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Tissue Iron Distribution and Urinary Mineral Excretion Vary Depending on the Form of Iron ($FeSO_4$ or NaFeEDTA) and the Route of Administration (Oral or Subcutaneous) in Rats Given High Doses of Iron

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Sodium iron ethylenediaminetetraacetate (NaFeEDTA) has considerable promise as an iron fortificant in food. However, effects of administering high levels of NaFeEDTA on tissue iron distribution and mineral excretion are not well understood. The objectives of this study were to assess nonheme iron distribution in the body and urinary excretion of Ca, Mg, Cu, Fe, and Zn after daily administration of high levels of iron to rats over 21 days. Iron was either given orally with food or injected subcutaneously, as either $FeSO_4$ or NaFeEDTA. Selected tissues were collected for nonheme iron analysis. Estimated total body nonheme iron levels were similar in rats fed NaFeEDTA or $FeSO_4$, but the tissue distribution was different: it was 53% lower in the liver and 86% higher in the kidneys among rats fed NaFeEDTA than among those fed $FeSO_4$. In contrast, body nonheme iron was 3.2-fold higher in rats injected with $FeSO_4$ than in rats injected with NaFeEDTA. Administering NaFeEDTA orally elevated urinary Cu, Fe, and Zn excretion compared with $FeSO_4$ (1.41-, 11.9-, and 13.9-fold higher, respectively). We conclude that iron is dissociated from the EDTA complex prior to or during intestinal absorption. A portion of intact FeEDTA may be absorbed via a paracellular route at high levels of intake but is mostly excreted in the urine. Metal-free EDTA may be absorbed and cause elevated urinary excretion of Fe, Cu, and Zn.

KEYWORDS: NaFeEDTA; iron distribution; mineral excretion; DMT-1; rat

INTRODUCTION

Iron deficiency (ID) is one of the most prevalent micronutrient deficiencies in the world, affecting one-third of the population globally (*1*). One intervention strategy for preventing ID is iron fortification. Plant-based foods that are the primary dietary staples in developing countries, or condiments that are regularly consumed, are frequently chosen as vehicles. Various forms of iron ranging from iron salts to iron chelates to elemental iron powders have been approved as iron sources for food fortification (*2*). In the past several decades, there has been renewed interest in using sodium iron(III) ethylenediaminetetraacetate (NaFeEDTA) for food fortification because of its high stability in long shelf-life foods, good solubility in low to near neutral pH aqueous environments, and superior iron bioavailability in foods containing iron absorption inhibitors compared with other fortificants such as ferrous sulfate ($FeSO_4$) (*3, 4*). NaFeEDTA-fortified condiments have been demonstrated to be efficacious in combating iron deficiency anemia (IDA) in humans. In China, IDA was reduced among adults and children 3 years or older when consuming NaFeEDTA-fortified soy sauce (29.6 mg of Fe/100 mL) for 18 months (*5*); 11–17-year-old anemic children benefited from daily intakes of 5 mg of Fe provided by NaFeEDTA-fortified soy sauce for 3 months (*6*). NaFeEDTA-fortified fish sauce (*7–9*), curry powder (*10*), and sugar (*11*) have all shown positive effects on iron status. A recent study in Kenya found that school children consuming NaFeEDTA-fortified maize porridge had greatly improved iron status compared with those consuming unfortified or elemental iron-fortified porridge (*12*). However, the authors also cautioned about consuming doses above the acceptable daily intake. Indeed, the high bioavailability of NaFeEDTA often raises concerns over possible iron overload, especially in iron adequate populations consuming it over a long period of time. In addition, mechanisms involved in the absorption, tissue distribution, and excretion of NaFeEDTA are not fully understood, hence the concern that the absorbed EDTA might chelate mineral ions in addition to iron, causing excessive excretion of other essential minerals such as calcium and zinc.

Findings from our previous study (*13*) indicated that iron may be mobilized by EDTA from its usual storage site, namely, the liver, and redistributed to other tissues such as the kidneys. However, we were unable to discern significantly higher iron content in the kidneys in rats fed 35 mg of Fe (as NaFeEDTA)/

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Table 1. Study Design: Forms and Concentrations of Iron Added to the Rat Diets

	group			
	I	II	III	IV
acclimation period (days −6 to 0)	35 mg of Fe (as $FeSO_4$)/kg of feed			
route of administration	subcutaneous (SC), iron delivered by daily injection		oral (OR), iron added in ID basal diet[a]	
loading period (days 1–21)[b]	ID basal diet, injected with $FeSO_4$ solution	ID basal diet, injected with NaFeEDTA	1200 mg of Fe (as $FeSO_4$)/kg of feed	1200 mg of Fe (as NaFeEDTA)/kg of feed
killing (day 22)	blood drawing and tissue harvesting[c]			
washout period (days 22–24)	35 mg of Fe (as $FeSO_4$)/kg of feed	35 mg of Fe (as NaFeEDTA)/kg of feed	35 mg of Fe (as $FeSO_4$)/kg of feed	35 mg of Fe (as NaFeEDTA)/kg of feed
killing (day 25)	blood drawing and tissue harvesting			

[a] ID basal diet: semipurified iron-deficient AIN-93G diets which contained 2 mg of Fe/kg of diet. [b] An estimated 0.9–1.7 mg of Fe per day as either $FeSO_4$ or NaFeEDTA was injected SC to rats in groups I and II on the basis of the amount of feed consumed by each rat. [c] Four out of eight of the rats from each group were sacrificed.

