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Original Article

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Differential bone and muscle recovery following hindlimb unloading in skeletally mature male rats

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Abstract

This study was designed to track the recovery of bone and muscle properties after 28 days of hindlimb unloading (HU) in skeletally mature male rats in order to quantify the degree and timing of the expected mismatch between bone and muscle properties. Outcome variables were *in vivo* plantarflexor peak isometric torque and proximal tibial volumetric bone mineral density (vBMD). Proximal tibia vBMD was significantly lower than age-matched controls (-7.8%) after 28 days of HU, continued to decrease through day 28 of recovery (-10%) and did not recover until day 84 of recovery. Plantarflexor peak isometric torque was significantly reduced after 28 days of HU (-13.9%). Further reductions of isometric torque occurred after 7 days of recovery (-15%), but returned to age-matched control levels by day 14. The functional relationship between bone and muscle (vBMD/isometric torque) tended to increase after 28 days of HU (+7.8%), remained elevated after 7 days of reloading (+9.1%) and was significantly lower than age-matched controls on day 28 (-13.6%). This relatively rapid return of muscle strength, coupled with continued depression of bone density at the proximal tibia metaphysis, may increase the risk for skeletal injury during recovery from prolonged periods of reduced mechanical loading.

Keywords: Disuse, Peripheral Quantitative Computed Tomography (pQCT), Muscle Strength, Bone Density

Introduction

Bone loss occurs rapidly with the removal of mechanical loads[1]. Prolonged periods of non-weightbearing activity (immobilization, bed rest, spaceflight) lead to reductions in bone density at a rate of 1-2% per month[2,3]. The inverse relationship between bone density and fracture risk suggests this change will negatively alter bone strength. Until countermeasures can successfully prevent spaceflight-induced bone loss, maximizing the recovery of skeletal mass/density and strength upon return to a normal gravity environment is an important research goal. This would help minimize the risk of skeletal injury during this time period.

Early Skylab missions first suggested recovery of bone was compromised following spaceflight. Os calcis mineral content of astronauts remained below baseline levels during recovery for a period exceeding the mission duration[4]. Despite rapid normalization of calcium metabolism within days post-flight[5,6], serum and urine biomarkers of bone formation/resorption necessitate weeks to normalize[7,8]. To date, recovery of bone density to pre-flight values has yet to be documented in astronauts/cosmonauts[3,9], even when follow-up has extended to 5 years post-flight[10].

Due to numerous limitations in studying skeletal adaptations to spaceflight, most existing data derive from earth-based models of microgravity, with the rodent hindlimb-unloading (HU) model the most widely-used[11]. In skeletally mature rats (≥5 months old), 28 days of unloading reduces bone mineral density[12], cortical and cancellous bone formation rates[12,13], and cancellous and cortical bone strength[12,14,15]. Fewer data exist, however, on the recovery of bone following unloading, particularly in skeletally mature rats. Six-month-old rats fail to recover unloading-induced loss of total tibia bone mineral content and cortical bone formation rate after a period of recovery greater than the unloading duration[16].

The authors have no conflict of interest.

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Additional studies have quantified recovery of bone properties in young rats[17-21]. However, as the mechanisms of bone loss during disuse appear to be age-dependent[13], it is likely that similar age-related differences exist with respect to skeletal recovery.

Similar to bone, the muscles of the lower limbs (soleus, gastrocnemius, etc.) lose significant mass when unloaded[22], contributing to decreased force production (for review see[23]). However, in direct contrast to bone, muscle mass and force production have been documented to recover quickly from periods of unloading[24-27]. Rapid muscle recovery, combined with a slow recovery of bone, has the potential to produce a mismatch between bone and muscle strength. The implications of such a mismatch could include increasing fracture risk, as muscles could produce high forces on a bone with compromised strength[28].

