Hindlimb unloading producing effects on bone biomechanical properties in mature male rats

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Abstract

Aging is associated with decline in muscle mass and strength and reduced bone density. Age-related bone loss is a primary factor in osteoporosis and all individuals are potential candidates for osteoporosis because bone loss with aging occurs in men and women, but less studied in men. To examine the appropriateness of hindlimb elevation, by tail suspension as a model for diminished mechanical loading, and to determine the influence of age on bone responsiveness to skeletal unloading, we use dual X ray absorptiometry (DXA) and digital radiographic images to analyze the response of the femur from mature rats to biomechanical loads. Femurs from male Wistar rats (9-mo-old) were scanned using DXA and DIGORA and measures obtained in ephipyseal and diaphyseal regions of interest. The mechanical testing was divided into compression load to fracture the head and a three-point bending load to fracture the femur middiaphysis. In femoral epiphysis from hindlimb unload (HU), animals presented significant differences between mineral bone content and density assessed by DXA. Detailed regions of femoral epiphysis (head, throcanteric fossa, throcanter and metaphysis) presented significant lower values from radiographic density. Only compressive load necessary to fracture the femoral head neck was also significantly diminished in HU animals. Disuse induced, as in elderly patients, deterioration of the trabecular bone architecture with critical effect on bone fragility. Rats with 21 days of hindlimb unloading can simulate disuse, suggesting that certain sub-regions of their aging bones are more susceptible to fracture, while other, i.e. diaphyses, are not.

Keywords: hindlimb suspension, osteoporosis, biomechanics, bone density, densitometry.

1 Introduction

With a consensus, osteoporosis was defined as a "systemic skeletal disease characterized by low mass and microarchitectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture". The bone loss is brought about by an imbalance between bone resorption and bone formation, coupled process that continuously occurs throughout the skeleton. Starting with resorption sites, their distribution is probably not random and seems to be related to mechanical function, since the absence of strain produced for example either by bed rest, paralysis or casting of a limb, increases resorption in the affected bone. Age-related bone loss is a primary factor in osteoporosis and all individuals are potential candidates for osteoporosis, a classic age-related disease (BASSO, BELLOW and HEERSCH, 2005; PIETSCHMANN, SKALICKY, KNEISSEL et al., 2007; PULKKINEN, JÄMSÄ, LOCHMÜLLER et al., 2007).

If bone receives a reduced force, the mechanical unloading will produce bone hypotrophy. Significant bone mineral content reductions in patients subjected to bed rest for long periods of time have been reported and bone loss and subsequent senile osteoporosis associated with aging has also received considerable attention. In elderly men it is a common but often neglected condition with poorly characterized pathophysiology. In this condition alterations occurring in bone quality, quantity, and microarchitecture affect the resistance of trabecular bones to local failure. The major risks associated with bone loss during hypoactivity or osteoporosis are reduction of bone strength, implicated in an increased incidence of senile femoral neck fractures. Although age-related development of skeletal fragility is well established, it is unclear how the local failure properties of bone change with age (ABRAM, KELLER and SPENGLER, 1988; DEHORITY, HALLORAN, BIKLE et al., 1999; NAGARAJA, LIN and GULDBERG, 2007; PIETSCHMANN, SKALICKY, KNEISSEL et al., 2007).

Bone fragility can be defined broadly as the susceptibility to fracture. However, bones fracture for different reasons, so there are several different biomechanical definitions of bone fragility. One function of bones is to carry loads and fractures occur when loads exceed the bone strength, so weakened bones should be considered fragile. During a traumatic loading, such as a fall to the ground, a fracture will occur if the energy from the fall exceeds the mechanical energy that bone can absorb. The biomechanical definition of bone fragility includes strength, brittleness and work to failure and stiffness is also used to assess mechanical integrity of bones, but is not a direct measure of fragility (TURNER, 2002).

Hindlimb suspension (HS) is a model that is frequently used to study the cellular and molecular mechanisms underlying skeletal muscle atrophy and bone loss. For the study of bone loss in a microgravity environment, as unloading model, produced by tail suspension, has long been used and it has been suggested that bone volume loss in this model was mainly due to a decline in bone formation and the suppression of osteoblast function (BLOOMFIELD, ALLEN, HOGAN, et al., 2002; DAVID, LAFAGE-PROUST, LAROCHE et al., 2006; NAGARAJA, LIN and GULDBERG, 2007; TURNER, 2002).

