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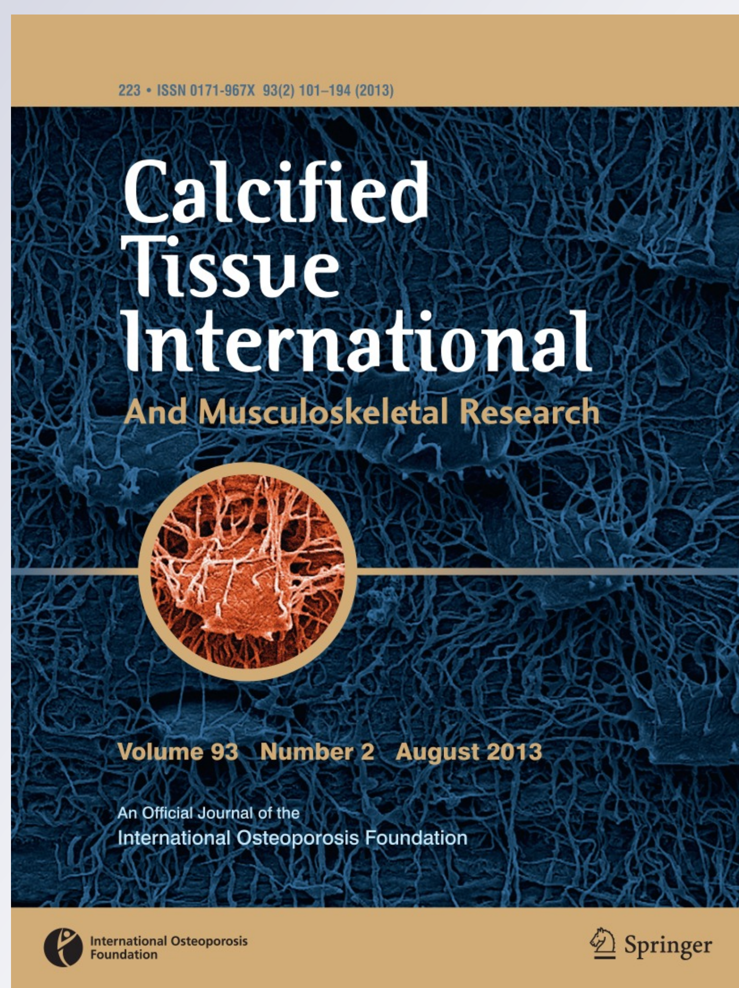

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ORIGINAL RESEARCH

Low Protein Intake Magnifies Detrimental Effects of Ovariectomy and Vitamin D on Bone

Clarisa Marotte · Macarena M. S. Gonzales Chaves · Gretel G. Pellegrini · Silvia M. Friedman · Fima Lifshitz · Patricia Mandalunis · Susana N. Zeni

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Abstract Protein-induced changes in bone and calcium homeostasis could potentially be greater in the elderly and in women at risk for osteoporosis. We hypothesize that a low protein intake would magnify the negative changes in bone metabolism seen in vitamin D (vitD) insufficiency and/or estrogen deficiency. The present study was undertaken to better understand how a low protein diet along with vitD insufficiency could affect bone metabolism using a rodent ovariectomized (OVX) model. Rats ($n = 60$) underwent ovariectomy (OVX) or sham operation. The first 15 days after surgery, all rats were fed a standard rodent diet. Thereafter, rats ($n = 10$/group) were fed a low protein diet (LP; 2.5 %) or a control diet (NP; 12.5 %) with 100 IU% vitD (+D; cholecalciferol) or without vitD (−D) for 45 days. The groups were as follows: SHAM + NP + D (control); SHAM + LP + D; SHAM + LP − D; OVX + NP + D; OVX + LP + D; OVX + LP − D. Body weight (BW) of control and OVX + NP + D groups increased while those feeding the LP diet, independently of vitD feedings, decreased ($p < 0.05$). The OVX + LP − D group presented the lowest serum Ca, phosphorus and osteocalcin levels and the highest CTX levels ($p < 0.05$). At the end of the study, total skeleton bone mineral content, proximal tibia bone mineral density, bone volume and trabecular number levels decreased as follows: SHAM + NP + D (controls) > SHAM + LP + D > OVX + NP + D > SHAM + LP − D > OVX + LP + D > OVX + LP − D ($p < 0.05$). A low protein diet negatively affected bone mass and magnified the detrimental effects of vitD and/or estrogen deficiencies.

Keywords Bisphosphonate · Bone · Low protein intake · Rats · Vitamin D insufficiency

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Nutrition plays an important role in bone health [1]. In this regard, calcium (Ca) and vitamin D (vitD) are the two main critical nutrients for accruing and maintaining skeletal mass [2]. The effect of Ca intake has been extensively studied in all age groups and its importance in maintaining mineral homeostasis is well established [3, 4]. VitD deficiency is common among elderly subjects, and hypovitaminosis D is very prevalent in both adults and children [5–7]. VitD insufficiency and/or inadequate dietary Ca intake stimulates parathyroid hormone (PTH) secretion, the primary regulator of bone remodeling, thus leading to bone resorption, which increases bone loss and consequent fracture risk [8]. Recently the Committee of Food and Nutrition Board of the Institute of Medicine established new recommended intakes for both nutrients according to age [9]. Recommendations for Ca and vitD intake were increased to 1,200 mg/day and to 800 IU/day, respectively, for people older than 70 years of age.

