Bone loss caused by iron overload in a murine model: importance of oxidative stress

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Introduction

Transfusion of red blood cells can be a life-saving therapy for patients with congenital or acquired anemias, such as the thalassemias, aplastic anemias, and myelodysplastic syndromes. Patients with sickle cell disease and severe vaso-occlusive complications may also be placed on regular transfusions. Each unit of transfused blood contains 220 to 250 mg of iron bound to hemoglobin, an amount much larger than the 1 to 2 mg of iron that is absorbed daily to maintain normal human iron homeostasis.1,2 Because there is no way of excreting iron in humans, chronic transfusions lead to progressive and pathologic iron accumulation. Nontransfusional iron overload can occur in conditions in which ongoing erythroid destruction leads to inappropriately high intestinal iron absorption. For example, iron overload is a feature in β-thalassemia intermedia, a condition for which most affected persons are not transfused. Other examples include congenital dyserythropoietic anemia and sideroblastic anemia. Finally, hereditary hemochromatosis, which is a genetic disorder characterized by high intestinal iron absorption, also results in increased tissue iron accumulation.1,3

Osteoporosis and fractures occur frequently in disorders associated with iron overload, such as the thalassemias4,5 and hereditary hemochromatosis.6 Whether this is caused by a direct effect of iron on bone or is the result of concomitant endocrine deficiencies, such as hypogonadism, ineffective erythropoiesis, other comorbidities that may affect bone metabolism, or the effect of chronic illness itself remains unclear.5,6 Recent limited animal and in vitro data support the idea that iron excess directly controls bone formation and/or remodeling.9,11

In this report, we developed a mouse model of iron overload in which C57/BL6 mice were injected with iron dextran. This approach resulted in increased iron content in various organs, including liver, spleen, and heart, similar to that seen in transfused patients with iron excess.12 We found that BL6 iron-overloaded mice have trabecular and cortical bone abnormalities that are related to the severity of tissue iron overload. In addition, our results ascertain the adverse role of iron in the development of low bone mass in states of iron excess through a process that involves increased oxidative stress (reactive oxygen species [ROS]), with accompanying increases in serum levels of the pro-osteoclastogenic cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6).

Methods

Animals

Groups (n = 8) of 2-month-old C57/BL6 male mice were treated intraperitoneally once a week for 2 months with low (0.1 g/kg) or high (1.0 g/kg) iron dextran [a complex of ferric hydroxide, Fe(OH)3] or placebo (phosphate-buffered saline). To evaluate the role of ROS, additional groups were given high-dose iron as above plus N-acetyl-L-cysteine (NAC; Sigma-Aldrich; 100 mg/kg per day) given subcutaneously 5 days/week.

At the time of death, blood was collected for measurement of serum cytokines, assayed by commercially available enzyme-linked immunosorbent assay (BioLegend). After death, one femur was stored in 90% ethanol and scanned by micro-computed tomography (micro-CT). The other femur was embedded in methyl methacrylate, and longitudinal 2-μm sections were mounted on barium fluoride (BaF2) infrared windows (Spectral Systems) for Fourier-transformed infrared spectroscopic imaging (FTIRI) analyses. Additional sections (5 μm) were used for histomorphometry. One tibia was fixed in 10% buffered formalin at 4°C, decalcified, and embedded in paraffin; 5-μm-thick deparaffinized sections were used for histology, whereas additional sections were stained for iron using the Perl Prussian method.
blue reaction. Liver, spleen, and cleaned vertebrae were collected for measurement of iron content. Liver, spleen, pancreas, and heart were also embedded in paraffin, sectioned, and stained with Pearl reaction for iron.

**Tissue and bone iron content**

Spleen, liver, and vertebral iron content was determined by atomic absorption spectroscopy. Tissue weight was determined and representative samples placed in termed porcelain crucibles. Samples were dried at 110°C overnight, dry weight was determined, after which they were ashed in a Muffle furnace at 550°C. The ash was dissolved in 3N HCl, and its total Fe content was determined by atomic absorption. Mineral content was calculated as the ratio of ash weight to dry weight.

