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Tissue Iron Distribution and Adaptation of Iron Absorption in Rats Exposed to a High Dietary Level of NaFeEDTA

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Although it has been shown that iron absorption from NaFeEDTA, a promising iron fortificant, is effectively down-regulated in iron-loaded rats, effects of prolonged exposure to high dietary levels of NaFeEDTA are not well understood. The objectives of this study were to determine whether rats can adapt to a high dietary level of NaFeEDTA by down-regulating iron absorption, and to determine effects on tissue iron distribution, with or without an iron absorption inhibitor. Male Sprague−Dawley rats were exposed to diets supplemented with $FeSO_4$ or NaFeEDTA at 1200 mg of Fe/kg of diet, with or without tea, for 27 days. Iron absorption measured by whole-body counting before and after exposure showed that rats adapted to the high dietary level of $FeSO_4$ or NaFeEDTA by down-regulating iron absorption to a similar extent. However, nonheme iron concentrations in liver and spleen were about 35−50% lower, whereas the concentration in kidney was about 300% higher in rats fed NaFeEDTA, compared to rats fed $FeSO_4$. Tea had no major impact on iron absorption or iron status, regardless of iron source. Our results showed that although iron absorption was down-regulated similarly, body iron distribution was markedly different between rats exposed to $FeSO_4$ and those exposed to NaFeEDTA. Further studies are warranted to determine the effects of prolonged exposure to dietary NaFeEDTA on kidney iron accumulation and kidney function.

KEYWORDS: Iron absorption; NaFeEDTA; rats; tea; kidney

INTRODUCTION

Iron bioavailability from NaFeEDTA is higher than from $FeSO_4$ when added to foods containing iron absorption inhibitors (*1*−*7*). We showed previously that iron absorption from NaFeEDTA is down-regulated to a similar extent as from $FeSO_4$ in iron-loaded rats (*8*). Hence NaFeEDTA appears to be no more likely than $FeSO_4$ to exacerbate iron overload in subjects with elevated body iron stores.

Accumulation of iron in liver, spleen and kidney increased with increasing iron intake in rats fed diets containing 35, 70, and 140 mg of Fe/kg of diet as either $FeSO_4$ or NaFeEDTA for 31 and 61 days, but iron from NaFeEDTA accumulated less efficiently in liver and spleen than iron from $FeSO_4$ (*9*). Similar dose-dependent responses were also observed in an acute toxicity study, but liver nonheme iron concentrations in rats fed high dietary iron levels were about 50% lower with NaFeEDTA when compared to $FeSO_4$, even though both compounds were found to have approximately the same level of lethal acute toxicity (*10*). Therefore, effects of prolonged exposure to high dietary levels of NaFeEDTA in intact animals on iron absorption and tissue iron distribution remain unclear.

The iron status of individuals, ranging from iron-deficiency anemia to iron overload, is presumably the most important physiological factor affecting iron absorption; absorption rate is inversely related to serum ferritin concentration, an index of body iron stores (*11*−*14*). In addition, humans may adapt to short-term increases in the concentration of bioavailable iron in diets by down-regulating iron absorption even before significant changes in total body iron develop (*15*). Hunt and Roughead (*16*) showed that iron-replete adult men down-regulated their iron absorption when fed diets with high iron bioavailability for 10 weeks and up-regulated iron absorption when fed diets with low iron bioavailability for the same period of time, even though there were no changes in serum ferritin concentrations.

The objectives of this study were therefore to determine whether rats can adapt to a high dietary level of $FeSO_4$ or NaFeEDTA by down-regulating iron absorption and to determine effects on tissue iron distribution, with or without tea. Tea, commonly considered an inhibitor of iron absorption, was added to permit a comparison between high and low bioavailability diets.