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Beyond Ca and vitD inadequacy, protein deficiency may also contribute to the increased incidence of age-related osteoporosis. Approximately half of bone volume and one-third of bone mass are made by protein [1, 10]. Although many early studies focused on the negative effects of a high protein intake on bone, recent data suggest that a low protein intake has a negative effect on Ca absorption and retention [11]. The elderly often have a decline in appetite and food intake leading to nutritional deficiencies that contribute to age related bone loss. Bone mineral density and body composition were altered in underweight elderly subjects [12]. Lower protein intake has been shown to be an important predictor of lower limb bone mass [13] and conversely an increased dietary protein in elderly people was considered important for optimal muscle and bone health [14].

The adverse effects of protein-induced changes in bone and Ca homeostasis could potentially be greater in the elderly and women who are at risk for osteoporosis [14]. We hypothesized that a low protein intake would magnify the negative changes in bone metabolism seen in vitD insufficiency and/or estrogen deficiency. The present study was undertaken to better understand how a low protein diet along with vitD insufficiency could affect bone metabolism using a rodent ovariectomized (OVX) model. On these bases, experiments were carried out on young adult female rats to determine whether low protein and/or vitD intake had a detrimental effect in bone during estrogens sufficiency or insufficiency.

Methods and Materials

Animals

A total of 60 two-month-old female Wistar rats (250–300 g) obtained from our laboratory (General and Oral Biochemistry Department, School of Dentistry, Buenos Aires University) were housed at controlled room temperature (21 ± 1 °C) and humidity of 55 ± 10 %, under 12-h light/dark cycles. Body weight (BW) and food consumption were assessed three times per week. Throughout the experimental period, rats had access to deionized water and food ad libitum. The rats were maintained in keeping with the National Institutes of Health Guide for the Care and Use of Laboratory Animals [15], and the protocol was approved by the Bioethics Committees of the Buenos Aires University.

Rats were randomly assigned to undergo either a bilateral ovariectomy (OVX) ($n = 30$) or a sham operation ($n = 30$) by a dorsal approach. Surgery was performed under anesthesia with ketamine hydrochloride (0.1 mg/100 g BW) and acepromazine maleate (0.1 mg/100 g BW). The OVX was confirmed at necropsy by the uterine horn atrophy.

The first 15 days after surgery, all rats were fed a standard rodent diet (Granave SA, Buenos Aires, Argentina). Thereafter, 10 rats per group were randomly assigned to receive one of the 3 experimental diets during 45 additional days in order to complete a 60-day period.

At the end of the experimental period rats were euthanized by CO_2 inhalation.

Diets

The 3 experimental isocaloric semisynthetic diets were prepared according to the American Institute of Nutrition Rodent Diets Recommendations (AIN-93) [16]. All diets contained the same type and concentration of Ca (0.5 % calcium carbonate) and phosphorus (0.4 % potassium phosphate monobasic). Diets varied in protein (potassium caseinate) and vitD (cholecalciferol) content. The composition of each diet is outlined in Table 1.

According to the treatment, the six studied groups were as follows: SHAM + NP + D (control): sham-treated rats fed a diet containing 12.5 % protein and 100 IU% vitD; SHAM + LP + D: sham-treated rats fed

Table 1 Nutrient composition of diets prepared according to AIN-93 to meet rat requirements and fed for a 45-day period

Diet	NP	LP + D	LP − D
Energy (kcal)	395	395	395
Protein (g)[a]	14.5	2.5	2.5
Lipids (g)[b]	7.0	7.0	7.0
Mineral mix[c]	3.5	3.5	3.5
Water-soluble vitamins[c]	0.25	0.25	0.25
Vitamin A	0.1 mL	0.1 mL	0.1 mL
Choline[d]	0.71	0.71	0.71
Fiber[e]	5.0	5.0	5.0
Carbohydrate[f]		To complete 100 g	
Vitamin D (cholecalciferol)	100 IU	100 IU	0 IU
Calcium (g)	0.5	0.5	0.5
Phosphorus (g)	0.4	0.4	0.4

[a] Potassium caseinate, Nestlé Argentina S.A., containing/100 g, 85.1 of protein and 0.095 g of Ca

[b] Commercial soy oil, Molinos Rio de la Plata, Argentina

[c] Manufactured by the Department of Food Science School of Biochemistry, University of Buenos Aires. Individual components from Sigma, St. Louis, MO, USA

[d] Choline citrate, 0.71 %, food grade, Anedra, Argentina

[e] Cellulose to meet rat requirements of fiber, according to AIN-93; $CaCO_3$, food grade individual components, Anedra, Argentina

[f] Corn dextrin from corn refinery, provided by Food SA Argentina, was added as carbohydrate source to achieve 100 g of diet

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a diet containing 2.5 % protein and 100 IU% vitD; SHAM + LP − D: sham-treated rats fed a diet containing 2.5 % protein without vitD (0 IU%); OVX + NP + D: OVX rats fed a diet containing 12.5 % protein and 100 IU% vitD; OVX + LP + D: OVX rats fed a diet containing 2.5 % protein and 100 IU% vitD; OVX + LP − D: OVX rats fed a diet containing 2.5 % protein without vitD (0 IU%).

