Journal of Food, Nutrition and Diet Science
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Original Research

Bovine lactoferrin is efficient for improving bone characteristics: a study based on a new preclinical model of marked bone fragility

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Abstract: Osteoporosis is associated with postmenopausal estrogen deficiency in women. Reduction of protein intake further decreases bone quality in these individuals. In this study, a new experimental model using ovariectomized mice with moderate alimentary protein restriction was used to evaluate the efficiency of lactoferrin, a protein present in milk, on bone mineral density (BMD), 3D-microarchitecture, and different physiological and biochemical parameters. Twelve-week-old female C3H mice were ovariectomized (OVX) or sham-operated (SHAM), and a group of mice were then fed for 3 months with a normal protein diet (NP: 20% of total energy as soy protein), while another group of mice were fed with a low protein diet (LP: 6% of total energy as soy protein) in which 10 g of soy protein was replaced by 10 g of bovine lactoferrin (bLF). Casein was used as a control for bLF. BMD was measured by dual energy X-ray absorptiometry, and the femurs of mice were imaged by microtomography. The effects of the OVX procedure, which were found to depend on the protein level in the diet, were notably significant reduction of BMD and bone quality in both conditions of protein supply. Indeed, a 35% and 70% reduction of the bone volume/tissue ratio were found for the NP OVX and LP OVX mice respectively. The greater bone loss observed in LP OVX mice did not correlate with a change in the ratio of bone formation or resorption, but with a decrease in circulating IGF-1 level. Supplementation of both diets with bLF significantly improved bone quality in OVX mice. In conclusion, the model of ovariectomized mice with moderate protein restriction appears suitable to mimic the marked alteration of bone quality in postmenopausal women with insufficient protein supply. In such a preclinical model, supplementation with bLF is efficient for improving bone characteristics.

Keywords: Dietary supplementation, Lactoferrin, Bone fragility, New preclinical model, Low-protein diet, Ovariectomy

Introduction

Osteoporosis is associated with estrogen deficiency and is characterized by decreased bone mass along with alterations of bone structure [1]. The quality of bone depends on the acquisition and maintenance of bone mass, as well as on the preservation of the structural integrity of the bone. When these parameters are optimal, they provide bones with optimal resistance to usual mechanical loading [2-4]. Adequate intake of key nutrients such as calcium, vitamin D and protein contributes significantly

Received: Sep.1, 2023; Revised: Oct.25, 2023; Accepted: Oct.31, 2023; Published: Nov.7, 2023

DOI: https://doi.org/10.55976//fnds.12023120033-43

to bone health [5-7]. Bone mineral density (BMD) and bone microarchitecture (trabecular number and thickness, cortical thickness) are 2 parameters that depend on the activities of osteoblasts and osteoclasts to allow adequate bone remodeling. Low bone mass may be linked to estrogen deficiency, which increases bone resorption over bone formation, but also to insufficient protein intake when compared with metabolic and physiological needs, thus reducing bone formation [8-9]. In fact, insufficient protein intake is associated with numerous negative consequences, not only on bone quality, but also on muscle mass. Indeed, osteoporosis and sarcopenia are often associated in the elderly, and these pathophysiological processes reduce quality of life and may even shorten life expectancy [10]. Therefore, osteoporosis is a major public health concern. We have previously shown in a model with mice fed a low protein diet (6% of total energy by soy protein) that this dietary condition reduces body weight associated with lower lean mass and BMD compared with mice receiving an adequate protein supply [11-12].

Protein intakes lower than estimated protein requirements are often observed in specific groups of individuals, particularly in elderly patients with osteoporosis. In preclinical studies, the utilization of dietary supplements in the form of specific proteins such as lactoferrin or individual amino acids has been shown to improve osteoblast activity and bone status in the situation of short supply of dietary proteins [12]. Lactoferrin (LF) is known to possess various biological properties, including anti-inflammatory and immunomodulatory effects [13]. Moreover, bovine lactoferrin (bLF) supplementation to mice beneficially modulates different biological processes, notably small intestine epithelial renewal [14], bone loss in ovariectomized (OVX) mice [15-16], and glucose tolerance [17]. In young rats, bLF promotes the differentiation of the small intestine epithelium, reinforces the barrier function of colonic gut, and increases bone mineral density [18]. In adult mice, bLF added to the diet was found to be resistant to major proteolytic degradation in the intestinal fluid and was transported from the intestinal lumen to the bloodstream [19]. Moreover, immunoreactive bLF can reach most peripheral tissues of animals after intestinal absorption [15]. bLF ingestion in OVX mice was correlated with decreased osteoclast activity and increased osteoblast activity, thus supporting a direct effect on bone physiology [16]. Finally, bLF supplementation may increase the plasma concentrations of several amino acids, thus presumably allowing for more efficient protein synthesis due to increased substrate availability [18].

As bLF appears to both improve bone physiology and support protein synthesis in experimental models, the aim of the present study was to compare the efficiency of lactoferrin supplementation in ovariectomized mice fed with a standard normoproteic diet, or with a low protein diet that resulted in mild dietary protein restriction and marked bone fragility.