MATERIALS AND METHODS

Chemicals. All chemicals were obtained from Sigma Chemicals (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ) unless stated otherwise. NaFeEDTA (food-grade) was a gift from Akzo Nobel Chemicals Research (Arnhem, The Netherlands). Water used in the preparation of reagents for rat tissue analyses was double deionized. Glassware

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Table 1. Forms of Iron Added to the Rat Diets for the Dosing, Retention and Adaptation Periods

	forms of iron in diets[a]			
group	1st dosing[b] (day 1)	1st retention/ adaptation (day 1–27)	2nd dosing[b] (day 28)	2nd retention (day 28–38)
$FeSO_4$, no tea	$^{59}FeSO_4$	$FeSO_4$	$^{59}FeSO_4$	$FeSO_4$
NaFeEDTA, no tea	$Na^{59}FeEDTA$	NaFeEDTA	$Na^{59}FeEDTA$	NaFeEDTA
$FeSO_4$, with tea[c]	$^{59}FeSO_4$	$FeSO_4$	$^{59}FeSO_4$	$FeSO_4$
NaFeEDTA, with tea[c]	$Na^{59}FeEDTA$	NaFeEDTA	$Na^{59}FeEDTA$	NaFeEDTA

[a] All test diets were based on the iron-deficient AIN-93G diet, with either $FeSO_4$ or NaFeEDTA added at 1200 mg of Fe/kg of diet. [b] Dosing was done in the morning following an overnight fast. [c] Test diets contained 20 g of tea leaves (PG Tips, Unilever Bestfoods UK, Brooke House, Crawley, England)/kg of diet.

and utensils were soaked in 10% HCl for no less than 4 h and rinsed with deionized water prior to use.

Diets. All diets were based on a commercial iron-deficient AIN-93G purified rodent diet (Dyets #115072, Dyets Inc., Bethlehem, PA) (*17*) and were prepared by adding different forms of iron to this base diet (Table 1). The iron sources were incorporated into the diet using a stainless steel mechanical mixer (Hobart Mfg. Co., Troy, OH).

Preparation of ^{59}Fe-Labeled Meals for Dosing. Radiolabeled $FeSO_4$ was prepared immediately before use by spiking a solution of unlabeled $FeSO_4$ at pH 1 with carrier-free ^{59}Fe (Perkin-Elmer, Norwalk, CT) in 0.1 mol/L HCl. For the preparation of radiolabeled NaFeEDTA, radiolabeled $FeCl_3$, which was prepared in the same way as radiolabeled $FeSO_4$, was mixed with a solution of disodium EDTA at an iron to EDTA molar ratio of 1:1 and then adjusted to pH 1 using 1 mol/L HCl. Freshly prepared aliquots of $^{59}FeSO_4$ or $Na^{59}FeEDTA$ were then transferred onto preweighed 2-g portions of the base diet and mixed thoroughly. Separate aliquots of $^{59}FeSO_4$ and $Na^{59}FeEDTA$ were also transferred into Eppendorf tubes, and their activities were counted each day during the dosing and retention periods to account for the radioactive decay of ^{59}Fe.

Animals. Forty weanling, male Sprague−Dawley rats with body weights of <50 g were purchased from Charles River (Wilmington, MA). They were housed individually in a temperature-controlled room in stainless steel cages, on a 12-h dark−light cycle. Upon arrival at the housing facility, rats were fed a diet containing 35 mg of Fe as $FeSO_4$/kg of diet for 7 days to acclimate them to the housing and feeding. This concentration of iron is sufficient for growth and achieving maximum hemoglobin concentration (*18*). All rats were given free access to food and deionized water during the acclimation period.

Experimental Design. On day 1 of the experiment, rats, blocked by their body weights, were divided into four groups of 10 rats. Each group was then offered one of the four ^{59}Fe-labeled meals (2 g of diets containing either $^{59}FeSO_4$ or $Na^{59}FeEDTA$, with or without tea, Table 1). The meals were given ad libitum for 3 h. After the 3-h dosing period, each rat was assayed for ^{59}Fe activity in a whole-body γ-spectrometer (Tobor Large Sample Gamma Counter, Nuclear Chicago Corp., Des Plaines, IL) to accurately determine the activity of the initial dose. Subsequently, whole-body ^{59}Fe activity was measured at 24-h intervals during the next 10 days. All rats had free access to their respective unlabeled diets during the first retention/adaptation period (day 1−27). On day 27, each rat was assayed again to determine the remaining ^{59}Fe activity from the first dose, which was subtracted from the measured ^{59}Fe activity during the second dosing period.

