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ORIGINAL CONTRIBUTION

Severe nutritional iron-deficiency anaemia has a negative effect on some bone turnover biomarkers in rats

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Abstract
Background The role of iron (Fe) in bone formation and disease have not received much attention, a fact that is interesting given the known biochemical role that this mineral has upon collagen maturation together with the high prevalence of Fe-deficiency anaemia worldwide.
Aim To investigate the changes in bone formation, resorption and mineral content in developing rats with induced nutritional Fe-deficiency anaemia.
Methods Thirty male Wistar rats were divided into two groups, a control group receiving AIN-93G diet with normal-Fe content and an anaemic group receiving AIN-93G diet with low-Fe content for 40 days. Both diets were prepared with an adequate calcium (Ca) and phosphorus (P) content. The most representative serum bone turnover biomarkers and femur and sternum calcium and phosphorus content, together with sternum Fe content were determined in both experimental groups.
Results In anaemic rats, bone matrix formation diminished as revealed by the lower amount of procollagen type I *N*-terminal propeptide. Bone resorption process increased in Fe deficiency as shown by the increase of serum parathyroid hormone, tartrate-resistant acid phosphatase and levels of degradation products from C-terminal telopeptides of type I collagen released to the serum. In addition, mineralization process was affected by Fe deficiency, because Ca and P content in femur decreased markedly.
Conclusions Fe-deficiency anaemia had a significant impact upon bone, affecting bone mineralization, decreasing the matrix formation and increasing bone resorption, therefore it is of great interest to assess bone status in situation of Fe-deficiency anaemia.

Keywords Iron-deficiency anaemia · Bone status · Rats

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Introduction

The role of (Fe) in bone metabolism has received little attention, and its mechanisms of action still remain unclear. Previous studies [1, 2] reported that Fe-deficient rats had decreased femur mineralization that was accompanied by higher levels of cortisol and parathyroid hormone, increasing bone fragility.

Dietary Fe deficiency causes not only anaemia but also several other health problems. Recently, some researchers have examined the relationship between dietary Fe intake and bone metabolism. Medeiros et al. [3–5] hypothesized that Fe exerts its influence on bone through collagen synthesis. Fe is a required cofactor for prolyl and lysyl hydroxylase enzymes, and this step is essential for lysyl oxidase activity, which then catalyses cross-linking of adjacent collagen fibres. In Fe deficiency, there may be less Fe available to the prolyl and lysyl hydroxylase enzymes, which could result in decreased cross-linking activity and, subsequently, weaker collagen fibres [6]. Previous studies of Fe deficiency in rats found significantly decreased growth rates compared with Fe adequate [3–5].

Furthermore, renal 25-hydroxyvitamin D 1-hydroxylase, which converts 25-hydroxyvitamin D into the active form of vitamin D, is a system that involves a flavoprotein, an iron-sulphur protein, and a cytochrome P-450 [7]. Therefore, in Fe-deficiency anaemia, these Fe-dependent enzymes might become inactive and abnormal metabolism of collagen and vitamin D might occur.

Fe-deficiency anaemia is characterized by the reduction or absence of Fe stores, low serum concentrations of Fe and haemoglobin (Hb), haematocrit reduction and increased platelets count [1]. It is not clear why the role of Fe in bone formation and disease has not received much attention given the known biochemical role that Fe has upon collagen maturation and the high prevalence of Fe-deficiency anaemia [4].

In previous studies, some mechanisms by which Fe deficiency induced bone loss were hypothesized; however, the details remain unclear. To investigate the changes in bone formation and resorption in rats fed Fe deficient diet compared with the bone metabolism of healthy rats, different markers of bone mineralization and bone turnover were measured. Serum concentration of 25-hydroxyvitamin D, which is considered to be the most reliable measure of overall vitamin D status and because of its importance in Ca and P metabolism [8] and serum parathyroid hormone as indicator of Ca trafficking between the bone compartment and plasma were determined. Aminoterminal propeptide of type I procollagen (PINP) as a biomarker of bone formation [9] and tartrate-resistant acid phosphatase (TRACP 5b) [10–14] and degradation products from C-terminal telopeptides of type I collagen as biomarkers of bone resorption [15] were also measured. In previous studies, we hypothesized that Fe deficiency could induced bone loss; however, the details and mechanisms remain unclear. Therefore, the aim of this study was to assess the manner in which dietary Fe deficiency may affect bone metabolism, assessing not only bone mineral content but also the major bone turnover biomarkers.

