Original article

Bone structure, strength and microhardness resulting after hindlimb unloading in rats

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Abstract

Introduction: Bone strength is influenced by a number of different determinants, such as mass, size, geometry and also by the intrinsic material properties of the tissue. Aims: The structure and mechanical properties of the femur were analyzed in aged (14 mo-old) animals submitted to hindlimb unloading (HU) for 21 days. Methods: Twenty Wistar rats were randomly divided into Control and HU groups and the femur was submitted to dual X ray absorptiometry (DXA), DIGORA radiographic density, mechanical compression testing and Knoop microhardness analyse in cortical and cancellous bone. Results: Femurs from HU group presented significantly lower failure load, decreased bone mineral density (BMD)/bone mineral content (BMC) in whole bone; proximal/distal epiphysis and middiaphyseal cortical bone measured by DXA were similar in the two groups; radiographic density from areas in proximal epiphysis was significantly lower in HU group, and microhardness measured at periosteal and endosteal levels were also significantly diminished in HU compared with Control group. Conclusion: Disuse induced damage in the trabecular femoral bone architecture with decisive effect on the head and trochanteric fossa, which became weaker. Bone diaphyseal cortical hardness also suffered effect of unloading, probably related to osteocyte/osteoblast activity.

Keywords: rat femur, mechanical strength, bone densitometry, bone microhardness.

1 Introduction

Aging is associated with decline in bone and muscle mass affecting their strength and bone density. Osteopenia has far-reaching consequences on the ability of the elderly to perform daily living tasks. Moreover, they are often subjected to periods of inactivity, such as bed rest. Evidences suggest that the increased bone fragility with aging is multifactorial and includes decreased bone mineral density, changes in bone microarchitecture and strength (PERRIEN, AKEL, DUPONT-VERSTEEGDEN et al., 2007) Osteoporosis is primarily a disease of bone fragility resulting from decreased bone mass and bone quality and it is widely recognized that loss of bone strength is accompanied by a corresponding rise in risk for osteoporotic hip fracture that rises exponentially with aging (TURNER, HSIEH, MÜLLER et al., 2001; WANG, BANU, McMAHAN et al., 2001). Bone strength is influenced by a number of different determinants, such as mass, size, geometry and also by intrinsic material properties of the tissue (BOIVIN, DOUBLIER, FARLAY et al., 2008). Bone mass is regulated through remodeling, involving endocrine/systemic regulators, and osteoporosis is a bone remodeling disease (DUCY, AMLING, TAKEDA et al., 2000). Osteocytes orchestrate bone remodeling, regulating both osteoblast and osteoclast activities, including in osteoporosis (TETI and ZALLONE, 2009).

The skeleton has evolved to adjust its bone mass and architecture in response to load. Loading increases bone mass but unloading results in bone loss. The complex lacunocanalicular network connecting all of the osteocytes within bone and cells on the bone surface supports the idea that these cells can sense loading on the skeleton or its absence and then translate those signals to biochemical signals of resorption or formation (BONEWALD, 2008; BONEWALD and JOHNSON, 2008). The size, shape and strength of bones are the result of activity of bone effector cells of the osteoblast and osteoclast lineages. While some aspects of local activity in the skeleton might be attributed to recognition of environmental disturbances, such as mechanical strains and mineralization level, others might involve signals generated by other cell types with sentinel functions in bone, and the osteocyte is a candidate for such a cell type (NOBLE, 2008).

The strength of bone is determined by its material composition and structure and bone must be stiff and able to resist deformation, and must also be flexible to absorb energy (SEEMAN and DELMAS, 2006). Microhardness assesses the resistance of a material to deformation, and has been used to investigate the structural and mechanical characteristics of bone on a microstructural scale, associating the degree of hardness and mineralization (BOIVIN, DOUBLIER, FARLAY et al., 2008; ZISSET, 2009). According to prevailing understanding, the feedback control system of bone rigidity perceives the incident loading-induced strain distribution within the bones and subsequently removes bone tissue from sites where the strains are marginal, while it

forms new bone tissue at sites subjected to increased strains (PAJAMÄKI, SIEVÄNEN, KANNUS et al., 2008). To understand the effects of disuse and the mechanism of age-related bone loss and bone mineralization in male animals, the purpose of this study was to evaluate the response of skeletally aged rats to hindlimb unloading.

