Iron Restriction Negatively Affects Bone in Female Rats and Mineralization of hFOB Osteoblast Cells

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We previously reported that severe iron deficiency negatively affects bone microarchitecture. Here we determined whether marginal iron restriction that reflects some human consumption patterns could have similar consequences. Thirty-two weanling female rats were randomly divided into four groups and fed the following diets for 10 weeks: (i) iron-adequate, calcium-adequate (FeA:CaA), (ii) calcium-restricted (FeA:CaR), (iii) iron-restricted (FeR:CaA), and (iv) both calcium- and iron-restricted (FeR:CaR) diets. DEXA analysis revealed that CaR decreased bone mineral density (BMD), and FeR decreased whole-body bone mineral content (BMC). Iron-restricted and calcium-restricted groups had lower BMD than did their adequate counterparts. All treatment-restricted groups had lower BMD in the fourth lumbar (L-4) vertebrae than the FeA:CaA group. Vertebrae BMD was lower in all treatment groups compared to the control group, and for BMC, the CaR groups were lower than the CaA groups and the FeR groups were lower that the FeA groups, and BMC were lower in iron- and calcium-restricted groups. The microarchitecture of the L-4 vertebrae was compromised in FeA:CaR, FeR:CaA, and FeR:CaR: (i) the connectivity density was reduced by FeR and by CaR; and (ii) trabecular number was decreased and trabecular separation was increased by FeR. Cortical thickness of the femur was reduced by both FeR and CaR. Finite element analysis revealed that L-4 vertebrae from the FeR:CaA group had greater internal stress with an applied force than the FeA:CaA group and, thus, would be more likely to break. Chelation of iron in cultured osteoblast cells impaired mineralization but had no impact upon Type I collagen deposition. Iron depletion, similar to that occurring among some human populations, reduced bone strength and microarchitecture based on the *in vivo* and *in vitro* results reported here. Impaired mineralization with iron depletion appears to be a possible mechanism for the observed bone abnormalities. Exp Biol Med 231:378–386, 2006

Key words: nutrition; calcium metabolism; animal models; rodents; bone histomorphometry; microarchitecture; iron; hFOB cells

Introduction

The Centers for Disease Control and Prevention (CDC) report that only 25% of females of childbearing age meet the recommended daily allowance for iron (1) and 12% meet the allowance for calcium (2). Adolescent females are especially at risk for both calcium and iron deficiency because of increased physiologic needs that accompany growth, the onset of menses (3), and consumption choices (4, 5). In 1998 the CDC stated that iron deficiency anemia had become a major health problem and that iron deficiency (either with or without anemia) was the foremost nutrient deficiency observed in females of childbearing age and worldwide (6). Furthermore, previous research has clearly established that a primary approach to preventing osteoporosis later in life is achieving peak bone mass during the adolescent years (7–14).

The role of iron in bone accrual has received little attention, and its mechanisms of action remain unclear. Medeiros *et al.* (15–17) hypothesized that iron exerts its influence on bone through collagen synthesis. Iron is a required cofactor for prolyl and lysyl hydroxylase enzymes, and this step is essential for lysyl oxidase activity, which then catalyzes cross-linking of adjacent collagen fibers. In iron deficiency there may be less iron available to the prolyl and lysyl hydroxylase enzymes, which could result in decreased cross-linking activity and, subsequently, weaker collagen fibers (18).

We previously reported that severe iron deficiency in the growing female rat has deleterious effects on bone morphometry and microarchitecture (15, 16), and severe calcium restriction exacerbates this effect (16). Previous studies of iron deficiency in rats found significantly decreased growth rates compared with iron-adequate controls (15–17). Medeiros *et al.* (15) reported results of a

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severe iron and calcium deficiency study that included a pair-fed group to determine whether the negative impact seen on bone was caused by the iron deficiency alone or by decreased caloric intake that accompanies iron deficiency anemia. Overall, the pair-fed group behaved similarly to the control group, which indicated that compromised bone integrity was caused by iron deficiency rather than decreased caloric intake. Harris *et al.* (19) showed in a study with postmenopausal women that there was a threshold effect regarding the influence of iron on bone mineral density (BMD). A maximal increase in BMD was reported with increasing iron intake only when calcium (Ca) intake was between 800 and 1200 mg (19). BMD also was influenced by hormone replacement therapy (20), which indicated a complex relationship between calcium, iron, and bone.

The current study used the rat model to assess the effects of marginal dietary depletion of calcium and iron on healthy bone. Given our previous studies (15–17) on iron deficiency and bone abnormalities, we needed to find a model to gain better insight with regard to what role iron could play in bone physiology. A whole animal has its limitations in that it is difficult to separate the secondary effects of iron restriction from the primary effect. As a first step toward gaining this better understanding, a cell culture model, which can circumvent secondary effects in an *in vivo* model, also was used, and in this model the impact of iron depletion upon Type I collagen deposition and mineralization was evaluated. To our knowledge, we are the first to investigate bone health under these conditions.

