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HEALTH SCIENCES

Caffeine and Ovariectomy Debilitate Bone Health in Rats on Calcium-deficient Diet

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Abstract: This study investigated the effects of ovariectomy and caffeine intake on bone health in rats on calcium-deficient diet. Forty adults female Wistar rats were divided into 4 groups in a 2x2 factorial design: Ovary (OVX/SHAM) and Caffeine (placebo/caffeine). The animals were housed in individual cages for 8 weeks, receiving 18-20g of AIN-93M diet per day, containing 50% of the daily recommended intake of calcium. The rats underwent ovariectomy (OVX) or laparotomy (SHAM) surgery. Caffeine groups received 6mg of caffeine/kg/day. After euthanasia, the tibia and femur were dissected to determine the calcium content and bone fracture strength, respectively. Blood sample was collected to determine serum Ostase. 24-hour urine was analyzed for excreted calcium and NTx. Reduced bone fracture strength and calcium content were observed in OVX and Caffeine groups. When observed separately, OVX group showed increased urinary NTx and lower bone weight, blood ostase, and urinary calcium. Caffeine groups showed elevated urinary calcium. There was a positive correlation between bone fracture strength and calcium content. NTx correlated negatively with bone calcium, fracture strength and thickness. In conclusion, both OVX and caffeine intake debilitate bone health in rats on calcium-deficient diet.

Key words: Estrogen deficiency, Calcium inadequacy, Fracture strength, Bone strength.

INTRODUCTION

Osteoporosis is a metabolic bone disease marked by reduced bone mineral content (BMC), reduced bone mineral density (BMD), and increased bone fragility and fracture risk due to unbalanced bone remodeling (Kapus et al. 2020, Kim et al. 2021, Zhu et al. 2020, Liu et al. 2022). Osteoporotic fractures seriously impact the quality of life of the population, with a mortality rate ranging from 21.5 to 30.0% (Oselame et al. 2016) after 12 months of osteoporotic fracture. This shows how much the aging of the world's population has turned osteoporosis into a serious public health issue, impacting millions of postmenopausal women (Xu et al. 2019, Li et al. 2021, He et al. 2022).

Menopause is a natural aging process for women that promotes metabolic and body changes, which increases the risk of cardiovascular and bone diseases, among others things (Franco et al. 2021, Kapus et al. 2020). The bone is a dynamic organ affected by various hormones. Postmenopausal estrogen deficiency can reduce BMC, BMD, and bone structure (Kapus et al. 2020, Sun et al. 2022), by interfering in bone remodeling balance and increasing its resorption mediated by osteoclastic activity (Kim et al. 2021, Zhu et al. 2020, Sapra et al. 2021). This process leads to greater bone fragility and increases its risk of fracture (Xu et al. 2019, Sapra et al. 2021, Hajisadeghi et al. 2021). The ovariectomy (OVX)-induced estrogen deficiency animal model is the most efficient and most used model for bone

remodeling studies (Long et al. 2022, Jang et al. 2022). The effects of estrogen deficiency on bone health may be exacerbated by dietary factors, such as the caffeine intake.

Caffeine (1,3,7-trimethylxanthine) is a plant alkaloid present in coffee, soft drinks, tea, chocolate, among other foods (Xu et al. 2019). The excessive ingestion these foods can be associated with loss of calcium and bone mass (Bierhals et al. 2019, de Souza et al. 2021) by reducing bone formation biomarkers (Xu et al. 2019) and increasing urinary calcium excretion (de Souza et al. 2021), promoting higher susceptibility to bone fracture (Dal-Fabbro et al. 2021, Guillan-Fresco et al. 2020). The combination of chronic caffeine intake with estrogen deficiency can aggravate the risks of fracture and bone health. However, no studies have evaluated its possible effects.

Inadequacy of dietary calcium intake is common among Brazilian women, who meet approximately 52% of calcium daily recommended intake (Oselame et al. 2016, Assis et al. 2018, Souza et al. 2020). Such food deficiency reduces circulating calcium (Silva et al. 2013), triggering an increase in parathyroid hormone (PTH) synthesis and its release in order to restore blood levels (Yadav et al. 2021). This mechanism can raise bone resorption and increase the risk of fracture (Yadav et al. 2021, Assis et al. 2018, Souza et al. 2020, Topolska et al. 2020).

