Goat milk during iron repletion improves bone turnover impaired by severe iron deficiency

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ABSTRACT

The effect of goat or cow milk-based diets, with either normal Fe content or an Fe overload, on bone turnover and the mineralization process was studied in control and anemic rats during chronic Fe repletion. One hundred eighty male Wistar rats were studied during a pre-experimental period of 40 d in which they were randomly divided into 2 groups, a control group receiving the AIN-93G diet with normal Fe content (45 mg/kg of diet) and the Fe-deficient group receiving the AIN-93G diet with low Fe content (5 mg/kg of diet) for 40 d. After the pre-experimental period, the rats were fed for 10, 30, or 50 d with goat or cow milk-based diets with a normal Fe content (45 mg/kg of diet) or an Fe overload (450 mg/kg of diet). In anemic rats, goat milk with normal Fe content increased levels of the biomarker of bone formation N-terminal propeptides of type I procollagen and diminished parathyroid hormone levels after only 10 d of supplying this diet, indicating the beginning of restoration of the bone demineralization induced by the anemia, which was not observed with cow milk. After 30 d of supplying the milk-based diets with normal Fe content or an Fe overload, biomarkers of bone formation and bone resorption were not different between control and anemic rats, indicating that the bone demineralization induced by the Fe-deficiency anemia had recovered, although the process of stabilization of bone turnover began earlier in the animals fed goat milk. In addition, a higher Ca deposit was observed in femur, which positively affects bone mineralization, as well as an increase of Fe in sternum, which indicates that the hematopoietic process essentially recovered earlier on the goat milk diet compared with the cow milk diet.

Key words: goat and cow milk, iron deficiency anemia, iron repletion, bone turnover

INTRODUCTION

Iron-deficiency anemia is characterized by the reduction or absence of Fe stores, low serum concentrations of Fe and hemoglobin, hematocrit reduction, and increased platelet counts (Campos et al., 1998). Iron overload is a frequent consequence of treatment for anemia and is less common than Fe deficiency (Parkkila et al., 2001). On the other hand, 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 anemia (Medeiros et al., 2002).

Previous studies (Campos et al., 1998, 2007) reported that Fe-deficient rats had decreased femur mineralization that was accompanied by higher levels of cortisol and parathyroid hormone (**PTH**), increasing bone fragility. Moreover, Katsumata et al. (2009) reported that severe Fe deficiency suppressed both bone formation and resorption, facts that encouraged us to extend our study of bone turnover. Some researchers have examined the relationship between dietary Fe intake and bone metabolism. Medeiros et al. (1997, 2002, 2004) hypothesized that Fe exerts its influence on bone through collagen synthesis. Iron is a required cofactor for prolyl and lysyl hydroxylase enzymes, and this mineral is essential for lysyl oxidase activity, which then catalyzes cross-linking of adjacent collagen fibers. In Fe deficiency less Fe may be available to the prolyl and lysyl hydroxylase enzymes, which could result in decreased cross-linking activity and, subsequently, weaker collagen fibers (Tuderman et al., 1977).

Most of the Ca in the body is stored in the bone and is found in serum in 2 forms: ionized and ready for use or not ionized and bound to protein (Campos et al., 2007). The maintenance of Ca concentrations is managed through hormonally coordinated action. Parathyroid hormone increases blood concentrations of Ca, stimulating production of the biologically active form of vitamin D_3, 1,25-dihydroxycholecalciferol [1,25 $(OH)_2D_3$], in the kidney. This hormone favors mobilization of Ca from bone and maximizes renal tubular reabsorption of Ca. Moreover, vitamin D facilitates Ca

absorption from the small intestine and enhancing its fluxes out of bone.

Bone is a connective tissue that consists essentially of a mineralized extracellular matrix and specialized cells. The principal organic component of the matrix is the type I collagen, which represents 90%; the remaining 10% is composed of a series of nonstructural proteins of minor size (Burgeson, 1988). The inorganic phase is composed of minuscule crystals of hydroxyapatite, inserted in the collagen fibers, allowing this form of stored Ca to be rapidly liberated in case of need when intake is inadequate. These elements play a crucial role in numerous physiological functions. The homeostasis of these minerals is regulated by mechanisms that control the formation and resorption of the bone tissue, processes that can be altered by different environmental, nutritional, genetic, and endocrine factors, giving place to abnormal compositions or pathologies of the bone (Ehrlich and Lanyon, 2002).

The interaction existing between Ca and Fe is well known. Divalent metal transporter-1, the principal mechanism by which nonheme Fe is taken up at the intestinal brush border, is shared also by Ca. Therefore, Ca acts as a low-affinity noncompetitive inhibitor (but not a transported substrate) of divalent metal transporter-1, explaining the inhibitory effect of dietary Ca on Fe bioavailability (Shawki and Mackenzie, 2010).

Milk has a high Ca bioavailability and therefore can affect in a positive way the process of bone mineralization (Campos et al., 2007). Goat milk improves Fe bioavailability in anemic rats, increasing Fe deposits in target organs and favoring the recovery of hematological parameters. Goat milk fat is richer in medium-chain triglycerides, which are rapidly absorbed and metabolized to obtain energy increasing the synthesis of carrier proteins and thus the absorption of Fe. Goat milk has higher contents of vitamin A and C, which would favor once again Fe absorption (Alférez et al., 2006). In addition, goat milk consumption improves the digestive and metabolic utilization of Ca and P and their deposits in the corresponding target organs, minimizing the interactions between Ca and Fe (Campos et al., 2007).

