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Biomechanics and structural adaptations of the rat femur after hindlimb suspension and treadmill running

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We microscopically and mechanically evaluated the femurs of rats subjected to hindlimb unloading (tail suspension) followed by treadmill training. Female Wistar rats were randomly divided into five groups containing 12-14 rats: control I (118 days old), control II (139 days old), suspended (tail suspension for 28 days), suspended-released (released for 21 days after 28 days of suspension), and suspended-trained (trained for 21 days after 28 days of suspension). We measured bone resistance by bending-compression mechanical tests of the entire proximal half of the femur and three-point bending tests of diaphyseal cortical bone. We determined bone microstructure by tetracycline labeling of trabecular and cortical bone. We found that tail suspension weakened bone (ultimate load = 86.3 ± 13.5 N, tenacity modulus = 0.027 ± 0.011 MPa·m *vs* ultimate load = 101.5 ± 10.5 N, tenacity modulus = 0.019 ± 0.006 MPa·m in control I animals). The tenacity modulus for suspended and released animals was 0.023 ± 0.010 MPa·m *vs* 0.046 ± 0.018 MPa·m for trained animals and 0.035 ± 0.010 MPa·m for control animals. These data indicate that normal activity and training resulted in recovered bone resistance, but suspended-released rats presented femoral head flattening and earlier closure of the growth plate. Microscopically, we found that suspension inhibited new bone subperiosteal and endosteal formation. The bone disuse atrophy secondary to hypoactivity in rats can be reversed by an early regime of exercising, which is more advantageous than ordinary cage activities alone.

Key words: Hindlimb suspension; Exercise; Biomechanics; Femur; Rat

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Introduction

Reduction of mechanical demands, such as those occurring during orthopedic immobilization, prolonged bed rest, or time spent in a microgravity environment (as experienced by astronauts) may affect all of the components of the locomotor system, leading to joint stiffness, muscular wasting, and osteopenia (1-3). Muscles become hypotrophic with interstitial proliferation of connective tissue, less resistant to fatigue (4), and mechanically weakened (5,6). Tendons are also affected by decreased maximal stress and energy absorption capacity (7). In joints, after a period of immobilization, stiffness results from several alterations that involve peri- and intraarticular structures.

Connective tissue retraction leads to capsular contracture (8) with myofibroblast proliferation; there is irregularity (9), thinning, and softening of the articular cartilage with proteoglycan depletion (10). With disuse, the skeleton seems to recognize that not all of its bone mass is needed to maintain structural integrity; bone mass decreases as a result of mineral density reduction (11), the bone architectural structure deteriorates, and osteopenia results. Weakening of the bone may lead to fractures or other musculoskeletal damage when physical activities are resumed (12,13).

Although there is no well-established countermeasure to maintain bone integrity during long periods of decreased physical activity or in a low-gravity environment (14), efforts have been made to minimize or neutralize the adverse effects that may follow. Trials aiming to maintain positive bone balance by administering drugs, especially bisphosphonates, have been conducted and the results have been positive for both humans and animals (15,16). However, drugs alone are unable to completely prevent or reverse the osteopenic process, and simultaneous mechanical stress may be required (16). There are some indications that physical rehabilitation programs can be of help in preventing side effects resulting from disuse since they encompass all of the musculoskeletal components (bone, joint, ligament, muscle) (17). Efforts have been made to establish safe yet efficient rehabilitation programs (18). For example, astronauts follow exercise programs during prolonged space flights, usually on treadmills or cycle ergometers; however, results are difficult to evaluate. Thus far, a validated exercise program does not exist for the microgravity side effects on the human locomotor system (16).

Rats are the animals most often used in experiments studying the effects of locomotor disuse; the tail-suspension method is the preferred method since it is practical and well tolerated and can simulate a weightless environment, immobilization, and changes due to prolonged bed rest (19-22). Swimming (23) and treadmill training (14) have been used for rats as rehabilitation models to investigate the response of the musculoskeletal system to exercise (24) because they are simple, relatively inexpensive, and have good animal compliance.