**Micro-CT**

Densitometric and morphometric micro-CT analysis was performed on cortical bone from mid-diaphyses of femurs and trabecular bone from the distal third of femurs. Bones were analyzed on a Scanco mCT 35 (Scanco Medical) system using a 6-µm voxel size, 55 KVP, and 0.36 degrees rotation step. The Scanco mCT software (HP, DEC windows Motif, Version 1.6) was used for three-dimensional reconstruction and data processing. A global threshold of 0.4 g/cm³ was used.

Cortical and trabecular regions of interest (ROIs) were first defined on all volumes using a utility of the processing system. In the femurs, the ROIs extended from 50 µm proximally to the end of the distal growth plate more than 1.7 mm toward the diaphysis. Trabecular ROIs were free-hand drawn on sequential slices to include the endosteal envelope, conforming to the endosteal contour on each slice. Cortical ROIs were defined as 1.7-mm segments of the mid-diaphysis of the femurs. Cortical ROIs resulted from digital subtraction of the respective trabecular ROIs from the whole bone volume. Bone mineral density measurements were performed on cortical and trabecular volumes after segmentation of bone voxels using the global threshold, to only include bone tissue. Cortical and trabecular morphometry was performed after binarization by the threshold assigned to each system dataset. Cortical bone morphometry included the cortical periosteal or outer perimeter, the cortical endosteal or inner perimeter, cortical thickness and area, and marrow area. The morphometric parameters defining trabecular bone mass and microarchitecture included bone volume fraction, trabecular thickness, trabecular number, and trabecular spacing.

**FTIRI**

Spectral images of proximal femur sections were obtained by FTIRI using the PerkinElmer Spotlight Imaging system (PerkinElmer Life and Analytical Sciences). Cortical and trabecular bone were examined separately. The spectral resolution was either 4 or 8 cm⁻¹, whereas the spatial resolution was approximately 7 µm. Background spectra were collected under identical conditions from the same BaF₂ windows. After correcting for background and the presence of polymethyl methacrylate, spectra were transformed to yield images corresponding to infrared band areas, peak height ratios, and the presence of polymethyl methacrylate, spectra were transformed to yield images corresponding to infrared band areas, peak height ratios, and subtraction of the respective trabecular ROIs from the whole bone volume. The spectroscopic results were expressed as histograms describing the pixel distribution of the measured spectroscopic parameters. Means and SD of the pixel distributions and corresponding color-coded images were generated at the same time using ISYS software. Means were averaged for multiple sites in each animal and among the 6 different animals for each age and genotype using Microsoft Excel 2007.

**X-ray diffraction**

Vertebral bone from iron-overloaded and control animals were ground to a fine powder in a liquid nitrogen cooled mill (Spex Industries) and subjected to wide-angle X-ray diffraction on a Bruker AXS diffractometer with Ni-filtered Cu Kα radiation. Scans were run from 4° to 50° 2θ. The particle size in the c-axis direction was estimated by line-broadening analysis of the 002 peak (25.85° 2θ) using DIFFRACCT software where the full width at half-maximum is linearly related to the crystallite size and perfection (t) based on the Debye Scherrer equation.

**Bone histomorphometry**

Longitudinal femur sections were stained using the von Kossa method and Goldner trichrome as previously described. Measurements were performed in cancellous tissue of the proximal metaphysis beginning 1 mm from the central region of the growth plate to exclude the primary spongiosa and 500 µm from the endocortical margins to exclude modeling bone. The Bioquant program (Bioquant-True Color Windows, Advanced Image Analysis Software, sVGA Frame Grabber Image Processing Board and Optronics DEI-470 Video Camera) was used to collect the raw data. Trabecular bone volume (BV/TV), osteocyte surface, and osteoid surface for bone surface (BS) were measured. Osteocyte number per BS (no/mm²) and BS covered by osteoblasts corrected for total BS (percentage) were measured using the Bioquant program. Osteoclasts and osteoblasts were identified in paraffin-embedded longitudinal tibia sections by staining for tartrate-resistant acid phosphatase (TRAP) using a commercially available kit (Sigma-Aldrich) and by immunostaining with the procollagen type I antibody clone SP1.D8 (Developmental Studies Hybridoma Bank, University of Iowa), respectively. Active osteoclasts and osteoblasts were identified as TRAP or procollagen type I positive cells on the BS, respectively.

