FREE ACTIVITY IN THE CAGE ASSOCIATED WITH BODY WEIGHT AND RESTORATION OF BONE STRUCTURAL AND MECHANICAL PROPERTIES IN GROWING RATS AFTER HINDLIMB UNLOADING

ATIVIDADE LIVRE NA CAIXA ASSOCIADA AO GANHO DE PESO E RESTAURAÇÃO DAS PROPRIEDADES ESTRUTURAIS E MECÂNICAS DE OSSO EM RATOS JOVENS APÓS HIPOSINESIA DE MEMBROS PÉLVICOS

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ABSTRACT: We investigated the importance of daily free activity in the cage and body weight gain during the recovering of bone structural and mechanical properties in growing rats after hindlimb unloading. Eight-week-old male Wistar rats were randomly divided into control (CG, n=24) and suspended (SG, n=24) groups. Animals from SG underwent a four-week hindlimb unloading period by tail-suspension. Animals from CG and those from SG after release were kept in collective cages and sacrificed at the age of 12, 16 and 20 weeks. Both femurs were removed and its area, bone mineral density (BMD), resistance to failure and stiffness were determined. Four-week hindlimb unloading decreased ($p<0.05$) body weight (CG, 373.00 ± 9.47 vs. SG, 295.86 ± 9.19 g), BMD (CG, 0.19 ± 0.01 vs. SG, 0.15 ± 0.01 g/cm^2), bone resistance to failure (CG, 147.75 ± 5.05 vs. SG, 96.40 ± 5.95 N) and stiffness (CG, 0.38 ± 0.01 vs. SG, 0.23 ± 0.02 N/m). Eight weeks of free activity in cage recovered ($p>0.05$) the body weight (CG, 472.75 ± 14.11 vs. SG, 444.75 ± 18.91 g), BMD (CG, 0.24 ± 0.01 vs. SG, 0.22 ± 0.01 g/cm^2), bone resistance to failure (CG, 195.73 ± 10.06 vs. SG, 178.45 ± 8.48 N) and stiffness (CG, 0.56 ± 0.02 vs. SG, 0.47± 0.03 N/m) of SG animals. Body weight correlated strongly with bone structural and mechanical properties ($p<0.0001$). In conclusion, free activity in the cage associated with body weight gain restored bone structural and mechanical properties in growing rats after hindlimb unloading.

KEYWORDS: Osteopenia. Bone mineral density. Bone stiffness

INTRODUCTION

Disuse osteoporosis a bone loss caused by reduced mechanical loading of the skeleton is an important clinical problem for patients with either marked weight loss (e.g. bariatric surgery) or chronic immobilization (e.g. long-term hospitalization). In humans and most animals, disuse leads to compromised bone architecture, loss of mineral density, reduced bone mechanical properties, and consequently, increased risk of bone fracture (KANIS; ODEN; JOHNELL, 2001; PEREIRA et al., 2007; MCGEE-LAWRENCE; CAREY; DONAHUE, 2008).

During growth the regulation of bone formation is governed by genetic and hormonal factors. However, also local factors such as mechanical stimulation from weight bearing and daily physical exercise can modulate the development of bone and influence the rate of growth and the shape, size, strength, and final anatomy of the musculoskeletal system in humans and animals (NORDSTRÖM; HÖGSTRÖM; NORDSTRÖM, 2008; IWANIEC et al., 2009).

Rodent partial immobilization by tail suspension, originally a model of simulated hypogravity, induces both cortical and cancellous bone loss in the hindlimbs (VICO et al., 2001; DAVID et al., 2006) and is widely used in the research field of bone remodeling. Studies on remobilization strategies (i.e. exercise) using this model are normally designed with a control group composed of animals being released free in the cage after a period of tail suspension while those animals from the test groups are submitted to the proposed treatments (e.g. JU et al., 2008; SHIMANO;

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VOLPON, 2009). Rats are physically active, exercise more voluntarily and gain body weight during growth which may affect bone remodeling (IWANIEC et al., 2009; ISAKSSON et al., 2009). Thus, it is important to address the influences of the animals' daily exertion in the cage and body weight gain on the bone structural and mechanical parameters of growing rats.