Materials and methods

Chemicals

Casein, vitamins, minerals, micronized cellulose, sucrose, choline chloride, L-cystine and methionine were purchased from Farmusal Chemical (Granada, Spain) and were of high purity food grade. Sodium pentobarbital was purchased from Sigma Diagnostics, (St. Louis, MO. USA). Analytical grade reagents were used, together with ultrapure water of 18 MΩ/cm specific resistivity obtained by the Milli-Q purification system (Millipore Corp., Bedford, MA, USA).

Animals

Male Wistar albino rats recently weaned, 21 days old, ($n = 30$) purchased from the University of Granada Laboratory Animal Service were used for this study. Animal care procedures and experimental protocols were approved by the Ethics Committee of the University of Granada in accordance with the European Community guidelines.

Experimental design and diets

Thirty male Wistar rats were divided into two groups, a control group received AIN-93G diet with normal-Fe content (45 mg/kg diet) [17] and an anaemic group received AIN-93G low-Fe content (5 mg/kg diet) [16] diet for 40 days (Table 1). Both diets were prepared with an adequate Ca (5,000 mg/kg diet) and P (3,000 mg/kg diet) content. After analysis, the Ca, P and Fe content in the diets were as follows: 5,132 mg Ca/kg diet, 3,075 mg P/kg diet and 44.7 mg Fe/kg diet in the normal-Fe diet; 5,146 mg Ca/kg diet, 3,100 mg P/kg diet and 6.3 mg Fe/kg diet in the low-Fe diet.

Animals were placed in individual metabolic cages in an environmentally controlled room with a constant temperature of 22 ± 1 °C, a 12 h light–dark cycle and 55 ± 10% humidity. Diet and mineral-free water were available ad libitum. On day 40, after fasting overnight, the rats were weighed and anesthetized by an intraperitoneal injection of 5 mg of sodium pentobarbital/100 g of body weight (Sigma Diagnostics, St. Louis, MO. USA). After median laparotomy, the rats were totally bled out by cannulation of the abdominal aorta; one aliquot of blood was collected using EDTA as anticoagulant for haematological analysis

Table 1 Composition of experimental diets

Component	g/kg diet (dry weight)
Protein (casein)	210
Fat (olive oil)	100
Fibre (micronized cellulose)	51
Mineral supplement[a]	36
Vitamin supplement[b]	10
Choline chloride	2
Wheat starch	538
Sucrose	100
L-cystine	3.0
Energy (kJ/kg)	17,940

[a] The mineral premix was prepared according to the recommendations of the American Institute of nutrition for control rats (normal-Fe: 45 mg/kg diet) [17] and low-Fe (5 mg/kg diet) for anaemic rats [16]

[b] The vitamin premix was prepared according to the recommendations of the American Institute of nutrition [17] for growing rats

and the remaining blood was used to obtain serum which was separated by centrifugation at 1,500×g for 15 min at 4 °C and stored at −40 °C to measure ferritin, transferrin saturation, total iron binding capacity (TIBC) and bone turnover parameters such as aminoterminal propeptides of type I procollagen (PINP), 25-hydroxyvitamin D, tartrate-resistant acid phosphatase (TRACP 5b), degradation products from C-terminal telopeptides of type I collagen and parathyroid hormone (PTH). Finally, the sternum and femur were removed immediately and stored at −40 °C until further analysis of mineral content.

Dry matter

Water content in the diet, femur and sternum was determined by drying the material at 105 ± 2 °C until the weight remained constant (~48 h).

