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Citric Acid Mediates the Iron Absorption from Low Molecular Weight Human Milk Fractions

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ABSTRACT: Previously, we have demonstrated increased iron absorption from low molecular weight (LMW) human milk whey fractions. In the present study, we investigated the effect of heat denaturation, zinc (a competitor of iron), duodenal cytochrome *b* (DcytB) antibody neutralization and citrate lyase treatment on LMW human milk fraction (>5 kDa referred as 5kF) induced ferric iron reduction, solubilization, and uptake in Caco-2 cells. Heat denaturation and zinc inhibited the 5kF fraction induced ferric iron reduction. In contrast, zinc but not heat denaturation abrogated the ferric iron solubilization activity. Despite inhibition of ferric iron reduction, iron uptake in Caco-2 cells was similar from both native and heat denatured 5kF fractions. However, iron uptake was higher from native compared to heat denatured 5kF fractions in the cells preincubated with the DcytB antibody. Citrate lyase treatment inhibited the ferric iron reduction, solubilization, and uptake in Caco-2 cells. These findings demonstrate that citric acid present in human milk solubilizes the ferric iron which could be reduced by other heat labile components leading to increased uptake in intestinal cells.

KEYWORDS: *bioavailability, Caco-2 cells, citric acid, DcytB, human milk, iron*

INTRODUCTION

Growth spurts and increases in body volume during infancy demand adequate nutrient supplies and thus makes infants susceptible to nutritional deficiencies particularly iron deficiency anemia. The prevalence of iron deficiency anemia was reported to be lower in exclusively breast fed infants compared to that in the formula fed infants during early age,[1,2] despite the low iron content in breast milk (0.2.−0.4 mg/L). Fractional absorption of extrinsically added iron and its incorporation into hemoglobin is higher in breast milk fed children compared to that in cow's milk or formula fed children.[3−7] Similarly, the absorption of extrinsically added iron from breast milk is also higher in fasted adults compared to that from cow's milk,[1] suggesting the presence of iron absorption enhancing factors in human milk. However, stable isotopic studies demonstrated much lower fractional absorption of iron from human milk compared to that in early studies, but it was still higher than that in cow's milk.[3,7,8]

Subsequent in vitro studies demonstrated that low molecular weight (LMW) human milk whey fractions can efficiently solubilize and enhance the transport of iron across polarized Caco-2 cells.[9,10] Further, iron in breast milk is predominantly distributed in LMW fractions which could be bound to peptides, amino acids, or other components.[11] Similar to that of iron, zinc absorption was also reported to be high from human milk,[12] wherein citric acid or picolinic acid were identified as the potential zinc binding ligands.[13,14] We have previously demonstrated the reduction, solubilization, and enhanced uptake of ferric iron in Caco-2 cells in the presence of <10 kDa human milk whey fractions.[15] On gel filtration chromatography, these activities were eluted at 1000−1500 Da,[15] which is close to the exclusion limit of the column. Therefore, it is likely that these activities are mediated by a single or multiple small molecular weight ligands.

Reduction of ferric iron is a prerequisite for iron absorption in enterocytes.[16,17] Duodenal cytochrome *b* (DcytB) is known to function as ferric reductase on the mucosal surface facilitating iron absorption through divalent metal ion transporter-1 (DMT1).[18,19] Further, inhibiting the expression of DcytB or the presence of neutralizing antibody was reported to inhibit the intestinal absorption of iron.[19−21] In the absence of information on the ontogenic regulation of DcytB, we speculated that reduction of the ferric iron by the LMW human milk fraction might complement DcytB expression during the neonatal period.[15]

In the present study, we demonstrate that the citric acid present in the LMW human milk fractions mediates ferric iron solubilization and aids in its intestinal uptake.

MATERIALS AND METHODS

Materials. All of the chemicals, reagents, and enzymes were obtained from Sigma Chemical Co. (Bangalore, India) unless otherwise mentioned. $^{59}FeCl_3$ (carrier free) was obtained from Board of Radiation and Isotope Technology (BRIT), Mumbai, India.