The aims of this study were to document the time course of recovery for a functional bone and muscle unit following disuse and to identify the time period when the mismatch between bone and muscle properties was greatest. To accomplish these aims, two separate, but related studies were undertaken. In experiment 1, skeletally mature male rats were hindlimb unloaded for 28 days and then allowed normal cage ambulation for 7, 14, or 28 days. *In vivo* measures of bone and muscle were quantified in separate sets of animals at each time point to assess tissue recovery. As bone parameters remained significantly suppressed through day 28 of recovery, experiment 2 tracked animals longitudinally for 84 days after hindlimb unloading with *in vivo* assessment of bone parameters every 28 days in each animal. This second experiment allows the determination of the time frame for compromised bone parameters, and therefore the period when the bone muscle unit will be altered. Our working hypothesis was that recovery of muscle strength (plantarflexor peak isometric torque) would occur more rapidly than bone density of the associated proximal tibia, resulting in a mismatch between bone density and muscle strength during the first 28 days of recovery.

Materials and methods

Experiment 1

This study protocol and all animal procedures were in compliance with the Texas A&M University Laboratory Animal Care Committee rules and regulations. Male Sprague-Dawley proven breeder rats (5 months old) were obtained (Harlan, Indianapolis IN) and housed individually in a temperature-controlled room with a 12:12 hour light/dark cycle. Proven breeder animals were used to complement previous studies in our laboratory. One week after arrival rats were randomly assigned to either cage control (CC) or hindlimb unloaded (HU) + recovery (R) groups (Figure 1). HU animals were provided free access to food and water throughout the unloading and recovery periods while control animals were pair fed to match daily food intake with HU/R animals until day 7 of recovery. After 7 days of recovery, HU animals' spontaneous food intake routinely returns to baseline levels (unpublished observation) and therefore after day 7 control animals were allowed free access to food.

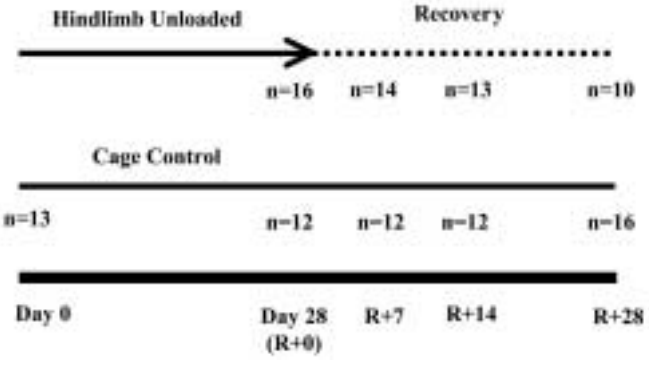

Figure 1. Experiment 1 protocol. Animals were randomly assigned to either hindlimb unloaded (HU) or cage control (CC) groups at the start of the experiment. All HU animals were unloaded for 28 days, after which time they were either sacrificed or removed from HU and allowed normal cage activity for 7, 14, or 28 days. Age-matched CC animals were sacrificed at similar times as HU-recovery animals, with an additional group sacrificed at the start of the experiment as a baseline control. In analyses where group n's differ from those stated in this figure, they are individually noted.

On day 0 (baseline) and the day of sacrifice, all animals underwent *in vivo* scanning of the left tibia using peripheral quantitative computed tomography (pQCT). Additionally, all animals underwent pQCT scanning on R+0, the day HU animals were removed from tail suspension and began normal cage ambulation. Prior to pQCT on the day of sacrifice, all animals underwent *in vivo* muscle function testing of the left leg plantarflexors (gastrocnemius, soleus, plantaris). An anesthetic regimen of fentanyl/droperidol and diazepam, agents that permit normal muscle function[29], was used during all pQCT and muscle function testing. Following pQCT on the day of sacrifice, animals were exsanguinated and left leg plantarflexor muscles (soleus, plantaris, and gastrocnemius) were dissected free and wet weights were recorded.