With its high precision, dual X ray absorptiometry (DXA) has established itself as the most widely used method to measure bone mineral density (BMD) because of its advantages of high precision, short scan times, low radiation dose and stable calibration; however, it does not elucidate trabecular bone structure which is important in maintaining bone integrity and mechanical strength (PULKKINEN, JÄMSÄ, LOCHMÜLLER et al., 2007). DXA allows scanning of the spine and hip, usually regarded as the most important measurement sites because they are frequent sites of fracture that cause substantial impairment of quality of life, morbidity and mortality (BASSO, BELLOW and HEERSCH, 2005).

With regard to the choice of animal models for studying age-related bone loss in men, clinically relevant bone sites, such as the vertebra and the femoral neck, should be used. Wistar rats have been suggested to be a viable model of age-related bone loss in men. Rats are commonly used as animal models of human diseases because they are easily available, relevant and appropriate. The reason for using the rat model as study of age-related bone loss in men in this project is, in part, because the Wistar rat is relatively small and is conveniently available, and much is known about male rats (WANG, BANU, MCMAHAN et al., 2001).

To examine the appropriateness of hindlimb elevation as a model for diminished mechanical loading in adult rats, and to determine the influence of age on bone responsiveness to skeletal unloading, we use DXA and digital radiographic images, analyzing the response of the femur from hindlimb unloaded (HU) mature rats to biomechanical forces, submitting the head femur to compression and the middiaphyseal region to three-point bending load to failure.

2 Material and methods

The study protocol (2006-005743) and all animal procedures were in compliance with the São Paulo State University/Araçatuba School of Dentistry Care Committee rules and regulations in their Ethical Principles for Animal Experiments.

2.1 Animals

Male Wistar rats (9-mo-old at sacrifice) were housed in a temperature-controlled room (21 ± 2 °C) with a 12/12 hour light/dark cycle. Animals were provided standard rat chow (containing 1.2% calcium and 0.74% phosphorus) and water *ad libitum*. The animals were assigned in their plastic cage (3 rat/cage) (control group n = 10 and HU n = 9). Unloading of the hindlimbs was achieved by tail suspension and there is no increase in loading of the forelimbs in this model (DEHORITY, HALLORAN, BIKLE et al., 1999). Anesthetized with a ketamine-xylazine cocktail, their tails were cleaned with liquid soap, dried, received Povidine solution and a layer of adhesive spume (Espuma Reston; 3M do Brasil), covering the proximal two thirds of the tail (the distal third was removed). An elastic tape was adhered to the spume and coupled to this we created a cotton plait to attach the tail at the top of the cage. The forelimbs of the animal maintained contact with the cage bottom, allowing the rat full access to the entire individual cage. On day 21 of the suspension, the animals were killed (with an overdose of ketamine), the right and left femur were removed, cleaned of adherent muscles and other tissues, and stored in a saline solution soaked gauze in a –20° until analysis.

2.2 Bone mineral densitometry

Each right femur was scanned using DXA to determine bone mineral density (BMD) and content (BMC). Bones were thawed to room temperature (23 °C), placed in a plexiglass container filled with deionized water and scanned in a bone densitometer (Lunar DPX Alpha, Madison, WI, USA) with a small-animal software coupled to a computer. The region of interest rectangle was moved to cover a portion: total bone, middiaphyseal area, total epiphysis and metaphysis area.

Conserved in saline solution, the femur was put on imaging plates and with a GE Mobile 100 X ray (Milwaukee, WI, USA), 50 kV, 10 mA, 40 cm focal-film distance, 0.2 seconds exposure time, radiographs were obtained and scanned in a DIGORA intraoral digital imaging system (Soredex, Finland). Standardized femoral epiphyseal areas, in pixels (pi), were determined in the image on computer monitor and we obtained the values of bone optical density from each one.

