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ORIGINAL ARTICLE

Body adiposity and bone parameters of male rats from mothers fed diet containing flaxseed flour during lactation

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Obesity and osteoporosis may have their origins in early postnatal life. This study was designed to evaluate whether flaxseed flour use during lactation period bears effect on body adiposity and skeletal structure of male rat pups at weaning. At birth, male Wistar rats were randomly assigned to control and experimental (FF) groups, whose dams were treated with control or flaxseed flour diet, respectively, during lactation. At 21 days of age, pups were weaned to assess body mass, length and composition by dual-energy X-ray absorptiometry. The animals were then sacrificed to carry out analysis of serum profile, intra-abdominal adipocyte morphology and femur characteristics. Differences were considered significant when $P < 0.05$. The FF group displayed the following characteristics ($P < 0.05$): higher body mass, length, bone mineral content, bone area and concentrations of osteoprotegerin, osteocalcin and high-density lipoprotein cholesterol; higher levels of stearic, α-linolenic, eicosapentaenoic and docosapentaenoic acids and lower levels of arachidonic acid and cholesterol; smaller adipocyte area; and higher mass, epiphysis distance, diaphysis width, maximal load, break load, resilience and stiffness of femur. Flaxseed flour intake during lactation period promoted adipocyte hypertrophy down-regulation and contributed to pup bone quality at weaning.

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Key words: adiposity, bone, diet, flaxseed, lactation, rat

Introduction

Early postnatal life is the period of maximal physical development,[1] where the mother's milk is the primary nutrition source. Changes in milk nutrients pose risk to future metabolic disturbances.[2] Thereby, interests have focused on the influence of lipid consumption during breastfeeding on child growth and its effects on obesity development and osteoporotic fracture origins.[3]

Adipose tissue and osteoblasts come from a common progenitor – the mesenchymal stem cell.[4] The effects of fat tissue on bone health are far from clear: investigators have suggested that fat mass may or may not be associated with bone mass, directly via mechanical loading and indirectly via hormonal production by adipocytes.[5,6] Clinical and experimental studies have reported on the importance of α-linolenic acid (ALA, 18:3n-3)-rich diets for adiposity accumulation prevention and promotion of bone formation. Conversely, linoleic acid (LA, 18:2n-6) contributes to bone reabsorption and adipocyte hypertrophy.[7,8]

In this context, the flaxseed (*Linum usitatissimum*) has been described as an excellent ALA source, presenting an average of 30% lipids in its composition, with 51–55% corresponding to ALA.[9,10] Furthermore, because of the presence of dietary fiber, high-quality proteins, antioxidants and minerals, a number of which offer synergistic health benefits and are part of basic nutrition, flaxseed is included in the following categories: functional foods and bioactive food.[11,12] In previous experimental models, our group evidenced protective effects of diet containing 25 out of 100 g flaxseed flour on glycemia, cardiovascular risk and lipid profile reduction.[13–15] Nevertheless, little data are available on flaxseed flour effects on the relationship between adipose tissue and bone development during early life stages.

Thus, this study was designed to evaluate whether maternal flaxseed use during lactation period has effects on body adiposity and skeletal structure of male rat pups at weaning.

Materials and methods

The protocol used for dealing with experimental animals was approved by the Ethics Committee on Animal Research of the Federal Fluminense University, Niteroi-RJ, Brazil (protocol 209/2012). All the procedures were performed in accordance with the Brazilian Society of Laboratory Animal Science and the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes

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of Health (NIH Publication No. 85-23, revised in 1996) provisions.

Wistar rats were maintained in a temperature-controlled (23 ± 1°C) and humidity-controlled (60 ± 10%) room, with artificial dark–light cycle (lights on from 7 am to 7 pm). Virgin female rats were caged with male rats (3 months old, respectively). After mating, each female was placed in an individual cage with free access to water and standard laboratory food (Nuvilab®, Paraná, Brazil).

