MECHANICAL BEHAVIOR OF RATS' FEMORAL PROXIMAL THIRDS AFTER A PERIOD OF TAIL SUSPENSION AND EXERCISES

MARCOS MASSAO SHIMANO[1], JOSÉ BATISTA VOLPON[2]

SUMMARY

Bone remodeling can be stimulated by mechanical forces present in normal physical activities. In the present research, we investigated the mechanical behavior of the proximal femur of rats previously maintained in tail suspension and later, submitted to physical exercise on a treadmill. Sixty-six Wistar rats were used. Firstly, the animals were raised until the age of ninety days and then divided into five groups (two control groups and three experimental groups). The animals allocated to Control I group were killed at 118 days of age. In the S group, the animals were suspended by tail for 28 days. In Control II group, the animals were killed at 139 days of age. In group S-R (suspended and released) the rats were kept free for 21 days after tail suspension. In group S-T (suspended and trained), after tail suspension period, the rats were trained in a treadmill for 21 days. For analysis of the mechanical behavior of the bone, force was applied on the femoral head until failure. The fracture was evaluated by x-ray. Suspension caused a decrease of the maximum load and, treadmill training and post-suspension release caused the recovery of mechanical properties. But, the fracture line pattern did not show any difference among the experimental groups.

Keywords: Weightlessness Simulation; Physical Conditioning, Animal; Biomechanics; Femur; Rats.

Citation: *Shimano MM, Volpon JB. Mechanical behavior of rats' femoral proximal thirds after a period of tail suspension and exercises. Acta Ortop Bras. [serial on the Internet]. 2007; 15(5):254-257. Available from URL: http://www.scielo.br/aob.*

INTRODUCTION

According to Bikle et al.[1], osteogenesis can be stimulated by small deformations on bone architecture, provoked by mechanical forces applied during usual physical activity, which can directly act on bone remodeling rate. In turn, hypokinesia (reduced level of activities) leads to a process that reduces that rate [2]. Thus, an environment with lower levels of mechanical stimuli produces changes to bone structure. Examples of this situation are seen in space trips (astronauts' exposure to an environment where gravitational forces are lower), orthopaedic immobilizations, and when patients stay in bed for a long time, which, according to Holick[3], may cause significant mineral density and bone mass losses. In these cases, skeleton does not require all its bone mass to keep structural integrity, developing osteopenia, which can lead to bone fractures after normal physical activities are resumed [4].

During space trips lasting more than a month, astronauts are submitted to significant bone mass and mineral density losses on portions of the skeleton supporting body weight, especially spine and lower limbs. Studies show that these losses are resultant from weight sub-load found in environments with lower gravitational force [3,5].

The idea of using rats' lower limbs suspension to study the consequences of unloading and ulterior loading occurred during and after space trips was first suggested in the 1970's by *National Aeronautics and Space Administration (NASA)-Ames Research Center*.

In 1987, Wronski and Morey-Holton[6] compared hip suspension and tail suspension in rats. They concluded that skeletal system response varies according to the method of suspension. The model that was closest to the effects caused by lower gravity was tail suspension, also being the one causing fewer problems to fixation system.

Rats' lower limbs unloading, by tail suspension, is an accepted model by scientific community to simulate the effects of space trips. The standard operating procedure of unloading in young and adult rats was updated and approved by the *National Aeronautics and Space Administration (NASA)*, *Ames Research Center (ARC), Institutional Animal Care and Use Committee* in August, 2001[7].

Despite of the serious implications of losing bone mass and, consequently, human skeleton pre-susceptibility to fractures when resuming normal activities, no established countermeasure exists yet to keep bone integrity during long period of weight sub-loading over bone structure[8]. Physical exercise is the countermeasure most commonly studied to minimize or prevent muscular atrophy and bone mass loss. Animal studies, requiring drill on treadmills report increase of bone mass[9,10].

Hip is a very important joint and is directly involved on issues concerned to bone mass loss, because it is a body site showing higher incidence of fractures[11]. Therefore, the objective of this study was to assess femoral proximal thirds of rats submitted to tail suspension and subsequent drills on treadmill, by means of mechanical assay and X-ray imaging.