2.3 Mechanical testing

Structural and material properties of femurs were determined by mechanical testing divided into compression test to fracture the femoral head and by a three-point bending test to fracture the middiaphyseal femoral region. The right femur epiphyses were placed with their distal two-thirds vertically into a plastic tube, firmed with acrylic resin, and the femoral necks were tested with a vertical load applied to the top of the femoral head, parallel to their long axis, until failure occurred. This compression force to the epiphysis was applied with Universal Testing Machine (EMIC, DL 3000 model, São José dos Pinhais [PR]), with a load cell at 2000 N (speed of 5 mm/minutes). The left femur was thawed to room temperature (23 °C), placed on a special holding device with two supports located at a distance of 20 mm. Until failure occurred, a three-bending load was applied at the femoral middiaphyses in the same Universal Testing Machine, with the same load cell at the same speed specified above. The data were automatically recorded and stored in a desktop computer interfaced to the materials testing device.

From the load-deformation curve the following values were calculated: maximum load to compression and flexion until fracture occurred in femur; the linear portion of the curve represents the elastic region, and the slope of this part of the curve is used to derive the stiffness of the bone. The nonlinear portion of the curve represents the plastic region in which the bone will be permanently deformed by the load and tenacity of the energy derived from this area (BASSO, BELLOW and HEERSCH, 2005).

3 Results

The comparative analysis of the variables evaluated is shown in Table 1,2. In the femoral epiphyseal region of HU animals, we observed significant differences between bone mineral content and density assessed by DXA measurements. Detailed regions of femoral epiphysis (head, throcanteric fossa, throcanter and metaphysis) presented significant lower values from digital radiographic density (Table 1). Compressive load (and associated stiffness/tenacity) necessary to fracture the femoral head neck was also significantly diminished in HU animals. The complete parameters from biomechanical and geometrical data are presented in Table 2.

4 Conclusion

There was a significant difference in final body mass between control and HU mature male animals at the moment of sacrifice. These data were also demonstrated (BILEZIKIAN, RAISZ and RODAN, 2002; DAVID, LAFAGE-PROUST, LAROCHE et al., 2006) in rats subjected to hindlimb unloading for 14 days, and this can represent an acute loss from combination of diminished appetite and loss of fluids as a consequence of the cephalad fluid shift induced by head-down tilt in the model. Allen and Bloomfield (2003) observed that body mass in female 6-mo-old decreased 6% 28 days after hindlimb suspension and Basso et al. (2005) registered a decrease of 10% in body mass of Fisher rats after 14 days of suspension. In our male Wistar mature rats we observed that the change for the individual cage (during the suspension period), the stress in the suspension and the

Table 1. Bone parameters of right femurs assessed by DXA and radiographic density parameters assessed by DIGORA.

	Control	HU
DXA		
Total bone area (mm^2)	2.044 (0.092)	1.985 (0.090)
BMC Total bone (g)	0.533 (0.02)	0.481* (0.03)
BMD Total bone ($g.cm^2$)	0.266 (0.015)	0.243* (0.017)
BMC Proximal epiphysis (g)	0.124 (0.006)	0.110* (0.009)
BMD Proximal epiphysis ($g.cm^2$)	0.263 (0.013)	0.234* (0.0019)
BMC Middiaphysis (g)	0.125 (0.102)	0.119 (0.0008)
BMD Middiaphysis ($g.cm^2$)	0.2771 (0.201)	0.252 (0.0157)
BMC Epiphyseal neck (g)	0.046 (0.003)	0.042 (0.006)
BMD Epiphyseal neck ($g.cm^2$)	0.302 (0.029)	0.259* (0.024)
DIGORA		
Head (34 x 34 pi)	188.04 (5.01)	182.06* (7.04)
Throcanter (30 x 30 pi)	182.71 (4.71)	177.27* (5.47)
Throcanteric fossa (40 x 40 pi)	182.26 (3.26)	173.00* (7.60)
Metaphysis (88 x 26 pi)	180.59 (3.84)	173.27* (7.60)
Medular diaphysis (30 x 100 pi)	175.80 (5.13)	171.97 (6.38)
Cortical diaphysis (100 pi)	183.54 (8.23)	184.18 (10.33)

Values are ± SD mean for each group; and *Significant difference ($p < 0,05$) versus. Control.

