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ORIGINAL CONTRIBUTION

Diet containing low n-6/n-3 polyunsaturated fatty acids ratio, provided by canola oil, alters body composition and bone quality in young rats

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Abstract
Purpose Adipocytes and osteoblasts were derived from a common progenitor, and canola oil intake may have an adipogenic and osteogenic effect. Thus, our objective was to evaluate the effect on adipocyte, lipid profile, glucose homeostasis, and bone of canola oil as main lipid source on the diet during development.
Methods After weaning, rats were divided into two groups ($n = 10$ per group): control (S) and experimental (C) diets containing 7 mL/100 g soybean or canola oil, respectively. At 60 days, body composition, liver and intra-abdominal fat mass, adipocyte morphology, serum analysis, femur and lumbar vertebras density by dual-energy X-ray absorptiometry and computed tomography were determined. Differences were considered significant with $P < 0.05$.
Results C group showed the following: lower liver (−12%) and intra-abdominal fat mass (−19%) area of adipocyte (−60%), cholesterol (−33%), insulin (−22%), lower total body (−9%) and spine (−33%) bone mineral content and bone area (−7 and −24%, respectively), femur mass (−9%), width of the diaphysis (−6%), femur (−10%) and lumbar vertebrae bone mineral density (−9%), and radiodensity of femoral head (−8%).
Conclusions The lower intra-abdominal adiposity could have more beneficial effects in a short term, since it can be associated with a better insulin sensitivity and lipid profile, than the small reduction in femur and lumbar vertebra density. However, it has to be considered the incremental effect of this reduction along the aging process.

Keywords Canola oil · Soybean oil · Adipocyte · Bone · Dual-energy X-ray absorptiometry · Computed tomography · Rats

Abbreviations

EFA	Essential fatty acids
SFA	Saturated fatty acids
PUFA	Polyunsaturated fatty acids
LA	Linoleic acid
ALA	Linolenic acid
S	Control group fed with diet containing 7 mL soybean oil/100 g
C	Experimental group fed with diet containing 7 mL canola oil/100 g
AIN	American Institute of Nutrition
CT	Computed tomography
DEXA	Dual-energy X-ray absorptiometry
BMD	Bone mineral density
BMC	Bone mineral content
HE	Hematoxylin–eosin
LV	Lumbar vertebra
CT	Computed tomography
HU	Hounsfield units
PPARα	Peroxisome proliferator-activated receptor alpha
SREBP-1c	Sterol regulatory element-binding protein
MUFA	Monounsaturated fatty acids

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Introduction

Fat has been traditionally regarded as an important calorie-dense nutrient and as a source of essential fatty acids (EFA). Fat and especially the EFA have been increasingly recognized as major biological regulators [1]. Organizations including the United States Department of Agriculture, American Heart Association, and National Academy of Sciences/Institute of Medicine have made dietary recommendations that focus not only on the quantity but also on the type of fats in the diet [2]. Dietary recommendations often advise to reduce the saturated fatty acids (SFA) intake and maintain or increase the intake of polyunsaturated fatty acids (PUFA) [3]. PUFA contains essential fatty acids, such as linoleic acid (LA, 18: 2n-6) and alpha linolenic acid (ALA, 18: 3n-3), and both are necessary every day, because they cannot be synthesized in the body [4, 5].

LA represents 80% of total PUFA energy in the United States and Brazil, by soybean oil intake, while ALA represents about 10%, and it is widely accepted that a large intake of ALA contributes to human health [4–9]. Canola oil, when compared to soybean oil, is characterized by a very low level of SFA (7% vs. 15%) and LA (21% vs. 54%). It contains 11% of ALA (vs. 8%) and a better balance of n-6 and n-3 fatty acid (n-6/n-3 1.90 vs. 6.75) [9]. However, canola oil represents less than 4% of the fat intake in American and Brazilian populations [6, 7].

Although the beneficial effects of polyunsaturated fatty acids intake, decreasing plasma cholesterol level and reducing the risk of coronary heart disease, have been extensively studied, the role of dietary fats on adipose tissue and bone growth has only emerged recently as an interesting area of research [10–17]. Adipocytes and osteoblasts were derived from a common progenitor—the mesenchymal stem cell. Extensive epidemiological data show that adipose tissue might influence bone density, through stresses caused by mechanical loading. And by indirect action, it influence bone through the production of leptin and adipokines and through the regulation of bone-active hormones as insulin [18, 19]. In addition, a higher ratio of n-6/n-3 fatty acids is associated with deleterious effects on bone health, while a lower ratio is associated with healthy bone properties [20]. These PUFA were acting directly on preadipocytes and increasing the rate of replication and/or differentiation, mainly in early stages of adipose tissue development [12, 21].