Because disuse atrophy weakens bone, mechanical testing is one of the best ways to characterize bone strength. This method can be used to evaluate the final effects of any adverse or positive interventions on bone structure.

Therefore, the objective of the present investigation was to study the effects of a rehabilitation program with treadmill training in rats after a period of hindlimb unloading by tail suspension, by assessing the microstructural and mechanical response of bone.

Material and Methods

The research design and all animal procedures were in accordance with the Institutional Principles of the Animal Experimentation Ethics Committee (CETEA #038/2003).

Female Wistar rats (*Rattus norvegicus albinus*) were used and randomly assigned prior to experimentation to the following groups: control I (12 animals, 118 days old), control II (12 animals, 139 days old), suspended (14 animals, 90 days old, suspended by the tail for 28 days), suspended-released (14 animals, 90 days old, suspended by the tail for 28 days and then released in their cage for 21 days), and suspended-trained (14 animals, 90 days old, suspended by the tail for 28 days and then trained on a treadmill for 21 days).

Animals belonging to the suspended, suspended-released, and suspended-trained groups were initially tail-suspended for 28 days. The routine animal preparation for tail suspension included anesthesia (30 mg/kg ketamine and 3 mg/kg xylazine), tail cleansing, and application of tincture of benzoin. Next, the proximal two-thirds of the tail were wrapped with a self-adherent foam pad (Reston 1560M, 3M, Brazil) for skin protection. A homogenously tensioned elastic bandage self-adherent wrap (Coban 1582, 3M, Brazil) was applied over the foam, encasing a 0.3-cm-wide strip that formed a free loop used to attach the animal to the suspension system. Before being suspended, rats were allowed to fully recover from anesthesia.

The suspension system consisted of a two-part cage and an animal fixation setup. The lower part of the cage was formed by a transparent open-topped acrylic box that was closed with an inverted metal grill cage, thus forming a 35-cm long, 35-cm wide, and 21-cm high compartment (floor area of 1225 cm^2). Therefore, the animal could be inspected daily without any interference from the suspension system. The strip loop was attached to a metal swivel hanging from a threaded cylindrical metal bar 5.0 mm in diameter, fixed to the upper part of the cage. Rod threading was necessary to increase attrition and to avoid excessive sliding with small animal movements. The range of animal excursion along the rod was restricted by two metal stops placed at the rod ends. Animal suspension was achieved by adjusting the bar height so that there was an approximately 30° angle of unloading (formed between the torso of the animal and the floor of the cage) (25), and the rat could bear weight on its forelimbs and rotate 360° or move around to reach water and food, but without any hindlimb contact with the bottom or walls of the cage. Hence, hindlimbs, pelvis, and the distal part of spine remained unloaded. Control animals were kept in regular cages (33 x 40 cm) with a floor area of 1320 cm^2. Animals were housed in a temperature-controlled room (23 ± 1°C) with 12-h light-dark cycles.

Animals assigned to the suspended-trained group were exercised on a motor treadmill, which consisted of six individual 15.0-cm high, 10.0-cm wide, and 50.0-cm long bays. We based the protocol for rat exercising on a study by Norman et al. (14). The training period began one day after the end of the suspension period, and started with 10 min of exercise, with daily 5-min increases until 60 min of

daily training was reached. The mean treadmill speed was 17.0 m/min on the horizontal plane. Animals underwent this protocol for 3 consecutive days, followed by 1 day with no training (rest/recovery).

Forty-eight hours before the end of the experimental period, we injected 50.0 mg/kg oxytetracycline hydrochloride intraperitoneally into all animals to label the newly formed bone (26). After euthanizing the rats with a thiopental overdose, we collected both femurs, cleaned them of soft tissue, weighed them, and stored them at -20°C.