Materials and Methods

Diets. Basal diets were made according to the 1980 recommendations of the American Institute of Nutrition (AIN; 21) to meet the nutritional needs of growing rats. Because of the high iron content of cellulose, 5% Avicel was used as the fiber source. Diets were obtained from Research Diets (New Brunswick, NJ).

Four diets were prepared. The iron-adequate:calcium-adequate (FeA:CaA) diet contained 40 mg Fe/kg of diet and 0.52% calcium (5.2 g Ca/kg diet), thereby meeting the iron and calcium requirements for growing rats. The restricted-calcium diet (FeA:CaR) had 0.25% Ca (2.5 g Ca/kg diet or 0.0625 mol/kg) and adequate iron; the restricted-iron diet (FeR:CaA) contained 12 mg Fe/kg diet with adequate calcium; and the doubly restricted group (FeR:CaR) matched the calcium and iron concentrations of the two singly restricted diets. Calcium and iron concentrations of each diet were verified analytically by flame atomic absorption (Model 5000; Perkin-Elmer, Norwalk, CT). The calcium salts used were low in trace mineral contaminants, as previously described, and phosphorus (P) levels were left constant among the diets (16). Briefly, the phosphorus content of the diet was maintained at 0.56% (5.6 g/kg P) by weight. In preparing the diet we assumed 0.16% (1.6 g/kg P diet) came from casein and 0.4% (4.0 g/kg P diet) came from phosphate.

Experimental Design. Thirty-two 3-week-old weanling female Long-Evans rats were obtained from Charles River Laboratories (Wilmington, MA). We used female rats in this study because the female population of childbearing age in the United States is an at-risk population for both inadequate iron intake (with and without iron deficiency anemia) and inadequate dietary calcium intake.

Animals were randomly assigned to one of the four diet groups (eight rats per group) described above and were individually housed in stainless-steel cages. At the beginning of the study, there were no differences among body weights (45–50 g) of the four treatment groups. Animals had free access to food and water, and the room was on a 12:12-hr light:dark cycle. Body weights were recorded weekly throughout the 10-week study.

At end of the study, final body weights were taken and the animals were given intraperitoneal injections of sodium pentobarbital (50 mg/kg body wt), as approved by the Kansas State University Animal Care and Use Committee. After anesthetization, whole-body DEXA images were obtained. An incision was then made into the thoracic cavity, and approximately 1 ml of blood was removed *via* cardiac puncture and placed in heparinized tubes. The blood was used immediately to measure hematocrit.

Right and left femurs and tibias were dissected and the muscle tissue was removed. Bones were defatted in hexane for 18 hrs, weighed, then dried and weighed again. Lumbar vertebrae 1 through 6 (L1–6) were removed from each rat and left in the articulated form for microstructural analysis.

The right femurs were used for mechanical testing. Then these femurs and the tibias were used for mineral analyses. The left long bones and vertebrae were sent to Oklahoma State University for DEXA, micro-computed tomography (micro-CT), and finite element analysis.

Mechanical Testing of Femurs. A three-point breakage test was conducted using an Instron Universal Testing System (model 4466; Instron, Dayton, OH), as previously described (16). By using a vernier caliper, the midpoints of the femurs were determined by measuring the length of each bone and dividing that length by two. The length of the femur was determined by measuring from the highest point on the head to the lowest point of the inner condyle. The Instron was interfaced with the company's Series IX software for Windows to measure bone strength and stress.

Mineral Analysis of Bone and Diet. The right femurs and tibias were subjected to wet ashing, as previously described by our laboratory (15). Briefly, the bones were digested in 15 ml of trace metal–grade nitric acid until approximately 1 ml of acid was remaining, and then samples were diluted to 10 ml with deionized distilled water. For the calcium analysis, samples were further diluted with 1% lanthum chloride to break the calcium-phosphorus bonds. Iron concentration was assessed from the

original 10-ml sample. Analysis of both calcium and iron was conducted by flame atomic absorption (Model 5000, Perkin-Elmer). Certified standards for calcium and iron were obtained from Fischer Scientific Co. (Fairlawn, NY).

Micro-CT and Finite Element Analysis. The DEXA small-animal high-resolution scan module (Hologic QDR-4500A) was used to determine the bone mineral content (BMC) and BMD of fourth lumbar (L-4) vertebrae of the L1–6 articulated vertebrae and of left femurs. Micro-CT (Micro-CT 40, Scanco Medical, Basserdorf, Switzerland) was used to evaluate the three-dimensional architecture of the vertebrae, as previously described (15). The vertebrae were placed in 16-mm tubes, and images were taken at every 0.0165 μm. The trabecular area was outlined, and three-dimensional values were determined by using a contouring algorithm. Analyses of bone volume fraction, connectivity, and trabecular number, thickness, and separation were conducted. The cortical bone at the midshaft of the femur also was analyzed by micro-CT. Total volume, bone volume, porosity, and cortical thickness were determined.

Finite element software (Scanco Medical) allowed us to simulate compression properties of the vertebrae in a similar manner to that previously reported (15). Total compression force, size-independent stiffness, and von Mises stresses were determined for each L-4 vertebra.