Materials and methods

Animals

Eight-week-old female C3H/HeN mice (Harlan) were housed (4 animals per cage) at 22 °C under a 12-h light cycle. The mice were fed with a standard AIN-93M diet containing 20% of total energy in the form of soy protein without phytoestrogens (to avoid interference of these phytochemicals with bone metabolism) during the first 4 weeks of habituation. The design of this study was approved by the French government (APAFIS#24484-202002191121731v3). Subsequently, 40 mice were ovariectomized (OVX) and divided into 4 experimental groups of 10 mice each. Twenty other mice were sham-operated (SHAM) and divided into 2 experimental groups of 10 mice each. Mice were anesthetized with isoflurane and morphine before surgery. After surgery, the animals were fed with the different experimental diets for up to 3 months. Mice in the SHAM group keep on consuming the 20% protein diet used as a control group with normal protein (NP), while LP mice were shifted to a moderate low protein (LP) diet containing 6% of total energy as protein. OVX NP mice were fed with the NP diet, while the OVX NP-bLF mice were fed with the NP diet including 10 g bLF/kg of diet, and the OVX LP mice were fed with the LP diet. Another group of mice was fed with the LP diet containing 10 g bLF/kg of diet (OVX LP-bLF). The composition of the main diets used in this study is shown in Table 1. Casein was used as control protein. To maintain an equal amount of energy in the different diets between the animals fed with the NP or LP diets, the amounts of starch and sucrose were increased in the 6% protein diet compared to the 20% protein diet. In addition, to avoid the protein leverage effect in our study, and thus to allow equal energy consumption, we pair-fed all LP animal groups compared to the NP control group. At the end of the experiments, the mice were anesthetized, blood was rapidly drawn by cardiac puncture, and the mice were immediately euthanized by decapitation. Body composition (lean and fat masses) was then determined by dissection. Liver, uterus, spleen, kidneys, and pancreas, as well as four white adipose tissue (WAT) pads (periovarian, retroperitoneal, mesenteric, and total subcutaneous), interscapular brown adipose tissue (BAT), and the carcass (muscle and bone) were recovered and weighed.

Body composition

Body composition was measured at the beginning of the experiments, after 1 and 2 months, and at the end of the study using dual energy X-ray absorptiometry (DEXA) with a Lunar PIXImus densitometer (DEXA-GE PIXImus). To control the stability of the device, the measurement of a phantom was duly performed before each session. Mice were anesthetized by isoflurane

inhalation to allow optimal measurement. The software provided with the device (Lunar PIXImus v2.10) was used to analyse the images, using automatic thresholding.

Table 1. Composition of the diet

Ingredients (g/kg of diet)	NP	LP	NP+bLF	LP + bLF
Soy protein[a]	173	41	173	41
LF[b]	0	0	10	10
Casein[c]	10	10	0	0
Corn starch[d]	584	698	584	698
Sucrose[e]	95	114	95	114
Soybean oil[f]	40	40	40	40
Alpha Celluose[g]	50	50	50	50
AIN 93M mineral mix[h]	35	35	35	35
AIN 93M Vitamins[h]	10	10	10	10
Choline[i]	2.3	2.3	2.3	2.3

[a] MP Biomedicals, Irvine, CA, USA, [b] Armor proteins, Saint-brice en Coglès, France, [c] Ingredia, Arras, France, [d] Cargil, MN, USA, [e] CristalCo Pro, Paris, France, [f] Lesieur, Asnière-sur-Seine, France, [g] Prat Dumas, Couze Saint Front, France, [h] ICN Pharmaceuticals, Orsay, France, [i] Jefo Nutrition, Saint-Hyacinthe, Québec, Canada

Biochemical analysis

At the end of the experiments, the blood of 12h fasted animals was recovered to allow biochemical analysis. After 1- and 2- months of dietary intervention, blood was recovered from the tail vein for additional analyses. N-terminal propeptides of type I procollagen (PINP) and C-terminal cross-linking telopeptides of type I collagen (CTx) were measured by enzyme immunoassay (EIA) (Immunodiagnostic Systems). Total IGF-1 levels were measured by using enzyme-linked immunosorbent assay (ELISA) after inactivation of IGF binding proteins (Immunodiagnostic Systems).

Bovine lactoferrin concentration was determined in the blood plasma using a bovine lactoferrin ELISA kit (Bethyl Laboratories, Montgomery, TX, USA). TNFα and IL-6 in plasma were determined using a sandwiched ELISA kit (Thermo Scientific, Courtaboeuf, France).

Oral glucose tolerance test

Oral glucose tolerance tests (OGTT) were performed under the different experimental conditions at the end of the experiment with 6 hours fasting mice, exactly as previously described [20]. Briefly, a glucose bolus (2g/kg) was administered into the stomach via a gavage needle. Blood samples were then collected from the tail at different times. Blood samples (25 µl) were collected before gavage (t0) and then 15, 30, 60 and 120 min after gavage for measurement of glycemia and insulinemia. Blood glucose was measured using One Touch Vita system (LifeScan, Issy-les-Moulineaux, France). Then, the remaining blood was centrifuged (3000g, 15 min, 4°C) and plasma was stored at -80°C until assayed for insulin using a Mercodia Mouse Insulin Elisa kit (Mercodia AB, Sweden).

Muscular strength

A few days before anesthesia, grip tests were carried out to evaluate the muscle strength of the mice using the BIO-GS3 Grip Strength Test device (Bioseblab, France) and the average values of the force were expressed in newtons (N) after 5 tests.

Measurement of bone microarchitectural parameters

To determine the microarchitecture of the femurs, we used a high-resolution X-ray Micro-CT device (Quantum FX Caliper, Life Sciences, Perkin Elmer, Waltham, MA, United States) provided by the PIPA Platform, EA2496 in Montrouge, France. The X-ray source was set at 90 V and 160 µA. Samples up to 65 mm in diameter and 200 mm in length were imaged with a Full 3D high-resolution system. Raw data were obtained by rotating both the X-ray source and the flat panel detector 360° around the sample, with a rotation step of 0.1° and a scan time of 3 minutes. The image projections were then automatically reconstructed (RigakuSW software, Caliper) into a Dicom stack of files using standard back-projection techniques. The multiplanar reconstruction tools used in this analysis allow images to be displayed in axial orientation and allow to visualize bone microstructure. Scans for the trabecular bone were obtained from the distal femoral growth plate. The different aspects of the trabecular bone were finally quantified at a 3D isotropic voxel size of 10x10x10 μm^3. The structural indices were evaluated using numerous parameters including the ratio of segmented bone volume to the total volume of the region of interest (BV/TV, %) and trabecular number (Tb.n, 1/mm). Trabecular thickness (Tb.Th, µm) was calculated on 3D images using CtAn® Skyscan software and a method previously described by Hildebrand and Ruegsegger (29, 30). Cortical thickness and diameter (mm) were measured manually on the image at the mid-diaphysis level. Regarding cortical bone area (B.Ar, mm^2) and moment of inertia (mm^4), these parameters were measured at the mid-diaphysis level as previously described [21]. Details of these techniques have been previously published in Lespessailles et al. [22].