The positive action of adipose tissue on bone health has been related in several obesity or high-fat diet intake studies [22–25]. To the best of our knowledge, there are no studies about experimental model young rats fed with diet containing canola oil on bone metabolism. Than, the aim of this study was to compare the effects of feeding young adult animals, after weaning, with normal-fat diets containing higher or lower n-6/n-3 ratio, provided by soybean or canola oil, respectively, on body composition.

Materials and methods

Animals and diets

The protocol to use and handling the experimental animals was approved by the Ethical Committee of the Biology Institute of the State University of Rio de Janeiro, which based their analysis on the principles adopted and promulgated by the Brazilian Law that concerns the rearing and use of animals in teaching and research activities in Brazil [26].

Wistar rats were kept in a room with controlled temperature (23 ± 1 °C) and with an artificial dark–light cycle (lights on from 0700 to 1900 hours). Virgin female rats (3 months old) were caged with male rats, and after mating, each female was placed in an individual cage with free access to water and food. Within 24 h of birth (day 0), excess pups were removed, so that only six male pups were kept per dam. This procedure maximizes lactation performance [27]. During 21 days of lactation, rat dams were continued on an ad libitum diet of standard laboratory food (Nuvilab®, Paraná, Brazil).

Male Wistar rats were randomized and divided on postnatal day 21, from different litters, to receive a diet containing either 7 mL of soybean (S control group, $n = 10$) or canola oil (C group, $n = 10$), 20 g of casein, and 54 g of cornstarch/100 g. The groups received the same amounts of vitamins and minerals per gram of diet (Table 1). The diets were manufactured each 7 days and stored in pellets at 4 °C in agreement with American Institute of Nutrition (AIN-93G) recommendations [28, 29]. The animals had free access to diet and water during the course of experimental period. Food intake (g), body mass (g), and length (cm, measured as the distance from tip of the nose to the tip of the tail) were evaluated every 4 days.

Body composition analysis

At the end of the nutritional period, 60-days-old rats, after 8 h of fasting, were anesthetized with Avertin® (*Tribromoethanol*, 300 mg/kg) and subjected to dual-energy X-ray absorptiometry (DEXA) [30–32], using a Lunar DEXA 200368 GE instrument (Lunar, Wisconsin, USA) with specific software (encore 2008. Version 12.20 GE Healthcare). The evaluation was blind, since the DEXA technician did not know the experimental protocol. Total lean (g), fat mass (g), trunk fat mass (g), and bone analysis (bone mineral density—BMD (g/cm^2); bone mineral

Table 1 Composition of experimental diets

Ingredient (g/100 g)	S	C
Casein	20	20
Cornstarch	52.95	52.95
Sucrose	10	10
Soybean oil	7	–
Canola oil	–	7
Fiber	5	5
AIN-93G mineral mix	3.5	3.5
AIN-93 vitamin mix	1	1
L-Cystine	0.3	0.3
Choline bitartrate	0.25	0.25
Energy		
kJ/g	19.7	19.7
kcal/g	4.7	4.7
Protein (% of energy)	17	17
Carbohydrate (% of energy)	65	65
Fat (% of energy)	17	17

Formulated to meet the American Institute of Nutrition AIN-93G recommendation for rodent diets [28]

S control group fed with diet containing 7 mL/100 g soybean oil, and *C* experimental group fed with diet containing 7 mL/100 g canola oil

Casein; Mineral and Vitamin Mix; L-Cystine; Choline Bitartrate: Agroquímica®; Cornstarch: Cargill®; Fiber: Natural Pharma®; Soybean and Canola oil: Proquímios®; Commercial Sucrose: União®

content—BMC (g); and bone area (cm^2)) were measured for each rat.

The intra-abdominal fat depots and liver were dissected and weighed (g). For morphological analyses, samples of retroperitoneal fat were fixed in formaldehyde. The fixed tissues were embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin–eosin (HE). The sectional area of the adipocytes (μm^2) was determined on digital images acquired at random (TIFF format, 36 bit color, 1,360 × 1,024 pixels) with an Optronics CCD video camera system and Olympus BX40 light microscope and analyzed with the software Image-Pro Plus version 5.0 (Media Cybernetics, Silver Spring, MD, USA).

