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ORIGINAL CONTRIBUTION

Low dietary calcium and obesity: a comparative study in genetically obese and normal rats during early growth

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Abstract
Purpose A low calcium intake (LCaI) may predispose to obesity, and excessive fat mass may be detrimental to bone. The impact of Ca inadequacy would be greater in subjects predisposed to obesity. LCaI effect on obesity development during the rapid growth period was compared in two strains of rats: spontaneously obese IIMb/β (O) and Wistar (W). Pregnant rats were fed 0.5 % (N) or 0.2 % (L) of Ca (OLCa, ONCa, WLCa and WNCa). Male pups were fed the maternal diet until day 60.
Methods Body composition, lipid profile, glucose homeostasis, 25 hydroxyvitamin D, Ca-phosphorus, and bone metabolism were evaluated.
Results BW and body fat were higher, whereas body protein was lower in OLCa versus ONCa ($p < 0.05$). OLCa presented the highest body fat, glucose, non-HDL and total cholesterol, TGL, insulin levels, and HOMA-IR, liver weight, and adipose perigonadal plus retroperitoneal pads ($p < 0.05$). WLCa did not exhibit an increase BW and only showed a slight change in body composition with minor biochemical alterations compared to WNCa ($p < 0.05$). Osteocalcin, CTX, and proximal tibia and lumbar spine BMDs were lower in O than in W rats fed the same Ca diet ($p < 0.05$). Body ash and Ca content, and total skeleton BMC/BW were lower in OLCa and WLCa versus their corresponding NCa groups ($p < 0.05$).
Conclusion The negative effect of a low Ca diet on fat mass accumulation and lipid profile may be more evident in rats predisposed to obesity. Nevertheless, low CaI interferes with the normal glucose homeostasis leading to an increase in insulin resistance. Low CaI during early growth may be an obesogenic factor that may persist into adult life and may account for the development of obesity and some of its co-morbidities.

Keywords Low calcium diet · Obesity · Osteocalcin · Insulin resistance · Bone remodeling · Bone mass

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Introduction

Emerging data suggest that excessive fat mass accumulation may be detrimental to calcium (Ca) metabolism in animals and in humans [1, 2]. Several factors related to obesity may negatively affect bone mass. First, marrow adipogenesis may be inversely related to osteoclastogenesis because adipocytes and osteoblasts are derived from a common multipotent-mesenchymal stem cell [3, 4]. Obesity is also associated with chronic low-grade inflammation, which increases inflammatory cytokines that enhance osteoclastogenesis [5]. Finally, parathormone

(PTH), the main calciotropic hormone that regulates bone remodeling, is increased in obesity [6]. Increased body fat mass (FM) during growth may have a negative effect on peak bone mass and optimal bone mineral content (BMC) attainment [7, 8]. Despite the relationship between obesity and bone health, as well as the mechanisms underlying the relation, still being poorly understood, it is clear that obese children are at significantly increased fracture risk [9].

In addition, it has been suggested that low Ca intake (CaI) may contribute to the development of obesity through the modulation of adipocyte lipid metabolism and energy partitioning between adipose tissue and lean body mass [10, 11]. In this regard, low CaI alters the PTH/vitamin D axis, inducing metabolic changes that inhibit lipolysis and stimulate lipogenesis inside the adipocytes [12, 13]. Several previous studies have linked the deficit in CaI to obesity in adults, though their results are controversial [14–16]. However, there are few studies in children [17–19] or in experimental animals during the high growth period [20].

The role of dietary Ca in obesity development was previously demonstrated in transgenic mice expressing the *agouti* gene or in rats susceptible to develop obesity [21, 22]. However, the impact of a Ca deficient diet on the development of obesity and its co-morbidities in normal rats during growth, particularly during the transitional stage from breast feeding to a mixed diet when mammals exhibit rapid growth, has not been studied.

Based on the above study, the hypothesis of the present study was that the effects of low CaI on energy metabolism should be influenced by the predisposition to obesity development. Moreover, we also hypothesized that not only low CaI but also the increment in fat mass during early growth could be detrimental to bone mass accretion. To address these issues, we comparatively investigated the effect of Ca inadequacy during early growth on body weight gain, body composition, insulin resistance, bone remodeling, and bone mass, in two different strains of rats: obese IIMb/β and Wistar rats.

Materials and methods

Animals

Wistar (W) and genetically predisposed obese IIMb/β (O) rats were obtained from the School of Pharmacy and Biochemistry, Buenos Aires University and from the School of Medicine, Rosario National University, respectively. The IIMb/β rats were obtained from Wistar rats by genetically environmental maladjustment and a high degree of inbreeding [23]. This strain of rats develops obesity with hypertriacylglycerolemia without hypercholesterolemia, and their glucose intolerance progresses to type II diabetes [24, 25].