kg of diet within the 12-day experimental period, compared with those fed a diet containing the same amount of iron as $FeSO_4$. Therefore, the main objective of this study was to assess tissue distribution of nonheme iron in rats administered high levels of iron, in the form of either $FeSO_4$ or NaFeEDTA for 21 days. In addition, we compared the urinary excretion of Ca, Mg, Cu, Fe, and Zn in these rats. The two routes of administration, oral (OR) or subcutaneous (SC), provided us an opportunitiy to study the effect of the digestive system on the availability of iron. We provide evidence that, by increasing the administrated iron level as well as the duration of the treatment, we could make inferences on the following questions: (1) Is the iron from NaFeEDTA and that from $FeSO_4$ distributed in the body similarly? (2) Does the route of administration affect iron utilization from these two iron sources? (3) Is the body mineral profile altered due to high levels of NaFeEDTA consumption?

MATERIALS AND METHODS

Chemicals. All chemicals were obtained from Sigma Chemicals (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ) unless stated otherwise. Water used in the preparation of reagents was 18 MΩ ultrapure water. For the preparation of glycine buffer with a final concentration of 0.1 mol/L, glycine powder was dissolved in 0.1 mol/L HCl. Subsequently, this glycine solution was filtered through a 0.22 μm PTFE (polytetrafluoroethylene) membrane filter and stored in sterile 50 mL polypropylene centrifuge tubes. Solutions of $FeSO_4$ and NaFeEDTA for injection were prepared immediately before use by dissolving either $FeSO_4$ or NaFeEDTA in 0.1 mol/L glycine buffer (pH = 1). The final concentration of both of the stock solutions was 100 mmol/L.

Study Design. Thirty-two weanling, male Sprague-Dawley rats were purchased from Charles River (Willmington, MA). They were housed in a temperature-controlled room on a 12 h dark–light cycle. Stainless steel metabolism cages configured to separate and collect urine were used. Rats were acclimated for 7 days to accustom them to the cage and diet. During this acclimation period, they were fed a semipurified, iron-deficient diet (AIN-93G; Dyets, Inc., Bethlehem, PA) fortified with 35 mg of Fe/kg of diet as $FeSO_4$ (*14*). This level of iron has been shown to be sufficient to support growth and meet iron requirements without inducing iron loading (*15*). On the last day of the acclimation period (day 0), rats were blocked by body weight and randomly assigned to one of four treatment groups of eight rats per group.

Starting on day 1 (and continuing to day 21) of the experiment, two groups of rats (groups III and IV) received diets containing 1200 mg of Fe/kg of diet in the form of either $FeSO_4$ (for group III) or NaFeEDTA (for group IV). The other two groups (groups I and II) were given the unfortified AIN-93G semipurified iron-deficient diet (ID basal diet) containing about 2 mg of Fe/kg of diet (*16*, *17*) and received an iron source via a daily SC injection. The SC injection method was chosen as an alternative route to deliver the presumably intact FeEDTA complex gradually into the blood stream, bypassing the digestive steps in the gut. The site of the injection was on the back of the rat, about 2–4 cm below the head. The volume of the injected iron solution was calculated and adjusted every day to deliver an amount of iron equivalent to the amount of iron the oral groups were absorbing. Specifically, we assumed that 7% of the iron in the oral treatments was absorbed throughout the study. This estimate was made on the basis of previous studies (*17*, *18*) in which about 60% of orally administered iron was absorbed by rats before iron loading and only 2% absorbed afterward. An average of 7% absorption was therefore established when the dosage and the duration of iron loading were also taken into consideration. As a result, 0.9–1.7 mg of Fe per day as either $FeSO_4$ or NaFeEDTA was adminstered SC to rats in groups I and II on the basis of the amount of feed consumed by each rat. The SC injection for groups I and II and the iron-loading diet for groups III and IV were stopped on day 21.

During the entire iron-loading period, each rat's 24 h urine output was collected. The urinary mineral contents were analyzed using an inductively coupled argon plasma/atomic emission spectrophotometer (ICAP 61E Thermal Jarrell Ash Trace Analyzer; Jarrell Ash Co., Franklin, MA). Specific sample preparation steps for the ICAP analysis were described by Kosse et al. (*16*). Because of the low volume of urine, urine output from day 4 to day 13 (10 samples) from each rat was combined, and results were expressed as average daily mineral excretion (micrograms) in the urine.

On day 22, half of the rats from each group were sacrificed. Rats were anesthetized with CO_2. Blood samples were obtained by heart puncture. Rats were then exposed to excess CO_2 until they expired. Tissues including the liver, spleen, kidneys, femur bone, the muscle around the femur, and skin (with hair) were collected, weighed, and analyzed for nonheme iron content. A small portion (less than 0.5 g) of the liver and one-half of a kidney were fixed with 10% formalin buffer for histological analysis. The remaining rats (half of the rats in each group) were switched back to diets containig 35 mg of Fe/kg of diet. For example, rats in groups I and III received a diet containing 35 mg of Fe as $FeSO_4$/kg of diet, and those in groups II and IV received 35 mg of Fe as NaFeEDTA/kg of diet.

On day 25, the remaining rats were sacrificed, and blood and tissue samples were collected following the same procedures described for day 22. This 3 day washout period (days 22–24) was designed to monitor changes in iron status shortly after iron loading.