Human Milk Fractionation. Aliquots of surplus human milk were collected during the first 3 to 6 days of lactation after obtaining consent from all of the donors, pooled, and immediately cooled on ice. Collection of human milk samples in this manner was approved by the Committee on the Use of Human Subjects in Research (BS01/27/11/07) of Gandhi Hospital, Secunderabad. Aliquots of pooled human milk (50 mL) were fractionated immediately as described,[15] except that 5

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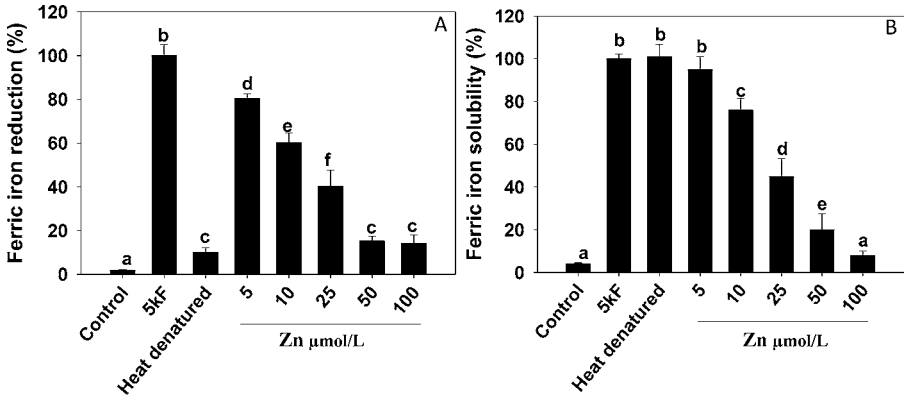

Figure 1. Effect of heat denaturation and zinc on low molecular weight human milk fraction induced ferric iron reduction and solubility. Ferric iron reduction (A) and solubility (B) were assayed in native and heat denatured 5kF fractions in the presence or absence of 0−100 μmol/L Zn as described in Materials and Methods. The % reduction and solubility were computed by considering the activity in native 5kF fractions as 100%. The bars indicate the mean + SD, and bars with different superscripts are significantly different ($P < 0.05$).

kDa MW-cutoff filters were used for the isolation of LMW fractions instead of 10 kDa cutoff filters. The protein concentration in various milk fractions was assessed by a micro BCA kit method (Sigma Chemical Co.) using BSA as a standard.

Iron and Zinc Estimation. Aliquots (10 mL) of pooled human milk samples ($n = 4$, collected on different days) and their respective 5 kDa filtrate (5kF) fractions (10 mL) were lyophilized to dryness and subjected to microwave digestion (Mars Xpress) in H_2O_2 and ultrapure nitric acid (1:2 ratio). The mineral content in the digest was estimated by atomic absorption spectrometry (Shimadzu AA-7000, Japan).

Heat Denaturation and Zinc Treatment. The 5kF fraction was incubated in a boiling water bath for 30 min and cooled on ice and tested immediately for ferric iron reduction, solubilization activities, and iron uptake in Caco-2 cells in the presence and absence of zinc as described below.

Ferric Reductase Assay. Ferric iron reduction was measured as described previously.[15] Briefly, the reaction mixture contained 100 μmol/L ferric iron ($FeCl_3$), ferrozine (500 μmol/L, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p*′-disulfonic acid) in 50 mmol/L 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer at pH 6.5 and was incubated in the presence and absence of 100 μL of the 5kF fraction (before and after heat denaturation) and/or 0−100 μmol/L $ZnSO_4$. The reaction was carried out at 25 °C for 10 min in a microplate based assay, while continuously monitoring absorbance at 562 nm using microplate reader (BioTek, Model# Powerwave HT-1). The percent (%) ferric iron reduction was calculated by assuming the total activity in 5kF fractions as 100%.