The aim of this study was to determine, through a longitudinal follow-up, the importance of daily free activity in the cage and body weight gain during the recovery of compromised bone structural and mechanical properties in growing rats after hindlimb unloading using the tail suspension model. We hypothesized that daily free activity in the cage and body weight gain would be sufficient enough to restore the impaired bone structural and mechanical properties induced by tail suspension of rats during growth.

MATERIAL AND METHODS

Young male Wistar rats were eight weeks old and weighed on average 320 g at the onset of the experiment. Animals were randomly divided into two experimental groups: Control (CG, n=24) or Suspended (SG, n=24). Animals from SG were submitted to four weeks of tail suspension as previously described (SHIMANO; VOLPON, 2009). Briefly, animals were anesthetized (30 mg/kg ketamine and 3 mg/kg xylazine), the tail was cleaned with liquid soap, dried, and then received povidine solution, and a layer of adhesive foam (Reston; 3M, Brasil) covering the proximal two thirds of the tail. An elastic tape was adhered over the foam, and in addition a cotton plait was created to attach the tail to the top of the cage. The rat could bear weight on its forelimbs and rotate 360° and move around to reach water and food, but without any hindlimb contact with the bottom or walls of the cage.

Animals from CG and those from SG after their release from suspension (12 weeks old) were housed in polypropylene cages (33 x 40 cm - four animals per cage) with a floor area of 1320 cm^2 during the experimental period. All animals were kept in a temperature-controlled room (23 ± 1°C) with 12-h light–dark cycles and were provided with standard rat chow (containing 1.2% calcium and 0.74% phosphorus) and water *ad libitum*.

The study protocol and all procedures involving animals were in compliance with the Federal University of Viçosa Animal Care and Use Committee rules and regulations contained in their Ethical Principles of Animal Experimentation and were in accordance with nationally accepted ethical principles concerning the care and use of laboratory animals and the Declaration of Helsinki.

Eight animals of each experimental group were euthanized per time-point (12, 16 and 20 weeks of age), the femurs removed, cleaned of adherent muscles and other tissues, frozen in saline solution at –20 °C until analysis. This treatment procedure has been shown not to affect the biomechanical properties of the bone (PAJAMÄKI et al., 2008).

The femurs were scanned using dual energy X ray absorptiometry (DEXA) to determine bone mineral density (BMD) and content (BMC). The cleaned bones were thawed at room temperature (23 °C), placed in a plexiglass container filled with deionized water and scanned using the Lunar DPX Alpha (Madison, USA) with small-animal software coupled to a computer. The bones were measured after selecting specific area of interest (whole bone) that was defined by drawing a box around the selected area.

Following the BMD measurements the bones were then placed on a computer-controlled EMIC universal testing machine (DL 3000; São José dos Pinhais, Brazil), with a 2000 N load cell (speed of 5 mm/min). For the three-point bending of the femoral shafts, the femurs were placed on their posterior surface. The first support was placed just distal to the trochanter minor, and the second just proximal to the condyles of femur. Then a bending load using a brass crossbar was applied to the midshaft perpendicularly to the long axis of the bone until the failure of the specimen. The measured data were converted to a load–displacement curve. Breaking resistance was defined as the measured bending load at failure. Stiffness was calculated from the slope of the linear (elastic) part of the load–displacement curve (ISAKSSON et al., 2009; BASSO; BELLOW; HEERSCH, 2005).

The Kolmogorov-Smirnov test for normality was initially performed for all properties. Body weights, structural and mechanical properties at different stages were compared by one-way repeated measures analysis of variance (ANOVA). The t-test was used to compare control vs suspended groups. The Tukey test was applied to compare pairs of groups when a statistical difference was detected. Additionally, Pearson correlation coefficients were used to highlight relationships between body weight, structural and mechanical parameters. The level of significance adopted in all comparisons was 5%. Data analysis was performed using the statistical software program SigmaStat version 3.0 (SPSS, USA).