For dynamic histomorphometry, mice were injected with calcein (100 mg/kg) and xylene orange (90 mg/kg), respectively, at 7 and 3 days before death. Fluorochrome-based indices of bone formation were measured in unstained 5-µm longitudinal sections of distal femur. Double- and single-labeled perimeters were identified. Interlabel width of double-labeled regions was measured using the Bioquant program. The mineral apposition rate (MAR, µm/day) and bone formation rate (BFR/BS, µm²/µm²/day) were then determined.

**Quantitative reverse-transcription polymerase chain reaction**

RNA from crushed femurs of iron-overloaded animals and their controls was extracted after bone marrow was flushed out. Total RNA was prepared using TRIZol reagent (Sigma-Aldrich) according to the manufacturer’s instructions. First-strand cDNA was synthesized with a SuperScript First-Strand Synthesis System for reverse-transcription polymerase chain reaction using SuperScript II reverse transcriptase enzyme (Invitrogen). All reactions were performed in a Chromo4 thermocycler (MJ Research) using FastStart SYBR Green Master Mix (Roche Diagnostics). The primers used for expression analysis, cathepsin K (CTK; forward, 5′-CACAGAGTGTGGTGTCAGCTCA-3′ and reverse, 5′-ACACTGGCCCTGTTCCTGTGA-3′), receptor activator of nuclear factor-κ ligand (RANKL; forward, 5′-GGCCACACAGGCTTCTCAG-3′ and reverse, 5′-GAGTGACTTTATGGGAACCCCGAT-3′), osteoprotegerin (OPG; forward, 5′-AGTGCTGAAGCGTGGTGGAA-3′ and reverse, 5′-TGTCAGAGTGCGCCGAGAT-3′), osteopontin (OPN; forward, 5′-GAAACTCTTCAAGCCATTCT-3′ and reverse, 5′-GGACTAGCTTGTCCTTGTGG-3′), and mouse HPRT (forward, 5′-ATGTTGGATACAGGCCAGAC-3′ and reverse, 5′-GGACTAGCTTGTCCTTGTGG-3′) were purchased from Invitrogen. All primer pairs amplified a single band with the expected molecular weight as analyzed by agarose gel electrophoresis. The quantities of all samples were normalized to the mouse HPRT housekeeping gene.
Determination of oxidative stress (ROS)

Bone marrow was flushed from femurs of placebo and high-dose iron dextran-treated mice, and total protein was extracted. The carbonyl content of total protein, which reflects oxidized protein,19 was determined by Western blot using the OxiBlot Detection Kit (Chemicon International), normalized to β-actin.

Statistical analysis

Results are presented as mean plus or minus SD, each group including 8 animals. The differences between 2 groups (ie, high-dose iron overload vs placebo, placebo vs high-dose iron + NAC) were evaluated by Student t test. The differences among 3 groups (ie, placebo vs low-dose iron vs high-dose iron) were evaluated by one-way analysis of variance. Statistical analysis was performed using the STATA/IC software. Differences with P values less than .05 were considered significant.

Results

Bone abnormalities associated with iron overload

Administration of iron dextran (1 g/kg/wk) intraperitoneally for 2 months resulted in severe tissue iron overload. We first measured liver and spleen iron content because iron is cleared from plasma by spleen and liver macrophages.1 As expected, liver and spleen iron content were markedly increased (Figure 1A). In addition, we documented iron deposition in other tissues, such as pancreas and heart (supplemental Figure 1A, available on the Blood Web site; see the Supplemental Materials link at the top of the online article), similar to that observed in iron-overloaded humans.12 Although bone iron content was also increased in iron-treated mice compared with placebo (Figure 1A), the increase was not as pronounced as in liver and spleen. Pearl staining for iron of bone sections indicated that most of the metal was accumulated in the bone marrow, and not on the BS, and was present intracellularly in macrophage-like cells (supplemental Figure 1B).