Bone microscopic analysis

We performed bone microscopic analysis on non-demineralized bone sections obtained with a precision saw (ISOMET 1000, Buehler, USA) equipped with a diamond wafering blade. We prepared cross-sectional rings from the left femoral shaft and coronal sections from the proximal third of the right femur. After progressive dehydration in ethyl alcohol, the sections were embedded in orthophthalic polyester resin. After the resin had set the blocks were hand-ground on sandpaper to a thickness of 80 µm and mounted on glass slides. We analyzed the samples by epi-illumination under ultraviolet light.

Mechanical tests

Two types of mechanical tests were performed: bending-compression on the entire proximal segment of the femur, and bending tests on cortical bone samples taken from the femoral diaphysis. All mechanical tests were performed in a testing machine (model DL 1000, EMIC, Brazil) at room temperature (23-25°C), and the specimens were kept moist with saline.

After thawing, the distal end of the proximal femur segment was embedded in a cubic block of acrylic resin, with the bone kept in a vertical position. Care was taken to avoid overheating during cement setting by immersing the metal mold with the acrylic cement and the bone in ice water. Using a 500 N load cell, we applied a vertical downward force to the top of the femoral head (0.1 mm/min rate) until fracture occurred. Next, anteroposterior radiographs were taken and the fracture pattern was analyzed. We plotted the load versus deformation and determined the following mechanical properties from the curves: stiffness (curve slope during elastic deformation) and ultimate load (maximum load before failure).

For the three bending mechanical tests on standardized specimens, samples were collected from the anterior surface of the femoral diaphysis and shaped so that 12.0-mm long and 1.4-mm wide rectangular specimens were obtained. The 50 N load cell was used; specimens were placed on two metal supports 7.0 mm apart and a vertical force was applied to the periosteal surface at the middle portion of the sample at a rate of 0.1 mm/min until fracture. Data were normalized and the following mechanical parameters were determined: yield stress (applied stress at which irreversible plastic deformation is first observed), elastic modulus (measurement of the stiffness of a given material), ultimate stress (measurement of the greater stress), and tenacity modulus (energy absorption until the occurrence of fracture).

Statistical analysis

The Kolmogorov-Smirnov test for normality was initially performed for all properties (body mass and mechanical properties). Body mass after the different stages was compared by the paired *t*-test for the control I and the suspended groups (90 days and 118 days) and by one-way repeated measures analysis of variance (ANOVA) for the other groups (90 days, 118 days and 139 days). For the mechanical properties, the *t*-test was used to compare control I *vs* control II and control I *vs* suspended groups. One-way ANOVA was used for comparisons between control II *vs* suspended-released *vs* suspended-trained groups. The Tukey test was applied to compare pairs of groups when a statistical difference was detected. The level of significance used in all comparisons was 5%. The statistical software program SigmaStat version 2.03 (SPSS, USA) was used for data analysis.

Results

Body mass variation

At the beginning of the experimental period (90 days of age) one-way ANOVA did not show any statistical difference ($P = 0.347$) in body mass among the groups. Twenty-eight days later, body mass had increased significantly in control I from 318.0 ± 21.2 to 332.0 ± 19.3 g ($P < 0.001$; paired *t*-test). Conversely, after the same period, the suspended animals presented a significant body mass decrease from 310.0 ± 25.3 to 287.5 ± 23.7 g ($P = 0.003$; paired *t*-test).

The control II rats presented a significant variation in body mass ($P < 0.001$; one-way repeated measures ANOVA). At the beginning of the experiment (90-day-old animals), body mass was 316.7 ± 22.5 g and 28 days later (118-day-old animals) body mass reached a statistically significant difference of 331.1 ± 21.4 g ($P < 0.001$; Tukey test). At the end of the experimental period (139 days of age), body mass stabilized (334.0 ± 25.2 g; $P = 0.246$; Tukey test).

The suspended-released rats presented a significant variation in body mass ($P < 0.001$; one-way repeated

measures ANOVA) that decreased from 317.7 ± 16.2 g at the beginning of the experimental period to 300.3 ± 25.8 g after 28 days in suspension (P < 0.001; Tukey test). However, 21 days after being released from suspension, the same animals presented a significant recovery of body mass (330.2 ± 21.3 g; P < 0.001; Tukey test).