Within 24 h of birth, excess pups were removed, and only six male pups were retained per dam, which maximized lactation performance.[16] During the lactation period, pups were randomly assigned to the following groups: control (C, $n = 12$), whose dams were fed control diet containing 20 out of 100 g casein; experimental (FF, $n = 12$), whose mothers were fed diet containing 25 out of 100 g flaxseed flour. During 21 days of lactation, free access to water and diets was not evaluated, owing to difficulties in controlling pup food intake, especially in the 14–21-day period. Diets were manufactured and stored as pellets at 4°C in agreement with American Institute of Nutrition (AIN-93G) recommendations for rodent diets.[17] The amount of flaxseed flour included – 25 out of 100 g – aimed to meet the entire recommended fiber intake, and it was not necessary to add oil as flaxseed seed comprises a source of this component (Table 1).

Table 1. *Composition of experimental diets*

Ingredients (g/100 g)	C	FF
Flaxseed flour	–	25.0
Casein	20.0	15.0
Cornstarch	52.9	45.0
Sucrose	10.0	10.0
Soybean oil	7.0	–
Cellulose	5.0	–
AIN-93G mineral mix	3.5	3.5
AIN-93 vitamin mix	1.0	1.0
L-Cystine	0.3	0.3
Choline bitartrate	0.25	0.25
Tert-butylhydroquinone (mg)	14.0	14.0
Protein (% of energy)	17.0	17.0
Carbohydrate (% of energy)	54.0	49.0
Fat (% of energy)	7.0	7.0
Energy (kcal)	347.2	327.0

C, control diet; FF, flaxseed flour (experimental group); AIN, American Institute of Nutrition.

FF experimental diet containing 25 out of 100 g flaxseed flour. Mineral and vitamin mix; L-cystine; choline bitartrate: PragSoluções®; casein; cornstarch; cellulose: FARMOS®; soybean: Lisa®; and sucrose: União®. Flaxseed flour: ArmaZen® with 17% protein, 45% carbohydrate and 26% fat. Formulated on AIN-93G recommendations for rodent diets.

At 21 days of age, the pups were weaned, and after 2 h of fasting body mass and length (cm, measured as the distance between nose tip and tail tip)[5,6] were evaluated. They were then anesthetized with Thiopentax (Sodium thiopental, 0.1 mg out of 100 g) and subjected to dual-energy X-ray absorptiometry (DXA)[18] using a Lunar DXA 200368 GE instrument (Lunar, with specific software encore 2008 version 12.20; GE Healthcare, Madison, WI, USA). The evaluation was carried out in a blinded manner, as the DXA technician did not know the experimental protocol. Total lean mass (g), total fat mass (% and g), trunk fat mass (g) and bone analysis [bone mineral density (BMD, g/cm^2); bone mineral content (BMC, g/cm^2); total bone area (cm^2)] were measured for each rat.[6,19]

Blood was collected by cardiac puncture following DXA procedures. Samples were centrifuged, and the serum samples were stored at −80°C for later analysis. Concentrations of osteoprotegerin (OPG), osteocalcin and leptin (ng/ml, respectively) were measured using multiplex assay kits (Millipore, Billerica, MA, USA). Concentrations of cholesterol, high-density lipoprotein (HDL)-cholesterol and triglycerides (mg/dl, respectively) were measured by colorimetric method (Bioclin BS-120; Bioclin, Belo Horizonte, MG, Brazil).

