Original article

Femoral biomechanic and microtomography from male rats submitted to dietary restriction supplemented with sucrose

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Abstract

Food restriction reduces body weight and influence bone mass and also is correlated with bone mineral density (BMD). Mechanisms have been proposed for the loss of BMD after body weight reduction, including reduced energy intake. Growing 8 wk-old Wistar male rats were randomly divided into Control and Calorie restriction associated with sucrose 30% (CRS). These animals were subjected to intermittent food restriction during 8 weeks and had free access to tap water and sucrose30% in distilled water. The rats were euthanized at the end of week 8, blood collected from abdominal aorta artery, femurs cleaned of adherent soft tissues, scanned using dual energy X-ray absorptiometry, structural and material properties determined by three-point bending testing in the mid-diaphyseal region, bone surface tested in a microhardness tester and microstructure was assessed in a microcomputer tomography. In CRS animals body weight decreased significantly relative to the Control animals. There was a clear option for high-sucrose beverage in CRS animals. No difference was observed in biochemical, densitometric and biomechanical analyzes. Results from micro CT showed only significant difference in connectivity of trabecular bone. It has been suggested that rats submitted to food restriction consumed sugar not because of its inherent palatability, but in order to alter their macronutrient balance and animals need to meet energy demands in high-sucrose.

Keywords: bone mineral density, femoral biomechanics, femoral microtomography, food restriction.

1 Introduction

The most commonly used intervention to lose body mass in humans is restriction of energy intake. During energy restriction, mice and humans show sustained reductions in energy expenditure, which may predispose subjects to a positive energy balance when food is made available postrestriction (CAMERON and SPEAKMAN, 2011). Dietary restriction (DR) results have demonstrated several effects on health that occur during restriction and appear to have a direct link to increasing longevity. Their effects include reducing the metabolic rate and oxidative stress, altering neuroendocrine and sympathetic nervous system function, and improving insulin sensitivity.

Restricting energy intake while supplying adequate micronutrients slows aging, retards the development of age-associated diseases, and extends maximal lifespan in all species studied to date. The molecular and physiological effects of energy restriction occur within a few weeks of starting restriction diet and appear to be qualitatively similar whether the restriction diet is initiated during young adulthood or late in life. Energy restriction causes changes in the amount and distribution of fat that differ in some critical way from other weight loss modalities. This possibility would be consistent with studies demonstrating that not only the amount but also the location of fat is critical to health (COLMAN, NAM, HUCHTHAUSEN et al., 2007). It is believed that no single factor, but a combination of factors, is responsible for the observed effects of caloric restriction on aging and the biochemical basis for the beneficial effects of DR remains controversial (BROCHMANN, DUARTE, ZAIDI et al., 2003; WESTERBEEK, HEPPLE and ZERNICKE, 2008). The enriched diets fed to the restricted mice consolidated the term caloric restriction as a label for a dietary intervention involving a decrease in calorie intake without micronutrient limitation, which is associated with lifespan extension in many animal models (CERQUEIRA and KOWALTOWSKI, 2009).

Bone growth and development is a tightly coordinated process of bone formation and resorption. Bone formation is regulated by both the proliferation and differentiation rate of osteoprogenitor cells as well as the activity of mature osteoblasts; however, the rate of bone formation is more dependent on the number of osteoblasts present than on the activity of the individual osteoblasts (MAHAJAN, ALEXANDER, SEABOLT et al., 2011). Body weight is highly correlated with bone mass and bone mineral density (BMD), but the role of body composition in regulating bone formation and resorption is less clear (HAMRICK, DING, PONNALA et al., 2008). In fact, DR is associated with low body mass, one of the major determinants of BMD across the life span (BROCHMANN, DUARTE, ZAIDI et al., 2003). Several mechanisms have been proposed for the loss of BMD after body weight reduction, including reduced energy intake and weight loss resulting in a reduction in bone mass in humans and animal models with most studies

showing 1-2% bone loss with 10% weight loss (HAWKINS, CIFUENTES, PLESHKO et al., 2010).

