Aerobic Exercise as a Countermeasure for Microgravity-Induced Bone Loss and Muscle Atrophy in a Rat Hindlimb Suspension Model

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Background: Loss of bone and skeletal muscle atrophy resulting from non-weight-bearing are major concerns associated with microgravity environment and spaceflight deconditioning. The objective of this research was to address the fundamental issue of whether bone loss and muscle atrophy could be attenuated using weight-bearing aerobic exercise on a treadmill as a countermeasure in rats subjected to simulated weightlessness by hindlimb suspension. ***Method:*** Bone and muscle from control and hindlimb-suspended groups with and without exercise were evaluated by bone mineral density (BMD), mechanical tests, bone histomorphometry and muscle mass. ***Results:*** Femoral BMD of hindlimb-suspended (HS) rats subjected to treadmill exercise was significantly greater than femoral BMD of HS rats without exercise and also was equivalent to that of weight-bearing controls. Muscle mass from HS rats exercised on a treadmill was significantly greater than muscle mass from HS rats that did not exercise. Exercise did not result in muscle mass equal to that of controls, however. In addition, histomorphometric analysis of the metaphysis of the proximal tibia revealed that HS rats that exercised did not maintain bone formation equivalent to controls. No other bone parameters were found to vary significantly between groups. ***Conclusions:*** It was concluded that moderate aerobic exercise on a treadmill did attenuate bone loss and muscle atrophy due to simulated weightlessness by hindlimb suspension, however its effectiveness differed by tissue, anatomical site and parameter investigated.

Keywords: microgravity, bone, muscle, exercise countermeasure, hindlimb suspension, aerobic, weightlessness, spaceflight.

IT HAS BEEN previously established in numerous radiodensitometric and biochemical studies of human bone and bone from other animals that disuse (paralysis, immobilization and weightlessness) and sex hormone deficiency result in loss of bone mass, or disuse osteoporosis (2,23,27,28,32,39,42). Bone loss in humans leads to increased fracture risk and fragility (10). Loss of bone due to microgravity has raised considerable concern about the increased fracture risk of astronauts, especially post-menopausal females. Despite the serious implications of bone loss and fracture fragility of the human skeleton, no countermeasure has been established that would maintain bone mass during periods of weightlessness.

Skeletal muscle atrophy resulting from non-weight-bearing is one of the major changes associated with spaceflight deconditioning (8). Spaceflight deconditioning may produce a loss of skeletal muscle protein content, strength and overall physical capability during long-term missions and difficulty in adaptation to the gravitational environment of earth after completion of the spaceflight (8). Several countermeasures have been tested for their ability to ameliorate or prevent skeletal muscle atrophy during microgravity. The countermeasure most often investigated is exercise (9,14,16,29,35). No exercise countermeasure to date has completely prevented muscle atrophy during periods of weightlessness (17).

Exercise has also been found to have a positive effect on bone mass. Animal studies that have used treadmill exercise protocols have reported increased bone mass (5,7,24,26,31,37,44). Treadmill exercise has been shown to attenuate bone loss in estrogen-deficient rats (7,12,24,36). Calcium content, bone mineral content (BMC), and bone mineral density have also been shown to increase with exercise in normal and estrogen deficient rats (7,45,46).

The objective of this research was to address the fundamental issue of whether bone loss and muscle atrophy could be attenuated using weight-bearing aerobic exercise as a countermeasure in rats subjected to simulated weightlessness by hindlimb suspension. The response of bone and muscle of HS and control rats to

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continuous treadmill exercise was examined using BMD, mechanical tests, bone histology and muscle mass.

METHODS

Following approval by the Animal Care and Use Committee, mature, sexually naive female Sprague Dawley rats (age 26 wk) were obtained (Hilltop Lab Animals, Scottdale, PA). The rats were randomly assigned to four groups (N=8) tested in two series (N=4 each series): 1) ground-bearing controls, no exercise (C); 2) ground-bearing, treadmill exercised (CE); 3) hindlimb-suspended controls, no exercise (HS); and 4) hindlimb-suspended, treadmill exercised (HSE). Rats from each of the groups were housed in identical cages and under identical climate and reversed light control conditions (12 hours light, 12 hours dark). Food and water were provided ad libitum and food intake and mass were recorded on a daily basis. The rats were each given a 7–10 d acclimation period before the start of the hindlimb suspension or exercise protocol.