Cell Culture Study. An immortalized human fetal osteoblast cell line (hFOB 1.19; American Type Culture Collection, Manassas, VA) was used for the cell culture study. This cell line is temperature sensitive, where cells proliferated at 34°C and differentiated between 38° and 39°C. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) without phenol red, 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), and 3% G418 disulfate solution (Sigma-Aldrich, St. Louis, MO). Cells maintained in T-75 flasks were considered grower cells, and cells used for experiments were grown in 10-ml petri dishes. Cells were fed every 48–72 hrs, as previously described (32), and were transferred from 34°C to >38°C when they reached 90%–100% confluency.

When cells were passaged, media were removed from the T-75 flasks and cells washed one time with sterile phosphate-buffered saline (PBS). Two milliliters of 0.05% trypsin/53 m*M* EDTA (Cellgro by Mediatech, Inc., Herndon, VA) were applied to cells and allowed to incubate at 34°C for 5 mins. After incubation, 13 ml of media were added to the trypsin, and the cell mixture and the contents of the flask were transferred to a sterile 50-ml tube. The mixture was aliquoted to petri dishes and a new, sterile T-75 flask in 3-ml increments. The medium added to each container was measured as follows: 12 ml to the T-75 flask and 7 ml to the petri dishes. Two petri dishes were randomly assigned to the control group and two others to the iron-restriction group described below.

Iron Restriction. The chelator deferoxamine (DFO), at a concentration of 8 μ*M*, was used to restrict available iron within the media (Sigma-Aldrich). A DFO concentration was chosen for the study. The amount of DFO used was determined after preliminary studies showed that quantities greater than 10 μ*M* DFO dramatically decreased cell viability. DFO was added to media when the cells reached 90%–100% confluency. Total cell lysates were collected and prepared as previously described (22, 23), and electrophoresis for Western analysis of the transferrin receptor and Type I collagen was conducted using 30 μg protein on Tris-HCl 10%–20% gradient gels (Bio-Rad Laboratories, Hercules, CA). The gels were run at 75 V until the dye fronts ran off the bottom of the gels. The protein was transferred to nitrocellulose membranes using a semidry transfer cell (Bio-Rad Laboratories, Richmond, CA) at 15 V for 20 mins. The membranes were placed in Ponceau S solution to verify the transfer. The membranes were soaked in 5% blocking buffer (nonfat powdered milk and PBS-Tween solution) overnight before probing with antibodies. The transferrin receptor fragment that served as a standard was a gift from Dr. John Beard (The Pennsylvania State University, State College, PA). For the transferrin receptor, primary antibody, mouse anti-human, was obtained from BD Biosciences (Franklin Lakes, NJ) and was allowed to incubate overnight on the blot and a concentration of 1 to 1000 in PBS-Tween. The secondary antibody (donkey, anti-mouse; Santa Cruz Technology, Santa Cruz, CA) was added to the blot at a dilution of 1:1000 in PBS-Tween and incubated for 1 hr before detection. High-molecular weight standards (Bio-Rad, Hercules, CA), Type I collagen primary antibody (mouse, anti-human; Santa Cruz Technology), and secondary antibody (donkey, anti-mouse; Santa Cruz Technology) were used for Type I collagen Western blot analysis, as previously described (22, 23). The employed dilutions for the Type I collagen were the same as those used for the transferrin receptor.

Mineralization. The early mineralization studies reported here were conducted after 48 hrs of treatment with DFO and after cells had differentiated. Alizarin red stain (Sigma-Aldrich) was applied to rinsed cells after 48 hrs at >38°C, whereupon the cells were lightly coated with formaldehyde and stored in the dark. After 24 hrs, images were taken at ×4, ×10, and ×20 magnification. Nodules were independently counted by two different individuals and the data statistically analyzed.

Statistical Analysis. All data were analyzed by two-way analysis of variance by using SAS for Windows (Version 8) with calcium and iron levels as main effects. P values of ≤ 0.05 for mean comparisons were determined by using a two-tailed test. The least significance differences method was used when significant interactions were calculated.

Results

Dietary treatments did not significantly affect final body weights. There was also no difference among the heart weights of the four groups, but analysis of heart to body

Table 1. Final Body Weight, Heart Weight, Heart to Body Weight, and Hematocrit of Rats Fed Experimental Diets for 10 Weeks[a,b]

Variable	FeA:CaA ($n = 7$)	FeA:CaR ($n = 8$)	FeR:CaA ($n = 8$)	FeR:CaR ($n = 8$)	Two-way ANOVA		
					Ca	Fe	Ca × Fe
Final body weight (g)	300 ± 16	297 ± 12	276 ± 14	290 ± 7	NS	NS	NS
Heart weight (g)	1.26 ± 0.10	1.26 ± 0.05	1.27 ± 0.07	1.39 ± 0.05	NS	NS	NS
Heart:body weight ($\times 10^{-3}$)	4.24 ± 4.11	4.26 ± 1.46	4.61 ± 0.14	4.78 ± 1.06	NS	0.048	NS
Hematocrit	42.8 ± 1.0	43.5 ± 0.9	39.7 ± 1.2	38.7 ± 0.9	NS	0.006	NS

