BJR

■ **BIOMECHANICS**

The 17β-oestradiol treatment minimizes the adverse effects of protein restriction on bone parameters in ovariectomized Wistar rats

RELEVANCE TO OSTEOPOROSIS AND THE MENOPAUSE

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Objectives

Insufficient protein ingestion may affect muscle and bone mass, increasing the risk of osteoporotic fractures in the elderly, and especially in postmenopausal women. We evaluated how a low-protein diet affects bone parameters under gonadal hormone deficiency and the improvement led by hormone replacement therapy (HRT) with 17β-oestradiol.

Methods

Female Wistar rats were divided into control (C), ovariectomized (OVX), and 17β-oestradiol-treated ovariectomized (OVX-HRT) groups, which were fed a control or an isocaloric low-protein diet (LP; 6.6% protein; seven animals per group). Morphometric, serum, and body composition parameters were assessed, as well as bone parameters, mechanical resistance, and mineralogy.

Results

The results showed that protein restriction negatively affected body chemical composition and bone metabolism by the sex hormone deficiency condition in the OVX group. The association between undernutrition and hormone deficiency led to bone and muscle mass loss and increased the fragility of the bone (as well as decreasing relative femoral weight, bone mineral density, femoral elasticity, peak stress, and stress at offset yield). Although protein restriction induced more severe adverse effects compared with the controls, the combination with HRT showed an improvement in minimizing these damaging effects, as it was seen that HRT had some efficacy in maintaining muscle and bone mass, preserving the bone resistance and minimizing some deleterious processes during the menopause.

Conclusion

Protein restriction has adverse effects on metabolism, leading to more severe menopausal symptoms, and HRT could minimize these effects. Therefore, special attention should be given to a balanced diet during menopause and HRT.

Article focus

■ Investigation of the effects of hormone replacement therapy (HRT) with 17β-oestradiol on bone parameters in a condition of hormonal deficiency and undernutrition.

Key messages

■ Protein restriction associated with hormone deficiency led to bone and muscle mass loss and increased the fragility of the bone compared with the controls.

■ The hormone replacement therapy (HRT) contributed to the maintenance of muscle and bone mass and bone resistance.

■ Particular attention should be given to a balanced diet during the menopause and HRT treatment.

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Strengths and limitations

- Bone health was analyzed through multiple bone parameters (bone mineral content (BMC), bone mineral density (BMD), bone fragility, and bone mineralogy).
- The model of hormone deficiency and hormone replacement therapy (HRT) in rats provides important insight but lacks direct clinical applicability.

Introduction

The acquisition of bone mass in children is dependent on the adequate intake of proteins, which influences the amount of bone accumulated by the end of the skeletal growth (known as peak bone mass).[1,2] Peak bone mass influences bone loss during ageing and is a significant factor for fracture risk in the elderly.[1] Furthermore, adequate protein intake is fundamental for bone maintenance in adulthood and ageing. It provides the structural matrix of bone, enhances insulin-like growth factor I (IGF-I) levels, suppresses parathyroid hormone, and increases calcium (Ca) absorption.[3-5] Indeed, several studies have positively correlated protein intake and bone mineral density (BMD).[1,2,4-10]

Protein intake is also essential to the acquisition and maintenance of muscle mass, increasing the absorption of amino acids that stimulate muscle protein synthesis.[11] Muscle mass is associated with bone mass during development and growth and is positively correlated with bone mass in ageing.[12] In the elderly, bone mass maintenance is strictly related to functional muscle mass; patients with a low protein intake present greater muscle spoliation and bone loss.[3,12] Therefore, insufficient protein ingestion may affect the maintenance of muscle and bone mass,[7-10] increasing the risk of osteoporotic fractures in the elderly.[3,4,12,13]

Menopause leads to loss of muscle and bone mass, aggravated by ageing. In postmenopausal women, the cessation of sex hormone secretion increases the risk of osteoporosis or fracture due to increased bone reabsorption.[14-19] In this condition, adequate protein intake has been correlated with a decrease in the rate of hip fracture.[2,4,5] Thus, insufficient protein intake during the menopause may further intensify the bone and muscle loss verified in this circumstance.

