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Flaxseed oil intake in post weaning period contributes to bone health of lactating rats

Carolina Ribeiro Pessanha[1*], Bianca Ferolla da Camara Boueri[2], Aline D' Avila Pereira[3], Danielle Cavalcante Ribeiro[4], Gilson Teles Boaventura[5], Carlos Alberto Soares da Costa[6]

[1-6] Laboratory of Experimental Nutrition, Department of Nutrition and Dietetics, Fluminense Federal University, Niterói, RJ, Brazil

Abstract
The aim of this study was to evaluate the effect of flaxseed oil on the composition and bone quality of lactating *Wistar* rats in the postweaning. *Wistar* rats females were randomly separated into control (C) and experimental (OL) groups. Group C received a control diet, during the 21-day lactation period and during 30 days after weaning. The OL group, as group C, continued the same period of study, however receiving an experimental diet with flaxseed oil in the composition. The end of the experiment, the animals were anesthetized, and were evaluated the bone composition through Dual-energy X-ray absorptiometry (DXA) and after, euthanasia was done. The femurs were collected for the analysis of bone composition by DXA, analysis of bone dimensions, computed tomography and biomechanical test. Higher values were found in the OL group in relation to total bone mineral content, serum calcium, bone mineral density of the femur, femoral mass, distance between the epiphyses and distance at the midpoint of the diaphysis of femurs. In the biomechanical test and tomography, higher values were found in the OL group when compared whit C group. In conclusion, fatty acid composition of flaxseed oil was able to promote positive effects in bone composition of female's rats in the post weaning period.

Keywords: rats, flaxseed, post weaning

Introduction
Pregnancy and lactation induce changes in women´s body that can have impact in later periods of life [1-3]. Several studies report adaptive physiological processes associated to bone metabolism during postpartum and post-lactation period, in order to restore the balance of bone process like remodeling and resorption [4-6]. However, remains inconsistent the capacity of certain foods to promote an effective bone quality after those periods. Thus, some studies have been investigating foods and nutrients that are capable not only to modulate metabolic processes but also compensate an increasingly frequent eating imbalances in many societies [7, 8]. The high consumption of industrialized products and changes in lifestyle increasingly influence the food choices of populations, especially women. Thus, an imbalance among the nutrients consumed is frequent [9-11].
There is an association between the high consumption of industrialized products rich in saturated fats and omega 6 (linoleic acid n-3) and negative consequences on the organism. Studies have reported increases in the ratio of linoleic acid (C18:2 n-6) to alpha linolenic acid (C18:3 n-3) in the diet, associated with decrease fruit and vegetable intakes and increased use of refined oils [12-15]. The estimated ratio of ingestion of n-6: n-3 currently ranges from 10:1 to 20:1, daily amounts [16, 17]. Adapt the ratio of essential fatty acids in the diet is an effective way to balance the metabolic processes that are involved in bone physiology [18, 20]. Nutritional factors play an important role in skeletal health during aging and most of them have your origin in the phase adult young. Avoiding the development of bone fragility is one way to prevent bone diseases [21].
For the purpose of balance these physiological processes through Nutrition, we highlight the flaxseed, one of the foods currently studied, due to the functional properties and it is an excellent source of omega 3 [22-24]. Named also as flax or winter flax, flaxseed is an herbaceous plant belonging to the Linaceae family. This plant is widely used in human´s diet as seed, flour or oil [24, 25].
Experimental studies have already investigated the possible benefits related to flaxseed consumption in *Wistar* rats. Mainly regarding the metabolism of fats and influence in the gain of weights and size of adipocytes. However, most of these studies focused on the effects of flaxseed on male rats not investigating about the interactions in female rat's bone activity [26-28].
The main indicators for assessing bone health are the amount of tissue and bone mineral density, whether in humans or rats [29, 30]. A steady decrease in bone mass during lactation period may negatively affect bone health, since lower bone mass and density are associated with an increased risk of fracture caused by brittleness [21]. Studies have evaluated specific situations a certain commitment to normal pregnancy adaptation mainly the recovery of bone mass lost during breastfeeding [9, 31]. Situations and conditions such as shorter breastfeeding time, gestational age, multiparity and lifestyles [31-33]. Several experimental models were employed in order to evaluate the effects of flaxseed in bone physiology however [19, 26, 38], most of them were with male rats. Therefore, the aim of the present study was to evaluate the effect of diet containing flaxseed oil in the bone structure at the 30 days after postweaning period of lactating *Wistar* rats.