Experiment 2

Based on incomplete recovery of bone properties in experiment one, a second experiment was undertaken to specifically determine the long-term recovery of bone properties. Male Sprague-Dawley proven breeder rats (5 months old) were assigned to either cage control (CC) or hindlimb unloaded + recovery (HU/R) groups (n=10 and 11, respectively). All animals underwent *in vivo* scanning of the left tibia using pQCT on day 0 (baseline) and every 28 days thereafter through day 84 of recovery (112 days in total).

	Baseline/ Age-matched controls	Hindlimb Unloaded	% change
Body mass, g	461 ± 7	453 ± 6	-1.7
Ankle plantarflexor muscles			
Total mass, g	3.27 ± 0.12	2.49 ± 0.11*	-23.3
Peak isometric torque, Nmm	0.36 ± 0.01	0.31 ± 0.01 *	-13.9
Proximal tibia metaphysis			
Total BMC, mg/mm	11.4 ± 0.15	10.5 ± 0.15 *	-7.9
Total vBMD, mg/cm^3	628 ± 5	580 ± 5 *	-7.6
Total cross-sectional area, mm^2	18.2 ± 0.3	18.2 ± 0.3	0
Cortical area, mm^2	8.4 ± 0.1	7.7 ± 0.1 *	-8.3
Total vBMD/peak torque, mg/cm^3/Nmm	1738 ± 95	1874 ± 47	+7.8

Body mass and bone parameters are *in vivo* measures, assessed longitudinally in the same animals at baseline and after 28 days of unloading (n=46 animals). Muscle parameters and bone/muscle ratios are *ex vivo* measures from baseline controls (n=12) compared to animals sacrificed after 28 days of unloading (n=14). BMC, bone mineral content; vBMD, volumetric bone mineral density; * $p < 0.05$ compared to baseline/age-matched controls.

Table 1. Effects of 28 days hindlimb unloading on bone and muscle parameters.

Hindlimb unloading, for 28 days, and pQCT procedures were similar to those in experiment 1. No muscle functional testing was performed in experiment 2 animals based on full recovery of these properties in experiment 1.

Hindlimb unloading

Unloading of the hindlimbs was achieved by tail suspension as previously described[12]. Animals were fully recovered from anesthesia prior to tail suspension. Animals in designated groups were removed from HU after 28 days (R+0) and allowed normal cage activity for up to 28 days (R+28).

Peripheral quantitative computed tomography

Bone scans were obtained by peripheral quantitative computed tomography (pQCT) using an XCT Research M (Stratec; Norland Corp., Fort Atkinson, WI). The proximal tibia metaphysis of each animal was scanned *in vivo* to quantify volumetric bone mineral density (vBMD) and geometry. The measure of bone density obtained using pQCT "volumetric" as opposed to other techniques such as DXA that provides "areal" BMD. Three contiguous slices (0.5 mm thick) were obtained at 5, 5.5, and 6 mm from the proximal tibia plateau using a voxel size of 0.10 x 0.10 x 0.50 mm. Using standard Stratec software (Version 5.40B), values of total bone mineral content, total volumetric bone mineral density, total cross-sectional area (all area within the periosteal perimeter, bone+marrow), and cortical area were obtained for each slice and those data were averaged to get a mean value. Reproducibility for *in vivo* pQCT measurements was assessed by scanning the same animals on 5 consecutive days. Co-efficients of variation were 1.24% and 1.95% for proximal tibia vBMD and total cross-sectional area, respectively.

In vivo muscle testing

In experiment one, contractile properties of the left leg plantarflexors were tested using an isokinetic dynamometer on the day of sacrifice[24]. Briefly, the left hindlimb was shaved and distal tendons of the left dorsiflexor muscles were cut to prevent antagonistic contraction of these muscles during the testing protocol. Two percutaneous needle electrodes were inserted at the upper thigh in close proximity to the sciatic nerve. Stimulation voltage was optimized using 0.1-ms pulses at 300 Hz to yield the maximal isometric torque. Next, a series of 13 isometric stimulations, with 45 seconds of rest between, were performed at the optimized voltage, increasing in frequency from 10 to 200 Hz. Peak isometric torque obtained during this test was recorded.