One of the treatments used to prevent osteoporosis in postmenopausal women is hormone replacement therapy (HRT).[14,15,20,21] According to the 2017 Hormone Therapy Position Statement of The North American Menopause Society (NAMS), HRT has been shown to prevent bone loss and fracture. Hormone replacement therapy is recommended to post-menopausal women under the age of 60 years, or those who are within ten years of menopause onset and have no contraindications, and who present high fracture risk.[20,21] It minimizes menopause symptoms such as hot flushes and sleep disturbances, improves altered lipoprotein profiles by increasing cholesterol-high density lipoprotein levels and diminishing cholesterol-low density lipoprotein levels, and prevents menopause-associated bone loss, thereby reducing the incidence of fracture.[14,15,20] Nevertheless, HRT has also been associated with an increased risk of breast and endometrial cancer.[14,15,20,22] The combination of progestin and oestrogen is reported to reduce the endometrial cancer risk, but there are contradictory data regarding breast and ovarian cancer.[15,22] Therefore, it is recommended to administer the lowest effective dose to alleviate the symptoms for the shortest amount of time in women with an intact uterus.[14,15,20,22]

The protein restriction model used in this present work was an isocaloric low-protein diet that has been reported to diminish bone mass.[2,6,23,24] Ovariectomized female rats were used to simulate the sex hormone deficiency. In this study, the experimental model has been used to mimic the postmenopausal state as it presents a similar pattern of bone mineral density, oestrogen deficiency, and HRT to that of postmenopausal women.[25-30] Considering that both sex hormone deficiency and protein restriction lead to loss of bone and muscle, increasing the risk of osteoporosis and of fracture, it is important to understand how those two factors combined could be modulated in the organism.[12,23] Moreover, it is imperative that we determine whether HRT can be influenced by the diet and whether the consequences of protein restriction could be minimized by the HRT effects, improving bone metabolism. Thus, this study evaluated how the effects of a low-protein diet on bone parameters would be altered in a condition of hormonal deficiency and how HRT with 17β-oestradiol could improve this state.

Materials and Methods

Animals and diets. The experimental protocol and procedures received the approval of the Institutional Ethics Committee on Animal Use. Female Wistar rats (28 days old), obtained from animal facilities at the University of Campinas, Sao Paulo, Brazil, were housed in collective cages at a mean 22°C (SD 2) with a 12-hour light/12-hour dark cycle, and given food and water *ad libitum* throughout the experiment. The semipurified diets were in accordance with AIN-93 from the American Institute of Nutrition.[31] The normoprotein diet (C) comprised 18% protein (20% casein). The low-protein diet (LP) was reduced in casein (8%) and contained 6.6% protein. The diets were isocaloric and contained the same amount of fat (7% soy oil), fibre (5% micro cellulose fibre), minerals (3.5%), and vitamin mix (1.0%). Both diets contained cornstarch (39.7% C; 44.4% LP), dextrin (13.2% C; 17.8% LP), and sugar (10% C; 14.9% LP). In addition, the diets were also supplemented with cystine (0.3% C; 0.1% LP) and choline (0.2% for both C and LP diets).

Experimental protocol. The animals were first divided into two groups according to the diet (C and LP groups).
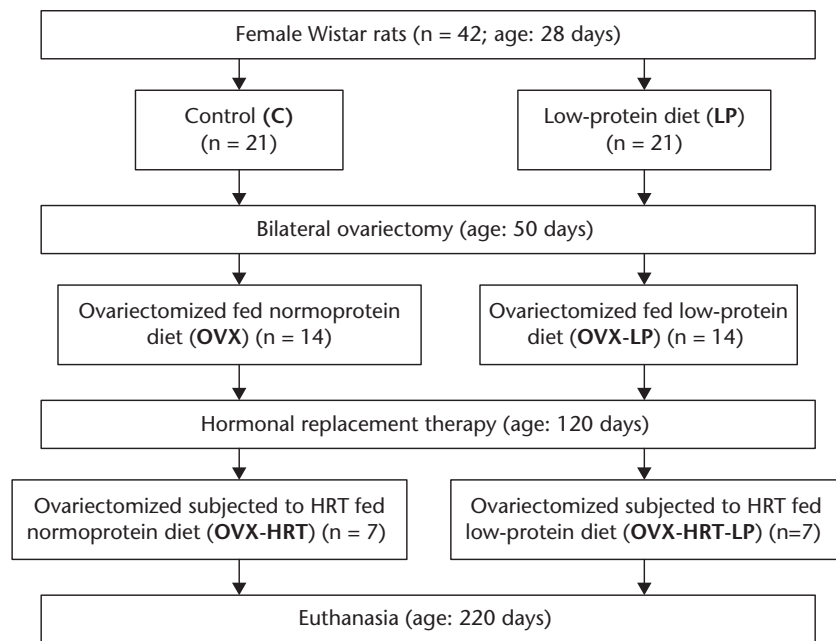
Fig. 1
Experimental protocol.