HS and HSE animals were suspended according to established protocols (14). After cleaning and applying adhesive (tincture of Benzoin) to the tail, two strips of adhesive tape were applied to the ventral and dorsal proximal half of the tail, allowing the exposed distal half of the tail to properly thermoregulate. The dorsal tape was then threaded through a metal connector and three strips of tape were loosely applied circumferentially for reinforcement. Gauze sprayed with Chew-Guard was wrapped around the proximal half of the tail to discourage chewing. The animal was then suspended for 21 days at a 30° head-down angle by a swivel apparatus in a plexiglass cage measuring 12 x 12 x 12 in (30.5 cm^3). The animals had 360° of mobility around a central axis, with no weight-bearing by the hindlimbs and with ready access to food and water ad libitum. In order to facilitate measurement of bone growth at the epiphyseal growth plate, all animals were labeled with 15 $mg \cdot kg^{-1}$ of demeclocycline intraperitoneally at the onset of the experiment (46) and with 8 $mg \cdot kg^{-1}$ of calcein 24 h before sacrifice.

Animals were removed from the study if they displayed any of the following: 1) excessive weight loss (15% weight loss); 2) tail necrosis or excessive discomfort; 3) chromodacryorrhea (excessive red tears); or 4) excessive hair loss. All four HSE rats in the first series were removed from the study as the result of excessive weight loss believed to be caused by the combination of hindlimb suspension and too much exercise. As a result, the exercise protocol was modified from daily exercise to the 3:1 cycle described below and four additional HSE rats were added. One HS rat was also removed from the study due to tail necrosis and discomfort.

The continuous treadmill exercise protocol consisted of exercising animals on a treadmill with the suspension dressing attached to low friction rails to prevent the dressings from dragging. The exercise period was increased from 10 $min \cdot d^{-1}$ in 5 $min \cdot d^{-1}$ increments to 60 $min \cdot d^{-1}$. Based on the average speed of previously published protocols, the treadmill was set at a speed of 17 $m \cdot min^{-1}$ and 0% grade for the entire training regimen. Lagging animals were encouraged to run via a microcurrent coil. The rats were exercised in the dark coinciding with the 12-h dark cycle. Throughout the 21-d period, animals were exercised for 3 consecutive d followed by 1 d of ground-bearing only, equal in time to the exercise period of the previous day. The purpose of the ground-bearing day was to reduce stress to the animals and prevent overtraining. This 3:1 cycle of exercise to active rest is identical to the Russian countermeasure program on MIR (19) chosen to avoid accumulation of weakness and fatigue.

BMD was measured for the left tibias and femurs of each animal. All measurements were done on a Lunar DPX-L densitometer (Lunar Corporation, Madison, WI). The software used was for small animals, version 1.0c. This was done under the direction of V. Matkovic, M.D., of the Ohio State University Bone Study, Davis Research Center, Columbus, OH. Each bone was scanned for area and bone mineral content (BMC). From this information, BMD was derived using BMD = BMC/Area of Scan.

Bone density of the tibias and femurs was determined using the wet mass and volume of whole bones according to Yeh et al. (45). Soft tissue was removed from the tibias and femurs of all rats and each bone placed in a volumetric flask filled with deionized water. The flask was placed in a dessicator connected to a vacuum for approximately 2 h to remove trapped air from the bone. The wet mass of the blotted bone was recorded and weighed again with the bone suspended by a fine wire in the water-filled flask. The difference in mass divided by the water density was the bone volume. The bone density was calculated as milligram wet mass per microliter bone volume.

The proximal tibias were used to determine trabecular bone volume [Bone Volume (BV)/Total Volume (TV)] according to methods proposed by Sogaard et al. (33) and Tuukkanen et al. (37) The right tibias were divided at the mid-diaphysis and the proximal ends bisected in the coronal plane. The sections were stained with Sirius Red and Masson's trichrome and viewed under a light microscope (Olympus BH-2). BV/TV of the proximal ends was estimated by finding the ratio of trabecular bone over the total area using Image Analysis Software (Optimas, Edmonds, WA). The rectangular measurement area was taken from the epiphyseal zone as the upper limit to approximately 3 mm from that position as the lower limit. In addition, the distance between the epiphyseal growth plate and the demeclocycline label of the proximal tibia was measured (4). An average of five measurements from random fields was taken. Specimens in each group were evaluated histologically; however, histological preparations from the first series were not suitable for examination and were removed from the study. This resulted in the variation in the number of specimens in Table III.

