascorbic acid mixture, and FeSO_4 . In addition, the cytotoxicity of α -LAH-Fe and β -LGH-Fe complexes were measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The iron absorption and ferritin content were assessed using the coupled in vitro digestion/Caco-2 cell model. Results support that peptide-iron complexes can promote ferritin formation and it is possible to apply β -LGH-Fe complexes as iron-fortified supplements with high iron absorbability.

Key words: iron absorption, α -lactalbumin hydrolysate-iron complex, β -lactoglobulin hydrolysate-iron complex, Caco-2 cells, ferritin

INTRODUCTION

Iron is an essential trace mineral that plays an important role in physical metabolism, including oxygen transport, DNA synthesis, and electron transport in human bodies (Lieu et al., 2001). It can be found in a broad variety of foods. Heme iron, which only comes from hemoglobin and myoglobin in animal food, possesses high bioavailability (de Castro Cardoso Pereira and dos Reis Baltazar Vicente, 2013). Non-heme iron, which is present in dark green leafy vegetables, can be severely impaired by iron absorption inhibitors such as phytate (Hurrell et al., 1992; Abizari et al., 2012) and some phenolic compounds (Petry et al., 2010).

Iron deficiency leads to iron deficiency anemia, the most common and widespread nutritional disorder, according to the World Health Organization (Geneva, Switzerland). Iron fortification is used to increase dietary iron intake. Soluble iron salts (e.g., ferrous sulfate) are not good candidates due to their negative effects on product stability, undesired colored complexes with polyphenol, and adverse organoleptic changes in high-fat or polyphenol-containing food. The free solubilized iron is also a prooxidant (Letelier et al., 2010). Poorly water-soluble iron compounds are more inert than solubilized iron, but they tend to have lower bioavailability, as the iron must be released first by the action of gastric juice (Walczyk et al., 2013). Therefore, the development of iron supplement and iron-absorption-promoting ingredients is critical.

Mineral-binding proteins in foods that are rich in acidic clusters and with specific binding sites for minerals are able to sequester a large amount of divalent cation (Hettiarachchy et al., 2012). The mineral-binding peptides derived from food protein could increase the solubility of minerals at intestinal pH (Erba et al., 2002; Meisel and FitzGerald, 2003). The formation of soluble peptide-mineral complexes and resistance to proteolysis of peptides in the intestinal lumen as mineral carriers contributed to the bioavailability of minerals (FitzGerald, 1998). Whey proteins have been widely applied for commercial interest as an emulsifier and whipping agent in food and nutraceuticals with nutritive value (Yamauchi et al., 1980; Marshall, 2004; Marinova et al., 2009). Consumption of cow milk by infants and toddlers has adverse effects on their iron stores, which is probably due to its low iron content (Ziegler, 2011). It also has been reported that iron absorption was reduced by native milk protein and protein hydrolysis lessened this negative effect (Hurrell et al., 1989; Kibangou et al., 2005). Milk protein hydrolysate could improve iron uptake in the Caco-2 cells model (Argyri et al., 2009). The major organ for digestion and absorption of nutrients is the small intestine. Caco-2 cells, which derived from a human colon adenocarcinoma, spontaneously differentiate into cells with enterocyte morphology (Levy et al., 1995). As Caco-2 cells exhibit several morphological

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and functional characteristics of mature enterocytes, the monolayers are suitable as an in vitro model for absorption and have been applied for the assessment of iron absorption from food (Au and Reddy, 2000; Hendricks et al., 2001; Argyri et al., 2007). Ferritin in Caco-2 cells is involved in iron absorption, which is a protein polymer with a hollow shell structure that can store iron (Vanoaica et al., 2010). Ferritin formation could be used as an indicative index of cell iron uptake (Glahn et al., 1998). Ascorbic acid [vitamin C (**Vc**)], which possesses reducing and chelating properties, has been reported to increase iron absorption and hemoglobin and plasma ferritin concentrations (Monárrez-Espino et al., 2011).