When the animals were 50 days old, the rats were recategorized into four groups based on whether they underwent bilateral ovariectomy, corresponding to the following groups: C, control female rats; LP, rats fed a low-protein diet; OVX, ovariectomized rats fed a normoprotein diet; and OVX-LP, ovariectomized rats fed a low-protein diet. After approximately 70 days, randomly chosen animals from each OVX group (OVX and OVX-LP) commenced HRT treatment. At this point, the OVX rats were rearranged into two additional groups according to the HRT: OVX rats subjected to HRT and fed a normoprotein diet (OVX-HRT); and OVX rats fed a low-protein diet and subjected to HRT (OVX-HRT-LP). All groups contained a minimum of seven animals in each. The diagram in Figure 1 shows the experimental protocol. Body weight was assessed three times per week. The animals were euthanized at 220 days old, and blood and tissue (gastrocnemius muscles, femora, uterus) were collected.

Ovariectomy and hormone replacement therapy. The bilateral ovariectomy was realized under anaesthesia with intraperitoneal ketamine (80 mg/kg body weight) and xylazine (10 mg/kg body weight). Animals were given water with acetylsalicylic acid (0.70 mg/ml) ad libitum for a week after surgical procedures.

The HRT was administered through subcutaneous injections of 17β-oestradiol (0.1 μg/kg body weight; Sigma-Aldrich, St. Louis, Missouri, USA) diluted in mineral oil (Nujol; Mantecorp, Rio de Janeiro, Brazil) three times a week until the end of the experiment.

Metabolic rate. Metabolic rate was assessed when animals were 200 days old. Animals were weighed and put into a hermetic recipient for three minutes. Oxygen and carbon dioxide concentrations were measured with an Oxygen/Carbon Dioxide Analyzer Model 902D Dual Trak (Quantek Instruments, Grafton, Massachusetts, USA) at the initial moment just after closing the animal in the recipient, and following the first, second, and third minute of measurement. Metabolic rate was calculated based on the following formula, and expressed in kcal × min^{-1} × 100 g^{-1}:

$$[(VCO_2f - VCO_2i) / (VO_2i - VO_2 f) / time]$$
$$\times 4.85 \ kcal \times 100 \ Weight(g)$$

Body composition. The total body composition of the animals was determined by dual-energy x-ray absorptiometry (DXA), Discovery model (Hologic, Marlborough, Massachusetts) located in the Nuclear Medicine Service of the University of Campinas. With this methodology, we accessed the bone mineral content (BMC), bone mineral density (BMD), and total body fat percentage for each animal in each group.

Bone fragility. The collected femoral bone underwent a compression test with a servohydraulic test machine, MTS model 180 FlexTest 40 (MTS Systems Corporation, Eden Prairie, Minnesota, USA) at the Mechanical Testing Laboratory of the Faculty of Mechanical Engineering at the University of Campinas. We evaluated bone fragility, analyzing the bone elastic modulus, peak stress, and stress offset.

Bone minerals. After the compression test, femora were prepared for bone mineralogy analysis. Bone samples (150 mg) were digested in concentrated nitric acid and

Table I. Morphometric and serum parameters in different experimental groups

Parameter	Group						p-value*		
	C	OVX	OVX-HRT	LP	OVX-LP	OVX-HRT-LP	Hormonal effect	Diet effect	Interaction
Mean final body weight, g (SD)	330.8 (35.9)	377.2 (39.9)†	322.3 (17.6)‡	307.4 (46.3)	330.9 (54.5)§	276.3 (25.5)‡§	0.004¶	0.004¶	0.682
Mean relative muscle weight, % (SD)	0.63 (0.18)	0.52 (0.06)†	0.62 (0.07)	0.48 (0.06)§	0.49 (0.06)	0.51 (0.04)§	0.285	0.004¶	0.229
Mean total protein, g/dl (SD)	8.4 (0.9)	8.0 (1.2)	8.2 (1.2)	8.1 (1.2)	7.1 (0.5)†	7.2 (1.0)	0.158	0.020¶	0.558
Mean albumin, g/dl (SD)	1.5 (0.2)	1.4 (0.2)	1.5 (0.2)	1.5 (0.1)	1.3 (0.1)†§	1.4 (0.1)†	0.045¶	0.079	0.153