[a] FeA:CaA, iron adequate, calcium adequate; FeA:CaR, iron adequate, calcium restricted; FeR:CaA, iron restricted, calcium adequate; FeR:CaR, iron restricted, calcium restricted; NS, not significant.
[b] Mean values ± SEM. Significance indicated if $P \leq 0.05$.

weight ratios showed that both FeR groups had significantly ($P \leq 0.05$) higher heart to body weight ratios than did FeA groups, and hematocrit values of the FeR groups were lower than those of FeA groups ($P \leq 0.01$; Table 1). Rats fed the FeR diets also appeared to have a graying of their fur compared to those assigned to the FeA groups.

Analysis of the calcium composition of the femur revealed an iron and calcium interaction. Iron depletion in the presence of adequate calcium (FeR:CaA) resulted in lower ($P \leq 0.05$) calcium concentration of the femur (Table 2) compared to the other three groups. The femurs and tibias of the FeR groups had lower ($P \leq 0.0001$) iron concentration than did the FeA groups (Table 2). Whole-body DEXA analysis showed that the CaR groups had lower ($P \leq 0.01$) BMD than did CaA groups, and FeR groups had lower ($P = 0.05$) BMC than the FeA groups (Table 2). DEXA scans of the left femurs revealed a significant effect for both iron and calcium, with the FeR and CaR groups having lower ($P \leq 0.01$) BMD than the FeA and CaA groups, respectively. There was a trend ($P = 0.06$) for all three treatment groups to be lower than the control or FeA:CaA group. There was no significant effect of treatment on femur BMC. The three-point breakage test demonstrated that femurs from the FeA:CaA group handled more load ($P \leq 0.05$) and experienced less stress ($P \leq 0.05$) at the breaking point than all other groups (Table 2).

Micro-CT was used to analyze the L-4 vertebrae of all 31 animals. Figure 1 illustrates a three-dimensional trabecular core from a L-4 vertebrae representative of each group. The bone volume fraction (BV/TV) represents the percentage of the total volume of interest that is bone. We found a trend ($P = 0.07$) for an interaction between calcium and iron depletion, with the FeA:CaA group tending to be higher than all other groups (Table 3). Connectivity density was significantly reduced both by FeR and by CaR. The trabecular number was decreased by iron depletion (Table 3). Trabecular thickness was not significantly affected by treatment. Trabecular separation (Tb.Sp) is a measure of the space between the trabecular regions of the bone. Our data show that iron depletion significantly increased Tb.Sp in the

Table 2. Bone Mineral Content, Morphometric Measures, and Breakage Data of Rats Fed Experimental Diets for 10 Weeks[a,b]

Variable	FeA:CaA ($n = 7$)	FeA:CaR ($n = 8$)	FeR:CaA ($n = 8$)	FeR:CaR ($n = 8$)	Two-way ANOVA		
					Ca	Fe	Ca × Fe
Bone minerals, right femur							
Ca (mmol/g)	5.20 ± 0.24 a	4.48 ± 0.31 a	3.93 ± 0.37 b	4.57 ± 0.31 a	NS	NS	0.039
Fe (μmol/g)	2.11 ± 0.14	2.19 ± 0.08	1.00 ± 0.04	0.97 ± 0.31	NS	<0.0001	NS
Bone minerals, right tibia							
Ca (mmol/g)	5.32 ± 0.27	4.92 ± 0.30	4.89 ± 0.27	4.79 ± 0.35	NS	NS	NS
Fe (μmol/g)	1.98 ± 0.20	1.89 ± 0.12	0.76 ± 0.05	0.76 ± 0.04	NS	<0.0001	NS
Bone morphometric measures							
Whole-body BMD (g/cm^2)	0.188 ± 0.004	0.174 ± 0.001	0.182 ± 0.003	0.176 ± 0.004	0.004	NS	NS
Whole-body BMC (g)	10.39 ± 0.42	10.48 ± 0.81	9.33 ± 0.33	9.43 ± 0.35	NS	0.05	NS
Left femur BMD (g/cm^2)	0.236 ± 0.004	0.218 ± 0.003	0.217 ± 0.003	0.214 ± 0.004	0.01	0.006	NS[c]
Left femur BMC (g)	0.376 ± 0.012	0.332 ± 0.010	0.339 ± 0.003	0.337 ± 0.010	NS	NS	NS
Right femur bone breakage							
Load (kgf)	10.03 ± 0.41 a	8.31 ± 0.26 b	8.92 ± 0.26 b	8.91 ± 0.56 b	0.04	NS	0.04
Stress (kgf/mm^2)	5.96 ± 0.22 a	4.98 ± 0.15 b	5.34 ± 0.16 b	5.33 ± 0.34 b	0.04	NS	0.04

[a] FeA:CaA, iron adequate, calcium adequate; FeA:CaR, iron adequate, calcium restricted; FeR:CaA, iron restricted, calcium adequate; FeR:CaR, iron restricted, calcium restricted; BMD; bone mineral density; BMC, bone mineral content; NS, not significant.
[b] Mean values ± SEM. Means in same row without same lowercase letter differ; $P \leq 0.05$.
[c] Trend ($P = 0.06$) for the interaction effect in which FeA:CaA group was greater than the other three treatment groups.