Dietary restriction has emerged as an important avenue of investigation in the area of aging, specifically the prevention of aging (LAMBERT, LAMOTHE, ZERNICKE et al., 2005). Apart from prolonging life span, DR can influence the mechanical, material and geometrical characteristics of bone and it is important to examine the impact reduced food intake can have on the processes and structures of the body (COLMAN, NAM, HUCHTHAUSEN et al., 2007; WESTERBEEK, HEPPLE and ZERNICKE, 2008). Sufficient macronutrient intake is required for optimal skeleton acquisition and chronic undernutrition during the time of peak bone mass accrual, as seen in anorexia nervosa, is associated with low bone mass, thus a better understanding of how insufficient food intake affects young, rapidly growing individuals is critical (MISRA and KLIBANSKI, 2006; DEVLIN, CLOUTIER, THOMAS et al., 2010). There is also a renewed interest in weight loss even in normal-weight populations due to its recent association with longevity, thus properties that determine bone strength, its material composition and its structural design are being studied (SEEMAN and DELMAS, 2006; HAWKINS, CIFUENTES, PLESHKO et al., 2010). But how dietary restriction affects cortical and cancellous bone characteristics is controversial and it is important to understand how bone is influenced by dietary restriction supplemented with high caloric/low cost diet.

2 Materials and Methods

2.1 Animals and experimental design

All animal procedures were in compliance with the São Paulo State University/Araçatuba School of Dentistry Care Committee's rules and regulations on their Ethical Principles in Animal Experiments. Following the 1-week acclimation period, twenty four male Wistar rats, 8 weeks old, were randomly divided into Control [n = 10, 298.20 ± 17.4 g at day 1] (C) group and Dietary restriction supplemented with sucrose (DRS) group [n = 14, 297.86 ± 19.2 g at day 1]. The animals were grouped three per cage, and the environment was temperature controlled (22° ± 1 °C) and humidity-controlled (54-56%) in a closed cabinet with ventilation (12-h light/12-h dark cycle with lights on at 6:00 AM). The animals were allowed free access to a standard pelleted rodent diet (Labina, Purina Nutriments) for 8 weeks. Group C had free access to the commercial diet and tap water. During the same 8 week period, rats in the DRS Group were fed three times per week: Monday, Wednesday and Friday, when they were given free access to a commercial solid food (TATSUMI, ITO, ASABA et al., 2008). During the days of fasting, DRS animals had free access to tap water and 30% sucrose. The sucrose beverage was composed of commercially available pure cane sugar (Docito, Brazil) in distilled water, prepared on Tuesday, Friday and Saturday at the same time (8:00-9:00 AM) and provided in 500 mL glass bottles with rubber stoppers. Animal body weight was determined once a week (on the same day and time at 8:00-9:00 AM). Solid chow, water and sucrose consumption were measured daily (between 8:00-9:00 AM).

2.2 Biochemical measurements

At the end of week 8 of the study, the rats were euthanized after fasting overnight, under ketamine (0.07 mL/100g) - xylazine (0.03mL/100g) anesthesia and exsanguination. After being anesthetized, animal blood was collected from the abdominal aorta artery, placed in heparin-treated tubes and kept on ice until centrifuged. The plasma was separated and frozen at –80 °C for subsequent calcium, phosphorus, triacylglycerol, total cholesterol, and fructosamine analyses using the manufacturer's instructions provided with the kits (Labtest, São Paulo, Brazil). The femurs were harvested, cleaned of adherent soft tissues and stored in saline solution at –20 °C.

2.3 Densitometry

Each left femur was scanned using dual energy X-ray absorptiometry (DXA) to determine bone mineral density (BMD). Bones were thawed at room temperature (23 °C), placed in a plexiglass container filled with deionized water, aligned in an anterior-posterior position, and scanned using a Lunar DPX Alpha (Madison, U.S.A.) with small-animal software coupled to a computer. The region of interest was moved to cover whole bone, proximal and distal epiphysis.