Iron Solubilization. Radio labeled ferric iron (^{59}Fe) solubilization was measured as described previously.[9,15] Aliquots of 100 μL aliquots of the 5kF fraction (either native or heat denatured) were diluted with 50 mmol/L MES buffer at pH 6.5 to 1 mL and supplemented with 100 μmol/L $FeCl_3$ (traced with 50 nCi $^{59}FeCl_3$) in the absence and presence of 0−100 μmol/L $ZnSO_4$ (at the highest Zn concentration, the molar ratio of iron to zinc is 1), and incubated for 30 min at 37 °C. At the end of incubation, the samples were centrifuged at 15,000*g* at 4 °C for 15 min, and the supernatant solution (800 μL) was mixed with 5 mL of Brays mixture and counted in a liquid scintillation counter (Perkin-Elmer Model# TRICARB 2900TR). The percent (%) solubilization of iron was calculated by assuming the solubility of iron in the presence of the 5kF fraction as 100%.

Caco-2 Cell Iron Uptake. Caco-2 cells were grown in 6-well culture plates and used for the experiments between 13 and 14 days postconfluence as described previously.[15,22] Ferric chloride (10 mmol/L stock in 10 mmol/L HCl traced with 50 nCi $^{59}FeCl_3$) was diluted to a final concentration of 100 μmol/L with MES buffer (50 mmol/L, pH 6.5) in the absence (blank) or presence of 100 μL of 5kF and/or $ZnSO_4$ (100 μmol/L), then fed to the differentiated Caco-2 cells for a period of 2 h. The zinc concentration was chosen based on our previous observation that zinc inhibits iron absorption at 1:1 molar ratio.[22] After incubation, the monolayers were washed with ice-cold phosphate buffered saline containing 10 mmol/L bathophenanthroline (to remove nonspecifically bound iron), harvested by scraping, and the ^{59}Fe radioactivity counted in a liquid scintillation counter. The % uptake was calculated assuming the uptake in control cells (only $FeCl_3$) as 100%.

DcytB Neutralization. DcytB neutralization studies were done as described previously.[21] Differentiated Caco-2 cells grown on 6-well plates were treated with 0.5 mL of minimum essential medium (MEM) in the absence or presence of the DcytB antibody (1:50 dilution, ADI# DCYTB11-A, Texas, USA) for 60 min. At the end of incubation, 1.5 mL of fresh minimum essential media (consisting of 100 μmol/L $FeCl_3$, traced with 50 nCi $^{59}FeCl_3$) with or without 100 μL of 5kF milk fractions were added to the respective wells. The cells were further incubated for 2 h. The cell associated ^{59}Fe radioactivity was assessed as described above. The % uptake was calculated assuming the uptake in control cells (only $FeCl_3$) as 100%.

Citrate Lyase/Oxaloacetate Decarboxylase Treatment of the 5kF Fraction. Citrate lyase (*Klebsiella pneumoniae*, E.C. No. 232-740-7, Sigma#C0897)/oxaloacetate decarboxylaase (*Pseudomonas* sp. E.C. No. 4-1-1-3, Sigma#O4878) digestion was carried out as described previously with modifications.[23] The 5kF fraction was diluted 1:1 (v/v) with 20 mM HEPES buffer at pH 7.8, followed by the addition of 2 units each of citrate lyase and oxaloacetate decarboxylase (predissolved in 10 mmol/L HEPES buffer at pH 7.8). The samples were incubated for 28 h at room temperature. At the end of incubation, the ferric iron reduction, solubility, and Caco-2 cell iron uptake were assessed as described above with the citrate lyase digested 5kF fraction and 100 μmol/L of standard citric acid.

Estimation of Citric Acid. The citric acid content in 5kF fractions before and after treatment with citrate lyase/oxaloacetate decarboxylase treatment was estimated by the pyridine−acetic anhydride method.[24]

Gel Filtration and RP-HPLC. Gel filtration chromatography was performed on a TSK-2000 SW (Altex, CA, USA) column connected to HPLC (Agilent, Model: 1100, Pala Alto, CA, USA). Briefly, 100 μL of the 5kF fraction or standard citric acid (12.5 μg) was subjected to size fractionation using 0.9% NaCl mobile phase, at a flow rate of 1 mL/min, and elution was monitored at 220 nm. Similarly, RP-HPLC of 5kF fractions before and after treatment with citrate lyase/oxaloacetate decarboxylase, standard citric acid (12.5 μg), and pyruvic acid (12.5 μg) was performed on a C-18 column (Thermo-Hypersil ODS, 5 μm, 250 × 4.6 mm) equilibrated and eluted with 2% acetonitrile (0.1% (v/v) triflouroacetic acid) at 1 mL/min flow rate while monitoring optical density at 220 nm.