To establish whether iron excess induces bone abnormalities, we performed micro-CT studies of the iron-overloaded mouse (1 g/kg/wk for 2 months) and its control. Micro-CT analysis of trabecular bone (distal femur) revealed a decreased bone volume fraction, decreased number of trabeculae and trabecular thickness, and increased trabecular spacing in iron-overloaded mice compared with placebo (Table 1; Figure 1B). Analysis of cortical bone (mid-diaphysis femur) in the iron-overloaded animals showed thinner cortices and decreased cortical area compared with controls (Table 1; Figure 1B).

FTIR analysis was undertaken to determine whether iron overload induced changes in bone composition, and the results are summarized in Table 2. Mineral-to-matrix and carbonate-to-amide I ratios were decreased in both cortical and trabecular bone of iron-overloaded animals, indicating changes in mineralization and an increase of the nonmineralized matrix. The carbonate-to-mineral ratio was decreased, reflecting increased bone turnover. No changes in collagen or hydroxyapatite structure were found.

Dynamic histomorphometry was then performed to determine changes in bone formation with iron. Based on fluorochrome-derived indices, MAR and BFR were calculated (Figure 2A; supplemental Figure 2) and were found unchanged with iron treatment, suggesting that osteoblast function was unaltered. Thus, we turned to static histomorphometry to obtain additional information on bone remodeling. Using the von Kossa method to stain the mineralized matrix, we observed decreased BV/TV in iron-overloaded animals (Figure 2A; supplemental Figure 2), a finding consistent with our micro-CT results. Bone resorptive surfaces were increased with iron (Figure 2A). In addition, osteoclasts were
identified as TRAP-positive cells on the BS, and their numbers were increased with iron dextran (Figure 2A; supplemental Figure 2; TRAP stain). Taken together, these results indicate increased resorption with iron excess. BS covered by active osteoblasts was identified using the procollagen I stain (Figure 2A; supplemental Figure 2). Consistent with the results of dynamic histomorphometry, no differences were found between treatment groups, indicating no changes in osteoblast number. Despite these last findings, we observed a significant increase in osteoid (Figure 2A; supplemental Figure 2), suggesting a mineralization defect.

To further evaluate bone turnover, mRNA from the femurs of iron-overloaded and placebo-treated animals was extracted after bone marrow was flushed. We observed increased expression of genes involved in bone resorption (CTK, OPG, and RANKL), associated with increased ROS and increased resorption.20,21 Both control animals, as these cytokines are elevated in other conditions3,12 and in iron overload,12 we determined the levels of ROS in bone marrow protein extracted from placebo and iron dextran-treated mice using the OxiBlot Detection Kit. The method detects the carbonyl content of proteins, and we observed increased carbonyl content in a number of bone samples from placebo and iron dextran-treated animals.

Bone abnormalities induced by iron overload correlate with an inflammatory process

Because iron was found to be abundant in the bone marrow of the iron-overloaded animals, and iron overload has been implicated in the development of increased ROS in other systems,3,12 we determined the levels of ROS in bone marrow protein extracted from placebo and iron dextran-treated mice using the OxiBlot Detection Kit. The method detects the carbonyl content of proteins, and the concentration of which correlates with ROS.19 An overlapping series of bands were present in the specimens from iron-overloaded animals, indicating increased carbonyl content in a number of bone marrow proteins consistent with increased ROS in the bone marrow of these mice (Figure 3A). We also measured TNF-α and IL-6 concentrations in the serum of low- and high-dose iron treated and control animals, as these cytokines are elevated in other conditions associated with increased ROS and increased resorption.20,21 Both serum TNF-α and IL-6 concentrations were increased with iron overload.

Table 1. Micro-CT analysis of mice treated with placebo, high-dose iron dextran (1 g/kg per week), and low-dose iron dextran (0.1 g/kg per week).