BW and Food Intake

Food cups were refilled once a day, and food consumption was measured with a Mettler scale PC 4,000 (accuracy ± 1 mg). Daily food intake was recorded (g/100 g BW/day). BW was measured every 4 days after a fasting period of 2–4 h.

Biochemical Determinations

Blood samples were obtained from the tail vein under light anesthesia in a fasting state. Serum Ca, phosphate (P), 25 hydroxyvitamin D (25OHD), CTX and osteocalcin levels were evaluated at the end of the experiment while bone alkaline phosphatase (bALP) levels were longitudinally evaluated at 0, 25, 45 and 60 days of the experiment as previously described [17].

Serum Ca (mg/dL) was determined by atomic absorption spectrophotometry. Lanthanum chloride (6,500 mg/L in the final solution) was added to avoid interference. The serum P (mg/dL) was determined by UV spectrophotometry by a commercial kit (BioSystems, Argentina). The bALP was measured by a colorimetric method (Boehringer Mannheim, Germany) after bone enzyme isoform precipitation with wheat-germ lectin. Serum 25OHD (ng/mL) was assayed by a competitive protein binding method (Diasorin, Minnesota) with an intra-assay coefficient variation of 9 %. The osteocalcin (ng/mL) and CTX (ng/mL) were measured by immunoassay (ELISA) (Rat-osteocalcine and Rat-laps, Osteometer BioTech, Herlev, Denmark), with a 6 % intra-assay variation coefficient.

DXA Measurements

Total skeleton (TS) BMD and bone mineral content (BMC) were assessed *in vivo* at the beginning ($T = 0$) and at the end ($T = 60$) of the experiment using a total body scanner with a software specifically designed for small animals (DPX Alpha 8034, Small Animal Softer, Lunar Radiation Corp., Madison, WI) as previously described [18].

All rats were scanned under light anesthesia using an identical scan procedure. The software precision in determining BMD was assessed by measuring one rat five times after repositioning between scans both on the same and on different days. The coefficient of variation was 0.9 % for TS BMD and 3.0 % for BMC. Different body sub-areas were analyzed by using the image of the animal on the screen, using a ROI for each segment. The BMD coefficient of variation for the different studied areas was 1.8 % for lumbar spine (LS) and 3.5 % for proximal tibia (PT). All analyses were carried out by the same technician to eliminate inter-observer variation differences.

Histological Determinations

At the end of the study, the right tibiae were removed and cleaned of soft tissue, fixed by immersion in buffered formalin for 48 h, decalcified in 10 % ethylene-diamine-tetraacetic acid (EDTA) (pH 7.0) for 25 days and then embedded in paraffin. Two 8- to 10-µm-thick longitudinally oriented sections of subchondral bone were obtained at the middle third level, including primary and secondary spongiosa. The sections were microphotographed (AXIO-SKOP, Carl Zeiss) to perform histomorphometric measurements on the central area of the metaphyseal bone displayed on the digitalized image.

The following static histomorphometric parameters were measured according to Dempster et al. [19]: Bone volume fraction (BV/TV) (%): the percentage of cancellous bone within the total measured area; osteoblast surface (Ob.S/BS) (%): the fraction of trabecular bone surface covered with osteoblasts; eroded surface (ES/BS): the fraction of trabecular surface covered with lacunae (including "active" lacunae with osteoclasts and lacunae in reversal phase); osteoclast number (Oc.N/B.Ar, mm^2): the number of osteoclasts in the total studied area; trabecular number (Tb.N, 1/mm); thickness (Tb.Th, um) and spacing (Tb.Sp, um).

Statistical Analyses

Results were expressed as mean ± standard error (SE). Data were analyzed using two-way factorial analysis of variance (ANOVA), and a multiple comparisons test was performed when significant differences were encountered among the six studied groups. The bALP and BW data were evaluated by one-way ANOVA followed by Bonferroni's multiple comparisons test to determine significance among the data at different stages of the experiment. Statistical analyses were performed by SPSS for Windows 11.0 (SPSS, Chicago, IL). A p value of <0.05 was considered statistically significant.

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Results

Effect of Feeding a Low Protein Diet

There were no differences in food consumption expressed as percentage of BW (g/100 g BW) between SHAM + NP + D and SHAM + LP + D (8.16 ± 0.37 vs. 8.59 ± 0.44, respectively).