Statistical analysis

All statistical analyses were run using SAS software (Version 6.12). In experiment 1, unloading effects were assessed using paired t-test analysis for data collected longitudinally (body mass, pQCT parameters) and unpaired t-tests for variables collected at sacrifice (muscle mass, torque, bone/muscle ratio). Bone and muscle recovery data from experiment 1

were assessed using unpaired t-tests within time and one-way analysis of variance within group. Experiment 2 longitudinal pQCT data were assessed using 2-way analysis of variance (time vs. group) with repeated measures on time. When a significant main effect was found, Duncan post-hoc analyses were performed to determine individual group differences. A significance level of $p<0.05$ was deemed significant and is the level of significance of all results unless otherwise noted. All data in text, tables, and figures are reported as mean ±SE.

Results

Experiment 1

Unloading effects on bone and muscle. Hindlimb unloading of skeletally mature animals for 28 days resulted in significant changes in both bone and muscle (Table 1). Plantarflexor muscle mass (-23%) and peak isometric torque (-13.9%) were both significantly lower after 28 days of HU compared to age-matched controls. Proximal tibia bone mineral content (-7.9%) and volumetric bone mineral density (-7.6%) were significantly lower compared to baseline. Total cross sectional area was unchanged yet cortical area was significantly reduced (-8.3%). The combined changes in bone and muscle resulted in a non-significant increase (+7.8%; p=0.17) in the ratio of vBMD/isometric torque after 28 days of unloading.

Recovery of bone. Proximal tibia vBMD of hindlimb unloaded + recovery (HU/R) animals remained significantly lower than age-matched controls (-6 to -10%) through day 28 of recovery (Figure 2B). Bone mineral content differences between CC and HU/R groups followed a pattern of change similar to vBMD (Table 2). Total cross-sectional area was not significantly different between groups at any time point over the 28 day recovery period, while cortical area was significantly lower in HU/R animals through day 14 of recovery (Table 2).

Recovery of muscle. Hindlimb unloaded animals gained less body mass compared to age-matched controls at all time points through day 14 of recovery (Table 2). There was no significant difference between groups by day 28 of recovery. Total muscle mass of the plantarflexors (gastrocnemius, soleus, plantaris) was lower than age-matched controls only through day 7 (-17%). Similarly, plantarflexor peak isometric torque (Figure 2A) was reduced at day 7 of recovery compared to age-matched controls (-15%). There was no difference in muscle torque between HU/R and age-matched controls after day 7 of recovery. Additional data detailing the functional recovery of muscle properties from these animals can be found elsewhere[24].

Bone/muscle relationship during recovery. To assess the functional relationship between bone and muscle, the ratio of proximal tibia vBMD to plantarflexor peak isometric torque was computed for each animal (Figure 2C). This ratio was non-significantly higher (+7.8%; p=0.17) in HU animals after 28 days of unloading and 7 days of recovery (+9.1%; p=0.11). The ratio of vBMD/torque was significantly lower (-13.6%) compared to age-matched controls on day 28 of recovery.

