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High-fat diet and caffeine interact to modulate bone microstructure and biomechanics in mice

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ABSTRACT

Aims: Although excessive fat and caffeine intake are independent risk factors for bone microstructural and functional disturbances, their association remains overlooked. Thus, we investigated the impact of high-fat diet (HFD) and caffeine alone and combined on serum lipid profile, bone microstructure, micromineral distribution and biomechanical properties.

Methods: Forty female C57BL/6 mice were randomized into 4 groups daily treated for seventeen weeks with standard diet (SD) or HFD (cafeteria diet) alone or combined with 50 mg/kg caffeine.

Key findings: The association between HFD and caffeine reduced the weight gain compared to animals receiving HFD alone. Caffeine alone or combined with HFD increases total and HDL cholesterol circulating levels. HFD also reduced calcium, phosphorus and magnesium bone levels compared to the groups receiving SD, and this reduction was aggravated by caffeine coadministration. From biomechanical assays, HFD combined with caffeine increased bending strength and stiffness of tibia, a finding aligned with the marked microstructural remodeling of the cortical and cancellous bone in animals receiving this combination.

Significance: Our findings indicated that HFD and caffeine interact to induce metabolic changes and bone microstructural remodeling, which are potentially related to bone biomechanical adaptations in response to HFD and caffeine coadministration.

1. Introduction

Dietary profile has a remarkable ability to modify cell metabolism and trigger molecular reprogramming, including the modulation of genes that regulate cell structure, function and survival [1,2]. These characteristics have been proven for hypercaloric diets, especially those rich in ultra-processed carbohydrates and lipids [3]. Accordingly, high-fat diets (HFD), including cafeteria diet, has been systematically applied in classical preclinical models of dyslipidemia and obesity, diseases closely correlated with excessive intake of ultra-processed foods [3,4]. By replicating eating habits of Western populations, cafeteria diet is useful for investigating the effects of sugar and fat overload on the metabolism and structure of multiple tissues and organs, especially adipose tissue, heart, liver and bones [4,5].

By predisposing to systemic inflammation, oxidative stress, atherosclerosis and ischemia, excessive adiposity and dyslipidemia also play a critical role in bone homeostasis [6]. Once bone ischemia reduces the nutrient flow from the arteries responsible for periosteum and endosteum nutrition, it negatively affects bone metabolism by impairing osteogenesis in favors of osteolysis [7]. Additionally, the abnormal

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accumulation of lipids in plasma and bone marrow predisposes to disturbances in redox metabolism. In this scenario, pro-oxidant events, such as lipid peroxidation, impairs osteoblasts and osteoclasts function and bone remodeling, thus favoring bone reabsorption and microstructural fragility [8]. In more serious cases, disturbances in lipid metabolism are coupled with extensive bone remodeling, in such a way that progressive reduction in bone mineral density evolves to osteopenia, osteoporosis and increased risk of fractures [9–11].

Unlike carbohydrate and lipid overload, caffeine dietary supplementation has been associated with antiobesogenic potential and protection against non-alcoholic fatty liver disease. Such effects are potentially mediated by a marked stimulation of cellular energy metabolism (i.e., β-oxidation) by this methylxanthine alkaloid [12–15]. The primary mechanism that explains this effect is its antagonistic profile to adenosine receptors, resulting in the inhibition of adenylyl cyclase enzyme and upregulation of cyclic adenosine monophosphate (cAMP) levels. Together, these events trigger antiobesogenic responses by stimulating catecholamine circulating levels and the lipolysis from fatty acid oxidation [14,16].

Due to its impact on mineral metabolism (especially calcium), caffeine intake has also been associated with reduced bone mass and increased risk of fractures in humans [17] and animals [18–21]. In this sense, Kwak et al. [20] showed higher renal calcium excretion, reduced body weight gain and bone mineral content in rats supplemented with 120 and 180 mg/kg/day caffeine for 63 days. Moreover, Olchowik et al. [19] observed that rats receiving 30 mg/kg/day caffeine for 21 days exhibited reduced bone mass and evident biomechanical instability, which are systematically described as determining factors for increased risk for fractures [22,23].

Despite its antagonistic obesogenic effect, HFD and caffeine intake share a potential negative impact on bone metabolism. However, it remains poorly understood whether and to what extent HFD and caffeine can interact to modify energy metabolism, bone structure and biomechanical function. Thus, we used a murine model to investigate the impact of the cafeteria diet and caffeine supplementation administered alone and combined on lipid profile, bone mineral composition, microstructure and biomechanical properties.

2. Material and methods

2.1. Animal diet and groups

Forty 6-week-old C57BL/6 mice were kept under controlled environmental conditions (temperature 22 ± 2 °C, air humidity 60–70% and 12/12 h daily light/darkness cycles). The animals were randomized into 4 groups, with 10 animals in each group. The groups were treated with HFD and standard diet (SD) alone or combined with caffeine, as follows: *Group 1*: SD; *Group 2*: SD + 50 mg/kg caffeine; *Group 3*: HFD; *Group 4*: HFD + 50 mg/kg caffeine. Both diets have similar content in protein (21 versus 17 g/100 g, respectively) and carbohydrate energy (219 versus 194 kcal/100 g, respectively), but differed in fat energy (366 versus 572 kcal/100 g, respectively).

Diet composition and energy content are indicated in Table 1. The HFD consisted of the following ingredients: ham pate, straw potatoes, white chocolate, bacon fat, stuffed biscuit and standard diet for mice in the following proportions: 2:1:1:1:1:1 [24]. Diet and water were provided ad libitum. In addition to control and experimental diets, animals in the groups 2 and 4 received 50 mg/kg/day caffeine (Sigma-Aldrich, St. Louis, MO, USA) diluted in 0.9% saline solution intraperitoneally (i.p.) [25], for 119 days. Control animals received saline solution by the same administration route. Twenty-four hours after the last application, the animals were anaesthetized with tribromoethanol (250 mg/kg, i.p.) and euthanized by exsanguination via cardiac puncture. Blood was collected for biochemical assays, while the tibia was removed for biomechanical, morphometric and mineral analysis. The use of animals in this study was approved by the Institutional Ethics Committee on the Use of Animals (Protocol: 047/2016).

Table 1
Nutritional composition and energy content of experimental diets.