Statistics. All of the experiments were performed in triplicate and repeated at least once to generate 6 observations. The mean and SD were calculated using Microsoft Excel, and the data were analyzed

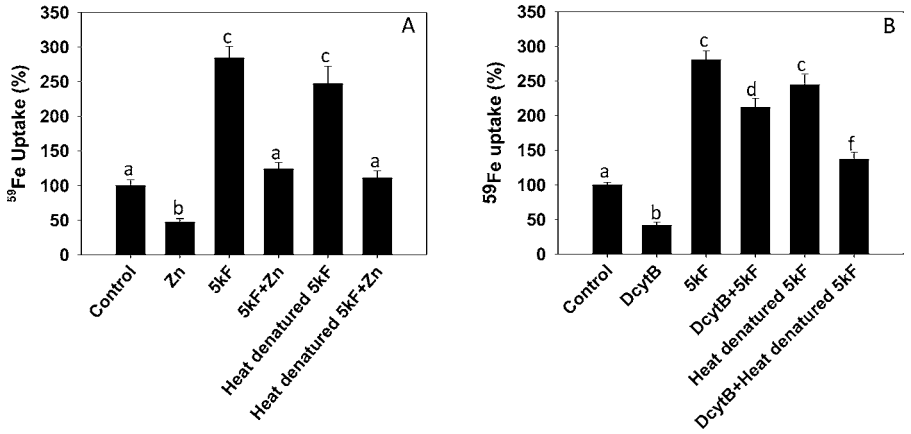

Figure 2. Effect of heat denaturation and zinc on LMW human milk fraction induced iron uptake in Caco-2 cells. (A) Differentiated Caco-2 cells were incubated with 100 μmol/L $FeCl_3$ in the absence (control) or presence of 100 μL of 5kF milk fractions and/or zinc (100 μmol/L) for 2 h. (B) ^{59}Fe uptake studies were done in differentiated Caco-2 cells either in the presence or in the absence of DcytB antibodies. At the end of incubation, the cell associated ^{59}Fe radioactivity was counted as described in Materials and Methods. The percent uptake was calculated by assuming the uptake in the control (only $FeCl_3$) as 100%. The bars indicate the mean + SD of 6 observations, and the bars with different superscripts differ significantly ($p < 0.05$).