<table>
<thead>
<tr>
<th>FTIRI parameter</th>
<th>Placebo</th>
<th>High-dose iron</th>
<th>Low-dose iron</th>
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<tr>
<td>Mineral-to-matrix</td>
<td>7.7 ± 0.4</td>
<td>6.3 ± 0.7</td>
<td>.002</td>
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<tr>
<td>Carbonate-to-mineral</td>
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<td>0.006 ± 0.001</td>
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<td>Crystallinity</td>
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<td>.06</td>
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<td>Mineral-to-matrix</td>
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<td>Carbonate-to-matrix</td>
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<td>3.4 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Crystallinity</td>
<td>1.054 ± 0.014</td>
<td>1.050 ± 0.01</td>
<td>NS</td>
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</table>

Table 2. Changes in mineral parameters from iron overload.

<table>
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<th>Iron dextran</th>
<th>P</th>
</tr>
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Values are mean ± SD. NS indicates not significant.
overload (Figure 3B). Furthermore, the increase in serum cytokines occurred in a dose-dependent fashion related to the severity of iron overload.

Because our data indicate that iron overload in the mouse is associated with increased ROS, we sought to determine whether ROS are prime mediators of the bone abnormalities that we observed. Thus, we treated mice with placebo versus iron dextran (1 g/kg/wk given intraperitoneally) versus iron dextran (1 g/kg/wk given intraperitoneally) plus the antioxidant NAC (100 mg/kg/d, subcutaneously, 5 d/wk) for 2 months. The micro-CT analysis and pictures (shown in Figure 4) reveal that NAC administration largely prevented the bone abnormalities of iron overload, confirming that these changes are

![Figure 2](image1)

**Figure 2.** Iron-overloaded mice exhibit decreased bone mass, increased resorption, and increased deposition of osteoid. (A) First row: No changes in MAR and BFR by double-label technique in the iron-overloaded mice compared with placebo. Decreased BV/TV (von Kossa stain) with iron excess. Second row: Increased osteoclast surface (Goldner trichrome stain) and increased osteoclast numbers (n/mm) by TRAP in iron-treated mice. No changes were found in osteoblast numbers between placebo and iron-treated mice using stain for procollagen type I. Increased osteoid was present in the iron-treated animals. Data are mean ± SD. *P < .05. **P < .01. (B) In vivo expression of genes associated with bone resorption (CTK, OPG, and RANKL) in iron-overloaded mice. No changes in expression of OC and OPN. Data are mean ± SD. *P < .01.

![Figure 3](image2)

**Figure 3.** Iron overload induces inflammatory changes. (A) Western blot for protein carbonyl content from bone marrow of placebo and iron dextran-treated mice. An overlapping series of bands were present in the iron-overloaded animals, indicating increased carbonyl content in a number of bone marrow proteins consistent with increased ROS in the bone marrow of these mice. (B) Serum TNF-α and IL-6 concentrations in mice treated for 2 months with high-dose or low-dose iron dextran and placebo. Serum cytokines increase in a dose-dependent fashion. Data are mean ± SD. *P < .01.
mediated at least in part by ROS. In agreement with these observations, serum TNF-α and IL-6 in animals given the antioxidant were similar to those in controls (TNF-α: 98 ± 15 vs 193 ± 8 vs 112 ± 8 pg/mL, placebo vs iron vs iron + NAC; IL-6: 165 ± 12 vs 337 ± 12 vs 182 ± 11 pg/mL, placebo vs iron vs iron + NAC). As expected, liver and spleen iron content from animals treated with iron dextran + NAC was increased compared with placebo, with levels similar or even higher than the iron dextran mice (liver iron content 0.38 ± 0.15 vs 59.0 ± 7.5 vs 58.5 ± 8.2 μg/mg dry weight, placebo vs iron vs iron + NAC, P < .001; spleen iron content: 6.1 ± 3.5 vs 35 ± 9.08 vs 48 ± 12.3 μg/mg dry weight, placebo vs iron vs iron + NAC, P < .001).