The cross-sectional morphometry of right femurs was measured from a 250-μm thick slice taken from the mid-diaphysis of each femur using a low-speed saw. Each slice was polished and mounted on a slide and viewed under a light microscope. The periosteal cross

TABLE I. BODY MASS (G) AND FOOD CONSUMPTION (G), MEAN (±SD).

	C	CE	HS	HSE
Initial Body Weight	295.34[a] (8.83)	**287.10[a]*** (9.38)	292.57 (4.60)	295.68[a] (7.80)
Final Body Weight	311.53[a] (20.41)	304.7[a] (16.44)	**276.21[a]** (18.14)	293.80 (16.51)
Initial Food Consumption	20.01[a] (4.47)	20.31[a] (6.00)	**9.77[a]** (4.32)	**10.98[a]** (2.93)
Final Food Consumption	23.1 (7.45)	26.56[a] (3.79)	**20.44[a]** (7.12)	26.73[a] (2.38)

*Bolded entries are significantly different ($p < 0.05$) than non-bolded entries with the superscript "a."

section area (PA) and the endosteal cross-section area (EA) or marrow area were measured using the Image Analysis Software from which the cortical cross-section area (CA = PA - EA) was calculated. The length of the femurs and tibias was also measured for each rat.

Mechanical testing was performed to measure breaking load of the femoral neck. The femurs were divided at the mid-diaphysis and the mid portion of the proximal end embedded in a cylinder 2 cm long and 1.5 cm in diameter. Using polymethylmethacrylate, the specimen was inserted into a fixture that allowed the load to be applied to the femoral neck along an axis parallel to the shaft on the Materials Testing System (Minneapolis, MN) servo hydraulic testing machine. The load was gradually applied at a rate of 12 mm · min^{-1} to the femoral neck until fracture of the neck occurred according to work by Tuukkanen et al. (37).

The soleus, plantaris and gastrocnemius muscles of the hindlimb were rapidly excised after exsanguination. The tissues were rinsed with cold saline, trimmed of excess fat and connective tissue, blotted dry and wet weighed separately.

All biological and mechanical parameters tested were analyzed to compare the four groups using a one-way analysis of variance (ANOVA). Results are expressed in terms of mean ± standard error and $p < 0.05$ was considered significant. Multiple comparison procedures were also used to examine differences between means to determine the effect of hindlimb suspension and the effect of exercise using Fisher's Least Significant Difference (LSD). Body mass and food consumption were also analyzed using a 4 group (C,CE,HS,HSE) x2 (Pre, Post) trial ANOVA with repeated measures on trial.

RESULTS

There were no significant differences in any of the variables investigated between the four CE rats exercised daily (first test series) and the four CE rats exercised on the 3:1 cycle (second test series). Therefore, results from both CE groups were pooled for the subsequent statistical analysis.

Average body mass of control and control exercise groups increased over the 21-d test duration (**Table I**) to approximately 105.5% and 106.1% of initial values, respectively. Average body mass of HSE rats decreased over the first 8 d and then increased to a level roughly equivalent to initial mass. HS rats without exercise lost weight over the entire duration of the test, with a finishing mass approximately 6% less than starting mass. There was a significant ($p < 0.0007$) difference among groups with respect to change in body mass over the test period. C and CE showed an increase while HSE did not change and HS showed a decrease. There was also a significant difference in the change in the amount of food consumption, with HS and HSE having a greater change (increase) in consumption than C and CE. After body mass was linearly adjusted for food consumption, there were no significant differences in body mass due to hindlimb suspension.

TABLE II. MUSCLE MASS (G), MEAN (±SD).