The optimum passage range of Caco-2 cells for experimental purposes was 28 to 65 passages. During these passages, the Caco-2 cells became increasingly columnar in appearance, the brush border became more apparent and more uniform, and intercellular spaces became more well defined after prolonged incubation (Briske-Anderson et al., 1997). Transepithelial electrical resistance (**TEER**) values of Caco-2 cells have been reported as a good indication of the tightness of junction structure (Lu et al., 1996; Briske-Anderson et al., 1997; Leonard et al., 2000). Cytotoxicity to Caco-2 cells can be used to evaluate the dose-dependent toxic potential with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (**MTT**) assay. The MTT assay, which is based on the conversion of MTT into formazan crystals by living cells, determines mitochondrial activity. The excess iron cannot be actively excreted by the human body. Excess of redox active iron leads to oxidative stress and tissue damage, as a result of the formation of free radicals (Okada, 1996; Papanikolaou and Pantopoulos, 2005). In addition, a higher concentration of Fe loading leads to disruption of the cell membrane and enhancement of genome instability and cancer risk (Huang, 2003; Prá et al., 2012).

β -Lactoglobulin and α -LA are the major proteins in whey protein. Our previous study showed that β -LG hydrolysate (**β -LGH**) obtained with alcalase exhibited the highest iron-binding capacity. Compared with β -LGH, the formed β -LGH-Fe complexes exhibited new absorption peaks in Fourier-transform infrared spectroscopy, which indicated the formation of peptide-iron complexes (Zhou et al., 2012). Whether the chelated complexes possess higher mineral absorbability still awaits further investigation. Therefore, the objectives of this study were to explore the feasibility of promoting iron absorption by α -LA hydrolysate (**LAH**)-Fe and β -LGH-Fe complexes, and compare the in vitro absorption efficiency of α -LAH-Fe and β -LGH-Fe complexes using Caco-2 monolayer cells.

MATERIALS AND METHODS

Materials

Alcalase [endoproteinase from *Bacillus licheniformis*; 2.4 Anson units (AU)/g] was donated by Novozymes North America Inc. (Franklinton, NC). Pepsin (EC3.4.23.1; 800–2,500 U/mg of protein) from porcine gastric mucosa and pancreatin from porcine pancreas [P7545; 8 $\times$ United States Pharmacopeia (USP) specification] were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Bovine α -LA (Arla-20; protein 88–94%) was obtained from Arla Foods Ingredients (Viby, Denmark). Bovine β -LG isolate (97.6% protein on a dry basis, 93.2% purity) was provided by Davisco Foods International Inc. (Eden Prairie, MN).

Preparation of α -LAH and β -LGH

α -Lactalbumin (5%, wt/vol) and β -LG (5%, wt/vol) were hydrolyzed with alcalase [enzyme:substrate ratio (E/S) = 5%] at 50°C for 6 h, respectively. The pH of dispersion was adjusted to 8.0 and maintained with 1 M NaOH during the hydrolysis process. After hydrolysis, the solutions were heated in an 85°C water bath for 15 min to inactivate the enzyme. Then, the hydrolysates were cooled to room temperature and ultrafiltered using membrane with a 10,000-Da molecular weight cutoff (Millipore Corp., Bedford, MA) to remove the enzyme and unhydrolyzed protein. The permeated α -LAH and β -LGH were freeze-dried, sealed in plastic bags, and stored at 4°C for the in vitro experiment. The degree of hydrolysis of the obtained α -LAH and β -LGH was 18.53 and 23.78%, respectively.

Formation of α -LAH-Fe Complexes and β -LGH-Fe Complexes

The α -LAH-Fe complexes and β -LGH-Fe complexes were prepared according to Zhou et al. (2012). Binding of iron to the freeze-dried α -LAH and β -LGH was performed by mixing them with ferric chloride solution (hydrolysate:Fe mass ratio = 40:1) at 25°C and pH 7.0, respectively. The reaction was carried out in a shaker (DSHZ-300A; Taicang Experimental Instrument Co. Ltd., Jiangsu Province, China) for 30 min. Free iron was precipitated and removed by centrifugation at $3,000 \times g$ for 20 min at 25°C after the binding process. The soluble peptide-bound iron complexes were collected in the supernatant.