2 Material and methods

The study protocol and all procedures involving animals were in compliance with the São Paulo State University/ Araçatuba School of Dentistry Animal Care and Use Committee rules and regulations contained in their Ethical Principles of Animal Experimentation.

2.1 Animals

Male Wistar rats (14-mo-old) were housed in a temperature-controlled room (22 ± 1 °C) with a 12/12 hour light/dark cycle. Animals were provided with standard rat chow (containing 1.2% calcium and 0.74% phosphorus) and water *ad libitum*. The animals (Control group n = 10 and HU n = 10) were assigned to plastic cages (3 rats/cage). Unloading of the hindlimbs was achieved by tail suspension and there was no increase in loading of the forelimbs (CARVALHO, LOUZADA and RISO, 2007). Animals were anesthetized with a ketamine (0.07 mL.100 g^{-1}) – xylazine (0.03 mL.100 g^{-1}) cocktail, their tails were cleaned with liquid soap, dried, and then received povidine solution, and a layer of adhesive foam (Espuma Reston; 3M, Brasil) covering the proximal two thirds of the tail (the distal third was surgically removed). An elastic tape was adhered over the foam, and in addition to this a cotton plait was created to attach the tail to the top of the cage. The animal's forelimbs remained in contact with the cage floor, allowing the animal full access to the individual cage. On day 21 of suspension the animals were killed, the femurs were removed, cleaned of adherent muscles and others 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, SIEVÄNEN, KANNUS et al., 2008).

Before being sacrificed, the animals were weighed and anesthetized, and 8-10 mL of blood was collected from the abdominal aorta, put into a centrifuged heparinized tube. The plasma was separated by centrifugation (5000 rpm/10 minutes) and frozen at –80 °C. Calcium, phosphorus, total proteins and creatine phosphokinase (CPK) concentrations were determined in accordance with the manufacturer's instructions (Labtest kits, São Paulo, Brasil).

2.2 Densitometry

Right femurs were scanned using dual energy X ray absorptiometry (DXA) 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 regions of interest (whole bone, middiaphyseal area, proximal and distal epiphysis) that were defined by drawing a box around the selected area.

After DXA measurement, the right femur was put on imaging plates and with a GE Mobile 100 X ray (Milwaukee, USA), 50 kV, 10 mA, 40 cm focal-film distance, 0.2 seconds exposure time, radiographs were obtained and scanned in a DIGORA digital imaging system (Soredex, Tuusula, Finland) coupled to a computer. Standardized femoral epiphyseal and diaphyseal areas were determined in the bone image on a computer monitor to obtain the density.

2.3 Mechanical testing

Structural and material properties of the femoral head were determined by destructive mechanical compression testing. The bones were slowly thawed at room temperature at least 12 hours before actual mechanical testing, kept wrapped in saline-soaked gauzes except during measurements, and 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). The distal two-thirds of the right femoral epiphysis was placed vertically into a specially constructed round metal tube (5.0 cm long, 2.7 cm wide), fixed in the center of the tube with six adjustable screws and the femoral neck was tested with a downward vertical load applied parallel to the bone long axis, on the top of the femoral head, using a small concave steel cup at its end (PAJAMÄKI, SIEVÄNEN, KANNUS et al., 2008), until fracture.