Serum analysis

Blood was collected by cardiac puncture after DEXA procedures. Samples were centrifuged, and serum was stored at −20 °C for posterior analysis of glucose, triglycerides, cholesterol, HDL-cholesterol, calcium, phosphorus, and albumin by colorimetric method (Bioclin, Belo Horizonte, MG, Brazil). Serum hormones concentrations were analyzed by RIA. Insulin kit (Linco Research, Inc., St Charles, MO, USA) determined an assay sensitivity of 0.1 ng/mL and a range of detection from 0.1 to 1.0 ng/mL, and the intra-assay CV was 8.5%. Leptin kit (Millipore, Billerica, MA, USA) determined an assay sensitivity of 0.5 ng/mL and the intra-assay CV of 4.8%.

Bone analysis

Right femur and lumbar vertebra (LV2–LV6) were collected and cleaned of soft tissue and preserved in saline solution (0.9% of NaCl) until analyzed. Bone dimension: the distance between epiphysis, the distance between the greater and lesser trochanter [33], the length of the entire LV2–LV6 [10] and LV4, and the medial point width of the diaphysis were measured using calipers with a readability of 0.01 mm. After drying overnight at 95 °C, femur and LV4 were weighed [34]. Before, the bones were submitted to DEXA and computed tomography (CT) analyses. In the same way for body composition, the DEXA or CT technician did not know about the experimental protocol.

Bone mineral density (BMD) in femur, LV2–LV6, and LV4 were determined by DEXA using a modification of previously described procedure [10]: In order to mimic soft tissue conditions, excised bones were fixed on constant volume of rice in a plastic container. After DEXA, bones were analyzed by a single-scan computed tomography (CT. Helicoidally model HISPEED, GE®). The images of femur and LV4 were obtained through axial cuts of thickness of 1 mm. The radiodensity (expressed as Hounsfield units, HU) of femoral head and vertebral body regions (R1 and R2, respectively, Fig. 1) were measured with a computerized analyzer software system (eFilm Lite, 2.0, 2003, Milwaukee, USA), by manual measurement Tool-Ellipse.

Statistical analysis

Statistical analyses were carried out using the Graph Pad Prism statistical package version 5.00, 2007 (San Diego, CA, USA). Food intake, body mass, and length were analyzed by two-way ANOVA, followed by Bonferroni post-test. The other data were analyzed by Student's *t* test. All results are expressed as means ± SEM with significance level of 0.05.

Results

During the nutritional period, food intake did not differ between groups. After 60 days, S and C groups showed similar body mass and length, although C group showed a slightly but significant higher body mass and length between 33–41 and 25–53 days, respectively (Fig. 2). The intra-abdominal fat mass and area of adipocytes were lower in C group (C: 4.39 ± 0.35 g vs. S: 5.45 ± 0.23 g and C: 241.60 ± 19.01 μm^2 vs. S: 611.90 ± 20.94 μm^2, $P < 0.05$ and $P < 0.0001$, respectively) (Fig. 3).