Rats were housed in individual stainless steel cages and were maintained on a 12-h-light/-dark cycle in a temperature- and humidity-controlled room (21 ± 1 °C and 55 ± 10 %, respectively). Throughout the experimental period, rats were allowed access to deionized water and food ad libitum. They were maintained in keeping with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the Bioethics Committees of the Universities of Buenos Aires and Rosario.

Diets

The two isocaloric diets were prepared according to the American Institute of Nutrition Rodent Diets Recommendations settled in 1993 (AIN′93) [26]. The composition of both diets was identical except for the Ca content: the normal Ca diet provided 0.5 % Ca (NCa) and the low Ca diet (LCa) contained 0.2 % Ca, as shown in Table 1.

Table 1 Centesimal composition of the experimental diets prepared according to AIN 93G to meet rat requirements during growth

DIET	LCa	NCa
Energy (KJ)	1,675	1,675
Protein (g)[b]	17.0	17.0
Lipids (g)[c]	7.0	7.0
Calcium-free salts mixture	4.0[a]	4.0[a]
Water-soluble vitamins[d]	0.25[a]	0.25[a]
Fat-soluble vitamins[d]	0.50[a]	0.50[a]
Choline[f]	0.15	0.15
Cellulose[g]	5.0	5.0
Dextrin[h]	To complete 100 g	
Vitamin D (Cholecalciferol) (IU)	100	100
Calcium (g)[e]	0.2	0.5
Phosphorus (g)	0.6	0.6

[a] Manufactured by the Department of Food Science School of Biochemistry, University of Buenos Aires

[b] Potassium Caseinate, Nestlé Argentina S.A., containing/100 g, 85.1 of protein and 0.095 g of Ca

[c] Comercial soy oil. Molinos Rio de la Plata. Argentina

[d] Vitamins (individual components from Sigma, Missouri, USA)

[e] $CaCO_3$ (food grade individual components, Anedra, Argentina); CO_3Ca (Analytical grade, Anedra, Argentina)

[f] 0.71 % choline citrate (food grade, Anedra, Argentina)

[g] Cellulose to meet rat requirements of fiber, according to AIN 93G

[h] Corn dextrin from corn refinery, provided by Food SA Argentina was added as carbohydrate source to achieve 100 g of diet

Experimental design

A total of 16 female rats (8 O and 8 W), approximately 5 months old, with an average body weight of 275 ± 25 g, were mated by placing one male rat in a cage with four females. The first day of pregnancy was confirmed by the presence of sperm in vaginal smears. At that moment, dams were randomly assigned to receive one of the diets, which only varied in Ca content: NCa or LCa. Maternal body weight (BW) was recorded once a week.

Within 24 h after delivery, BW and sex of the pups were registered, and the litter size was adjusted to 8–9 per dam to avoid malnutrition derived from too large litter sizes. In order to maintain such number of pups, if necessary, both male and female offspring were kept with the dams during lactation. At weaning, eight male pups per group continued feeding the maternal diet until post-natal day 60. The remaining male pups were sacrificed, dried at 100 °C for 72 h, and powdered in order to assess the body composition at weaning. Pup BW was registered twice a week (Fig. 1).

Fasting blood samples were collected from the vein tail under anesthesia (ketamine hydrochloride 0.1 mg/100 g BW and acetopromazine maleate 0.1 mg/100 g BW) at the end of the study ($T = 60$). The animals were then killed by CO_2 inhalation, and intraperitoneal and retroperitoneal fat and liver were removed and weighed.

Apparent food efficiency, Ca, and fat absorption

From weaning to the end of the study, food consumption and BW were recorded 3 times per week, and food efficiency (g/g) was calculated according to the following equation: Food efficiency = Food intake (g)/increase in BW (g).

During the last 3 days of the study, the animals were individually lodged in plastic metabolic cages, and food consumption (I) and feces (F) were collected to calculate Ca and fat absorptions (CaA and FatA, respectively) (mg/d). Apparent absorptions, expressed as a percentage of Ca or fat intake, respectively (A %), were calculated according to the following equation: $A\ \% = (I-F/I) \times 100$.