MATERIALS AND METHODS

This study was conducted as per the ethical principles of animal experiments as adopted by the Committee of Ethics in Animal Experiments (CETEA) of the Medical College of Ribeirão Preto, University of São Paulo. (protocol nr. 038/2003).

Study conducted at the Department of Biomechanics, Medicine and Locomotive Apparatus Rehabilitation (Bioengineering Laboratory), Medical College of Ribeirão Preto, University of São Paulo.
Correspondences to: Av. Bandeirantes, 3900 - Laboratório de Bioengenharia- Ribeirão Preto – São Paulo - CEP: 14049-900 - e-mail: mmshimano@gmail.com, jbvolpon@fmrp.usp.br
1. Ph.D. in Medical Sciences, Department of Biomechanics, Medicine and Locomotive Apparatus Rehabilitation, Medical College of Ribeirão Preto, University of São Paulo.
2. Chairman of the Department of Biomechanics, Medicine and Locomotive Apparatus Rehabilitation (Bioengineering Laboratory), Medical College of Ribeirão Preto/ USP.

Received in: 01/08/07; approved in: 03/19/07

Matrix Wistar female rats (*Rattus norvegicus albinus*), at later pregnancy phase, were separated and , after delivery, six female broods were selected from each mother rat, the remaining being disregarded. Mother rats remained in individual cages, together with brood, during the period until weaning (21 days).
At the age of 90 days, the animals were divided into the following groups: -Control Group-I (CONT-I) – the animals were raised and followed-up until the age of 118 days; -Suspended Group (S) – the animals were suspended by tail for 28 days, and then, sacrificed at 118 days of age; -Control Group II (CONT-II) – the animals were kept up to 139 days of age; -Suspended-Released Group (S-R) – animals were suspended by tail for 28 days and then released into the cages for additional 21 days. Following, they were sacrificed at 139 days of age;
-Suspended-Trained group (S-T) – the animals remained suspended by tail for 28 days, and then submitted to exercise on treadmill, being subsequently sacrificed, at 139 days of age.

Animal suspension

The animals included on groups S, S-R and S-T were suspended by tail for 28 days, and all procedures for hanging the animals by the tail were based on the study by Silva and Volpon[12], who used a suspension model developed from a description by Kasper et al.[13].
For preparing animals' tails for suspension, the rats were anesthetized by intramuscular injection of a combination of 30mg/kg and 3mg/kg ketamine and xylazine, respectively. Under anesthesia, rats' tails were washed and stained with benzoin all over the skin. Then, tails were wrapped with adhesive foam (Reston®), from its origin to two proximal thirds, intending to protect skin. Over the foam, an elastic bandage Coban (model 1582, 3M brand), homogeneously tensioned, so that the foam could be fully covered. On the elastic band wrap, a narrow cord fixated by additional bandages was placed in order to form a loop that served to secure the animal to the suspension system by means of a clasp. With the animal anesthetized, unwrapped distal end of the tail was amputated, in order to avoid necrosis. After the procedures for tail preparation, intramuscular ketoprophen (analgesic and anti-inflammatory agent) was applied on the lower limb at a dosage of 3.5mg/kg. This was repeated once a day, for three consecutive days.
Suspension system was constituted of a two-part cage and an animal fixation system. The bottom of the cage was constituted of a transparent acrylic box in order to enable animal visualization and control, with open top. On that box, a metal-bar cage was placed on an inverted position, with side fittings to assure fixation to the acrylic box. The animal fixation system was connected on the upper portion of the cage and was composed by a clasp, an axis, washers and screws. In that system, the animals could move to reach water and food supplies, with suspension height being adjusted to allow animals to lean on upper limbs to touch the ground (Figure 1).

