Are skeletally mature female rats a suitable model to study osteoporosis?

Ratas esqueleticamente maduras são um modelo satisfatório para estudar osteoporose?

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ABSTRACT

Objective: To analyze if female Wistar rats at 56 weeks of age are a suitable model to study osteoporosis. **Materials and methods:** Female rats with 6 and 36 weeks of age (n = 8 per group) were kept over a 20-week period and fed a diet for mature rodents complete in terms of Ca, phosphorous, and vitamin D. Excised femurs were measured for bone mass using dual-energy x-ray absorptiometry, morphometry, and biomechanical properties. The following serum markers of bone metabolism were analyzed: parathyroid hormone (PTH), osteocalcin (OC), osteoprotegerin (OPG), receptor activator of nuclear factor κappa B ligand (RANKL), C-terminal peptides of type I collagen (CTX-I), total calcium, and alkaline phosphatase (ALP) activity. **Results:** Rats at 56 weeks of age showed important bone metabolism differences when compared with the younger group, such as, highest diaphysis energy to failure, lowest levels of OC, CTX-I, and ALP, and elevated PTH, even with adequate dietary Ca. **Conclusion:** Rats at 26-week-old rats may be too young to study age-related bone loss, whereas the 56-week-old rats may be good models to represent the early stages of age-related changes in bone metabolism. Arq Bras Endocrinol Metab. 2012;56(4):259-64

Keywords
Aging; bone metabolism; osteoporosis; menopause

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RESUMO

Objetivo: Avaliar se ratas Wistar com 56 semanas de idade são um modelo satisfatório para estudar osteoporose. **Materiais e métodos:** Ratas com 6 e 36 semanas de idade (n = 8 por grupo) foram criadas por um período de 20 semanas e alimentadas com dieta completa em Ca, fósforo e vitamina D para ratas adultas. Os fêmures foram analisados quanto à massa óssea pela técnica de absortiometria por dupla fonte de raios-X, morfometria e propriedades biomecânicas; os marcadores séricos do metabolismo ósseo analisados foram paratormônio (PTH), osteocalcina (OC), osteoprotegerina (OPG), fator receptor ativador nuclear κappa B ligante (RANKL), peptídeos C-terminal de colágeno tipo I (CTX-I), cálcio total e atividade da fosfatase alcalina (FA). **Resultados:** As ratas com 56 semanas de vida apresentaram uma importante diferença no metabolismo ósseo quando comparadas ao grupo das ratas jovens, como, por exemplo, maior energia para quebrar a diáfise do fêmur, menores níveis de OC, CTX-I e ALP e maiores níveis de PTH mesmo com dieta adequada em cálcio. **Conclusão:** As ratas com 26 semanas de vida podem ser consideradas muito jovens para estudar a perda óssea relacionada à idade, porém, as ratas com 56 semanas de vida podem representar um bom modelo dos estágios iniciais das alterações associadas à idade no metabolismo ósseo. Arq Bras Endocrinol Metab. 2012;56(4):259-64

Descritores
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INTRODUCTION

Life expectancy in industrialized nations continues to increase, raising concerns over various health issues related to aging. It is estimated that, in the next 30 years, the proportion of people over the age of 65 years will double from 12% to 22%-25% of the population in the USA and Canada (1). Similarly, in Europe, it is estimated that this age group exceeds one in every thousand people, and will grow from 21.4 million in year 2000 to 35.7 million in year 2025. The most dramatic population growth is in the oldest age group, over 80 years of age, and is mainly represented by women. In Europe, for example, there are 15.2 million women aged 80 years old and above, compared with only 6.2 million men; this age group has virtually doubled from 7.2 million women and 3.3 million men since 1970 (2).

Estrogen metabolism is extremely important for the regulation of bone metabolism (3,4). With the aging of the female population around the world, and consequently prolonged deficiency of this hormone with menopause, the prevalence of osteopenia and osteoporosis is expected to increase markedly (2). Owing to the rapid decline in estrogen with menopause, rates of bone loss accelerate during menopausal years, resulting in accelerated rates of osteoporosis. After the menopausal years, the rate of bone loss in women slows slightly and becomes more comparable with that seen in older men (5).

According to a WHO Study Group, by the age of 75 years, approximately 30% of Caucasian women may be classified as having osteoporosis. This is related to declining BMD at the femoral neck, which is predictive of fractures with severe clinical consequences (1,2).