2.4 Microhardness

Fresh femoral cortical middiaphyseal bone and cancellous head bone was cut perpendicular to the bone long axis and embedded under pressure (150 kgf) and temperature in a thermoplastic mounting powder (Extec, Enfield, USA). The bone surface was ground and polished with grinding paper (400, 600, 800, 1200 grit abrasive paper, Buehler Paper Discs, Lake Bluff, USA) and with diamond suspension (0.25 μm, Metadi Diamond Suspension, USA). Tests were performed with a Shimadzu (HMV-2000; Kyoto, Japan) microhardness tester equipped with a Knoop indenter, a 25 g load and a dwell time of 10 seconds (cortical bone), and a 5 g load and dwell time of 10 seconds (cancellous bone), measured at 50× magnification (CAMS-WIN, NewAge, USA). Indentations were made at a distance of 100 and 300 μm from the periosteal surface in cortical and cancellous bone. On the diaphyseal cortical bone 20 indentations (separated by a distance of ± 120 μm), and on the femoral head cancellous bone 12 indentations (separated by a distance of ± 150 μm) were made in the sample. Knoop microhardness (HK) was expressed as mean of these measures.

Data are presented as the mean ± SD. The Student's-*t* test was used to compare the means.

3 Results

There was a significant ($p < 0.0001$) difference between final body weight of Control group (543.30 ± 28.84 g) and HU group (446.60 ± 53.64 g) at sacrifice. During the twenty one days of suspension the body weight of the animals diminished by 13.10%.

The differences in biochemical measures (calcium, phosphorus and total proteins) were not significant between groups, except ($p < 0.05$) for creatine phosphokinase (Control group 532.99 ± 339.11 and HU group 208.01 ± 142.52).

DXA measures showed no differences in BMC/BMD (Table 1) between the groups. Mechanical test also presented significantly decreased results in HU group (Table 1). Radiographic densitometry from HU group compared with Control group (Table 2) presented significantly lower maximum, minimum and median density measurements in the proximal epiphysis regions (head, trochanteric fossa and trochanter), reflecting their bone composition and architecture. Microhardness of trabecular bone was shown to be lower than cortical bone and results of cortical and cancellous bone sites (Table 3) showed lower measurements in HU animals than Controls, near the periosteal and endosteal surfaces, also expressing differences in bone material composition/mineralization.

4 Discussion

Hindlimb unloading significantly affected the metabolism of animals submitted to tail suspension. Decreased body weight in the HU group can be attributed to diminished appetite, change to individual cage during the suspension period, the size of the cage and stress caused by the disuse

Table 1. DXA and biomechanical parameters in the femurs.

Groups	Control	HU
Whole bone area (mm^2)	2.052 (0.110)	1.937 (0.019)
Whole bone BMC (mg)	507 (37)	480 (66)
Whole bone BMD ($mg.cm^{-2}$)	247 (12)	247 (19)
Proximal epiphysis BMC (mg)	124 (10)	117 (13)
Proximal epiphysis BMD ($mg.cm^{-2}$)	246 (20)	233 (27)
Diaphysis BMC (mg)	102 (7)	107 (11)
Diaphysis BMD ($mg.cm^{-2}$)	256 (19)	267 (20)
Distal epiphysis BMC (mg)	129 (6)	130 (13)
Distal epiphysis BMD ($mg.cm^{-2}$)	257 (3)	260 (20)
Maximum load to failure (N)	192.33 (46.71)	146.98* (46.43)
Stiffness ($N.mm^{-1}$)	296.92 (116.00)	180.92* (120.71)
Tenacity ($N.mm^{-1}$)	92.43 (52.61)	52.29* (18.19)

BMC - bone mineral content; BMD - bone mineral density. Difference from control: *$p < 0.05$.

Table 2. Results of DIGORA radiographic density.