2.4 Mechanical testing

The structural and material properties of the femur were determined by destructive three-point bending testing in the mid-diaphyseal region. The bones were slowly thawed at room temperature at least 12 h before tests and kept wrapped in saline-soaked gauzes except during tests, and then placed on a computer-controlled EMIC DL 3000 universal test machine (São José dos Pinhais, Brazil), with a 2000 N load cell (speed of 5 mm/min). The femur was tested by three-point bending metal supports located at a distance of 20 mm. Load was applied on the femoral cortex at its longitudinal midpoint that is, at mid-diaphysis in the anterior-posterior plane, until failure occurred.

2.5 Bone microhardness

Fresh undecalcified femoral mid-diaphyseal cortical bone was cut (5mm) perpendicular to the bone long axis. Bone marrow was irrigated and samples were then embedded in a thermoplastic mounting powder (Extec, Enfield, U.S.A.), under pressure (150 kgf/cm^2) and temperature for complete penetration of the resin into marrow cavity. The bone surface was then carefully polished under constant water irrigation, with special carbide grinding paper of decreasing particle size (400, 600, 800, 1200, Buehler Carbimet Paper Discs, U.S.A.) and finally polished using felt paper wet with diamond solution (1/4 µm, Metadi Diamond Suspension, U.S.A.). Knoop bone hardness testing was carried out with use of a Buhler Microhardness Tester (model 5100, Japan) with a Knoop diamond indenter, set at a 25-g load for 10 sec and measured at 500x magnification. Indentions were made at 100 and 300 µm distant from periosteal surface. Around the sample 20 indentions (distanced 100 µm) were made in cortical bone and measured. Knoop hardness (KH) was expressed as the mean of these measures.

2.6 Bone microstructure

Trabecular bone microstructure was assessed in the femoral distal epiphysis. After mechanical testing, the distal epiphysis

was removed (12 mm from distal end), placed in a holder and scanned using a Skyscan 1172 microcomputer tomography (micro-CT, Skyscan, Aartselaar, Belgium) with X-ray source power of 100 kV and 100 µA, with a resolution of 12 × 12 × 12 µm (TATSUMI, ITO, ASABA et al., 2008; DEVLIN, CLOUTIER, THOMAS et al., 2010) for nondestructive three dimensional (3D) evaluation of bone architecture from the binarized volume of interest. The region of interest (ROI) in the cancellous bone was manually interpolated, beginning in the thinner slice (above the cartilaginous growth plate). A direct three-dimensional evaluation of trabecular bone parameters was performed in a ROI that consisted of a volume of interest (VOI) with 120 slices. The reconstruction and 3D quantitative analyses were performed with Skyscan software. The following 3D parameters in the defined VOI were analyzed: tissue volume (TV), bone volume (BV), percent bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), bone surface (BS), bone surface/volume ratio (BS/BV), bone surface density (BS/TV), trabecular pattern factor (Tb.Pf) and structure model index (SMI).

3 Results

Table 1 summarizes results of biometrical, consumption and biochemical determinations after the 8-weeks study period. A manifest option for sucrose was observed in the DR animals, when water and sucrose was given simultaneously *ad libitum* (Table 1). Body weight decreased significantly as a consequence of DR40% from week 1 to week 8 and final body weight of these animals was significantly lower in comparison with that of the Control group. During the experimental period diet chow consumption in animals from DRS group was 40.87% when compared with the Control group, however during the fasting no hyperphagia was observed. Interestingly, no difference was observed in biochemical or femoral (trabecular or cortical fractions) BMD (Table 2) from DRS animals, in spite of their lower body weight during the experimental period. Ultimate load (N) and toughness (N.mm) presented no difference in the femoral three bending point test on the mid-diaphysis. Only significantly ($P < 0.05$) higher stiffness (N/mm) was observed in the DRS group (239.79 ± 51.41 vs. 150.16 ± 41.28 N/mm in Control group).