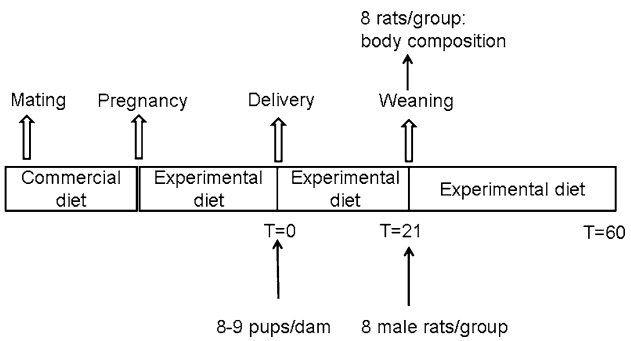

Fig. 1 Experimental design

Densitometry

At the end of the study and before killing the rats, total skeleton BMC (mg) and bone mineral density (BMD) (mg/cm^2) were determined in vivo under light anesthesia with a total body scanner by dual energy X-ray absorptiometry (DXA) using a software specifically designed for small animals (DPX Alpha, Small Animal Software, Lunar Radiation Corp. Madison WI) as previously described [27]. In brief, all rats were scanned using an identical scan procedure. Precision was assessed by measuring one rat five times with repositioning between scans, on the same and on different days. The coefficient of variation (CV) was 0.9 % for total skeleton BMD and 3.0 % for BMC. Two skeletal subareas were analyzed on the image of the animal on the screen, using a specific investigation area (ROI) for each segment. The BMD CV for the different studied areas was 1.8 % for lumbar spine (LS) and 3.5 % for the proximal tibia (PT). To avoid the interference of changes in BW, total skeleton BMC was expressed as a percentage of BW (mg/100 g BW). All analyses were carried out by the same technician in order to minimize interobserver variation.

Analytical procedures

Body composition was determined according to the Association of Official Analytical Chemists (AOAC) methods as previously described [28]. The Ca concentration in diets, serum, feces, and ashes was determined by atomic absorption spectrophotometry. Lanthanum chloride (6,500 mg/L in the final solution) was added to avoid interferences. Serum levels of phosphorus (P) and ashes were evaluated according to Gomori's method.

Serum glucose, total cholesterol and non-HDL cholesterol, and triglyceride (TGL) levels were determined by conventional enzymatic methods, and insulin was determined by enzyme immunoassay (Rat/Mouse Insulin ELISA Kit, Millipore, Billerica, MA, USA). The degree of insulin resistance was determined using the homeostatic model assessment for insulin resistance [HOMA-IR = (insulin × glucose)/22.5] [29]. The 25 hydroxyvitamin D (25OHD) levels were assayed by a competitive protein binding method (Diasorin, Stillwater, MN, USA). The intraassay coefficient of variation was 9 %. Serum levels of bone alkaline phosphatase (b-ALP) were measured using a colorimetric method (Boehringer Mannheim, Germany) after bone enzyme isoform precipitation with wheat germ lectin. Osteocalcin (ng/mL) and C-terminal telopeptide of type I collagen (CTX) levels (ng/mL) were

measured by immunoassay (ELISA) (Rat-osteocalcine and Rat-laps, respectively, Osteometer BioTech, Herlev, Denmark), with a 6 % intraassay variation coefficient.

Statistical methods

Results were expressed as mean ± standard error (ES). Data were analyzed using two-way analysis of variance (ANOVA), and Bonferroni multiple comparisons test was performed when significant differences were encountered. The linear association was analyzed by Pearson's correlation coefficients (r) and multivariate linear regression. Statistical analyses were performed using SPSS for Windows 19.0 (SPSS, Inc. Chicago, IL). A value of p below 0.05 ($p < 0.05$) was considered significant.

Results

Effect of low Ca intake at birth

The mean offspring number was lower in O rats as compared to W groups, with no differences according to CaI. Indeed, pup number was 9 ± 2 and 9 ± 4 in the OLCa and ONCa groups, respectively, and 13 ± 1 and 12 ± 2 in the WLCa and WNCa groups, respectively. No differences were found in the sex ratio of the pups (male:female): 4:5 and 4:5, and 7:6 and 6:6, respectively. No differences in BW or body composition were observed among the four studied groups at delivery (data not shown).

Effect of low Ca intake on obese IIMb/β rats

BW was higher in the OLCa group at birth, although the difference did not reach statistical significance. The OLCa group had a significantly higher food consumption than the ONCa group throughout the entire experiment ($p < 0.05$); however, no differences in food efficiency were observed between the two O groups studied here (Table 2).

Total body composition is shown in table 2, and body composition adjusted for BW is shown in Table 3. No differences in total body water content or percentage of water were observed between OLCa and ONCa, either at weaning or at the end of the study. The OLCa group showed a higher total fat and percentage of body fat content than the ONCa group. The differences in total fat content only reached statistical significance at the end of the study ($p < 0.05$) (Table 2), whereas fat content adjusted for BW was higher at the studied time points ($p < 0.01$) (Table 3). No differences in total body protein or ash content were observed at weaning; nevertheless, the ONCa group showed significantly lower values than OLCa pups at the end of the study ($p < 0.05$) (Table 2). OLCa had lower BW-adjusted protein content and higher BW-adjusted ash content than ONCa; the differences in protein content were statistically significant at both studied time points, whereas the differences in ash content reached statistical significance at the end of the study only ($p < 0.05$) (Table 3).

Serum levels of glucose, non-HDL and total cholesterol, TGL, insulin, and HOMA-IR, as well as liver weight and adipose perigonadal plus retroperitoneal pads were significantly higher in the OLCa than in the ONCa group ($p < 0.01$) (Table 4).

CaA % was lower in the ONCa group as compared to the OLCa group, but the difference did not reach statistical significance. No differences in fat absorption were observed between the OLCa and ONCa groups (Table 5).