The determination of fatty acid composition by gas chromatography was carried out using serum samples. Derivatization of lipid extract was performed according to AOAC Official Methods 996.06 with some modifications.[20] Aliquots of 0.2 ml from each serum sample were added to a screw-cap test tube and 5 mg pyrogallic acid, 0.025 ml standard (5 mg/ml tritridecanoin C13:0 in chloroform), 0.1 ml ethanol, 0.5 ml HCl 8.3 M and a number of glass beads were added. The tubes were placed in a water bath at 75°C for 40 min and then cooled at room temperature. Subsequently, 1 ml ethylic ether and 1 ml petroleum ether were added, and the samples were centrifuged at 10,000 rpm for 5 min. The top phase was transferred to another tube and ether was evaporated under N_2 in a water bath (below 40°C). Methylation was performed by adding 1 ml BF_3 (7% in methanol) and 0.5 ml toluene and subsequent boiling at 100°C for 45 min. After cooling at room temperature, 2.5 ml water, 1 ml hexane and ~0.5 g Na_2SO_4 anhydrous were added. The tubes were left to rest to allow phase separation, and then the top phase was transferred to a vial containing anhydrous Na_2SO_4 and evaporated under N_2. Before injection into the chromatograph, 0.1 ml hexane was added to each sample. Samples were analyzed using gas chromatography on a GC 17A (Shimadzu) equipped with a flame-ionization detector, automatic injector AOC-20 and a Workstation Class GC10. Fatty acid separation was achieved using a fused-silica column SP-2560 (bis-cyanopropyl polysiloxane) (100 m × 0.25 mm × 0.2 μm; Supelco, Bellefonte, PA, USA). The column temperature was programmed as follows: 140°C for 5 min; heating at 4°C/min up to 240°C; and 240°C for 30 min. The injector and detector were maintained at 250°C, and helium was used as the carrier gas at a flow rate of 1 ml/min. The split ratio was 1/10. Two microliters of derivatized lipid extract were injected, and the fatty acid methyl ester peaks were identified by comparison of retention times of fatty acid methyl ester standards and the chromatograms viewed using the Ce 1h-05 methods.[21]

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Intra-abdominal adipose tissue samples were dissected and weighed (g). For morphological analyses, retroperitoneal fat samples were collected and fixed in buffered formaldehyde (mesenteric and epididymal fat were not analyzed because of procedural difficulties). Tissues were embedded in paraffin, cut into 5-μm sections and stained with hematoxylin–eosin. For morphometric analyses, profiles with at least 100 adipocytes were randomly selected and captured for each animal. Sectional adipocyte area (μm^2) was determined based on digital images acquired (TIFF format, 36 bit color, 1360 × 1024 pixels) with an Optronics CCD video camera system and Olympus BX40 light microscope, analyzed using the U.S. National Institutes of Health IMAGE-J software (http://rsbweb.nih.gov/ij/).[5,6]

The right femur was collected and cleaned of soft tissue, and was preserved in −20°C for later analysis. Bone dimension – distance between the epiphysis (mm, distance between great trochanter and lateral condyle) and middle-point diaphysis width (mm) – was measured using calipers with a readability of 0.01 mm. After drying overnight, the femur was weighted (g) using an analytical balance (Sartorius TE214S; Sartorius, Chicago, IL, USA).[12]

Femur biomechanical properties were measured using the three-point bending test by means of a universal test machine (Instron model 4444; Instron, Canton, MA, USA), with a load cell of 100 kgf capacity. The extremities of the bone were supported on two rollers with 3-mm diameter and a distance of 21.70 mm. The load was applied to each bone's central region.[22] At the beginning of the test, a 10 N pre-load was applied in the posterior–anterior direction (perpendicular to longitudinal axis) to stabilize the femur. After 1-min accommodation and stabilization period, force was applied likewise, with constant velocity of 0.5 cm/min up to fracture instant. As a result of the force applied on the femur, Instron software (series IX) generated a load strain graph; in this graph, the main biomechanical properties were obtained: maximal load (higher load withstood by the femur, kN), maximal deformation (mm), break load (the load that fractured the bone, kN), break deformation (mm), resilience (J), tenacity (J) and stiffness (N/mm).

Statistical analyses were performed using GraphPad Prism statistical package (version 5.0, 2007 San Diego, CA, USA). The results were analyzed using Student's *t*-test. All results are expressed as means ± S.E.M. with significance level of $P < 0.05$.

Results

The body mass of pups was similar between groups at birth. However, pups whose dams were treated with diet containing flaxseed flour showed higher body mass and length (+8 and +6%, $P < 0.05$, respectively) at 21 days of age. Body composition analyzed by DXA showed total lean mass, total fat mass, trunk fat mass and BMD to be similar between groups. BMC (+42%, $P < 0.05$) and bone area (+33%, $P < 0.05$) were higher in the FF group (Table 2).