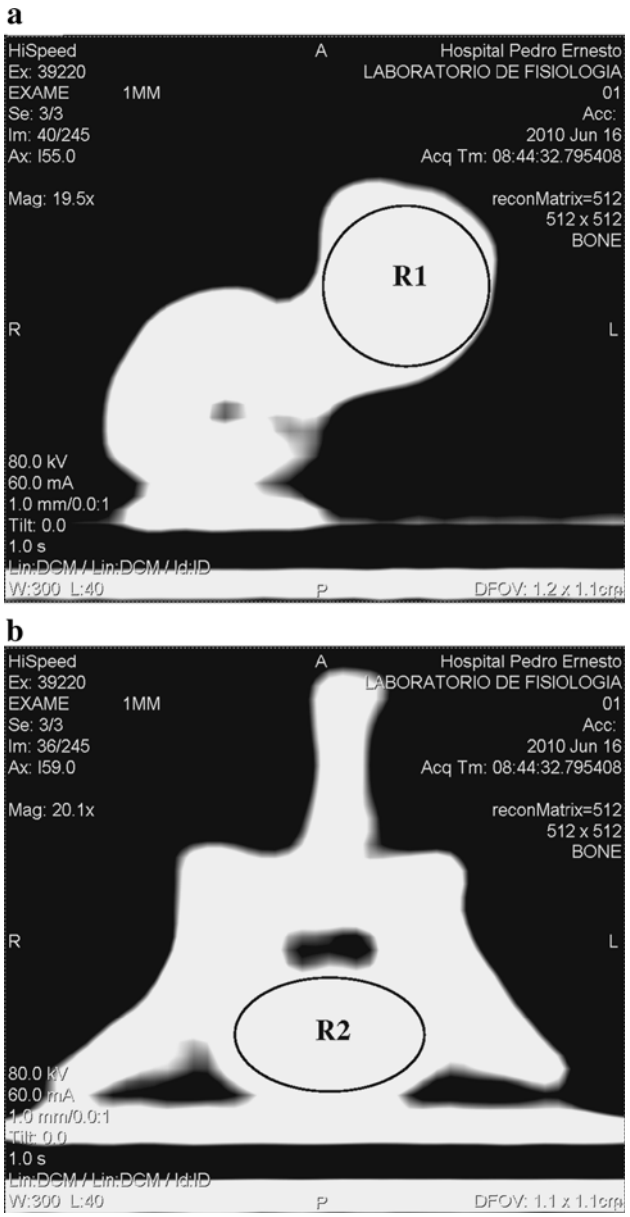

Fig. 1 Computed tomography image of femur (**a**) and lumbar vertebra (LV4, **b**). The regions of interest were shown by R1 (*femoral head*) and R2 (*vertebral body*)

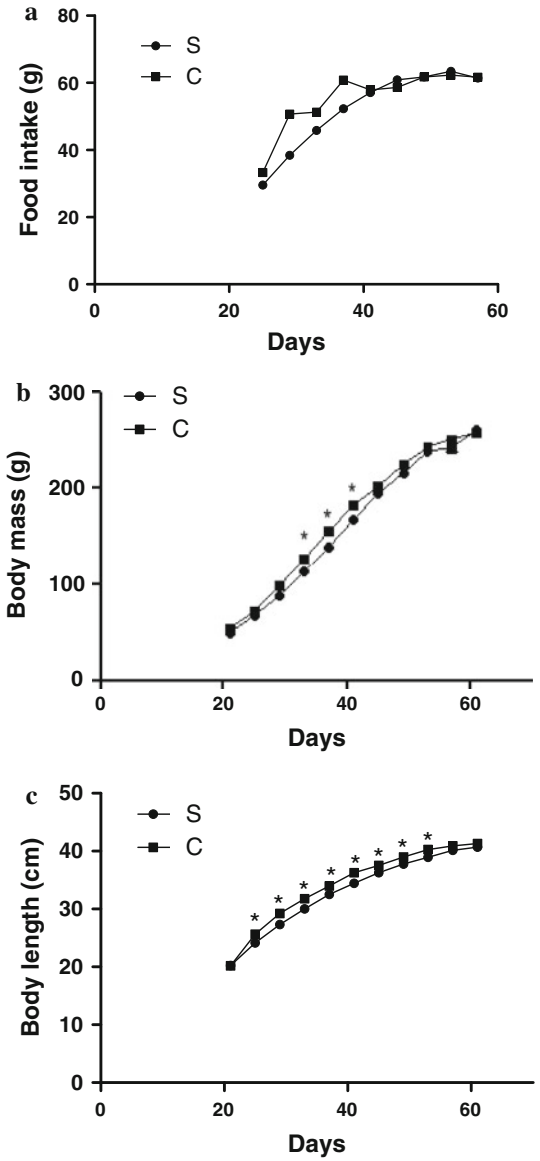

Fig. 2 Food intake (**a**), body mass (**b**), and length (**c**) postweaning until 60 days. Control group, fed with diet containing 7 mL of soybean oil (*filled circle*, S, $n = 10$) and experimental diet, containing 7 mL of canola oil (*filled square*, C, $n = 10$). Values are means. *Significantly different from the control group (two-way ANOVA, $P < 0.05$)

Serum analyses showed lower concentrations of cholesterol (−33%, $P < 0.05$) and insulin (−22%, $P < 0.05$) in C group, which has also the lower, but not different, concentrations of triglycerides (−15%). No differences were observed in glucose, HDL-cholesterol, calcium, phosphorus, albumin, and leptin (Table 2).