Composition	Standard diet	High-fat diet
Proteins (g/100 g)	20.73	17.14
Carbohydrates (g/100 g)	54.68	48.43
Total fat (g/100 g)	7.17	34.43
Protein Energy (kcal/100 g)	82.92	68.56
Carbohydrate Energy (kcal/100 g)	218.72	193.71
Fat Energy (kcal/100 g)	64.53	309.88
Total Energy (kcal/100 g)	366.20	572.15

2.2. Food intake and clinimetric parameters

Food intake and body mass were recorded weekly during 19 weeks of the experimental protocol. The mean food intake was used to estimate the daily ingestion by animal group and to obtaining parameters such as dietary intake (g/day), energy intake (kcal/day), which were adjusted by body mass (g). The Energy intake was estimated based on the dietary intake, using the reference values provided by each chemical component of the diet (i.e., 4.0 kcal/g carbohydrate, 4.0 kcal/g protein and 9.0 kcal/g fat) [26].

2.3. Triacylglycerol and cholesterol serum levels

The lipid profile was evaluated by quantifying triacylglycerol, total cholesterol, LDL cholesterol and HDL cholesterol serum levels. These parameters were analyzed on a spectrophotometer, using commercial kits, following the manufacturer's instructions (Bioclin, Belo Horizonte, Minas Gerais, Brazil) [27].

2.4. Biomechanical assays

The biomechanical assays were performed in a universal mechanical testing machine (Dual Column Tabletop Model testing system DL 3367, Instron, Grove City, PA, USA). For this purpose, the right tibia of animals from all experimental groups was used for the analysis of structural and material biomechanical properties. We performed a three-point bending test until failure, at a speed of load application of 1 mm/min, with preload of 1 N, load cell of 500 N, settling time of 30 s and distance between the points of 10 mm [28,29]. The bone was positioned so the supports were under the curve in the proximal tibia and at the distal tibia so that when the load was applied at the mid-point between the supports it was through the point of insertion of the fibula. During the settling time, preliminary tests were performed and found that this position was stable, reproducible and that the tibia did not rotate during the test. The data for the bone structural properties (ultimate load, yield load, and stiffness) were measured from load-displacement curves. The ultimate load is the highest force (N) a specimen can withstand, whereas the yield load represents a force (N) at which a specimen starts to experience permanent structural damage. Stiffness is the slope of the linear portion of the load-displacement curve [30]. The data were obtained through servo-hydraulics equipment (MTS model 810/TestStar II) and recorded in a computer coupled to the machine [30–32].

2.5. Mineral microanalysis

The bone mineral content was investigated by energy-dispersive X-ray spectroscopy (EDS) using a scanning electron microscope (Leo 1430VP, Carl Zeiss, Jena, Thuringia, Germany) with an attached x-ray detector system (Tracor TN5502, Middleton, WI, USA). Bone fragments were dehydrated in ethanol, submitted to critical point drying and coated with evaporated carbon (Quorum Q150 T, East Grinstead, West Sussex, England, UK). The EDS microanalysis was performed at 1000 × magnification, 20 kV accelerating voltage and 10 mm working distance.

The proportion of the chemical elements calcium (Ca), phosphorus (P), manganese (Mn), copper (Cu), zinc (Zn), selenium (Se), Magnesium (Mg), and sulfur (S) were expressed in percentages [33,34].

2.6. Anatomical dimensions of tibia

After complete removal of the soft tissue of the left tibia, the bone anatomical dimensions were measured, using a universal analogue caliper (Vonder, 150 mm, graduation 0.05 mm, Brazil) and the support of a magnifying glass, according to the technique described by Lammers et al [35] For each measurement, a minimum distance between two reference points in the bone was considered. The following measures were taken perpendicular to the length of the tibia: tibia length; distance between the proximal and distal epiphyses; mean diameter of tibia diaphysis (i.e. anteroposterior/medio-lateral thickness measured at the midpoint between proximal and distal epiphyses); mean diameter of the proximal tibia (i.e. anteroposterior/medio-lateral thickness of the proximal epiphysis), and mean diameter of the distal tibia (i.e. anteroposterior/medio-lateral thickness of the distal epiphysis). Surface area (SA) of the tibia was estimated as an approximation of the cylinder surface area as follows: $SA = 2 \times (\pi \times [r]2) + (2 \times \pi) \times (r \times h)$; where r is the mean radium obtained from tibia diameters (diaphysis, proximal and distal epiphyses), and h is the tibia length.

2.7. Histopathological processing and cancellous bone morphology

The distal epiphyses were decalcified by immersion in a decalcification solution containing formic acid (20% diluted in distilled water) for a minimum period of 60 days until the complete descaling of the material. Then, the epiphyses were rinsed in distilled water, dehydrated in crescent ethanol series and cleared in xylene. The anteroposterior thickness of the epiphysis was the measured and was sectioned in its medial portion. Both fragments positioned with the cutting base downwards were embedded in paraffin in a vertical orientation relative to the longitudinal axis. Four-micrometer thick histological sections were stained with hematoxylin-eosin (H&E) and mounted on glass slides. To avoid repeated analysis of the same histological area, the sections were evaluated in semi-series, using one in every 20 sections [36]. The slides were visualized using a 40× objective lens and histological images were captured using a light photomicroscope (Axioscope A1, Carl Zeiss, Germany). To evaluated the cortical bone thickness, twelve histological sections (1 section = 1 field) stained with H&E were prepared. The images were captured using a 4× objective lens and the cortical bone area (cross-section area - medullary area) as well as the mean cortical thickness were measured from the Image Pro-Plus 4.5 image analysis software (Media Cybernetics, Silver Spring, MD, USA) [37].

The volume densities occupied by trabecular bone (Vv[*bone*], %) and bone marrow (Vv[*marrow*], %) were estimated by point counting, according to the formula: $Vv[\textit{trabeculae or bone marrow}] = \Sigma P_P[\textit{trabeculae or bone marrow}]/\Sigma P_T$, where ΣP_P[trabecula or bone marrow] is the number of points that hit the bone trabeculae or bone marrow, and ΣP_T is the total number of test points, here 77 [36]. Seventy randomly sampled microscopic fields (magnification 400×) and a total bone area of 96.6×10^4 μm^2 were analyzed for each group. Trabecular width and separation were estimated using a sphere-fitting method from the image analysis software ImageJ (National Institutes of Health) [38] with the Bone J plugin [39]. We randomly selected and digitalized 10 regions of interest (ROI) for each animal, using the 40× objective lens in photomicroscope. Each ROI represented one area of the 12×10^4 μm^2 of the trabecular bone. To estimate trabecular width (Tb.Wi) and trabecular separation (Tb.Sp), each ROI was segmented in a black and white image, and these parameters were automatically calculated from the from Bone J algorithm [38,40]. The basic approach is to determine the diameter of the largest possible circle that can be fitted while completely contained within the bone trabeculae, and then, to average these diameters [41,42].