Surprisingly results from micro-CT showed significant difference only in connectivity of trabecular bone (Table 3), in spite of the lower body mass during the experimental period. Tb.Pf is a connectivity index developed to calculate the index of concavity or convexity of the total bone surface. Concavity indicates connectivity, and convexity indicates isolated disconnected structures. As a result, lower Tb.Pf signifies better connected trabecular lattices and prevalence of enclosed cavities and concave surfaces pushed the Tb.Pf into negative values (Skyscan CT-analyzer software instructions). It should be noted that the concave surfaces of enclosed cavities represent negative convexity to the SMI parameter and regions of bone containing a prevalence of enclosed cavities can have negative SMI values.

The added sugar intake in relation to energy is, in general, higher among children than in other population groups, and generally, an energy intake and consumption of sugar-sweetened drinks above the limit of 10% could lead to

Table 2. Femoral BMD and cortical microhardness results from male Wistar rats (2 mo-old) submitted to a solid dietary restriction of 40% supplemented with 30% sucrose beverage for a period of 8 weeks.

Parameters	C Group	DRS Group
Whole femur (g/mm²)	0.217 ± 0.02	0.227 ± 0.01
Proximal epiphysis (g/mm²)	0.224 ± 0.31	0.233 ± 0.16
Distal epiphysis (g/mm²)	0.222 ± 0.06	0.254 ± 0.03
Diaphysis (g/mm²)	0.210 ± 0.03	0.217 ± 0.01
Periosteal Knoop hardness	54.04 ± 2.56	52.68 ± 5.10
Endosteal Knoop hardness	58.30 ± 3.15	45.75 ± 4.32*

Values are means ± SD. Differences were tested by Student's-*t* test.
* P< 0.05 versus Control.

Table 1. Biometrical, average weekly solid and liquid intake, and biochemical parameters from male Wistar rats (2 mo-old) submitted to a dietary restriction of 40% supplemented with 30% sucrose beverage (commercial solid food = 3.5 kcal/g; cane sugar = 4.0 kcal/g) for a period of 8 weeks.

Parameters	C Group	DRS Group
Initial body weight (g)	298.20 ± 17.4	297.86 ± 19.21
Final body weight (g)	458.20 ± 36.21	411.43 ± 27.48*
Food intake (g/wk/animal)	198.13 ± 10.63	81.35 ± 9.19*
Food intake (kcal/wk/animal)	693.48 ± 57.39	284.73 ± 32.19*
Water consumption (mL/wk/animal)	384.90 ± 59.08	127.87 ± 18.52*
Sucrose consumption (mL/wk/animal)		385.24 ± 47.73
Sucrose intake (g/wk/animal)		115.29 ± 14.34
Sucrose intake (kcal/wk/animal)		462.29 ± 57.27
Calcium (mg/dL)	9.33 ± 1.06	9.48 ± 1.03
Phosphorus (mg/dL)	7.59 ± 1.14	7.39 ± 0.62
Triacylglycerol (mg/dL)	62.14 ± 21.36	47.93 ± 17.06
Total cholesterol (mg/dL)	94.40 ± 21.77	86.31 ± 16.38
Fructosamine (µmol/L)	369.55 ± 9.89	357.99 ± 23.49

Values are means ± SD. Differences were tested by Student's-*t* test. * P < 0.05 versus Control.

Table 3. Micro-CT results from cancellous bone in distal femoral epiphysis of male Wistar rats (2 mo-old) submitted to a dietary restriction of 40% supplemented with 30% sucrose beverage for a period of 8 weeks.

