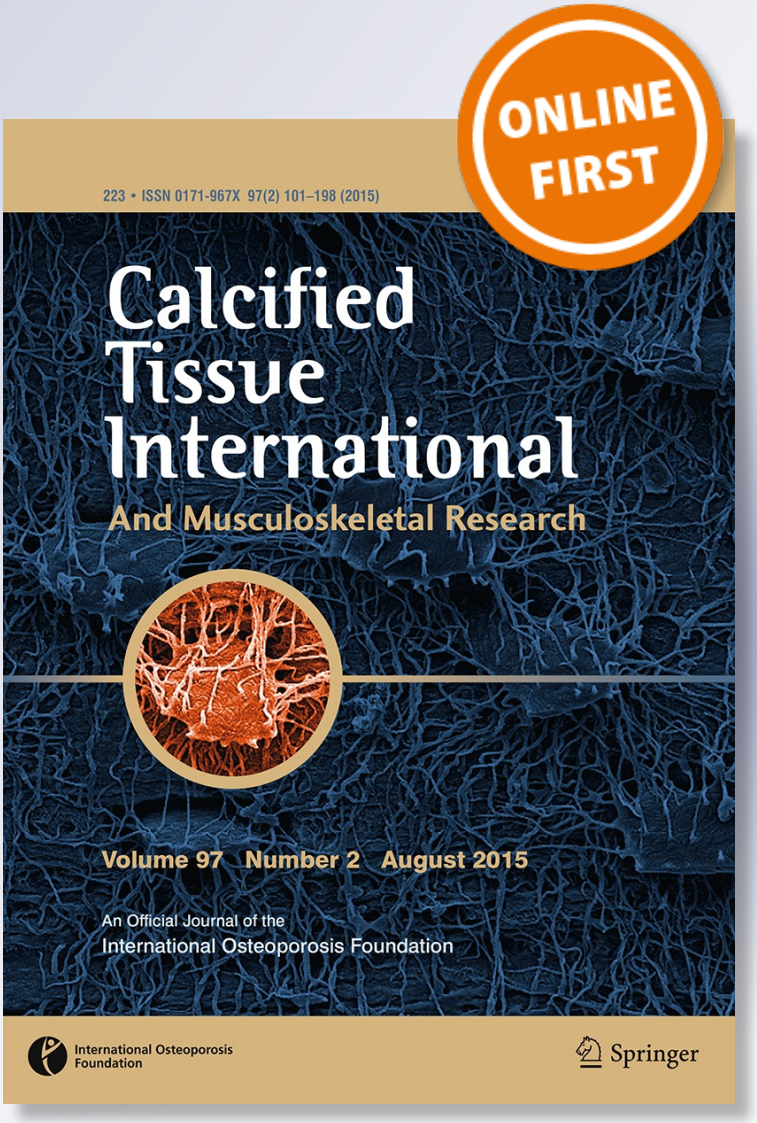
*Effects of Yerba Mate (*Ilex paraguariensis*) on Histomorphometry, Biomechanics, and Densitometry on Bones in the Rat*

Lucas R. Brun, María L. Brance, Mercedes Lombarte, María Cielo Maher, Verónica Elina Di Loreto & Alfredo Rigalli

Calcified Tissue International
and Musculoskeletal Research

ISSN 0171-967X

Calcif Tissue Int
DOI 10.1007/s00223-015-0043-0



Springer

Calcif Tissue Int
DOI 10.1007/s00223-015-0043-0

ORIGINAL RESEARCH

Effects of Yerba Mate (*Ilex paraguariensis*) on Histomorphometry, Biomechanics, and Densitometry on Bones in the Rat

Lucas R. Brun[1,2,4] · María L. Brance[1,2] · Mercedes Lombarte[1,2] · María Cielo Maher[1] · Verónica Elina Di Loreto[1] · Alfredo Rigalli[1,2,3]

Received: 19 March 2015 / Accepted: 20 July 2015

Abstract Yerba mate (*Ilex paraguariensis*) is a xanthine-containing beverage, which is also rich in caffeine. Because caffeine has a negative impact on bone mineral density (BMD) mainly associated with low calcium (Ca) diets, there would be expected a negative effect of yerba mate on bone. In this study, Sprague-Dawley rats were used and randomly assigned into four groups ($n = 6$/group): Control + Ca 0.2 g %; Control + Ca 0.9 g %; Yerba + Ca 0.2 g %; Yerba + Ca 0.9 g %. At the end of the experiment, tibias and femurs were obtained for BMD, morphometric, histomorphometric, and biomechanical analyses. While there was no difference in bone parameters between rats with and without yerba mate consumption, a negative effect of low Ca diet was observed in BMD, morphometric, histomorphometric, and biomechanical results. Interaction between Ca content in the diet and yerba mate was only found in trabecular bone volume, which would indicate that the negative effect of low Ca intake on bone volume is reversed in part by yerba mate infusion. However, yerba mate was not able to reverse the negative effect of low Ca content on biomechanical properties and trabecular connectivity. In summary, at least in our study, yerba mate would not have a negative effect on bone and would be safe for the bone health of consumers.