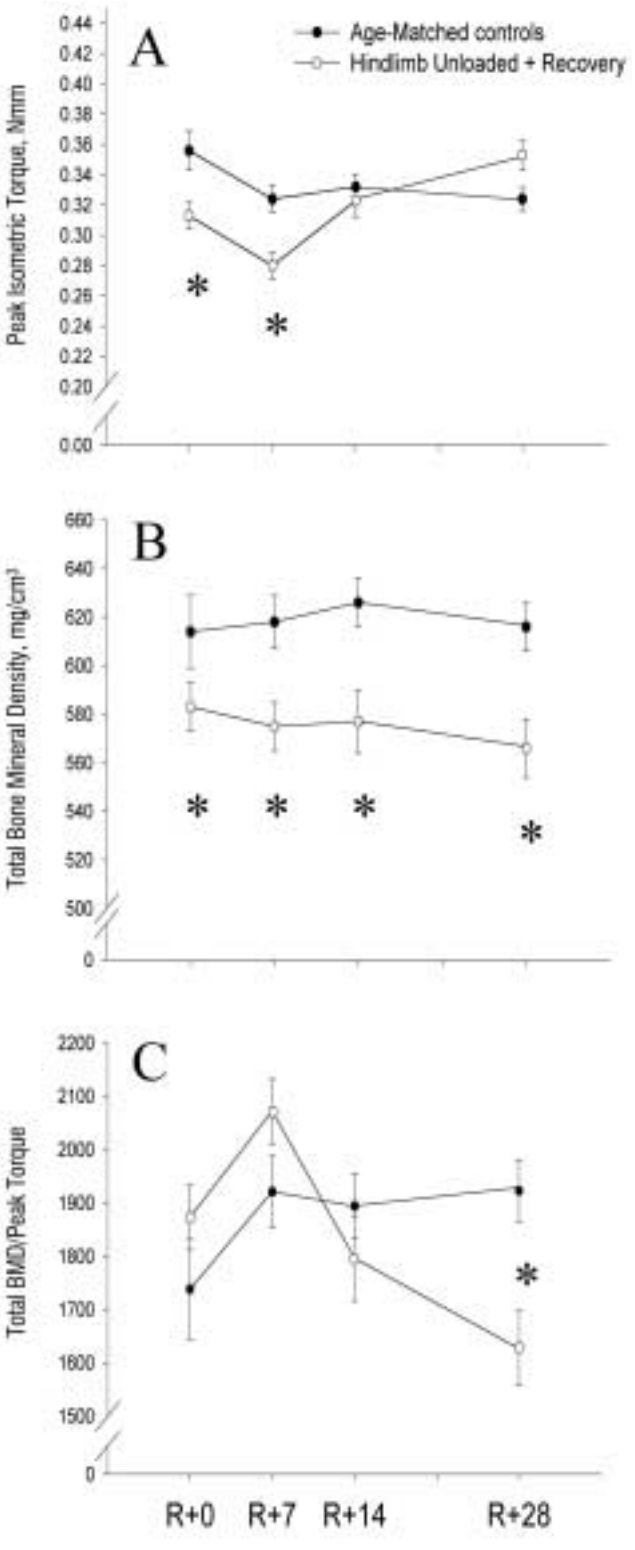

Figure 2. Bone and muscle adaptations during recovery from hindlimb unloading (Experiment 1). Results were measured *in vivo* on day of sacrifice and are presented as mean ± SE with significance (*) identifies as different from age-matched controls within a time point ($p < 0.05$). (A) Ankle plantarflexor peak isometric torque, (B) proximal tibia metaphysis total volumetric bone mineral density, (C) ratio of proximal tibia volumetric bone mineral density to peak isometric torque on day of sacrifice.

		R+7	R+14	R+28
N	CC	12	12	16
	HU	14	11	9
Body mass change from baseline, g	CC	37 ± 12	48 ± 22	61 ±17
	HU	-21 ± 26 *	16 ± 44 *	45 ± 25
Ankle plantarflexor mass, g	CC	3.31 ± 0.05	3.29 ± 0.05	3.33± 0.09
	HU	2.74 ± 0.11 *	3.08 ± 0.05	3.26 ± 0.08
N	CC	10	12	13
	HU	12	11	9
Bone mineral content, mg/mm	CC	11.2 ± 0.2	11.9 ± 0.3	11.1 ± 0.2
	HU	10.7 ± 0.3	10.7 ± 0.3 *	10.9 ± 0.4
Total cross-sectional area, mm^2	CC	18.3 ± 0.78	19.0 ± 0.64	19.1 ± 0.35
	HU	18.4 ± 0.58	18.5 ± 0.64	19.2 ± 0.71
Cortical area, mm^2	CC	8.43 ± 0.15	8.88 ± 0.21	8.39 ± 0.15
	HU	7.74 ± 0.18 *	7.77 ± 0.23 *	8.06 ± 0.24

CC: age-matched cage controls, HU: hindlimb unloading, N: number of animals, R: recovery. Values for R+0 and baseline time points can be found in Table 1 or Figure 2. Values presented as mean ± SE. * $p < 0.05$ versus cage control within time point.