Trabecular connectivity was estimated in 2D images from 3D deconvolution performed in the ImageJ software [38]. Based on these images, the principle of Marrow Star Volume (V*marrow) was applied, which is defined as the mean volume of all the parts of an object when seen unobscured along uninterrupted straight lines in all directions, from random points inside the object. In cancellous bone, V*marrow provides an estimate of the mean size of the marrow space in three-dimensions and reflects connectivity. V*marrow was calculated as follows: $V^*marrow = (\pi/3) \times \ell$ [3], where ℓ is the average length of an intercept with random orientation through a random point [43]. The surface density of bone trabeculae was estimated according to the following formula: $Sv[bt] = (2 \times \Sigma I[bt])/L_T$, where ΣI [*bt*] represents the total number of intersections between the cycloid arcs (i.e., 12) and the surface of bone trabeculae, and L_T is the total length of the cycloid arcs system (i.e., 1220 μm) [36].

2.8. Statistical analysis

To minimize research bias, the analyses were performed blindly, and only at the time of statistical analysis were the data identified and analyzed by one of the authors who had no contact with the measurements. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). The normality of the data distribution was verified by the Shapiro-Wilk test. The data were expressed as mean and standard deviation (mean ± S.D.). Data with parametric distribution were analyzed using two-way analysis of variance (two-way ANOVA), followed by the Student-Newman-Keuls *post-hoc* test. Data with non-parametric distribution were transformed by logarithmic transformation before the analysis in order to normalize the distribution. All results with $P \leq 0.05$ were considered statistically significant. All animals were included in the analysis.

3. Results

3.1. Feeding and clinimetric parameters

The data about dietary intake and biometric parameters are shown in Table 2. All groups presented similar food intake during the experiment. Total energy intake was higher in the group HFD + caffeine (G4), compared to the group receiving SD and caffeine (G2) ($P < 0.05$). Final body mass was higher in animals receiving HFD alone (G3) compared to other groups ($P < 0.05$). The biggest weight gain was observed in the group that received HFD alone (G3) ($P < 0.05$). The group HFD + caffeine exhibited reduced weight gain, which was similar to the groups receiving SD.

Table 2
Dietary intake and biometric parameters in mice treated with standard diet (SD) or high-fat diet (HFD), supplemented or not with caffeine.

Groups	Diet intake (g/day)	Energy intake (kcal/day)	Initial body mass (g)	Final body mass (g)	Weight gain (g)
G1	21.21 ± 4.94^{a}	71.29 ± 16.60^{b}	18.39 ± 1.20^{a}	21.32 ± 0.62^{a}	2.94 ± 0.77^{a}
G2	20.10 ± 3.39^{a}	67.59 ± 11.40^{b}	17.78 ± 1.59^{a}	22.63 ± 0.83^{a}	4.86 ± 1.10^{b}
G3	16.51 ± 6.82^{a}	94.48 ± 38.99^{ab}	17.58 ± 1.74^{a}	28.60 ± 3.67^{b}	11.02 ± 3.68^{c}
G4	17.77 ± 5.78^{a}	101.68 ± 33.06^{a}	18.86 ± 1.58^{a}	22.51 ± 1.27^{a}	3.65 ± 1.06^{ab}

G1: SD, G2: SD + 50 mg/kg caffeine, G3: HFD, G4: HFD + 50 mg/kg caffeine. n = 10 animals/group. The results were described as mean and standard deviation. ^{a,b,c} Different letters in the columns indicate statistical difference between groups ($P < 0.05$).

3.2. Triacylglycerol and cholesterol plasma levels

Triacylglycerol and LDL levels were similar in all groups (Fig. 1A and D). Total cholesterol presented high levels in the group treated with HFD alone (G3) ($P < 0.05$), compared to the groups receiving SD (G1) and SD + caffeine (Fig. 1B, G2). HDL levels were increased in all groups treated with HFD and caffeine alone or combined compared to the group receiving SD alone ($P < 0.05$) (Fig. 1C). Similar HDL levels were observed in all groups receiving caffeine, regardless of the diet received ($P > 0.05$) (Fig. 1C). The interaction between HFD and caffeine kept these serum biomarkers high ($P < 0.05$).

3.3. Bone biomechanical properties

Bending strength of tibia diaphysis were higher in animals that receiving HFD + caffeine (G4), compared to the other groups ($P < 0.05$) (Fig. 2A). All showing high values of stiffness compared to SD (Fig. 2B). The association HFD + caffeine presented significantly increase in stiffness compared to SD + caffeine and HFD alone (Fig. 2B). Displacement at maximum load was similar in all groups ($P > 0.05$). When associated, HFD and caffeine kept these variables significantly high ($P < 0.05$).

3.4. Mineral distribution in bone tissue

Fig. 3A shows Ca, P, Mn, Cu, Zn, Se, Mg and S distribution in bone tissue. Mn and Cu distribution was reduced in animals receiving HFD alone compared to the others groups ($P < 0.05$). Conversely, Se levels were higher in this same group (G3) ($P < 0.05$). Bone Mg was reduced in all groups treated with caffeine, regardless of the diet received ($P < 0.05$). All groups presented increased S compared to the animals receiving SD alone (G1) ($P < 0.05$). Calcium and Phosphorus levels were showed in Fig. 2B. Ca and P distribution was reduced in the group HFD (G3) compared to the groups receiving SD alone or combined with caffeine (G1 and G2) ($P < 0.05$). The interaction between HFD and caffeine (G4) impair minerals distribution, inducing marked reduction in Ca and P bone content ($P < 0.05$).

3.5. Bone macro and microstructural characteristics

Representative photomicrographs of tibia diaphysis and morphometric parameters are showed in Figs. 4 and 5. HFD alone (G3) showed increase the thick cortical bone compared to other experimental groups (Fig. 4A). Reduced cortical bone thickness was observed in animals treated with HFD + caffeine (G4).

Proximal mean diameter of the tibia and bone weight were similar in all groups ($P > 0.05$) (Fig. 5A and C). However, the mean diameter of the distal tibia was reduced in animals that received HFD alone (G3) or combined with caffeine (G4) compared to the other groups ($P < 0.05$) (Fig. 5B). All groups receiving caffeine (G2 and G4) presented reduced mean diameter of the diaphysis compared to other groups ($P < 0.05$) (Fig. 4B). Tibia length was reduced in animals receiving HFD + caffeine (G3) compared to the groups SD (G1) and SD + caffeine (G2) ($P < 0.05$) (Fig. 5D). Mean surface area of the tibia was reduced in the groups G2, G3 and G4 compared to G1 ($P < 0.05$), and in the group G4 compared to G2 and G3 ($P < 0.05$) (Fig. 4C). The interaction between HFD and caffeine potentiated structural remodeling in tibia length, mean diameter and surface area ($P < 0.05$) (Figs. 4 and 5).