Ca, P and Fe measurement

The concentration of Ca and Fe in the diet and bones was determined by atomic absorption spectrophotometry (PerkinElmer AAnalyst 1100B spectrometer with WinLab32 for AA software, Massachusetts, USA). The concentrations of P were analysed by visible spectrophotometry (Perkin-Elmer UV/VIS spectrometer lambda 16, Massachusetts, USA) using the Fiske and Subbarow technique [18]. The samples had been previously mineralized by wet method in a sand bath (J. R. Selecta, Barcelona, Spain). Samples of skimmed milk powder and lyophilized bovine liver (certified reference materials CRM 063R and BCR 185; Commission of the European Community Bureau of Reference, Brussels, Belgium) were simultaneously used to test the Ca recovery (skimmed milk powder) and Fe recovery (bovine liver) (Ca value = 13.88 ± 0.10 mg/g; Fe value = 210 ± 3.0 mg/kg, mean, SEM of five determinations, certified values: Ca = 13.49 ± 0.10 mg/g; Fe = 214 ± 5.0 mg/kg).

Haematological test

Haemoglobin concentration (Hb), red blood cells (RBC), mean corpuscular volume (MCV), haematocrit and platelets of fresh, blood samples treated with EDTA as anticoagulant were measured using an automated haematology analyzer Sysmex K-1000D (Sysmex, Tokyo, Japan).

Serum ferritin

Serum ferritin concentration was determined using the Rat Ferritin ELISA Kit (Biovendor Gmbh, Heidelberg, Germany). The absorbance of the reaction mixtures was read at 450 nm using a Bio-Rad microplate reader (Bio-Rad Laboratories Inc., California, USA). Colour intensity developed was inversely proportional to the concentration of serum ferritin.

Serum iron, total iron binding capacity (TIBC) and transferrin saturation

To calculate the rate of transferrin saturation, serum Fe concentration and TIBC were determined colorimetrically and enzymatically using Sigma Diagnostics Iron and TIBC reagents (Sigma Diagnostics, St. Louis, MO, USA). The absorbance of samples was read at 550 nm on a Bio-Rad microplate reader (Bio-Rad Laboratories Inc., California, USA). The rate of transferrin saturation was subsequently calculated using the following equation:

$$\text{Transferrin saturation } (\%) = \text{serum Fe concentration } (\mu g/L)/\text{TIBC } (\mu g/L) \times 100.$$

25-hydroxyvitamin D

The 25-hydroxyvitamin D was measured using the 25-hydroxyvitamin D enzymeimmunoassay (Immunodiagnostics System Ltd, Boldon, UK) from serum samples. The absorbances of the reactions were read at 450 nm using a Bio-Rad microplate reader (Bio-Rad Laboratories Inc., California, USA). Colour intensity developed was inversely proportional to the concentration of 25-hydroxyvitamin D.

Aminoterminal propeptides of type I procollagen (PINP)

PINP was determined in the Rat/Mouse PINP enzymeimmunoassay (Immunodiagnostics System Ltd, Boldon, UK) from serum samples. The absorbances of the stopped reactions were read at 450 nm using a Bio-Rad microplate reader (Bio-Rad Laboratories Inc., California, USA). Colour intensity developed was inversely proportional to the concentration of PINP, the most specific and sensitive marker of bone formation [19].

Tartrate-resistant acid phosphatase (TRACP 5b)

TRACP 5b was measured in rat serum using The RatTRAP^{TM} assay (Immunodiagnostics System Ltd., Boldon, UK) which uses a highly characterized, specific monoclonal antibody prepared using baculovirus generated recombinant rat TRACP as antigen [20]. The absorbances of the reactions were read at 405 nm in a microplate reader (Bio-Rad Laboratories Inc., California, USA). Colour intensity was directly proportional to the activity of TRACP 5b present in the sample.