Groups		Control	HU
Femoral head (34 × 34 Pi)	Mx	208.20 (6.08)	203.90* (2.16)
	Mn	166.20 (4.05)	155.00* (11.64)
	Me	189.62 (2.62)	185.40* (3.05)
Trochanteric fossa (20 × 30 Pi)	Mx	193.50 (6.09)	186.27* (4.05)
	Mn	164.10 (4.45)	157.63* (4.29)
	Me	177.48 (2.43)	171.95* (3.75)
Trochanter (30 × 30 Pi)	Mx	206.60 (10.02)	198.72 (3.13)
	Mn	162.10 (7.39)	155.90* (7.86)
	Me	183.19 (4.25)	178.74* (4.12)
Cortical diaphysis (100 Pi)	Mx	204.30 (4.30)	204.36 (3.50)
	Mn	187.90 (4.53	188.09 (7.06)
	Me	196.17 (4.19)	195.92 (5.20)
Distal epiphysis (60 × 60 Pi)	Mx	222.40 (3.02)	219.40 (4.20)
	Mn	172.90 (5.76)	169.70 (7.86)
	Me	197.91 (3.45)	196.17 (4.35)

Pi - pixels; Mx - maximum density; Mn - minimum density; Me - median density. Difference from control: *$p < 0.05$.

Table 3. Results of Knoop microhardness in cortical bone and cancellous bone measured from the periosteal surface.

Groups	Control	HU
Periosteal cortical bone	62.31 (3.82)	56.64* (2.75)
Endosteal cortical bone	65.43 (4.63)	48.11* (15.09)
Periosteal cancellous bone	33.78 (3.99)	27.12* (3.32)
Endosteal cancellous bone	35.15 (5.33)	30.99 (4.84)

Difference from control: *$p < 0.05$.

model (CARVALHO, LOUZADA, RISO et al., 2007). On the other hand, no differences between groups were observed for calcium, phosphorus and total protein concentrations, except for the CPK, which could express fragility in mass and strength of hindlimb muscles that can perform dynamic contractions without load.

DXA from the femur areas showed no differences with regard to BMC and BMD, probably related to the information on the amount of bone, but it does not elucidate trabecular structure and its importance in maintaining bone integrity and mechanical strength (PULKKINEN, JÄMSÄ, LOCHMÜLLER et al., 2008). DXA may not be sensitive enough to reflect differences, because DIGORA measurements of bone density from the proximal epiphysis were significantly lower in cancellous bone (head, trochanteric fossa and trochanter). The results of the present study also showed higher difference between maximum and minimum density in the femoral head region when the groups were compared, showing evidence of altered bone structure or material composition. This demonstrates that more X ray photons reach the imaging plates and that there could be an increase in trabecular separation associated with small trabecular thickness and/or trabecular number (IWASAKI, YAMATO, MURAYAMA et al., 2002; PIETCHSMANN, SKALICKY, KNEISSEL et al., 2007). Conversely, the small maximum-minimum differences observed in the density of the cortical diaphysis reflected its material composition and structure/architecture when cortical and cancellous bone were compared.

Based on the results of the present study, cancellous bone mineral density and bone volume were significantly decreased in the proximal epiphysis, affecting the architecture of cancellous bone in male animals of the HU group without alteration in middiaphyseal cortical bone mineral density. According to Rittweger, Simunic, Bilancio et al. (2009) immobilization-induced bone losses are found to be larger in regions rich in trabecular bone than from regions rich in compact or cortical bone, and in seeming agreement bed rest-induced bone losses seem to be three or four times larger from the epiphysis than from the tibia shaft. This is very important for understanding the present results of biomechanical compression testing of femoral head in the HU group, with significantly decreased load to fracture, reduced stiffness and capacity to absorb energy (toughness). It is supposed that the proximal epiphysis presented a similar aspect to that of the microtomographic results of Pietchsmann, Skalicky, Kneissel et al. (2007), who observed reduced bone volume (71%) in aged animals (23 mo-old), related to substantial decrease in trabecular number and the loss of trabecular elements with aging, resulting in increased trabecular separation associated with increased structure module index.