The above study design is summarized in **Table 1**. All rats had free access to their assigned diet and distilled water during this period. Rats were observed daily throughout the study for signs of abnormalities. Their body weights were recorded at the beginning of the study and before they were killed. The experimental protocol (no. 05-20) was approved by the Institutional Animal Care and Use Committee (IACUC) of Cornell University.

Measurement of Iron Content in Tissues. Nonheme iron concentrations in collected tissues were determined by the colorimetric method described by Schricker et al. (*19*, *20*) with modifications reported by Rhee and Ziprin (*21*) for minimizing the breakdown of heme pigments into nonheme iron. Results were expressed as micrograms of nonheme iron per gram of tissue (wet weight) and were used as indices of the iron status in rats. The hemoglobin concentrations of the collected blood samples were determined by the cyanomethemoglobin method (*22*).

Statistical Analysis. All statistical analyses were done using Minitab Release 14 (Minitab Inc., State College, PA). The effect of the 3 day washout period (day 22–24) on hemoglobin concentrations and iron

Table 2. Body Weights and Blood Hemoglobin Concentrations[a]

	group				
	I, SC-$FeSO_4$	II, SC-NaFeEDTA	III, OR-$FeSO_4$	IV, OR-NaFeEDTA	
initial body wt (g)	80.41 (2.38)	80.38 (2.34)	80.33 (3.17)	80.41 (1.91)	$p = 0.999$
final body wt (g)	232.8 (5.70)	235.0 (6.69)	259.7 (10.4)	244.2 (10.6)	$p = 0.135$
hemoglobin concn (g/L)	176.5 (4.91)	144.3 (6.37)[b]	193.2 (4.36)	185.3 (3.79)	$p < 0.001$

[a] Results for each parameter are shown as the mean (SEM), $n = 8$. [b] The blood was collected at the end of the study. Only the mean hemoglobin concentration in group II was significantly lower than the other three groups.

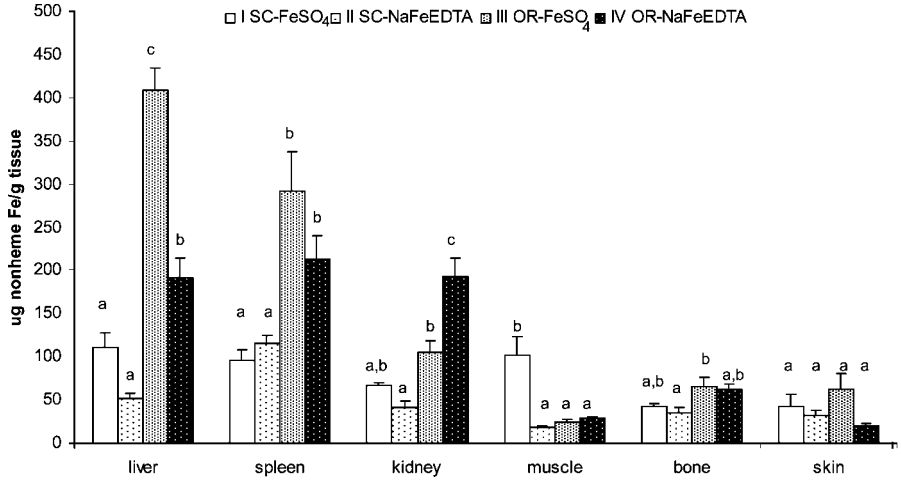

Figure 1. Nonheme iron concentrations in tissues following oral (OR) or subcutaneous (SC) administration, expressed as micrograms of Fe per gram of tissue (wet weight). Within a tissue category, bars not sharing the same letter are significantly different ($p < 0.05$) from each other (two-way ANOVA). Values are the mean + SEM ($n = 8$).

levels in tissues was compared using two-sample *t* tests. The effect of the treatments on nonheme iron concentrations in rat tissues, total body iron, and the excretion of other minerals was analyzed by two-way ANOVA. The body weights before iron loading and the body weights and hemoglobin concentrations after iron loading were analyzed by one-way ANOVA. A *p*-value of less than 0.05 was considered significant.

RESULTS

The 3 Day Washout Period. Two-sample *t* tests were conducted to analyze any changes in iron status during the 3 day washout period. Hemoglobin values among rats killed on day 22 (four per group) were not significantly different from those killed on day 25 (four per group) in each group ($p = 0.39$, 0.46, 0.58, and 0.39, respectively, for groups I–IV; data not shown). No significant differences were found for nonheme iron in the tissues of each group collected on days 22 and 25 (data not shown). It is therefore assumed that the iron status in the body did not change significantly between the two killings. Data gathered on days 22 and 25 were therefore combined to increase the power of the tests, and the following analyses are reported with eight rats per group.

Body Weights and Blood Hemoglobin Concentrations. The mean body weights at the beginning of the loading period, as well as the mean body weights and the blood hemoglobin (Hb) concentrations at the end, are summarized in **Table 2**. There were no significant differences in body weight among the four groups of rats either at the beginning (day 0, $p = 0.999$) or at the end (day 21, $p = 0.135$) of the loading period. Further analysis using Tukey's comparison showed that the mean Hb level was significantly lower in rats injected with NaFeEDTA (SC-NaFeEDTA, group II), while there were no significant differences in Hb concentrations among the other three groups. Using the same assay and hemoglobin standards, hemoglobin levels obtained from groups I, III, and IV were similar to those reported in previous studies (*17*, *18*) with iron-adequate rats, suggesting that rats in group II were anemic.