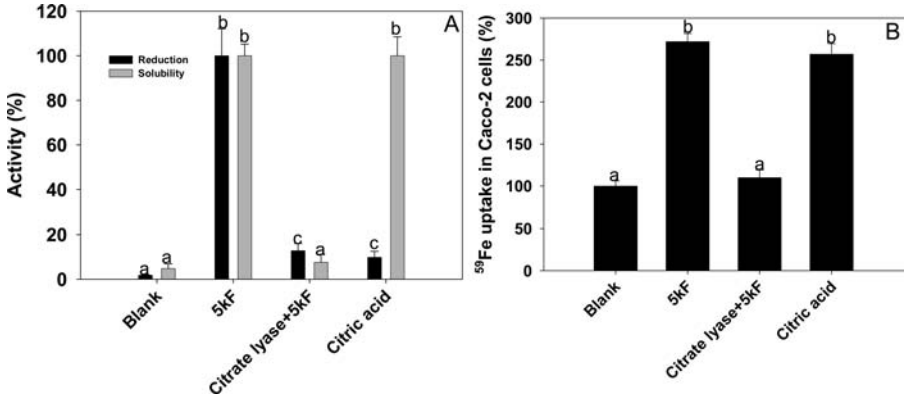

Figure 3. Effect of citrate lyase/oxaloacetate decarboxylase digestion on low molecular weight human milk fraction induced ferric iron reduction, solubilization, and uptake in Caco-2 cells. The 5kF milk fraction was diluted to 1:1 (v/v) with 20 mmol/L HEPES buffer at pH 7.8, followed by the addition of 2 units each of citrate lyase and oxaloacetate decarboxylase reconstituted in 10 mmol/L HEPES at pH 7.8. The solutions were incubated at room temperature for a period of 28 h. At the end of incubation, ferric iron reduction, solubilization (A), and Caco-2 cell uptake (B) were measured as described in Materials and Methods. The % reduction and solubility was computed by assuming the activity in the untreated 5kF fraction as 100%. The % uptake of ferric iron was computed by assuming the uptake in the control (only $FeCl_3$) as 100%. The bars indicate the mean + SD of 6 observations, and the bars with different superscripts differ significantly ($p < 0.05$).

using one-way ANOVA followed by the least significant differences (LSD) test, using an SPSS package (version 2007). The results were considered significant if the $p < 0.05$.

RESULTS

Isolation and Characterization of Low Molecular Weight (LMW) Human Milk Fractions. The protein content in the 5kF fraction (filtrate obtained by ultrafiltration of human milk whey through a 5 kDa cutoff membrane) was 1.12 mg/mL, while it was 1.9 mg/mL in a 10kF fraction. The specific activity of ferric iron reduction and solubilization was higher with the 5kF (~89%/mg protein) fraction compared to that in the 10kF fraction (~53%/mg protein). Therefore, the 5kF fraction was used for all of the subsequent studies. The 5kF human milk whey fraction contained 42% iron (9.24 ± 2.1 μg/100 mL) and 17% zinc (40.1 ± 4.3 μg/100 mL) in the whole human milk (28 ± 11 μg/100 mL iron and 241 ± 18 μg/100 mL zinc, respectively).

Effect of Heat Denaturation and Zinc on LMW Human Milk Fraction Induced Ferric Iron Reduction and Solubilization. 5kF fractions increased the reduction (100% ± 5.2) and solubilization (100% ± 2.1) of ferric iron compared to those in the control, $FeCl_3$ (1.8% and 4.2%, respectively; Figure 1). Heat denaturation inhibited the 5kF induced ferric iron reduction but not iron solubilization activity. In contrast, addition of 5−100 μmol/L zinc dose dependently inhibited the 5kF induced ferric iron reduction and solubilization (Figure 1). Similarly, zinc also inhibited the ferric iron solubilization induced by heat denatured 5kF fractions (data not shown).

Effect of Heat Denaturation, Zinc, and DcytB Neutralizing Antibody on LMW Human Milk Fraction Induced Ferric Iron Uptake in Caco-2 Cells. Native (270 ± 21%) and heat denatured 5kF fractions (254% ± 28) increased the ferric iron uptake to the same extent in Caco-2 cells compared to that in the control (100% ± 10.5), as assessed by ^{59}Fe uptake (Figure 2A). Addition of 100 μmol/L zinc (1:1 ratio of iron to zinc) significantly inhibited the ^{59}Fe uptake in Caco-2 cells from the control, native, and heat denatured 5kF fractions (Figure 2A). Pretreatment of Caco-2 cells with DcytB

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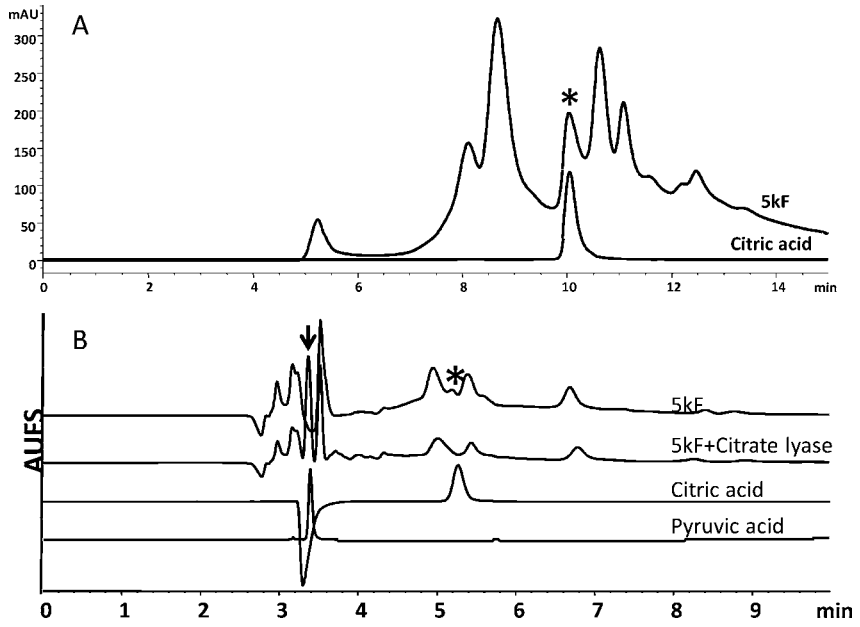