The suspended-trained animals presented a significant variation in body mass (P < 0.001; one-way repeated measures ANOVA). The body mass at the beginning of the experimental period was 314.0 ± 18.7 g and decreased to 297.5 ± 27.5 g after 28 days of suspension (P < 0.001; Tukey test), with a significant recovery 21 days later with treadmill training (335.0 ± 25.4 g; P < 0.001; Tukey test).

Mechanical properties obtained from the flexion-compression tests performed at the whole proximal femoral end

The mean stiffness for control I animals (209,367 ± 64,464 N/m) was statistically significant (P = 0.020; *t*-test) compared with suspended animals (189,809 ± 66,262 N/m; *t*-test), but no difference was found when control I was compared with control II (287,167 ± 77,155 N/m; P = 0.512; *t*-test). No difference was found when control II, suspended-released (269,130 ± 46,679 N/m) and suspended-trained (275,318 ± 62,726 N/m) groups (P = 0.432; one-way ANOVA) were compared.

The ultimate load for control I animals (101.5 ± 10.5 N) was statistically greater compared with the suspended group (86.3 ± 13.5 N; P = 0.012; *t*-test), but lower than control II animals (115.0 ± 12.6 N; P = 0.013; *t*-test). However, no difference was found when control II, suspended-released (104.9 ± 12.9 N) and suspended-trained (115.4 ± 15.5 N) groups (P = 0.183; one-way ANOVA) were compared.

Mechanical properties obtained from the three point flexion tests in cortical bone samples

The mean yield stress for control I animals was 147.5 ± 23.3 MPa, for the suspended group it was 133.4 ± 20.5 MPa, for control II animals it was 149.4 ± 19.0 MPa, for the suspended-released group it was 147.4 ± 27.0 MPa, and for the suspended-trained group it was 154.5 ± 21.1 MPa. Comparisons between control I *vs* suspended (P = 0.175; *t*-test) and control I *vs* control II (P = 0.833; *t*-test) showed no statistical differences. One-way ANOVA used to compare control II, suspended-released and suspended-trained groups showed no statistical difference (P = 0.766).

The elastic modulus in control I animals was 8.7 ± 1.3 GPa, 8.4 ± 2.5 GPa for the suspended group, 9.8 ± 1.7 GPa for control II animals, 9.7 ± 1.8 GPa for the suspended-released group, and 11.2 ± 1.2 GPa for the suspended-trained group. Comparisons between control I *vs* suspended (P = 0.795; *t*-test) and control I *vs* control II (P = 0.108; *t*-test) showed no statistical differences. One-way ANOVA used to compare control II, suspended-released and suspended-trained groups showed no statistical difference (P = 0.062).

The ultimate stress for control I animals was 152.9 ± 22.0 Mpa, which was statistically different (P < 0.001) compared with control II animals (199.6 ± 19.8 Mpa; *t*-test), but no difference was found for the comparison between control I animals and the suspended group (162.6 ± 35.5 MPa; P = 0.471; *t*-test). One-way ANOVA used to compare control II, suspended-released (176.7 ± 43.3 MPa) and suspended-trained (201.4 ± 28.0 MPa) groups showed no statistical difference (P = 0.361).

The tenacity modulus for control I animals was 0.019 ± 0.006 MPa·m, which was statistically different (P = 0.043) compared with the suspended group (0.027 ± 0.011 MPa·m; *t*-test) and between control I and control II groups (0.035 ± 0.010 MPa·m; P < 0.001; *t*-test). A statistical difference was found when comparing control II, suspended-released (0.023 ± 0.010 MPa·m) and suspended-trained (0.046 ± 0.018 MPa·m) groups (P = 0.003; one-way ANOVA). The Tukey test was applied and it was found that the suspended-trained group presented a tenacity modulus statistically different (P = 0.002) from suspended-released animals.