Keywords *Ilex paraguariensis* · Yerba mate · Bone

Introduction

Yerba mate (*Ilex paraguariensis*) is a xanthine-containing beverage which is very popular in South America. The highest consumption occurs in Uruguay (6–8 kg/person/year) and Argentina (5 kg/person/year) [1]. It is prepared with the dried, ground leaves and twigs of the shrub *Ilex paraguariensis*. *Ilex paraguariensis* extracts contain methyl xanthines, flavonoids, vitamin A, C, and B complexes, tannis, chlorogenic acid, quercetin, and rutin, among other compounds, which exert different pharmacological actions [1]. Thus, a hypolipidemic action, decreasing triglycerides, and total and LDL-cholesterol were observed in hypercholesterolemic rat [2], as well as an improvement on lipid profile in dyslipidemic patients [3]. Furthermore, an anti-obesity effect was demonstrated in mice fed with lipid-enriched diets together with yerba mate [4, 5]. Additional studies showed that yerba mate exerts anti-inflammatory and immunomodulatory effects by inhibiting proinflammatory factors [6–8] and also exhibits antifungal properties [9]. Moreover, provascular and angiogenic properties have been described [10].

It has been reported that caffeine consumption has a negative impact on bone mineral density (BMD) with accelerated bone loss [11] and increased risk of fractures [12], mainly when it is associated with low calcium diets [11, 13, 14]. A meta-analysis of ten prospective studies which collectively included more than 200,000 participants, indicated that coffee consumption is slightly but

✉ Lucas R. Brun
lbrun@unr.edu.ar

1 Bone Biology Laboratory, School of Medicine, Rosario National University, Rosario, Argentina

2 National Council of Scientific and Technical Research (CONICET), Buenos Aires, Argentina

3 Rosario National University Research Council, Rosario, Argentina

4 Cátedra de Química Biológica, Facultad de Ciencias Médicas, Santa Fe 3100, (2000) Rosario, Argentina

Published online: 30 July 2015

significantly associated to increased risk of fractures, especially in women, in a dose-dependent manner [15]. This negative effect was also observed in experimental animals [16, 17]. Caffeine administration enhanced osteoclastogenesis from bone marrow hematopoietic cells and bone resorption activity in vivo [18]. Moreover, caffeine enhanced the expression of the receptor activator of NF-κB ligand (RANKL) and reduced osteoprotegerin protein levels in MC3T3-E1 osteoblastic cells [18].

Because caffeine has a negative impact on BMD and increases fracture risk, it should be expected that yerba mate consumption is associated with a negative effect on bone due to its caffeine content. Although the concentration of caffeine in yerba mate infusion is low (330–480 mg/l) compared with coffee (average 600 mg/l), in Argentina, the 50 % of caffeine consumption in adults is from yerba mate infusion, followed by coffee (36 %), because the total caffeine intake depends not just on the concentration of caffeine but also on the ingested volume of each beverages [1, 19]. As a result, the mean caffeine intake for adults in Argentina is 288 mg/day; and it was shown to be higher in comparison with data reported for other countries such as USA, UK and Brazil [19].

In spite of the potential negative effect of yerba mate on bone mass based on the caffeine content, a recent publication showed 9.7 % higher lumbar spine BMD in postmenopausal women who drank at least 1 liter/day of yerba mate infusion versus controls who did not drink yerba mate infusion (0.952 vs 0.858 g/cm^2, respectively; $p < 0.0001$) [20]. Similar data were observed at femoral neck where BMD was 6.2 % higher in yerba mate consumers than in controls (0.817 vs 0.776 g/cm^2, respectively; $p = 0.0002$). In addition, a multiple regression analysis showed that yerba mate consumption showed a positive correlation with BMD at both the lumbar spine and the femoral neck suggesting a protective effect on bone.

Since investigations in yerba mate on bones of human beings could be influenced by the diversity of life styles and, also, the limitations of human studies per se, we aimed to evaluate the effect of yerba mate (*I. paraguariensis*) infusion on bone tissues of normal rats through methods of densitometric, morphometric, histomorphometric and biomechanical analyses. Ca absorption and Ca uptake were also evaluated in order to ensure that yerba mate did not affect intestinal Ca absorption.

Materials and Methods

Animals

Experiments were carried out in 30-day old, 60–80 g of body weight female Sprague-Dawley rats provided by the School of Medicine, Rosario National University (Argentina). Rats were housed in a room with 12-h light and dark periods, and constant temperature of 24 ± 1 °C.

Twenty-four rats were randomly assigned into two groups: Control and Yerba according as they received water or an infusion of yerba mate as ad libitum drink. A filtered yerba mate infusion (25 g of yerba mate in 1 l of water at 90 °C) containing 370 mg/l of caffeine was administered for 90 days. This infusion replaced the drinking water at the "Yerba" groups. The daily caffeine intake varied between 2–7 mg/100 g considered a low/moderate dose. It was not constant because it was administered in the drinking water, and the weight of the rats varied throughout the experiment. The dose of 2 mg/100 g is equivalent on the human daily consumption of four cups (240 ml) per day for a person weighing 60 kg. Control and Yerba groups were also divided according to the Ca content in the diet: 0.2 or 0.9 g %. Therefore, four experimental groups were formed ($n = 6$ per group): Control + Ca 0.2 g %; Control + Ca 0.9 g %; Yerba + Ca 0.2 g %; and Yerba + Ca 0.9 g %.

At the end of the experiment, rats were euthanized in a CO_2 chamber; and tibias and femurs were obtained. Duodenal cells were isolated for Ca-uptake measurement. The proximal epiphysis of the right tibia was processed for histomorphometric analysis of trabecular bone, as described below. BMD was immediately measured in the left tibia, and biomechanical tests were performed in the femurs, as described below.