The rat is commonly used in osteoporosis research. In comparison with human studies, rat studies are easily controlled, provide rapid results due to the short life span of the animals, and enable access to the skeleton. The adult rat skeleton has many similarities with the human skeleton. The long bones of both species elongate by epiphyseal growth (endochondral ossification) and increase in cross-sectional area by periosteal growth (secondary intramembranous ossification). Radial growth slows to very low rates in adult rats, as it does in humans. The secondary spongiosa in rats undergoes sequential remodeling, similar to that observed in human cancellous bone (6).

Despite the similarity between human and rat bone metabolism, studies to prevent, manage, and analyze osteopenia and osteoporosis mechanisms have focused heavily on the relatively young ovariectomized (OVX) rat model (7,8), rather than aged or skeletally mature female rats. Thus, the objective of this study was to analyze if skeletally mature female Wistar rats at 56 weeks of age are suitable models to study natural osteoporosis caused by aging.

MATERIALS AND METHODS

Animals and diets

Female Wistar rats at 6 and 36 weeks of age (n = 8 per group), weighing approximately 250 g and 350 g, respectively, were obtained from the Multidisciplinary Center for Biological Research at the Universidade Estadual de Campinas (Unicamp, Campinas, São Paulo, Brazil). Animals were housed individually in stainless steel hanging cages at controlled temperature (21-24°C) and humidity (55%), and with a 12:12h light:dark cycle throughout the study. Animals were kept over a 20-week period, during which they were fed *ad libitum* a nutritionally complete diet for mature rodents based on the AIN-93M formulation (9), containing the recommended amounts of Ca (5 g/kg of diet), phosphorous (3 g/kg of diet), and vitamin D (1000 U/kg of diet, Table 1). At the end of the study, animals were 26 (n = 8) and 56 (n = 8) weeks of age. The protocol of this study was approved by Animal Experimental Ethics Committee of the Universidade Estadual de Campinas.

Tissue collection

All animals were killed by decapitation, and trunk blood samples were collected and stored on ice until

Table 1. Diets formulated based on the AIN-93M (9)

Ingredients	(g/kg of diet)
Cornstarch	465.692
Casein (≥ 85% protein)	140.000
Dextrinized cornstarch (90%-94% tetrasaccharides)	155.000
Sucrose	100.000
Soybean oil	40.000
Cellulose	50.000
Mineral mix (AIN-93M-MX)	35.000
Vitamin mix (AIN-93-VX)	10.000
L-Cystine	1.800
Choline bitartrate (41.1% choline)	2.500
Tert-butylhydroquinone	0.008

they were centrifuged to obtain serum (3,000 rpm/10 min; FANEM Excelsa Baby I – model 206/São Paulo – Brazil). Serum samples were stored at -80°C until analysis. Femur and tibia were excised and stored at -20°C until analysis.

Dual-energy x-ray absorptiometry scans (DXA)

The right femur (whole femur, hip axis, and diaphysis) was scanned for bone area (BA), bone mineral content (BMC), and bone mineral density (BMD) in a water bath using DXA (4500A; Hologic Inc., Bedford, MA, USA; small animal software in high resolution mode QDR 12.3) (10). Precision errors (%) for triplicate scans of BA, BMC, and BMD were 25.6, 24.3, and 6.1, respectively, for the whole femur; 6.1, 6.2, and 6.0, respectively, for the hip axis; and, 3.9, 4.7, and 5.6, respectively, for the femur diaphysis.

Bone morphometry

After DXA analysis, right and left femurs were thoroughly cleaned of soft tissue. Morphometric measures were taken with a digital caliper, as described elsewhere (10), and recorded to the nearest 0.01 mm. All measures were recorded in triplicate by the same trained examiner, and included length, and head, neck, hip axis, diaphysis, and distal epiphysis width.

Biomechanical properties

The right femur diaphysis was selected to represent cortical bone, which is known as a site of bone loss caused by aging (11). Biomechanical properties included energy to failure (N), maximum load (N), resilience ($x10^{-3}$J), and stiffness ($x10^{3}$ N/m). Tests were conducted using a three-point bending rheolometer (Hydraulic Equipment Service, INC.; 810 TestStar II MTS; Houston, Texas/USA), as described elsewhere (8). Measurement conditions were the following: sample space, 18 mm; plunger speed, 2 mm/min; and load range, 100 kN.