Radiography

The fracture pattern observed was similar for all groups and consisted of a fracture line that started at the lateral third of the head and descended vertically toward the femoral neck. The fracture line was visible in most specimens, with no difference among the various groups.

Microscopic analysis

The coronal sections of the proximal femur from control rats showed a femoral head with a spherical shape and well-delimited secondary ossific nucleus, which was formed by well-preserved trabeculae with the outer surface outlined by a fluorescent layer. The growth plate was well defined, crossing the femoral head from one side to the other. We did not observe any difference between the suspended and control groups (Figure 1A).

In the suspended-released group, the main changes occurred in the femoral head, which had lost its spherical shape and presented a flat appearance. The ossification nucleus had a reduced height, with a collapsed and disorganized trabecular pattern and, as a result, loss of the intertrabecular space. The growth plate was also affected showing irregular boundaries and widened regions. In the

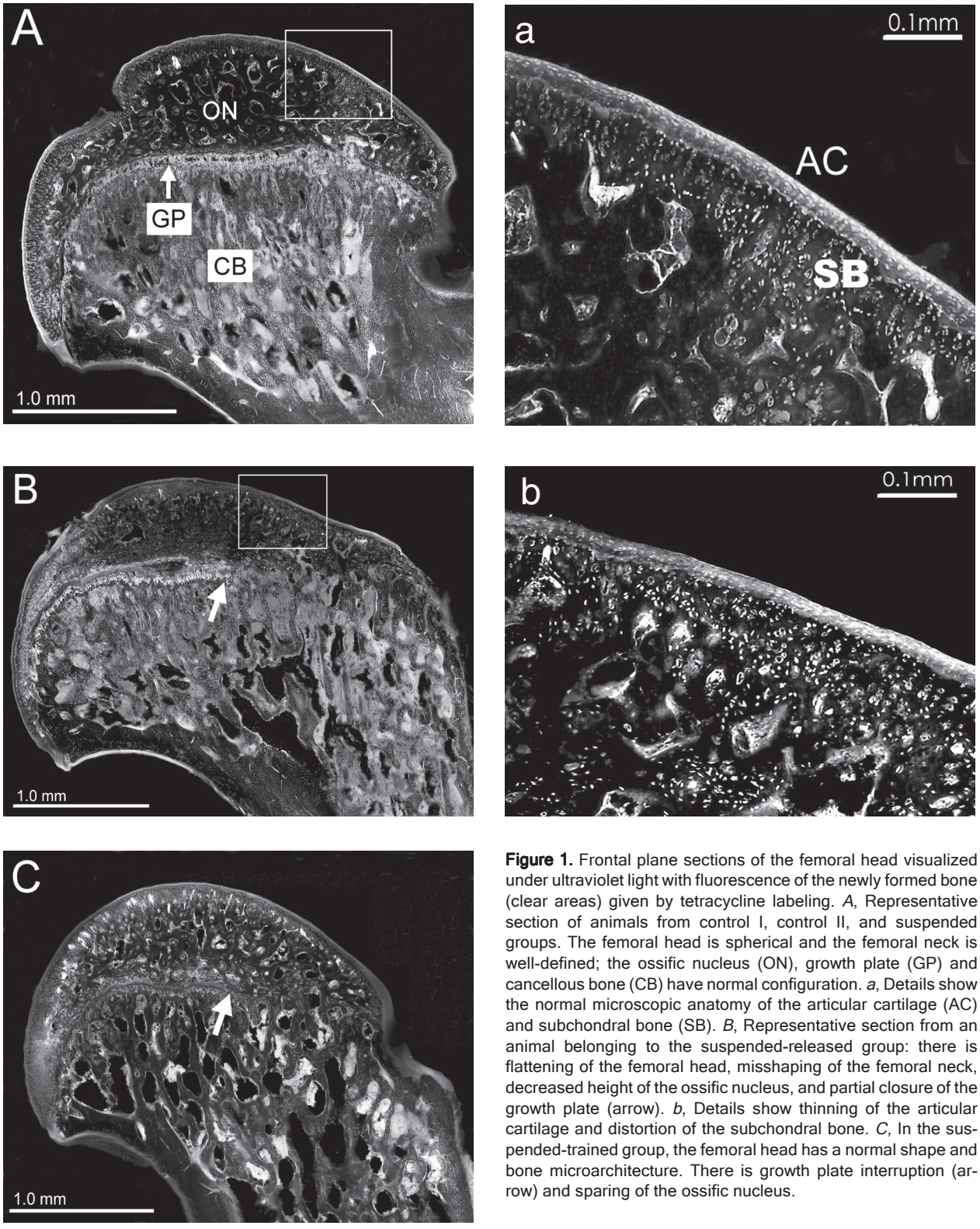

Figure 1. Frontal plane sections of the femoral head visualized under ultraviolet light with fluorescence of the newly formed bone (clear areas) given by tetracycline labeling. *A*, Representative section of animals from control I, control II, and suspended groups. The femoral head is spherical and the femoral neck is well-defined; the ossific nucleus (ON), growth plate (GP) and cancellous bone (CB) have normal configuration. *a*, Details show the normal microscopic anatomy of the articular cartilage (AC) and subchondral bone (SB). *B*, Representative section from an animal belonging to the suspended-released group: there is flattening of the femoral head, misshaping of the femoral neck, decreased height of the ossific nucleus, and partial closure of the growth plate (arrow). *b*, Details show thinning of the articular cartilage and distortion of the subchondral bone. *C,* In the suspended-trained group, the femoral head has a normal shape and bone microarchitecture. There is growth plate interruption (arrow) and sparing of the ossific nucleus.