The effects of a low-calcium diet on calcium metabolism and bone health may be potentiated by estrogen deficiency and caffeine intake, raising the risks of bone fragility and fracture. However, few studies have investigated the combination of these factors and their effects on direct measures of BMC and bone fracture resistance, as well as on bone remodeling biomarkers. In this context, the present study investigated the effects of OVX and caffeine intake on bone health in rats on a low-calcium diet.

MATERIALS AND METHODS

Experimental design

This study was conducted in forty 160-day-old adult female Wistar rats (weight: 274.6±19.9g; mean ± SD), sorted into 4 groups (n = 10). The animals were assigned to the treatments, in a 2x2 factorial design: SHAM Placebo (SP); OVX Placebo (OP); SHAM Caffeine (SC); OVX Caffeine (OC). The animals were housed in individual cages for 8 weeks, in a room with controlled temperature at 22±2ºC, relative humidity of approximately 60% and a light-dark 12-hour cycle. The rats received a daily dose of 18-20g of AIN-93M (Cao et al. 2019, Reeves et al. 1993) diet, made with a mixture of minerals containing 50% of the recommended calcium intake (0.25% of dietary calcium). Deionized water was supplied *ad libitum* (Franco et al. 2021). Food intake and body weight were monitored weekly.

Animal model

The animals were supplied by the University's vivarium (Biotério do Centro de Ciências Biológicas e Saúde da Universidade Federal de Viçosa, MG, Brasil). The experimental protocols were approved by the University's Veterinary Ethics Committee [Departamento de Veterinária da Universidade Federal de Viçosa, MG, Brasil (Technical Report no. 80/2007).

Three weeks before the experiment began, at 23 weeks of age, the animals underwent ovariectomy (OVX) or laparotomy (SHAM), anaesthetized with intramuscular ketamine (70mg/g) and Xylazine (8mg/kg). In the postoperative period, the animals received subcutaneous anti-inflammatory (Ketophen: 2 mg/kg for 3 days) and antibiotic (Ampicillin

Sodium: 30mg/kg for 5 days) administration (Franco et al. 2021).

Animal diet

The SC and OC groups received a daily dose of caffeine powder (6 mg/kg body weight) added to 18 to 20 g of AIN-93M diet (Cao et al. 2019) for 8 weeks. The dose of caffeine was weighed according to the average body weight of the animals in each group and is considered low to moderate in human and animal studies (Xu et al. 2019, Silva et al. 2013).

The SP and OP groups had no caffeine supplemented to their diet (Placebo), receiving only the AIN-93M diet.

The diet consumption of each animal was monitored weekly by weighing all leftovers on a digital electronic scale (Marte-Brazil). Weight gain was determined by the difference between the days of sacrifice and the first week.

Bone mineral content

At the end of the 8th week, the animals were placed in a chamber and euthanized with CO_2 inhalation. The right tibia was dissected, weighed, and digested in concentrated Nitric Acid for 16 hours. The digested product was transferred to a volumetric flask (50mL) with 0.5mL of Strontium Chloride, and the volume was made with deionized water (de Andrade et al. 2021) for further determination of calcium and zinc contents using atomic absorption spectrophotometry (GBC 908 AA Perkin Elmer-USA) (Pereira et al. 2021). Calcium and zinc contents were normalized by tibial weight and presented as a percentage of the tibial content.

Bone fracture strength

At euthanasia the right femur was dissected and weighed, and had its length and thickness measured using a caliper (Stainless Hardened - China). Bone fracture strength was measured using a three-point test in a machine (TA. HDi Texture Analyzer - USA) connected to a computer running the software Texture Expert® (Stable Micro System Inc - USA). In summary, the femur was settled transversally on two stainless supports 2cm apart, and the force (0.25N; Speed: 3mm/s; distance: 10mm) was applied downward on the bone midshaft to promote fracture (Xu et al. 2019, Yadav et al. 2021, Rosales Rocabado et al. 2018). The peak force required to achieve fracture was registered and the fracture force relative to the femur's weight was calculated.

Urinary calcium and bone biomarkers

24-hour urine was collected in the last experimental week in individual metabolic cages. The collected urine volume was made at 10mL by the adding deionized water and was centrifuged for 15 minutes at 2,865 x g (Excelsa-Fanem-Brazil). The urine received a 50%HCl (20µL/mL) solution and was stored at -80°C. An aliquot from the centrifuged urine supernatant was digested in concentrated Nitric Acid for 16 hours, from which the calcium content was determined (Silva et al. 2013). Another aliquot of urine (50µL) was pipetted into an Eppendorf tube, diluted with 500µL of deionized water to determine urinary creatinine by UV/VIS spectrometry method (Wettersten et al. 2021). The analyses were performed with Bioclin® kits in Alizé® automatic equipment (bioMérieux-France). Additionally, an aliquot of this urine was analyzed for N-terminal Collagen Type I (NTx) telopeptide by ELISA using the Osteomark® kit (EIA, Wampole Laboratories, Inc. - USA) (Fujita et al. 2017, O'Brien et al. 2018).