Muscle	C (n = 8)	CE	HS (n = 7)	HSE (n = 8)
Gastroc	1.7701[a] (0.1187)	1.8764[a] (0.0920)	**1.2549[a,b]*** (0.1603)	**1.4492[a,b]** (0.1796)
Soleus	0.1584[a] (0.0127)	0.1645[a] (0.0177)	**0.0922[a,b]** (0.0163)	**0.1209[a,b]** (0.0207)
Plantaris	0.3964[a] (0.0486)	0.4318[a] (0.0590)	**0.3154[a]** (0.0375)	0.3713 (0.0774)
Total	2.3249[a] (0.1707)	2.4727[a] (0.1467)	**1.6626[a,b]** (0.1981)	**1.9347[a,b]** (0.2557)

*Bolded entries are significantly different ($p < 0.05$) than non-bolded entries with the superscript "a." The superscript "b" signifies bolded entries that are significantly different ($p < 0.05$) from one another.

HSE rats had significantly greater ($p < 0.05$) muscle mass than HS rats (**Table II, Fig. 1**). Exercise did not result in muscle mass equal to that of controls (C and

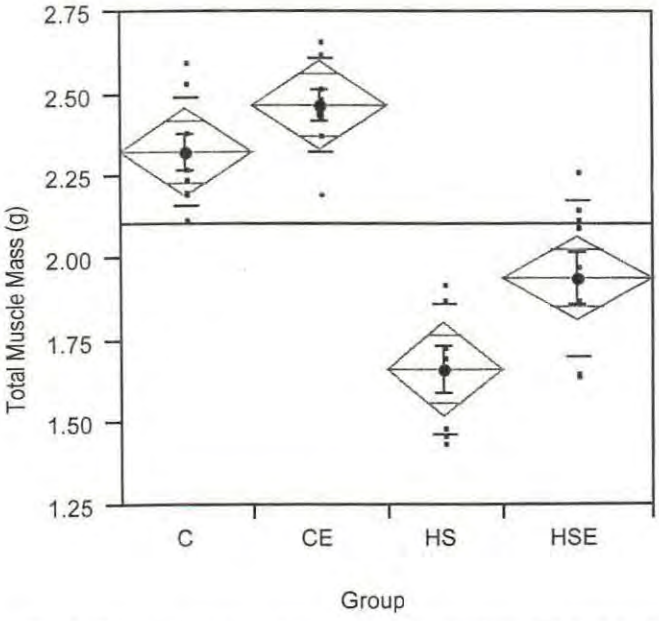

Fig. 1. Total muscle mass (mass of gastroc, plantaris and soleus) in hindlimb-suspended rats subjected to aerobic exercise on a treadmill (HSE) was significantly greater ($p < 0.05$) than total muscle mass of hindlimb-suspended rats without exercise (HS). Aerobic exercise of hindlimb-suspended total muscle mass, however, was less than the muscle mass of the control groups.

TABLE III. BONE PARAMETERS, MEAN (±SD).

Measurement	Location	C	CE	HS	HSE
BMD ($g \cdot cm^{-2}$)	Femur	0.2384[a]	0.2451[a]	**0.2087**[a†]	0.2509
		(0.0190)	(0.0157)	(0.0370)	(0.0096)
		8*	8	7	8
BMD ($g \cdot cm^{-2}$)	Tibia	0.2149	0.2106	0.2043	0.2140
		(0.0154)	(0.0191)	(0.0156)	(0.0122)
		8	8	7	8
Growth Plate Distance (μm)	Proximal Tibia	764.5[a]	684.9[a]	**330.0**[a]	385.1[a]
		(112.0)	(64.3)	(114.2)	(126.9)
		4	3	5	8
TBV (%)	Proximal Tibia	24.83	27.14	28.69	29.41
		(5.34)	(2.78)	(3.42)	(2.71)
		4	3	3	8
Fracture Load (N)	Femoral Neck	125.1[a]	114.6	114.0	102.7[a]
		(21.3)	(24.3)	(17.4)	(12.3)
		8	8	7	8
Stiffness ($N \cdot mm^{-1}$)	Proximal Femur	280.2	312.8	323.0	281.6
		(102.0)	(40.2)	(47.8)	(53.5)
		4	4	3	8
Length (mm)	Femur	37.18	37.59	36.91	37.33
		(0.90)	(0.89)	(1.18)	(0.64)
		8	8	7	8
Length (mm)	Tibia	41.06	40.98	40.79	40.45
		(1.02)	(0.83)	(1.20)	(0.92)
		8	7	7	8
Cortical Area (mm^2)	Femoral Mid-shaft	5.71	5.70	5.49	6.07
		(0.94)	(0.66)	(1.02)	(0.50)
		8	7	7	8
Density ($g \cdot cm^{-3}$)	Femur	1.72	1.67	1.66	1.68
		(0.13)	(0.07)	(0.04)	(0.08)
		8	8	7	8
Density ($g \cdot cm^{-3}$)	Tibia	1.68	1.66	1.61	1.64
		(0.07)	(0.05)	(0.04)	(0.15)
		8	7	7	8