These precedents led us to study the influence of goat or cow milk-based diets, with either a normal Fe content or Fe overload, and supplied for 10, 30, or 50 d, on the bone remodeling process impaired because of Fe-deficiency anemia, to determine if goat milk consumption could have a positive effect on this process.

MATERIALS AND METHODS

Animals

Male Wistar albino rats (n = 180) recently weaned (21 d old), weighing 40 ± 5 g, from the University of Granada Laboratory Animal Service (Granada, Spain) 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

After weaning, rats were randomly divided into 2 groups, a control group fed with the AIN-93G diet (Reeves et al., 1993) and an anemic group that received the same diet but with a low Fe content (5 mg of Fe/kg of diet) during the 40-d pre-experimental period (Pallarés et al., 1993). After induction of anemia (d 0 of the study), the experimental period (**EP**) began, in which the control and anemic groups were fed for 10, 30, or 50 d with 4 diets: goat or cow milk-based diet with normal Fe content (45 mg/kg) or Fe overload (450 mg/kg). The dose of 450 mg of Fe/kg is common for inducing chronic Fe overload in animal studies (Raja et al., 1994). The diets included ferric citrate ($C_6H_5FeO_7$) as the Fe source.

From the beginning of the study, animals were placed in individual metabolic cages in an environmentally controlled room with a constant temperature of 22°C $\pm$ 2, a 12-h light-dark cycle, and $55 \pm 10\%$ humidity. Mineral-free water was available ad libitum, but the diet intake was controlled by pair feeding all the animals to avoid any differences due to intake. The diet supplied was calculated as 80% of the average intake of each experimental period. On d 0 of the study, peripheral blood samples from the caudal vein were collected using EDTA as anticoagulant for hematological analysis from all rats to confirm that Fe-deficiency anemia has been reached. After fasting overnight, 10 animals per group (10 controls and 10 anemic) were weighed and anesthetized by an intraperitoneal injection of 5 mg of sodium pentobarbital (Sigma Diagnostics, St. Louis, MO) per 100 g of BW. After median laparotomy, the rats were totally bled out by cannulation of the abdominal aorta; the sternum and femur were removed immediately, and stored at −40°C until further analysis of mineral content.

Blood on d 10 was collected from caudal vein, because animals were not culled at this point. On d 30 and 50 animals were anesthetized and totally bled out by cannulation of the abdominal aorta (n = 80 on d 30 and n = 80 on d 50), and femur and sternum were removed. Bone turnover was assessed on d 10, 30, and 50 of the study by measuring N-terminal propeptides of type I procollagen (**PINP**), C-terminal telopeptides of type I collagen (**CTx**), tartrate resistant acid phosphatase 5b (**TRACP 5b**), and PTH in serum. Figure 1 shows the experimental design of the study.
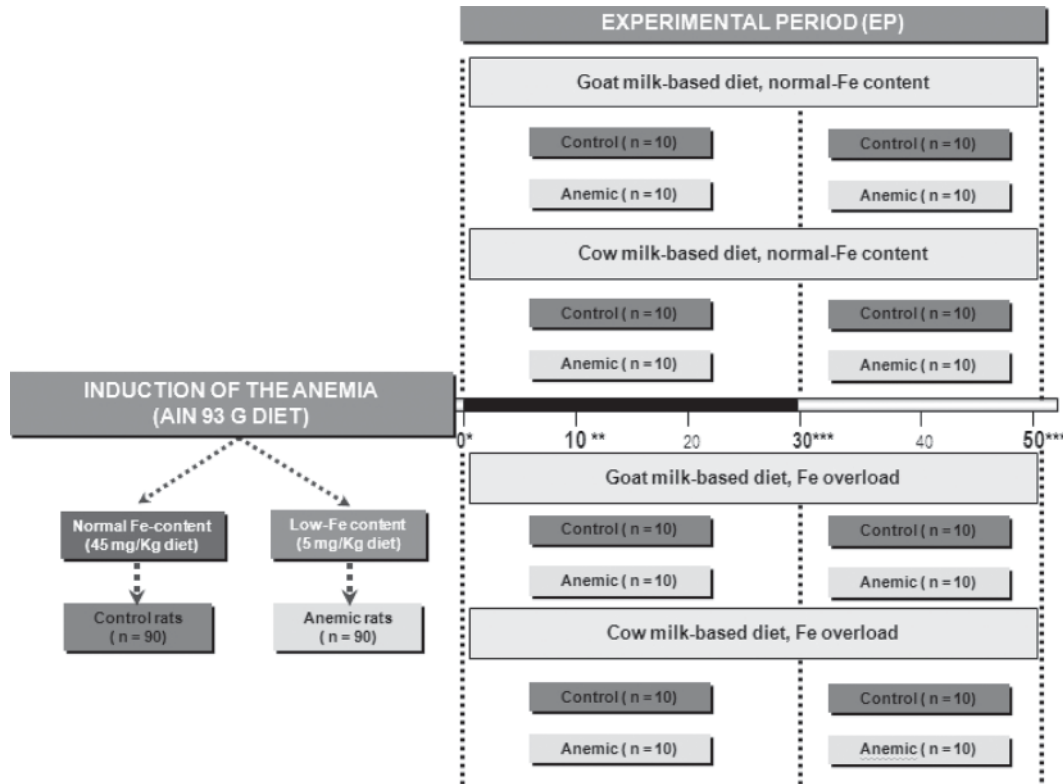
Figure 1. Experimental design of the study. On d 0 (*), blood from abdominal aorta (10 animals per group) was withdrawn to measure hematological parameters; femur and sternum were removed for mineralization analysis. On d 10 (**), blood samples from the caudal vein were obtained to measure bone turnover parameters. On d 30 and 50 (***), blood from the abdominal aorta was withdrawn to measure bone turnover parameters; femur and sternum were removed for mineralization analysis. AIN = American Institute of Nutrition.