Table 2. Bone and muscle parameters during recovery from unloading

Experiment 2

Long term recovery of bone. Due to the lack of complete vBMD recovery after 28 days, a second set of animals was unloaded for 28 days and allowed to recover for 84 days. No muscle data were collected in these animals. Proximal tibia vBMD was significantly lower in hindlimb unloaded + recovery (HU/R) animals after 28 days of unloading compared to baseline (-7.6%) and age-matched controls (-10.5%) (Figure 3A). Total vBMD remained significantly lower than age-matched controls at both day 28 (-8%) and 56 (-5%) of recovery; there was no significant difference between groups by day 84 (-2.5%; $p=0.20$). Total bone mineral content was significantly lower than age-matched controls through day 56 of recovery (-9 to -15%) (Figure 3B). Proximal tibia total cross-sectional area increased in controls over the course of the study (15% from baseline to day 84 of recovery) while HU/R animals increased 11.3%; there was no difference between groups at any time point (data not shown). Cortical area of the proximal metaphysis was significantly lower than age-matched controls at day R+0 (-15%), R+28 (-12%) and R+56 (-8%) (Figure 3C).

Bone and muscle changes from experiment one, and bone changes from experiment two are presented in Figure 4. The relative changes in proximal tibia vBMD and plantarflexor peak isometric torque predict that a bone/muscle mismatch exists following unloading and persists through recovery. The tissue mismatch during unloading and acute recovery favors bone, as muscle strength declines exceed those of bone density. Thereafter, the tissue mismatch favors muscle, with muscle strength recovering and bone density remaining reduced.

Discussion

The purpose of this study was to document the recovery profiles of bone and muscle, both individually and as a functional unit, following a period of disuse in the skeletally mature rat. Specifically, we wished to test the hypothesis that a significant mismatch in bone and muscle properties would occur early in the recovery period. Our results document that following 28 days of hindlimb unloading proximal tibia bone density requires nearly three times the duration of unloading to return to age-matched control levels. Conversely, peak isometric torque of the plantarflexor muscles exhibited complete recovery after one-half of the duration of unloading. These contrasting recovery rates result in a significant bone density/muscle strength mismatch, favoring muscle, by day 14 of recovery from 28 days of unloading (Figure 4). Based on these data, it appears that the bone/muscle complex is compromised following unloading, increasing the potential for musculoskeletal injury until normalization of bone properties can occur.