Table 2. Mechanical and geometrical (DIGORA) properties of femural epiphysis and middiaphysis.

	Control	HU
DXA		
Body mass (g)	546.20 (36.51)	490.67* (40.00)
Maximum compression load (N)	218.32 (20.82)	176.47* (22.54)
Compression tenacity (N.mm)	198.91 (121.69)	95.07* (19.61)
Compression stiffness (N.mm)	197.88 (61.23)	199.98 (27.27)
Maximum flexion load (N)	169.80 (28.46)	178.10 (16.95)
Bending tenacity (N.mm)	120.24 (29.04)	106.16 (20.44)
Bending stiffness (N.mm)	281.76 (86.49)	284.64 (45.13)
DIGORA		
Head angle	35.6° (3.86)	33.55° (3.43)
Maximun head diameter (mm)	4.39 (0.20)	4.44 (0.16)
Head neck diameter (mm)	3.23 (0.41)	3.54 (0.45)
Femoral neck axis length (mm)	11.51 (0.81)	13.01* (0.92)
Middiaphysis width (mm)	4.88 (0.23)	5.02 (0.41)
Middiaphysis trabecular width (mm)	3.02 (0.28)	3.25 (0.29)

Values are ± SD mean for each group; and *Significant difference ($p < 0.05$) versus. Control.

increased area of the cage influenced smaller food intake and the loss (10.16%) of animal body mass.

Bone changes in tail suspension rats in various studies have been controversial, probably due to differences in the animals ages and suspension periods. In order to model changes in bone strength and resistance to fracture in adult humans with exposure to microgravity, it is essential to use an animal model with a more mature skeleton to avoid complications in data interpretation introduced by rapid growth (BLOOMFIELD, ALLEN, HOGAN, et al., 2002). Cortical bone sites in the tibia of mature adult rats appear to be much slower to exhibit changes in bone mineral density, bone area and mechanical properties with hindlimb unloading than in young, rapidly growing animals.

However, cancellous bone in these mature animals appears to be very sensitive to changes in mechanical loading (BLOOMFIELD, ALLEN, HOGAN, et al., 2002). Pietschmann et al. (2007) reported that in aged SD rats, distinct age-related changes in bone density and structure were detected and cancellous bone mass decreased substantially in tibia of ageing male rats due to removal of trabecular elements, resulting in an increased trabecular spacing and unfavorable bone structure. Our results suggest that the femoral epiphyseal region, richer in cancellous bone than the diaphyseal region (with strong cortical sites), became a frailty area probably with new trabecular arrangement induced by ageing-disuse, and was significantly affected in its response to compressive loads, but not to bending load in the diaphysis.

Bone volume loss in the hindlimbs was evident in the tail suspension model, which has been mainly attributed to the decreased activity of osteoblasts in long bones. Seventeen-week-old rats subjected to hindlimb unloading during 4 weeks presented a significant decrease in: mineral apposition rate, bone formation rate per bone surface, bone volume per tissue volume, and trabecular number, thickness and spacing. On the other hand, the same animals presented an increased number of osteoclasts per bone perimeter and per bone surface (DEHORITY, HALLORAN, BIKLE et al., 1999; TURNER, 2002). Basso et al. (2005) observed similar results and decrease in the number of osteoblasts surface and number per bone surface in male mature rats with hindlimb unloading for 14 days. The decrease in bone volume observed in the femurs of 6-week-old rats unloaded for 14 days is probably caused by an effect of unloading on the formation of osteoprogenitors. These results suggest that decreased proliferative capacity of the progeny of these osteoprogenitors also plays a role and established that the effects of unloading were specific for osteoprogenitors and cells of the osteoblastic lineage (BASSO, BELLOW and HEERSCH, 2005).

With in vitro study from human femurs and testing their strength to a side impact simulating a sideway fall in a greater throcanter, Pulkkinen et al. (2007) reported that radiography can be used to analyze both trabecular structure and bone geometry, and information on bone density may be obtained by using the appropriate image analysis techniques. Our methodology showed that the excellent quality image on digital radiography can be applied to this analysis based on the fact that conventional radiography is widely available with low costs and the considerable interest to discover how well the mechanical competence of bone can potentially be determined using X ray technology (PULKKINEN, JÄMSÄ, LOCHMÜLLER et al., 2007). With our results we observed that this technology could evaluate bone structure and density accurately.