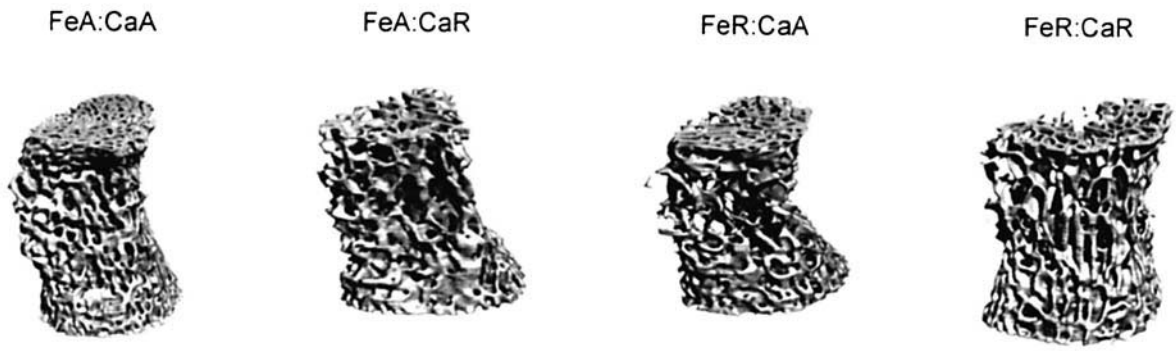


Figure 1. Images of L-4 trabecular bone of rats given adequate-iron and -calcium (FeA:CaA), calcium-restricted (FeA:CaR), iron-restricted (FeR:CaA), and both calcium- and iron-restricted FeR:CaR diets for 10 weeks, as determined by micro–computer tomography. The FeR:CaA vertebrae appeared to have the most honeycombed appearance of all groups, with greater porosity evident, followed by the other two treatment groups, as compared with the control FeA:CaA group.

vertebrae, indicating more fragile bone in the FeR rats (Table 3). BMD analysis of the L-4 vertebrae showed that CaR decreased BMD, but if iron was depleted, a CaA diet did not increase BMD to the level of the FeA:CaA group. Both CaR and FeR reduced BMC of the vertebrae. Bone mineral areas were lower in the FeR:CaA group compared with the other three groups (Table 3).

Micro-CT evaluation of the femur midshaft revealed that the CaA bones were larger and had more bone (Table 4), but the bone volume fraction was not significantly different (data not shown). Cortical thickness at the midshaft was significantly reduced by both CaR and FeR. Table 5 summarizes the findings of the Finite Element analysis. There was a trend for a calcium × iron interaction ($P = 0.07$) for force to fully compress the bone and for size-independent stiffness ($P = 0.08$). Iron deficiency in the presence of adequate calcium was detrimental. There was a significant calcium × iron interaction with von Mises stress. This is the internal stress in the bone when a constant force is applied. When calcium was adequate, iron depletion significantly increased internal stress.

Western blot analysis (Fig. 2) showed that 8 μM DFO resulted in increased expression of transferrin receptors in the treated cells compared to controls. Western blot analysis of Type I collagen expression after 48 hrs of treatment showed that there was no effect of DFO on protein expression (Fig. 3) Figure 4 depicts representative images taken after staining for calcium with Alizarin red. There was a significant ($P \leq 0.05$) decrease in the amount of calcium staining in the DFO-treated cells compared with the controls.

Discussion

The current daily recommended intake (DRI) for calcium for 9–18-year-olds is 1300 mg/day, and for 19–30-year-olds it is 1000 mg/day. For iron the DRI is 8 mg/day for 9–13-year-olds, 15 mg/day for 14–18-year-olds, and 18 mg/day for 19–30-year-olds (24). When we extrapolate the iron levels of our animal diets to human intakes, the level of iron consumed would be equivalent to 2.7 mg Fe/day for 9–13-year-old girls, 5.1 mg/day for 14–18-year-old girls, and for older females 19–30 years of age, the level

Table 3. Micro-CT Analysis and Small Animal DEXA of L-4 Vertebrae of Rats Fed Experimental Diets for 10 Weeks[a,b,c]