Discussion

The mouse model of iron overload described in this study had a pronounced bone phenotype, with abnormalities occurring in a dose-dependent fashion. Changes included trabecular and cortical thinning and alterations in the material properties of the bone. They were associated with increased resorption and oxidative stress. The bone loss was largely prevented by treatment with the antioxidant NAC, which replenishes intracellular glutathione levels, a central component in the cellular defense against oxidative stress.22 The prevention of bone abnormalities with NAC supports the hypothesis that oxidative stress is involved in the pathogenesis of bone loss in iron excess.

Iron has been implicated in the development of osteoporosis in diseases characterized by iron overload, such as the thalassemias and hemochromatosis, although the evidence is not consistent.5-7,23,24 Studies in patients with such disorders have been complicated by sample size limitations and multiple disease covariants that may also have an effect on the bone. Iron status has been so far assessed by serum ferritin, which is subject to variability and may not accurately reflect the severity of iron overload,25-29 explaining some of the inconsistencies in clinical studies. Certainly, the use of recent magnetic resonance imaging technology to assess liver iron stores is expected to shed light on the role of iron on bone remodeling.

The study of osteoporosis in thalassemia, in particular, is quite complex. The exact role of ineffective erythropoiesis in regularly transfused patients is unclear. Low ascorbate concentrations have been frequently described in regularly transfused patients and may contribute to bone loss.30-32 Alternatively, our findings of unaltered collagen synthesis suggest that levels of ascorbate have not decreased to the point of inhibiting hydroxylation of key proline residues in collagen, a rate-limiting step in collagen synthesis.33 Finally, the iron chelator desferrioxamine has been associated with

Figure 4. Treatment with an antioxidant prevents the bone loss induced by iron overload. (A) Micro-CT analysis of mice treated with placebo, iron dextran alone, or iron dextran plus NAC for 2 months: placebo (）， iron dextran （）， and iron + NAC （）。 Data are mean ± SD. NS indicates not significant. *P < .01. (B) Micro-CT images of placebo, iron dextran, and iron dextran + NAC animals. Trabecular bone at distal femur and cortical bone at mid-diaphysis femur.
bone lesions that resemble those of rickets. These facts demonstrate the complexities of the clinical studies, some of which can be addressed by animal experiments, which allow direct manipulation of individual factors. The iron overload achieved in the mice used in this study resulted from parental administration of iron and mimics transfusional siderosis. Similar to what is seen in humans, the iron-overloaded mouse has iron accumulation in multiple tissues, including liver, spleen, and heart. Hepatic iron concentrations reflect total body iron stores, as liver is the main site of iron storage. As expected, we observed higher iron content in the liver compared with other tissues. However, hepatic iron concentrations were much higher in the iron-overloaded mouse than are measured in transfused patients. Species differences and length of exposure must be taken into account when considering the relationship between severity of iron overload and development of osteoporosis.

The concept that iron overload leads to increased oxidative stress and inflammation is not new. In iron-overloaded states, the binding capacity of transferrin, the major iron transport protein, is surpassed. The resulting increase in non–transferrin-bound iron has been associated with increased ROS. In addition, the intracellular storage capacity of ferritin is exceeded and the labile pool of intracellular iron is increased. This metabolically active iron catalyzes the formation of free radicals, which can lead to cell damage and eventually death. Indeed, a number of in vitro and in vivo studies link iron to the generation of oxidative species. With regards to the situation in humans, a growing body of literature comes from studies of iron-overloaded patients with thalassemia, in whom increased biomarkers of oxidative stress, increased serum cytokines, and alteration in lymphocyte subsets have been described.

Although the ineffective erythropoiesis of thalassemia itself has been associated with increased oxidative stress in these studies, there is also evidence that the excess iron burden is an additional culprit. Immune alterations have also been described in hereditary hemochromatosis. Two studies have described an association between increased bone resorption and serum cytokine in states of iron excess. To our knowledge, however, ours is the first study directly linking iron-induced oxidative stress to low bone mass. We also observed increased serum inflammatory cytokines and provided evidence that their production is also regulated by oxidative stress. Further studies are needed to determine whether proinflammatory cytokines mediate bone loss in this model.