Table 2. *Body mass and length and dual-energy X-ray absorptiometry analysis at 21 days of age*

	C		FF	
	Mean	S.E.M.	Mean	S.E.M.
Body mass (day 0) (g)	6.48	0.10	6.44	0.10
Body mass (day 21) (g)	54.42	1.79	59.04*	0.36
Length (day 21) (cm)	18.79	0.22	19.96*	0.22
Total lean mass (g)	32.83	1.61	35.50	1.04
Total fat mass (%)	30.40	2.51	30.51	1.93
Total fat mass (g)	14.42	1.30	15.67	0.94
Trunk fat mass (g)	5.36	0.72	6.09	0.91
Total BMD (g/cm^2)	0.05	0.00	0.06	0.00
Total BMC (g)	0.64	0.03	0.91*	0.06
Total bone area (g/cm^2)	11.10	0.45	14.83*	1.09

C, control group; FF, flaxseed flour (experimental group); BMD, bone mineral density; BMC, bone mineral content.
Control group ($n = 12$) and experimental group ($n = 12$), whose dams were treated with control diet or 25 out of 100 g flaxseed diet, during lactation period, respectively. Values are means with their standard errors of mean.
*Significantly different from the control group (Student's *t*-test, $P < 0.05$).

Regarding serum analyses, the FF group showed higher concentrations of OPG ($P < 0.05$), osteocalcin ($P < 0.05$), HDL-cholesterol (+8%, $P < 0.05$) and lower concentrations of cholesterol (−12%, $P < 0.05$). Triglyceride and leptin concentrations were similar between groups. Concentrations of stearic, ALA, eicosapentaenoic (EPA) and docosapentaenoic acids were higher ($P < 0.05$), whereas arachidonic acid (AA) concentration was lower ($P < 0.05$) in the FF group (Table 3).

Intra-abdominal fat mass was similar between groups. However, the FF group showed lower adipocytes area (−40%, $P < 0.05$, FF: 1471 ± 175 *v.* C: 2486 ± 252 μm^2) (Fig. 1).

Femur measures showed higher mass (+27%, $P < 0.05$), distance between the epiphysis (+4%, $P < 0.05$) and diaphysis middle-point width (+11%, $P < 0.05$) in the FF group. Biomechanical characteristics such as maximal deformation, break deformation and tenacity were similar between groups. Meanwhile, maximal load (+44%, $P < 0.05$), break load (+71%, $P < 0.05$), resilience (+40%, $P < 0.05$) and stiffness (+33%, $P < 0.05$) were higher in the FF group (Table 4).

Discussion

A limitation of the present study lies in the fact that we were not able to evaluate flaxseed flour effects on gender. Nevertheless, we have observed that male pups whose dams were treated with diet containing flaxseed flour during the lactation period display higher BMC, bone area, bone quality and lower intra-abdominal adipocytes area at weaning. Probably, fatty acid profile in serum was a determining factor to outcomes regarding adiposity and bone structure.

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Table 3. *Serum hormonal, biochemical and fatty acids analysis at 21 days of age*

	C		FF	
	Mean	S.E.M.	Mean	S.E.M.
Osteoprotegerin (ng/ml)	1.19	0.38	7.55*	2.76
Osteocalcin (ng/ml)	88.01	12.0	223.4*	39.0
Leptin (ng/ml)	7.02	1.28	6.08	1.26
Cholesterol (mg/dl)	123.30	3.16	108.60*	2.97
HDL-cholesterol (mg/dl)	35.09	0.53	38.09*	0.95
Triglycerides (mg/dl)	75.62	6.54	86.44	7.45
Stearic acid (mg/100 ml)	63.7	5.07	118.65*	22.44
α-Linolenic acid (mg/100 ml)	3.26	0.59	28.22*	2.10
Arachidonic acid (mg/100 ml)	74.37	8.31	36.92*	5.92
Eicosapentaenoic acid (mg/100 ml)	1.46	0.27	25.74*	1.85
Docosapentaenoic acid (mg/100 ml)	3.20	0.37	10.03*	0.92

C, control group; FF, flaxseed flour (experimental group); HDL, high-density lipoproteins.