Nonheme Iron Concentrations in Tissues. Nonheme iron concentrations in various tissues are summarized in **Figure 1**. In general, the two SC-injected groups had lower nonheme iron concentrations in tissues. The only exception was observed with the SC-$FeSO_4$ group (group I) in the muscle, where the nonheme iron concentration was 5.6-, 4.3-, and 3.6-fold higher than the SC-FeEDTA, OR-$FeSO_4$, and OR-FeEDTA groups, respectively. The OR-$FeSO_4$ group (group III) had the highest nonheme iron concentrations in five out of the six tissue categories tested. Compared with SC-$FeSO_4$ (group I), nonheme iron concentrations in group III were 3.7-fold higher in the liver and 3.0-fold higher in the spleen. Although still significantly higher than the two injected groups, the nonheme iron concentration in the OR-FeEDTA group (group IV) was 53.1% lower in the liver and 27.2% lower in the spleen, compared with the OR-$FeSO_4$ group. On the other hand, group IV had the highest nonheme iron concentration in the kidneys: 85.9% higher than group III and more than double the levels of the two injected groups.

Organ Weights and Body Iron. Total organ weights were either measured (for the liver, spleen, and kidneys) or calculated (for the muscle, bone, and skin). We estimated total body iron by summing up iron from hemoglobin and nonheme iron from all major iron-containing tissues (**Table 3**). The livers of rats given NaFeEDTA (groups II and IV) were significantly heavier than those given $FeSO_4$ (groups I and III), and the route of administration (OR vs SC) did not affect liver weight. The total body nonheme iron in rats injected with NaFeEDTA (group II) was 47.8%, 62.9%, and 69.2% lower than that in group IV, III, and I, respectively. The same trend was also observed for the total body iron.

Table 3. Total Organ Weights and Total Body Iron[a]

	group				
	I, SC-$FeSO_4$	II, SC-FeEDTA	III, OR-$FeSO_4$	IV, OR-FeEDTA	
liver (g)	13.12[a] (0.77)	16.70[b] (0.76)	13.18[a] (0.41)	17.03[b] (1.20)	$p = 0.002$
spleen (g)	1.06 (0.12)	0.92 (0.07)	1.09 (0.06)	0.96 (0.06)	$p = 0.405$
kidneys (g)	2.18 (0.08)	2.44 (0.08)	2.19 (0.10)	2.31 (0.19)	$p = 0.424$
muscle (g)	104.8 (2.56)	105.8 (3.01)	116.9 (4.69)	109.9 (4.75)	$p = 0.135$
bone (g)	12.55 (0.33)	12.69 (0.39)	14.14 (0.61)	13.23 (0.62)	$p = 0.132$
skin (g)	27.62 (0.73)	27.91 (0.86)	31.11 (1.35)	29.11 (1.36)	$p = 0.132$
total nonheme iron (mg)	14.01[c] (2.11)	4.31[a] (0.15)	11.62[b,c] (1.06)	8.26[a,b] (0.49)	$P < 0.001$
total heme iron (mg)	8.40 (0.35)	6.91 (0.35)	10.23 (0.46)	9.23 (0.45)	$P < 0.001$
total iron (mg)	22.41[b] (2.17)	11.22[a] (0.41)	21.85[b] (1.34)	17.50[a,b] (0.65)	$P < 0.001$

[a] Each parameter was expressed as the mean weight (SEM). Each category was analyzed using two-way ANOVA, $n = 8$. Parameters with no letters in common are significantly different within each category. The whole organ of the liver, spleen, and kidneys was collected and weighed. The weights of the other three organs were calculated on the basis of body mass (M_{body}): the total weight of muscle = 45%M_{body} (*32*); the total weight of skeletal bone (M_{sk}) = $0.0343M_{body}^{1.083}$ (*33*); the total weight of skin = $2.2M_{sk}$ (*34*). Total nonheme iron, an estimate of nonheme iron in major iron-containing tissues, was calculated using the nonheme iron concentration (**Figure 1**) and the organ weight. Total heme iron, an estimate of iron in hemoglobin, was calculated on the basis of M_{body} and the hemoglobin concentration of each rat. The total iron was calculated by adding the total nonheme and heme iron.