Parameters	C Group	DRS Group
Tissue Volume (mm^3)	20.32 ± 2.39	21.22 ± 2.67
Bone Volume (mm^3)	6.32 ± 1.89	6.35 ± 1.79
% Bone Volume	30.60 ± 6.31	29.48 ± 4.66
Trabecular Thickness (μm)	95.98 ± 6.86	98.81 ± 8.75
Trabecular Number (mm^{-1})	3.17 ± 0.50	2.98 ± 0.39
Trabecular Separation (μm)	415.79 ± 75.11	355.46 ± 75.11
Trabecular Pattern Factor (mm^{-1})	–4.30 ± 4.25	–0.34 ± 3.84*
Structure Model Index	0.10 ± 0.57	0.62 ± 0.49*
Bone Surface (mm^2)	213.61 ± 47.13	217.41 ± 47.94
Bone Surface/Volume Ratio (mm^{-1})	34.66 ± 4.04	34.77 ± 3.19
Bone Surface Density (mm^{-1})	10.41 ± 1.30	10.15 ± 1.04

Values are means ± SD. Differences were tested by Student's-*t* test. * P< 0 .05 versus Control.

obesity, due to imprecise and incomplete compensation for energy consumed in liquid form (ERKKOLA, KRONBERG-KIPPILÄ, KYTTÄLÄ et al., 2009). However it has been well established that when animals are offered tasty and calorically rich diets high in fat and/or sugar, they overeat and eventually become obese. Although a variety of factors may influence the consumption of fat and/or sugar, palatability is thought to be an especially important one. Sweet taste, in particular, is a stimulus that promotes ingestion in animals, and investigators have reported that rats offered a sugar solution, in addition to chow and water, increase their caloric intake and body weight when compared with controls fed only chow (SCAFLANI and XENAKIS, 1984; NOVELLI, DINIZ, GALHARDI et al., 2007; LONDON, LALA, BERGER et al., 2007; CAO, GREGOIRE and GAO, 2009; IONOVA-MARTIN, DO, BARTH et al., 2010). In the results of the present study, dietary restriction significantly reduced body weight, although sucrose ingestion was high in the DRS group. Consistent with a previous study, the body mass of DRS animals was significantly lower than the *ad libitum* animals during the 56 days of the experiment (LAMBERT, LAMOTHE, ZERNICKE et al., 2005; WESTERBEEK, HEPPLE and ZERNICKE, 2008; LEVAY, TAMMER, PENMAN et al., 2010).

Biochemical parameters presented no difference related to increased consumption of beverages with low satiety but with impact on body weight and energy intake. Studies exploring the effects of beverage consumption on appetitive sensations have revealed that they are less satiating than solid foods. Fluids stimulate weak appetitive and compensatory dietary responses compared with energy-matched semisolid or solid items and findings also document a strong orosensory effect because oral-liquid stimulation led to more rapid gastric-emptying and orocecal transit times. The prevailing mechanisms linking sugar beverage intake to weight gain are low satiety of liquid calories and incomplete compensatory reduction in energy intake at subsequent meals, leading to an increase in total energy intake (VASANTI and HU, 2011; CASSADY, CONSIDINE and MATTES, 2012).

According to the results of the present study as regards body weight, solid food consumption and sucrose consumption, assuming that food volume or weight are salient satiety signals, the finding that a large volume of sucrose beverage elicits the same appetitive response as a much smaller solid load is evidence of the weak satiety value of the fluid (MATTES, 2006). It has been proposed that rats submitted to dietary restriction consumed sugar not because of its inherent palatability, but in order to alter their macronutrient balance (SCAFLANI and XENAKIS, 1984), and animals need to meet energy demands in sucrose (LEVAY, TAMMER, PENMAN et al., 2010). Beverages have lower expected satiety value, lower demand for oral processing, shorter gastrointestinal transit times and the energy they contain has greater inaccessibility and bioavailability. Each of these attributes has been associated with weaker effects on appetite and dietary compensation (MOURÃO, BRESSAN, CAMPBELL et al., 2007). This is evident in the DRS animals in this study, in which sucrose consumption was elevated during the absence of solid food. It has been suggested that food shortage results in an adaptive redirection of resources to somatic maintenance (LEVAY, TAMMER, PENMAN et al., 2010). Thus, it was conjectured that the animals from this group used calories/energy from high sucrose and low solid diet optimizing and maintaining their biological mechanisms of life, including bone metabolism. With reduced chow intake, animals on DR attempt to balance total caloric intake with high-sucrose consumption, equivalent to that of Control group fed solid chow.