Material and Methods
Ethics statement
The protocol used to deal with experimental animals was approved by Ethics Committee on Animal Research of Fluminense Federal University, Niteroi-RJ, Brazil (protocol 887/2017). All procedures were in accordance with the Brazilian Society of Science and Laboratory Animals

provisions and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication N 85-23, revised in 1996).

Study design

Wistar rats from the Laboratory Animals Center of the Fluminense Federal University were housed in a temperature-controlled room (23 ± 1 ºC), humidity (60 ± 10%) with an artificial dark-light cycle (lights on from 7 am to 7 pm). 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 standard laboratory food (Nuvilab®, Paraná, Brazil). Within 24 h of birth, excess pups were removed so that only six were kept per mother. This procedure maximizes lactation performance. The dams were randomly separated into control (C, n = 6) and experimental (OL, n = 6) groups. Group C received a control diet, during the 21-day lactation period and during 30 days after weaning, totaling 51 days of experiment. The OL group, as group C, continued the same period of study, however receiving an experimental diet with flaxseed oil in the composition.

The diets presented the same amount of nutrients and were isocaloric [35]. Up to the 21st day of the experiment, lactation period of the rats was offered the version the feed diet based on AIN-93G, providing an adequate amount of nutrients for this physiological moment. During the 30 days of postweaning, the version of the AIN-93M diet was used, which is adequate for the maintenance period [36, 37]. The main difference between control and experimental ration was the lipid source offered and the adjustment in the percentage of proteins and minerals. The control ration contained soybean oil, while the experimental ration was flaxseed oil (Table 1).

Table 1: Diets Composition

Ingredients (g/100g)	AIN-93G		AIN-93M	
	C	OL	C	OL
Casein	20	20	14	14
Cornstarch	52.95	52.95	62.07	62.07
Sucrose	10	10	10	10
Soybean oil	7	-	4	-
Flaxseed oil	-	7	-	4
Cellulose	5	5	5	5
Mineral mix (AIN-93M)	-	-	3.5	3.5
Mineral mix (AIN-93G)	3.5	3.5	-	-
Vitamin mix	1	1	1	1
L-Cystine	0.3	0.3	0.18	0.18
Choline bitartrate	0.25	0.25	0.25	0.25
Tert-Butylhydroquinone	0.014	0.014	0.008	0.008

C, control diet; OL, flaxseed oil diet. Formulated based on the American Institute of Nutrition AIN-93G and AIN-93M recommendation for rodent diets. C and OL, fed a diet containing Casein, Mineral and Vitamin mix, L-cystine, Choline bitartrate, Pragsolucões®; Cornstarch, Cellulose, FARMOS®; Soya bean oil, Liza®; Sucrose, União®; Flaxseed oil: Giroil Agroindustria Ltda.

At the end of 51 days, the experimental rats were fasted for six hours and anesthetized by intraperitoneal injection of Thiopentax® at 5% (0.1 mg / 100g of body mass). Afterwards, they were submitted to the evaluation of bone composition by Dual-energy X-ray absorptiometry (DXA) equipament, Lunar IDXA 200368 GE instrument (Lunar, Wisconsin, USA). The analysis was performed with the aid of specific software (encore 2008. Version 12.20 GE Healthcare) for small animals. Bone mineral content (BMC), bone mineral density (BMD) and total bone area (AO) were analyzed in each rat.