Figs. 6, 7 and 8 show tibia microstructures in all experimental groups. Marked reduction in bone trabecular area was observed in animals receiving caffeine (G2 and G4) compared to the control animals (G1). The worst microstructural results occur when HFD and caffeine were combined (G4) ($P < 0.01$) (Fig. 6). This association also reduced trabecular separation (Tb.Sp), marrow space volume (V*), surface density of bone trabeculae (SV) and volume density of bone marrow (Vv marrow) compared to the other groups (Fig. 8A, B, C and D) ($P < 0.05$). Conversely, volumetric density of bone tissue (Vv bone) and trabecular width (Tb.Wi) were significantly increased in the groups G4 compared to the other groups ($P < 0.05$) (Figs. 7, 8E and F).

4. Discussion

The impact of high-fat diet and caffeine alone and combined was investigated in this study. Aligned with previous evidence [8,19,20], animals exposed to both risk factors exhibited some degree of metabolic and bone adaptations. Interestingly, our findings indicated that the association of HFD and caffeine induced a differential response pattern in

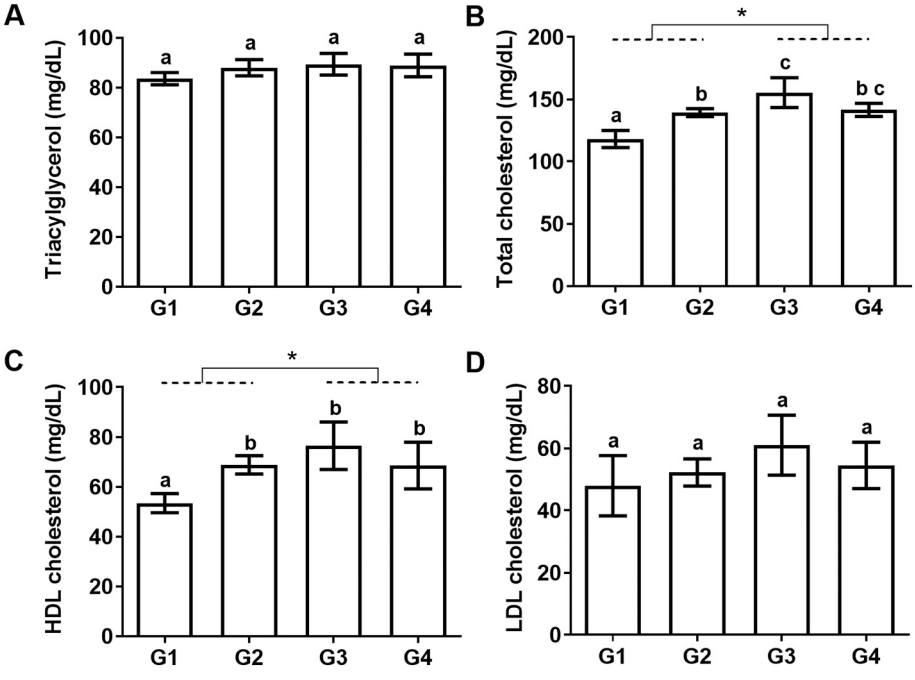

Fig. 1. Triacylglycerol (A), total cholesterol (B), high-density lipoprotein (HDL) (C), and low-density lipoprotein (LDL) (D) plasma levels in control mice and those treated with standard diet (SD) or high-fat diet (HFD), supplemented or not with caffeine. G1: SD, G2: SD + 50 mg/kg caffeine, G3: HFD, G4: HFD +50 mg/kg caffeine. $n = 10$ animals/group. The results were described as mean and standard deviation. ^{a,b,c} Different letters in the columns indicate statistical difference between groups ($P < 0.05$). *Denotes significant interaction between HFD and caffeine ($P < 0.05$).

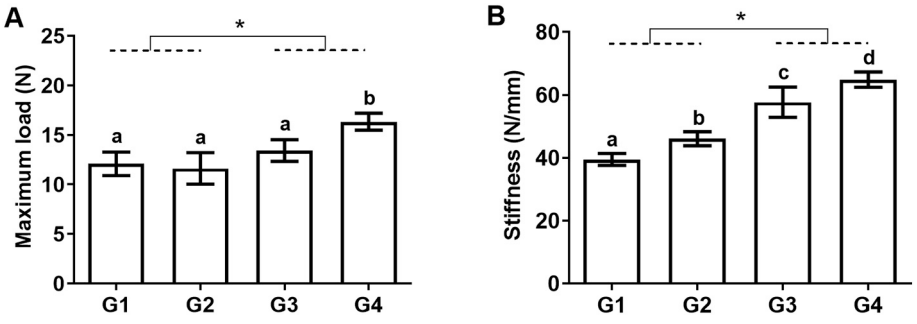

Fig. 2. Biomechanical properties of the tibia in mice treated with standard diet (SD) or high-fat diet (HFD), supplemented or not with caffeine. A: Maximum load. B: Stiffness. G1: SD, G2: SD + 50 mg/kg caffeine, G3: HFD, G4: HFD +50 mg/kg caffeine. $n = 10$ animals/group. The results were described as mean and standard deviation. [a,b,c] Different letters in the columns indicate statistical difference between groups ($P < 0.05$). *Denotes significant interaction between HFD and caffeine ($P < 0.05$).

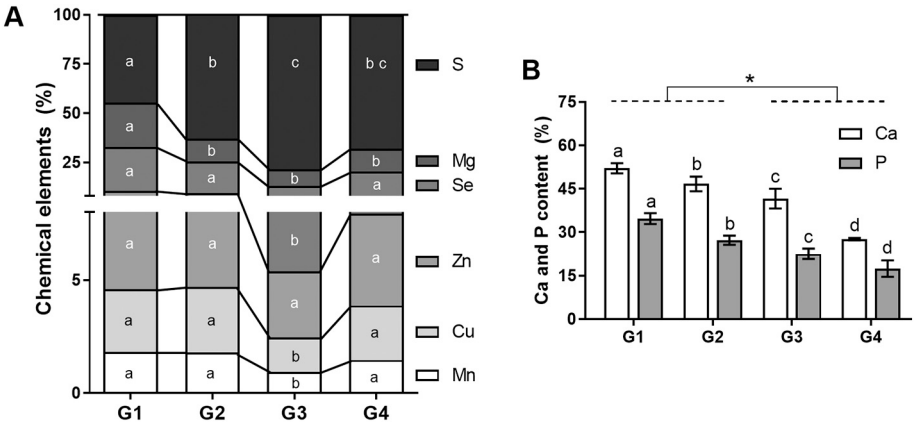

Fig. 3. Mineral content in the tibia of mice treated with standard diet (SD) or high-fat diet (HFD), supplemented or not with caffeine. A: Chemical elements. B: Ca and P content. G1: SD, G2: SD + 50 mg/kg caffeine, G3: HFD, G4: HFD +50 mg/kg caffeine. S: sulfur, Mg: magnesium, Se: selenium, Zn: zinc, Cu: copper, Mn: manganese, Ca: calcium, P: phosphorus = 10 animals/group. The results were described as mean and standard deviation. [a,b,c,d,e] Different letters in the columns indicate statistical difference between groups ($P < 0.05$). *Denotes significant interaction between HFD and caffeine ($P < 0.05$).