The bone loss that was observed in our model was associated with increased resorption, which is most probably related to the presence of increased ROS. The proinflammatory cytokines TNF-α and IL-6, which were increased in the sera of iron-treated mice, increase osteoclast formation. Increased resorption is responsible for the bone loss seen in a wide range of conditions, including joint inflammation, estrogen withdrawal, and aging. ROS are known to activate NF-κB, a key factor involved in osteoclastogenesis. Increased resorption has also been described in patients with thalassemia and in iron-overloaded rats. In contrast, the literature on the effect of iron on osteoblastogenesis is limited and conflicting. A negative effect of iron on mineralization and bone formation has been described in vitro and in iron-overloaded pigs, whereas the opposite has been reported in rats. In our study, we did not observe a significant decrease in bone formation by dynamic histomorphometry.

Although our data provide evidence that increased oxidative stress leads to the described bone phenotype, it is feasible that iron, as a heavy metal, may also act directly on bone. Aluminum toxicity, for example, has been reported to cause mineralization defects similar to rickets. Indeed, we found that the bone iron content was increased in the iron-overloaded animals compared with controls. Both our histology, showing a large increase of deposited nonmineralized osteoid, and FTIRI data indicate a defect in mineralization that supports the idea of a direct effect of iron on specific aspects of osteoblast function, an area in which we provide no insights. Our studies, using FTIRI and x-ray powder diffraction to better understand changes in bone chemical properties with iron, failed to detect changes in hydroxyapatite crystal size and perfection.

There are some limitations to our study that must be indicated. First, the interactions between the immune system and bone constitute the focus a new rapidly growing field of osteoimmunology. Results must be considered in light of the expanse of information being developed. It is already known, however, that cytokines, such as TNF-α and IL-6, mediate bone loss primarily by increasing bone resorption. There is additional evidence that TNF-α mediates bone loss in cases of increased oxidative stress. The second limitation is that our controls did not receive dextran because the iron dextran that was used in our experiments contained dextran of low molecular weight (~ 5000) a nonimmunogenic form that was not available for injection into the controls. This is in contrast to the high molecular weight dextrans, which are immunogenic and are part of certain iron dextran preparations.

Finally, in terms of the antioxidant studies, treatment of iron-overloaded mice with NAC showed a different response of the trabecular versus cortical bone. The alterations of the trabecular bone were completely rescued with NAC, whereas the cortical bone continued to demonstrate abnormalities. Cortical and trabecular bone are known to have differential turnover, which may explain these results. Higher doses of NAC or its daily administration instead of 5 days per week, as done in other animal studies, may have yielded comparable results. Regardless, this is the first experiment to suggest an alternative or an adjunctive therapy to iron chelation in iron-overloaded states and carries the promise of significant translational applications.

In conclusion, taking the literature and our results into account, we propose a model where iron excess leads to increased oxidative stress. Oxidative stress then induces inflammatory changes, again in a dose-dependent fashion, which then mediate bone loss through changes in bone remodeling. Further studies are needed to confirm this model in animals as well its relevance in humans. Consistent with the proposed model of systemic inflammation driving the bone abnormalities in the iron-overloaded mouse, iron was primarily localized intracellularly in the bone marrow cavity instead of on the BS or within the bone.

Acknowledgments

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Authorship

Contribution: J.T. performed research in the areas of micro-CT and FTIRI analysis, analyzed data, and contributed to the writing of the paper; Z.Y., R.C., and H.L. performed research in the areas inflammation; F.P.R., P.M.-K., P.J.G., and A.L.B. participated in the
study design; S.C.-R. contributed to the study design in the area of inflammation; S.B.D. performed the bone histology and contributed to the study design; R.W.G. measured tissue iron measurements and contributed to the study design; and M.G.V. designed and performed research, analyzed data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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