On day 28, rats were given the ^{59}Fe-labeled meals for the second time following the same procedures used on day 1 for dosing and whole-body counting. Rats were returned to their respective diets and then assayed at 24-h intervals during the second retention period (day 29−38).

On day 39, rats were sacrificed and blood, liver, spleen, and kidney samples were collected. Rats were first anesthetized with CO_2 and blood samples were obtained by cardiac puncture. Immediately following blood sampling, the rats were killed with an overdose of CO_2. Liver, spleen, and kidneys were removed and weighed portions were analyzed for nonheme iron concentrations.

Table 2. Iron Absorption in Rats (mean ± SEM, $n = 10$) Determined by Whole-Body Counting before and after Exposure to $FeSO_4$ or NaFeEDTA at 1200 mg of Fe/kg of Diet, with or without Tea, for 27 days

	iron absorption (%)		down	
group	day 1	day 28	regulation (%)	*P*-value[a]
$FeSO_4$, no tea	14.9 ± 0.5	2.2 ± 0.2	−85.4	<0.0005
NaFeEDTA, no tea	14.1 ± 0.6	1.8 ± 0.1	−87.5	<0.0005
$FeSO_4$, with tea	19.8 ± 0.7	2.0 ± 0.2	−89.9	<0.0005
NaFeEDTA, with tea	12.6 ± 0.6	1.6 ± 0.1	−87.1	<0.0005

[a] Results of analyses by paired *t*-test of day 1 vs day 28 for each group.

Rats were observed daily during the whole study for signs of abnormalities. The body weights of the rats were recorded weekly. Animal care procedures and experimental protocols were approved by the Institutional Animal Care and Use Committee at Cornell University.

Calculations of Iron Absorption. Whole-body ^{59}Fe activity in the rats at the end of each 24-h interval was determined and expressed as a percentage of the initial dose. Retention data were used to calculate iron absorption as previously described (*8*, *19*−*24*).

Tissue Analyses. Nonheme iron concentrations in liver, spleen, and kidney samples were determined by the colorimetric method described by Schricker et al. (*25*) with modifications reported by Rhee and Ziprin (*26*). Results were expressed as μmol of Fe/g of tissue (wet weight). The hemoglobin concentrations of the collected blood samples were determined by the cyanmethemoglobin method (*27*).

Statistical Analyses. All statistical analyses were done using Minitab (Minitab Inc., State College, PA). Differences in iron absorption percentages in rats before and after exposure to the test diets were analyzed by paired *t*-test. The effects of iron source and tea on tissue nonheme iron concentrations in rats were analyzed by ANOVA, followed by Fisher LSD procedures if appropriate. A p-value of <0.05 was considered significant.

RESULTS AND DISCUSSION

Body Weight and Blood Hemoglobin Concentration. Mean (±SEM) body weight and blood hemoglobin concentration were 357 g (±3.9 g) and 176 g/L (±2.7 g/L) at the end of the study, respectively, with no significant differences among the four groups of rats.

Others have shown that rats fed AIN-93G diets supplemented with 15, 45, 650, and 1500 mg of Fe as $FeSO_4$/kg of diet ad libitum for 28 days resulted in the same body and liver weights (*28*), suggesting that diets containing high levels of iron (up to 1500 mg/kg of diet) have no adverse effects on growth rate. In the present study, rat body weights were not different, regardless of the form of dietary iron, suggesting that NaFeEDTA fed at 1200 mg of Fe/kg of diet for 39 days has no noticeable adverse effects on growth rate, when compared to $FeSO_4$.

Iron Absorption. Iron absorption decreased significantly in all four groups of rats after exposure to the test diets (Table 2). The extent of reduction in iron absorption percentages (i.e., down-regulation) ranged from −85.4% to −89.9%.