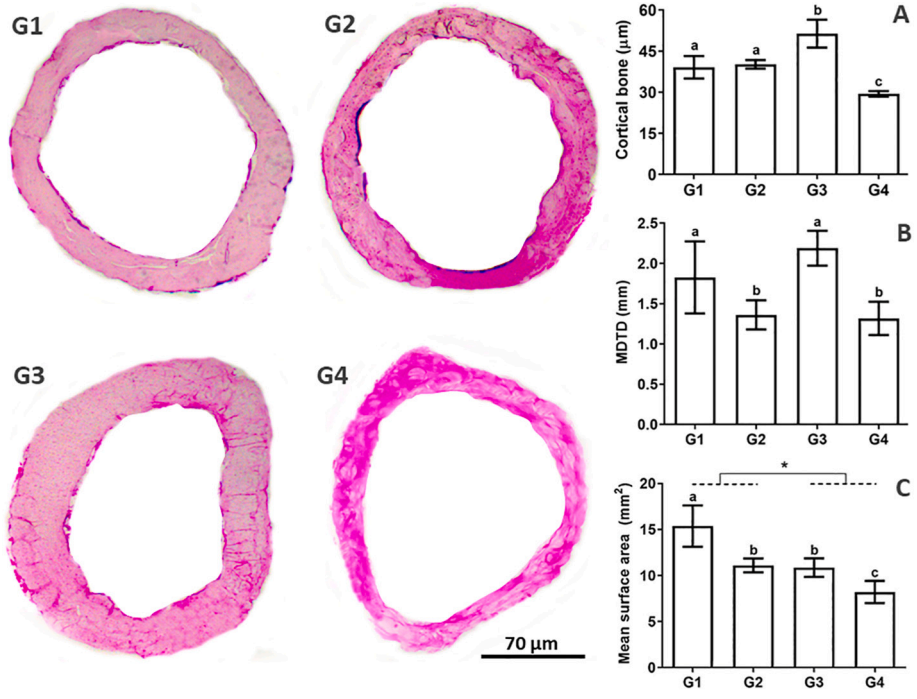

Fig. 4. Representative photomicrographs of tibia diaphysis (H&E staining, ×100 magnification) in mice treated with standard diet (SD) or high-fat diet (HFD), supplemented or not with caffeine. Morphometric parameters of the tibia. A: Cortical bone thickness, Mean diameter of the tibia diaphysis (MDTD), C: Mean surface area. G1: SD, G2: SD + 50 mg/kg caffeine, G3: HFD, G4: HFD +50 mg/kg caffeine. $n = 10$ animals/group. The results were described as mean and standard deviation. [a,b,c] Different letters in the columns indicate statistical difference between groups ($P < 0.05$). *Denotes significant interaction between HFD and caffeine ($P < 0.05$).

relation to weight gain and serum lipid profile. This association was also effective in modifying morphofunctional bone integrity in mice, whose changes in bone microstructure and mineral content were coherent with marked adaptations in bone biomechanical properties.

Previous studies have shown a regulatory effect of caffeine on the central nervous system, with impact over food intake, body weight and lipid metabolism [44–46]. As expected, despite similar food consumption among all groups observed in our investigation, the HFD contributed to increase body weight gain in mice. Conversely, caffeine contributed to reduce weight gain in the HFD-treated mice, although this group presented higher energy consumption when compared to the group treated with SD and caffeine. By contrast, animals receiving SD

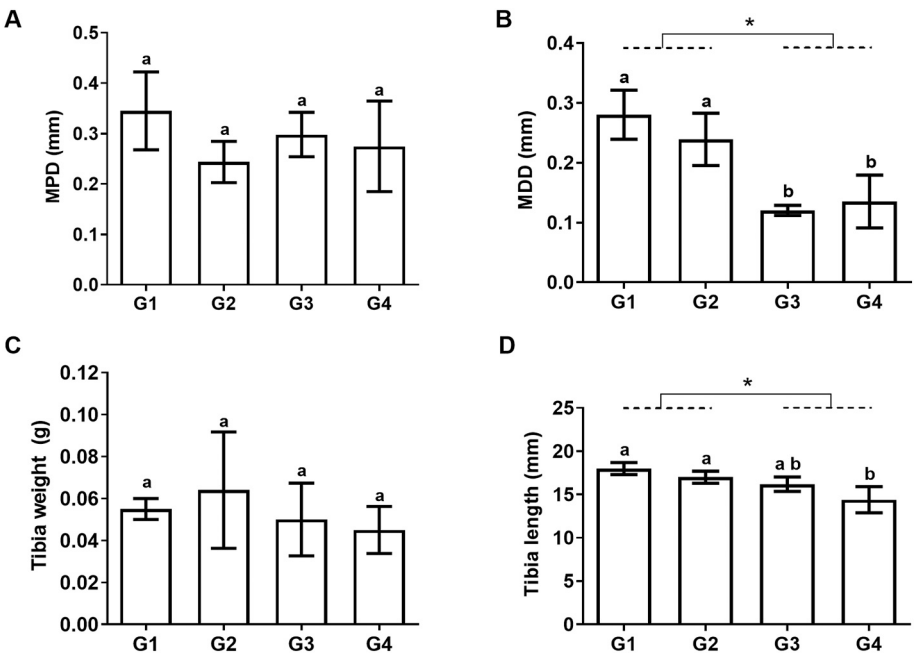

Fig. 5. Morphometric parameters of the tibia in mice treated with standard diet (SD) or high-fat diet (HFD), supplemented or not with caffeine. G1: SD, G2: SD + 50 mg/kg caffeine, G3: HFD, G4: HFD +50 mg/kg caffeine. n = 10 animals/group. A: Mean proximal diameter (MPD), B: Mean distal diameter (MDD), C: Tibia weight, D: Tibia length. The results were described as mean and standard deviation. [a,b,c] Different letters in the columns indicate statistical difference between groups ($P < 0.05$). *Denotes significant interaction between HFD and caffeine ($P < 0.05$).