Training

Training was made on a motor treadmill, constituted of six individual 15.0-cm high, 10.0-cm wide inside and 50.0-cm long stalls.
For rats exercising on treadmill, the protocol recommended by Norman et al.[8] was followed. Training sessions began

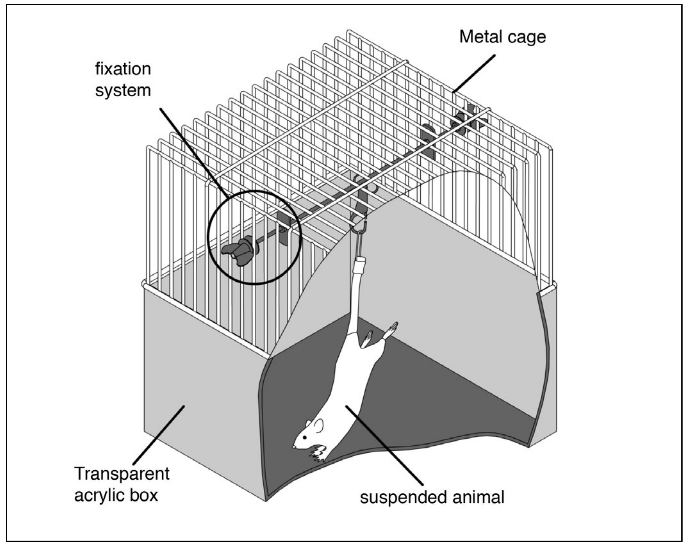

Figure 1 - *Schematic illustration of an animal suspended by tail in the cage.*

on the day after suspension release, for 10 minutes. Five minutes were added a day up to 60 minutes of daily training. The average treadmill speed was 17m/min, at horizontal position. The animals were submitted to the protocol above for 3 consecutive days, followed by one training-free day (rest/ recovery).

Mechanical analysis

After the experiment period, the animals were weighted and then submitted to euthanasia, with an excessive dose of thiopental. Left femurs were removed, cleaned from soft parts, weighted and identified, and subsequently involved with saline solution wetted gauze and preserved at –20°C.
Twelve hours before mechanical assay preparation processes started, the bones were removed for freezer and kept in refrigerator at an average temperature of 1°C and, two hours earlier, they were allowed to unfreeze at room temperature (range: 25°C - 27°C). For conducting assays, inclusion of femoral distal ends in acrylic was required for vertical position fixation.
For that, a metal device for inclusion in acrylic of up to six femurs was prepared and built (Figure 2). The device was built with brass and aluminum. Previous to inclusion procedures, the accessory was greased with mineral oil in order to avoid acrylic adherence to the metal.

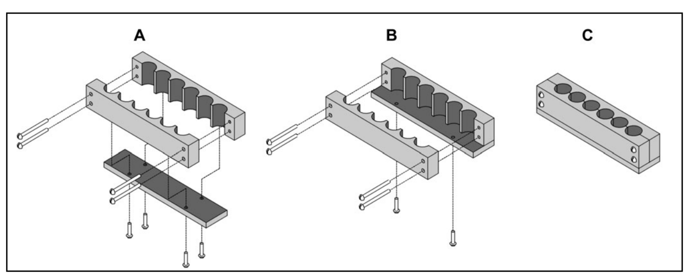

Figure 2 - *Schematic illustration of the accessory built for including rats' distal femoral portion into acrylics. (A) Disassembled accessory. (B) First assembly for gluing and alignment of femurs. (C) Assembled accessory.*

Femurs were fixated onto the base with epoxy glue (5 minutes) and secured with elastic bands to keep them at vertical position until glue was dried. Then, the elastic bands were sectioned and the accessory was closed to receive acrylics. The self-polymerizing acrylics "JET-Classic" in a ratio of 2.0

ml powder for 1.0 ml solvent; the ingredients were mixed and poured into the accessory. Full polymerization occurred within approximately six minutes, and, throughout the process, the set was refrigerated within a container filled with cold water, also keeping the exposed femoral portion moistened. After acrylics polymerization was completed, the accessory was opened and the acrylic-bone set was removed.

Mechanical assay

Assays were performed on a universal assay machine EMIC®-10000N. For capturing the forces exerted, a load cell with maximum capacity of 50kgf was used, and deformations were identified by built-in shift sensors on the machine.
The acrylic-bone set was secured in a bench vise connected to the universal assay machine base. A vertical force was applied with a 2-mm wide accessory on femoral head until fracture occurred. Force application speed was 0.1mm/min (Figure 3).
From these assays, graphs of force vs. deformation were obtained, from which we could detect stiffness and maximum force as mechanical properties.