*Number of specimens, n, are indicated by last row of each measurement.
†Bolded entries are significantly different ($p < 0.05$) than non-bolded entries with the superscript "a."

CE), however. BMD of the femur in the HSE rats was significantly greater than BMD of HS rats (**Table III, Fig. 2**). HSE rats also maintained BMD equivalent to that of weight-bearing controls.

Histomorphometric analysis of the metaphysis of the proximal tibia showed that the distance between the epiphyseal growth plate and the distal stained region was significantly greater ($p < 0.05$) in the controls (C and CE) compared to the hindlimb-suspended (HS and HSE) rats. However, there were no significant differences between the HS and HSE groups (Table III). No additional bone parameters were found to vary significantly between groups (Table III).

DISCUSSION

Most of our understanding on the mechanical adaptations of human muscle and bone are derived from studies conducted on other animals, primarily rats. Rat models have been approved by the FDA and are accepted by the scientific community as a reliable model for human bone loss and other human skeletal problems as it mimics bone loss over short periods of time. Morey et al. (21) and Wronski and Morey-Holton (43) developed a hindlimb suspension model using the rat to simulate the weightless environment of space. The hindlimb suspension model, used to remove the weight-bearing function of the hindlimb, has been shown to elicit changes in muscle size, biochemistry and function similar to changes associated with spaceflight (13,20). Spaceflight also induces similar functional, morphological, metabolic and biochemical changes in rodents and humans (6). Although rat bone is a generally accepted model to study human bone, it is clear that there are differences between the behavior of rat tissue and human tissue (e.g., bone remodeling rate) which imposes certain limitations to transfer to humans.

Utilizing the hindlimb suspension model to study changes in skeletal muscle, our results show that muscle mass was significantly reduced in HS animals compared to weight-bearing controls. This result is not surprising as numerous studies have documented muscle atrophy resulting from microgravity or disuse (11,35). It was also found that even though HSE rats did not result in muscle mass equal to that of controls, they had significantly ($p < 0.05$) greater muscle mass than HS rats. This finding is also not surprising as the benefits of exercise on muscle have been reported in numerous studies (3,18,34,41).

Significant changes were also detected in BMD in the current study. BMD was significantly reduced in HS rats for the femur and tibia. However, BMD of the femur of HSE rats was significantly greater than that of HS rats. HSE rats also maintained BMD equivalent to that of weight-bearing controls, C and CE. Although we do expect a difference between the absolute values of

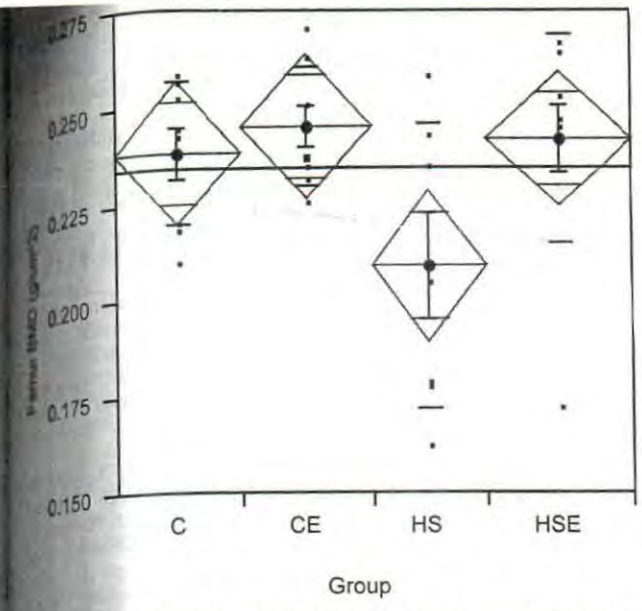

Fig. 2. Bone mineral density (BMD) in hindlimb-suspended rats subjected to aerobic exercise on a treadmill (HSE) was significantly greater ($p < 0.05$) than BMD of hindlimb-suspended rats without exercise (HS). Aerobic exercise of hindlimb-suspended rats also maintained BMD equivalent to control groups.