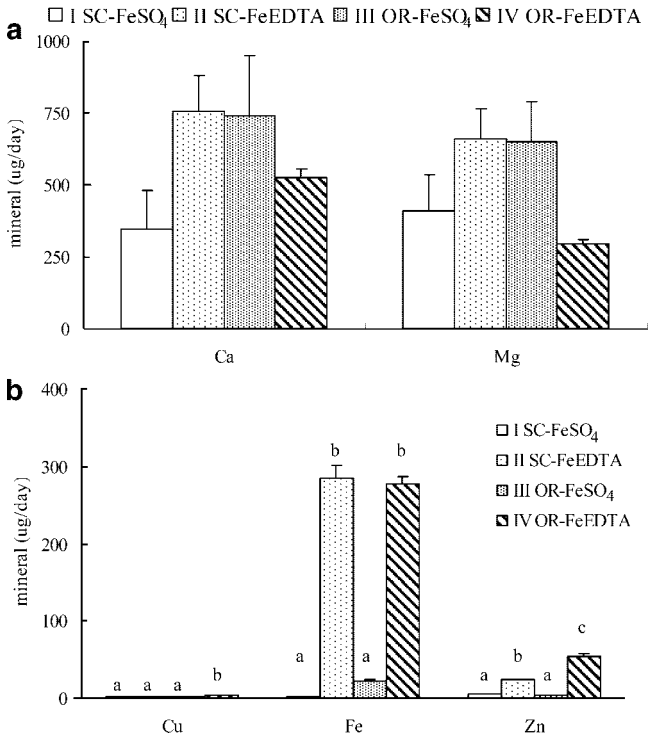

Figure 2. Daily urinary mineral excretion, expressed as micrograms of mineral output. (**a**) Daily urinary Ca and Mg excretion. There were no significant differences among the four groups. (**b**) Daily urinary Cu, Fe, and Zn excretion. Columns in each mineral category with no letters in common represent significant differences ($p < 0.05$). Values are the mean + SEM ($n = 8$). On the basis of the average body mineral concentrations for rats (Cu, 2 ppm; Fe, 60 ppm; and Zn, 33 ppm), the percent of the mineral excreted in group IV was calculated to be 0.7%, 1.9%, and 0.7% for Cu, Fe, and Zn, respectively. Relative to the level of administration, 25% of iron was excreted (assuming that on average 1.1 mg of iron was injected daily) in group II and 1.8% was excreted (assuming that on average rats consumed 13 g of diet daily) in group IV.

Excretion of Minerals in the Urine. The urinary output of Ca, Mg, Cu, Fe, and Zn, expressed as total mineral (in micrograms) excreted per day is illustrated in **Figure 2**. There were no significant differences in the excretion of Ca and Mg among the four groups of rats. Significant differences in Cu, Fe, and Zn excretion were detected, but the excretion pattern for each mineral was different. Rats fed with NaFeEDTA (group IV) had the highest urinary Cu concentration, which was on average 2.3 times higher than the other three groups. The total iron content in the daily urine output was similar for the two FeEDTA groups, which was 208 times higher than the SC-$FeSO_4$ group and 13 times higher than the OR-$FeSO_4$ group. In comparison with the daily administered iron, group II excreted on average about 25% of the injected NaFeEDTA, and group IV excreted 1.8% of the oral dose. The total zinc excreted from group IV was the highest. The total urinary zinc excretion in the SC-FeEDTA group (group II) was also significantly higher than that in the other two $FeSO_4$ groups but was 56.3% lower than that in group IV.

DISCUSSION

The hemoglobin concentration in rats injected with NaFeEDTA was significantly lower than the concentrations in the other three groups (**Table 2**), and the liver nonheme iron concentration was also the lowest (**Figure 1**), indicating that this group of rats suffered from iron deficiency anemia despite a high level of iron administration. Although not significant, the mean body weight in this group was the lowest among four groups at the end of the study, suggesting that anemia was affecting growth. These findings corroborate those of our previous rat study in which about 80% of radioactivity was excreted in the urine after a single SC injection of $Na^{59}FeEDTA$ (*13*). On the other hand, the mean hemoglobin concentration in rats fed NaFeEDTA was normal and similar to that in rats fed $FeSO_4$. Taken together, our results suggest that iron from NaFeEDTA cannot be efficiently utilized by the body if injected subcutaneously regardless of the dose of the iron, presumably because most of the iron is still in the chelated form and therefore is not available for incorporation into iron-containing proteins. In contrast, if administered orally, at least a portion of iron dissociates from the EDTA complex prior to or during intestinal uptake. Only this dissociated portion of iron is then reduced by a ferrireductase Dcytb (*30*), taken up by a divalent metal transporter (DMT-1) (*29*), transported into the blood, and utilized by the body. This DMT-1 pathway is recognized as the transporting mechanism for ionic nonheme iron such as $FeSO_4$, which supports our finding that iron from $FeSO_4$ and from NaFeEDTA was utilized similarly in the two oral groups.

The two OR groups showed elevated liver, spleen, and kidney nonheme iron concentrations (**Figure 1**), which corresponds to successful iron loading. However, the patterns of distribution for $FeSO_4$ and NaFeEDTA were distinctively different. The iron concentration of the oral-fed $FeSO_4$ group was significantly

higher in the liver and spleen than that in the kidneys, whereas in rats fed NaFeEDTA, the liver, spleen, and kidney nonheme iron concentrations were about equal. As a result, nonheme iron concentrations were 53.1% lower in the liver and 85.9% higher in the kidney in the OR-NaFeEDTA group, compared with the OR-$FeSO_4$ group. Similar differences in liver nonheme iron have been reported in previous studies of rats fed NaFeEDTA compared with those fed $FeSO_4$ (*18, 23, 24*), but few hypotheses were proposed to explain mechanisms behind this phenomenon. Overall, the two NaFeEDTA groups accumulated less iron in the liver and had less nonheme iron in the body than the $FeSO_4$ groups at these high levels of administration (**Table 3**). These findings diminish the concern that the highly bioavailable NaFeEDTA may cause iron overload especially in iron-adequate subjects and suggest that NaFeEDTA, as a food fortificant, is no more likely to cause iron overload than $FeSO_4$. The nonheme iron accumulation in the kidneys seems to be unique to rats fed high levels (i.e., 1200 mg of Fe/kg of diet) of NaFeEDTA. For example, the kidney nonheme iron concentration did not increase significantly among rats fed 140 mg of Fe as NaFeEDTA/kg of diet (*23*), which is 12% of the dosage used in our study, nor did it increase when rats were loaded with 30000 mg of Fe as elemental iron/kg of diet (*17*). This difference in nonheme iron distribution may be explained by the separate absorption of free EDTA once Fe^{3+} dissociates from the EDTA complex before being taken up by the enterocytes. In fact, it has been shown that 5–10% of EDTA in the form of a disodium salt may be absorbed and eliminated via the kidneys (*25, 26*). As a result, one possibility is that at high levels of NaFeEDTA administration, the amount of absorbed free EDTA ligand is high enough to cause a significant quantity of iron to be mobilized from the liver. This chelated iron is transported to the kidneys, filtered by the glomerular tubule, released from EDTA, reabsorbed, and deposited in the kidneys. It is also possible that, at high levels of intake, intact FeEDTA is absorbed across the epithelial cell barrier via a paracellular route in addition to the DMT-1 pathway for ionic iron (*27*). Some of this chelated iron then dissociates from the EDTA in the blood and in the kidneys and is reabsorbed.