Cell Cultures

Human epithelial colorectal adenocarcinoma Caco-2 cells [American Type Culture Collection (ATCC)] with

passage numbers between 40 and 50 were seeded into microporous polycarbonate membrane filters (Transwell, 3402; Corning Inc., Corning, NY) at a density of 50,000 cell/cm². The cells were grown in Dulbecco's modified Eagle's medium (12100-046; Gibco Products International Inc., Langley, OK) with 10% (vol/vol) fetal bovine serum (16000; Gibco Products International Inc.), 1% (vol/vol) NEAA (11140; Gibco Products International Inc.), and 1% (vol/vol) antibiotic antimycotic solution (Gibco Products International Inc.). The cells were cultured in an incubator with 5% CO₂ and 95% air atmosphere at 37°C and the medium was replaced every 2 d.

Measurement of TEER Values

The Caco-2 monolayers grown in chambers were applied for transport studies when the cells had differentiated to form the intact monolayers, as checked by measuring TEER. The TEER values of the cells were measured at 8 d postseeding. After washing both the apical and basolateral chambers with Hanks' balanced salt solution (HBSS; 14175-095; Gibco Products International Inc.), the sample solutions were added to the apical chambers with serum-free medium. The TEER measurements were made using a Millicell ERS device (Millipore Corp.) and chopstick-style electrodes. The growth medium was removed and the differentiated monolayers were gently washed twice with HBSS and finally placed in 0.5 mL of HBSS. The growth medium was also removed from the basolateral chamber and replaced with 1.5 mL of HBSS. Measurements were made at room temperature (25°C).

MTT Assay

The Caco-2 cells were grown in 96-well plates until subconfluent. The FeSO₄, α -LAH-Fe, and β -LGH-Fe complexes were then added to the cells at defined concentrations (10, 20, 30, 40, 50, and 60 μ g of Fe/mL) and incubated for 24 h. After incubation, the medium was discarded and 20 μ L of 5 mg/mL MTT assay stock solution was then added to each well. Then, the plate was incubated further for 4 h in the incubator. After incubation, the medium was discarded from the wells and 150 μ L of dimethyl sulfoxide was added to solubilize the formed formazan crystals. The optical density was measured at 490 nm.

Iron Transport Measurement

A series of samples (α -LAH-Fe complexes, β -LGH-Fe complexes, α -LAH and Fe mixture, and β -LGH and Fe mixture) and controls (FeSO₄ and FeSO₄ and Vc

mixture at 20:1 molar ratio) were studied for their effects on iron uptake by Caco-2 cells. The mixtures, with the same mass ratio as the complexes, were mixed and then in vitro digested immediately. In vitro enzyme preparation methods were operated using the procedure by Glahn et al. (1998) with some modifications in the digestion, in that the final pH was adjusted to pH 7.5 after adding pancreatin-bile extract mixture. The digests were centrifuged at 10,000 $\times g$ for 25 min at 4°C and the supernatants were filtered through a 0.22- μ m membrane (Millipore Corp.) for iron uptake by Caco-2 cells. Then, 0.5 mL in vitro digest was added to the upper chamber and 1.5 mL of HBSS to the lower chamber. The uptake was measured at 37°C for 1 h in 5% CO₂ and 95% air atmosphere.

Analysis of Iron Absorption and Ferritin Content

Iron in filtered supernatant, kept in cells and transported by Caco-2 cells, was quantified by inductively coupled plasma atomic emission spectrometry (ICP-AES, Optima 2000 DV; PerkinElmer Inc., Waltham, MA). Absorbed iron was quantified by the amount of iron kept within the cell monolayers and iron transported cross the cell model. Dialyzable iron refers to the iron of in the vitro digestion supernatant that could pass through the 0.22- μ m filter membrane:

$$\text{Iron absorption (\%)} = \frac{(\text{absorbed iron})}{(\text{dialyzable iron})} \times 100.$$

Ferritin content in Caco-2 cells was measured by the ferritin assay (Beijing Chemclin Biotech Co. Ltd., Beijing, China).