There was a significant effect of the LP diet on BW. At the beginning, the control and SHAM + LP + D groups had similar BW; however, a significant reduction of BW was observed by feeding the low protein diet as compared with baseline values and the control group at the end of the study ($p < 0.05$) (Fig. 1).

At the end of the study, the low protein intake did not induce significant changes in serum Ca, P and 25OHD levels; conversely, significantly higher levels of osteocalcin and CTX were observed as compared to the levels detected in the control group ($p < 0.05$) (Table 2). In addition, the levels of bALP remained unchanged throughout the study (Fig. 2).

A low protein intake significantly decreased TS BMC and PT BMD ($p < 0.05$) (Figs. 3a, b, respectively).

The histological findings showed that a low protein intake induced a reduction in BV/TV% (Table 3), in the trabecular number and thickness compared to the control group ($p < 0.05$). Meanwhile, osteoclast number, osteoblast surface and eroded surface increased significantly ($p < 0.05$), without differences in trabecular separation (Table 3; Fig. 4a, b).

Effect of Feeding a Low Protein Intake-Deficient vitD Diet

There were no differences in food consumption expressed as percentage of BW (g/100 g BW) between SHAM + LP + D and SHAM + LP − D groups (8.59 ± 0.44 and 8.32 ± 0.51, respectively).

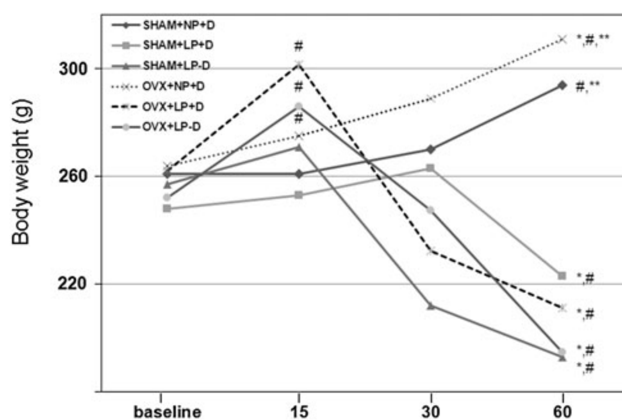

Fig. 1 The effect of diet on BW was performed by 1-way ANOVA (Bonferroni's multiple comparisons test) to show differences among points: $*p < 0.01$ compared to SHAM + NP + D; $**p < 0.05$ compared to OVX + LP + D; $^{\#}p < 0.05$ compared to baseline

There was a significant effect of the low protein-free vitD diet intake in BW. At the beginning, the average BW of each group was similar. However, at the end of the experimental period, the BW was significantly lower in the SHAM + LP − D as compared with SHAM + LP + D group ($p < 0.05$) (Fig. 1).

At the end of the study, serum levels of Ca, P, 25OHD and osteocalcin decreased, while CTX and bALP levels increased in SHAM + LP − D as compared with SHAM + LP + D group ($p < 0.05$) (Table 2; Fig. 2).

The TS BMC, PT BMD (Fig. 3a, b), BV/TV% (Table 3) and trabecular number decreased while osteoclast number, eroded surface and trabecular separation increased in SHAM + LP − D as compared to SHAM + LP + D ($p < 0.05$) (Table 3; Fig. 4b, c). However, no differences in trabecular thickness and osteoblast surface were found (Table 3). In addition, no signs of osteomalacia were observed (data not shown).

Effect of Ovariectomy in a Normal Protein–vitD Diet

No differences in food consumption expressed as percentage of BW (g/100 g BW) were observed between SHAM + NP + D and OVX + NP + D (8.32 ± 0.51 vs. 8.47 ± 041, respectively).

At baseline the average BW was similar. As expected, OVX + ND + D group gained more BW than SHAM + NP + D rats after the study period ($p < 0.05$) Fig. 1).

Although serum Ca, P and 25OHD levels were unchanged, OVX increased bone turnover. Ovariectomy significantly increased osteocalcin and CTX levels compared with the control group (Table 2). Moreover, bALP levels of OVX + NP + D increased from baseline to day 45 and decrease thereafter to the end of the study ($p < 0.05$) (Fig. 2).

Ovariectomy significantly decreased TS BMC and PT BMD ($p < 0.05$) (Fig. 3a, b, respectively).

Ovariectomy induced a significant decrease in BV/TV, trabecular number and thickness ($p < 0.05$) with a significant increase in osteoblast surface, osteoclast number, eroded surface and trabecular separation ($p < 0.05$) (Table 3).

Effect of Feeding a Low Protein Diet to Ovariectomized Rats

There were no differences in food consumption expressed as percentage of BW (g/100 g BW) between OVX + NP + D and OVX + LP + D groups (8.47 ± 041 and 8.53 ± 0.42, respectively).