*Significant difference accounted for hormonal, diet, or interaction effect, analyzed by two-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) method
†Hormonal effect: p ⩽ 0.05 versus control-matched group (C or LP)
‡p ⩽ 0.05 versus OVX-matched group (OVX or OVX-LP)
§Diet effect: p ⩽ 0.05 versus control hormonal-matched group (C, OVX, or OVX-HRT)
¶Statistically significant
C, control; OVX, ovariectomized rats; OVX-HRT, ovariectomized rats subjected to HRT and fed a normoprotein diet; LP, low-protein; OVX-LP, ovariectomized rats subjected to a low-protein diet; OVX-HRT-LP, ovariectomized rats subjected to HRT and fed a low-protein diet

hydrogen peroxide. The samples were heated for 15 minutes at 100°C in a digestion block and then heated for an additional 30 minutes at 130°C. After cooling, the sample quantities of sodium (Na), potassium (K), magnesium (Mg), zinc (Zn), Ca, and phosphorus (P) were determined using inductively coupled plasma-optical emission spectrometry (ICP-OES) (Optima 2000DV; PerkinElmer, Waltham, Massachusetts, USA), coupled with a parallel-path pneumatic nebulizer (Mira Mist; Burgener Research, Mississauga, Canada) and a quartz cyclonic spray chamber. All determinations were realized in duplicates. K, Mg, Zn, and P quantifications were accessed with the axial view, and Ca and Na quantifications were made with the radial view. The whole system was controlled by the software WinLab32 (PerkinElmer). The parameters used for the ICP-OES operation are described in Supplementary Table i.

Serum assays. Blood was collected by cardiac puncture and then centrifuged at 10,000 × g for 15 minutes at 4°C to obtain the serum. Total protein and albumin concentrations were determined using commercial colorimetric methodology kits and then read by the spectrophotometer (Hidex, Zug, Switzerland).

The hormone serum content was measured using Multiplex commercial kits (MilliporeSigma, Burlington, Massachusetts, USA) for fluorescence flow cytometry and read with Luminex (MilliporeSigma). Levels of serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were measured to verify the sex hormonal deficiency.

Statistical analysis. The results are reported as mean and standard deviation. The data were analyzed using two-way analysis of variance (ANOVA) tests, followed by Fisher's least significant difference (LSD) method to detect the effects of diet and the animals' hormonal condition (non-OVX, OVX, and OVX-HRT). Outliers were treated using the extreme studentized deviate (ESD) method (k = 1, only one round performed for each outcome).

All statistical analysis calculations were performed using GraphPad Prism 7.0 software (GraphPad Software, San Diego, California), and values of p ⩽ 0.05 indicate statistical significance.

Results

Morphometric, serum, and body composition parameters. The oestrogen deficiency induced by ovariectomy led to an increase in final body weight (Table I), whereas HRT-treated groups had maintained body weight similar to the control group, with a significant decrease compared with the OVX-matched groups (hormonal effect accounted for 22% of the total variance; p = 0.004, two-way ANOVA) (Table I). Ovariectomy also led to a reduced relative muscle weight in OVX, whereas the relative muscle weight in OVX-HRT was similar to the control (C) (OVX < C; p = 0.043, two-way ANOVA tests followed by Fisher's LSD) (Table I). Protein restriction also significantly reduced final body weight in OVX animals and more specifically in HRT-treated animals (OVX-LP < OVX and OVX-HRT-LP < OVX-HRT) (diet effect accounted for 17% of total variance; p = 0.004, two-way ANOVA) (Table I). In addition, protein restriction negatively affected relative muscle weight in all LP groups (LP, OVX-LP and OVX-HRT-LP), representing 20% of total variance (p = 0.004, two-way ANOVA test) (Table I). Indeed, the total lean body mass was also negatively affected by protein restriction (diet effect accounted for 44% of total variance; p < 0.001, two-way ANOVA test) (Figure 2a), being more severe in the OVX-HRT-LP group (p < 0.001 vs OVX-HRT, two-way ANOVA test followed by Fisher's LSD) (Figure 2a).