The SC-$FeSO_4$ group had one of the highest body nonheme iron levels among the four groups (**Table 3**), indicating that the iron injections successfully induced iron loading. This high level of nonheme iron is expected because the body lacks mechanisms to excrete iron, and therefore, almost 100% of the injected ionic form of iron is retained in the body. Unlike the orally administered $FeSO_4$ which accumulated mostly in the liver, the injected $FeSO_4$ was found mainly in the muscle (**Figure 1**). This may be explained by the circulation of the blood. Iron absorbed from the small intestine is collected into the blood in the portal vein which passes directly to the liver, whereas the injected iron enters the cardiovascular system near the heart after going through the lymphatics and reaches the skeletal muscles first through the systemic circulation (*35*). This deposition of nonheme iron in the muscle is unique to the injected $FeSO_4$ group but is not seen in the injected FeEDTA group, indicating that only the ionic form of iron can be efficiently taken up by the muscle. The SC-FeEDTA group had about 70% less nonheme iron in organs compared with the SC-$FeSO_4$ group (**Table 3**), and the urinary iron was high (**Figure 2**), further supporting the hypothesis that the chelated iron from NaFeEDTA cannot be utilized by the body effectively unless dissociated. These findings also suggest that the strong chelating effect of EDTA directs the destination of the iron. On the other hand, about 30% of the injected iron was retained in the body, indicating that this small portion of iron was dissociated from EDTA at some point in the body. The dissociation could be the result of a combination of forces including the change of pH, the competitive iron binding from transferrin in the blood, the competitive EDTA binding from other minerals such as Ca in the blood, and/or glomerular filtration and DMT-1 uptake of iron in the kidney tubules.

The average liver weights of rats administered NaFeEDTA (groups II and IV) were significantly heavier than those administered $FeSO_4$ (groups I and III). Microscopic examination did not reveal histopathological changes that could be related to either the type of iron or the route of administration (data not shown). Therefore, the mechanism for this weight difference is not clear. No similar liver weight differences have been reported previously. In a study by Oates et al. (*28*), no significant differences in liver weights of rats fed a 30000 mg of Fe/kg of iron-loading diet (as carbonyl iron) were found compared with the controls.

Administering NaFeEDTA increased the excretion of Cu, Fe, and Zn, with distinctive patterns associated with each mineral. In general, the amount of the minerals excreted from the body was small, about 0.7%, 1.9%, and 0.7% of the total body Cu, Fe, and Zn, respectively, over the 21 day period (**Figure 2**). The daily excretions of Cu, Fe, and Zn were all significantly elevated in rats fed NaFeEDTA, presumably because of the absorption of free EDTA, which mobilizes some liver Cu and Zn in addition to Fe, and some of the EDTA-chelated minerals are thereby excreted. The daily excretion of Cu was not changed in rats injected with NaFeEDTA, whereas the excretion of Zn was elevated. This may be explained by the different availability of Cu and Zn in the blood. For example, Zn^{2+} mainly binds nonspecifically to albumin, while Cu^{2+} is mainly carried by ceruloplasmin, an enzyme dependent on Cu for its activity. Therefore, the binding affinity of these minerals and their carrier proteins directly affects the efficiency of the competitive binding from EDTA.

The daily excretion of Fe was similar in the injected and oral-fed NaFeEDTA groups, indicating that rats fed 1200 mg of Fe as NaFeEDTA/kg of diet also excreted greatly elevated amounts of iron in the urine. This was not seen previously (*13*) when rats were fed 35 mg of Fe as NaFeEDTA/kg of diet. This finding suggests a paracellular route for intestinal iron uptake; namely, intact iron chelates cross the epithelial barrier by moving between cells rather than being transported by DMT-1 or other transcellular mechanisms. The paracellular route may become significant only when high levels of iron are given orally. Specifically, in addition to the iron that is dissociated from the EDTA complex and is reduced and taken up by the DMT-1 pathway, some of the intact FeEDTA may diffuse passively across the intestinal epithelium via the paracellular route. Once inside the body, the majority of this chelated iron cannot be retained as efficiently as the ionic iron, causing an increase in urinary iron excretion in the OR-FeEDTA group. It should be noted that FeEDTA absorbed via this paracellular route is unlikely to contribute significantly to the nonheme iron concentrations in tissues and, therefore, is unlikely to cause or exacerbate NaFeEDTA-induced iron overload. It is not clear whether this paracelluar route is specific to the chelated form of iron. It is also possible that the permeability of the enterocytic apical membrane was altered as a result of high levels of iron intake, which may be of interest for future research.