Uptake into intestinal enterocytes of iron from $FeSO_4$, an ionic form of iron, is primarily via the divalent metal transporter (DMT-1), which is located at the apical surface of the intestinal mucosal cell (*29*). Since DMT-1 expression is responsive to changes in iron status (*30*−*32*), iron absorption from $FeSO_4$ should be down-regulated when high levels are present in the diet over the long term (*15*). Our results showed that iron absorption in rats fed diets containing either high levels of $FeSO_4$

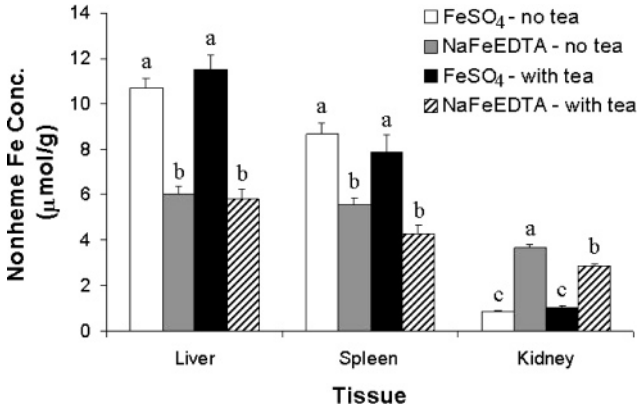

Figure 1. Effects of prolonged exposure to $FeSO_4$ or NaFeEDTA, with or without tea, on nonheme iron concentrations in rat tissues. Error bars indicate standard error of mean ($n = 10$). Bars within a tissue group without a common letter differ ($p < 0.05$).

or NaFeEDTA decreased significantly on day 28 when compared to day 1, suggesting that rats adapted to both forms of iron after exposure by down-regulating iron absorption to a similar extent. In our previous study, rats exposed to a high level of elemental iron to attain iron-overload status also down-regulated subsequent iron absorption from $FeSO_4$ and NaFeEDTA to a similar extent (*8*). These results taken together suggest that absorption of iron from NaFeEDTA, a chelated form of iron, is regulated as effectively as iron from $FeSO_4$.

Tissue Nonheme Iron Concentration. Liver and spleen nonheme iron concentrations were about 35−50% lower, but kidney nonheme iron concentration was about 300% higher in rats fed diets containing NaFeEDTA, when compared to rats fed diets containing $FeSO_4$ (Figure 1).

Liver and, to a lesser extent, spleen nonheme iron concentrations have been routinely used as indicators of body iron status in rats (*28*, *33*−*36*). Rats fed diets containing NaFeEDTA showed liver and spleen nonheme iron concentrations that were significantly lower than their $FeSO_4$ counterparts, suggesting that NaFeEDTA is not as effective as $FeSO_4$ in elevating iron storage. This occurred even though percentages of absorption and down-regulation from these two forms of iron were comparable. It has been shown that rats given intravenous injections of a radiolabeled mixture of $FeCl_3$ and EDTA had lower ^{59}Fe activities in the blood, liver, and spleen but higher activities in the skin, bones, and muscles initially than rats injected with radiolabeled $FeCl_3$ alone (*37*). Subsequently, a rapid excretion of 60% of the isotopic activity was observed within 1 h following the injection, but the excretion rate declined so much that only about 10% more of the activity was excreted during the next 3 days. In another study, when EDTA alone was given intraperitoneally to rats daily for 6 days, iron excretion in urine within a 24-h period postinjection increased by about 100%, although urinary iron excretion was low in both groups (*38*). Also, rats given ^{59}Fe−EDTA via isolated small intestinal loops absorbed 9% of the administered dose and EDTA led to lower retention in the blood and liver but higher retention in kidney and higher excretion via the urine after 2 h, compared to rats given unchelated $^{59}FeCl_3$ (*39*). These studies together suggest that the lower liver and spleen nonheme iron concentrations found in rats fed NaFeEDTA could be due to a different postabsorptive distribution pattern for iron chelated with EDTA. In other words, the amount of iron being retained in the liver and spleen may differ significantly, even if rats absorb the same amount of iron from diets containing $FeSO_4$ or NaFeEDTA.