Control group ($n = 12$) and experimental group ($n = 12$), whose dams were treated with control diet or 25 out of 100 g flaxseed diet, during lactation period, respectively. Values are means with their standard errors of mean.

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

Table 4. *Mass, distance between the epiphysis, middle-point diaphysis width and biomechanical analysis of the right femur at 21 days of age*

	C		FF	
	Mean	S.E.M.	Mean	S.E.M.
Mass (g)	0.18	0.00	0.23*	0.00
Distance between epiphysis (mm)	0.70	0.00	0.73*	0.00
Middle-point diaphysis width (mm)	0.09	0.00	0.10*	0.00
Maximal load (kN)	0.09	0.01	0.13*	0.01
Maximal deformation (mm)	4.36	0.06	4.64	0.24
Break load (kN)	0.07	0.01	0.12*	0.01
Break deformation (mm)	4.77	0.11	4.81	0.23
Resilience (J)	0.005	0.00	0.007*	0.00
Tenacity (J)	0.11	0.01	0.12	0.01
Stiffness (N/mm)	33.58	1.81	44.81*	2.35

C, control group; FF, flaxseed flour (experimental group).

Control group ($n = 12$) and experimental group ($n = 12$), whose dams were treated with control diet or 25 out of 100 g flaxseed diet, during lactation period, respectively. Values are means with their standard errors of mean.

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

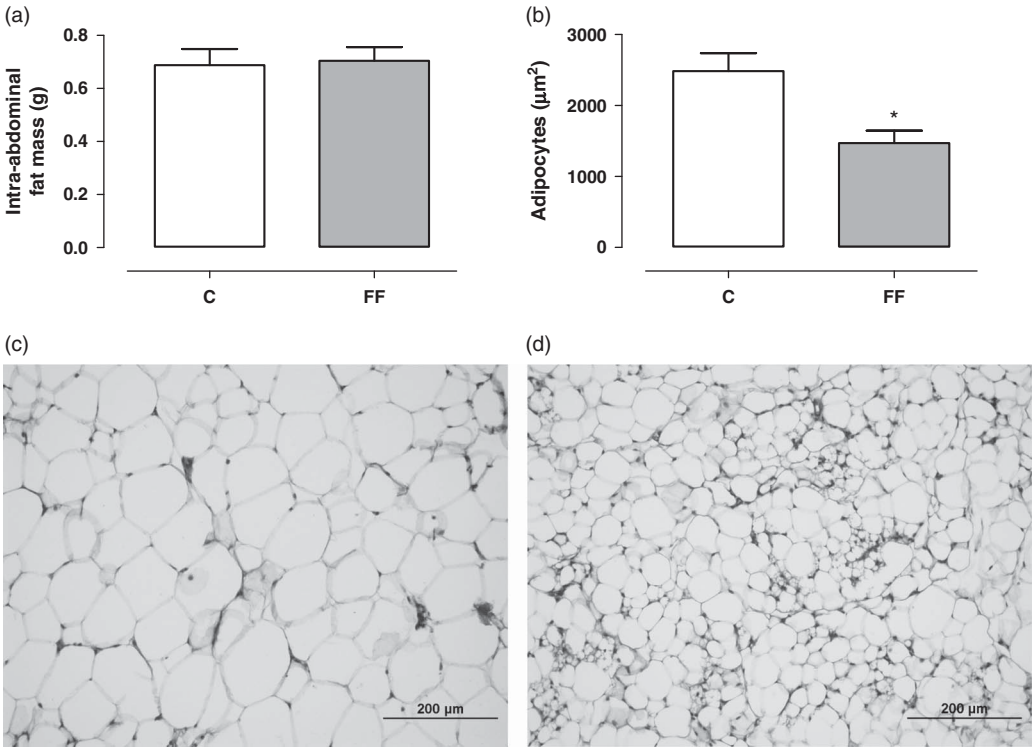

Fig. 1. Intra-abdominal fat mass (*a*) and adipocyte morphometry (*b*, sectional area) at 21 days of age. Control group (C, $n = 12$), whose dams were treated with control diet, and experimental group [flaxseed flour (FF), $n = 12$], whose dams were treated with diet containing 25 out of 100 g flaxseed flour, during lactation, respectively. *Significantly different from control group (Student's *t*-test, $P < 0.05$). Photomicrographs of adipose tissue (original magnification 200×). (*c*) Control group, usual adipocyte aspect and (*d*) experimental group, lower adipocyte area.