Serum total protein was also negatively affected by protein restriction (diet effect accounted for 13% of total variance; p = 0.021, two-way ANOVA test) (Table I). In particular, ovariectomy associated with protein restriction had a further reduction in serum total protein (p = 0.055, two-way ANOVA test followed by Fisher's LSD) (Table I), and serum albumin was significantly decreased
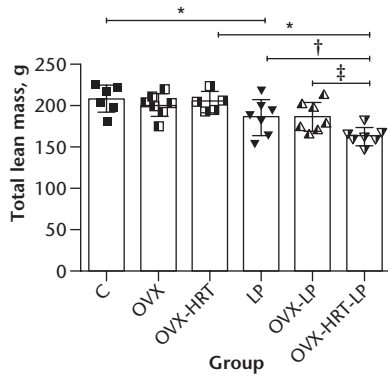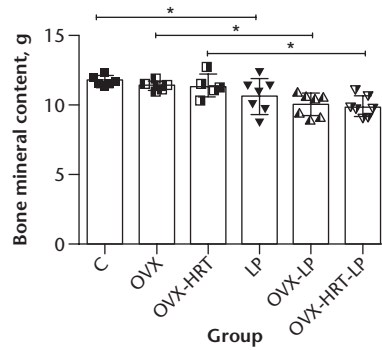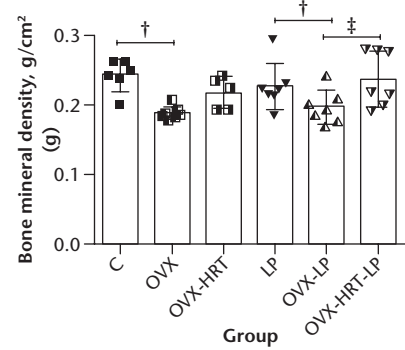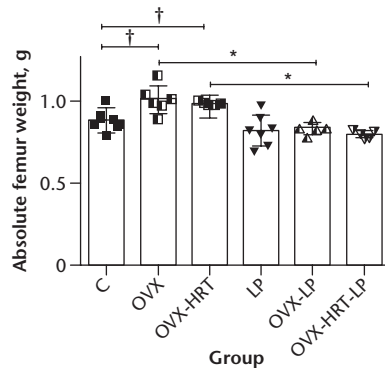
Fig. 2a
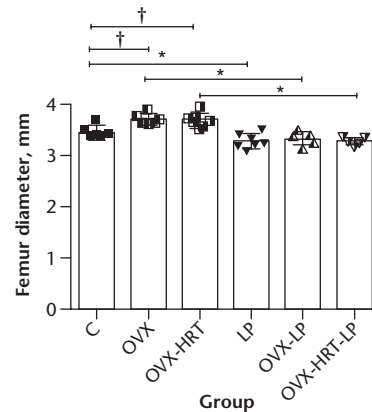

Fig. 2b

Fig. 2c

Fig. 2d

Fig. 2e

Charts showing: a) total body lean mass; b) bone mineral content; c) bone mineral density (both b) and c) parameters were assessed by dual-energy absorptiometry (DXA)); d) absolute femoral weight; and e) femoral diameter from different experimental groups. Results are expressed as the mean (SD). Significant difference accounted for hormonal, diet, or interaction effect, analyzed by two-way analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD) method. *Diet effect: $p \leqslant 0.05$ versus control hormonal-matched group (C, OVX or OVX-HRT); hormonal effect: †$p \leqslant 0.05$ versus control-matched group (C or LP); ‡$p \leqslant 0.05$ versus OVX-matched group (OVX or OVX-LP). C, control; OVX, ovariectomized rats; OVX-HRT, ovariectomized rats subjected to HRT and fed a normoprotein diet; LP, low-protein; OVX-LP, ovariectomized rats subjected to a low-protein diet; OVX-HRT-LP, ovariectomized rats subjected to HRT and fed a low-protein diet.

in the OVX-LP group ($p = 0.003$; hormonal effect accounted for 14% of total variance; $p = 0.045$, two-way ANOVA test followed by Fisher's LSD) (Table I).

Additional results that reinforced the effects of ovariectomy are presented in Supplementary Table ii and Supplementary Figure a.

Bone parameters. Protein restriction negatively affected BMC, as the diet effect accounted for 41% of total variance (LP < C; $p = 0.002$; OVX-LP < OVX; $p = 0.005$, two-way ANOVA test followed by Fisher's LSD) (Figure 2b). In particular, the OVX-HRT-LP group had the lowest BMC value ($p = 0.003$ vs OVX-HRT, using two-way ANOVA test followed by Fisher's LSD) (Figure 2b). Bone mineral density was also reduced in the OVX groups (OVX vs C: $p = 0.001$; OVX-LP vs LP: $p = 0.051$, using two-way ANOVA test followed by Fisher's LSD) (Figure 2c), but was higher in the OVX-HRT-LP group (hormonal effect accounted for 33% of total variance; $p = 0.001$, two-way ANOVA test) (Figure 2c).

The absolute femoral weight and femoral diameter were negatively affected by protein restriction and were significantly reduced in OVX-LP and OVX-HRT-LP compared with OVX and OVX-HRT (diet effect accounted for 47% of total variance; $p = 0.001$; diet effect accounted for 56% of total variance; $p < 0.001$, two-way ANOVA test) (Figures 2d and 2e), although they were greater in OVX and OVX-HRT compared with the C group ($p = 0.003$ and $p = 0.002$ for OVX and OVX-HRT, two-way ANOVA test followed by Fisher's LSD) (Figures 2d and 2e). On the other hand, the hormone replacement increased the relative femoral weight significantly (Supplementary Table ii).