Table 1 summarizes the diets supplied during the EP. The milk-based diets were made with skim cow (Holstein breed) or goat milk (Murciano-Granadina breed) powder. Goat or cow skim milk samples were formulated to provide 20% of protein in the diets. Because the fat content in the milk was undetectable (<0.01%), fat was added to reach a diet with 10% fat, obtained by purification of skim or butter (from goat or cow milk, respectively). The mineral contents in the normal Fe diets used during the EP by analysis were 40.5 mg of Fe/kg, 5,136 mg of Ca/kg, and 3,067 mg of P/kg (goat milk-based diet) and 39.6 mg of Fe/kg, 5,146 mg of Ca/kg, and 3,127 mg of P/kg (cow milk-based diet). Mineral content in the Fe overload diets were 476.4 mg of Fe/kg, 5,093 mg of Ca/kg, and 3,105 mg of P/kg (goat milk-based diet) and 482.0 mg of Fe/kg, 5,100 mg of Ca/kg, and 3,181 mg of P/kg (cow milk-based diet).

DM and Determination of Protein

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). The nitrogen content in the skim milks and diets was determined by the Kjeldahl method, using a protein conversion factor of 6.38 (Kjeldahl, 1983).

Measurement of Ca, P, and Fe

The concentrations of Ca and P were measured in femur and Fe was determined in sternum. In addition, the same minerals were measured in the diet. The concentrations of Ca and Fe in the diet and bones were determined by atomic absorption spectrophotometry (AAnalyst 1100B spectrometer with WinLab32 for AA software, PerkinElmer, Beverly, MA). The concentration of P was analyzed by visible spectrophotometry (UV/VIS spectrometer lambda 16, Perkin-Elmer) using the Fiske and Subbarow (1925) technique. The samples had been previously mineralized by wet method in a sand bath (J.R. Selecta, Barcelona, Spain). Samples of skim milk powder and lyophilized bovine liver (certified reference material CRM 063 R and BCR 185; Commission of the European Community Bureau of Reference, Brussels, Belgium), were used simultaneously to test the Ca (skim milk powder) and Fe recovery (bovine

Table 1. Composition of the diets supplied during the experimental period

Diet[1]	Amount, g/kg
Goat milk diet (normal Fe or Fe overload)	
Goat milk protein	200
Goat milk lactose	341
Goat milk fat	100
Wheat starch	160
Constant ingredients[2]	199
Cow milk diet (normal Fe or Fe overload)	
Cow milk protein	200
Cow milk lactose	353
Cow milk fat	100
Wheat starch	148
Constant ingredients[2]	199

[1]The diets were prepared according to the recommendations of the American Institute of Nutrition (AIN; Reeves et al., 1993) for control (normal Fe), and Fe overload was induced according to recommendations of Raja et al. (1994).

[2]The constant ingredients consisted of (g/kg of diet): fiber (micronized cellulose) 50, sucrose 100, choline chloride 2.5, L-cystine 1.8, mineral premix 35, vitamin premix 10. The vitamin premix was prepared according to the recommendations of the AIN (Reeves et al., 1993). The mineral premix for goat and cow milk-based diets was formulated taking into account the mineral content of the skim milk powder supplied to meet the recommendations of the AIN (Reeves et al., 1993). All the diets were isocaloric (17,226 kJ).

liver): Ca value = 13.88 ± 0.10 mg/g; Fe value = 210 $\pm$ 3 µg/g; mean $\pm$ SEM of 5 determinations; certified values: Ca = 13.49 ± 0.10 mg/g; Fe = 214 ± 5 µg/g).

Hematological Tests

Hemoglobin (**Hb**) concentration, red blood cells (**RBC**), hematocrit, mean corpuscular volume (**MCV**), and platelets were measured using an automated hematology analyzer Sysmex K-1000D (Sysmex, Tokyo, Japan).

PINP

N-Terminal propeptide of type I procollagen is the most specific and sensitive marker of bone formation (Claudon et al., 2008). The concentration of PINP was determined by using a rat/mouse PINP enzyme immunoassay (Immunodiagnostics System Ltd., Boldon, UK) from serum samples. Calibrators, controls, and samples were added to the wells of the microtiter plate followed by PINP labeled with biotin; the plate was incubated for 1 h at room temperature before being aspirated and washed. Horseradish peroxidase-labeled avidin was added and bound selectively to complexed biotin; following a further wash step, the color was developed using a chromogenic substrate (3,3′,5,5′-tetramethylbenzidine). The absorbance of the stopped reac-

tion mixtures was read in a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA) at 450 nm (reference 650 nm) within 30 min of adding the stop solution. The color intensity developed was inversely proportional to the concentration of PINP.