In regard to body composition, total lean, fat mass, trunk fat mass and body, and spine BMD did not differ between S and C groups. But C group showed lower values ($P < 0.05$) of body BMC (−9%), spine BMC (−33%), body bone (−7%), spine area (−24%), and liver mass (−12%, $P < 0.05$) (Table 3).

Bone measures and mineral density analyses showed lower femur mass (−9%), width of the diaphysis (−6%), BMD (−9%), and radiodensity of femoral head (−8%) in C group. No differences between the groups were found in the distance between epiphysis and that between trochanters (Table 4). The lumbar vertebra showed lower BMD of LV2–LV6 (−6%), LV4 (−9%), and LV4 mass (−6%) in C group, while no differences were found in LV2–LV6 and LV4 length and radiodensity of LV4 vertebral body (Table 5).

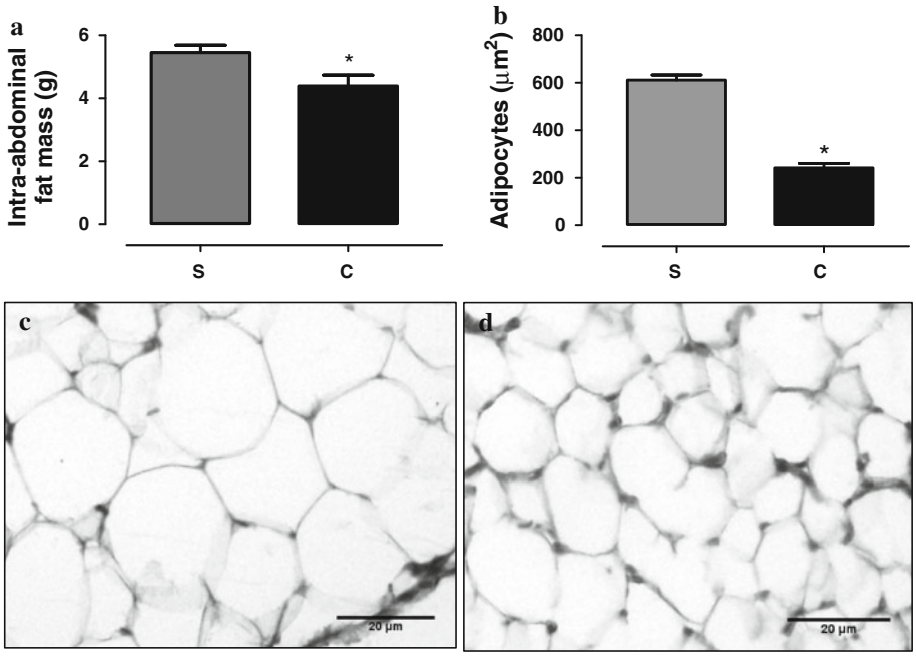

Fig. 3 Intra-abdominal fat mass (**a**) and adipocytes morphometry (sectional area, **b**), after 60 days. Control group, fed with diet containing 7 mL of soybean oil (S, $n = 10$) and experimental diet, containing 7 mL of canola oil (C, $n = 10$). *Significantly different from the control group (Student's t test, $P < 0.05$). Photomicrographs of the adipose tissue staining with HE (original magnification 200×): (**c**) S group, usual aspect of adipocyte and (**d**) C group, lower adipocyte area

Table 2 Serum analyses, at 60 days

	S (n10)		C (n10)	
	Mean	SEM	Mean	SEM
Glucose, mg/dL	122.90	13.30	145.40	4.98
Triglycerides, md/dL	34.40	2.49	29.46	4.36
Cholesterol, mg/dL	69.39	6.47	46.40*	5.68
HDL-cholesterol, mg/dL	23.40	3.88	24.12	2.54
Calcium, mg/dL	9.05	0.47	9.13	0.20
Phosphorus, mg/dL	5.18	0.57	6.16	0.71
Albumin, g/dL	3.26	0.65	3.46	0.42
Insulin, μUI/mL	35.42	3.52	27.55*	0.87
Leptin, ng/mL	1.68	0.13	1.56	0.08

Postweaning groups fed with control diet, containing 7 mL of soybean oil (S, $n = 10$) or experimental diet, containing 7 mL of canola oil (C, $n = 10$), until 60 days

* Significantly different from the control group (Student's t test, $P < 0.05$)