Serum 25OHD (ng/dl) and Ca levels (mg/dl) were similar in the two studied O groups; however, serum P levels were significantly higher in the OLCa group as compared to the ONCa group ($p < 0.05$) (Table 6).

Serum bALP levels tended to be higher in OLCa as compared to ONCa rats ($p = 0.056$). Osteocalcin levels were significantly higher, and CTX levels were significantly lower in the OLCa group than in the ONCa group ($p < 0.05$) (Table 6).

As observed with ash content, total body Ca content and total skeleton BMC/BW were significantly lower in OLCa as compared to ONCa animals ($p < 0.05$). No significant differences in total skeleton BMD were observed; conversely, PT and LS BMDs were significantly lower in the OLCa group ($p < 0.05$) (Table 5).

Effect of low Ca intake on Wistar rats

No differences in BW were observed between WLCa and WNCa throughout the experiment. In addition, no differences in food consumption and efficiency were observed between the WLCa and WNCa groups (Table 3).

Total body composition is shown in Table 2, and BW-adjusted body composition is shown in Table 3. No differences in BW-adjusted total water content or body water were observed between WLCa and WNCa, either at weaning or at the end of the study. Total body fat content and BW-adjusted body fat were higher in WLCa than in WNCa animals, but the difference only reached significance at weaning ($p < 0.05$). Total protein content and body protein percentage were significantly lower in WLCa as compared to WNCa at weaning ($p < 0.05$) and tended to be higher at the end of the study ($p = 0.058$). Total ash content and the percentage of body ash content were significantly lower in the WLCa than in the WNCa group ($p < 0.05$) (Tables 2 and 3).

The biochemical alterations detected in the W groups were 4 to 5 times lower than those observed in O rats (Table 3). Serum levels of glucose, total cholesterol,

Table 2 Total body composition at weaning and at the end of the study

	OLCa	ONCa	WLCa	WNCa
Water (g)				
At weaning	22.3 ± 1.7	24.9 ± 1.6	28.4 ± 0.4	29.3 ± 1.5
At the end of the study	125.8 ± 5.1	118.8 ± 6.5	108.1 ± 6.3	102.3 ± 6.9
Body fat (g)				
At weaning	5.5 ± 0.9	4.7 ± 0.8	3.8 ± 0.5	2.7 ± 0.5**
At the end of the study	45.1 ± 3.1	38.6 ± 1.9*	29.9 ± 1.1	28.3 ± 1.3
Protein (N × 6.25) (g)				
At weaning	5.9 ± 0.4	6.3 ± 0.5*	5.1 ± 0.2	5.9 ± 0.2**
At the end of the study	41.1 ± 2.8	30.3 ± 2.7*	28.6 ± 1.1	32.5 ± 1.6
Ashes (g)				
At weaning	0.8 ± 0.1	0.7 ± 0.1	1.2 ± 0.1	1.8 ± 0.1**
At the end of the study	5.5 ± 0.4	6.2 ± 0.3*	4.6 ± 0.7	6.3 ± 1.0**

The statistical effects were assessed by two-way ANOVA (multiple comparisons test). Data are expressed as mean ± SE

* $p < 0.05$ OLCa as compared to ONCa; ** $p < 0.05$ WLCa as compared to ONCa

Table 3 Food consumption and efficiency, body weight and body composition in obese IIMb/β (O) and Wistar (W) rats, at birth, at weaning and at the end of the study

	OLCa	ONCa	WLCa	WNCa
Food consumption (g/d)	18.9 ± 0.7	15.9 ± 0.7*	16.9 ± 0.6*	16.7 ± 0.5*
Food efficiency (g/g)	2.9 ± 0.3	3.0 ± 0.5	3.1 ± 0.3	3.0 ± 0.4
BW at birth (g)	5.9 ± 0.6	5.4 ± 0.3	5.6 ± 0.3	5.2 ± 0.5
BW at weaning (g)	56.1 ± 5.5	50.7 ± 5.2*	44.5 ± 5.8*,#	44.2 ± 3.4*,#
Final BW (g)	278.8 ± 40.0	231.2 ± 42.0*	197.6 ± 22.6*,#	192.9 ± 9.0*,#
Body composition				
Water (g/100 g BW)				
At weaning	64.3 ± 3.5	65.6 ± 4.5	70.0 ± 4.0	69.1 ± 3.4
Final	67.3 ± 2.2	66.7 ± 2.8	66.9 ± 2.0	64.7 ± 3.3
Body fat (g/100 BW)				
At weaning	15.9 ± 2.8	12.6 ± 1.9*	9.5 ± 0.8*,#	7.2 ± 0.1*,**,#
Final	16.9 ± 1.5	13.1 ± 2.2*	11.1 ± 2.1*,#	10.4 ± 0.8*,#
Protein (N × 6.25) (g/100 g BW)				
At weaning	15.6 ± 0.9	18.3 ± 0.7*	17.4 ± 0.3*	19.8 ± 0.2*,**,#
Final	14.8 ± 0.8	17.3 ± 0.6*	18.1 ± 0.3*	19.6 ± 0.6*,#
Ashes (g/100 g BW)				
At weaning	1.9 ± 0.1	2.0 ± 0.9	2.4 ± 0.1*,*	3.4 ± 0.1*,**,#
Final	2.0 ± 0.2	2.6 ± 0.2*	2.2 ± 0.4*,#	3.2 ± 0.4*,**,#