Statistical Analysis

All treatments in the present study were performed in 3 replicates. Data were expressed as means $\pm$ standard deviations. Statistical analysis of the data was carried out with the Tukey post-hoc test to compare the various means of each series of experiments by Statistix 9.0 (Analytical Software, Tallahassee, FL). Differences were considered significant at $P < 0.05$.

RESULTS

TEER Value of Caco-2 Monolayer

To well establish the Caco-2 monolayer model with enterocyte morphology to mimic in vitro absorption, TEER values between 2 sides of the monolayer were measured, as shown in Figure 1. During the interval of

Figure 1. Effect of culture days on transepithelial electrical resistance (TEER) values of a Caco-2 cell monolayer. Each point represents the mean $\pm$ SD of triplicate experiments.

d 8 to 21, the TEER value increased as the incubation period was prolonged. At d 14, the TEER value was higher than 300 $\Omega\cdot\text{cm}^2$ and increased to $552.5 \pm 35 \Omega\cdot\text{cm}^2$ at d 21, which indicated the formation of tight junctions of the Caco-2 cell monolayer and, thus, the model could be used for the absorption experiment.

Cytotoxicity of α -LAH-Fe and β -LGH-Fe Complexes by the MTT Assay

The cytotoxic potential of α -LAH-Fe and β -LGH-Fe complexes with concentrations from 10 to 60 μg of Fe/mL on Caco-2 cell monolayers following 24 h of treatment was evaluated by the MTT assay, as shown in Figure 2. The MTT assays on Caco-2 cells showed that supplementation of α -LAH-Fe complexes with concentrations from 10 to 60 μg of Fe/mL did not impair cell viability significantly ($P > 0.05$). The MTT assays of β -LGH-Fe complexes with different concentrations demonstrated some minor differences, whereas no statistical decrease was observed in the viability of cells treated with β -LGH-Fe complexes with concentrations less than 50 μg of Fe/mL ($P > 0.05$).

Iron Absorption of α -LAH-Fe and β -LGH-Fe Complexes

Figure 3 documents the iron absorption of FeSO_4 , FeSO_4 and Vc mixture, α -LAH-Fe complexes, β -LGH-Fe complexes, α -LAH and Fe mixture, and β -LGH and Fe mixture at the same iron concentration of 0.6 μg of Fe/mL. The results indicate that β -LGH-Fe complexes significantly improved iron absorption compared with

Figure 2. Caco-2 cell viability [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay] treated with different concentrations of α -LA hydrolysate (LAH)-Fe complexes and β -LG hydrolysate (LGH)-Fe complexes. The asterisk (*) indicates a significant difference compared with 0 μg of Fe/mL ($n = 4$; $P < 0.05$).

FeSO_4 , α -LAH-Fe complexes, β -LGH and iron mixture, and α -LAH and Fe mixture ($P < 0.05$). The iron absorption of both α -LAH-Fe and β -LGH-Fe complexes was significantly higher than that of the corresponding hydrolysate and iron mixtures ($P < 0.05$).

Ferritin Content in Caco-2 Cell Treated with α -LAH-Fe and β -LGH-Fe Complexes

Figure 4 shows the ferritin content in Caco-2 cell monolayers treated with α -LAH-Fe complexes, β -LGH-Fe complexes, α -LAH and iron mixture, β -LGH and iron mixture, α -LA and iron mixture, and β -LG and iron mixture. The ferritin content in cells exposed to digest containing FeSO_4 in the presence of Vc at a 20:1 molar ratio of Vc to iron was significantly higher than that of other digests ($P < 0.05$; Figure 4a). Both α -LAH and β -LGH bound iron significantly elicited greater ferritin content in Caco-2 cells than FeSO_4 , hydrolysate and iron mixtures, and protein and iron mixtures ($P < 0.05$; Figure 4b). Interestingly, no significant difference was observed in the ferritin content between digests of complexes, between mixtures of hydrolysate and iron, and between FeSO_4 and β -LG and iron mixture ($P > 0.05$).