Variable	FeA:CaA ($n = 7$)	FeA:CaR ($n = 8$)	FeR:CaA ($n = 8$)	FeR:CaR ($n = 8$)	Two-way ANOVA Ca	Fe	Ca × Fe
BV/TV (%)	33.3 ± 2.8	27.6± 0.9	28.3 ± 1.4	29.7 ± 2.1	NS	NS	NS[d]
Conn.Dens. (1/mm^3)	90.5 ± 3.4	77.0 ± 2.5	67.3 ± 4.8	61.4 ± 2.6	0.01	<0.0001	NS
Tb.N (1/mm)	4.34 ± 0.24	3.81 ± 0.07	3.65 ± 0.15	3.66 ± 0.20	NS	0.02	NS
Tb.Th (mm)	0.078 ± 0.003	0.073 ± 0.001	0.076 ± 0.001	0.077 ± 0.002	NS	NS	NS
Tb.Sp (mm)	0.217 ± 0.017	0.247 ± 0.006	0.264 ± 0.011	0.263 ± 0.017	NS	0.02	NS
BMD (g/cm^2)	0.253 ± 0.005 a	0.231 ± 0.003 b	0.240 ± 0.004 b	0.236 ± 0.007 b	0.01	NS	0.05
BMC (g)	0.392 ± 0.019	0.326 ± 0.006	0.339 ± 0.010	0.344 ± 0.018	0.01	<0.001	NS
BMA (cm^2)	1.533 ± 0.0540 a	1.425 ± 0.0373 a	1.315 ± 0 .0731 b	1.489 ± 0.0181 a	NS	NS	0.029

[a] FeA:CaA, iron adequate, calcium adequate; FeA:CaR, iron adequate, calcium restricted; FeR:CaA, iron restricted, calcium adequate; FeR:CaR, iron restricted, calcium restricted; NS, not significant.
[b] Mean values ± SEM. Means in same row without same lowercase letter differ, $P \leq 0.05$.
[c] BV/TV, bone volume/total volume; Conn.Dens., connectivity density; Tb.N., trabecular number; Tb.Th, trabecular thickness; Tb.Sp., trabecular separation; BMD, bone mineral density by DEXA; BMC, bone mineral content by DEXA; BMA, bone mineral area.
[d] Trend ($P = 0.07$) for the FeA:CaA group to be greater than the other three groups.

Table 4. Micro-CT Analysis of Cortical Bone at Femur Midshaft of Animals Fed Experimental Diets for 10 Weeks[a,b]

Variable	FeA:CaA ($n = 7$)	FeA:CaR ($n = 8$)	FeR:CaA ($n = 8$)	FeR:CaR ($n = 8$)	Two-way ANOVA		
					Ca	Fe	Ca × Fe
TV (mm^3)	3.46 ± 0.12	3.18 ± 0.06	3.22 ± 0.07	3.15 ± 0.07	0.05	NS	NS
BV (mm^3)	3.41 ± 0.12	3.11 ± 0.06	3.16 ± 0.07	3.09 ± 0.07	0.03	NS	NS
Porosity (%)	1.53 ± 0.14	1.92 ± 0.16	1.94 ± 0.003	1.94 ± 0.19	NS	NS	NS
Cortical thickness (mm)	0.576 ± 0.010	0.544 ± 0.007	0.528 ± 0.008	0.499 ± 0.009	0.001	<0.0001	NS

[a] FeA:CaA, iron adequate, calcium adequate; FeA:CaR, iron adequate, calcium restricted; FeR:CaA, iron restricted, calcium adequate; FeR:CaR, iron restricted, calcium restricted; TV, total volume; BV, bone volume; NS, not significant.
[b] Mean values ± SEM.

Table 5. Finite Element Analysis of L-4 Vertebral Core of Rats Fed Experimental Diets for 10 Weeks[a,b]

Variable	FeA:CaA ($n = 7$)	FeA:CaR ($n = 8$)	FeR:CaA ($n = 8$)	FeR:CaR ($n = 8$)	Two-way ANOVA		
					Ca	Fe	Ca × Fe
Force (N)	16.9 ± 3.4	14.0 ± 1.2	11.9 ± 0.9	16.9 ± 2.2	NS	NS	NS[c]
Size-independent stiffness ($N/mm^2 \times 10^{-3}$)	1.23 ± 0.20	1.06 ± 0.07	0.88 ± 0.08	1.21 ± 0.15	NS	NS	NS[c]
von Mises stress (mPa)	21.1 ± 2.3 b	24.6 ± 1.6 ab	25.5 ± 1.1 a	21.9 ± 1.9 ab	NS	NS	0.05

[a] FeA:CaA, iron adequate, calcium adequate; FeA:CaR, iron adequate, calcium restricted; FeR:CaA, iron restricted, calcium adequate; FeR:CaR, iron restricted, calcium depleted; NS, not significant.
[b] Mean values ± SEM. Means in same row without same lowercase letter differ, $P \leq 0.05$.
[c] Trend for FeR:CaA group to be lower than the other three groups ($P = 0.07$ for force and $P = 0.08$ for size-independent stiffness).

would be equivalent to 6.2 mg/day. Only less than 5% of the females within each age category would have levels of iron this low (2). However, the level of iron used in this study did not result in body weight change, which eliminates this potentially confounding variable. With respect to calcium, the level of intake for a 9–13-year-old girl and a 14–18-year-old girl would be 624 mg Ca/day (25th and 50th percentiles, respectively), and for adult women, the level of intake would be 480 mg/day. These levels of calcium represent the 25th to 50th percentiles for calcium intake (2) for those greater than 13 years of age and lie between the 10th and 25th percentiles for the 9–13-year-old age group.