RESULTS

At the onset of the experiment the initial body weight of animals from SG and CG were not statistically different (327.06 ± 9.87 g and 317.83 ± 11.59 g, respectively, P > 0.05). After four weeks of tail suspension rats from SG lost body weight as compared to CG (9, 54%, P < 0.05, Fig. 1A). Meanwhile animals from CG gained body weight (17.37 %). However, after four weeks of release and free activity in the cage rats from SG gained body weight (32.45 %) and those from CG kept increasing body weight (13.57 %), which was still higher than that of SG. By the end of the experiment, at the age of 20 weeks (eight weeks after release in the cage) the body weight of animals from SG was not statistically different from that of CG.

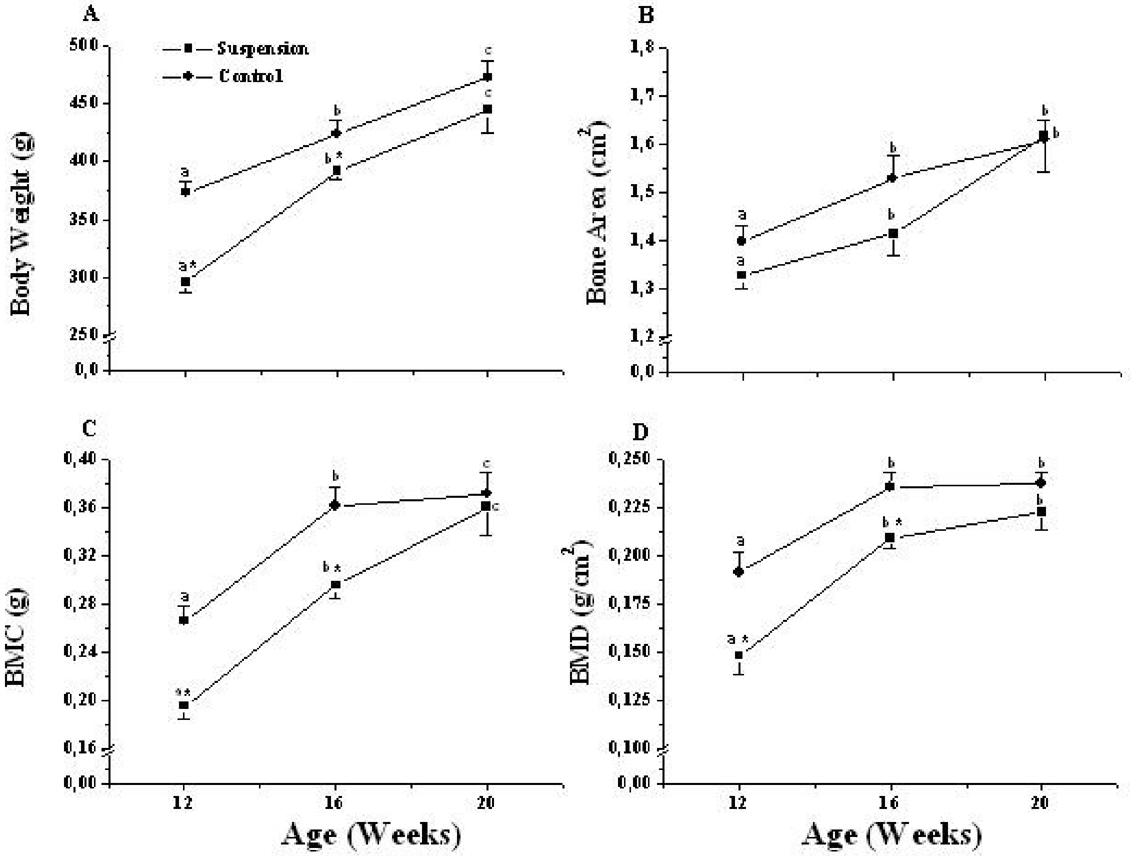

Figure 1. Body weight (A), bone area (B), bone mineral content (C) and bone mineral density (D) of femurs from control and suspended rats. BMC, bone mineral content. BMD, bone mineral density. *, denotes statistically significant difference (P<0.05) from control group for the same age. Different letters denotes statistically significant difference between ages for the same group. Data are mean ± SEM.