direct measurements and measurements made by a densitometer (30), directly measured density did not differ between groups whereas BMD did. This is yet unexplained; however, it may be related to the accuracy of the technique used to measure density.

Despite increases in BMD for the whole femur, the femoral neck fracture load was also not significantly different among the groups. There were also no correlations between femoral neck fracture load and whole bone BMD and femoral neck fracture load and whole bone density, which may be reflective of the level of comparison. Femoral neck fracture load depends on cortical and cancellous bone properties (density) and geometry at the neck. BMD and bone density measured in this study were whole or "global" bone properties that may not sufficiently reflect femoral neck "local" properties that influence fracture load.

A recent study by Vajda et al. (38) reported that the breaking strength of spaceflight bone was not significantly different than controls; these results are in agreement with the current study. Several other studies have investigated the influence of treadmill exercise on the strength of rat bones (1,22,24,25,33,37). These studies indicate that bone can be strengthened, weakened or unaffected by exercise, depending on the intensity of the training protocol.

The intensity of the training protocol used in the present study, although not measured, would be considered moderate and would correspond to a relative intensity of approximately 60 percent of maximum oxygen consumption ($\dot{V}O_{2max}$). Moderate intensity exercise with a similar duration as used in the present study has been shown to produce cancellous bone gain and trabecular structure reinforcement (5) whereas strenuous training (80% $\dot{V}O_{2max}$) for a much longer duration has been shown to reduce longitudinal bone growth, induce bone loss and reduce bone strength (4,40). Exercise of low intensity has also been shown to be insufficient to inhibit bone loss (5). It would appear that running 17 $m \cdot min^{-1}$ for up to 60 $min \cdot d^{-1}$ was sufficient to see some of the positive benefits of weight-bearing exercise, at least on BMD in rats subjected to hindlimb suspension. The beneficial effect of exercise may also differ in young vs. old rats (44). In the present study, the 26-wk-old rats may respond very differently to training compared with rats only 5 wk old that have not fully matured. In addtion, the 3:1 exercise cycle apparently reduced stress levels in HSE rats as compared to daily exercise. When HSE rats were exercised daily, we eliminated nearly every rat in that group due to excessive mass loss. However, this problem was eliminated with the 3:1 exercise cycle which supports the exercise philosophy on the MIR (19).

The distance between the epiphyseal growth plate of the proximal tibia and the distal stained region was significantly greater ($p < 0.05$) in the control rats (C and CE) compared to the hindlimb-suspended rats (HS and HSE). There were no significant differences, however, between control and exercise groups. Although metaphyseal growth was significantly greater in C and CE animals compared to HS and HSE in the tibia, the growth was small (micrometers) and the BMD of the whole tibia was not able to detect any differences between groups. Histomorphometric analysis of the femur was not conducted.

Additional bone parameters measured including stiffness of the proximal femur, length of the tibia and femur, cortical area of the femoral midshaft and density of the tibia and femur did not vary significantly between groups. It might be expected that bone parameters related to cortical bone would be less or undetectable over the 21-d period. A recent study (38) using flight rats and ground-based controls found no significant difference in static morphologic bone parameters.

From these results, we have shown that aerobic training reduced muscle atrophy and maintained BMD with no change to femoral neck strength and stiffness and no change in directly determined whole bone density (grams per cubic centimeter) in rats subjected to simulated microgravity. Several morphologic bone parameters were insensitive to the 21-d protocol. It remains to be seen if a similar or greater benefit would be obtained from resistance exercise in less time as has been indicated for soft tissue. Additionally, it has not been determined if a similar or greater benefit would be obtained with simple weight-bearing alone.

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