Degradation Products from CTx

Type I collagen accounts for more than 90% of the organic matrix of bone and is synthesized primary in bone (Burgeson, 1988). During renewal of the skeleton, bone matrix is degraded and fragments of type I collagen are released into the circulation. These fragments were measured using a RatLaps enzyme immunoassay (Immunodiagnostics System Ltd.) from serum samples. This assay is based upon the competitive binding of a polyclonal antibody to soluble RatLaps antigens or to immobilized RatLaps antigens. Briefly, the polyclonal antibody is raised against a synthetic peptide having a sequence specific for a part of the C-terminal telopeptide α-1 chain of rat type I collagen. For standardization of the kit, a synthetic peptide specific for the C-terminal telopeptide α-1 chain of type I collagen in rats was used. During the preincubation step, biotinylated peptide was immobilized by binding to the streptavidin-coated microtiter wells. The wells were emptied and washed. Standards, controls, and samples (rat serum) were pipetted into appropriate wells, followed by a solution of a primary antibody (polyclonal rabbit). Following the primary incubation step, the wells were emptied and washed. In the secondary incubation step, a solution of a goat anti-rabbit antibody conjugated with peroxidase (secondary antibody) was added and bound to the polyclonal rabbit antibody. After the third washing step, a chromogenic substrate (3,3′,5,5′-tetramethylbenzidine) was added and the color reaction was stopped with 0.18 M H_2SO_4. Finally, the absorbance was read using a Bio-Rad microplate reader. The absorbance level is inversely related to concentration of RatLaps antigens in the sample.

TRACP 5b

The concentration of TRACP 5b was measured in rat serum using the RatTRAP assay (Immunodiagnostics System Ltd.), which uses a highly characterized, specific monoclonal antibody prepared using baculovirus-generated recombinant rat TRACP as antigen (Kaija et al., 1999). The monoclonal antibody is incubated in anti-mouse IgG-coated microtiter wells. After washing, standard, control, and serum samples were incubated in the wells, and bound TRACP 5b activity was determined with a chromogenic substrate to develop color. The reaction was stopped and the absorbance of the

reaction mixture was read in a microplate reader (Bio-Rad Laboratories Inc.) at 405 nm within 30 min of adding the stop solution. Color intensity was directly proportional to the activity of TRACP 5b present in the sample. Because secreted TRACP 5b is indicative of the number of osteoclasts, these values can be used to replace histological determination of osteoclast number in rat bone (Alatalo et al., 2004; Rissanen et al., 2006).

PTH

Serum PTH was measured using a rat PTH immunoradiometric assay kit (ALPCO Diagnostics, Salem, MA). Two different goat antibodies to the N-terminal region (1–34) of rat PTH were purified by affinity chromatography. One of the antibodies was immobilized onto plastic beads to capture the PTH molecules and the other antibody was radiolabeled for detection. Serum samples were incubated simultaneously with an antibody-coated bead and the ^{125}I-labeled antibody. Both intact PTH (1–84) and N-terminal PTH (1–34) contained in the sample were immunologically bound by the immobilized antibody and the radiolabeled antibody to form a "sandwich" complex. At the end of the incubation period, the beads were washed to remove any unbound labeled antibody and other components. The radioactivity bound to the beads was then measured in a Wizard Automatic Gamma Counter (Perkin Elmer, Waltham, MA). The radioactivity of the antibody complex bound to the bead was directly proportional to the amount of rat PTH in the sample. A standard curve was generated by plotting the counts per minute against 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

Data are reported as means ± SEM. Differences between groups (control vs. anemic and normal Fe vs. Fe overload) were tested for statistical significance with the Student's t-test. One-way ANOVA was used to compare the different diets supplied to the 2 groups (control and anemic) during EP. Individual means were tested by pair-wise comparison using Tukey's post hoc multiple comparison test when main effects and interactions were significant. Data were analyzed by 2-way ANOVA to determine the effects of anemia, type of diet, and Fe content in the diet. A level of $P < 0.05$ was considered to indicate statistical significance. The SPSS software package, version 15.0 (2008, SPSS Inc., Chicago, IL) was used for data treatment and statistical analysis.

RESULTS AND DISCUSSION

At d 0, all hematological parameters in the anemic rats (that consumed the low-Fe diet) differed from those of the control rats. Most of the hematological parameters were lower: serum Fe (1,372 ± 121 µg/L in control group vs. 710 ± 58 µg/L in anemic group; $P < 0.001$), Hb concentration (124.3 ± 2.5 g/L in control vs.74.3 ± 2.4 g/L in anemic, $P < 0.001$), RBC (7.1 ± 0.15 × 10^{12}/L in control vs. 6.3 ± 0.2 × 10^{12}/L in anemic; $P < 0.001$), hematocrit (39.5 ± 0.7% in control vs. 26.9 ± 0.4% in anemic; $P < 0.001$), MCV (55.7 ± 0.3 fL in control vs. 40.0 ± 0.6 fL in anemic; $P < 0.001$), whereas platelets were higher (732 ± 25.5 × 10^9/L in control vs. 1,389 ± 66.1 × 10^9/L in anemic; $P < 0.001$). All these findings were expected and consistent with long-term induced Fe deficiency. The impaired hematological parameters recovered to baseline levels after feeding rats the milk-based diets for 30 or 50 d, as expected with consumption of normal or high-Fe diets (data not shown).

Mineral Content in Femur and Sternum at d 0

At the beginning of the study (d 0), evaluation of bone mineralization revealed that Ca and P contents in femur and Fe deposits in sternum were markedly lower for the anemic group ($P < 0.001$), which was in agreement with previous results (Campos et al., 2007), revealing a high degree of bone demineralization induced by the anemia. Medeiros et al. (1997, 2002, 2004) reported that Fe deficiency had a significant effect upon bone mineral density, content, and fragility in young rats fed an Fe- and Ca-restrictive diet. Hydroxylase enzymes require both ascorbate and Fe to function properly. Iron deficiency could lead to decreased hydroxylation of 1,25-dihydroxylcholcalciferol, the active form of vitamin D, resulting in diminished Ca absorption, because the 25-hydroxycholcalciferol-1- hydroxylase is a 3-component system: a flavoprotein, an Fe-sulfur protein, and a cytochrome P-450 (DeLuca, 1976). A lack of Fe could affect this enzyme system, leading to decreased renal reabsorption of Ca as well as decreased intestinal Ca absorption when Fe is limiting (Medeiros et al., 2004). On the other hand, as expected for consumption of a diet with low Fe content, a decrease was observed in the sternum Fe content of anemic rats ($P < 0.001$), which adversely influences the hematopoietic process in this group of animals and confirms the severe degree of Fe deficiency (Table 2).