A blood sample was collected by cardiac puncture and serum was obtained for the determination of bone alkaline phosphatase (Ostase®, Beckman Coulter) by indirect chemiluminescence technique (Beckman

Access Immunoassay Analyzer, New Jersey - USA) (Milinkovic et al. 2020).

Statistical analysis

After submitting the data to the Kolmogorov-Smirnov normality test, analysis of variance (two-way ANOVA) was applied to evaluate Ovary and Caffeine factors and their interactions according to body weight parameters, food intake, and bone, urinary, and blood parameters. The Holm-Sidak test was used to analyze multiple post-hoc comparisons. Pearson's correlation test was also applied to these parameters. The analyses were performed using Sigma Stat 3.0 software (Systat Software Inc., USA), using the significance level of $p<0.05$.

RESULTS

Body weight and diet consumption

There was no difference in the initial body weight (Fig. 1a). At Week 8, there was no significant interaction between Ovary and Caffeine factors in final body weight (Fig. 1b), weight gain (Fig. 1c), and diet consumption (Fig. 1d). However, a significant change was observed for the Ovary factor, where OVX rats showed higher final body weight and more weight gain than SHAM rats ($p<0.05$).

Bone mineral content

The results of bone mineral content analyses are presented in Figure 2. No interactions between Ovary and Caffeine were observed for tibial weight (Fig. 2a) and bone zinc contents (Fig. 2d). Significant interaction was observed in the tibial calcium content (Fig. 2c), where Caffeine and OVX showed reduced calcium content. When observed separately, OVX groups showed lowered tibial relative weight (Fig. 2b).