The statistical effects were assessed by two-way ANOVA (multiple comparisons test). Data are expressed as mean ± SE

* $p < 0.05$ compared to OLCa; ** $p < 0.05$ compared to WLCa; # $p < 0.05$ compared to ONCa

insulin, and HOMA-IR were higher in WLCa than in WNCa group ($p < 0.05$), with no differences in TGL and non-HDL cholesterol levels, liver weight, or adipose perigonadal plus retroperitoneal pads (Table 4).

CaA % was significantly lower in the WNCa than in the WLCa group ($p < 0.05$). However, no differences in fat absorption were observed between both studied W groups (Table 5).

No differences in serum 25OHD (ng/dl) or Ca levels (mg/dl) were observed between the two studied W groups; however, serum P levels were higher in WLCa than in WNCa animals ($p < 0.05$) (Table 6).

Serum bALP levels tended to be higher in the WLCa group as compared to the WNCa group ($p = 0.058$). Osteocalcin levels were significantly higher, and CTX levels were significantly lower in the WLCa group as compared to the WNCa group ($p < 0.05$) (Table 6).

As observed with ash content, total body Ca content and total skeleton BMC/BW were significantly lower in the WLCa than in the WNCa group ($p < 0.05$). No significant differences in total skeleton BMD were observed; however, PT and LS BMDs were significantly lower in WLCa than in WNCa rats ($p < 0.05$) (Table 5).

Comparative effect of Ca intake on obese IIMb/β and Wistar rats

Pup BW was similar in the four studied groups at birth. However, OLCa and ONCa already had a higher BW than the non-obese WLCa and WNCa pups, respectively, at

Table 4 Serum levels of fasting glucose, insulin, cholesterol, non-HDL cholesterol, and triglycerides; HOMA-IR index and liver and fat pads weights in obese IIMb/β (O) and Wistar (W) rats, at the end of the study

	OLCa	ONCa	WLCa	WNCa
Glucose (mg/dl)	252.0 ± 11.0	151.9 ± 21.9*	190.6 ± 27.7*,#	98.7 ± 15.6*,**,#
non-HDL cholesterol (mg/dl)	119.0 ± 16.0	95.0 ± 4.4*	57.4 ± 12.1*,#	31.9 ± 14.7*,**,#
Cholesterol (mg/dl)	122.4 ± 8.1	98.4 ± 4.9*	65.1 ± 9.1*,#	48.1 ± 8.9*,**,#
Triglycerides (mg/dl)	290.6 ± 42.9	225.0 ± 36.6*	71.9 ± 13.4*,#	79.1 ± 16.2*,#
Insulin (ng/ml)	6.9 ± 1.0	4.1 ± 0.6*	1.3 ± 0.9*,#	0.10 ± 0.01*,**,#
HOMA-IR	81.5 ± 12.2	31.0 ± 7.4*	12.2 ± 0.8*,#	0.5 ± 0.1*,**,#
Liver weight (g)	15.6 ± 0.2	12.3 ± 0.9*	8.2 ± 0.9*,#	7.7 ± 0.5*,**,#
% (Perig. + Retrop.) fat/BW	5.34 ± 0.08	4.36 ± 0.14*	1.33 ± 0.44*,#	1.32 ± 0.43*,#

Statistical effects were assessed by two-way ANOVA (multiple comparisons test). Data are expressed as mean ± SE

* $p < 0.05$ compared to OLCa; ** $p < 0.05$ compared to WLCa; # $p < 0.05$ compared to ONCa

Table 5 Ca and fat absorption, total body Ca content and total skeleton, proximal tibia and lumbar spine bone mineral densities referred to body weight in obese 11 Mb/β (O) and Wistar (W) rats, at the end of the study

	OLCa	ONCa	WLCa	WNCa
Apparent fat absorption %	97.6 ± 0.6	97.5 ± 0.5	91.3 ± 1.0	99.8 ± 0.9
Apparent Ca absorption %	91 ± 3	88 ± 1*	82 ± 2*	72 ± 3*,**,#
Body Ca (mg/100 g BW)	596.5 ± 30.4	802.3 ± 33.8*	567.4 ± 31.1#	852.9 ± 29.7*,**
BMC/BW (g/100 g BW)	0.84 ± 0.09	1.44 ± 0.12*	0.93 ± 0.10*,#	1.53 ± 0.09*,**
Total skeleton BMD (mg/cm^2)	225.3 ± 9.0	234.6 ± 7.2	235.0 ± 11.8	229.3 ± 4.3
BMD proximal tibia (mg/cm^2)	203 ± 4	212 ± 5*	218 ± 6*	230 ± 4*,**,#
BMD lumbar spine (mg/cm^2)	185 ± 3	209 ± 5*	214 ± 6*	243 ± 7*,**,#