After evaluation bone composition, rats, still anesthetized, were euthanized by total exsanguination. The blood was then collected through cardiac puncture and placed in tubes without anticoagulant to be centrifuged (Sigma Centrifuge) at 3500 rpm for 15 minutes to obtain the serum and after the samples were storage in freezer -80°C for future biochemical analysis. Calcium, osteocalcin and osteoprotegerin were analyzed in the serum. Calcium assays were performed using an automated biochemistry analyzer (Bioclin BS120, Quibasa - Química Básica Ltda / Belo Horizonte - MG). Osteocalcin and osteoprotegerin were analyzed with the aid of an ELISA microplate reader (TP Reader NM, Thermo Plate, Hexasystens / Taboão da Serra-SP).

Subsequently the collection of blood, femurs were collected and cleaned of soft tissue and preserved and stored at -80 ° C for further analysis. In the right femur, the bone mass was measured by a precision analytical balance. A digital caliper with a readability of 0·01mm was used to analyze the distance between the epiphyses were analyzed and the mean point for measuring the width of the diaphysis. After the femurs were submitted to the DXA for analysis of the bone composition (BMC, BMD and AO). The femur's analysis was performed using a three-point universal bending test at the Universal Testing Machine (DL 2000, EMIC, São José dos Pinhais, SP, Brazil) [28]. The ends of the bones were supported on two rolls with a diameter of 3 mm and a radius of 21.70 mm. The maximum force (N), the rupture force (N) and the elastic modulus (MPa) were obtained by software.

In order to evaluate the radiodensity of the femoral head, bones were analyzed by a CT scanner (HISPEED, GE ® model) [38]. The images were distributed through axial cuts of 1 mm. The radiodensity (expressed in units of Hounsfield, HU) was measured using a computer-based software analyzer system (Film Lite, 2.0, 2003, Milwaukee, USA) by Tool-Ellipse tools.

Statistical analysis

For statistical analysis the program Graph Pad Prism (San Diego, CA, EUA) was used. Data was analyzed by Student's t test and results were expressed in mean ± standard error of mean (SEM) considering significance level $p < 0.05$.

Results

As shown in table 2 the DXA analysis of total bone mineral density and bone area were similar between the C and OL groups, showed no significantly differences. Bone mineral content showed higher values in the experimental group ($p < 0.05$) when compared to control, in the total DXA analysis.

Table 2: Bone composition by DXA at 51 days of experiment

	C		OL	
	Mean	SEM	Mean	SEM
BMD (g/cm^2)	0.149	0.001	0.151	0.003
BMC (g)	7.700	0.105	8.157*	0.172
Area (cm^2)	51.710	0.747	54.000	0.690

Control group (C, n = 6) received control diet and Experimental Group (OL, n = 6) received increased diet of linseed oil during 30 days of experiment after the lactation period. Bone analysis in

DXA (dual-energy X-ray absorptiometry): BMD (bone mineral density) and BMC (bone mineral content). Significantly different from C group according to Student's t Test considering * ($p<0.05$). SEM, standard error of the mean.

Regarding the serological analysis, the animals those have received the experimental diet had a significant difference in blood calcium values, showing values higher in the OL group ($p<0.0001$) than in the C group. Other analyzes of osteocalcin and osteoprotegerin show no significant difference between groups (Table 3).

Table 3: Serum analysis at 51 days of experiment

		C		OL
	Mean	**SEM**	**Mean**	**SEM**
Calcium (mg/dL)	9.800	0.0756	10.820***	0.083
Osteocalcin (ng/mL)	55340	6579	70500	6730
Osteoprotegerin (ng/mL)	6714	3994	9290	4267

Control Group (C, n = 6) fed with control diet and Experimental Group (OL, n = 6) fed with diet of flax during 30 days of experiment after the lactation period. Significantly different to group C according to Student's t test considering: *** ($p <0.0001$). SEM, standard error of the mean.