BMD Measurement by X-ray Absorptiometry

At the end of experiment, bone mineral density (BMD, mg Ca/cm^2) was measured by an X-ray equipment (Work Ray 70 kV, Workman SRL, Argentina) simultaneously with an aluminum step wedge, which was previously calibrated with known Ca concentrations [21]. The BMD was determined in a 2-mm^2 area at 1 mm from growth plate–metaphyseal junction similar to the measurement of histomorphometric parameters.

Bone Histomorphometry

The proximal epiphysis of the right tibias were fixed in 10 % phosphate-buffered formaldehyde and decalcified in 10 % EDTA before embedment in paraffin. 5-μm longitudinal sections were stained with hematoxylin and eosin, and four sections per rat were examined using a light microscope (Leitz, Wetzlar, Germany). Digital images were obtained at a ×40 magnification (Olympus SP-350, China) of proximal epiphysis. Analyses were performed in a 2-mm^2 area at 1 mm from growth plate–metaphyseal junction. The following measurements were performed

with ImageJ 1.40 software (NIH, Maryland, USA), as described by Parfitt et al. [22]: (1) total tissue volume, TV (μm^2); (2) trabecular bone volumen, BV (μm^2); and (3) trabecular bone surface, BS (μm). With these values, histomorphometric variables were calculated: (1) bone volume, BV/TV (%) = [BV*100/TV]; (2) trabecular thickness, Tb.Th (μm) = [2/(BS/BV)]; (3) trabecular number, Tb.N (1/mm) = [(BV/TV)/(Tb.Th)]; and (4) trabecular separation, Tb.Sp (μm) = [(1/Tb.N) − Tb.Th].

Trabecular Connectivity Assessment

The analysis of trabecular interconnectivity was performed as previously published [23], and the following parameters were measured by means of ImageJ 1.40 software (NIH, Maryland, USA): total number of nodes (Nd), the number of node-to-node branches (NNd), the number of node-to-termini branches (NNdTm), the number of trees (*T*), the number of terminals (Tm), and the number of branches with two terminals (NTm). With these parameters, we proceeded to calculate the interconnectivity parameters: the interconnectivity index [ICI = Nd*NNd)/T*(NNdTm + 1)]; the node-to-termini ratio [*R* = Nd/Tm]; the mean size of branches [Dist (mm) = $\sum$ branches size/(NNdTm + NNd + NTm)]; and the new interconnectivity index [NDX (%/mm) = (*R**NNd*(BV/TV)*Tb.Th)/(NTm*NNdTm*Dist*Tb.Sp)].

Cortical Bone Measurements

For morphometric analysis of cortical bone, cross Sects. (500 μm in thickness) of right femoral diaphyses were cut with a low speed saw (IsoMet. Buehler Ltd. Illinois, USA). A digital image was obtained at 40x magnification, and measurements were performed using ImageJ 1.40 (NIH, Maryland, USA) for the (1) cross-sectional area (CS.Ar: the area of bone and marrow cavity bounded by the periosteal surface, mm^2); (2) medullary area (Me.Ar: the area delineated by the endocortical surface, mm^2); (3) cortical bone area (CB.Ar: calculated as the difference between the CS.Ar and Me.Ar, mm^2); (4) periosteal perimeter (mm); (5) endosteal perimeter (mm); (6) cortical width (mm); (7) periosteal diameter (mm); and (8) medullary diameter [24]. Cross-sectional moment of inertia (CSMI, mm^4) was calculated as ([periosteal diameter/2]^4 − [medullary diameter/2]^4) * π/64.

Mechanical Testing

Femurs were stored at −20 °C wrapped in saline-soaked gauze until tested and were thawed at 37 °C. The cortical bone strength at the femoral mid-diaphysis was determined using a three-point bending test, and the trabecular bone strength was evaluated by a compression test in distal femur [25, 26]. Mechanical testing was performed on a machine designed at the engineering department of the Bone Biology Laboratory, with a 300-N load cell with 0.01 N of discrimination and an accuracy of 10 μm in displacements. The two-bar distance for the three-point bending test was 14 mm. The compression test was performed with a compression cone of 7.07 mm^2 on a 2.5-mm thickness transversal section from distal epiphysis of the same bone. For both tests, the speed was 0.01 mm/s, and was monitored on a computer. Load versus displacement plots were recorded using software Biomedical Data Acquisition Suite 1.0 (Argentina, 2011) to determine bone properties. The software data acquisition rate was 10 Hz. The ultimate load (N) was defined as the highest load, and the fracture load (N) was recorded just before the first decline in load. The stiffness (N/mm) was determined as the slope of the linear portion of the load versus displacement curve. Absorbed energy (mJ) was defined as the area under the curve until the fracture load point. The material properties were determined by employing classic beam theory and transforming the data of load and displacement in stress [stress = load * two-bar distance * outer radius/(4 * CSMI) and strain [strain = (12 * outer radius * displacement * 10^6)/(two-bar distance)^2], respectively. The ultimate stress (MPa) was defined as the highest stress, and the modulus of elasticity or Young's modulus (GPa) was calculated using the linear portion of the stress versus strain curve.

In Vivo Net Ca Absorption

Animals were placed in individual metabolic cages, and the amount of food eaten in 24 h was measured. Feces were collected and incinerated at 550 °C. Calcium concentrations in food and feces were determined by atomic absorption spectroscopy (Arolab MK II, Buenos Aires, Argentina), and 24-h Ca intake and 24-h fecal Ca were calculated every 30 days. The percentage of Ca absorption (%Ca) for each animal was calculated as %Ca = (24 h-Ca intake − 24 h-fecal Ca) * 100/24 h-Ca intake.