The treatments in this study contained extraordinarily high levels of iron and EDTA. On average, the 1200 mg of Fe/kg of diet provided the rats with 90 mg of Fe and 470 mg of EDTA/

kg of body weight per day, which is 113- and 188-fold higher than the acceptable daily intake (ADI) (*31*), respectively. Elevated body iron status was achieved successfully by using these treatments, but we should note that it is extremely unlikely to find such high levels in NaFeEDTA-fortified foods or in intervention programs. No specific tissue damages associated with either $FeSO_4$ or NaFeEDTA, administered either OR or SC, were found from the liver and kidney histology slides. Previous findings suggested that NaFeEDTA would have no detrimental effect on the metabolism of Zn, Cu, and Ca when used as a food fortificant (*36*); however, the level used was low (31.6 mg of Fe/kg of diet), and the duration of the study was short (4 days). Therefore, findings from this study such as the elevated urinary excretion of Cu, Fe, and Zn, and different tissue nonheme iron distribution patterns associated with high NaFeEDTA administration, need to be examined closely. Future studies should focus on elucidating mechanisms involved in the transportation and dissociation of intact FeEDTA absorbed via the paracellular route and the long-term physiological consequences of the altered tissue nonheme iron distribution pattern in humans.

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LITERATURE CITED

(1) UNICEF/UNU/WHO/MI. Preventing iron deficiency in women and children. Technical consensus on key issues. **1999**, 1–60.

(2) Hurrell, R. F. Preventing iron deficiency through food fortification. *Nutr. Rev.* **1997**, *55*, 210–222.

(3) Hurrell, R. F.; Reddy, M. B.; Burri, J.; Cook, J. D. An evaluation of EDTA compounds for iron fortification of cereal-based foods. *Br. J. Nutr.* **2000**, *84*, 903–910.

(4) Layrisse, M.; Martinez-Torres, C. Fe(III)-EDTA complex as iron fortification. *Am. J. Clin. Nutr.* **1977**, *30*, 1166–1174.

(5) Chen, J.; Zhao, X.; Zhang, X.; Yin, S.; Piao, J.; Huo, J.; Yu, B.; Qu, N.; Lu, Q.; Wang, S.; Chen, C. Studies on the effectiveness of NaFeEDTA-fortified soy sauce in controlling iron deficiency: a population-based intervention trial. *Food Nutr. Bull.* **2005**, *26*, 177–186 (discussion 187–189).

(6) Huo, J.; Sun, J.; Miao, H.; Yu, B.; Yang, T.; Liu, Z.; Lu, C.; Chen, J.; Zhang, D.; Ma, Y.; Wang, A.; Li, Y. Therapeutic effects of NaFeEDTA-fortified soy sauce in anaemic children in China. *Asia Pac. J. Clin. Nutr.* **2002**, *11*, 123–127.

(7) Garby, L.; Areekul, S. Iron supplementation in Thai fish-sauce. *Ann. Trop. Med. Parasitol.* **1974**, *68*, 467–476.

(8) Van Thuy, P.; Berger, J.; Nakanishi, Y.; Khan, N. C.; Lynch, S.; Dixon, P. The use of NaFeEDTA-fortified fish sauce is an effective tool for controlling iron deficiency in women of childbearing age in rural Vietnam. *J. Nutr.* **2005**, *135*, 2596–2601.

(9) Thuy, P. V.; Berger, J.; Davidsson, L.; Khan, N. C.; Lam, N. T.; Cook, J. D.; Hurrell, R. F.; Khoi, H. H. Regular consumption of NaFeEDTA-fortified fish sauce improves iron status and reduces the prevalence of anemia in anemic Vietnamese women. *Am. J. Clin. Nutr.* **2003**, *78*, 284–290.

(10) Ballot, D. E.; MacPhail, A. P.; Bothwell, T. H.; Gillooly, M.; Mayet, F. G. Fortification of curry powder with NaFe(III)EDTA in an iron-deficient population: report of a controlled iron-fortification trial. *Am. J. Clin. Nutr.* **1989**, *49*, 162–169.

(11) Viteri, F. E.; Alvarez, E.; Batres, R.; Torun, B.; Pineda, O.; Mejia, L. A.; Sylvi, J. Fortification of sugar with iron sodium ethylenediaminotetraacetate (FeNaEDTA) improves iron status in semirural Guatemalan populations. *Am. J. Clin. Nutr.* **1995**, *61*, 1153–1163.

(12) Andang'o, P. E.; Osendarp, S. J.; Ayah, R.; West, C. E.; Mwaniki, D. L.; De Wolf, C. A.; Kraaijenhagen, R.; Kok, F. J.; Verhoef, H. Efficacy of iron-fortified whole maize flour on iron status of schoolchildren in Kenya: a randomised controlled trial. *Lancet* *369*, 1799–1806.

(13) Zhu, L.; Yeung, C. K.; Glahn, R. P.; Miller, D. D. Iron dissociates from the NaFeEDTA complex prior to or during intestinal absorption in rats. *J. Agric. Food Chem.* **2006**, *54*, 7929–7934.