Bone characteristics

The left femur of each mouse was recovered by dissection, and after careful separation from muscles, was fully dried overnight at 110°C. After weighing, the bones were incinerated at 550°C for 48 hours, and the weight of the ashes was finally measured. The protein fraction of the bones was finally indirectly estimated by measuring difference between the dry weight of the bones and the weight of the corresponding ashes.

Statistical analysis

All results obtained in this study were expressed as mean ± SD along with the number of independent experiments. Comparison of results between the different experimental groups receiving the NP or LP diets was performed using

a one-way analysis of variance (ANOVA) and a Tukey multiple comparison test. In addition, a t-Test was done to compare the Sham NP with the Sham LP. Differences were considered significant when p was less than 0.05. All statistical analyses were performed using Prism® Version 6.05 (GraphPad Software Inc.).

Results

Effect of the OVX and protein level on body composition

The low protein diet reduced weight gain in the mice, as previously shown. The average weight of the mice was 21.6 ± 0.9 g at T0 and 35.7 ± 2.4 g in the NP and 25.0 ± 1.0 g in the LP group at the end of the experiment. Figure 1A shows that the OVX procedure induced significant weight gain when the mice consumed the NP diet, but when the mice consumed the LP diet, no such significant weight gain was observed. After bLF supplementation, the LP diet resulted in greater weight gain, which correlated with an increase in fat and lean mass (Figure 1B and 1C). As shown in Figure 1D, the adiposity index of the mice ingesting the NP diet was higher in the OVX group than in the OVX+LF group. For the mice fed with the LP diet, no significant changes of the adiposity index were observed.

A more detailed analysis of body composition is presented in Table 2. The LP diet reduced fat mass by more than 60%. No significant differences were observed in fat mass between the different groups in the NP group, but an increase in carcass weight was observed in the OVX and OVX+LF compared with the Sham group. When the mice consumed the LP diet, the OVX procedure induced an increase in fat mass without change of the carcass weight. However, bLF supplementation to the LP diet was able to increase the carcass weight, supporting that bLF might favor protein synthesis. Moreover, the body weight reduction of the LP mice was also associated with a reduction of the weight of the liver, kidney, and spleen.

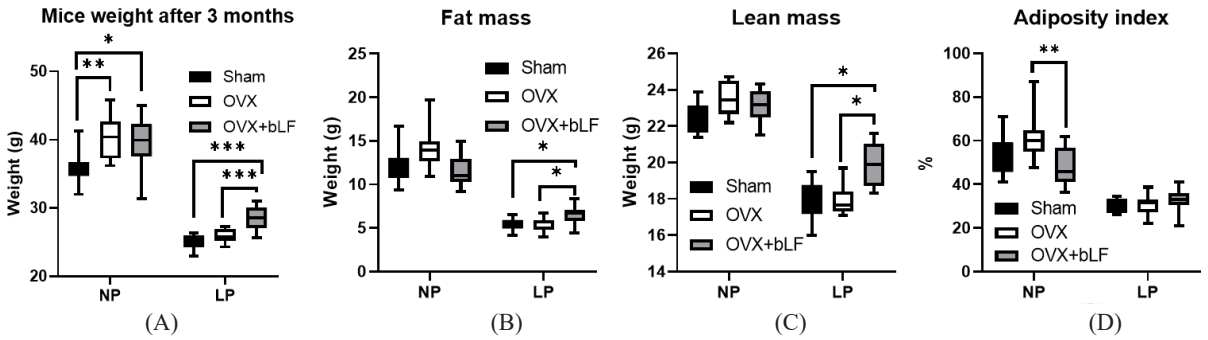


Figure 1. Effect of ovariectomy (OVX) and restricted protein diet on body weight and body composition. The mice were fed for 3 months with either normal protein (NP) diet (20% soy protein) or with a 6% soy protein diet (low protein [LP]) and mice weight (A), fat mass (B), lean mass (C), and adiposity index (D) were determined. Data are presented as box and whiskers, (n = 10). Values in the different groups were compared using a one-way analysis of variance (ANOVA) and a Tukey multiple comparison test. (*p < 0.05, **p < 0.01, ***p < 0.001).

Table 2. Body composition

Parameters	NP			LP		
	Sham	OVX	OVX+LF	Sham	OVX	OVX+LF
Total fat mass (g)	9.20±0.69	9.52±0.53	10.19±0.33	3.45±0.12^{a}	4.02±0.16^{b}	4.62±0.24^{b}
Internal fat (g)	5.23±0.40	5.22±0.18	5.53±0.21	1.83±0.18^{a}	2.44±0.09^{b}	2.60±0.12^{b}
Subcutaneous fat (g)	4.02±0.30	4.30±0.25	4.73±0.11	1.46±0.07^{a}	1.58±0.08^{a,b}	2.01±0.13^{b}
Carcasse (g)	10.65±0.17^{a}	12.18±0.27^{b}	12.0±0.22^{b}	8.39±0.1^{a}	8.55±0.1^{a}	9.84±0.1^{b}
Kidney (mg)	316 ± 4^{a}	377 ± 8^{b}	387 ± 10^{b}	238 ± 4^{a}	251 ± 11^{b}	291 ± 4^{c}
Liver (g)	1.15±0.03	1.25±0.03	1.27±0.07	0.87±0.02^{a}	0.96±0.03^{a,b}	0.98±0.02^{b}
Spleen	111 ± 8^{a}	83 ± 12^{b}	76 ± 3^{b}	62 ± 5	67 ± 4	60 ± 4

The values are means ± SEM (n = 10). Values in the different (NP or LP) groups were compared using a one-way analysis of variance (ANOVA) and a Tukey multiple comparison test. Values with different letters are significantly different (p<0.05).