Figure 4. Gel filtration and RP-HPLC of the 5kF fraction. (A) The 5kF milk fraction (100 μL) or citric acid (12.5 μg) was fractionated on a TSK-2000 column at a flow rate of 1 mL/min (B). The 5kF fraction before and after digestion with citrate lyase/oxaloacetate decarboxylase, citric acid (12.5 μg), and pyruvic acid (12.5 μg) was fractionated on a reverse phase column (c-18) with isocratic elution with 2% acetonitrile at a flow rate of 1 mL/min. The elution was monitored at 220 nm. The peak showing iron solubilization activity is marked with "*". The pyruvic acid peak in the 5kF fraction digested with citrate lyase was marked with "↓". The digestive enzymes and BSA did not elute from the column during isocratic elution with 2% acetonitrile and thus were not shown.

neutralizing antibody reduced the ^{59}Fe iron uptake in the control (42% ± 4.4), native (212% ± 12.6), and heat denatured 5kF fraction (137% ± 9.6) compared to that in the respective controls with out an antibody (Figure 2B). However, the extent of inhibition in ^{59}Fe iron uptake due to DcytB neutralization was lower in the native 5kF fraction (21%) compared to that in the heat denatured 5kF fraction (44%).

Effect of Citrate Lyase/Oxaloacetate Decarboxylase Digestion of LMW Human Milk Fraction and Citric Acid on Ferric Iron Reduction, Solubilization, and Uptake in Caco-2 Cells. Citric acid content of the 5kF fraction was 242 μg/mL but was reduced to undetectable levels after enzymatic digestion. Citrate lyase/oxaloacetate decarboxylase treatment significantly inhibited the 5kF fraction induced ferric iron reduction, solubilization, and ^{59}Fe iron uptake in Caco-2 cells (Figure 3). However, standard citric acid (1:1 molar ratio of iron to citric acid) significantly increased the iron solubility and uptake in Caco-2 cells without reducing the ferric iron (Figure 3).

Gel Filtration and RP- HPLC of 5kF Fractions. The iron solubilization activity of the 5kF fraction and citric acid coeluted during gel filtration (Figure 4A) or RP-HPLC (Figure 4B) in a single peak at 10 and 5.2 min, respectively. RP-HPLC of citrate lyase/oxaloacetate decarboxylase treated 5kF fraction led to the disappearance of the citric acid peak (eluting at 5.2 min), while a new peak with retention similar to that of standard pyruvic acid (3.2 min) was observed (Figure 4B).

DISCUSSION

Bioavailability of iron from human milk was reported to be high, but the associated mechanisms are not clear. In this study, we demonstrate that a heat stable iron chelator and a heat labile ferric iron reducing component present in the LMW human milk fraction synergistically act to reduce and solubilize the ferric iron. We also demonstrate that citrate lyase/oxaloacetate decarboxylase digestion of the LMW human milk fraction decreases the citric acid content and inhibits the ferric iron reduction, solubilization, and uptake in intestinal cells. These results suggest that citric acid mediates the LMW human milk fraction induced iron uptake in intestinal cells.