In Vitro Calcium Uptake

Immediately after euthanasia, duodenal cells were isolated as described previously [27] to determine Ca uptake. Cell viability was assessed by Trypan blue exclusion. Exclusion of the dye in >90 % of the cells was observed for at least 90 min after isolation. A total of 200,000 cells were incubated for 10 min at 37 °C with the uptake buffer (1 mM-Tris, 1 mM $MgCl_2$, 50 mM $CaCl_2$, ^{45}Ca [1 μCi/ml, New England Nuclear, Research products, Boston, MA,

USA]) ($n = 10$). After 10 min, the cell suspension was diluted 25-fold in ice-cold medium (phosphate-buffered saline [PBS] + 0.02 mM $CaCl_2$, 4 °C). The cells' suspensions were spun down at 750 g for 1 min, resuspended in 1 M NaOH, and radioactivity (^{45}Ca) was measured using a scintillation counter (Beckman LS 100C, USA). Higher radioactivity indicates higher Ca uptake [28].

Ca Concentration on Yerba Mate Infusion

The Ca concentration on yerba mate infusion was evaluated throughout the yerba mate infusion preparation by atomic absorption spectroscopy (Arolab MK II, Buenos Aires, Argentina). The Ca concentration was measured at every 10° between 20 and 90 °C.

Statistical Analysis

Data were expressed as mean ± SEM. Two-way analysis of variance (ANOVA) was performed with Ca diet (0.2 or 0.9 g %) and infusion (yerba mate or water) as analyzed factors. The multiple comparison LSD posttest analysis was performed if significant interaction was found. Differences were considered significant if $p < 0.05$. Statistical analyses were performed using the stat, base, and agricolae packages of the software version R 2.14.0 [29].

Results

BMD Measurement

BMD was only affected by Ca content in the diet ($p = 0.001$, Two-way ANOVA). The yerba mate infusion did not affect the BMD, and no interaction between Ca diet and yerba mate was observed (Fig. 1).

Bone Histomorphometry

Ca content in the diet, but not yerba mate infusion, had effects on histomorphometric parameters (Table 1). Further, a significant interaction between Ca diet and yerba mate infusion was found only for BV/TV ($p = 0.048$, Two-way ANOVA). The lower BV/TV in Control + Ca 0.2 % compared to Control + Ca 0.9 % was due to a decrease in the trabecular thickness and an increase in trabecular separation without changes on trabecular number. No differences were observed in BV/TV between Yerba groups.

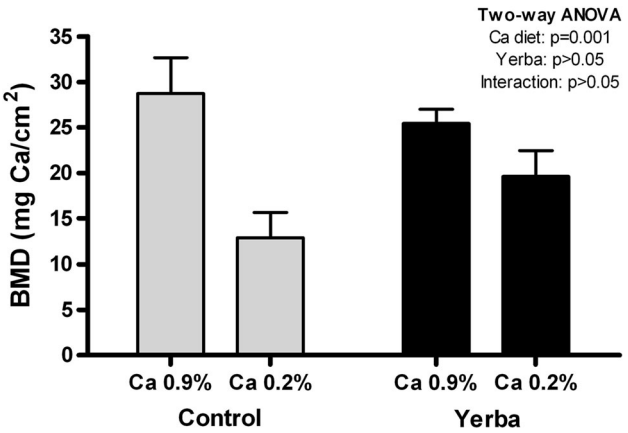


Fig. 1 Bone mineral density (BMD) in Control and Yerba groups. BMD was only affected by Ca content in the diet (p = 0.001, Two-way ANOVA)

Trabecular Connectivity

Connectivity parameters were not affected by yerba mate consumption, but an effect of Ca content in the diet was observed (Table 2). No interaction between Ca diet and yerba mate was found. Lower NDX values were found in groups with low Ca content in the diet. The NTm parameter, which provides information about loss of connectivity in the bone architecture, was also found increased in groups that received Ca 0.2 g %. However, this negative effect on NTm was compensated with a decrease in Dist.

Cortical Bone Measurements

The diet Ca content, but not yerba mate consumption, had effect on morphometric parameters without interaction between Ca diet and yerba mate (Two-way ANOVA) (Table 3). An increase in medullary area with no change in cross-sectional area, which led to a decrease in the cortical area was observed in the groups that received Ca 0.2 %. Also, increased endosteal perimeters without changes in periosteal perimeter leading to a decrease in the cortical width were observed in the groups fed with Ca 0.2 %-diet.

Mechanical Testing

Biomechanical parameters of bone analyzed with three-point bending test were not affected by Ca content of the diet or yerba mate infusion (Table 4). Also, no interaction between Ca diet and yerba mate infusion was observed. On the other hand, trabecular biomechanical parameters were affected by the Ca content of the diet (Table 4), while no

Table 1 Bone histomorphometric measurement

	Control		Yerba		Two-way ANOVA		
	Ca 0.9 g %	Ca 0.2 g %	Ca 0.9 g %	Ca 0.2 g %	Ca diet	Yerba	Interaction
BV/TV (%)	25.25 ± 3.71	10.68 ± 1.59*	20.50 ± 1.54	16.31 ± 2.59	$p < 0.05$	ns	$p < 0.05$
Tb.Th (μm)	59.31 ± 6.51	44.48 ± 3.57	55.64 ± 3.50	45.07 ± 3.68	$p < 0.05$	ns	ns
Tb.N (1/mm)	4.37 ± 0.64	2.38 ± 0.29	3.76 ± 0.30	3.78 ± 0.67	ns	ns	ns
Tb.Sp (μm)	202.80 ± 38.52	434.40 ± 87.65	230.30 ± 25.97	278.00 ± 62.97	$p < 0.05$	ns	ns