Effects of OVX and dietary protein intake on the glucose and insulin responses to OGTT

As there were significant differences between the fat masses of the mice fed with the low protein diet compared with the mice fed with the normal protein diet, an oral glucose tolerance test (OGTT) was performed at the end of the study. The results (Figure 2) show that in mice ingesting the NP diet, the OVX procedure increased the fasting blood glucose (90 ± 15 versus 145 ± 13 for the Sham and OVX mice, respectively). In addition, the glucose area under the curve (AUC) was more importantly increased for the OVX group. In the LP group, fasting blood glucose was similar in the three groups, and the values were similar to those measured in the Sham NP group. However, blood glucose levels were increased after 15 minutes in the OVX and OVX+LF mice (202 ± 10, 289 ± 17, and 260 ± 14 for the Sham, OVX, OVX+LF mice, respectively). The AUC of blood glucose was significantly lower in the Sham-LP group compared with the Sham-NP group (19105 ± 279 and 15617 ± 460 for the Sham-NP and Sham-LP groups, respectively), and a significant increase in the AUC of blood glucose was observed in the OVX mice, whatever the diet ingested. However, bLF ingestion tended to reduce the AUC of glucose.

From the measurement of blood insulin, it can be presumed that insulin secretion from pancreatic islet beta cells was more important for the mice in the Sham NP group than for the animals in the Sham LP. Furthermore, the OVX procedure was found to increase insulinemia in mice in both groups (Figure 3A and B). Insulin AUC values equal to 137 ± 13, 213 ± 14, and 182 ± 9 were measured in mice in the NP group (Sham, OVX, and OVX+LF), respectively (Figure 3C). A similar effect was observed for insulin AUC when the mice consumed the LP diet. In fact, values equal to 63 ± 6, 89 ± 8, and 88 ± 6 were measured for the Sham, OVX and OVX+LF mice, respectively.

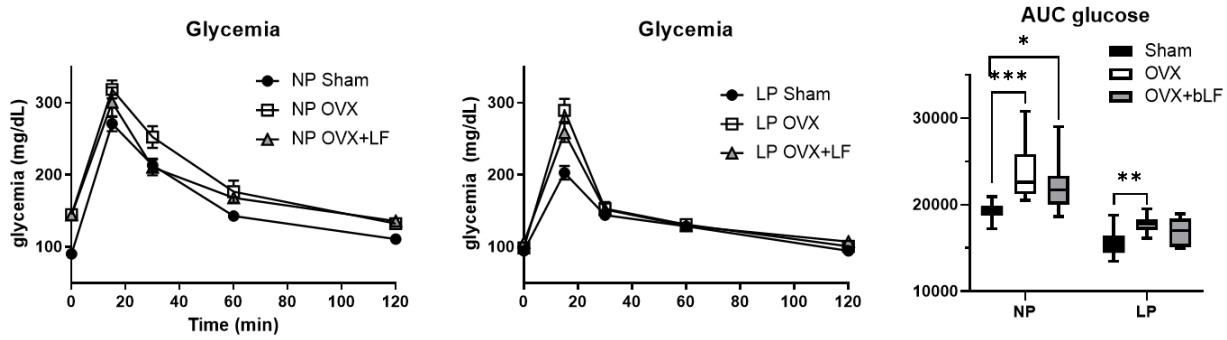
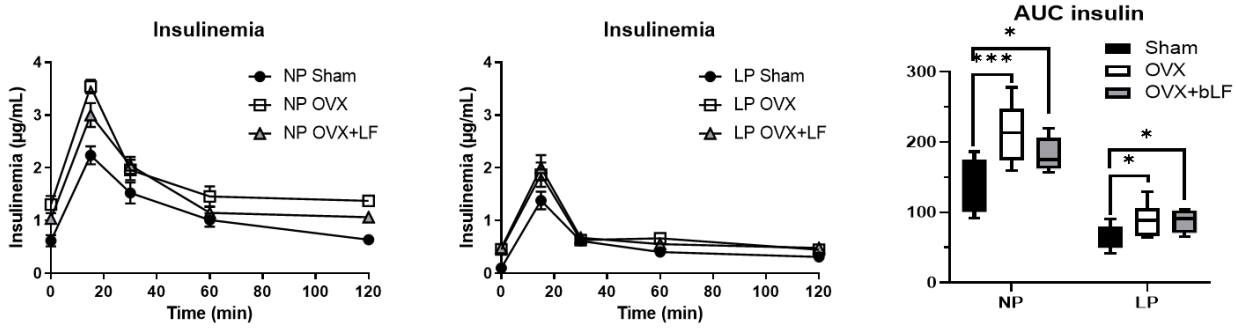


Figure 2. Effect of ovariectomy (OVX) and restricted protein diet on glycemia. Evolution of glycemia concentrations during the oral glucose tolerance test for mice fed for 3 months with the normal protein (NP) diet (20% soy protein), and mice fed with a 6% soy protein diet (low protein [LP]). The values are means ± SEM (n = 10). Glucose AUC were calculated using the trapezoidal rule. Data are presented as box and whiskers (n = 10). Values in the different groups were compared using a one-way analysis of variance (ANOVA) and a Tukey multiple comparison test. (*p < 0.05, **p < 0.01, ***p < 0.001).