Four weeks of tail suspension did not affect bone area (Fig. 1B). After four weeks of release from tail suspension (16 week-old rats) bone area did not change in SG while in CG it increased by 9.44 %. From week 16 to 20, bone area increased significantly in SG (14.57 %) only. At the age of 20 weeks bone area was similar in animals from both SG and CG groups.

After four weeks of tail suspension animals from SG showed lower BMC (Fig. 1C) and BMD (Fig. 1D) compared to those of CG. Then, four weeks after release and free activity in the cage 16 week-old rats from SG showed increased BMC (51.28 %) and BMD (41.22 %), but it was still lower compared to those of CG. During this period rats from CG also showed increased BMC (35.71 %) and BMD (23.56 %). After eight weeks of release BMC and BMD increased in rats (20 week-old) from both CG and SG returning to control levels in SG animals.

Four weeks of tail suspension was sufficient enough to significantly reduce bone mechanical properties in growing rats. Animals from SG showed lower femoral resistance to failure (- 34.75 %, Fig. 2A) and stiffness (- 39.58 %, Fig. 2B) compared to CG at the age of 12 weeks. After four weeks of release and free activity in the cage bone resistance and stiffness increased significantly in CG only (25.33 % and 28.38 %, respectively) and were still higher in CG compared to SG (16 week-old). By the end of the experiment (eight weeks of release) the femoral resistance and stiffness of animals from SG were restored to control levels.