A low protein diet consumed by OVX rats induced a significantly reduction in BW as compared to baseline and

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Table 2 Serum calcium (Ca), phosphorus (P), osteocalcin, collagen type I C-terminal telopeptide (CTX) and 25 hydroxyvitamin D (25OHD) profile at the end of the experimental period (day 60)

Characteristic	SHAM + NP + D	SHAM + LP + D	SHAM + LP − D	OVX + NP + D	OVX + LP + D	OVX + LP − D
Ca (mg/dL)	10.0 ± 0.2	10.0 ± 0.1	8.9 ± 0.2[b,c]	9.5 ± 0.5	9.9 ± 0.1	9.1 ± 0.1[b]
P (mg/dL)	5.1 ± 0.3	5.6 ± 0.3	4.7 ± 0.2[b,c]	5.8± 0.4[b]	5.3 ± 0.2	4.4 ± 0.4[b,d]
Osteocalcin (ng/mL)	148 ± 14	194 ± 9[b]	175 ± 10[b,c]	177 ± 10[a]	130 ± 18[c,d,e]	84 ± 12[b,c,e,f]
CTX (μg/mL)	15.2 ± 3.7	23.6 ± 2.3[b]	31.4 ± 5.2[b,c]	26.6 ± 3.9[b]	23.1 ± 5.7[b,e]	67.0 ± 13.8[b,c,e,f]
25OHD (ng/mL)	29.3 ± 4.0	32.0 ± 5.6	8.6 ± 1.7[b,c]	30.1 ± 3.7	29.0 ± 4.4[c,e]	10.4 ± 4.4[b,c,f]

[a] Data are expressed as mean ± ES. Analysis of the effect of diets was performed by 2-way ANOVA (multiple comparisons test)

[b] $p < 0.05$ compared to SHAM + NP + D

[c] $p < 0.05$ compared to SHAM + LP + D

[d] $p < 0.05$ compared to OVX + NP + D

[e] $p < 0.05$ compared to SHAM + LP − D

[f] $p < 0.05$ compared to OVX + LP + D

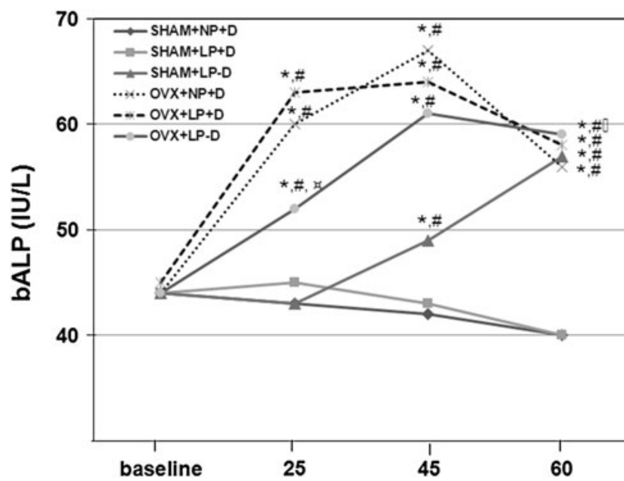

Fig. 2 The effect of diet on levels of bALP was performed by 1-way ANOVA (Bonferroni's multiple comparisons test) to show differences among points: *$p < 0.05$ compared to baseline; #$p < 0.05$ compared to SHAM + NP + D and SHAM + LP + D; ¤$p < 0.05$ compared to OVX + LP + D

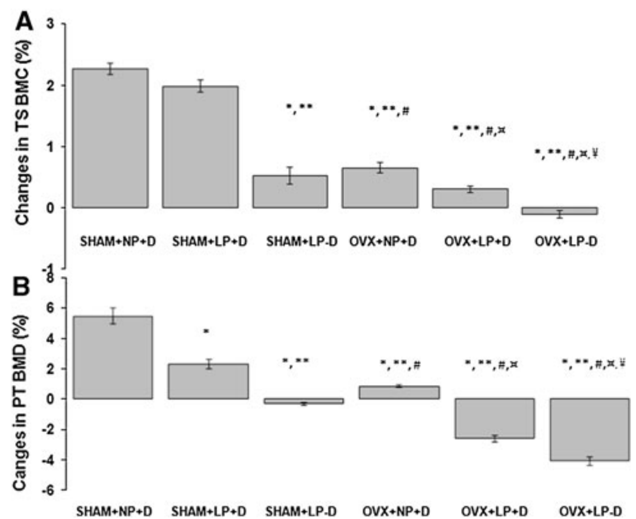

Fig. 3 Percentage of changes. **a** Total skeleton bone mineral content (TS BMC). **b** Proximal tibia bone mineral density (PTBMD) induced by feeding the 5 experimental diets during a 45-day period and the effect of the antiresortive OPD treatment. Data are expressed as mean ± ES. *$p < 0.05$ compared to SHAM + NP + D; **$p < 0.05$ compared to SHAM + LP + D; #$p < 0.05$ compared to SHAM + LP − D; ¤$p < 0.05$ compared to OVX + NP + D; ¥$p < 0.05$ compared to OVX + LP + D

OVX + NP + D group ($p < 0.05$) (Fig. 1). At the end of the study, serum Ca, P, 25OHD and osteocalcin levels were decreased meanwhile CTX levels were increased as compared with OVX + NP + D group ($p < 0.05$).