BV/TV bone volume, *Tb.Th* trabecular thickness, *Tb.N* trabecular number, *Tb.Sp* trabecular separation

* $p < 0.05$ compared to Control + Ca 0.9 % (Two-way ANOVA, LSD posttest)

Table 2 Trabecular connectivity assessment

	Control		Yerba		Two-way ANOVA		
	Ca 0.9 g %	Ca 0.2 g %	Ca 0.9 g %	Ca 0.2 g %	Ca diet	Yerba	Interaction
ICI	0.40 ± 0.15	0.11 ± 0.04	0.28 ± 0.11	0.26 ± 0.19	ns	ns	ns
R	0.23 ± 0.05	0.12 ± 0.02	0.22 ± 0.04	0.12 ± 0.04	$p < 0.05$	ns	ns
NDX (%/mm)	0.53 ± 0.21	0.02 ± 0.008	0.30 ± 0.16	0.11 ± 0.10	$p < 0.05$	ns	ns
Dist (mm)	0.19 ± 0.05	0.12 ± 0.02	0.19 ± 0.02	0.09 ± 0.02	$p < 0.05$	ns	ns
NTm	10.17 ± 2.18	14.33 ± 2.84	9.00 ± 1.51	21.00 ± 6.48	$p < 0.05$	ns	ns

ICI interconnectivity index, *R* node-to-termini ratio, *Dist* mean size of branches, *NDX* new interconnectivity index, *NTm* number of branches with two terminals

Table 3 Morphometric parameters

	Control		Yerba		Two-way ANOVA		
	Ca 0.9 g %	Ca 0.2 g %	Ca 0.9 g %	Ca 0.2 g %	Ca diet	Yerba	Interaction
Cross-sectional area (mm^2)	8.91 ± 0.19	9.12 ± 0.22	9.04 ± 0.30	8.93 ± 0.26	ns	ns	ns
Medullary area (mm^2)	3.23 ± 0.15	4.56 ± 0.16	3.41 ± 0.25	4.48 ± 0.17	$p < 0.05$	ns	ns
Cortical bone area (mm^2)	5.68 ± 0.25	4.56 ± 0.27	5.63 ± 0.23	4.45 ± 0.25	$p < 0.05$	ns	ns
Periosteal perimeter (mm)	11.48 ± 0.15	11.76 ± 0.17	11.62 ± 0.20	11.54 ± 0.17	ns	ns	ns
Endosteal perimeter (mm)	6.82 ± 0.14	8.20 ± 0.14	7.12 ± 0.26	8.06 ± 0.14	$p < 0.05$	ns	ns
Cortical width (mm)	0.71 ± 0.03	0.50 ± 0.03	0.69 ± 0.03	0.49 ± 0.03	$p < 0.05$	ns	ns

effects of yerba mate consumption or interactions between Ca diet and yerba mate were observed. The compression test showed lower values in fracture load, stiffness, and Young's modulus in Ca 0.2 %-fed rats compared to Ca 0.9 %, independent of yerba mate consumption.

In Vivo Net Ca Absorption and In Vitro Calcium Uptake

The net Ca absorption was only affected by the Ca content in the diet (Table 5) without interaction between Ca content and yerba mate infusion.

Ca uptake of isolated enterocytes was not affected by Ca content in the diet or by the presence of yerba mate (Table 5). In addition, no interaction between Ca content and yerba mate infusion was found.

Ca Concentration on Yerba Mate Infusion

The concentration of Ca on yerba mate infusion was measured, and it was demonstrated that the concentration is dependent of the temperature of the process. The Ca concentration in yerba mate infusion was significantly increased from 1.06 ± 0.04 mg/dl at 20 °C to 2.85 ± 0.04 mg/dl at 80 °C and 2.87 ± 0.08 mg/dl at 90 °C (Wilcoxon test, $p < 0.05$). However, the contribution of Ca from yerba mate to total Ca intake was insignificant: 1.82 % in the groups with low Ca intake and 0.40 % in the normal Ca groups.

Table 4 Mechanical testing

	Control		Yerba		Two-way ANOVA		
	Ca 0.9 g %	Ca 0.2 g %	Ca 0.9 g %	Ca 0.2 g %	Ca diet	Yerba	Interaction
Three-point bending test							
Fracture load (N)	64.70 ± 3.98	67.52 ± 3.90	58.93 ± 4.28	61.05 ± 3.43	ns	ns	ns
Ultimate load (N)	70.50 ± 6.41	68.44 ± 1.88	65.85 ± 3.77	71.50 ± 3.77	ns	ns	ns
Stiffness (N/mm)	206.50 ± 30.10	124.40 ± 19.18	186.10 ± 25.75	168.80 ± 28.47	ns	ns	ns
Absorbed energy (mJ)	73.25 ± 9.50	59.76 ± 1.32	92.56 ± 4.09	77.35 ± 6.91	ns	ns	ns
Ultimate stress (MPa)	74.76 ± 2.59	82.09 ± 2.90	72.94 ± 4.01	84.42 ± 7.99	ns	ns	ns
Young's modulus (GPa)	1.97 ± 0.14	1.71 ± 0.14	2.11 ± 0.18	2.09 ± 0.30	ns	ns	ns
CSMI (mm^4)	5.77 ± 0.55	4.89 ± 0.20	5.63 ± 0.36	5.08 ± 0.43	ns	ns	ns
Compression test							
Fracture load (N)	31.39 ± 6.06	10.59 ± 2.33	23.14 ± 3.85	10.42 ± 2.80	$p < 0.05$	ns	ns
Stiffness (N/mm)	54.16 ± 17.57	16.51 ± 3.61	52.07 ± 8.86	16.68 ± 4.81	$p < 0.05$	ns	ns
Absorbed energy (mJ)	4.43 ± 1.31	2.16 ± 1.00	2.99 ± 0.71	1.58 ± 0.74	ns	ns	ns
Young's modulus (GPa)	0.019 ± 0.006	0.006 ± 0.001	0.018 ± 0.003	0.006 ± 0.002	$p < 0.05$	ns	ns