Serum markers of bone metabolism

Serum parathyroid hormone (PTH), osteocalcin (OC), osteoprotegerin (OPG), receptor activator of nuclear factor κappa B ligand (RANKL), and C-terminal peptides of type I collagen (CTX-I) were measured by enzyme-linked immunosorbent assays specific for rats (Rat Bone Panel 3 – PTH and Osteocalcin; Rat Bone Panel 1 – osteoprotegerin; Rat Bone Panel 2 – RANKL, Millipore, Massachusetts, USA; CTX-I of the Immunodiagnostic Systems Ltd., Arizona, USA). Serum total calcium and alkaline phosphatase (ALP) activity were measured by colorimetric method (Calcium CAT no. 00800, Laborlab – São Paulo/Brazil; alkaline phosphatase activity LB 170123-800, Biodiagnostic – Paraná/Brazil) in Beckman Coulter DU-70 spectrophotometer (Beckman Coulter Inc., California, USA).

All biochemical assays were performed according to the manufacturer's instructions, in duplicate. Assay sensitivity, and intra-assay and inter-assay coefficient of variation (% CV) were respectively: PTH = 0.3 pg/mL, 3.5% CV and 8.1% CV; OC = 1.6 pg/mL, 2.9% CV and 4.6% CV; OPG = 2.3 pg/mL, 3.1% CV and 3.4% CV; RANKL = 1.1 pg/mL, 3.1% CV and 9.0 %CV; CTX-I = 2 ng/mL, 5.6% CV and 10.5% CV, ALP = 24 U/I, 1.2% CV and 0.99% CV.

Statistical methods

Differences between age groups were tested using Student's t-test. Criteria of normal and equal variances between groups were met with a level of significance of 0.05, using GraphPad Prism 5.0 (GraphPad, Inc.; La Jolla, CA, USA). All data are recorded as means and standard errors of the mean (SEM).

RESULTS

Feed intake and body weight

Body weight was greater ($p < 0.05$) in the female rats at 56 weeks of age than at 26 weeks of age (233.5 ± 2.5 g *vs.* 333.9 ± 0.9 g) upon arrival at the facility. Daily feed intake during the experiment was not different between age groups (26 weeks: 15.2 ± 0.6 g; 56 weeks: 15.6 ± 0.6 g).

Bone mass (DXA) and morphometry

Femur diaphysis BA, BMC and BMD, whole femur BMD, hip axis BA, and femur wet weight, length, and head, diaphysis, and distal epiphysis width results were greater ($p < 0.05$) for female rats at 56 weeks of age than for female rats at 26 weeks of age (Tables 2 and 3). However, hip axis BMC and BMD, and neck and hip axis widths were not different ($p > 0.05$) between age groups.

Biomechanics properties

Energy to failure (endpoint of the elastic region) was decreased ($p < 0.05$) for 56 weeks of age, while maximum load, resilience and stiffness were not different ($p > 0.05$) between the age groups (Table 4).

Table 2. Bone area (BA), mineral content (BMC), and bone mineral density (BMD) of femur and mandible of female rats at 26 and 56 weeks of age

	26 weeks of age rats (n = 8)	56 weeks of age rats (n = 8)	P value
Whole femur			
BA (cm^2)	1.22 ± 0.08	1.20 ± 0.11	0.5447
BMC (g)	0.271 ± 0.017	0.280 ± 0.023	0.1812
BMD (g/cm^2)	0.221 ± 0.004[a]	0.237 ± 0.004[b]	0.0135
Hip axis			
BA (cm^2)	0.28 ± 0.008[a]	0.31 ± 0.003[b]	0.0011
BMC (g)	0.066 ± 0.003	0.073 ± 0.002	0.0648
BMD (g/cm^2)	0.233 ± 0.005	0.236 ± 0.004	0.6492
Femur diaphysis			
BA (cm^2)	0.31 ± 0.003[a]	0.44 ± 0.007[b]	< 0.0001
BMC (g)	0.070 ± 0.002[a]	0.108 ± 0.002[b]	< 0.0001
BMD (g/cm^2)	0.225 ± 0.005[a]	0.243 ± 0.004[b]	0.0062

Data are presented as means (SEM). BA: bone area; BMC: bone mineral content; BMD: bone mineral density. Within rows, values with different letters indicate significant differences ($p < 0.05$) between the age groups.

Table 3. Morphometry measurements of femur and mandible of female rats 26 and 56 weeks of age

Measurements	26 weeks of age rats (n = 8)	56 weeks of age rats (n = 8)	P value
Wet weight (mg)	488.4 ± 16.0[a]	690.3 ± 6.7[b]	< 0.0001
Length (mm)	30.3 ± 0.2[a]	33.8 ± 0.2[b]	< 0.0001
Head (mm)	3.55 ± 0.09[a]	3.82 ± 0.04[b]	0.0101
Neck (mm)	2.03 ± 0.04	2.06 ± 0.02	0.4630
Hip axis (mm)	7.45 ± 0.05	7.65 ± 0.09	0.0632
Diaphysis region (mm)	3.67 ± 0.09[a]	4.27 ± 0.04[b]	< 0.0001
Distal epiphysis region (mm)	5.40 ± 0.05[a]	5.59 ± 0.05[b]	0.0164

Data are presented as means (SEM). Within rows, values with different letters indicate significant differences ($p < 0.05$) between the age groups.