Besides, histological findings showed a higher decreased in BV/TV%, trabecular number and higher increased in osteoclast number, eroded surface and trabecular separation than OVX + NP + D ($p < 0.05$); however, osteoblast surface decreased ($p < 0.05$) while no changes in trabecular thickness were observed (Table 3).

The low protein diet in OVX rats induced an additional decrease in TS BMC and PT BMD as compared with OVX + LP + D ($p < 0.05$) (Fig. 3a, b, respectively).

Effect of Feeding a Low Protein-Deficient vitD Diet to Ovariectomized Rats

There were no differences in food consumption expressed as percentage of BW (g/100 g BW) between OVX + LP + D and OVX + LP − D groups (8.53 ± 0.42 and 8.39 ± 0.39, respectively).

No additional changes in BW were observed in OVX rats by feeding the low protein diet lacking of vitD (Fig. 1).

As expected, OVX + LP group not receiving dietary vitD presented significantly lower 25OHD levels than

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Table 3 Histological parameters obtained at the level of the middle third: osteoblast surface (Ob.S/BS) (%), osteoclast number (Oc.N/B.TA), trabecular number (Tb.N) (1/mm); trabecular thinner (Tb.Tn) (μm); and trabecular separation (Tb.Sp.) (μm) at the end of the experiment

Characteristic	SHAM ± NP ± D	SHAM ± LP ± D	SHAM ± LP − D	OVX ± NP ± D	OVX ± LP ± D	OVX ± LP − D
Bone volume (BV/TV)	25.0 ± 1.2	14.4 ± 1.3^{b}	$6.3 \pm 0.8^{b,c}$	$7.4 \pm 1.0^{b,c}$	$3.1 \pm 0.4^{b,c,d}$	$1.0 \pm 0.1^{b,c,d,e}$
Osteoblast surface (Ob.S/BS) (%)	34.0 ± 2.8	40.1 ± 3.2^{b}	40.7 ± 5.4^{b}	48.2 ± 1.9^{b}	$32.4 \pm 6.3^{c,d}$	$27.8 \pm 4.5^{c,d,e}$
Eroded surface (ES/BS) (%)	1.03 ± 0.59	3.55 ± 1.69	6.71 ± 1.77	6.27 ± 3.05^{b}	7.53 ± 3.37	11.61 ± 1.88
Total osteoclast number (Oc.N/B.Ar) (mm2)	0.49 ± 0.31	0.84 ± 0.26^{b}	$1.81 \pm 0.50^{b,c}$	1.19 ± 0.11^{b}	$2.40 \pm 0.89^{c,d}$	$8.60 \pm 5.61^{b,c,d,e}$
Tb.N (1/mm)	3.70 ± 0.79	2.61 ± 0.36^{b}	$1.8 \pm 0.53^{b,c}$	1.93 ± 0.42^{b}	$0.83 \pm 0.52^{c,d}$	$0.54 \pm 0.33^{b,c,d,e}$
Tb.Th (mm)	0.040 ± 0.007	0.034 ± 0.009^{b}	0.030 ± 0.003^{b}	0.033 ± 0.006^{b}	0.031 ± 0.002^{b}	0.033 ± 0.003^{b}
Tb.Sp (mm)	0.24 ± 0.06	0.28 ± 0.07	$0.97 \pm 0.46^{b,c}$	1.24 ± 1.01^{b}	$1.71 \pm 1.52^{b,c,d}$	$2.73 \pm 1.99^{b,c,d,e}$

[a] Data are expressed as mean ± ES. Analysis of the effect of diets was performed by 2-way ANOVA (multiple comparisons test)