CSMI cross-sectional moment of inertia

Table 5 In vivo net Ca absorption and in vitro calcium uptake

	Control		Yerba		Two-way ANOVA		
	Ca 0.9 g %	Ca 0.2 g %	Ca 0.9 g %	Ca 0.2 g %	Ca diet	Yerba	Interaction
Net Ca absorption (%)	41.17 ± 7.81	96.61 ± 0.80	57.11 ± 7.80	94.70 ± 2.06	$p < 0.05$	ns	ns
Ca uptake (kBq/mg protein)	26.49 ± 3.19	32.90 ± 4.24	37.42 ± 5.75	31.83 ± 3.01	ns	ns	ns

Discussion

Because caffeine has a negative impact on bone, it could be expected that yerba mate consumption is deleterious for the bone, due to its caffeine content, at least when combined with a low Ca diet. It was previously demonstrated a negative association between caffeine (more than 200–300 mg/day = ~400–500 ml of coffee) and BMD, which was attenuated in women with high Ca intake (>750 mg/day) [14]. Current evidence indicates that individuals consuming a minimum 800 mg Ca/day would not be significantly affected by daily caffeine intakes below 400 mg [30].

This study shows lower BV/TV values in Control + Ca 0.2 % compared to Control + Ca 0.9 % without differences between Yerba groups. The interaction between Ca contents in the diet and yerba mate infusion (BV/TV: $p = 0.048$) would indicate a protective effect of yerba mate on bone volume when the Ca content of the diet is below the requirements. While yerba mate infusion was administered in the drinking water and not in the usual way of administration in humans, the daily dose of caffeine was equivalent to humans' doses. Despite the fact that in the study of Conforti et al. there were no significant differences in Ca intake between yerba mate drinkers and nondrinkers, in both groups the Ca intake was under Ca requirement (600 mg/day [range 450–700] and 500 mg/day [range 450–650], respectively). Therefore, the higher lumbar spine and femoral neck BMD observed in individuals drinking yerba mate—similar to what was found in the current study in rats—could be attributed to the effect of yerba mate infusion on bone volume when the Ca content of the diet is below the requirements. However, this effect would not be biologically important because other bone parameters as trabecular connectivity and mechanical properties were not affected by yerba mate.

The disruption parameter (NTm) was found to be increased in Yerba + Ca 0.2 %, which could explain why yerba mate infusion was not able to prevent the effects of low Ca content in the diet on the mechanical properties of the trabecular bone. Therefore, despite a protective effect of yerba mate on BMD found by Conforti et al. [20], we found that bone structural and biomechanical parameters were not preserved, suggesting that it cannot prevent the effect of low Ca diet on fracture risk. However, this is an important outcome as this study shows no negative effect

of yerba mate on bone that could be expected due to the caffeine content. In addition, acute toxicity and subchronic toxicity studies using yerba mate-dried extract in Wistar rats showed no effects on survival, clinical observations, macroscopic and histopathological examination of organs, body weight or food, and water consumption. Moreover, most of biochemical and hematological parameters remained unchanged [31]. Taken together, the results indicate that yerba mate is safe for bone, as well as general health.

It is important to consider not only the differences in the concentration of caffeine between coffee and yerba mate, but also the concentration of flavonoids and other organic compounds which have favorable effects on bone [32]. The presence of polyphenols [1, 33, 34] with favorable effect on bone tissue can mitigate the negative effect of caffeine. Tea (*Camellia sinensis*), another common xanthine-containing beverages, has been reported to have a protective effect on bone [35–37]. Tea contains fluoride (0.54–2.21 mg/l) [38], and this mineral has been proven to have osteogenic activity [39, 40], especially when it is administered in low doses for long periods of time. The opposite effect of tea could be the consequence of the interaction of caffeine and fluoride. Yerba mate is also a rich source of flavonoids (gallocatechin and gallic acid) which may benefit bone density both through the inhibition of bone resorption and increasing osteoblast proliferation [41–43]. Chlorogenic acid is the most abundant polyphenol bioactive compound of *Ilex paraguariensis* and has the property of inhibiting osteoclast differentiation and bone resorption [44]. Therefore, the combination of agents that are beneficial and detrimental to bone renders yerba mate infusion neutral on bone mass and strength.

In summary, the consumption of yerba mate does not affect bone structure parameter or bone resistance in normal rats. Although the negative effect of low Ca intake that affects bone volume can be prevented at least in part by the yerba mate, the yerba mate infusion is not able to reverse the negative effect of low Ca intake on biomechanical properties and trabecular connectivity. In summary, our results suggest that yerba mate does not have a negative effect on bone, and it is safe for the bone health of the consumers.