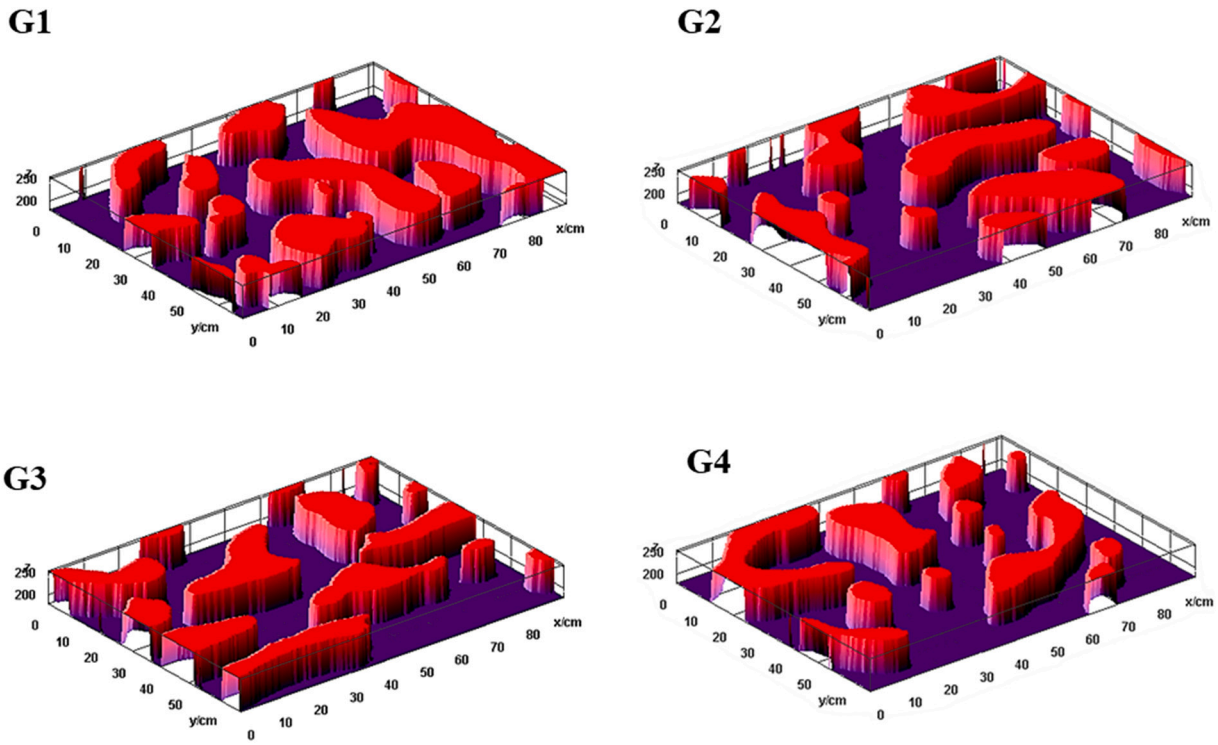

Fig. 6. Representative three-dimensional images of the cancellous bone in mice treated with standard diet (SD) or high-fat diet (HFD), supplemented or not with caffeine. Red color: bone trabecula; Purple color: medullary space. G1: SD, G2: SD + 50 mg/kg caffeine, G3: HFD, G4: HFD +50 mg/kg caffeine. $n = 10$ animals/group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

presented lower final body weight and weight gain compared to the HFD fed groups, an effect possibly mediated by the thermogenic potential of caffeine on lipid metabolism [47]. In line with this response profile, Mechanick et al. [48] pointed out that caffeine potentiates energy expenditure by favoring fatty acid catabolism as an energy substrate [48,49]. According to Harpaz et al. [47], the antagonistic properties of caffeine on adenosine receptors is one of the mechanisms that explain this effect. There is evidence that, by connecting to these receptors, caffeine triggers a cascade of reactions whose final product is the release of catecholamines, which perform as direct endogenous modulators of lipolysis. In this process, intracellular cAMP levels increases and activates the enzyme lipase, which hydrolyzes the adipocyte stocks of triacylglycerol into fatty acids [50].

A direct consequence of excessive triacylglycerol and fatty acid metabolism is the biosynthesis and release of lipoproteins into the bloodstream, causing an imbalance of plasma lipid concentration such

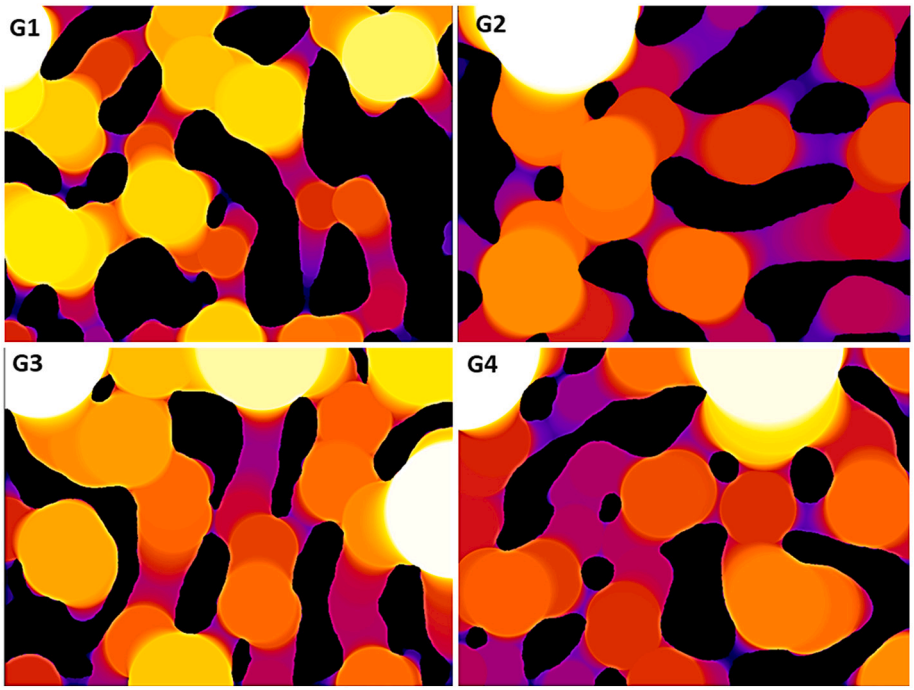

Fig. 7. Three-dimensional deconvolution of the cancellous bone in mice treated with standard diet (SD) or high-fat diet (HFD), supplemented or not with caffeine. Different colors represent the overlapping of spheres positioned over the spaces between the trabeculae. G1: SD, G2: SD + 50 mg/kg caffeine, G3: HFD, G4: HFD +50 mg/kg caffeine. n = 10 animals/group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