Statistical effects were assessed by two-way ANOVA (multiple comparisons test). Data are expressed as mean ± SE

* $p < 0.05$ compared to OLCa; ** $p < 0.05$ compared to WLCa; # $p < 0.05$ compared to ONCa

Table 6 Serum 25 hydroxyvitamin D (250HD), calcium, phosphorus, bone alkaline phosphatase (bALP), osteocalcin and collagen type I C-terminal telopeptide (CTX) levels in obese IIMb/β (O) and Wistar (W) rats, at the end of the study

	OLCa	ONCa	WLCa	WNCa
250HD (ng/dl)	20.8 ± 1.8	19.0 ± 1.7	18.9 ± 0.8	17.9 ± 0.9
Calcium (mg/dL)	10.4 ± 0.2	10.3 ± 0.2	8.3 ± 0.3*,#	8.2 ± 0.3*,#
Phosphorus (mg/dL)	11.9 ± 0.6	10.5 ± 0.5*	9.2 ± 0.2*,#	8.2 ± 0.3*,**,#
bALP (nkat/L)	3,134 ± 149	2,884 ± 133*	1,350 ± 149*,#	1,184 ± 100*,#
Osteocalcin (ng/mL)	514 ± 23	375 ± 18*	1,051 ± 47*,#	840 ± 36*,**,#
CTX (ng/mL)	70 ± 2	83 ± 3*	87 ± 3*	94 ± 6*,**,#

Statistical effects were assessed by two-way ANOVA (multiple comparisons test). Data are expressed as mean ± SE

* $p < 0.05$ compared to OLCa; ** $p < 0.05$ compared to WLCa; # $p < 0.05$ compared to ONCa

weaning ($p < 0.05$). In addition, the OLCa group attained the highest BW and food consumption throughout the entire study ($p < 0.05$), whereas the other 3 groups consumed a similar amount of food. However, no differences in food efficiency were observed among the four studied groups (Table 2).

No differences in body composition were observed among the four studied groups at birth (data not shown). No differences in water content adjusted for BW were observed among the four studied groups, either at weaning or at the end of the study (Table 3). The significantly highest body fat percentage was observed in the OLCa group ($p < 0.05$); the ONCa group also showed a significantly higher body fat content than the two studied W groups throughout the study ($p < 0.05$). The O groups showed a significantly higher fat percentage and a significantly lower protein percentage than their matched W group fed the same dietary Ca content ($p < 0.05$).

The significantly highest serum levels of glucose, non-HDL and total cholesterol, TGL, insulin, and HOMA-IR,

as well as liver weight and adipose perigonadal plus retroperitoneal pads were observed in the OLCa group ($p < 0.05$). In addition, the ONCa group also had significantly higher levels than the two W groups ($p < 0.05$); although these alterations were less marked than those observed in the OLCa group (Table 4).

CaA % was significantly lower in the W groups than in the O groups fed the same experimental diet ($p < 0.05$). No significant differences in fat absorption were observed among all studied groups (Table 5).

When the individual data on apparent CaA (mg/day) were plotted against CaI (mg/day), a positive function was observed, with no differences between the W and O rats ($r = 0.985$, $p < 0.001$) (Fig. 2).

No differences in serum 25OHD (ng/dl) levels (mg/dl) were found among the four studied groups; nevertheless, serum Ca, P, and bALP levels were significantly higher in the O groups than in their corresponding W groups ($p < 0.05$) (Table 6).

Osteocalcin levels were significantly higher, and CTX levels were significantly lower in the W groups than in their matched O groups ($p < 0.05$) (Table 6).

No significant differences in total body Ca content and total skeleton BMC/BW were observed between the O and the W rats fed the same dietary Ca content. BMC/BW was higher in the WLCa group than in the OLCa group ($p < 0.05$), and no differences were observed between the ONCa and WNCa groups. In addition, no significant differences in total skeleton BMD were observed among the four studied groups; however, PT and LS BMDs were significantly lower in the OLCa and ONCa groups than in WLCa and WNCa animals, respectively ($p < 0.05$) (Table 5).