Acknowledgments The authors thank Candela Retamozo, Damián Lescano, and Hilda Moreno for their technical assistance. This work was funded by a grant from Alberto J. Roemmers Foundation.

Compliance with Ethical Standards

Conflict of interest Lucas R. Brun, María L. Brance, Mercedes Lombarte, María Cielo Maher, Verónica Elina Di Loreto, and Alfredo Rigalli have reported no conflict of interest.

Human and Animal Rights and Informed Consent All the experiments were conducted in accordance with international guidelines for animal care [45] and have been approved by the Bioethics Committee of School of Medicine, Rosario National University.

References

1. Bracesco N, Sanchez AG, Contreras V, Menini T, Gugliucci A (2011) Recent advances on *Ilex paraguariensis* research: minireview. J Ethnopharmacol 136:378–384
2. Bravo L, Mateos R, Sarriá B, Baeza G, Lecumberri E, Ramos S, Goya L (2014) Hypocholesterolaemic and antioxidant effects of yerba mate (*Ilex paraguariensis*) in high-cholesterol fed rats. Fitoterapia 92:219–229
3. de Morais EC, Stefanuto A, Klein GA, Boaventura BC, de Andrade F, Wazlawik E, Di Pietro PF, Maraschin M, da Silva EL (2009) Consumption of yerba mate (*Ilex paraguariensis*) improves serum lipid parameters in healthy dyslipidemic subjects and provides an additional LDL-cholesterol reduction in individuals on statin therapy. J Agric Food Chem 57:8316–8324
4. Arçari DP, Bartchewsky W, dos Santos TW, Oliveira KA, Funck A, Pedrazzoli J, de Souza MF, Saad MJ, Bastos DH, Gambero A, Carvalho Pde O, Ribeiro ML (2009) Antiobesity effects of yerba maté extract (*Ilex paraguariensis*) in high-fat diet-induced obese mice. Obesity (Silver Spring) 17:2127–2133
5. Arçari DP, Santos JC, Gambero A, Ribeiro ML (2013) The in vitro and in vivo effects of yerba mate (*Ilex paraguariensis*) extract on adipogenesis. Food Chem 141:809–815
6. Puangpraphant S, de Mejia EG (2009) Saponins in yerba mate tea (*Ilex paraguariensis* A. St.-Hil) and quercetin synergistically inhibit iNOS and COX-2 in lipopolysaccharide-induced macrophages through NFkappaB pathways. J Agric Food Chem 57:8873–8883
7. Pimentel GD, Lira FS, Rosa JC, Caris AV, Pinheiro F, Ribeiro EB, Oller do Nascimento CM, Oyama LM (2013) Yerba mate extract (*Ilex paraguariensis*) attenuates both central and peripheral inflammatory effects of diet-induced obesity in rats. J Nutr Biochem 24:809–818
8. Arçari DP, Bartchewsky W Jr, dos Santos TW, Oliveira KA, DeOliveira CC, Gotardo ÉM, Pedrazzoli J Jr, Gambero A, Ferraz LF, Carvalho Pde O, Ribeiro ML (2011) Anti-inflammatory effects of yerba mate extract (*Ilex paraguariensis*) ameliorate insulin resistance in mice with high fat diet-induced obesity. Mol Cell Endocrinol 335:110–115
9. Filip R, Davicino R, Anesini C (2010) Antifungal activity of the aqueous extract of *Ilex paraguariensis* against *Malassezia furfur*. Phytother Res 24:715–719
10. Strassmann BB, Vieira AR, Pedrotti EL, Morais HN, Dias PF, Maraschin M (2008) Quantitation of methylxanthinic alkaloids and phenolic compounds in mate (*Ilex paraguariensis*) and their effects on blood vessel formation in chick embryos. J Agric Food Chem 56:8348–8353
11. Harris SS, Dawson-Hughes B (1994) Caffeine and bone loss in healthy postmenopausal women. Am J Clin Nutr 60:573–578
12. Kiel DP, Felson DT, Hannan MT, Anderson JJ, Wilson PW (1990) Caffeine and the risk of hip fracture: the Framingham Study. Am J Epidemiol 132:675–684
13. Barrett-Connor E, Chang JC, Edelstein SL (1994) Coffee-associated osteoporosis offset by daily milk consumption. The Rancho Bernardo Study. JAMA 271:280–283
14. Ilich JZ, Brownbill RA, Tamborini L, Crncevic-Orlic Z (2002) To drink or not to drink: how are alcohol, caffeine and past smoking related to bone mineral density in elderly women? J Am Coll Nutr 21:536–544