as triglycerides, total, LDL and HDL cholesterol [47,51]. According to Harpaz et al. [47], this process may be triggered by neuroendocrine action in response to chronic caffeine use. In our investigation, although caffeine did not contribute to increase lipids or lipoprotein plasma levels, we observed the potential of HFD to increase total cholesterol and HDL cholesterol levels in mice. Li et al. [52] evidenced increased cholesterol, triglycerides and LDL cholesterol serum levels in Wistar rats treated with SD and 120 mg/kg caffeine during the prenatal period. In this study, this effect was associated with the activation of the hypothalamus-pituitary-adrenal axis by caffeine, with consequent upregulating lipid metabolism and lipid fractions into the bloodstream. However, caffeine was not successful in causing similar effects, both in SD- or HFD-treated animals. Although studies on caffeine association are scarce, Liu et al. [53] showed that when combined with catechin (phytonutrient of polyphenols family with antioxidant effect), caffeine exerts an inhibitory effect on atherosclerosis evolution. In a mechanistic approach, this effect was associated with the inactivation of tumor necrosis factor-alpha (TNF-α) biosynthesis and release during atherogenesis, as well as marked suppression of oxidized low-density lipoprotein receptor 1 (LOX-1) production, which reduced endothelium inflammation and atherosclerotic plaque formation [53].

Although HDL cholesterol serum levels were unaffected by the dose of caffeine administered, HFD contributed to the increase of this lipoprotein, compared to SD-treated animals. There is consistent evidence of a complex and heterogeneous response profile in LDL and HDL circulating levels in experimental models based on nutritional manipulation [53,54]. Surprisingly, HFD-treated animals showed high HDL levels in the present investigation, compared to mice receiving SD alone. This finding was aligned with the results reported by Higa et al. [54] and Zeeni et al. [54] which pointed out that although cafeteria diet predisposes to an increased serum lipid profile, this does not always occur to the detriment of falling HDL levels. Thus, it is believed that the amount of fat consumed by the animals in the present study was unable to exert greater stimulatory effects on the blood lipid profile, whose lipid control was potentially influenced by caffeine intake in animals receiving the combined treatment.

Excess serum lipids and caffeine have been independently associated with the impairment of bone tissue cell metabolism. Under both conditions, the negative impact on cellular microstructure and osteogenic processes is often related to molecular imbalance and disturbances in bone biomechanical properties [55,56]. Interestingly, the proposition that the association between HFD and caffeine could be potentially harmful to bone integrity was not corroborated by our findings. Conversely, this association was unable to impair bone biomechanics, as evaluated from maximum load and stiffness. In agreement with these findings, Brun et al. [57] evaluated the effect of *Ilex paraguariensis* (containing 50 mg/kg/caffeine) on Wistar rats during pregnancy. In this study, the authors observed that treated animals exhibited increased activity of their offspring's osteoprogenitor cells, which was related to the increased alkaline phosphatase, a fundamental enzyme for the activation of osteoblasts. These cells, in turn, promote high synthesis of type I collagen, osteocalcin, sialoproteins and osteopontin, which are essential for bone matrix mineralization. In addition, previous evidence indicates a relationship between HDL levels and bone biomechanics, which may partially affect the greater bone stiffness and strength found in our investigation. In this regard, Soares et al. [37] showed that high levels of this lipoprotein have been associated with protective mechanisms in bone tissue cells, with direct impact on the preservation of microstructural and bone mechanical integrity.

Ca and P balance contributes to the integrity of the bone matrix and stiffness. The homeostasis of these minerals is regulated by hormonal stimulation (i.e. parathyroid hormone (PTH) and calcitriol) and is essential for the maintenance of bone tissue mineralization. As these hormones are independently affected by caffeine and lipid profile, bone disturbances such as osteopenia and osteoporosis can be triggered by metabolic overloads of these molecules [58–60]. In the present study, HFD associated with caffeine drastically reduced Ca and P distribution in bone tissue. However, the levels of these minerals remained higher when SD and caffeine were combined. This result indicates a potential interaction between diet and caffeine, with a direct effect on bone mineralization. Lu et al. [61] demonstrated that caffeine may affect bone mineral balance through the excitability of the sympathetic nervous