The material properties of bone control, its strength as a tissue, and studies investigating the structure and properties use mechanical testing to obtain strength, stiffness and toughness values. The strength of a whole bone depends on the amount of bone tissue and on its outside diameter, shape, cortical thickness and the distribution of cortical and trabecular bone (FROST, 1997; RITCHIE, KOESTER, IONOVA et al., 2008). The present study results demonstrated the significantly lower load necessary to fracture the hip at the head neck in HU animals, which reflected changes in the stiffness and in the ability of this region to absorb energy (toughness), probably because its bone structure/architecture was decisively affected by unloading. In HU, the proximal femoral epiphysis with alterations in its stiffness and energy absorbing capability became a weaker region (SEEMAN and DELMAS, 2006).

Cortical bone is used to build long bones, and long bones are levers needed for loading and movement, with rigidity being favored rather than flexibility (SEEMAN and DELMAS, 2006). The degree of bone mineralization (DMB) remained unchanged or increased with advancing age and subtle changes in DMB led to large modifications in bone ability to resist fracture (WU, BERGOT, JOLIVET et al., 2009). Considering the microhardness results, it was observed that cortical and cancellous bone composition were modified near the periosteal or endosteal surfaces, and middiaphyseal cortical bone from HU animals presented lower microhardness. One supposed that these animals had localized changes in the rate of bone remodeling reflected in the structure/material composition and hardness, based on Teti and Zallone (2009) who stated that periosteocytic osteolysis was observed in rats immobilized for 10 days, in which destruction of lacunar wall, fragmentation of collagen fibers and loss of mineral crystals occurred. According to Zisset (2009) the difference in hardness did therefore not depend exclusively on the degree of calcification but also on the arrangement of the collagen fibers. An interesting observation is that the mean radiographic density in the middiaphyseal cortical from 14 mo-old animals was extremely increased when compared with that of 9 mo-old (CARVALHO, LOUZADA, RISO, 2007) and 4 mo-old (data not shown) animals. These results are in agreement with Zioupos, Currey and Casinos (2000) and Pietchsmann, Skalicky, Kneissel et al. (2007) who observed increased bone material density with age. Boivin, Doublier, Farlay et al. (2008) pointed out that secondary mineralization, a slow increase in mineral content during which the number and size of crystals increase (WU, BERGOT, JOLIVET et al., 2009), appears to be the major cause of change in the microhardness of bone.

The degree of bone mineralization not only influences the mechanical resistance of bone but also partly determine the bone mineral density (BOIVIN, DOUBLIER, FARLAY et al., 2008). Pietchsmann, Skalicky, Kneissel et al. (2007) showed that bone mineral density increased with ageing, detected in cortical and cancellous bone, and reported that newly formed bone is less mineralized and becomes fully mineralized over the course of time, which confirms the results of the present study. Osteoblasts turn into osteocytes after they have been surrounded by bone matrix and they are poor in organelles, indicating functions other than matrix synthesis and mineralization. This process could, in some manner, be related to the alteration in cortical bone response to unloading, observed in the femur from mature animals submitted to disuse in this experiment, because continuously changing functional demands require permanent adaptation of bone structure and microarchitecture (RAUNER, SILPOS and PIETSCHMANN, 2007). In the absence of loading, bone is lost and in the presence of loading, bone is either maintained or increased and the skeleton is unique in its ability to adaptively remodel on perception of mechanical loading (BONEWALD and JOHNSON, 2008).

Older bone, more highly mineralized than younger bone, is weaker, irrespective of porosity and a decreased capacity for absorbing energy before fracturing, has been suggested as a major risk for fracture (WU, BERGOT, JOLIVET et al., 2009). The mineral content (mineral-to-matrix ratio) was correlated with the animal age in both old (interstitial) and newly formed bone tissue, showing for the first time that age-related changes in BMC can be explain by an alteration in the mineralization process itself and not only by an imbalance in the remodeling process (GOURION-ARSIQUAUD, BURKET, HAVILL et al., 2009). The results of the present study also showed also that microhardness in control 14 mo-old male Wistar rats was 74.29% (at 100 μm) and 72.41% (at 300 μm from the periosteal surface) higher when compared with control 4 mo-old animals (data not shown). This can denote significant changes in bone mineralization and that average degree of mineralization is higher in a state of low bone formation (middle-aged animals) than a state of quick bone formation (growing animals).