Statistical analysis

For statistical analysis, the SigmaStat® v.2.03 software was used. Before applying statistical tests, normality and variance equality analysis were made. For comparing groups CONT-I *vs.* CONT-II and groups CONT-I *vs.* S, the Student's t-test was employed. We also compared the groups CONT-II *vs.* S-R *vs.* S-T; in this case, the one-way variance analysis test (ANOVA) was used for parametric data. In all analyses, a significance level of 5% was adopted.

X-ray analysis

Following mechanical assays, the acrylic-bone set was reproduced on dental X-ray equipment, at anteroposterior plane, with 0.15 second exposure time. Maximum nominal tension of the equipment was 70kVp and the maximum nominal current was 10mA. A periapical - occlusal - M2 (5cm x 7cm) X-ray film was used. The distance between X-ray focus and film was 5 cm.
For developing the X-ray film, routine procedures were followed taking care to assure that all films were soaked with developer and fixator for the same time period. X-ray images were digitalized with 1200 dpi resolution. The image of the proximal femoral third was magnified and the fracture was assessed for trace.

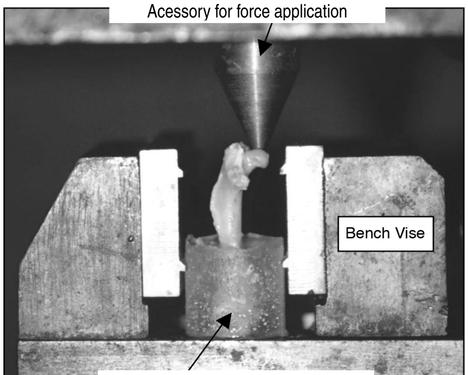

***Figure 3** – Assembly details for flexion-compression assays on rat's proximal femoral region.*

RESULTS

For endpoints, all animals that did not experience problems during experimental procedures have been considered. These problems included: weight significantly below average at the beginning of procedures (one animal in group CONT-I, one animal in group S, and one animal in group S-R); death of unknown causes (one animal in group CONT-I); tail rupture during suspension (three animals in group S, two animals in group S-R, and one animal in group S-T); excessive weight loss during procedures (one animal in group S-R) and two animals in group S-T failed to adjust to training protocol. In addition, two results were disregarded (one from group S-R, and one from group S-T) for having presented a very different mechanical behavior compared to other specimens. Therefore, ten (10) animals in group CONT-I, ten (10) animals in group S, twelve (12) animals in group CONT-II, nine (9) animals in group S-R and ten (1) animals in group S-T were considered for analysis.
When comparing both control groups (CONT-I and CONT-II), significant differences were detected for stiffness and maximum force values. When comparing group CONT-I and group S, a significant difference was found only for maximum force, and, when comparing the remaining three groups together, no statistical difference was found (Table 1).

	Groups				
	CONT-I	S	CONT-II	S-R	S-T
Stiffness ($x10^3$ N/m)	209.4 ±64.5	189.8 ±66.3	287.2 ±77.1*	269.1 ±46.7	275.3 ±62.7
Maximum force (N)	101.5 ±10.5	86.3 ±13.5*	115.0 ±12.6*	104.9 ±12.9	115.4 ±15.5

*statistical difference to group CONT-I

***Table 1** - Average ± standard deviation of the mechanical properties obtained on assays performed on animals' left femoral proximal third.*

Fracture pattern seen on X-ray images was similar in all groups and, basically, constituted by a trace starting from the lateral third of the head top following a vertical orientation towards femoral neck. In some X-ray images, the trace could be easily visualized and accompanied by a small fragment shift. In some other images, the trace was hard to visualize, but, in one case, a full split of fragments was found (Figure 4).

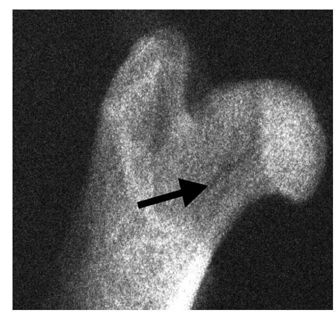

***Figure 4** - Detail of an X-ray image obtained after flexion-compression assay on the proximal third of left femurs, showing fracture (arrow).*

DISCUSSION

During space trips lasting more than a month, astronauts are submitted to significant bone mass and mineral density losses on major skeletal portions supporting body weight, especially spine and lower limbs[2]. Studies showed that those losses are consequences of weight sub-loading found in environments of lower gravity[3,14].
Bone mass decrease may provoke an increased risk of fractures, not during the sub-loading period, but when resuming normal physical activities. Therefore, it is crucial to know the mechanical behavior of skeletal structures responsible for supporting body weight after a period of hypokinesia. This idea led to an interest towards performing a mechanical analysis on animals' bones that had their lower limbs precluded from touching the ground, and subsequently

exercised on treadmills.