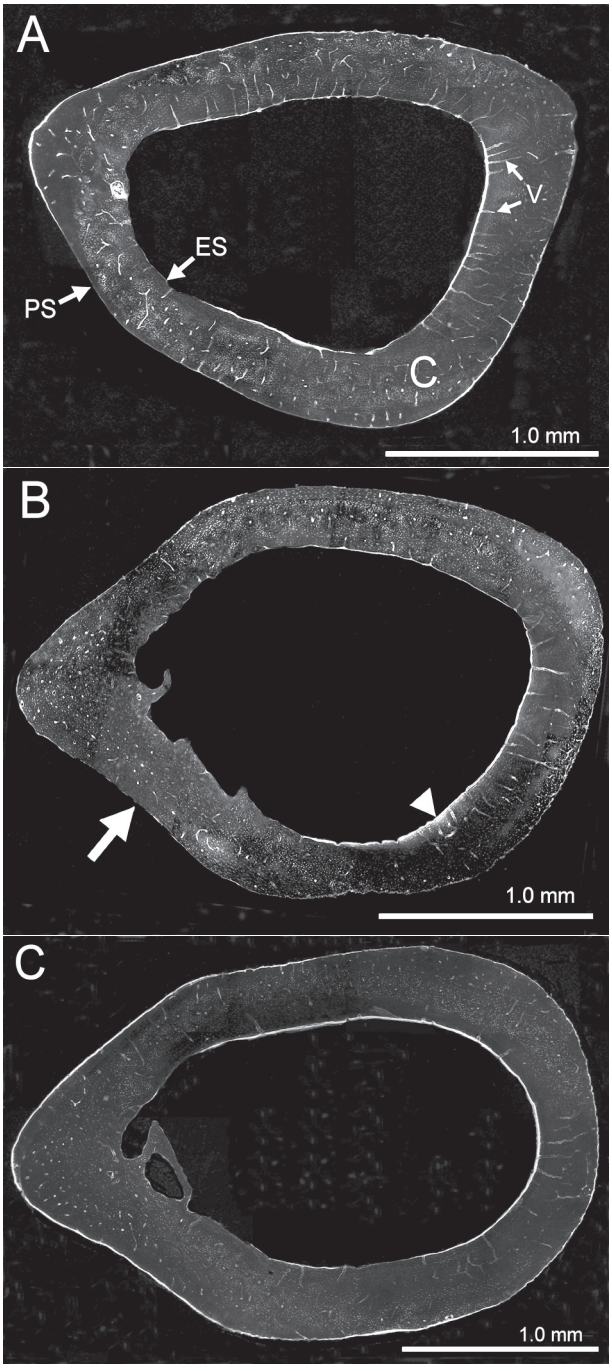

Figure 2. Cross sections of the femoral diaphysis seen under ultraviolet light, with fluorescence of the newly formed bone (clear areas) given by tetracycline labeling. *A*, Representative section of control I, control II, and suspended-trained animals. The periosteal (PS) and endosteal (ES) surfaces are outlined with bone fluorescence and the bone cortex (C) is traversed by vessel channels with fluorescent walls (v). *B*, In the suspended group, weaker subperiosteal (arrow) and endosteal (arrow head) fluorescence was observed. *C*, In the suspended-released animals, recovery of bone formation on the periosteal and endosteal surfaces was observed.

lateral part of the femoral head, the growth plate had disappeared, thus establishing union between the metaphyseal and the epiphysial bone (Figure 1B). In this group, the articular cartilage was ill-defined, and the osteocytic lacunae in the subchondral bone were irregularly arranged compared to control (details in Figure 1a,b).

In the suspended-trained group, all morphological aspects of the ossification nucleus were preserved, such as sphericity, shape, height, and trabecular arrangement in the femoral head. The growth plate was visible, although it had partially disappeared at the lateral portion of the femoral head (Figure 1C).

For the diaphyseal cross-sections of the femur, both control groups showed a narrow, well-defined fluorescent ring on the outer surface (subperiosteal bone formation), and another ring lining the medullary canal (endosteal osteogenesis). Inside the cortex, the fluorescent areas followed the tract of intraosseous blood vessels, crossing from one cortex to the other, which corresponded to Volkmann's vascular system. Osteocytic lacunae appeared as weak, nonspecific fluorescent spots (Figure 2A).

The femoral diaphysis of suspended animals showed weaker fluorescence on both the periosteal and endosteal surfaces (Figure 2B). For the suspended-released group, the subperiosteal fluorescent layer was more irregular and had a jagged appearance (compared with control groups; Figure 2C). We did not observe any difference when we compared the suspended-trained group with the control groups.

Discussion