[b] $p < 0.05$ compared to SHAM + NP + D

[c] $p < 0.05$ compared to SHAM + LP + D

[d] $p < 0.05$ compared to SHAM + LP − D

[e] $p < 0.05$ compared to OVX + LP + D

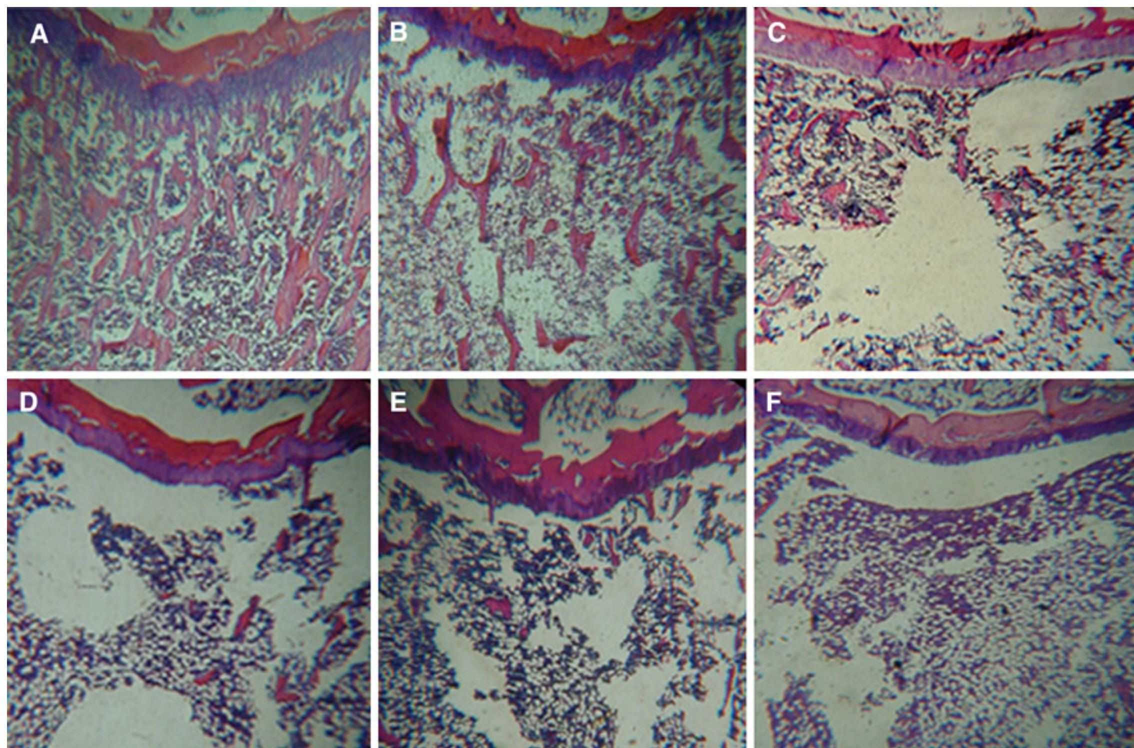

Fig. 4 **a** SHAM + NP + D. **b** SHAM + LP + D. **c** SHAM + LP − D. **d** OVX + NP + D. **e** OVX + LP + D. **f** OVX + LP − D. Hematoxylin–eosin staining shows trabeculae stained in *red*. Note the decrease in the number of trabeculae in OVX + LP + D and OVX + LP − D groups. Original magnification ×50 (Color figure online)

those fed the diet containing 100 UI% of vitD ($p < 0.05$); however, serum Ca and P levels remained unchanged. Moreover, osteocalcin and CTX levels presented an additional reduction and increment, respectively, as compared to OVX + LP + D group ($p < 0.05$) (Table 2). As a consequence, the OVX + LP − D group reached the lowest osteocalcin and the highest CTX levels (Table 2).

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The bALP levels of the studied LP-OVX groups increased from baseline to day 45 and decreased thereafter to the end of the study, independently of vitD status ($p < 0.05$).

The lack of vitD in the OVX group feeding the low protein diet induced the greatest alterations in densitometric and histological parameters (Figs. 3, 4) The pattern of the changes in TS BMC and PT BMD was as follows: SHAM + NP + D (controls) < SHAM + LP + D < OVX + NP + D < SHAM + LP − D < OVX + LP + D < OVX + LP − D ($p < 0.05$) (Fig. 3a, b, respectively). Such reduction order was also observed in BV/TV% (Table 3) and in trabeculae number (Table 3; Fig. 4).

In addition, osteoclast number, trabecular separation and eroded surface increased in the same order of magnitude as detected for bone densitometric measures ($p < 0.05$). No additional reduction in trabecular thickness was observed (Table 3). In addition, no signs of osteomalacia were observed (data not shown).

Discussion

The main finding of the present study is that a low protein intake magnified the negative changes in bone metabolism of VitD insufficiency and/or estrogen deficiency. Indeed, a low protein intake exaggerated the effects in bone mass of VitD inadequacy and/or estrogen withdrawal.

Age-related increase in bone resorption can be attributed to several factors, including protein nutrition [12–14]. With advancing age there is a reduction in food consumption, changes in dietary habits, and malabsorption; in addition, older adults lack sunlight exposure and frequently receive medications that could negatively affect bone health [20, 21]. Ca and/or vitD inadequacy are perhaps the most widespread deficient conditions in developed nations, even in young adults [3, 5]; however, bone remodeling requires continuous supply of protein because a substantial fraction of certain amino acids are not reutilized in the synthesis of new bone type I collagen molecules.

Reduced protein intake negatively affects muscle mass, an important component of BW and decreases bone mass [21]. Throughout the experiment, despite similar food intake, BW and BMC were negatively affected in rats fed isocaloric low protein diets. It has long been recognized that an inadequate dietary protein intake might lead to a negative nitrogen balance [22]. It could also interfere with Ca absorption and in achieving the structural organic matrix of type I collagen that ensures bone formation. In agreement with previous studies in aged male rats and in young Sprague Dawley female rats [23, 24], the present report confirmed that protein inadequacy induces changes in bone remodeling that decrease bone mass. In addition, our model also addressed the relative importance of other common factors such as estrogen deficiency and/or poor vitD status. Our data demonstrated that the effect of both manipulations seemed to be additive in rats consuming low protein diets.