We chose rats as experimental animals because of the ease to keep them in a laboratory environment, because of the low costs, and, particularly, because of the potential to simulate sub-loading.

Suspension for 28 days was based on studies performed with animals hanged for the same period of time; that was enough time to cause significant changes to bone structure [15-17].

Tail was fully amputated after suspension, as a result of the severe injuries caused by the fixation system. Morey-Holton and Globus[7] , and, more recently, Knox et al.[18] introduced different fixation systems for suspension-by-tail, all of these intending to minimize tail compression by strips. Thus, damages to animals' tails remain a problem, although few studies have reported them. For future studies, testing new fixation systems should be considered in an attempt to avoid damages to tails.

There are several studies with different protocols of exercises on treadmills, but we followed the protocol recommended by Norman et al.[8] because those authors trained rats on treadmills after a 21-day period of suspension by tail. And, according to them, the rest day was important for animals to recover and avoid damages associated to excessive drills.

The conventional method for mechanically testing femoral proximal third in rats is based on applying an axial force onto femoral head; however, in osteoporotic bones, most of the fractures occurring at that site result from high flexed moments, generated by lateral forces or torsional forces [19]. In this study, we selected the traditional method for not requiring special accessories, for its satisfactory results, and, also, for the well-defined assay methodology. The major issue found in this kind of assay was femoral alignment during inclusion, but, in studies using the same kind of assay [9,17,19], the authors did not describe how they aligned the femurs.

The use of an inclusion accessory optimizes the process, once, in other studies, inclusions were individually made, thus demanding more time. Therefore, we could conduct mechanical assays on the same day of inclusion, not requiring re-freezing.

Macroscopically, after assays, femoral head crushing was observed, confirming the reports by Bloomfield et al. [17]. Therefore, according to those authors, the mechanical property that best represented the behavior of assayed region was maximum force.

Another point to be commented is that, in this case, the assayed structure is formed by cortical bone filled with trabecular bone, that is, it is not homogeneous, making the interpretation of results difficult. Furthermore, on this femoral portion, format is very complex and causes an irregular forces distribution.

Comparing groups CONT-I and CONT-II, significant changes were noticed on stiffness and maximum force, therefore, no comparisons were made between group S (suspended) and groups S-R (suspended-released) and S-T (suspended-trained).

By comparing CONT-I to group S, a significant reduction of maximum force was found, showing that suspension for 28 days caused structural changes, a fact that was also reported by Bloomfield et al.[17].

By comparing the properties of study, no statistical differences were found between groups CONT-II, S-R and S-T. Thus, the simple release (group S-R), as well as training sessions, fostered maximum force recovery.

In X-ray images, no difference was found on fracture patterns, probably because the changes fostered by suspension, suspension-release and suspension-training were not sufficient to change it, but applying a different force (lateral or torsional) could generate other kinds of fractures and even a different mechanical behavior.

Mechanical properties differences between group CONT-I and group CONT-II suggest, as per the study by Burstein et al.[20], that differences are present on the microstructural composition of bones of animals in different age groups. Therefore, we couldn't compare group S (suspended) to groups S-R (suspended- released) and S-T (suspended-trained).

For future studies, performing X-ray control before assays would enable the identification of geometric changes that might be occurred on the femoral proximal third region (femoral neck angle and femoral head flattening). These changes can occur as an adaptation to different loads imposed when resuming normal activities (release) or during training.

From this study, it is evident that further studies are required with results that, added to those presented here, could lead to a better understanding about the phenomena occurring on bone tissues when these are temporarily not mechanically demanded, and then re-demanded again.

CONCLUSION

Our findings indicate that the sub-loading of rats' lower limbs caused significant damages on maximum force supported by femoral proximal third, and by resuming normal activities - with or without training - the mechanical behavior of that region could be restored.