Furthermore, 21 days of hindlimb unloading significantly modified the rate of mineralization of cortical bone, as demonstrate by the microhardness results, and bone near the endosteal surface can undergo constant remodeling, and was shown to be less mineralized than bone near the periosteal surface. Pietchsmann, Skalicky, Kneissel et al. (2007) concluded that in older male rodent animals the determinants of bone turnover markers indicated that with ageing bone formation decreases and is exceeded by bone resorption, despite blunted osteoclast generation. It is clear that high strain stimuli (such experienced when activity is increased) initiate increases in mass or changes in architecture to increase bone strength and low strain stimuli (due to reduced activity, such as disuse) cause bone loss or alterations in architecture that reduce strain (SKERRY, 2008), as experienced by the HU animals in this study.

The bone cells with the potential for sensing mechanical strain and translating these forces into biochemical signals include bone lining cells, osteoblasts and osteocytes. Due to their distribution throughout the bone matrix and extensive interconnectivity, osteocytes are thought to be one of, if not the major bone cell type responsible for sensing mechanical strain and orchestrating resorption and formation signals (BONEWALD and JOHNSON, 2008; SEEMAN and DELMAS, 2006). Not only do these cells communicate with those others on the bone surface, but their dendritic processes are in contact with bone marrow, giving them the potential to recruit osteoclast precursors and to regulate mesenchymal stem cell differentiation (BONEWALD and JOHNSON, 2008). Studies have demonstrated the inability of osteocytes to resorb bone in vitro and in vivo but they are clearly capable of modifying the matrix environment around them, since the perilacunar matrix is structurally unique (NOBLE, 2008). It is appropriate consider cortical bone and cancellous bone as separate compartments (WANG, BANU, McMAHAN et al., 2001), and it is thought that this can be applied to Wistar rats; that age and the level of cortical bone surrounding periosteal or endosteal surfaces modify the biological response, altering osteocytes and mechanical strain sensing, and influencing their role in bone homeostasis (TETI and ZALLONE, 2009).

Osteoporosis can result from any imbalance in bone turnover, leading to excess of osteoclast activity over osteoblast activity (RAUNER, SILPOS and PIETSCHMANN, 2007). This bone cell activity imbalance decisively influences the composition and architectural arrangement responsible for bone strength, primarily reflecting integration of bone density and bone quality, and their lower resistance to mechanical load in the adult animals (CARVALHO, LOUZADA and RISO, 2007). The cells responsible for performing the regulatory function of the adaptive mechanism to overload or disuse are the osteocytes/osteoblasts and osteoclasts are effectors instructed and regulated by osteoblasts and osteocytes (SKERRY, 2008). If the osteocyte is the primary sensor of mechanical effects on bone, then the osteoblast or lining cell is the effector of that response, either by forming new bone or recruiting new osteoblasts or osteoclasts to the bone surface, in a situation such as a short period of disuse (SKERRY, 2008).

5 Conclusion

It was observed that disuse induced significant alterations in bone structure, related to composition, architecture, and strength in the proximal and distal femoral epiphysis, according to data obtained when middle-aged male rats were submitted to 21 days of hindlimb unloading. HU animals presented higher susceptibility to fracture in femur head neck with degraded femoral mechanical properties. Densitometric radiographic density of the proximal epiphysis can demonstrate frailty in cancellous bone in this region, not registered by DXA measurements. Microhardness of the cortical and cancellous bone tissues were significantly lower in HU animals at the level of periosteal and/or endosteal surface in femoral middiaphysis, showing evidence of age dependent disarrangement of bone composition/strength, in the analyzed regions.

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