(A) (B) (C)

Figure 3. Effect of ovariectomy (OVX) and restricted protein diet on insulin concentrations during the oral glucose tolerance test for mice fed for 3 months with the normal protein (NP) diet (20% soy protein), and mice fed with a 6% soy protein diet (low protein [LP]). The values are means ± SEM (n = 10). Insulinemia is shown on panels A and B. Insulin AUC were calculated using the trapezoidal rule (C). Data are presented as box and whiskers (n = 10). Values in the different groups were compared using a one-way analysis of variance (ANOVA) and a Tukey multiple comparison test. (*p < 0.05, ***p < 0.001).

Effect of the restricted dietary protein intake and the OVX procedure on IGF-1, muscle strength and uterus weight

Figure 4A indicates that, as previously shown, the dietary protein restriction reduced plasma IGF-1 level, and bLF supplementation to the LP diet was able to increase such IGF-1 level. Protein restriction also slightly reduced muscular strength compared with the NP diet (1.48 ± 0.04 and 1.38 ± 0.02 N ($p<0.0001$) for the Sham NP and Sham LP, respectively). The OVX procedure reduced muscular strength more significantly, whereas this strength was significantly increased by bLF supplementation, up to the level measured for the NP group (1.38 ± 0.02, 1.18 ± 0.03, and 1.54 ± 0.06 N for the Sham, OVX, and OVX+LF groups, respectively) (Figure 4B).

As previously shown, OVX procedure and protein restriction reduced uterus weight. However, bLF supplementation was able to mitigate the weight loss of uterus (Figure 4C). As the OVX procedure is known to increase the TNFα expression, we evaluated TNFα and IL-6 plasma concentrations. The plasma levels of these two cytokines were not changed by protein restriction (Table 3) and OVX procedure did not modulate IL-6 plasma level. However, we measured an increase of the TNFα plasma level after OVX procedure, such increase being reduced by bLF ingestion.

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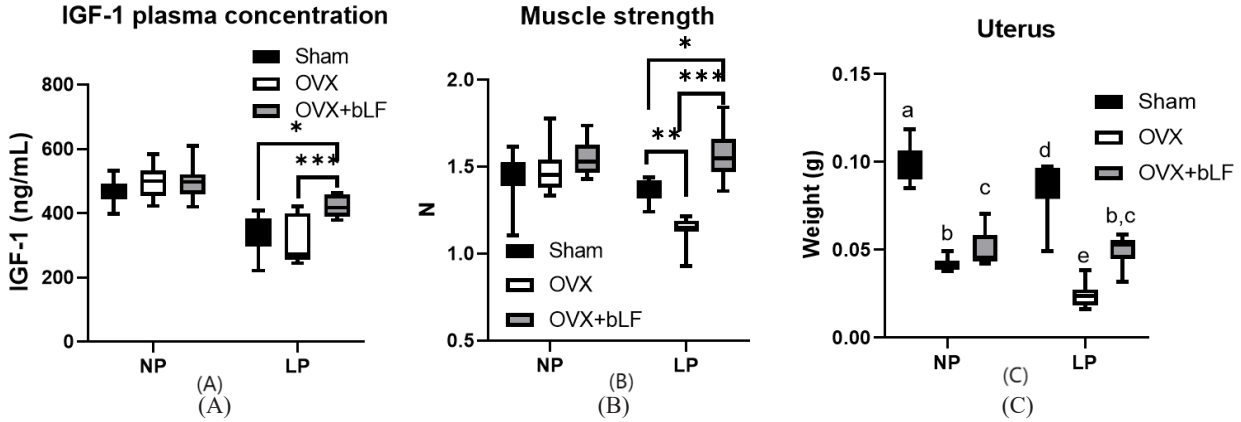

(A) (B) (C)

Figure 4. Effect of ovariectomy (OVX) and restricted protein diet on Insulin like growth factor 1 (IGF-1) plasma concentration, muscle strength and uterus weight. The mice were fed for 3 months with either normal protein (NP) diet (20% soy protein) or with a 6% soy protein diet (low protein [LP]) and IGF-1 plasma concentrations (A), muscle strength (B), and utérus weight (C) were measured. Data are presented as box and whiskers, (n = 10). Data obtained in the different groups for IGF-1 plasma concentration and muscle strength were compared using a one-way analysis of variance (ANOVA) and a Tukey multiple comparison test. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Data obtained in the different groups for uterus weight were compared using a one-way analysis of variance (ANOVA) and a Tukey multiple comparison test. Means that are significantly different ($p < 0.05$) according to the Tukey multiple comparison test have different letters.

Table 3. Plasma cytokine concentrations

Parameters	NP			LP		
	Sham	OVX	OVX+LF	Sham	OVX	OVX+LF
TNFα	2.52±0.27[a]	5.96±0.65[b]	3.04±0.46[a]	2.62±0.34[a]	6.18±0.16[b]	3.47±0.38[a]
IL-6	3.75±0.56	2.65±0.40	4.27±0.52	3.06±0.71	3.84±0.81	2.88±0.45

The values (pg/mL) are means ± SEM (n = 10). Values in the different (NP or LP) groups were compared using a one-way analysis of variance (ANOVA) and a Tukey multiple comparison test. Values with different letters are significantly different ($p<0.05$).