Bone Turnover Biomarkers at d 10

On d 10 of the study, some important changes in bone metabolism were observed, after supplying milk-based

Table 2. Mineral content in femur and sternum of control and anemic rats on d 0 of the study[1]

Mineral content	Normal Fe (control group)	Low Fe (anemic group)
Ca in femur, mg/g of DM	167.44 ± 3.61	98.76 ± 3.58*
P in femur, mg/g of DM	124.75 ± 2.67	101.54 ± 2.62*
Fe in sternum, μg/g of DM	78.53 ± 4.24	62.05 ± 2.83*

[1]Data are means ± SE of 10 animals.

*Significantly different ($P < 0.001$) from the control group by Student's t-test.

diets with normal Fe content or Fe overload (Table 3). The concentration of PINP increased in the anemic group compared with controls when a goat milk-based diet with normal Fe content was supplied ($P < 0.05$) and in these animals compared with those fed cow milk-based diet ($P < 0.001$). The beneficial effect of goat milk on mineral metabolism has been well documented: it improves Fe bioavailability (Alférez et al., 2006; Nestares et al., 2008), and the Fe is available for hydroxylation processes and then in the biosynthesis of vitamin D. It is known that 25-hydroxycholcalciferol-1-hydroxylase is located within the renal mitochondria and this enzyme is Fe-dependent (DeLuca, 1976). Moreover, Fe exerts its influence on bone turnover by affecting type I collagen synthesis and maturation. Iron is an essential cofactor for prolyl and lysyl hydroxylases, enzymes that catalyze ascorbate-dependent hydroxylation of prolyl and lysyl residues, essential steps before crosslinking by lysyl oxidase (Tuderman et al., 1977). Therefore, an improvement in Fe bioavailability

would contribute to increase cross-linking activity and, subsequently, stronger collagen fibers. This fact supports the increase in PINP found in animals fed with goat milk-based diets, reflecting the higher amount of bone matrix developed (Cons-Molina, 2003).

Serum levels of bone resorption biomarkers were lower in anemic animals fed a goat milk-based diet with Fe overload compared with cow milk-based diet ($P < 0.01$ for CTx and $P < 0.001$ for TRACP 5b), so the increase in the resorption process induced by the anemia seemed to stop in this point following the consumption of goat milk, because the bone remodeling process maintains skeletal mass practically invariable. The increase in TRACP 5b in control and anemic animals fed a cow milk-based diet with Fe overload compared with those fed a goat milk-based diet ($P < 0.001$) revealed an active process of bone resorption. In this context, Irie et al. (2000) proposed a functional correlation of the TRACP 5b activity in osteocytes with osteocytic osteolysis. In osteoclasts, TRACP 5b has been suggested to generate free radicals and to directly participate in bone resorption (Hayman and Cox 1994), to dephosphorylate osteopontin and bone sialoprotein, and to serve as an osteoclast detachment factor (Ek-Rylander et al., 1994). The osteoclasts are mobilized toward the zone of destruction and then adhere tightly to the bone surface, beginning the process of resorption. This negative effect in the process of bone turnover was not observed when the rats consumed goat milk-based diets with Fe overload, indicating once again a positive effect in the process of recovery of bone demineralization induced by

Table 3. Bone turnover biomarkers at d 10 of the study in control and anemic groups fed with goat- or cow-based diets with normal Fe content or Fe overload[1]

Bone turnover biomarker[2]	Goat milk diet		Cow milk diet		P-value (2-way ANOVA)		
	Control	Anemic	Control	Anemic	Anemia	Diet	Fe overload
PINP, ng/mL							
Normal Fe	3.38 ± 0.48	5.10 ± 0.36[A]*	2.96 ± 0.09	2.90 ± 0.11[B]	<0.05	<0.001	0.15
Fe overload	4.40 ± 0.46	4.12 ± 0.59	3.68 ± 0.38	3.18 ± 1.11	0.09	0.08	
CTx, ng/mL							
Normal Fe	21.78 ± 1.55	22.82 ± 0.59	23.36 ± 1.00	23.96 ± 0.95	0.23	0.31	0.07
Fe overload	24.60 ± 1.09	22.58 ± 1.13[A]	24.32 ± 5.63	28.32 ± 2.03[B]	0.09	<0.05	
TRACP 5b, U/I							
Normal Fe	0.72 ± 0.02	0.75 ± 0.05	0.75 ± 0.03	0.78 ± 0.04	0.45	0.56	<0.05
Fe overload	0.50 ± 0.01[a]†	0.51 ± 0.01[A]†	0.83 ± 0.09[b]	0.71 ± 0.02[B]	0.54	<0.05	
PTH, pg/mL							
Normal Fe	28.48 ± 1.63[a]	16.68 ± 1.72[A]*	16.96 ± 1.05[b]	27.76 ± 2.60[B]*	<0.001	<0.05	<0.05
Fe overload	14.16 ± 1.81[a]†	20.00 ± 2.98	24.38 ± 2.54[b]†	22.36 ± 3.88	0.08	<0.01	

[a,b] or [A,B]Mean values among groups of controls (lowercase) or anemic (uppercase) rats with unlike superscript letters in the same row were significantly different ($P < 0.05$, Tukey's test).