Assessing the impact of caloric restriction on bone, as was done in other study (LAMBERT, LAMOTHE, ZERNICKE et al., 2005), the protocol of the present study included dietary restriction on 2 mo-old male Wistar rats with a moderate level of solid food restriction (40.87% in comparison with the Control) during a period that was not longer (8 weeks). Peak bone mass is achieved by approximately 10 months of age in rats, thus the male Wistar rats in the present study grew throughout the study. In fact, in this study the DRS animals continued to gain body mass during the restriction period, although at a significantly lower rate in comparison with the control animals. Another study pointed out that caloric restriction had an age-dependent relationship between the time of initiating restriction and bone adaptation (WESTERBEEK, HEPPLE and ZERNICKE, 2008). It is possible that an early adaptive response related to growth and high caloric

beverage compensation in the young rats confers protection from the deleterious effects of dietary restriction on bone, and it is possible the DRS femora grew to accommodate the accumulating low weight resulting in bone quality similar to that of the Control group (LAMBERT, LAMOTHE, ZERNICKE et al., 2005; IONOVA-MARTIN, DO, BARTH et al., 2010).

Chronic undernutrition during the time of peak bone mass accrual, as seen in anorexia nervosa, is associated with low bone mass, increased fracture risk, and high rates of osteoporosis (DEVLIN, CLOUTIER, THOMAS et al., 2010). However, high caloric beverage consumption in the DRS animals in the present study positively influenced bone mineral density, bone periosteal microhardness and trabecular bone microstructure viewed in micro-CT. Although energy restriction resulted in bone loss at most sites and trabecular bone appears to be especially vulnerable (HAWKINS, CIFUENTES, PLESHKO et al., 2010), the micro-CT results from the present study are able to support the idea that energy from consuming a high-sucrose beverage balanced the low intake of solid food by the DR animals. In addition, cortical bone reacted positively to dietary restriction supplemented with sucrose, evidenced by biomechanical/DXA parameters measured in the cortical mid-diaphysis.

Body weight, per se, is a relatively poor predictor of total body bone mineral content whereas body composition, expressed either as percent fat or relative muscle mass, is a more robust determinant of whole body bone mass (HAMRICK, DING, PONNALA et al., 2008). One supposed that 30% sucrose supplemented beverage affected these soft tissues influencing the mid-diaphyseal cortical bone compartment. Corticosterone and insulin, involved in the high consumption of sucrose, play complex roles in the amount and composition of calories ingested, and the use and deposition of this energy, determining the intake, form, and compartmentalization of energy both independently and interactively (WARNE, AKANA, GINSBERG et al., 2009). On the hand, both leptin and insulin-like growth factor-1 are essential for normal skeletogenesis and starvation, as in young women with anorexia or other undernutrition conditions, decreases levels of these nutritionally dependent hormones during peak skeletal acquisition, contributing to low bone mass (DEVLIN, CLOUTIER, THOMAS et al., 2010). Thus, others studies are necessary to elucidate how these mechanisms could be involved in dietary restriction.

4 Conclusion

This study supported the hypothesis that structural and densitometric characteristics of femoral cortical and trabecular bone from a 40% restriction of solid food consumption in male Wistar rats is balanced with a 30% sucrose beverage and there is reason to suspect that DR supplemented with sucrose has beneficial effects during skeletal growth.

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