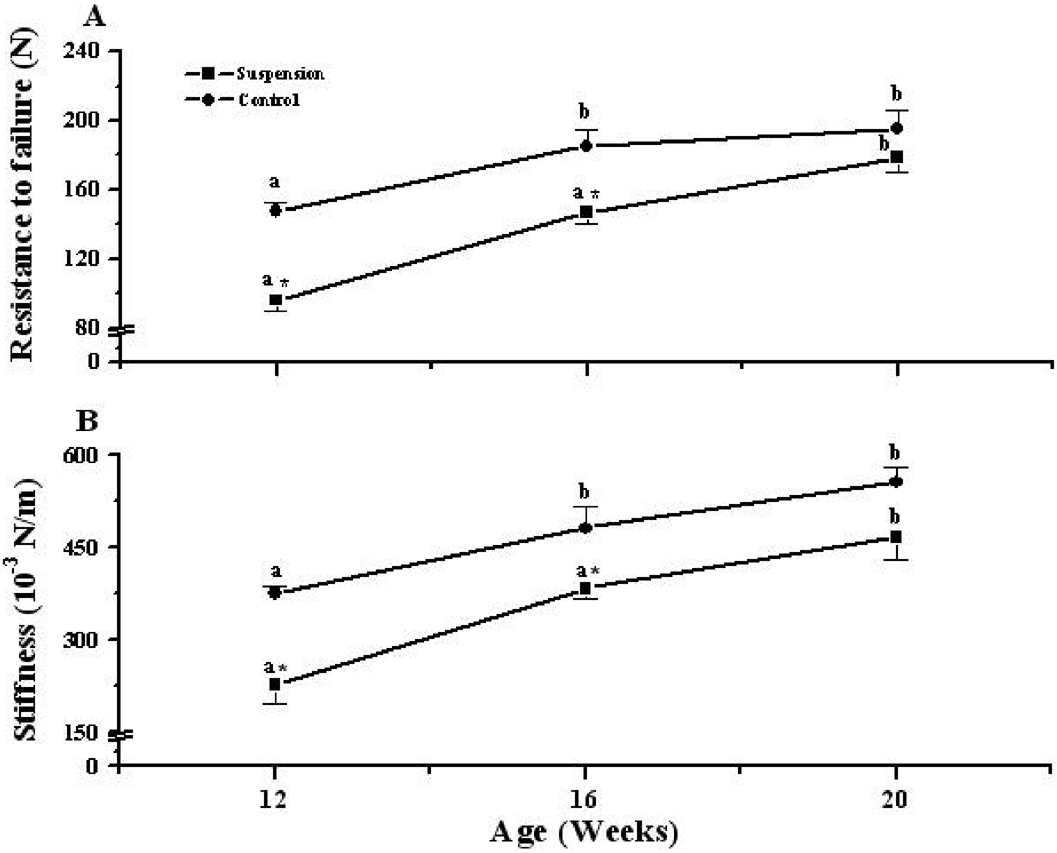

Figure 2. Resistance to failure (A) and stiffness (B) of femurs from control and suspended rats. BMC, bone mineral content. BMD, bone mineral density. *, denotes statistically significant difference (P<0.05) from control group for the same age. Different letters denotes statistically significant difference between ages for the same group. Data are mean ± SEM.

Associations between BM, femoral structural and mechanical properties were investigated. High correlations were found between BM and bone properties: the correlation between BM and BMD for all animals was $r^2 = 0.81$ (P<0.0001). There were also strong correlations between BM and femoral resistance to failure ($r^2 = 0.85$, P<0.0001) and bending stiffness ($r^2 = 0.80$, P<0.0001); and between BMD and femoral resistance to failure ($r^2 = 0.85$, P<0.0001) and bending stiffness ($r^2 = 0.80$, P<0.0001).

DISCUSSION

Our data showed that daily free activity in the cage associated with body weight increase was sufficient enough to restore the structural and mechanical properties of the femur of growing rats submitted to four weeks of hindlimb unloading.

The hindlimb unloading method used by our group led to significant reduction in bone area, mineral content and hence BMD. In fact, limited weight bearing causes bone loss as in the case of spaceflight (MOREY-HOLTON et al., 2000). The reduction of mechanical loading results in diminished bone formation and leaves the bone re-absorption activity temporarily without opposition to deposition (MOREY-HOLTON, GLOBUS, 2002; CHOW et al., 1998).

Four weeks of hindlimb unloading also impaired the mechanical properties of the femur of young rats. Suspended rats had the bone resistance to failure and stiffness decreased by 34.75 and 39.58 %, respectively. It has been reported that a combination of bone geometry deterioration and reduction of mineral content decreases the whole-bone strength and stiffness (JU et al., 2008; DONNELLY et al., 2010). In the present study the mechanical properties correlated strongly with BMD.

The bone structural and mechanical impairment observed in suspended rats was restored after eight weeks of release in the cage. The animals were allowed to move freely in the cage which involves reloading the hindlimbs and thus promoting the increase in bone formation and hence mechanical function. It is noteworthy that in the present study the suspended animals were housed in collective cages after release. Group housing affords physical-social interaction between animals, which may be sufficient to physically load the skeleton and promote the increase in bone formation in young rats (MOREY-HOLTON et al., 2000). The mineral phase of bone controls the stiffness, whereas the collagen is believed to contribute to the ultimate strength and toughness of bone (BOIVIN et al., 2008). Although we have not measured collagen, bone mineral and collagen matrix is affected by increased mechanical stimulation as a result of exercise, especially during growth and maturation (ISAKSSON et al., 2009).