Table 4. Biomechanical properties of femurs of female rats 26 and 56 weeks of age

	26 weeks of age rats (n = 8)	56 weeks of age rats (n = 8)	P value
Energy to failure (N)	87.3 ± 1.7[a]	81.3 ± 0.8[b]	0.0051
Maximum load (N)	95.3 ± 1.2	93.8 ± 1.1	0.3850
Resilience ($x10^{-3}J$)	12.3 ± 0.7	11.6 ± 1.3	0.6757
Stiffness ($x10^3$ N/m)	330.4 ± 16.6	319.1 ± 36.3	0.5357

Data are presented as means (SEM). Within rows, values with different letters indicate significant differences ($p < 0.05$) between the age groups.

Serum markers of bone metabolism

Serum PTH and OPG concentrations were greater ($p < 0.05$) in rats at 56 weeks of age, and serum OC, CTX-I, and ALP activity were lower ($p < 0.05$) in the same animals; serum RANKL concentration, and serum total calcium were not different ($p > 0.05$) between the groups (Table 5).

Table 5. Serum markers of bone metabolism of female rats at 26 and 56 weeks of age

Serum markers	26 weeks of age rats (n = 8)	56 weeks of age rats (n = 8)	P value
PTH (pg/mL)	2.47 ± 0.30[a]	6.56 ± 0.57[b]	< 0.0001
OC (ng/mL)	4.12 ± 0.38[a]	2.82 ± 0.36[b]	0.0275
CTX-I (ng/mL)	25.12 ± 1.86[a]	11.98 ± 0.50[b]	< 0.0001
ALP (U/L)	70.56 ± 8.14[a]	46.22 ± 7.61[b]	0.0464
OPG (ng/mL)	0.39 ± 0.06[a]	3.03 ± 0.57[b]	0.0004
RANKL (pg/mL)	57.55 ± 4.31	80.63 ± 23.75	0.3551
Calcium (mg/dL)	9.66 ± 0.44	8.77 ± 0.25	0.1123

Data are presented as means (SEM). PTH: parathyroid hormone; OC: osteocalcin; OPG: osteoprotegerin; RANKL: receptor activator of nuclear factor κappa B ligand; CTX-I: bone-related degradation products of C-terminal peptides of type I collagen; ALP: alkaline phosphatase (ALP) activity. Within rows, values with different letters indicate significant differences ($p < 0.05$) between the age groups.

DISCUSSION

By examining our DXA and morphometry bone results, we can suggest that rats at 56 weeks of age did not show aged cortical and cancellous bone depletion. Kiebzak and cols. (12) evaluated cortical (femur midshaft tissue area) and trabecular (femur distal metaphysis tissue area) bone BMC and BMD of young (24 weeks of age), mature adult (48 weeks of age), and senescent (96 weeks of age) male and female rats. These investigators also concluded that cortical and trabecular bone BMC and BMD, and width of the femur increased progressively with advancing age. However, maximum BMD was found in mature adult rats (48 weeks of aged), and this value decreased slightly only in femurs of senescent rats (96 weeks of age). Li and cols. (13) showed that Sprague-Dawley female rats also began to show decreased cancellous bone (metaphysis tissue area) at 48 weeks of age.

Despite the fact that our femur diaphysis BMD values were greater ($p < 0.05$) in rats at 56 weeks of age, energy to failure (endpoint of the elastic region) of this bone region was lower ($p < 0.05$) in the same group. Akkus and cols. (14) also observed an age-related reduction on energy to failure in femur diaphysis of rats. These results can be explained by Ammann and Rizzoli (15), who stated that despite BMD being a major determinant of bone strength, some changes, such as perforation and/or disappearance of cancellous bone

without major repercussions on BMD, could account for the modifications in bone strength. The cortical sheath is connected to cancellous bone and consists of interconnected rods and plates. This structure maximizes strength, while minimizing weight. Loss in structure occurs with advancing age, and rates of fracture also increase markedly with age. Thus, this result is extremely important because early osteoporosis is not usually diagnosed and remains asymptomatic; it does not become clinically evident until fractures occur, giving rise to significant morbidity and some mortality (2).