Table 3 Body compartments analyzed by DEXA, at 60 days

	S (n10)		C (n10)	
	Mean	SEM	Mean	SEM
Total lean, g	169.10	4.71	154.80	6.28
Fat mass, g	69.50	1.70	68.60	1.72
Trunk fat mass, g	45.80	2.03	42.67	3.43
Body BMD, g/cm^2	0.11	0.01	0.11	0.01
Body BMC, g	6.58	0.08	5.95*	0.16
Body bone area, cm^2	54.80	0.51	51.00*	0.73
Spine BMD, g/cm^2	0.11	0.01	0.10	0.03
Spine BMC, g	1.47	0.07	0.97*	0.10
Spine area, cm^2	12.70	0.70	9.67*	0.86

Postweaning groups fed with control diet, containing 7 mL of soybean oil (S, $n = 10$) or experimental diet, containing 7 mL of canola oil (C, $n = 10$), until 60 days

* Significantly different from the control group (Student's t test, $P < 0.05$)

Discussion

To our knowledge, this is the first study to evaluate the effect of a substitution of soybean per canola oil on the rat diet, after weaning. Our results showed that canola oil diet was associated with a leaner intra-abdominal adiposity, lower body BMC, normal body BMD, but lower mass, and BMD of femur and lumbar vertebras. But the groups had similar food intake and body growth. These results demonstrate that despite protein, calcium, phosphorus, fat, and energy intake constant in groups, the polyunsaturated fatty acids composition of diets was decisive for the outcomes observed in this experimental model.

There has been a considerable interest in the role of amount and nature of fatty acids in the diet and body fat accumulation and adipogenesis [35, 36]. In the present study, the intake of diet containing canola oil contributed to lower accumulation of intra-abdominal fat mass, associated with lower adipocytes area. The lowest n-6 intake prevents the formation of mature adipocytes and hypertrophy [37]. The n-3 intake inducing the fatty acid oxidation genes through PPARα (Peroxisome proliferator-activated receptor alpha) and the suppression of lipogenic genes through SREBP-1c

Table 4 Femur mass, dimensions, bone mineral density (BMD), and radiodensity of femoral head, at 60 days

	S (*n*10)		C (*n*10)	
	Mean	SEM	Mean	SEM
Femur, mg	404.30	6.33	365.90*	7.61
Distance between epiphysis, mm	34.42	0.11	33.77	0.09
Distance between trochanters, mm	9.24	0.08	9.18	0.08
Width of the diaphysis, mm	4.36	0.05	4.11*	0.06
BMD, g/cm^2	0.114	0.022	0.103*	0.002
Femoral head, Hu	782.90	10.22	720.70*	9.54

Postweaning groups fed with control diet, containing 7 mL of soybean oil (S, $n = 10$) or experimental diet, containing 7 mL of canola oil (C, $n = 10$), until 60 days

* Significantly different from the control group (Student's t test, $P < 0.05$)

Table 5 LV2-LV6 and LV4 dimensions, bone mineral density (BMD), and radiodensity of LV4 vertebral body, at 60 days

	S (*n*10)		C (*n*10)	
	Mean	SEM	Mean	SEM
LV2–LV6:				
Length, mm	35.00	3.10	35.17	2.90
BMD, g/cm^2	0.130	0.001	0.121*	0.003
LV4:				
Mass, mg	119.50	21.10	111.60*	23.68
Length, mm	7.25	0.06	7.32	0.03
BMD, g/cm^2	0.106	0.001	0.096*	0.003
Vertebral body, Hu	742.40	16.72	698.10	34.87

Postweaning groups fed with control diet, containing 7 mL of soybean oil (S, $n = 10$) or experimental diet, containing 7 mL of canola oil (C, $n = 10$), until 60 days

* Significantly different from the control group (Student's t test, $P < 0.05$)

(Sterol regulatory element-binding protein) [38] decreased the size of adipocyte. These pathways help to explain the decrease in intra-abdominal fat mass and adipocyte area, because canola oil when compared to soybean oil is characterized by a low level of n-6 (21% vs. 54%, respectively) and great level of n-3 (11% vs. 8%, respectively) [9].

Additionally, the groups showed similar trunk fat. The substitution of soybean oil per canola oil confirmed that dietary fats do not increase body fat compartments equally [39–41]. Probably, associated with decreased visceral fat mass, the C group showed a compensatory increase in subcutaneous fat mass (that was not collected due to procedure difficulties). The fact could explain why the groups have no differences in serum leptin concentration, since the circulating leptin is positively correlated with body fat and body mass index in human subjects and rodents [42–44].