Femur analysis presented in OL group showed higher ($p<0.05$) bone mineral density. Femur mass, distance between epiphysis and the middle point width of the diaphysis were higher ($p<0.05$) in OL group regarding to control group. In biomechanical test, OL group showed higher ($p<0.05$) elastic force vs. C. However, maximum strength and rupture strength were similar between groups. Computed tomography showed a higher ($p<0.05$) radiodensity of femoral head in OL group compared to C group (Table 4).

Table 4: Femur parameters and analysis by DXA, biomechanical test and tomography at 51 days of experiment

	C		OL	
	Mean	**SEM**	**Mean**	**SEM**
DXA femur				
BMD femur	0.1462	0.0013	0.1567**	0.0027
Femur parameters				
Femur mass (g)	0.681	0.013	0.748***	0.0139
Distance between epiphysis (mm)	34.990	0.149	35.850**	0.181
Middle point width of the diaphysis (mm)	3.879	0.061	4.126*	0.073
Biomechanical teste				
Maximum force (N)	108.0	3.994	109.8	2.798
Breaking strength (N)	93.86	6.794	95.50	3.096
Ridigity (MPa)	566900	11720	645600**	15220
Femur tomography	808.4	31.41	1050**	47.82

Control Group (C, n = 6) fed with control diet and Experimental Group (OL, n = 6) fed with diet of flax during 30 days of experiment after the lactation period. Bone analysis in DXA (dual-energy X-ray absorptiometry); BMD (bone mineral density). Significantly different from C group according to Student's t test considering: * ($p <0.05$); ** ($p <0.001$); *** ($p <0.0001$). SEM, standard error of the mean.

Discussion

Alpha-linolenic acid is a polyunsaturated fatty acid considered essential because the human's body is not able to produce it, therefore your ingestion through the diet is necessary. This fatty acid when related to bone metabolism shows ability to stimulate an increase of the production of osteoblastic cells that are mainly responsible for the processes of bone formation and remodeling [20, 23, 25]. The benefit of remodeling is the renewal of the bone without any microfracture. An excellent balance in metabolic bone processes contributes to the recovery and healthy maintenance of bone tissue [39]. The importance of bone maintenance and strengthening correlates with an improved long-term quality of life. Previous research has already linked the women's consumption of alpha-linolenic acid with a lower risk of fracture due to bone fragility in later life [30]. Considering that during pregnancy and postpartum periods the woman has her nutritional needs increased, it would be interesting to enrich her diet with source food of nutrients that can bring benefits to her recovery after these periods, such as linseed oil rich in acid alpha-linolenic [23, 33, 41].

There are several ways to evaluate and monitor bone composition and quality. The densitometry body assessment method has been used for decades in both human and rat research. The validation of DXA for small animals is well described in the scientific literature and is considered the gold standard for bone-related diagnoses [42, 43]. Regarding the DXA analysis (Table 2), higher values of bone mineral content found in the experimental group may indicate a greater activity of the bone's osteoblastic cells. The bone mineral content is composed mainly of crystals of calcium phosphate (hydroxyapatite) that give the bone hardness and resistance to compression. In this context we consider that alpha-linolenic acid has been able to promote a greater recruitment of osteoblasts stimulating the increase of the process of bone formation. These findings corroborate other studies as Costa and Ribeiro that found a beneficial effect on the addition of flaxseed in the *Wistar* diet [27, 28].

Concerning the serological results, OL group presented increases in serum calcium levels (Table 3), when compared to the control, indicate a positive effect of flaxseed oil in relation to the metabolism of this mineral [44, 45]. The bone tissue functions as a reservoir of calcium and other minerals that are used by other tissues in body. When the calcium source of diet is not adequate or during pregnancy, when a maternal calcium diversion occurs to milk production, the calcium is removed from the bone for adequate maintenance of serum levels [45, 46]. Therefore, when we associate this result with the other analysis in the present research, we can suggest that animals in experimental group presented higher values of calcium than control, it could mean that there was a greater preservation of the calcium even though the rats had passed through the lactation process [47, 48]. This finding may be interesting when applied to the clinical part, since women with genetic predisposition to bone diseases could benefit from supplementation with flaxseed oil in the postpartum period in order to prevent future complications as well as fracture due to bone fragility in the long-term [49,-51].