In addition, suspended animals recovered the body weight to the levels of control animals after release. Such increase in body weight also correlated strongly with BMD, resistance to failure and stiffness. Bone mass is also correlated with body weight in growing mice (IWANIEC et al., 2009), but the mechanisms for the positive relationship between body weight and bone mass are not clear. Increased skeletal loading may be a contributing factor however obese individuals often have increased bone mass at non-weight bearing as well as weight bearing sites (KUWAHATA et al., 2008). Also, ob/ob mice, in spite of being morbidly obese have smaller bones with locally increased trabecular density than their lean wild type counterparts (HAMRICK et al., 2004). Thus, the specific contributions of body weight on bone balance remain unclear.

The animals' body weight reduction after suspension observed in the present study is in accordance with other studies (SHIMANO; VOLPON, 2009) meaning that it is unlikely that any of the animals completely adapted to suspension. Morey-Holton and Globus (2002) noted that body weight is an important parameter that reflects the health of the rat, and stated that weight loss not exceeding 10% is an acceptable limit (9.54 % in the present study). The observed weight loss in the present study may be secondary to decreased food intake (not recorded) or to a stress state (not measured) in response to tail suspension. In the present investigation, we used still immature adult rats (mean body weight of 320 g) as shown by the gain in body weight with time and increased bone resistance to failure and stiffness with age in the control group. At the end of the suspension period, the animals in the current study recovered the body weight, thus indicating that they were able to spontaneously adapt after the suspension period (WESTERLIND et al., 1998). Unfortunately we did not measure stress surrogates such as corticosterone and it is known that it has strong influence on bone metabolism. Therefore, we take this lack of data as a study limitation inasmuch as feeding behavior, stress and hence hormonal regulation might have also influenced bone characteristics.

In conclusion, hindlimb unloading reduces bone mineral density, resistance to failure and stiffness in rats which was restored by free activity in the cage associated with body weight increase. These finding highlight that unscheduled exercise associated with weight bearing is able to restore the bone structural and mechanical properties loss arising from the lack of use and that rehabilitation programs may be established regardless the use of expensive equipment.

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RESUMO: Investigou-se a importância das atividades diárias na caixa e o ganho de peso durante a recuperação das propriedades ósseas estruturais e mecânicas em ratos jovens após hiposinesia de membros pélvicos. Ratos Wistar com oito semanas de idade foram divididos em grupos controle (CG, n=24) e suspensos (SG, n=24). Animais do SG permaneceram quatro semanas suspensos pela cauda. Animais do CG e aqueles do SG após a liberação foram alojados em caixas coletivas e sacrificados com 12, 16 e 20 semanas de idade. Foram mensuradas área, densidade mineral (DMO), resistência de fratura e rigidez do fêmur. Quatro semanas de hiposinesia reduziu ($p<0.05$) o peso corporal (CG: 373,00 ± 9,47 vs. SG: 295,86 ± 9,19 g), DMO (CG: 0,19 ± 0,01 vs. SG: 0,15 ± 0,01 g/cm^2), resistência de fratura (CG: 147,75 ± 5,05 vs. SG: 96,40 ± 5,95 N) e rigidez óssea (CG: 0,38 ± 0,01 vs. SG: 0,23 ± 0,02 N/m). Oito semanas de atividade na caixa recuperou ($p>0.05$) o peso corporal (CG: 472,75 ± 14,11 vs. SG: 444,75 ± 18,91 g), BMO (CG: 0,24 ± 0,01 vs. SG: 0,22 ± 0,01 g/cm^2), resistência de fratura (CG: 195,73 ± 10,06 vs. SG: 178,45 ± 8,48 N) e rigidez óssea (CG: 0,56 ± 0,02 vs. SG: 0,47± 0,03 N/m) do SG. Peso corporal correlacionou fortemente com as propriedades ósseas, estrutural e mecânica, (p<0.0001). Concluiu-se que a atividade livre na caixa associada ao ganho de peso restaurou as propriedades ósseas estruturais e mecânicas em ratos jovens após hiposinesia dos membros pélvicos.

PALAVRAS-CHAVE: Osteopenia. Densidade mineral óssea. Rigidez óssea.

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