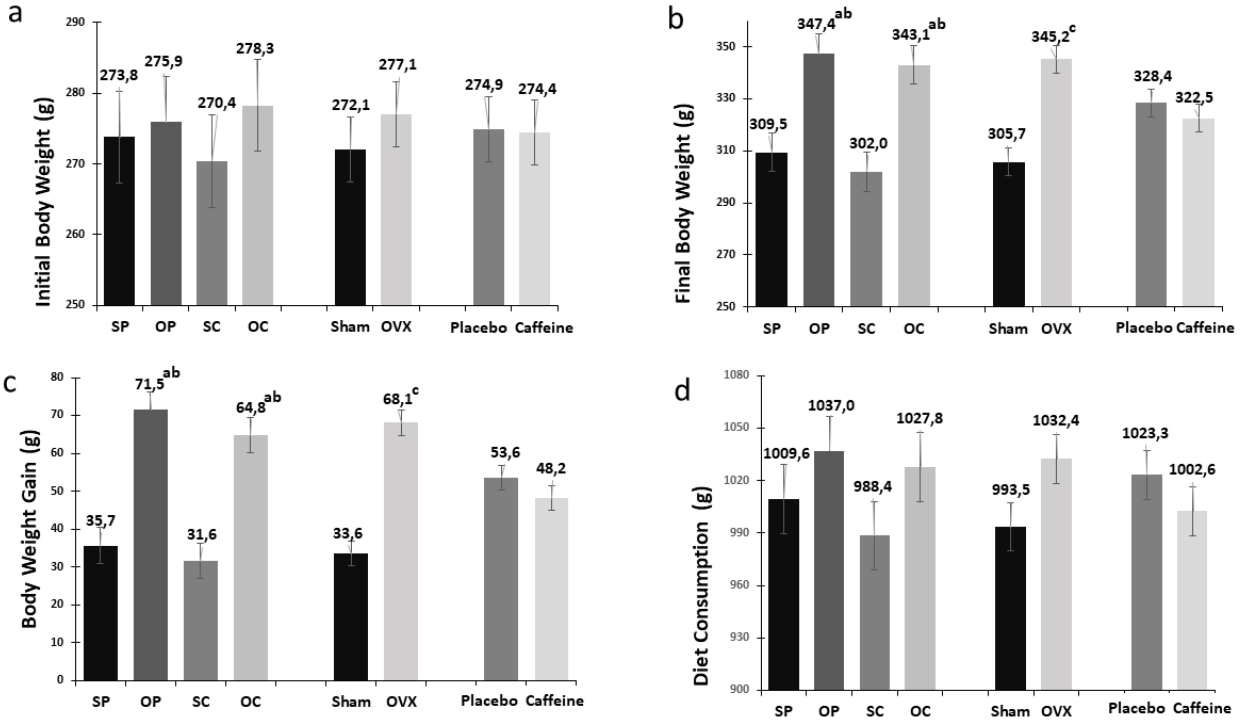

Figure 1. Initial body weight (a), final body weight (b), weight gain (c) and diet consumption (d). Values in mean ± SEM. Significance ($p<0.05$): [a] *vs.* SP, [b] *vs.* SC, [c] *vs.* SHAM, [d] *vs.* Placebo for Two-Way ANOVA followed by the Holm-Sidak test. SP (SHAM Placebo); OP (OVX Placebo); SC (SHAM Caffeine); OC (OVX Caffeine); SHAM (SP + SC); OVX (OP + OC); Placebo (SP + OP); Caffeine (SC + OC).

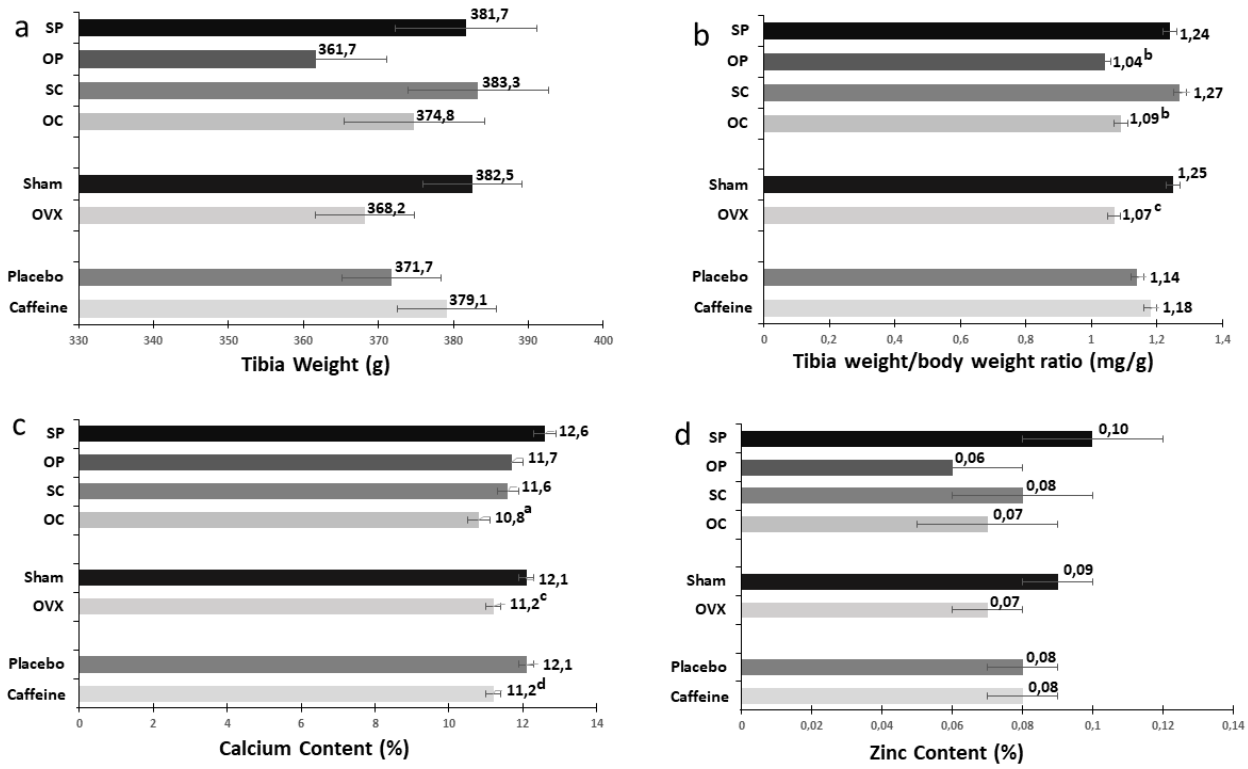

Figure 2. Bone mineral content in the tibia: tibia weight (a), tibia weight/ body weight ratio (b), calcium content (c) and zinc content (d). Values in mean ± SEM. Significance (p<0.05): [a] *vs.* SP, [b] *vs.* SC, [c] *vs.* SHAM, [d] *vs.* Placebo for Two-Way ANOVA followed by the Holm-Sidak test. SP (SHAM Placebo); OP (OVX Placebo); SC (SHAM Caffeine); OC (OVX Caffeine); SHAM (SP + SC); OVX (OP + OC); Placebo (SP + OP); and Caffeine (SC + OC).