The concentrations of osteocalcin and osteoprotegerin were evaluated for being considered blood bone markers [48]. They can signal when there is an imbalance between the processes of bone renewal and resorption. Osteocalcin is a protein derived from osteoblastic cells that acts in the bone matrix aiding mineralization and preventing this process from occurring in excess [39]. Osteoprotegerin is a protein synthesized by osteoblasts that acts as a receptor to regulate the activity of osteoclast cells [39, 52]. When there is a decrease in the activity of osteoprotegerin there is an increase in the bone resorption process, which in excess can

be detrimental to bone quality, correlating with bone fragility. No significant difference was found between groups in the values of these proteins (Table 3). However, as there is no apparent decrease in its activity, we believe there is no reason to associate flaxseed oil with a negative effect when evaluated from the perspective of bone cell activity.

The results of femur analysis (Table 4) are the complement of the findings described above and corroborate much of what has been described in the scientific literature [38, 53, 54]. The femur is considered the largest bone presented in the body and has been the subject of clinical and observational studies related to fractures from frailty and bone diseases [39]. The DXA analysis found significant difference in C group in bone mineral density which signify the concentration of tissue in a determined volume. Increased values in the OL group when compared to C group suggest that the experimental group had a stronger bone than the control. Regarding bone parameters, we found a significant difference in the OL group in the three markers: mass, distance between the epiphyses and the mean point of the diaphysis. Higher values of the femoral mass in the OL group correlate with an increase in the amount of bone mineral content present and the effectiveness of the bone consolidation process that occurs in the adult phase [6, 40]. Femur is considered a long bone being formed by compact tissue and spongy or trabecular tissue that is mainly found in the extremities of these long bones. Trabecular tissue provides a large surface area that is exposed to the circulation of bone marrow fluids. In addition, it is surrounded by a larger number of cells than cortical bone tissue, being more sensitive to hormonal changes and nutritional deficiencies [20, 52]. Thus, the reduction of trabecular bone tissue is one of the main factors responsible for the occurrence of fractures. Increased values at the midpoint of the diaphysis and distance between the epiphyses in the OL group (Table 4) suggest a larger and stronger bone that can be due to a greater amount of trabecular bone tissue, which is beneficial for the protection of the skeletal structure.

The Biomechanical test was performed to evaluate the bone for maximum force, breaking strength and rigidity, measured by a three-point bending test, the resistance capacity of the bone is verified when it is submitted to a tension. We consider that bone tissue is constituted by a mineral phase, in the form of hydroxyapatite organized collagenous matrix (organic phase). The combination of the mineral with the organic phases gives the bone tissue a unique biomechanical properties [55]. The collagen fibers give an elasticity to bone tissue, as well as some resistance to cracking. The hydroxyapatite crystals provide high hardness and compressive strength. Thus, the experimental group presented higher values of rigidity compared to the control group, suggesting a greater elastic capacity of the bone, that is, the greater capacity of the bone to deform due to a pressure but without rupture [55, 56]. The other forces evaluated represent maximum force, the force that the bone tolerates until the fracture occurs, and the force of rupture, the force enough to cause the bone to rupture. In relation to computed tomography, this test is used in rat studies also for the evaluation of densitometric data and geometric properties of the bone [56, 57, 58]. The significant statistical difference in the OL group (Table 4) added to the other data of the study suggests a better composition and bone structure of the experimental group.

Conclusion

In conclusion, the findings of our study suggest that fatty acid composition of flaxseed oil was able to promote positive effects in bone composition of female's rats in the post weaning period. Furthermore, studies are needed to guide the way ingestion of flaxseed oil and its effects on bone quality in both rats and humans.

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Conflict of interests

The authors have declared no conflict of interest.

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