Effects of dietary protein restriction and bLF supplementation on bone mineral density

After 3 months of consumption of the different experimental diets, BMD are shown in Figure 5. The LP diet significantly reduced the whole body, femoral, and vertebral BMD. The OVX procedure reduced the BMD whatever the diet ingested, and bLF supplementation was efficient for restoring the BMD both in NP and LP animals

Effects of the OVX procedure, dietary protein restriction, and bLF supplementation on plasma concentrations of bone-remodeling markers

CTX plasma concentration (Figure 6), used as an osteoclast activity marker, was increased by the OVX procedure, whatever the diet given to mice. bLF ingestion significantly reduced CTX plasma level only when the mice ingested the LP diet. The increase of CTX plasma concentration was associated with an increase of PINP, the osteoblast marker activity, only when the mice ingested the NP diet. The LP diet was able to reduce the PINP

level. Moreover, the OVX mice (for which we reported an increase of the CTX plasma level) were apparently not able to compensate for such increase induced by a modification of the PINP level. After 3 months, the CTX plasma level was still increased by the OVX procedure (Figure 6), and bLF ingestion significantly reduced CTX plasma level whatever diet given to the mice. The PINP level was still lower when the mice received the LP diet. However, the bLF supplementation was able to increase the PINP level when compared with the mice without supplementation.

Effect of OVX procedure, dietary protein restriction, and bLF supplementation on bone microarchitecture

The combination of OVX procedure and protein restriction induced a significant reduction (75%) of the trabecular bone (BV/TV) compared with the Sham NP mice (Figure 7). Protein restriction was detrimental with respect to most of the parameters evaluated. When bLF was added to the diet, bone quality was improved whatever the dietary protein level was. Evaluation of cortical bone quality (Figure 8) showed that the combination of protein restriction and OVX procedures had deleterious effects that were not observed when the mice were subjected to only one of the two procedures. However, under these conditions, bLF supplementation was able to restore cortical bone quality.

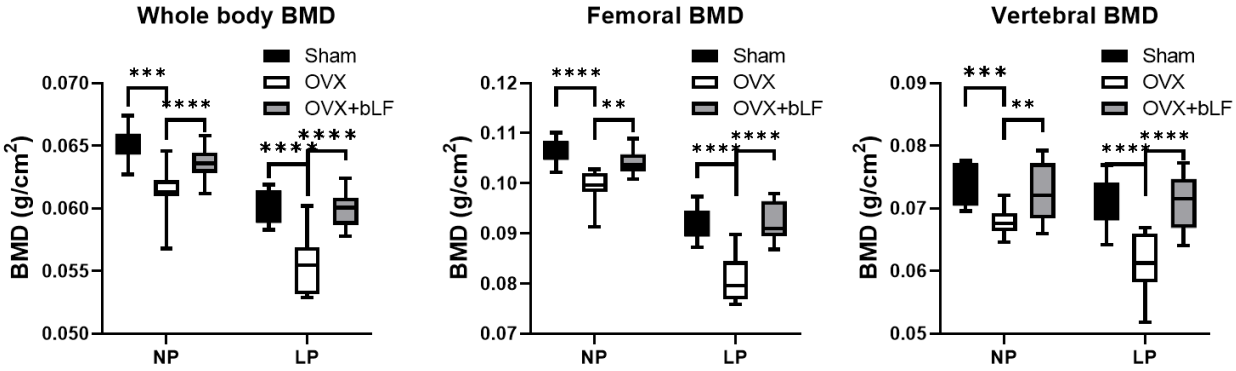

Figure 5. Effect of ovariectomy (OVX) and restricted protein diet on bone mineral density (BMD). The mice were fed for 3 months with either normal protein (NP) diet (20% soy protein) or with a 6% soy protein diet (low protein [LP]). Data are presented as box and whiskers, (n = 10). Values in the different groups were compared using a one-way analysis of variance (ANOVA) and a Tukey multiple comparison test. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

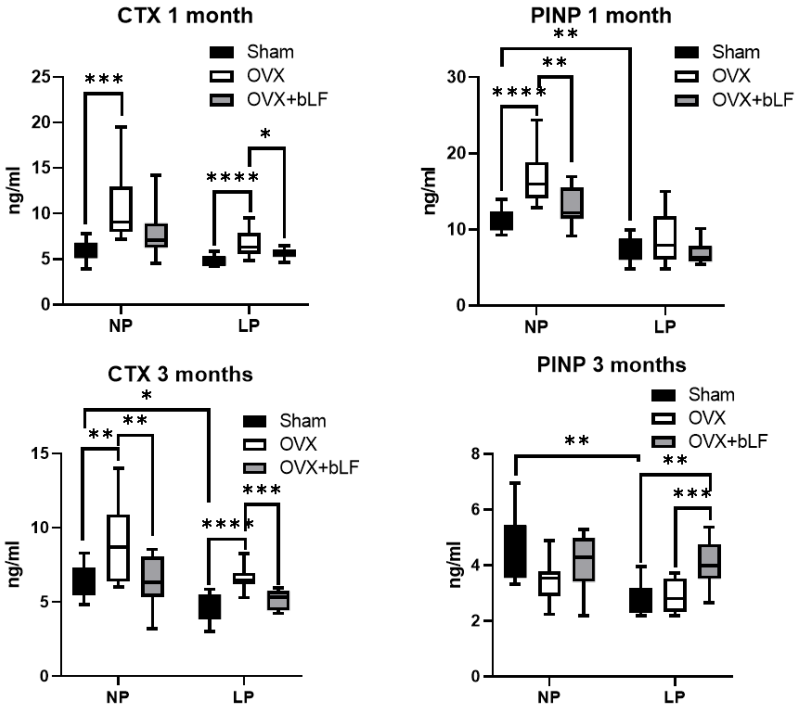

Figure 6. Effect of ovariectomy (OVX) and restricted protein diet on marker of osteoclast (CTX) and osteoblast (PINP) activities. The mice were fed for 3 months with either normal protein (NP) diet (20% soy protein) or with a 6% soy protein diet (low protein [LP]) and CTX activity was measured after 1 month and after 3 months. PINP activity was also measured after 1 month and after 3 months. Data are presented as box and whiskers, (n = 10). Values of all the groups are compared using a one-way analysis of variance (ANOVA) and a Tukey multiple comparison test. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