To our knowledge, only one previous study has tracked

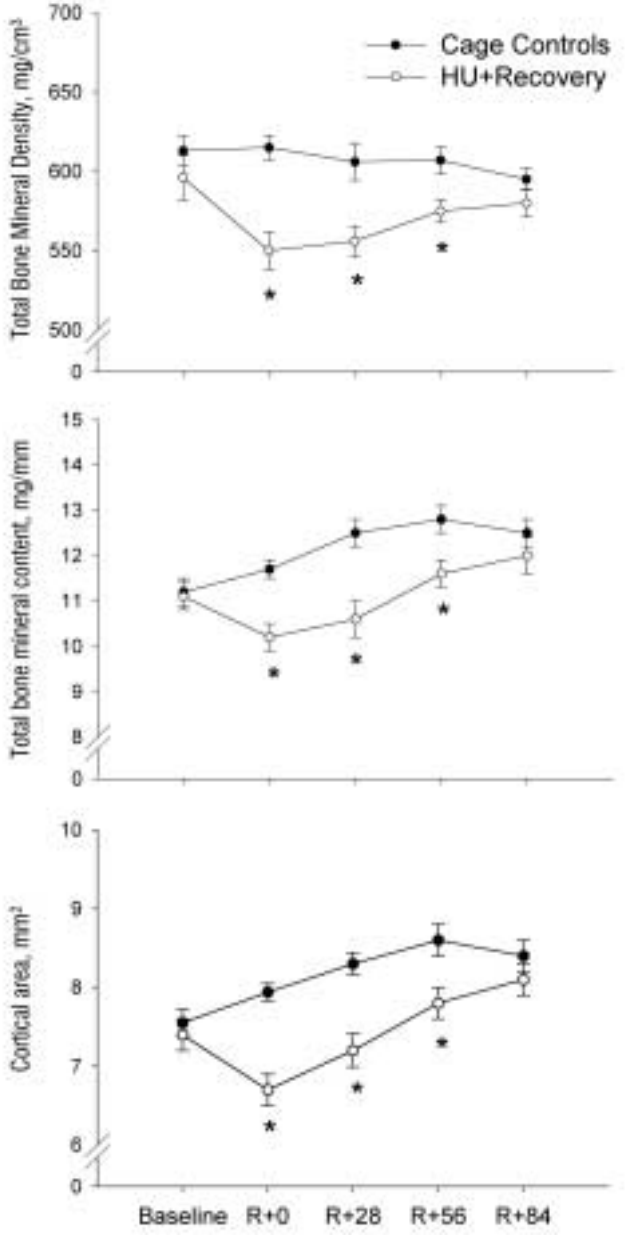

Figure 3. Long-term assessment of bone recovery following hindlimb unloading (Experiment 2). Animals were HU for 28 days and then scanned *in vivo* using pQCT on day 28, 56, and 84 of recovery. Age-matched cage controls were subjected to the same protocol without having been hindlimb unloaded. Data are mean ± SE with significant (*) identified as different from age-matched controls within a time point ($p < 0.05$) (A) proximal tibia volumetric bone mineral density, (B) proximal tibia bone mineral content, (C) proximal tibia cortical area. All parameters were significantly lower in HU/R animals compared to age-matched controls at days R+0, R+28, and R+56.

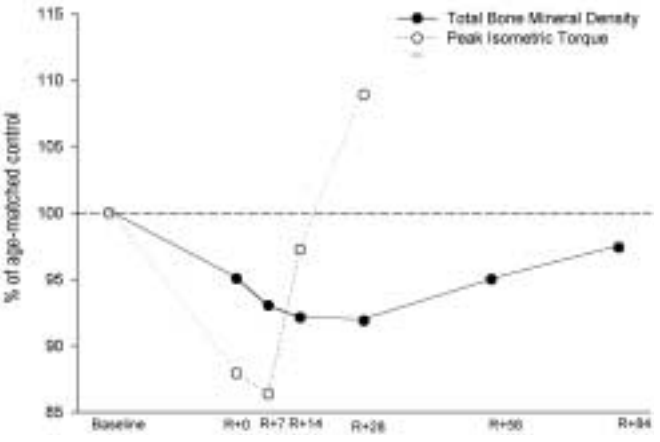

Figure 4. Summary graph of bone and muscle changes with unloading and recovery. Data from Experiments 1 and 2 expressed as percentage of age-matched controls for proximal tibia total volumetric bone mineral density (vBMD) and ankle plantarflexor peak isometric strength.