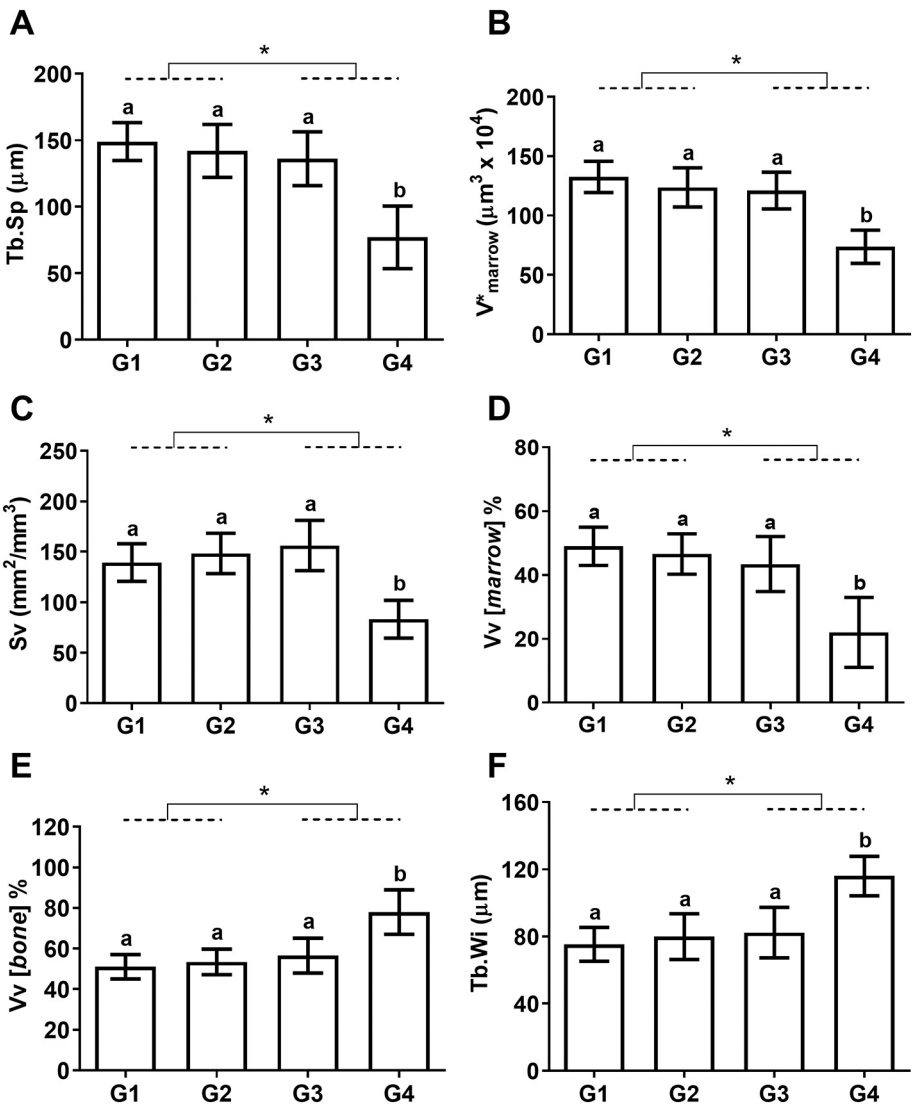

Fig. 8. Microstructural parameters of the cancellous bone in mice treated with standard diet (SD) or high-fat diet (HFD), supplemented or not with caffeine. Tb. Sp: trabecular separation (A), V*: Volumetric density of marrow (B), Sv: surface density of bone trabeculae (C), Vv marrow: Volume density of bone marrow (D), Vv bone: Volumetric density of bone tissue (E), Tb. Wi: trabecular width (F). G1: SD, G2: SD + 50 mg/kg caffeine, G3: HFD, G4: HFD +50 mg/kg caffeine. n = 10 animals/group. The results were described as mean and standard deviation. [a,b] Different letters in the columns indicate statistical difference between groups ($P < 0.05$). *Denotes significant interaction between HFD and caffeine ($P < 0.05$).

system, which exacerbates PTH responses in bone cells inducing matrix degradation. Besides, PTH acts on the kidneys and intestines, limiting the absorption of Ca and P and reducing the deposition of these minerals in bone tissue. In rats fed a HFD for 10 weeks, Song et al. [62] found increased PTH levels and reduced calcitriol production, which are central regulators of Ca and P balance in bone tissue. They also showed that insufficient calcitriol plasma levels reduce intestinal and renal Ca absorption, which increases the activation of osteoclastic cells, with consequent reduction of both Ca and P mineral content and bone density [62].

Besides the relevance of Ca and P availability for bone microstructure and function, minerals such as Mn, Cu, Zn, Se, Mg and S are also essential for maintaining the metabolic activity of bone cells and preserving bone integrity [63]. Currently, the interaction of these elements and the mechanism by which they maintain an adjusted metabolism and balanced bone biomechanical properties remain poorly understood and require further investigation. In the present study, HFD contributed to a marked reduction of Mn, Cu, Zn and Mg tissue levels, compared to the group receiving SD, which suggests the contribution of dietary lipids to this response profile. In agreement with these findings, Aslam et al. [64] showed that C57BL/6 male mice receiving a high-fat diet and supplemented with a micromineral mix for 18 weeks presented an accelerated loss of Se, B, Zn, Mn and Mg, with a negative impact on tibia and femur bone mineral density, compared to SD-treated animals.

Although dietary lipids contributed to the reduction of some chemical elements in bone tissue, the percentage of S and Se were proportionally increased in the groups receiving HFD alone. Specifically, the sulfur has been positively related to the animal growth process. It is essential for sulfur amino acid composition and the maintenance of SH groups functionality during the cellular respiration process. It is also a component of biotin, thiamine, coenzyme A and insulin [65]. Van Donkelaar et al. [65] also hypothesize the relevance of S element in hypertrophic areas of cartilage and the process of chondrocyte maturation in epiphyseal discs during the growth process.

Disturbances in the dynamics of chemical elements may also reflect negatively on the structural dimensions of long bones. Tan et al., [66] observed altered bone growth due to the inhibition of extracellular matrix synthesis in growth plates and, consequently, alterations in the distal and proximal width, length and weight of long bones in the offspring of rats receiving 120 mg/kg caffeine during pregnancy. Despite this fact, in our study, the caffeine dose administered not induced macroscopic morphometric alteration in most of these parameters. However, when HFD and caffeine were combined, decreased tibia width, cortical bone thickness, diaphysis width and tibia length were observed. In addition, important changes in the bone microstructure were also observed with this combination. It has been proven that HFD and caffeine exert independent negative effects on bone trabeculae microstructure, modifying parameters such as trabecular separation,

marrow space volume, surface density of bone trabeculae, volumetric density of bone tissue and trabecular width [67–71]. Although these effects are mainly associated with the isolated use of HFD and caffeine [67–71], we identified that morphofunctional bone adaptations can be potentiated when HFD and caffeine are combined. According to He and Cronstein [55], this fact may be partially attributed to the caffeine activity on adenosine receptor activation in osteoblasts (A1R), with consequent activation of osteoprogenitor cells, which hamper the bone resorption process.

In summary, our findings indicated that HFD and caffeine alone or combined change lipid metabolism by increasing total and HDL cholesterol circulating levels. Alone, lipid and caffeine overload were unable to induce bone biomechanical adaptations. However, biomechanical changes were especially observed when HFD and caffeine were combined, an effect potentially associated with a more severe reduction in calcium and phosphorus distribution, as well as macrostructural and microstructural remodeling of the cortical and cancellous bone from mice simultaneously receiving lipid and caffeine dietary overload. Thus, our findings indicate that HFD and caffeine are two independent risk factors that can interact to modulate bone mineral distribution, structure and biomechanical function, effects whose mechanism should be detailed in further investigation.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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CRediT authorship contribution statement

Fernanda Batista de Souza: Writing - original draft, Conceptualization, Methodology, Validation, Formal analysis, Investigation. **Rômulo Dias Novaes**: Data curation, Conceptualization, Supervision, Writing - review & editing. **Cynthia Fernandes Ferreira Santos**: Investigation, Writing - review & editing. **Franciele Angelo de Deus**: Investigation, Writing - review & editing. **Felipe Couto Santos**: Formal analysis, Investigation, Writing - review & editing. **Luiz Carlos Maia Ladeira**: Investigation, Writing - review & editing. **Reggiani Vilela Gonçalves**: Writing - review & editing. **Daniel Silva Sena Bastos**: Formal analysis, Investigation, Data curation, Writing - review & editing. **Ana Cláudia Ferreira Souza**: Investigation, Writing - review & editing. **Mariana Machado-Neves**: Conceptualization, Resources, Writing - review & editing. **Eliziária Cardoso dos Santos**: Conceptualization, Validation, Supervision, Resources, Funding acquisition, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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