Hindlimb rat suspension is considered to be the model of choice for simulating the effects of hypoactivity and weightlessness, with its use having been described in >800 articles (17). With respect to the skeletal system, tail suspension is an appropriate model for bone disuse studies, as shown by Bloomfield et al. (3), who established a correlation between suspension and bone morphometry (3). An additional advantage of the model is its reversibility, which allows studies to be performed during the recovery period, in contrast to irreversible methods such as sciatic nerve division (27) and damage to the spinal cord (28). Nevertheless, some authors have also called attention to the drawbacks and limitations of the suspension model. Because it causes differential muscle atrophy of the postural and other extensor muscles, it may not be a good model for muscle disuse (29). In addition, some divergences between rats and humans must be considered, such as the different rates of biological tissue remodeling (14).

Morey-Holton and Globus (17) focused on some important technical and methodological details of the model, such as the angle of unloading (i.e., the angle formed between the torso of the animal and the floor of the cage), which should maintain a 30° tilt (17,25). This position provides adequate weight bearing on the forelimbs, with unloading of the hindlimbs and the lumbar vertebrae, but not of the cervical vertebrae (30). Furthermore, the head-down position causes a cephalic fluid shift and mimics many cardiovascular and metabolic spaceflight changes (31). Another detail is the environment and animal stress control, indicators of which include corticosterone level, thymus and adrenal weight, food consumption, and body weight. During hindlimb suspension, these indicators should not be markedly different from those of control animals (17).

In our experimental design, we took into consideration many of the recommendations of Morey-Holton and Globus (12). For example, we used more than one control to correctly interpret the results. Two control groups were created (baseline controls) because we used young adult animals that were still developing and could present differences caused by maturation during the experimental period (3). Control I was used as a control group for suspended animals (118 days old at sacrifice) and control II served as a control group for the suspended-released and suspended-trained animals (139 days old at the end of the experiment). The differing results of ultimate stress between the two control groups reinforce the correctness of our decision.