15. Liu H, Yao W, Zhang W, Zhou J, Wu T, He C (2012) Coffee consumption and risk of fractures: a meta-analysis. Arch Med Sci 8:776–783
16. Huang TH, Yang RS, Hsieh SS, Liu SH (2002) Effects of caffeine and exercise on the development of bone: a densitometric and histomorphometric study in young Wistar rats. Bone 30:293–299
17. Lacerda SA, Matuoka RI, Macedo RM, Petenusci SO, Campos AA, Brentegani LG (2010) Bone quality associated with daily intake of coffee: a biochemical, radiographic and histometric study. Braz Dent J 21:199–204
18. Liu SH, Chen C, Yang RS, Yen YP, Yang YT, Tsai C (2011) Caffeine enhances osteoclast differentiation from bone marrow hematopoietic cells and reduces bone mineral density in growing rats. J Orthop Res 29:954–960
19. Olmos V, Bardoni N, RidolfiAS Villaamil-Lepori EC (2009) Caffeine levels in beverages from Argentina's market: application to caffeine intake assessment. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 26:275–281
20. Conforti AS, Gallo ME, Saraví FD (2012) Yerba mate (*Ilex paraguariensis*) consumption is associated with higher bone mineral density in postmenopausal women. Bone 50:9–13
21. Moreno H, Lombarte M, Di Loreto VE (2009) Bones and bone tissue. In: Rigalli A, Di Loreto V (eds) Experimental surgical models in the laboratory rat, vol 1. CRC Press, Routledge, pp 229–232
22. Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR (1987) Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Miner Res 2:595–609
23. Harrar K, Hamami L (2012) An interconnectivity index for osteoporosis assessment using X-ray images. J Med Biol Eng 33:569–575
24. Brun LR, Pera L, Rigalli A (2010) Bone morphometry and differences in bone fluorine containing compounds in rats treated with NaF and MFP. Biomed Pharmacother 64:1–6
25. Turner CH, Burr DB (1993) Basic biomechanical measurements of bone: a tutorial. Bone 14:595–608
26. Hogan HA, Ruhmann SP, Sampson HW (2000) The mechanical properties of cancellous bone in the proximal tibia of ovariectomized rats. J Bone Miner Res 15:284–292
27. Picotto G, Massheimer V, Boland R (1997) Parathyroid hormone stimulates calcium influx and the cAMP messenger system in rat enterocytes. Am J Physiol 273:C1349–C1353
28. Brun LR, Brance ML, Lombarte M, Lupo M, Di Loreto VE, Rigalli A (2014) Regulation of intestinal calcium absorption by luminal calcium content. Role of intestinal alkaline phosphatase. Mol Nutr Food Res 58:1546–1551
29. R Development Core Team (2011). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/
30. Nawrot P, Jordan S, Eastwood J, Rotstein J, Hugenholtz A, Feeley M (2003) Effects of caffeine on human health. Food Addit Contam 20:1–30
31. de Andrade F, de Albuquerque CA, Maraschin M, da Silva EL (2012) Safety assessment of yerba mate (*Ilex paraguariensis*) dried extract: results of acute and 90 days subchronic toxicity studies in rats and rabbits. Food Chem Toxicol 50:328–334
32. Dew TP, Day AJ, Morgan MR (2007) Bone mineral density, polyphenols and caffeine: a reassessment. Nutr Res Rev 20: 89–105
33. Bojić M, Simon Haas V, Sarić D, Maleš Z (2013) Determination of flavonoids, phenolic acids, and xanthines in mate tea (*Ilex paraguariensis* St.-Hil.). J Anal Methods Chem 2013:658596
34. Bastos Deborah H, Fornari AC, Queiroz YS, Torres EA (2006) Bioactive compounds content of chimarrão infusions related to the moisture of yerba maté (*Ilex paraguariensis*) leaves. Braz Arch Biol Technol 49:399–404
35. Devine A, Hodgson JM, Dick IM, Prince RL (2007) Tea drinking is associated with benefits on bone density in older women. Am J Clin Nutr 86:1243–1247
36. Chen Z, Pettinger MB, Ritenbaugh C, LaCroix AZ, Robbins J, Caan BJ, Barad DH, Hakim IA (2003) Habitual tea consumption and risk of osteoporosis: a prospective study in the Women's Health Initiative observational cohort. Am J Epidemiol 158: 772–781
37. Johnell O, Gullberg B, Kanis JA et al (1995) Risk factors for hip fracture in European women: the MEDOS Study. Mediterranean Osteoporosis Study. J Bone Miner Res 10:1802–1815
38. Fung KF, Zhang ZQ, Wong JW, Wong MH (1999) Fluoride contents in tea and soil from tea plantations and the release of fluoride into tea liquor during infusion. Environ Pollut 104: 195–205
39. Lau KH, Farley JR, Freeman TK, Baylink DJ (1989) A proposed mechanism of the mitogenic action of fluoride on bone cells inhibition of the activity of an osteoblastic acid phosphatase. Metabolism 38:858–868
40. Farley JR, Wergedal JE, Baylink DJ (1983) Fluoride directly stimulates proliferation and alkaline phosphatase activity of bone-forming cells. Science 222:330–332
41. Delaissé JM, Eeckhout Y, Vaes G (1986) Inhibition of bone resorption in culture by (+)-catechin. Biochem Pharmacol 35:3091–3094
42. Vali B, Rao LG, El-Sohemy A (2007) Epigallocatechin-3-gallate increases the formation of mineralized bone nodules by human osteoblast-like cells. J Nutr Biochem 18:341–347
43. Ko CH, Lau KM, Choy WY, Leung PC (2009) Effects of tea catechins, epigallocatechin, gallocatechin, and gallocatechin gallate, on bone metabolism. J Agric Food Chem 57:7293–7297
44. Kwak SC, Lee C, Kim JY, Oh HM, So HS, Lee MS, Rho MC, Oh J (2013) Chlorogenic acid inhibits osteoclast differentiation and bone resorption by down-regulation of receptor activator of nuclear factor kappa-B ligand-induced nuclear factor of activated T cells c1 expression. Biol Pharm Bull 36:1779–17786
45. Canadian Council on Animal Care Guidelines. Guide to the care and use of experimental animal. 2nd ed. 1998