Effect of Complex Concentrations on Iron Absorption

Figure 5 summarizes the effect of increasing the iron concentration of α -LAH-Fe and β -LGH-Fe complexes

Figure 3. Iron absorption by Caco-2 cells treated with α -LA hydrolysate (LAH)-Fe complexes, α -LAH and Fe mixture, β -LG hydrolysate (LGH)-Fe complexes, and β -LGH and Fe mixture. Different letters (A–D) indicate significant differences at $P < 0.05$. Vc = vitamin C.

from 0.6 to 3.0 μg of Fe/mL in the digest on iron absorption. No significant difference existed among different concentrations of α -LAH-Fe complexes ($P > 0.05$). When the addition concentration of β -LGH-Fe complexes was 0.6 μg of Fe/mL, iron absorption was higher ($P < 0.05$), whereas no significant difference was observed when the β -LGH-Fe concentration was 1.2, 1.8, 2.4, or 3.0 μg of Fe/mL ($P > 0.05$).

Figure 6 shows the iron absorption of β -LGH-Fe complexes with concentration less than 0.6 μg of Fe/mL. The iron absorption of β -LGH-Fe complexes increased with concentration enhancement from 0.24 to 0.6 μg of Fe/mL. No significant difference was observed in iron absorption when the β -LGH-Fe concentration was 0.24, 0.36, or 0.48 μg of Fe/mL ($P > 0.05$).

DISCUSSION

In this experiment, passages 40 to 50 were applied for the iron transport experiment. At d 21 of the period, the TEER value was high enough to symbolize the integrity of the Caco-2 cell monolayer and development of enterocyte morphology, which indicated that it could be applied to the in vitro absorption experiment.

Cytotoxicity to Caco-2 cells was determined to evaluate the dose-dependent toxic potential of α -LAH-Fe and β -LGH-Fe complexes. The cell viability assay performed on Caco-2 cells showed that a certain degree of decrease in viability existed in the cells incubated with higher concentrations of β -LGH-Fe complexes. This could be due to iron overloading resulting in oxidative damage to lipids, which is associated with the impairment of membrane-dependent functions of mitochondria

Figure 4. Ferritin content of Caco-2 cells treated with α -LA hydrolysate (LAH)-Fe complexes and β -LG hydrolysate (LGH)-Fe complexes. Different letters (A–D and a–d) indicate significant differences at $P < 0.05$. Vc = vitamin C.

and lysosomes (membrane integrity, fluidity, and pH) (Britton et al., 2002). The MTT assay indicated that α -LAH-Fe and β -LGH-Fe complexes with concentrations less than 50 μg of Fe/mL could be applied for further study.

Iron solubilization is a primary step before iron is absorbed in the intestine. The greater iron solubility in the small intestine indicates that more iron was available for absorption (Kim and Atallah, 1993). It has been reported that peptides can prevent the formation of insoluble iron complexes in the intestinal environment, which is important for bioavailability of food iron (Argyri et al., 2009). The present study demonstrated that α -LAH-Fe complex and β -LGH-Fe complex could improve iron absorption compared with the mixture of hydrolysate and iron (Figure 3). Peptides contained a higher content of negative charges than protein, with the breakdown of peptide bonds and the formation of

Figure 5. Iron absorption by Caco-2 cells treated with different concentrations of α -LA hydrolysate (LAH)-Fe complexes and β -LG hydrolysate (LGH)-Fe complexes. Different letters (A–D) indicate significant differences at $P < 0.05$.

Figure 6. Iron absorption by Caco-2 cells treated with different concentrations of β -LG hydrolysate (LGH)-Fe complexes which were lower than 0.60 μ g of Fe/mL. Different letters (A and B) indicate significant differences at $P < 0.05$.

carboxyl groups, which will effectively bind with divalent cations and form the soluble complexes (Vegarud et al., 2000). Hegsted et al. (1949) suggested that glutamic acid may increase the absorption or deposition of iron. α -Lactalbumin hydrolysate and β -LGH contain 11.85 and 12.66% glutamic acid and 7.66 and 5.95% aspartic acid, respectively. Although peptides and proteins can interact with iron via the peptide bond, as the glutamic acid and aspartic acid have more free carboxyl groups than other amino acids, they can form iron complexes through carboxylate bonding and enhance iron binding (Storcksdieck genannt Bonsmann and Hurrell, 2007). Furthermore, the Fourier-transform infrared spectra in our previous study also confirmed the transformation of carboxylate groups and the formation of C–O–Fe bonds in peptide-iron complexes (Zhou et al., 2012).