Mechanical stress is important in remodeling bone architecture and bone mass, through dynamic loads imposed by muscle and passive loads by whole body weight [25, 45]. We verified that S and C groups showed similar body lean and body fat mass. Although the effect of fat distribution on BMD is far from clear [46], the lower loads imposed by intra-abdominal fat mass, in group fed with diet containing canola oil, could be related with lower bone parameters verified.

The increase in intra-abdominal fat mass and adipocyte size is associated with peripheral insulin resistance and dyslipidemia. Those are related to lipid metabolism, including triglycerides storage and fatty acids release, participating in ectopic accumulation of lipids in liver [47, 48]. In present study, rats fed with canola oil showed lower serum insulin and cholesterol concentrations associated with a lower intra-abdominal adiposity. Our results corroborate with the literature that suggests a benefic effect of diets enriched in polyunsaturated fatty acids, such as α-linolenic acids, to reduce serum cholesterol [14, 15, 49, 50]. Canola oil is an important source of oleic acid (18:1n-9), a monounsaturated fatty acid (MUFA), when compared to soybean oil (61% vs. 23%, respectively) [9]. It is known that high-MUFA diets reduce cholesterol without reducing HDL-cholesterol or increasing serum triglycerides [51]. Thus, the fatty acids composition of canola oil could explain the health lipid profile and the lower liver mass that could be related with lower accumulation of lipids, preventing the development of nonalcoholic fatty liver disease. By the other hand, it seems that canola oil also prevents intra-abdominal adiposity due to decreased insulin serum concentrations. But, these effects of canola oil require more studies.

Insulin is a potent regulator of bone growth, based on the fact that osteoblasts have insulin receptors, and in vitro, insulin directly stimulates osteoblasts proliferation [46, 52]. Contrarily, insulin deficiency is associated with a decreased bone formation, bone mass, and BMD [53]. The present study has not any data to confirm the osteoblasts activity, but, the lower serum insulin concentration observed in C group, associated with the lower loads imposed by intra-abdominal fat mass, contributed to impair mass and BMD of femur and lumbar vertebras.

The DEXA is considered the ultimate reference method for body composition determination and has been used successfully in studies of the whole body and regional bone in rats [30–32]. Using this technique, we verified that the lower n-6/n-3 ratio induced lower total body bone area and body BMC, which is reflected in the lower spine area and spine BMC. Bone characteristics are represented by two factors: the volumetric development that is associated with the skeleton area and the calcium storage determining the BMD [54]. However, in this study, S and C groups did not

differ for body BMD and spine BMD. It was necessary to examine specific bones, because there are several reports of regional differences [8, 10, 18, 33, 34].

Mineral density increases predominantly at trabecular site (i.e., vertebral body) than at cortical site (i.e., proximal femur), and these regional differences can be explained by bone remodeling [55]. However, when we evaluated each bone individually, we found that canola oil intake promotes a similar trend in femur and lumbar vertebra, with low body and regional bone development. Additionally, canola oil induces a lower femur mass, width of the diaphysis and LV4 mass, although both groups have similar body mass and length. The proximal femur radiodensity evaluated by CT, in the canola oil group, is compatible with the DEXA results, both showing a reduction, while the vertebral body was similar between the groups. These analyses indicate that the use of CT as method of bone density measuring allowed a better differentiation between compartments of lumbar vertebra, not possible for DEXA technique. In addition, if the radiodensity of vertebral body and femoral head of C group are compared, it corroborates with the well-known definition of higher remodeling rate in predominantly trabecular sites [55].

The mechanism of action of PUFA on adipocytes and bone growth is complex and involves several signaling pathways [56, 57]. Nevertheless, the present experimental model corroborates the hypothesis of association between body fat mass and bone density. In the same way, a recent study demonstrated that low body fat content is associated with low BMD, in growing children and adolescents [58]. Apparently, the lower intra-abdominal adiposity could have more beneficial effects in a short term, since it can be associated with a better insulin sensitivity and lipid profile, than the small reduction in femur and lumbar vertebra density. However, it has to be considered the incremental effect of this reduction along the aging process. Further studies evaluating bone strength are required to elucidate whether those changes can be deleterious.

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