We and others have previously demonstrated the reduction and solubilization of ferric iron with <10 kDa human milk fractions, and both of these activities are coeluted between 1500 Da and 1000 Da on the gel filtration column.[9,10,15] Therefore, we replaced the 10 kDa membrane with the 5 kDa membrane and found that the ferric iron reducing and solubilization activity/mg protein was higher in the 5kF compared to that in the 10kF. These results suggest that the ferric iron reducing activity of human milk fractions is indeed resident in the <5 kDa human milk fractions. The endogenous iron and zinc content in human milk and their distribution in the LMW fraction obtained in the present study were similar to the published results.[9,11]

The poor solubility of ferric iron limits its intestinal absorption.[25] The solubility of ferrous iron is higher compared to that of ferric iron at near neutral pH, but the solubility of ferric iron could be improved by specific chelators such as EDTA, citric acid, etc.[25] Higher solubility of ferric iron in LMW human milk fractions therefore could be due to the reduction and/or chelation of ferric iron mediated by single or multiple factors. Heat denaturation specifically inhibited the 5kF induced ferric iron reduction but not the ferric iron solubilization activity, implying the involvement of different factors in iron reduction and solubilization. In contrast to the heat denaturation, zinc dose dependently inhibited both the reduction and solubilization activities of the 5kF fraction. Since zinc is a known inhibitor of ferric reductases and a potential competitor for iron in physiological systems,[26,26−28] it might inhibit the reduction of iron or displace the bound iron from

specific components of 5kF fractions. Interestingly, zinc also inhibited the iron solubilization activity of the heat denatured 5kF fraction (with out ferric iron reducing activity), implying the presence of a specific iron chelator in LMW human milk fractions. The fact that heat denaturation abrogated only the reduction of iron while zinc abrogated both of the activities suggests that a heat labile electron donor (reducing component) and a heat stable iron binding component (to which zinc competes) act together to reduce and solubilize the ferric iron in 5kF human milk fractions.

It is well established that the reduction of ferric iron is a prerequisite for iron absorption at the enterocytes.[16,17] However, iron uptake from heat denatured 5kF, without ferric iron reducing activity, was similar to that of native 5kF fractions possessing ferric iron reducing activity. DcytB at the apical surface of the intestinal cells reduces the ferric iron and thereby facilitates its absorption via DMT1.[19−21] It is therefore likely that the higher solubility of ferric iron in heat denatured 5kF fractions might facilitate its reduction by DcytB and thus masking the differences, if any, in iron uptake between native and heat denatured 5kF fractions. In agreement with these observations, the solubility of iron salt is positively associated with its absorption at the enterocyte regardless of the oxidation state.[25,28] For instance, iron absorption from a soluble ferric iron compound (NaFe (III) EDTA) is similar to that of ferrous iron salt, and its absorption is also mediated by DcytB.[28] In agreement with this notion, neutralization of DcytB in Caco-2 cells led to greater inhibition of iron uptake from heat denatured 5kF fractions compared to that in native 5kF fractions. Because of the fact that the solubility of iron in native and heat denatured 5kF fractions is similar, the observed higher iron uptake in DcytB neutralized Caco-2 cells could be explained by the endogenous reduction of ferric iron in this fraction, which is complementary to intestinal DcytB. Additionally, higher iron uptake from heat denatured 5kF fractions compared to that in the control (only $FeCl_3$) could be explained by the higher solubility of ferric iron in this fraction, which increases the accessibility of ferric iron to DcytB (remaining after neutralization) for reduction and subsequent uptake by Caco-2 cells. This is further supported by the fact that Zn (which inhibited both reduction and solubilization of ferric iron) inhibited the uptake of iron from both native and heat denatured 5kF fractions. In addition, zinc also inhibited iron uptake compared to that in the control in the absence of 5kF fractions, consistent with its negative interaction on iron absorption reported in Caco-2 cells and in humans.[22,27]

Lonnerdal et al.,[13] demonstrated that zinc and copper binding ligands in the human milk fraction could be citrate, an endogenous component of milk. Similar to that of iron, the absorption of zinc was also reported to be high from human milk.[12] Since zinc inhibits 5kF induced iron reduction and solubilization, it is possible that the iron binding milk factor could also be citrate. Furthermore, the retention time of authentic citric acid is quite similar to that of the iron solubilization factor during gel filtration or RP-HPLC (Figure 4). These observations strongly suggest that the iron solubilization factor isolated from human milk might be citric acid. However, others reported that the metal chelating ligand in milk could also be picolinic acid,[14,29] necessitating the need for further characterization of the molecular identity of the citrate in 5kF fractions and its role in iron solubilization.