Our data show that the levels of calcium and iron restriction used in this study did compromise bone strength and microstructure. However, the CaR:FeR group responded in a unique way, with less apparent damage than expected and unlike what might have been anticipated based on our previous study of severe deficiency (16). The ratio of calcium to iron in the diet may be important in assessing the effects of these deficiencies on bone. Harris *et al.* (19) found that postmenopausal women with calcium intake of between 800 and 1200 mg/day experienced the greatest benefits in BMC and BMD with increasing amounts of iron. The use of hormone replacement therapy also plays a role in the ability of iron to positively influence BMD (20). To our knowledge, there are no data available regarding calcium thresholds, iron, BMC, and BMD during growth periods, but it is possible that similar parameters may apply during bone accrual.

The animals fed FeR diets had decreased iron status, which was validated in this study by decreased hematocrit levels and increased heart to body weight ratios, compared with those associated with animals fed FeA diets. The FeR

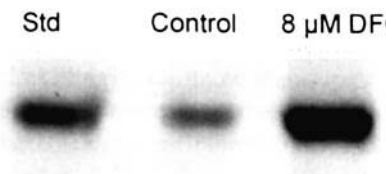
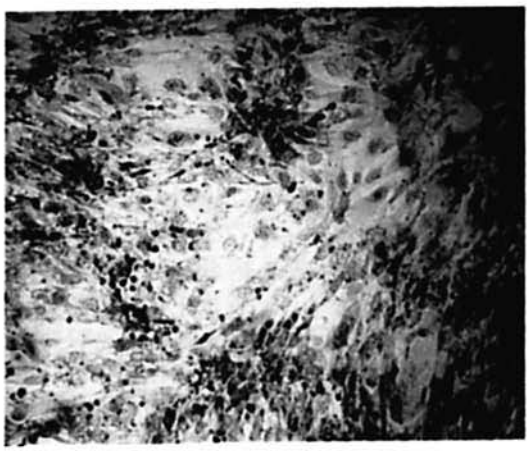


Figure 2. Image of Western blot of transferrin receptor fragment using 30 μg of protein/well of total cell lysates from control and deferoxamine (DFO)-treated (8 μ*M*) hFOB cells after 96 hrs of differentiation. Protein was separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane for probing with 1:1000 primary antibody against the transferring receptor fragment and a 1:1000 secondary antibody. The standard (Std) is the positive control. The DFO-treated cells have an enhanced band, indicating upregulation of the transferring receptor in response to iron chelation, an indication of iron-deficient cells.

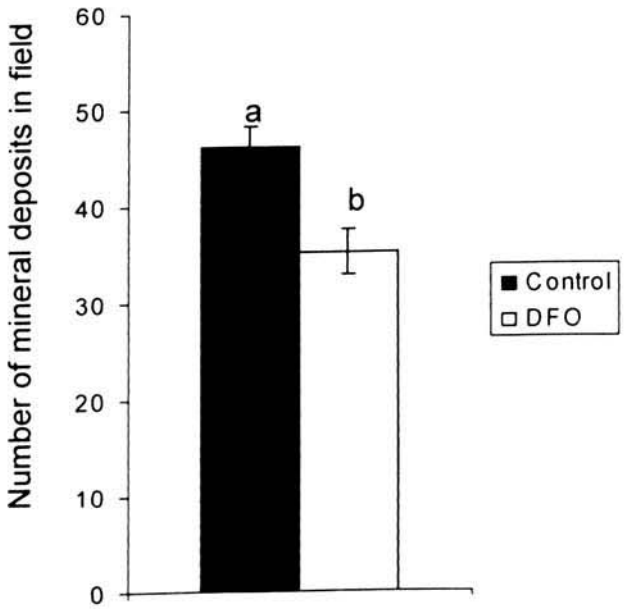
Figure 3. Representative images of Western blot for Type I collagen using 30 μg protein/well total cell lysates from control and deferoxamine (DFO)-treated (8 μ*M*) hFOB cells after 48 hrs of differentiation and treatment. Cells were collected from three passages in control-treatment pairs. Summary of densitometry analysis of Western blots revealed there were no significant differences ($P = 0.24$).

a) control

b) 8 μM DFO



c)



groups did not, however, have hematocrit levels reflective of severe iron deficiency anemia (hematocrits below 30), but they were low enough to lead to an increased heart to body weight ratio, which is indicative of volume overload hypertrophy. As expected, mineral composition of the long bones showed that iron restriction led to decreased iron concentrations. Also, in the CaA:FeR group, calcium levels were decreased compared to levels in the other three groups. This result parallels the findings in severe deficiency studies, in which tibias and femurs of Fe-deficient animals had lower calcium content (16, 17) than their Fe-adequate counterparts. It is well established that when iron stores are low, iron uptake into cells is improved through increased expression of transferrin receptors on cell surfaces (25–28). This increased expression and subsequent ability to bind more holotransferrin results in more iron being brought into the osteoblast, and it could possibly displace calcium.