Bone fracture strength

Figure 3 shows the results for femur mechanical analyzes. No interaction was observed between femoral length (Fig. 3a) and thickness (Fig. 3b). Significant interaction between Ovary and Caffeine was verified in bone fracture strength (Fig. 3d), where OVX and caffeine lowered relative fracture strength. When observed separately, OVX lowered femur weight (Fig. 3c).

Urinary bone biomarkers and calcium excretion

The results of bone biomarkers and urinary calcium excretion are shown in Figure 4. Significant interaction between factors (p<0.05) was identified in urinary calcium excretion (Fig. 4c), where caffeine raised and OVX lowered calcium excretion. For the Ovary factor, it was found that OVX animals lowered the rate of blood ostasis (Fig. 4a) but raised urinary NTx (Fig. 4b).

Correlation between parameters

The analysis of correlation between the parameters is shown in Table I. The main findings were a positive correlation between femoral fracture strength and tibial calcium content, and a negative correlation between NTx and femoral length. Urinary NTx correlated negatively with tibial calcium content and femoral thickness. Femoral weight correlated positively with femoral thickness and length, and tibial weight.

Values in correlation coefficient (r). Pearson correlation ([a] *vs.* p<0.05; [b] *vs.* p<0.001). Tibial Calcium (tibial calcium content); NTx (N-terminal collagen type I telopeptide); Ostase (bone alkaline phosphatase); Femoral Strength (femoral fracture strength relative to bone weight); Ca/Crn ratio (urinary calcium/creatinine content ratio).