Bloomfield et al. (2002) reported that mature male rats presented a significant decrease in cancellous bone mineral density after 21 days subjected to hindlimb suspension and the mechanical properties in femoral neck, rich in cancellous bone, is adversely affected by unloading, confirming our data obtained at DIGORA in the throcanteric fossa, which coincide with the femoral head neck in femur rat anatomy. Our results of significant decrease of BMD/BMC on the epiphysis region can reflect an imbalance between osteoblast/osteoclast activity in femur from HU animals. The data obtained in DIGORA measurements considered the throcanteric fossa as the site of elevated frailty in the epiphysis (BILEZIKIAN, RAISZ and RODAN, 2002; WANG, BANU, MCMAHAN et al., 2001). However, the femoral head and throcanter also presented significant lower values of radiographic density in HU animals. It is important to observe that all femurs fractured, under compressive load, in this region but in HU animals with smaller load than control ones. We also observed significant decreased tenacity (energy absorbed from elastic and plastic regions in load-deformation curve) suggesting that there was bone frailty in the femoral epiphysis, particularly in the throcanteric fossa, occurring with aging as well as changes in rat bone microarchitecture and strength, contributing to the increase in fracture risk associated with osteopenia developed by HU (NAGARAJA, LIN and GULDBERG, 2007).

Measuring the effects of unloading on cortical and cancellous compartments in the mature adult rats (5-mo-old), the suggestion is that unloading induces few changes in cortical bone mass, geometry and mechanical properties in the mature rodent skeleton. However, these properties are adversely affected by unloading in sites rich in cancellous bone, such as the femoral neck and proximal tibia (BLOOMFIELD, ALLEN, HOGAN et al., 2002; PERRIEN, AKEL, DUPONT-VERSTEEDEN et al., 2007; PIETSCHMANN, SKALICKY, KNEISSEL et al., 2007; YWASAKI, YAMATO, MURAYAMA et al., 2002). As in humans, the femoral necks of rats contain both cancellous and cortical bones, which have been shown to be decreased to differing degrees by aging (TURNER, 2002) and demonstrated that there is a clear need to focus on unloading-induced fracture risk at those sites with a more significant cancellous bone component (BILEZIKIAN, RAISZ and RODAN, 2002). Our results in more mature animals also support these observations in femoral epiphysis and indicate few alterations in mechanical properties at the femur middiaphyseal region after 21 days of hindlimb unloading.

According to Reddy et al. (2001), the alterations in plastic-elastic observed in biomechanical properties of bones may relate the changes in bone collagen metabolism and bone mineral composition. It is known that the mechanical properties in the elastic region of the load-deformation curve relate more to the mineral components of bone, whereas the plastic region relates more to the collagen component. Thus, the biomechanical change (tenacity) observed in our study, from the HU animals, can reflect the change in their collagen component of bone, representing the non-linear portion of the curve in which the bone will be permanently deformed by the load (BASSO, BELLOW and HEERSCH, 2005).

Our results confirmed that the middiaphyseal bone of 6-mo-old rats is unaffected by hindlimb unloading (BILEZIKIAN, RAISZ and RODAN, 2002). In humans, fractures of the hip usually begin in the metaphyseal-epiphyseal regions of the bone, which depend heavily on the trabeculae arrangement to support loads and osteoporotic fractures usually do not occur in the mid-shaft of the femur or radius which is mainly cortical bone (BASSO, BELLOW and HEERSCH, 2005). Consequently, the femoral head neck with its unique anatomy becomes prone to fracture under deforming forces and bone fragility leading to hip fractures in men may be the result of fracture site-specific deficits in volumetric BMD.