Micro-CT imaging provided evidence that marginal iron and calcium restriction negatively influenced vertebral structure and strength, which parallels previous findings in deficiency studies (15, 16, 29). The combined restriction, however, had a more complex effect, which is in contrast to our earlier, more-overt findings (16). Overall, there were main iron effects that resulted in more porous and fragile bones. Finite element analysis of the L-4 vertebrae indicated a trend in that animals fed the FeR:CaA diet tended to require the least total force for complete compression and experienced the greatest internal stress when an applied force was simulated. This indicates that adequate calcium was not able to maintain bone quality if iron was restricted. Collagen in osteoporotic bone has fewer collagen cross-links (30), and decreased lysyl oxidase has been associated with weaker bones (30, 31). Perhaps inadequate iron affects prolyl and lysyl hydroxylase activity (18).

Previously, we reported that severe calcium restriction dramatically decreased whole-body BMD (15, 16) and BMC (16). In the current study we found that FeR affected whole-body BMC, and CaR affected whole-body BMD, but analyses of femurs and vertebrae frequently showed calcium and iron interactions with BMD and BMC. This inconsistency may be caused by a difference in sensitivity between the two DEXA scans used in this study.

Analyses of femur strength showed that calcium depletion significantly reduced strength, but even when calcium was adequate, strength was significantly reduced in femurs from FeR animals. It seems that iron helps impart strength to the bone. Iwamoto *et al.* (32) conducted a 10-

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Figure 4. Cell mineralization was decreased in hFOB cells as a result of iron chelation. (a) Representative control cells stained with Alizarin red after 48 hrs of differentiation; (b) representative deferoxamine (DFO)-treated cells (8 μ*M* DFO) stained with Alizarin red after 48 hrs of treatment and differentiation; and (c) summary of statistical analysis of calcium mineralization in control and treated cells after 48 hrs of treatment ($P < 0.05$).

week study with 0.1% dietary calcium and found decreased mineralization of tibial trabecular, but not cortical bone, of the Ca-deficient group (32). They also found increased mineralization in the periosteal region of the cortical bone in the calcium-depleted group, but there was no difference between the mineralization of the endosteal region (32). It is possible that there are compensatory actions occurring within various regions of the bone. Our data indicate that iron chelation decreased calcium mineralization of hFOB cells, which further indicates the existence of a complex but not yet well understood relationship between calcium and iron in bone.

Our cell culture study showed that while iron restriction did affect calcium mineralization of the hFOB cells, it did not affect the expression of Type I collagen at the time point used in this study. In the animal study, we found that there were times when the FeR:CaR group behaved similarly to the FeA:CaA group, and yet other analyses revealed that the FeR:CaR group was definitely impaired by dietary restriction. Additional research on prolyl and lysyl hydroxylase activity and Type I collagen integrity may help us better understand the role of iron in bone health.

The unique and unexpected response of the FeR:CaR group in not demonstrating increased severity of pathology compared to the other groups may be related to the ratio of calcium to iron present in the diet. The potential importance of this ratio has been investigated with conflicting results (33–37). Variability of sample sizes, length of study time, and the type of calcium supplement used may contribute to some of the conflict regarding the effect calcium has on iron absorption. In our FeR:CaR diet, calcium and iron may have been present in the diet and absorbed into the bloodstream in comparable proportion to when both nutrients were adequately present. In other words, each may compete with one another more effectively for absorption when one is present in adequate amounts and the other in marginal amounts. When both are present in marginal amounts, there is a possibility that a greater absorption of each could be obtained. This could explain some of the inconsistent results we have found in the doubly deficient group.

A limitation of this study could be the use of the AIN-76a diet, since this diet has previously been shown to cause nephrocalcinosis in female rats, presumably as a result of the low Ca:P molar ratio in the diet (38). Any kidney damage could alter bone in calcium, and phosphorus balance is impaired. However, in that study, the diets were maintained for 16 weeks, far beyond the 10 weeks used in our study.

An interesting observation reported here but observed by us in other iron-restriction studies is the graying of the black fur in the FeR-treated rats. We believe that this could be due to a decrease in activity of the iron-dependent phenylalanine hydroxylase to generate tyrosine for subsequent melanin synthesis (39). Phenylketonuria patients who lack this enzyme have hypopigmentation as one of their classical symptoms (40).

The restrictions of iron and calcium employed in this study may have implications for the female population during the years of peak bone mass accretion. This study demonstrates that the restriction of iron, calcium, or a combination of both affects the quality of the long bones and vertebrae less dramatically than has been seen in severe deficiency studies and in a manner more reflective of the long-latency pathogenic nature of osteoporosis (14, 15). Subsequent studies should examine the impact of graded levels of dietary iron on these bone variables to determine a threshold requirement for this nutrient. Decreased bone mineralization appeared to be a factor in the *in vivo* and *in vitro* studies reported here. The importance of iron intake and status upon human bone health needs to be pursued further, as it may be an overlooked factor of significant public health consequence.

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