The compression test showed that ovariectomy associated with the low-protein diet led to an adverse effect, reducing the femoral elasticity in the OVX-LP group compared with the LP group (hormonal effect accounted for 20% of total variance; $p = 0.031$; OVX-LP vs LP: $p = 0.012$, two-way ANOVA followed by Fisher's LSD) (Figure 3a). Moreover, the femoral stress offset yield showed a deep decrease in the OVX and OVX-HRT groups (OVX < C; $p = 0.002$; and OVX-HRT < C; $p = 0.034$, using two-way ANOVA followed by Fisher's LSD) (Figure 3b), whereas the femoral peak stress was not significantly different among the experimental groups (Figure 3c).

Table II. Femoral mineralogy in different experimental groups

Mineral	Group						p-value*		
	C	OVX	OVX-HRT	LP	OVX-LP	OVX-HRT-LP	Hormonal effect	Diet effect	Interaction
Mean calcium, ppm (SD)	254 592 (3539)	247 892 (10 136)	245 400 (2194)†	256 157 (9090)	247 130 (8668)†	249 360 (6942)	0.021‡	0.546	0.775
Mean phosphorus, ppm (SD)	129 583 (8653)	130 150 (5788)	127 440 (2120)	130 807 (6720)	121 540 (3214)	125 510 (3989)	0.156	0.132	0.131
Mean sodium, ppm (SD)	6234 (406)	6286 (249)	5916 (806)	6688 (797)	5619 (208)†§	5709 (137)†	0.019‡	0.478	0.059
Mean potassium, ppm (SD)	3615 (587)	3326 (690)	2304 (253)†¶	3298 (550)	2426 (92)†§	2380 (96)†	< 0.001‡	0.021‡	0.093
Mean magnesium, ppm (SD)	4325 (127)	4562 (210)	4409 (322)	4159 (284)	3998 (66)§	4094 (117)§	0.846	< 0.001‡	0.081
Mean zinc, ppm (SD)	258.9 (14.6)	226.4 (15.7)†	221.2 (2.8)†	164 (7.6)§	154.4 (10.3)§	159.3 (10.8)§	< 0.001‡	< 0.001‡	0.006‡
Mean calcium/ phosphorus ratio (SD)	1.97 (0.12)	1.91 (0.06)	1.93 (0.03)	1.96 (0.05)	2.03 (0.02)†§	1.99 (0.02)	0.875	0.008‡	0.031‡

*Significant difference accounted for hormonal, diet, or interaction effect, analyzed by two-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) method
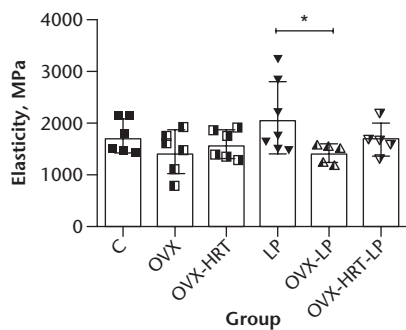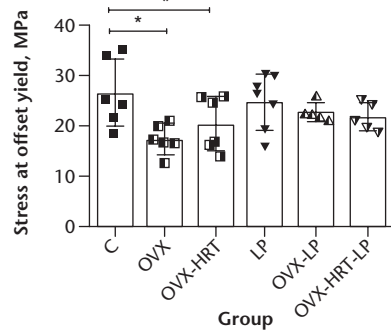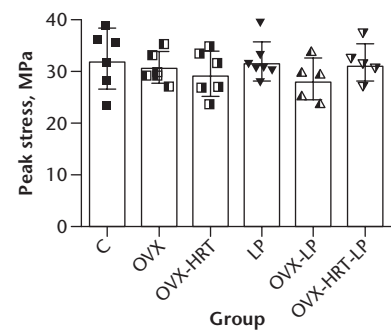†Hormonal effect: $p \leq 0.05$ versus control-matched group (C or LP)
‡Statistically significant
§Diet effect: $p \leq 0.05$ versus control hormonal-matched group (C, OVX, or OVX-HRT)
¶$p \leq 0.05$ versus OVX-matched group (OVX or OVX-LP)
C, control; OVX, ovariectomized rats; OVX-HRT, ovariectomized rats subjected to HRT and fed a normoprotein diet; LP, low-protein; OVX-LP, ovariectomized rats subjected to a low-protein diet; OVX-HRT-LP, ovariectomized rats subjected to HRT and fed a low-protein diet

Fig. 3a

Fig. 3b

Fig. 3c

a) Femoral elasticity; b) stress at the offset yield; and c) peak stress of different experimental groups. Results are expressed as the mean (SD). Significant difference accounted for hormonal, diet or interaction effect, analyzed by two-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) method. Hormonal effect: *$p \leq 0.05$ versus control-matched group (C or LP). C, control; OVX, ovariectomized rats; OVX-HRT, ovariectomized rats subjected to HRT and fed a normoprotein diet; LP, low-protein; OVX-LP, ovariectomized rats subjected to a low-protein diet; OVX-HRT-LP, ovariectomized rats subjected to HRT and fed a low-protein diet.