We assumed that disuse-induced, as in elderly patients, deterioration trabecular architecture have a critical effect on bone frailty and that rats with 21 days of hindlimb unloading can simulate disuse. In our study certain sub-regions from aging rat bone showed higher susceptibility to fracture than others, such as diaphysis. In these mature rats, with degraded femoral mechanical properties, decreased densitometric and radiographic densities of the epiphyseal region can demonstrate the bone frailty at this site. We demonstrated mechanically and radiographically that there was a potential risk factor for the experimental femoral neck fracture under compressive load. An imbalance in osteoblasts/osteoclasts activity in the trabecular bone, with decreased bone formation and exceeded bone resorption, decisively influence the architectural arrangement (shape, size and structure of bone) of the femoral head neck, throcanteric fossa and throcanter. These regions (mostly trabecular bone) in the male mature animals appear to be very sensitive to changes in the response to mechanical loading.

References

ABRAM, AC., KELLER, TS. and SPENGLER, DM. The effects of simulated weightlessness on bone and biomechanical properties in the maturing rat. *J. Biomechanichs* 1988, vol. 21, no. 9, p. 755-767.

ALLEN, MA. and BLOOMFIELD, SA. Hindlimb unloading has a greater effect on cortical compared with cancellous bone in mature female rats. *J. Appl. Physiol.* 2003, vol. 94, no. 2, p. 642-650.

BASSO, N., BELLOW, CG. and HEERSCH, JN. Effects of simulated weightlessness on osteoprogenitor cell number and proliferation in young and adult rats. *Bone* 2005, vol. 36, no. 1, p. 173-183.

BILEZIKIAN, JP., RAISZ, LG. and RODAN, GA. *Principles of bone biology*. 2nd ed. San Diego, CA: Academic Press, 2002.

BLOOMFIELD, SA., ALLEN, MR., HOGAN, HA. et al. Site- and compartment-specific changes in bone with hindlimb unloading in mature adult rats. *Bone* 2002, vol. 31, no. 1, p. 149-157.

DAVID, V., LAFAGE-PROUST, MH., LAROCHE, N. et al. Two-week longitudinal survey of bone architecture alteration in the hindlimb-unloaded rat model of bone loss: sex differences. *Am. J. Physiol.* 2006, vol. 290, no. 3, p. E440-E447.

DEHORITY, W., HALLORAN, BP., BIKLE, DD. et al. Bone and hormonal changes induced by skeletal unloading in the mature male rat. *Am. J. Physiol.* 1999, vol. 276, no. 1, p. E62-E69.

NAGARAJA, S., LIN, AS. and GULDBERG, RE. Age-related changes in trabecular bone microdamage initiation. *Bone* 2007, vol. 40, no. 4, p. 973-980.

PERRIEN, DS., AKEL, NS., DUPONT-VERSTEEDEN, EE. et al. Aging alters the skeletal response to disuse in the rat. *Am. J. Physiol.* 2007, vol. 292, no. 2, p. R988-R996.

PIETSCHMANN, P., SKALICKY, M., KNEISSEL, M. et al. Bone structure and metabolism in a rodent model of male senile osteoporosis. *Exp. Gerontol.* 2007, vol. 42, no. 11, p. 1099-1108.

PULKKINEN, P., JÄMSÄ, T., LOCHMÜLLER, EM. et al. Experimental hip fracture load can be predicted from plain radiography by combined analysis of trabecular bone structure and bone geometry. *Osteoporos.* Int. 2007, vol. 19, no. 4, p. 547-548.

REDDY, GK., STEHNO-BITTEL, L., HAMADE, S. et al. The biomechanical integrity of bone in experimental diabetes. *Diabetes Res. Clin. Pract.* 2001, vol. 54, no. 1, p. 1-8.

TURNER, CH. Biomechanics of bone: determinants of skeletal fragility and bone quality. *Osteoporos.* Int. 2002, vol. 13, no. 2, p. 97-104.

WANG, L., BANU, J., MCMAHAN, CA. et al. Male rodent models of age-related bone loss in men. *Bone* 2001, vol. 29, no. 2, p. 141-148.

YWASAKI, Y., YAMATO, H., MURAYAMA, H. et al. Maintenance of trabecular structure and bone volume by vitamin K in mature rats with long-term tail suspension. *J. Bone Miner. Metab.* 2002, vol. 20, no. 4, p. 216-222.

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