[1]Data are means ± SE of 10 animals.

[2]PINP = N-terminal propeptides of type I procollagen; CTx = C-terminal telopeptides of type I collagen; TRACP 5b = tartrate resistant acid phosphatase 5b; and PTH = parathyroid hormone.

*Mean values from the corresponding group of control rats were significantly different ($P < 0.05$, Student's t-test).

†Mean values from the corresponding group of rats fed with normal-Fe content were significantly different ($P < 0.05$, Student's t-test).

the Fe deficiency. In general, the results showing that osteoclastic activity (TRACP 5b) and, therefore, bone resorption were lower indicate that Fe overload in the diet for 10 d induced stabilization of bone turnover in anemic animals. This first experimental period (10 d of consumption of diets with normal or high Fe content) can be considered transitional, emphasizing that, in the animals fed goat milk-based diets, the process of bone remodeling had already begun to stabilize, although at the view of the short time of supply of the diets, a certain imbalance in the studied biomarkers was observed.

With regard to PTH levels, no significant differences caused by anemia were found in the groups that consumed either milk-based diet with Fe overload for 10 d, whereas feeding diets with normal Fe content resulted in a decrease of PTH in the anemic animals that consumed goat milk ($P < 0.001$) and an increase in those that consumed cow milk ($P < 0.001$). Iron overload produced a marked decrease of PTH levels in control animals that consumed goat milk-based diet ($P < 0.001$), whereas an increase was observed ($P < 0.05$) in those fed a cow milk-based diet. These results are in agreement with the increase in nutritive utilization of Ca, because of the greater absorption of this mineral, as previously reported Campos et al. (2007).

Bone Turnover Biomarkers at d 30 and 50

At d 30 of the study, no effect of anemia was observed for any of the diets as assayed by the biomarker of bone formation PINP or by the resorption biomarkers CTx and TRACP 5b, revealing that the bone demineralization induced by the lack of Fe was restored. The process of stabilization of turnover began earlier in the animals fed goat milk, as indicated by PINP levels only 10 d into the EP. The supply of diets extended for 50 d caused diminished CTx and TRACP 5b in anemic animals that received the goat milk diet with normal Fe content ($P < 0.001$; Table 4). The decrease of these resorption biomarkers in anemic rats at d 50 confirmed that the remodeling process was normalized after supplying goat milk, probably because goat milk minimizes the interactions between Fe and Ca (Barrionuevo et al., 2002; Campos et al., 2003). Moreover, as it shows the evolution of PINP, the bone formation is lower at 50 d of the study in comparison with the previous periods (10 and 30 d), because at this point the rats were evolved to the adult stage (McCutcheon and Marinelli, 2009), the active growth had finished and remodeling process had become stable.

In the anemic rats that consumed the goat milk-based diet with normal Fe content for 30 d, a marked decrease in PTH levels occurred compared with controls ($P < 0.001$; Table 4). This decrease in PTH levels was prob-

ably due to the increase in the nutritive utilization of Ca, following major absorption of this mineral (Campos et al., 2007). Nevertheless, for the cow milk-based diet with Fe overload, the levels of this hormone increased in anemic animals ($P < 0.05$), which could be due to the decrease in the serum levels of ionic Ca in situations of Fe deficiency (Masuyama et al., 2000). By effect of Fe overload, at d 30 and 50 of the study, PTH levels increased in all the experimental groups ($P < 0.001$; Table 4). This finding might be because PTH increases the speed of bone remodeling and the mobilization of Ca deposit in the organism, which is in agreement with Pawlotsky et al. (1999) and Dimitriadou et al. (2010), who found an increase in the serum levels of PTH in Fe overload conditions. After 50 d of feeding milk-based diets, PTH levels were not modified by the effect of anemia or type of diet in rats fed normal Fe diets, indicating that the speed of bone remodeling had decreased because of the complete recovery of bone metabolism.

According to Hale et al. (2007), PINP levels can be correlated directly to PTH levels, and recent studies reveal that at intermittent doses and within the physiological range, PTH favors bone formation through an increase of the number of osteoblasts (Cosman and Lindsay, 2004), because PTH stimulates the transformation of osteocytes in osteoblasts (Langub et al., 2001) and diminishes the apoptosis of these cells (Bringhurst, 2002).

Mineral Content in Femur and Sternum at d 30 and 50

In relation to Ca deposits in femur, after supplying the diets with normal or high Fe content for 30 or 50 d (Table 5), no effect of anemia was observed in animals that consumed goat milk-based diets. However, at d 30, Ca femur content was lower in anemic rats fed cow milk-based diets with normal Fe content ($P < 0.01$), and at d 50 in rats fed cow-milk-based diets with normal and high Fe contents ($P < 0.01$). Deposit of P in femur was not affected by anemia after 30 or 50 d. By effect of the diet, Ca deposits in femur increased in controls ($P < 0.001$) and anemic rats ($P < 0.001$) fed goat milk with normal or high Fe content at d 30 and 50, and also P deposits increased in controls ($P < 0.01$) and anemic rats ($P < 0.001$) that consumed goat milk-based diets with normal Fe content (Table 5).