ACKNOWLEDGEMENTS

We acknowledge FAPESP, Research Support Foundation of the State of São Paulo, for the financial support for acquiring a treadmill and further equipment. We also acknowledge CAPES, University Research Aid Committee, for its support and Ph.D. scholarship granting.

REFERENCES

1. Bikle DD, Sakata T, Halloran BP. The impact of skeletal unloading on bone formation. Gravit Space Biol Bull. 2003; 16:45-54.
2. Turner RT. Physiology of a microgravity environment. Invited Review: What do we know about the effects of spaceflight on bone? J Appl Physiol. 2000; 89:840-7.
3. Holick MF. Perspective on the impact of weightlessness on calcium and bone metabolism. Bone. 1998; 22:105-11.
4. Morey-Holton ER, Globus RK. Hindlimb unloading of growing rats: a model for predicting skeletal changes during space flight. Bone. 1998; 22:83-8.
5. Vico L, Lafage-Proust M-H, Alexandre C. Effects of gravitational on the bone system in vitro and in vivo. Bone. 1998; 22:95S-100S.
6. Wronski TJ, Morey-Holton ER. Skeletal response to simulated weightlessness: a comparison of suspension techniques. Aviat Space Environ Med. 1987; 58:63-8.
7. Morey-Holton ER, Globus RK. Hindlimb unloading rodent model: technical aspects. J Appl Physiol. 2002; 92:1367-77.
8. Norman TL, Bradley-Popovich G, Clovis N, Cutlip G, Bryner RW. Aerobic Exercise as a countermeasure for microgravity-induced bone loss and muscle atrophy in a rat hindlimb suspension model. Aviat Space Environ Med. 2000; 71:593-8.
9. Tuukkanen J, Peng Z, Väänänen HK. Effect of running exercise so the bone loss induced by orchidectomy in the rats. Calcif Tissue Int. 1994; 55:33-7.
10. Mathey J, Horcajada-Molteni M-N, Chanteranne B, Picherit C, Puel C, Lebecque P, et al. Bone mass in obese diabetic Zucker rats: influence of treadmill running. Calcif Tissue Int. 2002; 70:305-11.
11. Shen ML, Wronski TJ. Time course of femoral neck osteopenia in ovariectomized rats. Bone. 1997; 20:55-61.
12. Silva AV, Volpon JB. Modelo de simulação de microgravidade e seu efeito em algumas propriedades mecânicas do osso do rato. Acta Ortop Bras. 2004; 12:22-31.
13. Kasper CE, McNulty AL, Otto AJ, Thomas DP. Alterations in skeletal muscle related to impaired physical mobility: an empirical model. Res Nurs Health. 1993; 16:265-73.
14. LeBlanc AD, Schneider VS, Evans HJ, Engelbretson DA, Krebs JM. Bone mineral loss and recovery after 17 weeks of bed rest. J Bone Miner Res. 1990; 5:843-50.
15. Colleran PN, Wilkerson MK, Bloomfield SA, Suva LJ, Turner RT, Delp MD. Alterations in skeletal perfusion with simulated microgravity: a possible mechanism for bone remodeling. J Appl Physiol. 2000; 89:1046-54.
16. Garber MA, McDowell DL, Hutton WC. Bone loss during simulated weightlessness: a biomechanical and mineralization study in the rat model. Aviat Space Environ Med. 2000; 71:586-92.
17. Bloomfield SA, Allen MR, Hogan HA, Delp MD. Site and compartment-specific changes in bone with hindlimb unloading in mature adult rats. Bone. 2002; 31:149-57.
18. Knox M, Fluckey JD, Bennett P, Peterson CA, Dupont-Versteegden EE. Hindlimb unloading in adult rats using an alternative tail harness design. Aviat Space Environ Med. 2004; 75:692-6.
19. Zhang G, Qin L, Shi Y, Leung K. A comparative study between axial compression and lateral fall configuration tested in rat proximal femur model. Clin Biomech. 2005; 20:729-35.
20. Burstein AH, Reilly DT, Martens M. Aging of bone tissue: mechanical properties. J Bone Joint Surg Am. 1976; 58:82-6.