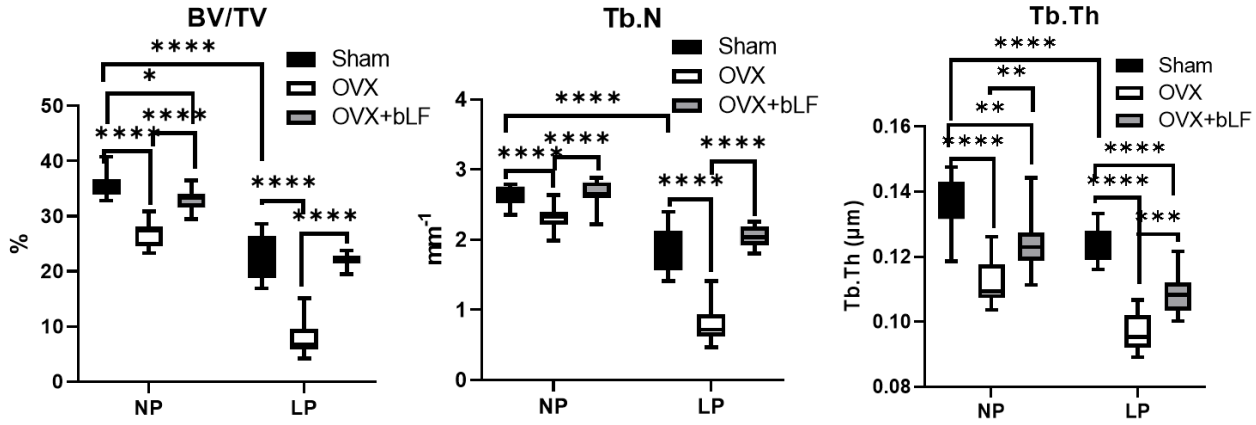

Figure 7. Effect of ovariectomy (OVX) and restricted protein diet on trabecular bone. The mice were fed for 3 months with either normal protein (NP) diet (20% soy protein) or with a 6% soy protein diet (low protein (LP)). Then the bone microarchitecture was determined. The bone volume to the total volume of the region of interest (BV/TV) was determined as well as the Trabecular number (Tb.N), and the Trabecular thickness (Tb.Th). Data are presented as box and whiskers, (n = 10). Values of all the groups are compared using a one-way analysis of variance (ANOVA) and a Tukey multiple comparison test. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

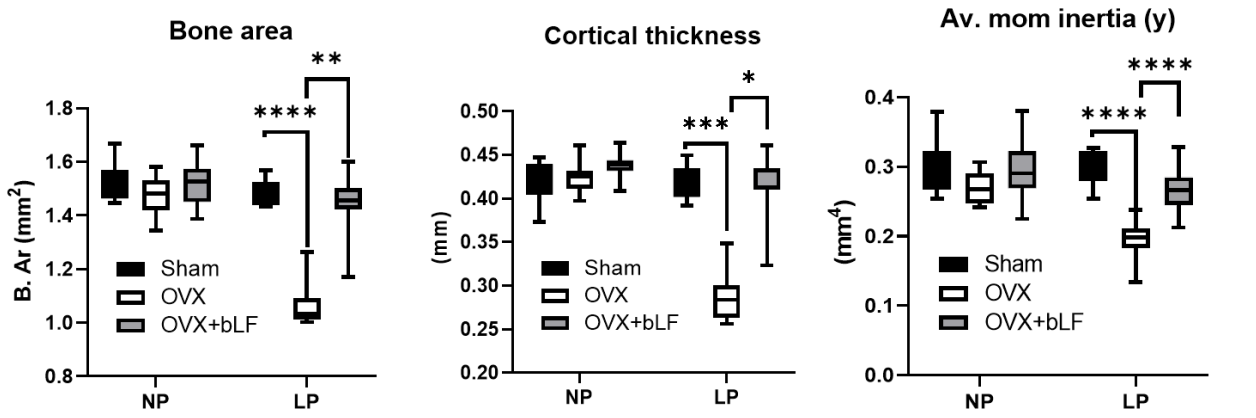

Figure 8. Effect of ovariectomy (OVX) and restricted protein diet on cortical bone. The mice were fed for 3 months with either normal protein (NP) diet (20% soy protein) or with a 6% soy protein diet (low protein (LP)). Then the bone microarchitecture was determined. The figure indicates the bone area, the cortical thickness, and the Average moment of inertia (Av. mom inertia). Data are presented as box and whiskers, (n = 10). Values of all the groups are compared using a one-way analysis of variance (ANOVA) and a Tukey multiple comparison test. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

Discussion

In the present preclinical study, we propose a new model of bone fragility induced by ovariectomy combined with moderate restriction of dietary protein supplied to mice. This experimental situation aimed to mimic the clinical situation of postmenopausal women with insufficient dietary protein intake. Such a situation can also be observed in "real life", as aged women often have lower dietary protein intake when compared with younger women [23] in a context of increased protein requirement [24]. Indeed, in individuals over 65 years, the dietary protein requirement is estimated to be 1.0-1.2 g protein per kg body weight per day [25], which is higher than the recommended protein intake for healthy young adults (that is 0.8 g protein per kg body weight per day [26]). The data obtained clearly show that moderate protein restriction further deteriorates bone quality that is already impaired by the ovariectomy procedure. Furthermore, in this new model of bone fragility, our data indicate beneficial effects of lactoferrin supplementation to improve bone characteristics, thus demonstrating the relevance of such a model for testing dietary interventions with supplements.