Several factors could account for the negative effect of protein inadequacy on bone. A low protein intake influences PTH-1,25 dihydroxyvitamin D [1,25(OH_2)D] axis by decreasing Ca absorption and insulin-like growth factor 1 (IGF1) levels [24–26]. A poor Ca absorption interferes with Ca homeostasis inducing a secondary hyperparathyroidism. Small increments in PTH levels over time cause a chronic increase in bone turnover that contributes to bone loss and increases fracture risk [27]. Moreover, PTH positively regulates the renal 1α,25 hydroxylase enzyme [28] that synthesizes 1,25$(OH)_2$D, the main regulator of PTH synthesis and responsible for increasing Ca absorption. A reduction in 1,25$(OH)_2$D and an increase in PTH levels were observed in intact female rats fed a protein deficient diet [24]. VitD nutritional status assessed by 25OHD levels could contribute to alter PTH/1,25$(OH)_2$D axis because PTH levels rise as 25OHD levels decrease [29].

The synthesis of 1,25$(OH)_2$D is influenced by IGF1 production, clearance and actions being regulated by protein intake. Although IGF1 levels normally decline with age [30], protein inadequacy increases IGF1 metabolic clearance rate, induces hepatic resistance to the action of growth hormone and induces resistance to this factor in organs less sensitive, such as bone [31]. Estrogen withdrawal could indirectly affect 1,25$(OH)_2$D synthesis because it down-regulates IGF1 transcription [30]. Although PTH, 1,25$(OH)_2$D and IGF1 were not measured in the present study, several other biochemical parameters determined herein suggest and allow inferring changes in the former.

A previous study showed a reduction in serum Ca with a low protein intake [24]. In contrast, we showed no changes in P–Ca homeostasis of intact female rats fed a low protein diet. Protein restriction limits Ca absorption; however a compensatory rise in PTH may maintain Ca–P levels by inducing changes in bone homeostasis. It has been shown that bone formation was depressed and bone resorption was increased in intact female rats fed a deficient protein diet [23]. Conversely, under our experimental conditions, histological and biochemical parameters showed that both processes of bone remodeling are increased during a protein deficient diet. The observed increments in osteoblast surface and osteoclast number were compatible with a 14 % increase in osteocalcin levels and with a 55 % increase in CTX without changes in bALP. The differences observed in osteocalcin and bALP behavior could be partially explained because these markers reflect different stages of bone formation.

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The uncoupling in bone remodeling resulted in bone mass loss without changes in total skeleton BMD, although a decrease in proximal tibia BMD was observed. Bone volume at the proximal tibia metaphysis level decreased because an increment in bone resorption that induced a reduction in trabecular number and thinning of the remaining trabeculae. These results indicate that there are different responses to protein deficiency that vary in accordance with the type of bone. Total skeleton is mainly composed of cortical bone while proximal tibia contains a significant proportion of trabecular bone which is a metabolically more active bone [13].

The P–Ca homeostasis in the intact rat was altered by low protein intake coupled with vitD insufficiency. The severe reduction in 25OHD levels could have led to secondary hyperparathyroidism with bone remodeling with a 100 % increase in bone resorption. An additional reduction in total skeleton bone mass, proximal tibia BMD and bone volume was observed in rats fed the low protein-normal vitD diet. As indicated by histological analyses, such reduction was the result of trabecular thinning and a dramatic decrease in trabecular number, which led to an increase in trabecular separation without the presence of osteoid.

The low protein intake did not induce changes in P–Ca homeostasis in OVX rats, but induced greater changes in bone turnover. A 100 % increment in CTX levels was observed, whereas osteocalcin levels did not change and bALP remained higher compared with intact rats fed the normal-protein diet. The histological parameters confirmed the uncoupling in bone remodeling with a greater bone mass and trabecular density deterioration. Estrogen is a well-known regulator of bone turnover through the control of pro-inflammatory cytokines release [32]. The higher negative effect on bone health could be the result of a reduction in Ca absorption in association with the lack of control by estrogen at each bone multicellular unit level. The additional vitD deficiency in OVX rats fed a low protein diet induced the greatest impairment in bone health. There was a high uncoupling in bone turnover, decreased bone formation and an increased resorption, and a diminution in both serum P and Ca levels.

One limitation of the present work is that PTH and IGF1 levels were not measured and the body composition was not evaluated.

In conclusion, the present study highlights the importance of adequate protein intake in the preservation of skeletal integrity. A low protein diet negatively impacted bone mass and magnified the detrimental effects of vitD and/or estrogen deficiencies. The experiments provide preclinical evidence of the effects of protein undernutrition on female bone health.

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Conflict of interest The authors report that they have no conflict of interest.

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