As for biochemical results, serum PTH was greater ($p < 0.05$) in rats at 56 weeks of age. This hormone is associated with increased bone resorption: typically, osteoblasts and osteoclasts cells respond to serum PTH elevation, with the net result being increased bone resorption and rapid release of Ca^{2+} from the bone matrix (16,17). Lips (18) and Mosekilde (19) described that upregulation of serum PTH is directly related with aging in mammals. Kiebzak and cols. (20) did not show age-related increases in PTH levels of female rats at 24, 48 and 96 weeks of age; however, immunoreactive PTH titers were slightly, but significantly, elevated when the data were analyzed by linear regression. In humans, Saraiva and cols. (21) evaluated the PTH levels in 177 inpatients (125 women and 52 men) with 76.6 ± 9.0 years of age, and 243 outpatients (168 women and 75 men) with 79.1 ± 5.9 years of age; these investigators showed PTH levels corresponding to secondary hyperparathyroidism in 61.7% of the inpatients and in 54% of the outpatients. Russo and cols. (22) also showed PTH levels as secondary hyperparathyroidism in 8% of post-menopausal women (n = 251) between 50-85 years of age. Saraiva and cols. (21) and Russo and cols. (22) did not show correlations between serum PTH and vitamin D (25OHD) levels. In our study, animals were fed a diet adequate in vitamin D that would support normal PTH metabolism.

Serum OPG level was also increased in rats at 56 weeks of age. This result is supported by Khosla and cols. (24), who measured serum OPG levels in an age-stratified, random sample of men (n = 346 age range, 23-90 years) and women (n = 304; age range 21-93 years), and showed that serum OPG levels increased with age in both men and women (171 ± 6 pg/mL; 134 ± 6 pg/mL, respectively). These investigators associated this age-related OPG elevation as a compensatory phenomenon to slow down enhanced bone resorption. Probably, this compensatory phenomenon occurs because OPG is a soluble factor produced by osteoblastic cells and it is considered a decoy receptor for RANKL. Indeed, OPG blocks the interaction between RANKL and RANK, inhibiting the terminal stage of osteoclastic differentiation, and then inhibiting bone resorption. Furthermore, the inhibitory effect of OPG on bone resorption can be explained not only as a decoy receptor function, but also as a modulator of RANKL half-life. In turn, RANKL controls the bioavailability of OPG and its internalization and degradation (25). In our study, serum RANKL level did not show age-related difference. However, Norian and cols. (26) described that RANKL may be a reliable metabolite that shows the state of bone metabolism only in the premenopausal period.

In this study, serum calcium concentration did not show age-related reductions. It is important to distinguish this model from humans in that rats were fed a nutritionally complete diet throughout aging, providing ample amounts of calcium to sustain calcium balance (9). Kiebzak and cols. (20) did not show age-related reductions on serum calcium concentration in female rats with 24, 48 and 96 weeks of age, either, using animals that were also fed a diet adequate in calcium. In addition, Takada and cols. (23) described that serum calcium concentration changes only in a critical situation, such as undernutrition or hyperparathyroidism.

Serum OC, CTX-I, and ALP bone markers were lower with aging in this study. OC and CTX-I is a biomarker of bone formation and degradation, respectively, and ALP is produced by osteoblasts and is proportional to bone remodeling rates (27). As the maintenance of bone mass depends on the balance between bone formation and bone resorption, these results suggest that the bone remodeling slows with aging in this rat model. Kiebzak and cols. (20) showed that serum OC decreased progressively from 24 to 48 weeks of age (21%) and from 48 to 96 weeks of age (23%) in female rats; however, these investigators did not show total serum alkaline phosphatase activity alteration with aging in the same female rats. Iida and Fukuda (28) showed decreased in the total ALP activity up to 36 weeks of age in Wistar female and male rats.

Initially, by examining our bone DXA and morphometry results, we can suggest that rats at 56 weeks of age are not a satisfactory model to evaluate age-related changes on bone metabolism. However, the biomechanical properties and biochemical results suggest that these rats showed important bone metabolism

differences when compared with the younger group; the physiological response to any intervention is likely to be different as well. For example, highest diaphysis energy to failure, lowest levels of OC, CTX-I, and ALP, and elevated PTH even with adequate dietary Ca might attenuate response to interventions designed to limit age-related bone loss. Additionally, 26-week-old rats may be too young to study age-related bone loss, whereas the 56-week-old rat may be a good model to represent the early stages of age-related changes in bones.

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