More precisely, the LP diet by itself was found to reduce body weight gain in C3H/HeN mice. In previous studies, the low protein diet was shown to reduce IGF-1 level, and the present study indicates that bLF supplementation was able to increase IGF-1 level, supporting the view that bLF favors protein synthesis in target tissues [27]. The bLF supplementation partially counteracted the BMD reduction induced by the OVX procedure whatever the NP or LP diets used. Indeed, evaluation of the bone markers indicates that both CTX and PINP levels were modulated depending on the diet used for mice feeding. Interestingly, the OVX procedure showed a significant effect on the CTX level whatever the NP or LP diet used. Our data show that the combination of ovariectomy and LP diet leads to a very significant reduction of the trabecular bone (75% reduction). Since trabecular bone is mainly found in long bone and vertebra, a large reduction of trabecular bone may increase the risk of fragility defects occurring in these bones [28]. It is worth noting that only the combination of LP diet and OVX procedure reduced the cortical bone. These data highlight the combination of deleterious effects of protein deficiency and oestrogen depletion on bone characteristics.

Regarding the metabolic characteristics of ovariectomized mice fed with the NP diet, the increased adiposity observed in these animals was associated with an increase of the fasting blood glucose, an increase of the glucose AUC, and an increase of insulin AUC. The LP diet reduced glucose and insulin AUC compared with the NP diet, indicating, as expected, a correlation between obesity and glucose tolerance. The OVX procedure was found to increase glucose and insulin AUC in both LP and NP dietary conditions. Thus, our data are in good accordance with the results of previous studies showing that a decrease in estrogen level is associated with central obesity, insulin resistance, and decreased energy expenditure ([29-30]).

As shown in previous experiments [11-12], LP diet reduced IGF-1 level. Our data show that bLF supplementation was able to increase several endocrine and physiological parameters, namely IGF-1 level, muscle mass, and muscle strength, thus once again supporting that bLF can increase protein synthesis in target tissues [31]. Moreover, the reduction of the uterus weight by the OVX procedure was thwarted by bLF supplementation, whatever the LP or NP diet fed to the animals. Previously, we reported that bLF supplementation to young rats increases the plasmatic concentration of several amino acids, such as the essential amino acids phenylalanine and tyrosine, and such increased availability may favor protein synthesis in the different tissues [18]. Further experiments are required to confirm this hypothesis, which is not the aim of the present study.

Evaluation of BMD of the whole body, femur and vertebra showed that the LP diet reduced BMD in the three compartments, as expected, and that bLF supplementation was able to thwart the BMD decrease induced by the OVX procedure. Estrogen withdrawal is associated with T cell activation, which produces essential osteoclastic factors such as RANKL and TNFα that contribute to osteoporosis [32-33]. The OVX procedure is associated with increased activity of the osteoclasts. Our data show that bLF supplementation was able to reduce CTX plasma level regardless of the LP or NP diet used. These data support the view that bLF ingestion reduces the immunological dysregulation induced by the OVX procedure and consequently bone loss [16]. Moreover, the bLF supplementation was able to increase PINP level in the OVX mice that ingested the LP diet. It is worth nothing that, bLF supplementation has been shown to increase the availability of trans-4-hydroxy-L-Proline, such compound being known to play a crucial role in collagen synthesis.

Finally, analysis of the bone microarchitecture shows that the effect of the LP diet is quantitatively as important as the OVX procedure, and when both conditions are associated, which is the condition of sarcopenia in elderly osteoporotic patients, the bone quality is significantly decreased. This may explain the higher risk of fracture in such population with insufficient dietary protein intake when compared to the need. As shown by the BMD analysis, BV/TV volume and Tb.N are largely preserved by bLF ingestion, whatever the diet consumed. What noteworthy is that the cortical bone of C3H mice was less sensitive to OVX or LP diet than the trabecular bone. These results are consistent with previous data obtained in our laboratory, showing that when Balb/C ingested LP diet, a reduction of the bone area and cortical thickness is observed. However, it is known that the characteristics of cortical bone of C3H mice is different from those of Balb/C mice [34], which probably explains the differences in the parameters obtained when comparing the two strains of mice. Indeed, in a previous study, we obtained values of to 0.974 ± 0.022 and 0.259 ± 0.013 for the bone area and the cortical thickness, respectively, in

Balb/C mice, and these values were 62% lower than those obtained for C3H mice. In other words, the strain of mice used in preclinical studies may yield different results in terms of the magnitude of the effects on bone physiology.

In conclusion, the results of the present study allow us to propose this new experimental model of ovariectomy in mice fed with a low protein diet as suitable for studying the marked deleterious effects of oestrogen deficiency and inadequate protein supply on bone quality, and as timely for studying a dietary supplementation aimed at limiting such deleterious effects in a context of high prevalence of osteoporosis in the aging population. Regarding this last point, our results indicate that supplementation of both diets with bLF significantly improved bone quality in OVX mice. Moreover, bLF supplementation to mice ingesting the LP diet preserved the lean body mass and increased IGF-1 level. The results reinforce the view that bLF dietary supplementation favors protein synthesis and is beneficial in terms of bone characteristics.

These experimental studies should stimulate future work testing other dietary components expected to improve bone quality in our new preclinical model and stimulate future clinical dietary interventions in volunteers.

Disclosure

The authors declare no conflict of interest.

Acknowledgments

The authors thank INRAe, AgroParisTech, Université Paris-Saclay, and Faculty of Dentistry in Tours (France) for their constant support.

Funding information

This work was performed with the financial help of AgroParisTech.

Authors' contribution

All three authors participated in the conception of the study and in the writing of the paper. AB and GYR performed the experiments.

Ethics approval

The design of this study was approved by the French Government (APAFIS#24484-202002191121731v3).

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