Treatment of 5kF fractions with citrate lyase/oxaloacetate decarboxylase reduced the citric acid content and inhibited ferric iron reduction, solubilization, and uptake in Caco-2 cells (Figure 3), implying that citric acid present in 5kF fraction mediates the LMW human milk fraction induced iron uptake in Caco-2 cells. In agreement with these observations, standard citric acid also increased the solubility and uptake of iron without reduction (Figure 3). Furthermore, the citric acid peak disappeared, and the pyruvic acid peak was observed in the RP-HPLC chromatogram of citrate lyase/oxaloacetate decarboxylase digested 5kF fraction (Figure 4), further confirming the identity of citric acid. The above results demonstrate that citric acid present in the LMW human milk fractions chelates and thus solubilizes the ferric iron, which may stimulate uptake in Caco-2 cells. In agreement with these results, citric acid was reported to enhance the ferric iron absorption in rat models and Caco-2 cells.[30−32] In addition, Glahn et al.[23] reported that citric acid enhances the iron absorption maximally when the relative concentrations of citric acid to iron are closer (2:1) but decreases the same when the citrate content exceeds that of iron (20:1). Further, citrate lyase digestion of infant formula also led to improved iron bioavailability, implying that excess citrate might inhibit iron absorption. Interestingly, such opposite effects of citric acid on iron absorption were also reported in human subjects.[33,34] Therefore, apart from the differences in experimental models and food matrix, the relative concentrations of citrate to iron appear to influence both the magnitude and direction of its effect on iron absorption. In the current study, the citric acid content in the 5kF fraction was 1.2 mmol/L, and the uptake study was carried out with nearly equimolar concentrations of iron to citric acid (1:1.2), explainingthe higher iron uptake in Caco-2 cells compared to that in ferric iron salt.

The above results demonstrate that citric acid mediates iron solubilization from LMW human milk fractions. Nevertheless, the role of iron reducing factor (heat labile) in the 5kF fraction and its role in enhancing iron uptake is perplexing. Unlike that of gel filtration chromatography wherein ferric iron solubilization and reduction activities are coeluted,[15] the ion-exchange chromatography of 5kF fraction led to the elution of iron solubilization activity in a single peak without ferric iron reducing activity (data not shown). It is possible that heat labile reducing components such as ascorbic acid, cysteine, or others present in the LMW human milk might reduce the citrate−iron complexes.[23,35] It has been reported that the uptake of iron from iron-citrate is further enhanced by ascorbic acid.[36] It is also possible that citric acid might improve the half-life of ferrous iron in solution by preventing its oxidation[37] and thus increasing its absorption. Moreover, we have previously demonstrated that the human milk 5kF fraction reduces the ferric iron from a variety of iron salts including ferric citrate.[15]

It is also noteworthy that the citric acid content of cow's milk is 10-fold higher than that in human milk,[23] and yet, the bioavailability of iron from human milk is high. Therefore, citric acid alone might not completely explain the higher bioavailability of iron from human milk compared to that in cow's milk. It has been demonstrated that iron (either endogenous or added) is uniquely distributed in whey fraction in human milk compared to that in cow's milk.[11,30] It is likely that the high amount of phosphorylated-casein (which chelates iron) and low content of iron absorption enhancers such as ascorbic acid and other organic acids in cow's milk[30,38] might account for the relatively low bioavailability of iron. In fact, the bioavailability of iron from a low casein diet is higher in rat pups.[30]

Together, these results demonstrate that citric acid present in the LMW human milk fraction solubilizes the ferric iron at neutral pH, while other heat labile components present in human milk reduce the ferric iron (complementary to intestinal DcytB) leading to enhanced intestinal cell uptake. Further, these studies also highlight the role of DcytB during iron absorption from soluble ferric iron chelates.

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Notes

The authors declare no competing financial interest.

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