Degradation products from C-terminal telopeptides of type I collagen

Type I collagen accounts for more than 90% of the organic matrix of bone and is synthesized primary in bone [15]. During renewal of the skeleton, bone matrix is degraded and consequently fragments of type I collagen are released into circulation. These fragments were measured using a RatLaps™ enzymeimmunoassay (Immunodiagnostics System Ltd, Boldon, UK) from serum samples. The absorbance was read using a Bio-Rad microplate reader (Bio-Rad Laboratories Inc., California, USA), which is inversely related to concentration of RatLaps antigens in the sample.

Parathyroid hormone (PTH) determination

Serum PTH was measured using a Rat PTH immunoradiometric assay kit (Alpco Diagnostics, Salem, Massachusetts, USA). The radioactivity was measured in a Wizard automatic gamma counter (Perkin Elmer, Waltham, Massachusetts, USA). The radioactivity of the antibody complex bound to the bead is directly proportional to the amount of rat PTH in the sample. A standard curve is generated by plotting the counts per minute versus the respective rat PTH concentration for each standard on logarithmic scales. The concentration of PTH in the samples was determined directly from this curve.

Statistical analysis

SPSS version 15.0, 2008 (SPSS Inc., Chicago, IL, USA) software has been used for data treatment and statistical analysis. Data are expressed as mean values with their standard errors of 15 rats per group. Student's t test for independent samples was used to determine significant differences between control vs. anaemic groups. A level of $P < 0.05$ was considered to indicate statistical significance.

Results

As expected, dietary Fe restriction for 40 days drastically impaired haematopoiesis, affecting all the haematological parameters in the anaemic group. Hb, RBC, MCV, haematocrit, serum ferritin, serum Fe and the percentage of transferrin saturation were low ($P < 0.001$), and platelets and TIBC increased markedly ($P < 0.001$), all consistent with severe anaemia (Table 2). Meanwhile, in the control rats, all haematological parameters studied were within normal limits for this species [1]. The food intake was lower in the anaemic group compared with their counterparts controls (16.6 ± 0.6 g/day in control rats versus 15.1 ± 0.4 g/day in anaemic rats) ($P < 0.05$).

Table 2 Haematological parameters in control and anaemic rats

	Normal-Fe Control group ($n = 15$)	Low-Fe Anaemic group ($n = 15$)
Hb (g/L)	126.5 ± 2.5	75.4 ± 2.3*
RBC (10^{12}/L)	7.0 ± 0.16	6.4 ± 0.18*
MCV (fl)	55.3 ± 0.2	39.1 ± 0.6*
Haematocrit (%)	39.0 ± 0.78	27.2 ± 0.45*
Platelets (10^9/L)	735 ± 25.5	1354 ± 65.6*
Serum ferritin (μg/L)	82.6 ± 2.6	50.3 ± 1.3*
Serum Fe (μg/L)	1380 ± 120	700 ± 57*
TIBC (μg/L)	2825 ± 199	17787 ± 735*
Transferrin saturation (%)	47.1 ± 7.0	3.6 ± 0.3*

Values are mean ± SEM

Fe iron, *RBC* red blood cells, *MCV* mean corpuscular volume, *TIBC* total iron binding capacity

* Values were significantly different ($P < 0.001$) from the control group by Student's *t* test

The bone mineralization study revealed that Ca and P content in sternum were unchanged in anaemic group versus control group; however, as it was expected by the consumption of a low-Fe diet during 40 days, the sternum Fe deposit diminished 21% ($P < 0.001$). In relation to Ca and P content in femur are lower for the anaemic group ($P < 0.001$) in comparison to the control group (41 and 24.6% respectively) (Table 3).