Goat milk has a high content of vitamin D (Alférez et al., 2006), which favors Ca energy-dependent transcellular saturable transport. Moreover, the lactose in this type of milk is more digestible compared with cow milk and easy to absorb. According to Heaney (1996), these factors favor Ca absorption via paracellular pathway, which explains the greater Ca deposits in the femurs of

Table 4. Bone turnover biomarkers at d 30 or 50 of the study in control and anemic groups fed with goat- or cow-based diets with normal Fe content or Fe overload[1]

Bone turnover biomarker[2]	Goat milk diet		Cow milk diet		P-value (2-way ANOVA)		
	Control	Anemic	Control	Anemic	Anemia	Diet	Fe overload
d 30 of the study							
PINP, ng/mL							
Normal Fe	3.53 ± 0.13^a	3.12 ± 0.19	2.46 ± 0.35^b	3.08 ± 0.09	0.47	<0.05	0.85
Fe overload	3.45 ± 0.61	3.13 ± 0.45	2.47 ± 0.24	3.90 ± 0.47	0.09	0.56	
CTx, ng/mL							
Normal Fe	22.48 ± 0.49	23.26 ± 0.33	19.06 ± 1.20	24.90 ± 2.47	0.34	0.43	0.23
Fe overload	26.85 ± 3.43	29.53 ± 2.99	22.83 ± 4.39	24.63 ± 2.52	0.31	0.25	
TRACP 5b, U/I							
Normal Fe	0.63 ± 0.04	0.75 ± 0.05	0.74 ± 0.06	0.84 ± 0.08	0.45	0.28	<0.01
Fe overload	0.61 ± 0.11	$0.50 \pm 0.05†$	0.63 ± 0.11	$0.56 \pm 0.03†$	0.87	0.65	
PTH, pg/mL							
Normal Fe	22.10 ± 0.39^a	$14.36 \pm 0.74*$	17.58 ± 0.71^b	15.96 ± 0.80	<0.01	<0.01	<0.001
Fe overload	$36.08 \pm 1.89^a†$	$34.55 \pm 9.12†$	$28.90 \pm 3.19^b†$	$37.76 \pm 3.38*†$	<0.05	<0.01	
d 50 of the study							
PINP, ng/mL							
Normal Fe	2.30 ± 0.20^a	2.26 ± 0.14^A	1.62 ± 0.21^b	1.42 ± 0.13^B	0.59	<0.001	0.23
Fe overload	2.13 ± 0.20	2.10 ± 0.10^A	2.20 ± 0.12	$1.54 \pm 0.25^B*$	<0.05	<0.05	
CTx, ng/mL							
Normal Fe	26.28 ± 1.82	$21.06 \pm 1.06*$	24.03 ± 2.12	23.58 ± 2.17	<0.05	0.25	<0.001
Fe overload	$15.43 \pm 1.89†$	$16.90 \pm 1.60†$	$16.43 \pm 1.84†$	$16.17 \pm 0.75†$	0.78	0.45	
TRACP 5b, U/I							
Normal Fe	0.79 ± 0.02	$0.69 \pm 0.02*$	0.69 ± 0.03	0.72 ± 0.04	<0.05	0.43	<0.05
Fe overload	$0.70 \pm 0.02†$	0.73 ± 0.11	0.66 ± 0.02	0.70 ± 0.02	0.69	0.92	
PTH, pg/mL							
Normal Fe	14.10 ± 1.9	19.89 ± 0.8	15.31 ± 1.9	20.62 ± 3.8	0.09	0.85	<0.001
Fe overload	$20.61 \pm 2.6†$	$26.32 \pm 2.1†$	$24.30 \pm 2.6†$	$30.22 \pm 3.6†$	0.07	0.016	

[a,b] or [A,B]Mean values among groups of controls (lowercase) or anemic (uppercase) rats with unlike superscript letters in the same row were significantly different ($P < 0.05$, Tukey's test).

[1]Data are means $\pm$ SE of 10 animals.

[2]PINP = N-terminal propeptides of type I procollagen; CTx = C-terminal telopeptides of type I collagen; TRACP 5b = tartrate resistant acid phosphatase 5b; and PTH = parathyroid hormone.

*Mean values from the corresponding group of control rats were significantly different ($P < 0.05$, Student's t-test).

†Mean values from the corresponding group of rats fed with normal-Fe content were significantly different ($P < 0.05$, Student's t-test).

rats fed goat milk diets with normal Fe or Fe overload. This could be because nutritive utilization of Fe from goat milk is better than from cow milk, thereby replenishing more efficiently the depleted body stores and, under these conditions, the quantity of available oxygen and ATP increases, recovering the carriers of Ca most efficiently, which favors Ca absorption via active transport (Conrad and Umbreit, 1993). Something similar happens with P, because the absorption of this element is performed, in part, via active transport (López-Aliaga et al., 1994). In addition, goat milk reduces the interactions between Ca and Fe, thus increasing the digestive utilization of both minerals (Barrionuevo et al., 2002; Campos et al., 2003). The lower interaction between Ca and Fe in animals fed goat milk diet can improve Ca nutritive utilization (Barrionuevo et al., 2002; Campos et al., 2003; Alférez et al., 2006). The higher Ca and P deposits in femur favor the formation of the inorganic component of the bone, because Ca and P are the principal constituents of the hydroxyapatite crystals that

inlay in the collagen fibers to form a material that has the suitable characteristics of hardness, flexibility, and resistance to constitute the inorganic phase of bone tissue.

After 30 and 50 d of feeding diets with Fe overload, lower Ca deposits in femur were observed in all experimental groups ($P < 0.01$ for control rats and $P < 0.001$ for anemic rats); P deposits showed the same tendency, although the differences were not significant. These results can be because PTH levels were increased with the Fe overload, which is in accordance with Pawlotsky et al. (1999) and Dimitriadou et al. (2010), and this hormone increases the mobilization of Ca and P bone deposit.