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Tinoco *et al.*[23] observed that ALA content in milk was associated with higher weight and length gain in premature infants. Although the present study has not assessed fatty acid composition in breast milk, we have observed that experimental diet contributes to higher body mass and pup length at weaning, because flaxseed is one of the richest plant ALA sources.[13–15]

The experimental and control diets provided the same energy percentage from fat, which justifies the similar body and intra-abdominal fat mass in both groups. However, as reported by McCullough *et al.*,[24] we observed that flaxseed intake significantly increased plasma and adipose ALA levels. Flaxseed flour, when compared with soybean oil (present in control diet), displays higher ALA levels and lower LA levels as well as LA/ALA ratio.[25] ALA is converted to EPA and docosahexaenoic (DHA) acid, which induces fatty acid oxidation genes through peroxisome proliferator-activated receptor alpha (PPARα), suppresses lipogenic genes through sterol regulatory element-binding protein (SREBP-1c) and decreases adipocyte size. However, LA is converted into AA, inducing mature adipocytes formation and hypertrophy.[5,6,26,27] These pathways help explain higher ALA, EPA and docosapentaenoic (DHA precursor) acid levels, lower AA in serum and lower intra-abdominal adipocyte area in the FF group.

In addition, the FF group showed higher serum stearic acid concentrations. Flaxseed is source of this saturated fatty acid.[28] Dietschy *et al.*[29] and Pearson *et al.*[30] have suggested that stearic acid bears neutral or even reduces cholesterol. Regarding HDL-cholesterol, some studies have not shown any improvement;[12,19] however, Daleprane *et al.*[13] and Pacheco *et al.*[10] have observed higher HDL profile after flaxseed intake. Experimental studies have reported that cholesterol interferes directly in osteoblast differentiation, by decreasing bone formation and increasing osteoclast bone resorption.[31,32] Meanwhile, Jeong *et al.*[33] have found a positive correlation between HDL-cholesterol and BMD, favoring bone formation. In this study, low cholesterol and high HDL-cholesterol levels may have had some relation with bone parameters, determined in the FF group at weaning, thus requiring further study.

Flaxseed flour diet, during the lactation period, contributed to higher BMC and bone area in the FF group at weaning. Diets containing high ALA levels are associated with receptor activator of nuclear factor kappa beta ligand (RANKL) down-regulation, lower osteoclast maturation and bone resorption. Moreover, such diets preserve bone mass by increasing the expressions of OPG and osteocalcin (as observed in the present study), enhancing pre-osteoblasts differentiation into mature osteoblasts and bone formation.[7,8,34]

DXA is considered a useful reference method for body composition determination, having been successfully employed in whole-body and regional bone studies in rats.[35] In order to complement bone analyses, we observed higher distance between epiphysis, width of diaphysis and femur mass in the FF group. Furthermore, we submitted the femur to three-point bending test in order to assess bone strength. There are only a few studies about bone quality in rats at weaning. Ward *et al.*[36] have observed that male rats treated with 10% flaxseed diet during lactation showed bone strength similar to our control group. In the present study, 25% flaxseed flour diet was associated with higher femur dimension and biomechanical strength improvement, which suggests resistance to fracture.

In short, our data evidence that flaxseed flour intake during the lactation period promotes adipocyte hypertrophy down-regulation and contributes to the bone quality of pups at weaning. Nevertheless, further studies are necessary to clarify flaxseed flour role in early stages of life and its effects on obesity and osteoporotic fracture prevention during adult life stage.

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Conflicts of Interest

None.

Ethical Standards

The protocol used to deal with experimental animals was approved by Ethics Committee on Animal Research of Fluminense Federal University, Niteroi-RJ, Brazil (protocol 597/2014). All procedures are in accordance with the provisions of Brazilian Society of Science and Laboratory's Animals and the Guide for the Care and Use of Laboratory Animals.

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