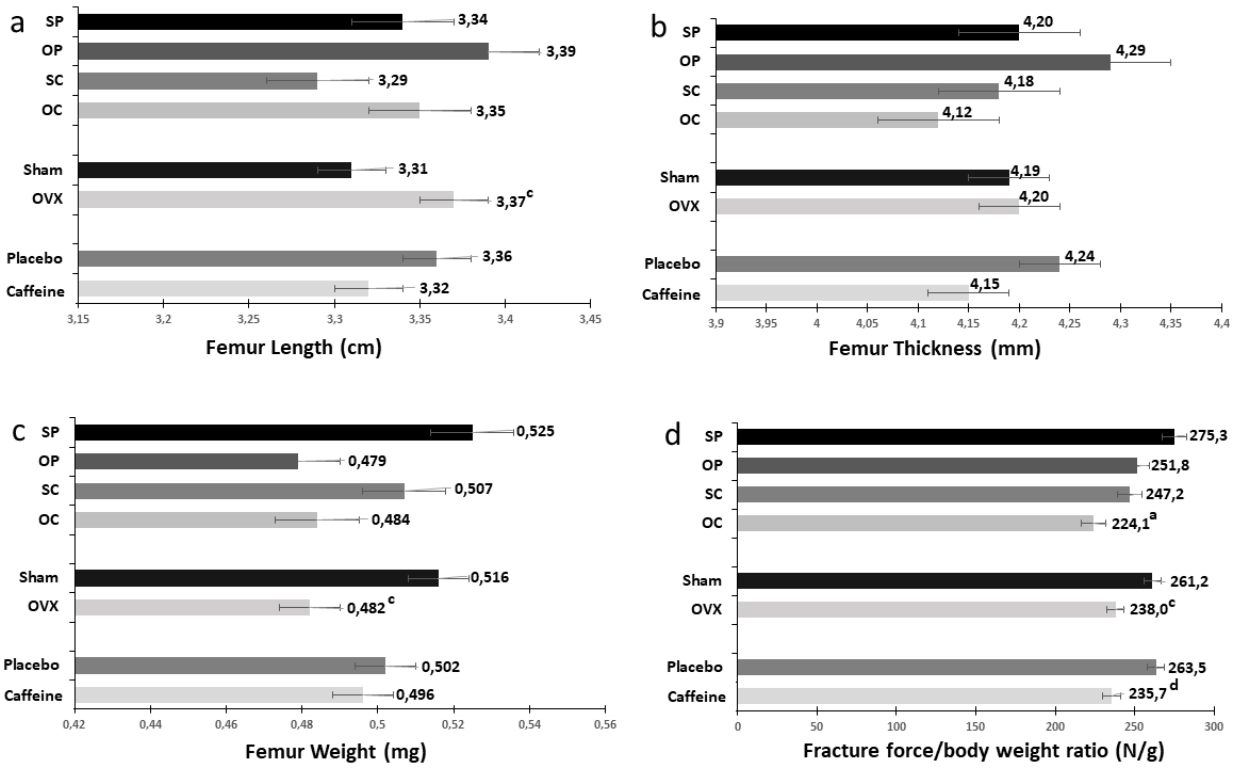

Figure 3. Femur fracture strength: femur length (a), femur thickness (b), femur weight (c) and fracture force/body weight ratio (d). Values in mean ± SEM. Significance ($p<0.05$): [a] *vs.* SP, [b] *vs.* SC, [c] *vs.* SHAM, [d] *vs.* Placebo for Two-Way ANOVA followed by the Holm-Sidak test. SP (SHAM Placebo); OP (OVX Placebo); SC (SHAM Caffeine); OC (OVX Caffeine); SHAM (SP + SC); OVX (OP + OC); Placebo (SP + OP); and Caffeine (SC + OC).

DISCUSSION

This study investigated the impact of ovariectomy and caffeine intake on bone health in rats on calcium-deficient diet. The main findings were that OVX and caffeine reduced femoral fracture strength and tibial calcium content; while OVX raised urinary NTx and lowered bone weight, ostase and urinary calcium; and caffeine augmented urinary calcium.

Our results also show that femoral fracture strength is directly linked to bone mass and has an inverse relation to bone resorption, being negatively impacted by OVX and caffeine intake.

We demonstrate significant interaction between Ovary and Caffeine factors, in which OVX-induced estrogen deficiency and caffeine intake potentiate the decline of calcium content and femoral fracture strength in animals fed 50% of daily calcium intake recommended. It is conceivable that the lowest bone calcium content reduced bone mass and, consequently, increased bone susceptibility to fracture. Such possibility was confirmed by the positive and moderate correlation observed between tibial calcium content and femoral fracture strength.

Previous studies reported isolated adverse effects of OVX and caffeine intake on bone structure, BMD, BMD and biomarkers (Zhu et al. 2020, Xu et al. 2019, Xie et al. 2020) however, our study identified enhancement of the effects. The study by Xu et al. (2019) shows that caffeine intake decreased the activity of alkaline and acid phosphatases, however, it did not affect bone length, BMD and biomechanical parameters.

Our results indicate that bone health was negatively impacted by ovariectomy and caffeine