The femoral mineralogy showed that bone Ca concentrations were affected by ovariectomy, with statistical significance when associated with protein restriction (OVX-LP < LP; hormonal effect accounted for 23% of total variance; $p = 0.021$, two-way ANOVA test) (Table II), but HRT did not reverse this reduction in bone Ca (OVX-HRT < C; $p = 0.053$, two-way ANOVA followed by Fisher's LSD) (Table II). Phosphorus concentrations had no differences among the experimental groups (Table II), whereas the ratio between Ca and P was significantly higher in the OVX-LP group compared with OVX and LP ($p = 0.002$ vs OVX; $p = 0.048$ vs LP, using two-way ANOVA followed by Fisher's LSD) (Table II). In relation to the other minerals, the ovariectomy led to a reduction of bone Zn concentrations in both OVX and OVX-HRT ($p < 0.001$ for OVX and OVX-HRT, using two-way ANOVA followed by Fisher's LSD) (Table II), and lower bone K concentrations only in OVX-HRT compared with the C group (Table II). On the other hand, ovariectomy associated with a low-protein diet led to a reduction of Na, K, Mg, and Zn concentrations in the OVX-LP and OVX-HRT-LP groups (Na: hormonal effect accounted for 21% of total variance; $p = 0.019$; K: hormonal effect accounted for 44% of total variance; $p < 0.001$; and diet effect accounted for 8% of total variance; $p = 0.021$; Mg: diet effect accounted for 45% of total variance; $p < 0.001$; Zn: diet effect accounted for 87% of total variance with $p < 0.001$; hormonal effect accounted for 6% of total variance with $p < 0.001$; and interaction effect accounted for 3% of total variance with $p = 0.006$, all using two-way ANOVA test) (Table II).

Discussion

The oestrogen deficiency caused by ovariectomy, as demonstrated by our results, is consistent with other studies,[7,10] and is similar to what happens in the menopausal state in women.[17,32-37] The increased final body weight associated with significant reduction in the relative muscle weight, as presented here in this experimental model, is also related to these effects observed in menopausal women, where oestrogen deficiency aggravates the metabolic dysfunction that occurs with ageing, increasing body weight and the adipose tissue, as well as increasing muscle loss.[38-41]

The lack of gonadal hormones also leads to impairment of the bone tissue in women and experimental animals.[26,28,42-46] Accordingly, our results could be correlated with an increased osteoporosis risk during menopause, because the ovariectomy negatively affected bone parameters, diminished BMD, and increased bone fragility, which highly characterizes osteoporosis.[42] In fact, the reduction of bone elasticity and stress at the offset yield, found in our results, indicated a lower mechanical resistance compared with nonovariectomized rats, being consistent with other studies where a reduction in the bone mechanical resistance in ovariectomized rats was verified.[28,42] Our results also showed that ovariectomy affected the bone mineral concentrations that are considered essential to bone formation and resorption, and mostly regulated by oestrogen. Similarly, some studies found that ovariectomy significantly diminished Ca, P, and Mg concentration in bone.[47-49] Although the specific role of minor and trace minerals in postmenopausal osteoporosis is still not completely clear, different studies with ovariectomized rats found significant changes in the concentration of elements after ovariectomy.[47-49] Similarly to our findings, some studies have shown that ovariectomy in rats diminished significantly bone concentrations of K and Zn, which is related to changes in bone metabolism.[47-49] Indeed, Zn is reported to affect bone metabolism directly by stimulating osteoblast activity while inhibiting osteoclast activity, whereas K is associated with positive Ca balance, indirectly affecting bone metabolism.[2,47,49,50] Those studies also correlate with data from the literature in which osteoporotic postmenopausal women presented significantly lower serum concentrations of Mg and Zn.[51,52]