With regard to bone matrix formation biomarker, it has found a substantial decrease ($P < 0.001$) in the PINP released to blood, revealing that bone turnover has been impaired in anaemic rats (Table 4). Not only serum PTH, but also TRACP 5b levels were increased in the anaemic group compared to their respective controls ($P < 0.001$ for both biomarkers). Moreover, levels of degradation products from C-terminal telopeptides of type I collagen increased

Table 3 Mineral content in sternum and femur of control and anaemic rats

	Normal-Fe Control group ($n = 15$)	Low-Fe Anaemic group ($n = 15$)
Sternum		
Ca (mg/g DM)	76.59 ± 2.42	75.91 ± 2.73
P (mg/g DM)	56.22 ± 2.10	54.87 ± 2.89
Fe (μg/g DM)	77.65 ± 4.35	61.90 ± 2.85*
Femur		
Ca (mg/g DM)	168.3 ± 3.5	99.4 ± 3.7*
P (mg/g DM)	122.8 ± 2.6	92.6 ± 2.5*

Values are mean ± SEM

* Values were significantly different ($P < 0.001$) from the control group by Student's *t* test

Table 4 Bone turnover biomarkers in control and anaemic rats

	Normal-Fe Control group ($n = 15$)	Low-Fe Anaemic group ($n = 15$)
PINP (ng/mL)	6.16 ± 0.20	4.48 ± 0.28**
C-terminal telopeptides of type I collagen (ng/mL)	30.14 ± 1.97	38.51 ± 3.30*
TRACP 5 b (UI/L)	0.51 ± 0.01	0.72 ± 0.08**
PTH (pg/mL)	12.98 ± 0.51	17.64 ± 1.16**
25 hydroxyvitamin D (nmol/L)	101.2 ± 8.6	105.4 ± 5.4

Values are mean ± SEM

Fe iron, *PINP* aminoterminal propeptides of type I procollagen, *TRACP 5 b* tartrate-resistant acid phosphatase, *PTH* parathyroid hormone

* Values were significantly different ($P < 0.05$) from the control group by Student's *t* test

** Values were significantly different ($P < 0.001$) from the control group by Student's *t* test

($P < 0.05$), revealing that bone status was negatively affected in the anaemic group. However, no differences were found in 25-hydroxyvitamin D levels between both experimental groups (Table 4).

Discussion

After Fe deprivation (5 mg/kg of diet) during 40 days, all the haematological parameters in the experimental group were different from those of the controls, due to progressive Fe depletion from body stores as it is reflected in the lower Fe content in sternum of anaemic rats, fact that influences adversely the haematopoietic process in this group of animals and confirms the severe degree of Fe deficiency as showed previously [1]. The food intake was lower in the anaemic animals, but because the rats were recently weaned at the beginning of the Fe-deficiency induction, diet was supplied ad libitum during 40 days to avoid interferences in growing and development of the animals. Moreover, differences in the food intake were just slightly significant, reason why we thought that this fact should not affect to a great extent bone mineralization because Ca and P intakes differences were not too apparent.

In relation to bone mineral deposit, Ca and P content in the femur of Fe-deficient rats were lower from their counterparts control, which is in accordance with Campos et al. [1, 2] and constituted the precedent which induced us to deepen in the study of bone turnover as a dynamic and global process. Moreover, it is noteworthy that it was observed an important bone fragility when the femur were removed.

In our study, it were observed important changes in bone metabolism due exclusively to severe Fe-deficiency anaemia, meanwhile, the studies of Medeiros et al. [3–5] reported that Fe-deficiency has significant impact upon bone mineral density, content and fragility in young rats, but in this case was due not only to Fe limitation but also to the Ca restriction in the diet.

An important step in the bone formation process is synthesis of type I collagen, which is the major organic component in bone matrix. During collagen synthesis, propeptides are released from both the N- and C-terminal parts of the procollagen molecule [9]. Fractionated products of this process are procollagen type I C-terminal propeptide (PICP) and PINP and these peptides indicate the amount of bone matrix developed [21]. At present, the most sensitive markers for bone formation is the PINP [19]. The noticeably decrease in this bone formation biomarker revealed that anaemic rats had an important bone mineralization impairment induced by Fe-deficiency, together with growth retardation as it was observed previously [1]. Several studies from Medeiros et al. [3–5] have reported that Fe exerts its influence on bone turnover by affecting type I collagen synthesis and maturation. Fe is a cofactor for prolyl and lysyl hydroxylases, enzymes that catalyse an ascorbate-dependent hydroxylation of prolyl and lysyl residues, essential steps prior to crosslinking by lysyl oxidase [6]. Therefore, Fe deficiency diminishes the amount of Fe available, leading to a diminishing in crosslinking of type I collagen which could result in decreased crosslinking activity and, subsequently, weaker collagen fibres.