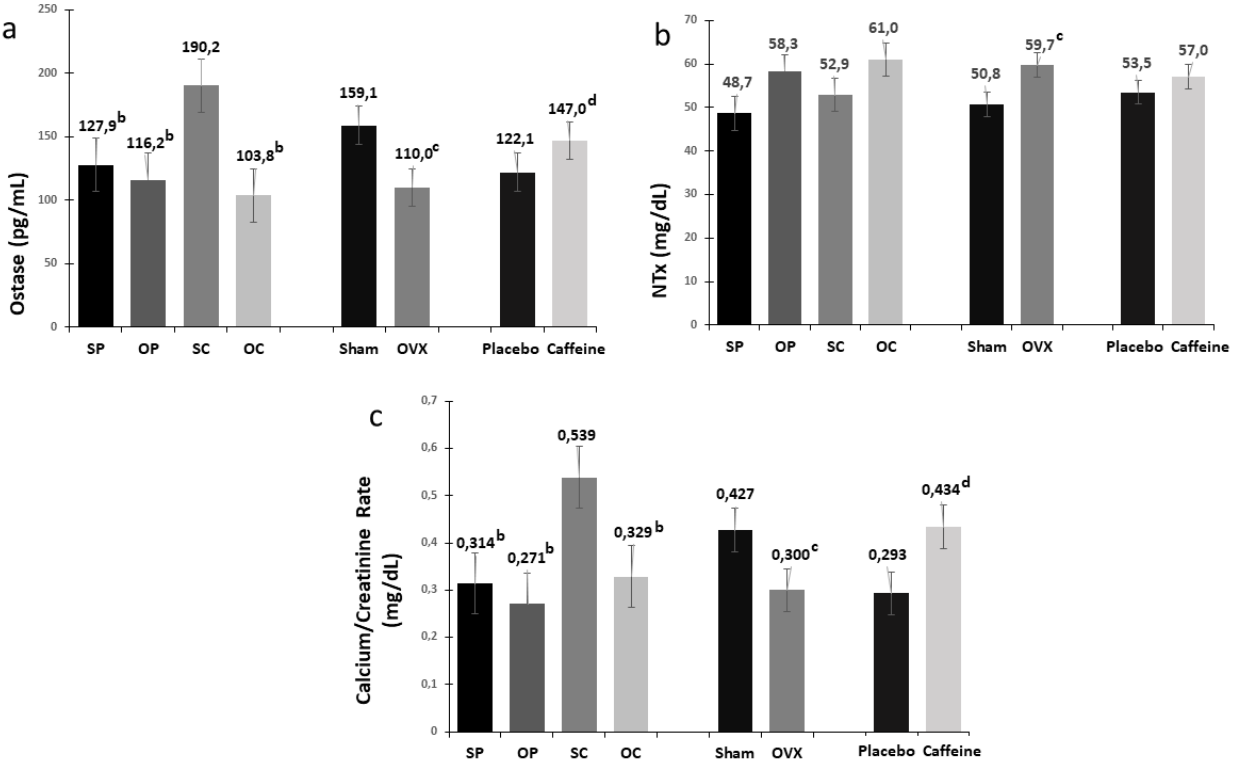

Figure 4. Urinary bone biomarkers: Ostase (a) and NTx (b) and calcium excretion (c). Values in mean ± SEM. Significance (p<0.05): [a] *vs.* SP, [b] *vs.* SC, [c] *vs.* SHAM, [d] *vs.* Placebo for Two-Way ANOVA followed by the Holm-Sidak test. Ostase (Bone Alkaline Phosphatase); NTx (N-terminal telopeptide of collagen type I); SP (SHAM Placebo); OP (OVX Placebo); SC (SHAM Caffeine); OC (OVX Caffeine); SHAM (SP + SC); OVX (OP + OC); Placebo (SP + OP); and Caffeine (SC + OC).

intake as it reduced bone fracture strength and mass and raised bone resorption in animals on a calcium-deficient diet. These findings suggest that low-calcium intake at postmenopausal stage associated with a caffeine-rich diet increase the risk of bone fractures. This is of particular concern for the Brazilian women, who have a dietary calcium intake between 240 to 625mg/day, which represents 20 to 52% of the recommendation (Bierhals et al. 2019, Oselame et al. 2016).

In the present study, OVX lowered tibial calcium content, femoral fracture strength and bone formation activity, and increased bone resorption activity in rats on a calcium-deficient diet. Previous studies revealed that OVX reduced BMC, BMD and bone microarchitecture (Zhu et al. 2020, Li et al. 2021, Sapra et al. 2021, Xie et al. 2020, Zheng et al. 2020). Li et al. (2021) showed that female mice had reduced bone mass after OVX surgery, and that OVX had bone resorption augmented by increasing the expression of RANK-L (Receptor activator of nuclear factor-kappa B ligand). According to the literature, RANK-L is a protein that activates a sequence of intracellular events that promotes the formation of osteoclasts, thus increasing bone resorption (Zhu et al. 2020, Dal-Fabbro et al. 2021, Zheng et al. 2020). The present study did not analyze the expression of RANK-L, but because an increase in bone resorption activity (NTx) was observed, it is possible that the RANK-L mechanism was activated and promoted the loss of bone mass and bone fracture strength.

The study by Topolska et al. (2020), showed that OVX rats ingesting 60% of the recommended

Table I. Correlation between urinary and bone parameters.

	Femoral Thickness	Femoral Length	Tibial Weight	Tibial Calcium	Ostase	NTx	Ca/Crn Ratio	Femoral Strength
Femoral Weight	0,347[a]	0,333[a]	0,802[b]	-0,138	0,033	-0,155	-0,084	-0,160
Femoral Thickness		0,364[a]	0,349[a]	-0,038	0,049	-0,314[a]	-0,369[a]	0,107
Femoral Length			0,383[a]	-0331[a]	-0,077	0,252	-0,179	-0,421[a]
Tibial Weight				-0453[a]	-0,075	-0,149	-0,023	-0,304
Tibial Calcium					0,041	-0,335[a]	0,125	0,576[b]
Ostase						0,129	0,385[a]	0,063
NTx							0,010	-0,311[a]
Ca/Crn Ratio								-0,109