(14) Reeves, P. G.; Nielsen, F. H.; Fahey, G. C., Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.* **1993**, *123*, 1939–1951.

(15) NRC National Research Council, Nutrient Requirements of Laboratory Animals. **1995**.

(16) Kosse, J. S.; Yeung, A. C.; Gil, A. I.; Miller, D. D. A rapid method for iron determination in fortified foods. *Food Chem.* **2001**, *75*, 371–376.

(17) Yeung, C. K.; Zhu, L.; Glahn, R. P.; Miller, D. D. Iron absorption from NaFeEDTA is downregulated in iron-loaded rats. *J. Nutr.* **2004**, *134*, 2270–2274.

(18) Yeung, C. K.; Zhu, L.; Glahn, R. P.; Miller, D. D. Tissue iron distribution and adaptation of iron absorption in rats exposed to a high dietary level of NaFeEDTA. *J. Agric. Food Chem.* **2005**, *53*, 8087–8091.

(19) Schricker, B. R.; Miller, D. D. Effects of Cooking and Chemical Treatment on Heme and Nonheme Iron in Meat. *J. Food Sci.* **1983**, *48*, 1340–1343.

(20) Schricker, B. R.; Miller, D. D.; Stouffer, J. R. Measurement and Content of Nonheme and Total Iron in Muscle. *J. Food Sci.* **1982**, *47*, 740–743.

(21) Rhee, K. S.; Ziprin, Y. A. Modification of the Schricker Nonheme Iron Method to Minimize Pigment Effects for Red Meats. *J. Food Sci.* **1987**, *52*, 1174–1176.

(22) Davidsohn, I.; Nelson, D. A. The blood. In *Clinical Diagnosis by Laboratory Methods*; Davidsohn, I., Henry, J. B., Eds.; W. B. Saunders: Philadelphia, PA, 1974; pp 110–310.

(23) Appel, M. J.; Kuper, C. F.; Woutersen, R. A. Disposition, accumulation and toxicity of iron fed as iron(II) sulfate or as sodium iron EDTA in rats. *Food Chem. Toxicol.* **2001**, *39*, 261–269.

(24) Whittaker, P.; Ali, S. F.; Imam, S. Z.; Dunkel, V. C. Acute toxicity of carbonyl iron and sodium iron EDTA compared with ferrous sulfate in young rats. *Regul. Toxicol. Pharmacol.* **2002**, *36*, 280–286.

(25) Candela, E.; Camacho, M. V.; Martinez-Torres, C.; Perdomo, J.; Mazzarri, G.; Acurero, G.; Layrisse, M. Iron absorption by humans and swine from Fe(III)-EDTA. Further studies. *J. Nutr.* **1984**, *114*, 2204–2211.

(26) Foreman, H.; Vier, M.; Magee, M. The metabolism of C14-labeled ethylenediaminetetraacetic acid in the rat. *J. Biol. Chem.* **1953**, *203*, 1045–1053.

(27) South, P. K.; Miller, D. D. Iron binding by tannic acid: Effects of selected ligands. *Food Chem.* **1998**, *63*, 167–172.

(28) Oates, P. S.; Jeffrey, G. P.; Basclain, K. A.; Thomas, C.; Morgan, E. H. Iron excretion in iron-overloaded rats following the change from an iron-loaded to an iron-deficient diet. *J. Gastroenterol. Hepatol.* **2000**, *15*, 665–674.

(29) Gunshin, H.; Mackenzie, B.; Berger, U. V.; Gunshin, Y.; Romero, M. F.; Boron, W. F.; Nussberger, S.; Gollan, J. L.; Hediger, M. A. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* **1997**, *388*, 482–488.

(30) McKie, A. T.; Barrow, D.; Latunde-Dada, G. O.; Rolfs, A.; Sager, G.; Mudaly, E.; Mudaly, M.; Richardson, C.; Barlow, D.; Bomford, A.; Peters, T. J.; Raja, K. B.; Shirali, S.; Hediger, M. A.; Farzaneh, F.; Simpson, R. J. An iron-regulated ferric reductase associated with the absorption of dietary iron. *Science* **2001**, *291*, 1755–1759.

(31) JECFA Joint FAO/WHO Expert Committee on Food Additives. 1993, FNP 52.

(32) Even, P. C.; Rolland, V.; Roseau, S.; Bouthegourd, J. C.; Tome, D. Prediction of basal metabolism from organ size in the rat: relationship to strain, feeding, age, and obesity. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2001**, *280*, R1887–R1896.

(33) DeMoss, D. L.; Wright, G. L. Sex and strain differences in whole skeletal development in the rat. *Calcif. Tissue Int.* **1998**, *62*, 153–157.

(34) Delp, M. D.; Evans, M. V.; Duan, C. Effects of aging on cardiac output, regional blood flow, and body composition in Fischer-344 rats. *J. Appl. Physiol.* **1998**, *85*, 1813–1822.

(35) Guyton, A. C.; Hall, J. E. Chapter 62: General principles of gastrointestinal function-motility, nervous control, and blood circulation. In *Textbook of Medical Physiology*; 10th ed.; W. B. Saunders: Philadelphia, PA, 2001; p 724.

(36) Hurrell, R. F.; Ribas, S.; Davidsson, L. $NaFe^{3+}EDTA$ as a food fortificant: influence on zinc, calcium and copper metabolism in the rat. *Br. J. Nutr.* **1994**, *71*, 85–93.

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