The present data show that the rats had a reduction in body mass after 28 days of suspension, which is in accordance with other studies (3,32), meaning that it is unlikely that any of the animals completely adapted to suspension. Morey-Holton and Globus (17) noted that body weight is an important parameter that reflects the health of the rat, and stated that weight loss not exceeding 10% or 2 SD from the mean is an acceptable limit. Weight loss may be secondary to decreased food intake or to a stress state. Differentiating between the two conditions would entail obtaining the weight of the adrenal gland for all animals and controlling access to food for the control groups such that the amount would be the same as that ingested by the suspended animals (17). In the present investigation, we used still immature adult rats (mean body mass of 315 g), as shown by the gain in body mass with time and increased bone stiffness with age in the control groups. When age is taken into consideration, younger animals are preferred by many researchers since older animals (>400 g) may have difficulty adapting to tail suspension (17). At the end of the suspension period, the animals in the current study showed complete recovery of body mass with or without training, thus indicating that the rat was able not only to spontaneously adapt after the suspension period (33), but also to comply with the physical training without exhaustion.

When mechanical tests are performed on an entire bone segment, they reflect the bone structural properties; when standardized samples are used, the resulting information reflects the characteristics of bone as a tissue (34,35). The mechanical tests on the proximal femur and cortical bone samples indicated that tail suspension for 28 days significantly weakened bone, in agreement with previous studies (3,34,36,37). These results support the idea that tail suspension is a good model for skeletal disuse atrophy. In the current study, the results of the mechanical tests were less apparent in the cortical bone samples, demonstrating that this type of bone may be less susceptible to hypoactivity than is the cancellous bone found in the epiphyseal and metaphyseal regions. These results are in contrast to those obtained by Allen and Bloomfield (32), but the difference in results could be due to the fact that those investigators tested the whole tibia and femur in three-point bending tests rather than in standardized samples as we did.

Our findings, obtained by microscopic analysis, are descriptive and show that suspension alone did not affect the bone microarchitecture of the proximal third of the femur. However, morphometric studies should be carried out to confirm or reject this observation, because other studies have shown quantitative changes in cancellous bone with suspension (3,14). Nonetheless, we found the most remarkable changes when we compared morphological aspects between the suspended-released and suspended-exercised animals. At the end of the animal release period, if there was no programmed physical activity, we observed a flattening of the femoral head with crushing of the ossific nucleus, trabecular collapse, degenerative changes of the articular cartilage, and premature closure of the growth plate. In contrast, the group that underwent training after suspension had preservation of the femoral head shape, a normal trabecular pattern, and only partial closure of the growth plate. Therefore, exercise protected bone morphology and microarchitecture. We were unable to find similar experiments in the literature, and the interpretation of these data is speculative, but restriction in cage of recently suspended animals may have led to continuous mechanical stress applied to the top of the femoral head as a result of the restricted mobility and sustained hip position in the cage.

The microscopic studies performed on cross sections of the femoral diaphysis showed that this area was similar in suspended trained animals and control animals. How-

ever, the suspended animals displayed less new bone formation on the endosteal and periosteal surfaces, as demonstrated by tetracycline labeling. Previous studies on the effects of space flight on rats showed that weightlessness inhibited periosteal new bone formation but did not affect endosteal ossification (17,32,37). Our results partially support these findings.

We found that suspension caused substantial weakening (mechanical behavior) of rat femurs, which was reversed by treadmill exercising. Recovery after suspension in regular cages, without any provocative activity, revealed that many bone structures (growth plate, ossific nucleus, and articular cartilage) were negatively affected.

Our results suggest that reversion of bone disuse atrophy is more responsive to early assisted loading and walking than the customary protective activity. Therefore, the study of new regimens of physical activities should be encouraged to establish the best balance between physical activity and bone response.

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