Kidney nonheme iron concentration in rats fed NaFeEDTA was markedly higher than in rats fed $FeSO_4$. This is in stark contrast to liver and spleen nonheme iron concentrations, which were significantly lower in rats fed NaFeEDTA. Reabsorption of urinary iron occurs primarily in the loop of Henle and distal convoluted tubules in the rat kidney (*40*), via the DMT-1 transport pathway (*29*, *40*). Presumably, iron would accumulate in the kidney cells if the rate of reabsorption exceeds the rate of iron returning to the bloodstream. This normally would not occur, as iron bound to transferrin in blood serum is too large in size to be filtered out by the glomerulus (*41*); hence, only a relatively insignificant amount of iron is filtered. However, as a large dose of EDTA in the bloodstream may dramatically increase iron concentration in the filtrate, reabsorption of iron will likely increase as well and potentially lead to a net deposit of iron in the kidney. It is therefore imperative in the future to determine the effects of dietary exposure to NaFeEDTA on the forms of iron accumulated in kidney as well as any abnormal changes in kidney function as a result of iron accumulation.

Owing to the high level of dietary iron used, it should be noted that the concerns raised in this study only apply to populations with continuously high exposure to NaFeEDTA, an unlikely scenario with fortified foods but a possible outcome with NaFeEDTA dietary supplements. However if NaFeEDTA is only to be used in intervention programs at the level proposed by the Joint FAO/WHO Expert Committee on Food Additives (*42*) or if NaFeEDTA is only to be used to fortify condiments such as fish and soy sauces (*43*, *44*), of which consumption is self-limiting, these concerns may not be important.

Adding tea to the diets appeared to have no impact except that kidney nonheme iron concentrations in rats fed NaFeEDTA without tea was slightly higher than those fed NaFeEDTA with tea (Figure 1). Tea is rich in phenolic compounds, which are potent iron absorption inhibitors (*45*). Nonetheless, data from the literature regarding the inhibitory effect of tea on iron absorption in rats have been inconsistent. When given to rats at the level of 20 g of tea leaves/kg of diet (the same level used in the present study), total liver iron and liver iron concentration decreased significantly, suggesting that tea may negatively affect iron status (*36*). Also, when beverage tea and dietary iron were consumed together by rats, a significant reduction in percent iron absorption was observed (*23*). In another study, tea solids obtained by freeze-drying brewed black tea decreased iron absorption in rats by 83% when fed in a single meal, but by only 39% when fed over a 10-day period, suggesting that rats can partially adapt to the inhibitory effect of tea (*46*, *47*). Nevertheless, direct comparisons of rat and human absorption trials indicated that while tea had a marked inhibitory effect on iron absorption in humans, iron absorption in rats fed meals similar to those fed to humans was not affected by tea (*48*). Both black and green teas, when given as the sole beverages, were also shown to have no effect on iron bioavailability to weanling rats (*49*). One possible explanation to these discrepancies is that the effect of tea on iron absorption in rats is dependent on the tea-to-iron ratio and a much higher ratio is required for the effect to be revealed in rats than in humans. If the dietary iron level is high, the capacity of tea phenolics to bind iron may be exceeded, leaving sufficient unbound iron for maximal uptake by the enterocytes. The high level of iron given to rats in the present study might be the reason our results showed no major impact of tea on the iron status of the rats.

In conclusion, our study provides further evidence that absorption of iron from NaFeEDTA is regulated similarly to iron from $FeSO_4$. Nonetheless, postabsorptive body iron dis-

tribution differs significantly between rats fed these two forms of iron at high intake levels. Further studies are warranted to evaluate the effects of prolonged exposure to different dietary levels of NaFeEDTA on kidney iron accumulation and kidney function.

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