Values are correlation coefficient (r). Pearson correlation ([a] *vs.* p<0.05; [b] *vs.* p<0.001). Tibial Calcium (tibial calcium content); NTx (N-terminal collagen type I telopeptide); Ostase (bone alkaline phosphatase); Femoral Strength (femoral fracture strength relative to bone weight); Ca/Crn ratio (urinary calcium/creatinine content ratio).

calcium intake had diminished BMC and increased urinary CTx (C-terminal peptide of collagen), but did not presented changes in bone alkaline phosphatase or BMD. The study conducted by Xu et al. (2019) suggests showed that rats after 15 weeks of OVX had reduced BMD and femoral deflection force. It is noteworthy that in the present study OVX rats under low calcium intake negatively affected calcium metabolism and unbalanced bone remodeling after only 10 weeks of OVX.

We demonstrate here that caffeine intake elevated urinary calcium excretion, which may have affected the reduced calcium content and bone fracture strength. De Souza et al. (2021) reported that caffeine affects the bone calcium balance by inhibiting parathyroid hormones (PTH) and calcitriol, thus impairing calcium absorption and deposition, and hence bone biomechanical properties. They studied female mice having 50mg/kg/day of caffeine for 17 weeks, and found reduced bone calcium levels, however, caffeine did not interfere with bone strength. Xu et al. (2019) described that caffeine intake in OVX rats reduced alkaline phosphatase activity and increased bone deflection force, but did not affect BMD, microarchitecture, and bone biomechanical properties.

Previous studies indicate that caffeine promotes bone loss and affects adversely bone remodeling (Zhu et al. 2020, Dal-Fabbro et al. 2021, Maia et al. 2020). According to Dal-Fabbro et al. (2021), caffeine could promote RANK-L expression and increase osteoclast formation, thereby activating bone resorption. Maia et al. (2020) stated that high doses of caffeine (15-20mg/kg/day, equivalent to approximately 6 cups of coffee/day), aggravated alveolar bone loss in periodontal disease, but did not induce alveolar bone resorption in adult-elderly rats. In epidemiological studies, these authors also observed that caffeine dose is a determining variable for the positive or negative effects of caffeine on bone health, where low doses of caffeine (6-10mg/kg/day) could offer bone protection. In the present study, found that rats receiving 6mg/kg of caffeine per day had bone calcium loss, reduction in fracture strength and increase in urinary calcium excretion. Nevertheless, the biomarkers of bone absorption and formation did not differ significantly. These results contrast with those presented by some studies (Zhu et al. 2020, Topolska et

al. 2020, Maia et al. 2020), showing that there is inconsistency in the dose of caffeine and its effects on bone health. Thus, further studies are suggested to evaluate the dose-response in biomarkers affected by caffeine and point out the mechanism of its action in bone tissue.

It is conceivable that in the present study, the effects of OVX and caffeine on bone health may have been exacerbated by the dietary calcium inadequacy. Low calcium intake could reduce intestinal content absorption and urinary loss as a protective mechanism of blood calcium levels for physiological functions. Low blood calcium levels would stimulate the synthesis of PTH, which mobilizes bone calcium via paracrine factors such as RANK-L (Silva et al. 2013, Yadav et al. 2021). The increase in bone calcium resorption would reduce its contents, thus promoting greater bone fragility.

Finally, the fact that estrogen levels after OVX surgery were not measured is considered a limitation. Nonetheless, OVX rats is an animal model that simulates the physiological and bone loss characteristics observed in postmenopausal women (Sun et al. 2022, Long et al. 2022). Studies have shown that OVX-induced estrogen deficiency can lead to increased osteoclast numbers, loss of cancellous bone, and unbalanced bone formation in rodents by several pathways (Liu et al. 2022, He et al. 2022, Long et al. 2022, Jang et al. 2022). Our study also indicates that OVX animals exhibited physiological and bone characteristics similar to those found in animals with estrogen deficiency. However, bone remodeling signals such as RANK-L, PTH and calcium isotopes were not analyzed in the present study. Such analyzes would help to elucidate the mechanisms by which OVX and caffeine intake would affect calcium metabolism under the condition of low calcium intake. On the other hand, it would be relevant to know how much additional calcium supplement in the diet could minimize the effects on bone metabolism and/or the mechanical properties of bones under the conditions of OVX and caffeine intake. These gaps need to be clarified in future studies.

The results of the present study make it possible to conclude that both OVX and caffeine intake debilitate bone health in rats on calcium-deficient diet.

Acknowledgments

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