By the effect of anemia, Fe deposits in sternum were lower at d 30 in the animals that consumed goat milk-based diet with normal Fe content ($P < 0.05$), whereas in animals fed cow milk diet with normal Fe content this decrease in sternum Fe deposits was observed at d 30 and 50 ($P < 0.001$). Therefore, it is evident that

Table 5. Mineral content in bone tissues at d 30 and 50 of the study in control and anemic rats fed goat or cow milk-based diets with normal Fe content or Fe overload[1]

Bone content	Goat milk diet		Cow milk diet		P-value (2-way ANOVA)		
	Control	Anemic	Control	Anemic	Anemia	Diet	Fe overload
d 30 of the study							
Ca in femur, mg/g of DM							
Normal Fe	293.8 ± 12.3^a	288.3 ± 10.2^A	243.2 ± 5.8^b	$223.9 \pm 2.1^{B*}$	<0.05	<0.001	<0.001
Fe overload	$260.7 \pm 4.8^a†$	$255.9 \pm 5.20^A†$	$207.3 \pm 13.2^b†$	$202.2 \pm 8.2^B†$	0.98	<0.001	
P in femur, mg/g of DM							
Normal Fe	134.9 ± 5.2^a	142.8 ± 5.7^A	118.2 ± 3.8^b	114.2 ± 3.8^B	0.42	<0.01	0.08
Fe overload	120.1 ± 5.9	117.1 ± 13.6	113.6 ± 4.1	105.9 ± 5.1	0.91	0.82	
Fe in sternum, µg/g of DM							
Normal Fe	146.1 ± 6.7	$128.4 \pm 2.6^{A*}$	137.6 ± 5.5	$107.2 \pm 3.9^{B*}$	<0.001	<0.05	<0.001
Fe overload	$194.3 \pm 5.3†$	$178.2 \pm 12.1†$	$177.2 \pm 6.6†$	$159.1 \pm 8.8†$	0.06	0.26	
d 50 of the study							
Ca in femur, mg/g of DM							
Normal Fe	285.0 ± 5.9^a	286.0 ± 7.1^A	238.9 ± 7.0^b	$215.5 \pm 2.6^{B*}$	<0.05	<0.001	<0.01
Fe overload	$247.0 \pm 2.8^a†$	$237.0 \pm 6.9^A†$	$193.9 \pm 5.4^b†$	$176.5 \pm 4.0^{B*}†$	<0.05	<0.001	
P in femur, mg/g of DM							
Normal Fe	127.2 ± 5.4^a	135.9 ± 4.6^A	108.4 ± 2.3^b	102.7 ± 4.8^B	0.68	<0.01	0.24
Fe overload	117.9 ± 5.9	119.7 ± 8.1	100.6 ± 10.3	96.4 ± 6.6	0.75	0.28	
Fe in sternum, µg/g of DM							
Normal Fe	142.5 ± 8.3^a	138.8 ± 7.2^A	121.2 ± 4.4^b	$99.7 \pm 4.1^{B*}$	<0.05	<0.001	<0.001
Fe overload	$204.9 \pm 4.9†$	$188.1 \pm 11.2†$	$196.9 \pm 3.5†$	$171.7 \pm 5.4†$	0.54	0.71	

[a,b] or [A,B]Mean values among groups of controls (lowercase) or anemic (uppercase) rats with unlike superscript letters in the same row were significantly different ($P < 0.05$, Tukey's test).

[1]Data are means ± SE of 10 animals.

*Mean values from the corresponding group of control rats were significantly different ($P < 0.05$, Student's t-test).

†Mean values from the corresponding group of rats fed with normal-Fe content were significantly different ($P < 0.05$, Student's t-test).

the repletion of Fe in sternum had begun earlier in rats fed the goat milk diet and it is noteworthy that this bone has an important hematopoietic function. If the incorporation of Fe in the synthesis of hemoglobin is fundamental in the process of development of erythroblasts to reticulocytes, it is at this stage of cell differentiation when the lack of Fe can damage the complete hemoglobinization of the erythrocytes, giving place to a functional Fe deficiency (Besarab et al., 2009). Iron overload increased Fe deposit in sternum in both periods in all the experimental groups ($P < 0.001$), as expected. After supplying the goat milk-based diet with normal Fe content for 30 or 50 d, the Fe deposit in sternum increased in anemic animals compared with animals fed cow milk-based diet ($P < 0.001$). Therefore, in view of the evolution of the concentration of Fe in sternum, it is possible to affirm that the hematopoietic process, altered as consequence of the Fe restriction to induce the Fe-deficiency anemia, was restored 50 d after supplying goat milk diet, whereas a longer time was necessary with the cow milk diet.

CONCLUSIONS

In only 10 d of supplying goat milk, bone demineralization induced by Fe deficiency began to recover, as evidenced by the increase of the bone formation biomarker PINP and diminishing PTH levels. If the consumption of milk-based diets was prolonged (for 30 or 50 d), the parameters of bone remodeling recovered with both milk-based diets, although bone remodeling was restored earlier in animals fed the goat milk-based diets, and feeding a goat milk-based diet favors the mineralization of femur and sternum, improving bone metabolism and hematopoietic process. Therefore, inclusion of goat milk is recommended in diets of humans who suffer nutritional Fe-deficiency anemia, because this type of milk helps to relieve the adverse effects produced in bone turnover due to the Fe deficiency.

ACKNOWLEDGMENTS

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