In addition, an interesting aspect of this study is that the 17β-oestradiol HRT dose used was lower (0.1 μg/kg body weight) than in other studies,[34,35,43,53,54] and was still effective in maintaining body and bone parameters. The hormonal therapy was indeed able to keep the uterus tissue, as well as body weight, in a healthier state, corroborating with the literature.[33-36] Moreover, hormone replacement correlated with the high metabolic rate observed in the HRT-treated groups, which supports the importance of oestrogen in the maintenance of energy expenditure.[55]

As expected, HRT also benefited bone metabolism. The HRT-treated animals presented higher BMD and bone mechanical resistance compared with ovariectomized rats, in accordance with the literature,[43,44] and in correlation with HRT in postmenopausal women, which is effective in preventing the loss of bone mass and in reducing the incidence of fracture.[14,15,20] The hormone treatment also influenced the mineral concentrations in bone. Significantly decreased levels of Ca, K, and Zn in the femur of the HRT-treated animals were verified, as well as non-significant reduction of P and Na levels. Ynsa et al[49] also verified decreased bone levels of Ca, P, K, and Zn in ovariectomized animals treated with 17β-oestradiol compared with controls, although Avila et al[48] found that the oestrogen treatment restored bone Ca, P, and Mg to control levels.[48,49]

Those results indicate that HRT has profound modulatory effects on bone composition, which should be better investigated to understand and prevent bone loss and fragility.

Considering the higher level of malnutrition in many countries and also the ageing of the population, it is essential that we improve our understanding of, and prevent the major problems related to, muscle-bone interaction with osteoporosis. Previous studies by our group, and also in the literature, showed that a 6% protein diet negatively affected growth, final weight gain, and muscle mass, as well as serum total protein and albumin, while simultaneously maintaining the total body fat due to insufficient calorific intake.[56-60] As shown here, the low-protein diet significantly affected the body parameters negatively but maintained the total body fat, corroborating the literature data. Moreover, as expected, protein restriction also impaired bone mass and mineral composition, diminishing BMC, the absolute femoral weight, and mineral elements such as Na, Mg, P, and Zn compared with controls.[2,6,23,24] In keeping with other studies,[8,10] the low protein intake, or even the protein quality, also changed the bone microarchitecture, increasing bone fragility and negatively affecting mechanical function.

Interestingly, the low-protein diet exacerbated the adverse effects of oestrogen deficiency, intensifying muscle and bone loss, and significantly reducing the bone elasticity, which likely suggests a severe osteosarcopenic state.[9,10,41,61] Furthermore, the low-protein nutrition associated with ovariectomy (menopause) affected most of the body and serum parameters that are likely related to the dysfunction of hypothalamic-pituitary axis regulation, controlling the body's energy source and also the bone cell signalling, further jeopardizing bone health.[9,10,62]

On the other hand, as shown by Wang et al,[63] the parathyroid hormone and other drugs can prevent osteoporosis,[63] and in the present study, we also showed that HRT, specifically using a low oestrogen dose, was able to minimize some adverse effects of protein restriction in ovariectomized animals. In fact, the maintenance of bone parameters such as elasticity, BMD, and relative femoral weight, as well as total body fat and the metabolic rate, were likely attenuated by HRT, which minimized the adverse effect produced by ovariectomy associated to malnourished state. These results are important in clinical treatment to minimize the effects of menopause, especially when linked to an unhealthy nutritional scheme. On the other hand, HRT linked to protein restriction was not able to maintain the total body lean mass or the BMC when compared with the ovariectomized groups. Thus, new studies are necessary to understand better the reason or reasons why, in our experimental conditions, the HRT was not enough to counter the effect of protein restriction on bone microarchitecture, as also shown by the reduced mineral concentrations on the OVX-HRT-LP group in the results of this study. Therefore, HRT can minimize the adverse effects of protein restriction in menopause, but the effectiveness of the hormone treatment may improve with a balanced diet.

In conclusion, the results demonstrated that protein restriction negatively affected body and bone metabolism when in a state of gonadal hormone deficiency. It led to bone and muscle mass loss, and increased the fragility of the bone. The HRT showed some improvements in minimizing those damaging effects imposed by ovariectomy when combined with protein restriction, since HRT had some efficacy to maintain muscle and bone mass, preserving bone resistance and minimizing some deleterious processes during the menopause. We concluded that protein restriction has an adverse effect on metabolism, which can worsen menopausal symptoms, but which HRT can attenuate. Therefore, special attention should be given to a balanced diet during menopause and HRT.

Supplementary Material

Tables showing bone mineral analysis conducted by inductively coupled plasma-optical emission spectometry and the effects of ovariectomy on body, morphometric, and serum parameters in various experimental groups. Figure showing the percentage of body fat and metabolic rate from these groups.

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