Iron is solubilized in the acidic pH of the stomach; it then becomes insoluble again and, thus, unabsorbable in the alkaline pH of the small intestine. Peptide-iron complexes are water soluble and biocompatible with the functional groups present in the peptides (Papst et al., 2013). Iron binding to peptides is also resistant to the changes in pH during digestion (Ait-Oukhatar et al., 2000). After comparing the iron absorption of the peptide and Fe mixture and peptide-Fe complexes, it is clear that the absorption of complexes was significantly higher than that of the mixtures, which indicates that β -LGH-bound iron possesses great potential to be applied as an iron-fortified supplement.

Iron is taken up by intestinal epithelial cells and then it reaches the body. Its absorption is regulated according to the iron status of storage proteins in intestinal

epithelia cells. Iron is more readily absorbed in the ferrous state (Fe^{2+}), but most of the dietary iron is in the ferric form (Fe^{3+}), which must be reduced to the ferrous state before absorption. Ascorbic acid as reducing substance can facilitate conversion of ferric to ferrous iron and promote iron absorption. Iron absorption from the small intestine is regulated according to the body's needs, which increases in iron-deficient and decreases in iron-replete conditions (Morgan and Oates, 2002). This might be the reason why the 0.6 μ g of Fe/mL β -LGH-Fe complexes had a significantly higher absorption rate than the concentrations of 1.2, 1.8, 2.4, and 3.0 μ g of Fe/mL, and the iron absorption increased with higher concentrations from 0.24 to 0.6 μ g of Fe/mL. Jin et al. (2009) used ferritin content to mark iron uptake by Caco-2 cells and found that ascorbic acid could promote the iron absorption and ferritin content significantly, which was in agreement with the present study. The ferritin molecule is the main iron storage molecule in all mammalian tissues, which is a hollow protein shell that can store potentially harmful and reactive iron (Aisen et al., 2001; Mackenzie et al., 2008). Once iron enters the mucosal cell, it will either encounter a ferritin molecule, which incorporates iron as Fe, oxidizing it to Fe (III) within the protein shell, or be transported to the basolateral membrane (Crichton et al., 2002). The accessible and non-toxic iron stored in ferritin is available for utilization by other functional proteins. In addition, the induction of ferritin synthesis is regulated by iron concentrations (White and Munro, 1988). Ferritin content has been used for predicting iron availability in *in vitro* digestion and iron uptake

in Caco-2 cell monolayers (Glahn et al., 1998; Puyfoulhoux et al., 2001; Tako et al., 2013). In iron deficiency, most iron entering the cell will transport across the cell to the basolateral membrane and mucosal cells and hardly produce any ferritin. In the normal situation, many ferritin molecules are available and much of the iron will be trapped in ferritin and retained in a low mucosal transfer of iron (Crichton et al., 2002). The ferritin formed by Caco-2 cells in the present study also indicates that it is possible to apply β -LGH-Fe complexes as iron-fortified supplements and a novel nutraceutical with high absorption of iron and induction of ferritin synthesis. The obtained peptides provide more flexibility in size and shape of molecules and more free carboxyls for iron chelation than native protein. The mechanism by which iron is donated to ferritin for storage remains elusive. More work is required to clarify the involvement of β -LGH-Fe complexes in iron absorption. The effect of ascorbic acid on the absorbability of iron bound to whey protein hydrolysate still awaits further investigation.

CONCLUSIONS

The present study shows that β -LGH-Fe complexes and α -LAH-Fe complexes can promote iron absorption and ferritin (iron storage protein) content in the in vitro Caco-2 model. The complexes with concentrations less than 50 μ g of Fe/mL were detected in Caco-2 cells and did not impair cell viability. It can be inferred that β -LGH-Fe complexes possess the potential to be applied as iron-fortified supplements with high iron absorbability, but the mechanism of this finding needs to be explored further.

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