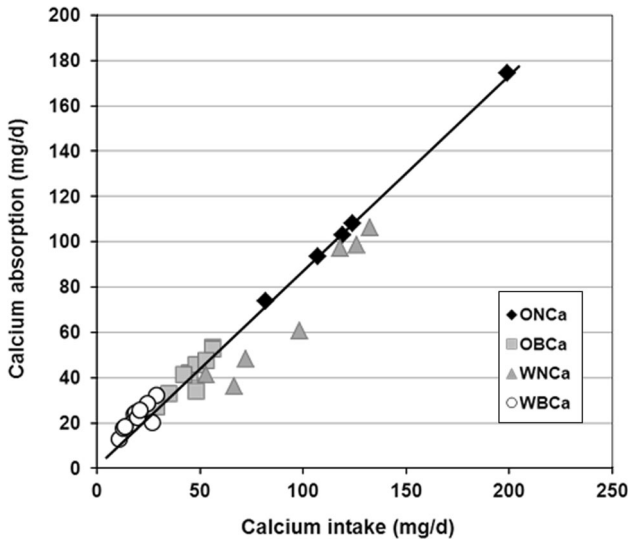

Fig. 2 Individual data of apparent Ca absorption as a function of Ca intake in obese IIMb/β (O) and Wistar (W) rats, during the last 3 days of study

Discussion

Obesity development is a complex multifactorial disease, in which environmental factors such as nutrition could interact with genetic susceptibility to gain weight. Our IIMb/β rats were obtained from ordinary Wistar rats by a high degree of inbreeding; they spontaneously develop obesity from puberty onwards [24, 25]. Therefore, IIMb/β rats would seem to be an optimal model of obesity to evaluate environmental factors, such as low CaI, that may potentially contribute to obesity development. The results of the present report strongly suggest that there is an association between low CaI and BW gain, including changes in body composition. This effect appears to be more evident in the IIMb/β strain of rats, which are susceptible to develop obesity. Indeed, analysis of the effects of low CaI in the two strains of rats in the present study showed that the OLCa group exhibited the most accelerated weight gain after birth. The increase in BW was accompanied by changes in body composition evidenced by an increase in body fat and a decrease in body protein accretion. Conversely, only slight changes in body composition were observed in the WLCa group. In this regard, a decrease in body protein accretion and a slight increase in body fat mass, with no changes in BW, were observed in this group. Moreover, the OLCa group also showed the most marked alterations consistent with metabolic syndrome, i.e., elevated glucose, lipids, insulin resistance, and liver and abdominal fat accumulation, whereas the WLCa group only showed slight signs of insulin resistance, i.e., elevated levels of glucose, insulin and HOMA-IR, and hypercholesterolemia without hypertriglyceridemia. These findings suggest the importance of genetic factors in the possible obesogenic role of low CaI during the rapid growth period.

According to the literature, the effect of Ca inadequacy in early life may be most notable and may predispose and/or program individuals to increase susceptibility to obesity and metabolic syndrome later in life [30–32]. In the present study, low CaI had no effect on BW or body composition in either of the studied strains of pups at delivery (data not shown). We hypothesized that the absence of marked changes during this period may be due to the short duration of rat pregnancy, together with the adjustments induced in the mother to ensure fetal health. In contrast, low CaI induced changes in body composition at weaning. It is important to take into account that the magnitude of such changes was higher in the O rats, which already evidenced an increment in BW at this point. Low CaI may be responsible for such changes during early life because pups begin to consume a mixed diet (milk plus solid) approximately the last week before weaning [33]. A clearly negative correlation between low CaI and obesity development

was observed later in both our strains of rats, from weaning to the end of the study. It must be pointed out, however, that the changes were more marked in the genetically susceptible rats than in the ordinary strain of W rats. Such effects were evidenced by higher fat stores and, namely, by visceral fat pad accumulation. The latter is associated with several features of obesity and is a strong predictor of metabolic syndrome [34].

In the present study, the highest mean food intake was observed in the OLCa group. It could be thought that the higher BW increases observed in this group are associated with their higher food consumption, since energy excess is the main cause of fat mass accumulation. However, food consumption in rats is BW dependent. OLCa had the highest BW values at birth and at weaning, though the difference compared to the remaining groups only reached statistical significance at weaning. Therefore, their higher BW would account for their higher food intake. In addition, it is well known that the transformation of consumed energy into BW (food efficiency) is similar in rats fed isocaloric diets, which supply the same percentage of protein, lipids, and carbohydrate [35]. Our results showed no differences in food efficiency, calculated as food intake/BW (g/g) ratio, or in mean food intake to BW increase ratio among the studied groups. This allows positing that the increase in BW observed in OLCa was not related to their higher food intake. It is also important to point out that, irrespective of the strain, the lowest body lean mass values were observed in the LCa groups, with the OLCa group exhibiting the lowest body protein content. These findings suggest a possible shift in energy away from fat stores. Although the process of fat and lean mass accumulation may have similar energy costs, lean mass per kg is metabolically more active and requires greater energy utilization than fat mass [36].