Bone remodelling is a series of complex processes of bone matrix formation, mineralization, and its resorption performed by the three types of bone cells. High amount of tartrate-resistant acid phosphatase (TRACP) is expressed by bone-resorbing osteoclasts and activated macrophages [10]. Two forms of TRACP circulate in blood, known as TRACP 5a and TRACP 5b [11]. TRACP 5b is derived from osteoclasts and TRACP 5a from inflammatory macrophages [22, 23]. Osteoclasts secrete TRACP 5b into the blood circulation as an active enzyme that is inactivated and degraded before it is removed from the circulation. Irie et al. [24] proposed a functional correlation of the TRAP activity in osteocytes with osteocytic osteolysis. Bonucci et al. [25] noted enhanced TRAP reactions in the osteocytes in Ca-depleted rats and suggested a correlation between the TRAP activity of osteocytes and the Ca levels in the body fluid. Recent studies have shown that secreted TRACP 5b indicates the number of osteoclasts rather than their activity [12–14], so it could be concluded that the increase in TRAPC 5b found under our experimental conditions in anaemic rats indicates an increase in the number of osteoclasts, accelerating the increase of the resorption process. Moreover, it has been found slightly

increased levels of degradation products from C-terminal telopeptides of type I collagen in the Fe deficient group. As mentioned above, Fe deficiency would lead to the reduction in crosslinking of type I collagen [6], and this would resulted in increased degradation of C-terminal telopeptides of type I collagen, weaker collagen fibres and increased bone fragility. These facts reveal once more that bone tissue is negatively affected in the anaemic group, which was directly related with the increased bone fragility found to remove the femur at the end of the study, compared with their counterparts controls.

Hydroxylase enzymes require both ascorbate and Fe to function properly. The results from the present study revealed that 25-hydroxyvitamin D was not affected by the Fe-deficiency; however, it may suggest that exists a decreased hydroxylation of 1,25-dihydroxylcholecalciferol, the active form of vitamin D, resulting in a diminishing of Ca absorption, because the 25-hydroxycholcalciferol-1-hydroxylase is located within the renal mitochondria and the enzyme is a three-component system: a flavoprotein, an Fe-sulphur protein and a cytochrome P-450 [7]. A lack of Fe could have an impact on this enzyme system, leading to decreased renal reabsorption of Ca as well as decreased intestinal Ca absorption when Fe is limiting [5]. The increase in serum levels of PTH contributes to the lower Ca and P deposit in femur of anaemic rats as it was mentioned previously. Moreover, the weakness of the collagen type I fibres lead to a decrease in the mineralization process, because hydroxyapatite crystals might not be deposited properly when the collagen fibres does not bring together the suitable characteristics of rigidity, flexibility and resistance [26].

In addition, other potential mechanisms through which Fe deficiency could affect bone metabolism would be at least partly modulated by the bone morphogenetic protein (BMPs) pathway. The signalling pathways involving BMPs are important in the development of cartilage, as well as post-natal bone development. In this sense, the BMPs induce hepcidin transcription [27], which is impaired by the Fe deficiency. Moreover, it has to be taken into account the possibility that severe Fe deficiency is a pro-inflammatory state in the affected organism and inflammatory cytokines produced in this situation are very strong inducers of hepcidin [28].

In conclusion, the present study indicates that severe Fe-deficiency anaemia has a significant impact upon bone status, decreasing bone formation and increasing its resorption.

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Conflict of interest The authors have no conflict of interest.

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