It is well known that inadequate CaI during growth is the main risk factor for osteoporosis development later in life. However, it has been suggested that low CaI may also be involved in BW regulation [13]. In this regard, it has been posited that the increment in parathyroid hormone (PTH) levels or the increase in PTH resistance induced by inadequate CaI may cause an increment in intraadipocyte Ca concentrations, which impairs lipolytic activity and stimulates lipogenesis [37–40]. The latter leads to the accumulation of adipose tissue stores and may contribute to the health consequences of obesity.

Two additional possible mechanisms are thought to be involved in the inverse association between CaI and the relative risk of obesity. One of these mechanisms is a decrease in the formation of fecal Ca-fatty acid soaps, leading to an increase in fat absorption and bioavailability [41]. According to some authors, dietary Ca content might affect body adiposity, modifying triacylglycerol absorption from the gastrointestinal tract through changes in total body lipid flux and fecal fat excretion [38]. The results of the present work showed no differences in the percentage of fat absorption between the two strains of rats fed the same dietary Ca content, or between the same strains of rats fed diets with different Ca content.

The other proposed mechanism is an increase in lipid oxidation [41]. According to previous studies, an adequate CaI may induce beneficial effects on lipid and lipoprotein profiles, with a decrease in LDL-cholesterol and triglyceride concentrations [42, 43]. However, other studies have failed to find a relationship between Ca and serum lipid levels [37]. In the present report, the non-HDL cholesterol levels, similar to LDL in humans, were higher in the LCa groups independent of the strain of rat. It should be kept in mind that our data evidenced that the OLCa group showed marked alterations in the serum lipid profile as well as the highest increase in hepatic weight. These findings suggest that the high fat accumulation observed in this group may be the result of fatty acid anabolism in the liver. Conversely, the WLCa group only showed a slight increment in lipid profiles and liver weight, with no changes in fat pad content. The differences observed between the two strains of rats studied here could partly explain the different results reported by other researchers regarding the effect of CaI on several parameters of lipid metabolism.

It is well known that low CaI negatively affects Ca homeostasis and bone health [44]. This effect was confirmed in the present report. Indeed, as Ca absorption was directly related to CaI, the rats fed the low Ca diet absorbed less amount of Ca and attained less mineral content in bone, regardless of the rat strain. It has also been suggested that through the generation of lipid peroxidation products, hyperlipidemia may also have a negative effect on Ca homeostasis and bone health by inducing PTH resistance. According to literature, PTH resistance appears to be independent of 25OHD levels [45]. As PTH levels are the main regulator of bone remodeling, PTH resistance should be concomitant to a decrease in bone turnover. In this regard, it has been suggested that subjects with more fat mass have lower levels of bone turnover biochemical markers, including CTX and osteocalcin [46]. However, other authors have reported an increase in bone resorption markers in humans [47] and in mice [48]. Although we did not measure serum PTH levels, the higher serum Ca and P levels as well as the low bone turnover rates in O rats could indirectly suggest a certain degree of PTH resistance. The results of the present study showed no differences in 25OHD levels among the four studied groups, lending support to the idea that PTH resistance would be independent of 25OHD levels.

The published findings regarding the effect of high fat mass on bone density are controversial. Some authors

associate high fat mass with a protective effect on trabecular bone, on cortical bone, on both types of bone, or with no effect at all [48–52]. Others, conversely, suggest an unfavorable effect of adiposity on BMD [1, 46, 53, 54]. Our findings are in agreement with those suggesting a negative effect of fat mass accumulation on bone density. Out results showed that the proximal tibia and lumbar spine density of spontaneously O groups were lower as compared to the corresponding W groups, with no differences in total skeleton density. Such differences could be due to the fact that the proximal tibia and lumbar spine have a higher percentage of trabecular bone than the total skeleton, which is composed of 80 % cortical bone. The present study showed obesity to have an impact on trabecular bone, which is metabolically more active, and no effect on the less active cortical bone.

There are same limitations in the present work. One of them is the relatively short duration of the study, which focused on evaluating the effect of a predisposition to develop obesity on the relation between low CaI and fat mass accrual in a growing rodent model. Further studies should be conducted to confirm whether the same results are observed in an adult rodent model. Another limitation is that PTH levels, which could clarify the presence of PTH resistance in the low Ca groups, were not measured. Finally, the third limitation was that the active form of vitamin D was not evaluated. In this regard, although no differences in 25OHD levels were observed, the assessment of 1, 25 dihydroxyvitamin D levels would have been physiologically more relevant to determine phospho-calcium homeostasis.

In summary, although further studies are necessary to clarify the mechanisms underlying the anti-obesity effect of an adequate CaI, the negative effect of a low Ca diet on fat mass accumulation and lipid profile appears to be more evident in rats predisposed to obesity. Nevertheless, regardless of such a predisposition, low CaI interferes with the normal glucose homeostasis leading to an increase in insulin resistance. Thus, it is possible that low CaI during early growth may be an obesogenic factor, in which case it would lead to permanent long-term changes that might account for the development of obesity and some of its co-morbidities.

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Conflict of interest The authors declare that there is no conflict of interest associated with this manuscript.

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