recovery of bone properties in skeletally mature rats following hindlimb unloading, quantifying an incomplete recovery of tibial vBMD after a period of recovery equal to twice that of unloading[16]. A greater number of studies have quantified recovery of bone following disuse in younger animals, documenting a slow recovery of bone structural variables (bone volume, strength, geometry)[16-18,30]. One previous experiment has documented the complete recovery of trabecular bone volume in young rats after unloading[20]. The reason for this alternative finding is likely related to the age of the animal. Evidence suggests the kinetics of bone loss during disuse may differ between young and skeletally mature animals[12,13,31] and therefore it is likely that animal age would similarly influence the kinetics of bone recovery. Independent of animal age, the current study recovery period extends beyond these previous publications and documents the sustained reduction of bone mineral content and density until 2-3 times the duration of unloading. This matches estimates of Jaworski et al. who showed the length of time necessary for recovery of bone lost to disuse was 3-times the period of unloading[31]. Alternatively there may be some absolute time period necessary for recovery, given that bone density of astronauts/cosmonauts fails to completely recover bone density when followed up to 5 years post-flight[3,9,10].

Reduced total vBMD with unloading appears to result from bone loss on the endocortical/cancellous surfaces. Following 28 days of unloading, there was minimal change in total cross-sectional area at the proximal metaphysis yet cortical bone area was significantly lower. This suggests endocortical bone resorption, a finding previously documented in humans after spaceflight[32] and rodents with prolonged disuse[33]. Beyond influencing total BMD, endocortical resorption likely serves to reduce bone strength especially in the absence of periosteal expansion, known to be suppressed in diaphyseal bone with unloading[12,34]. Periosteal apposition at the proximal tibia metaphysis has not been previously quantified with unloading, yet based on the lack of increase in

total cross-sectional area in the current study, it is likely suppressed similar to the mid-diaphysis. Upon reambulation, total cross-sectional area increases similarly in HU/recovery and age-matched controls, yet differences in cortical area between the two groups remain through day 56 (-8% vs. controls). We hypothesize this is due to a more rapid recovery of periosteal bone formation compared to endocortical surface bone formation. It is important to confirm these geometrical changes using histological assessment of periosteal and endocortical activity at this metaphyseal site.

Our data detailing the unloading-induced alterations in muscle mass/strength support previous studies on both humans and animals[23]. More detailed analyses of changes in functional muscle properties during recovery for the same animals from the current study can be found elsewhere[24]. The changes most pertinent for the discussion here are the further decrement in plantarflexor muscle torque during the first 7 days of reambulation, followed by a quick and complete recovery by day 14, one-half the duration of unloading. Reambulation-induced decrements in muscle strength have previously been reported in humans returning from space[35] as well as rats following hindlimb unloading[36]. While our data do not address the mechanisms of reloading-induced declines in muscle strength[24], they do clearly document that these adverse effects for a functional muscle group are short-lived. Plantarflexor peak isometric torque returns to age-matched control levels within 14 days of recovery. Such rapid recovery could be perceived as advantageous, as muscle-related risks would be short-lived during recovery. However, when coupled with compromised bone properties at the site of muscle insertion, fully recovered muscle strength may pose an increased injury risk during the recovery period. Such risk would likely be exacerbated if physical activity beyond normal weight-bearing occurs during this recovery period. Additionally, rapid muscle recovery may adversely affect the myotendinous junction, likely the weakest element in the muscle/bone unit. Although it is known that this junction is negatively influenced by unloading[37], its recovery during reambulation is unclear.

It has long been proposed that the rapid responsiveness of muscle to adapt to increased demand, coupled with the relatively slow adaptability of bone, contributes significantly to the development of skeletal injury[28,38]. This hypothesis has been generally supported by studies on military recruits (for review see[39]), a population in which gains in muscle strength can exceed bone strength and injury incidence is higher than normal. Data from animal models provide support for this theory. Disuse results in a compromised bone/ligament complex (reduced strength of complex, increased avulsion fractures)[37], changes that remain for months after resumption of normal weight-bearing activity[40]. Based on the current study's data, it would be predicted that the risk for muscle contraction-induced fractures at the proximal tibia would be significantly increased between days 14 and 28 of recovery, and likely last until between day 56 and 84 (Figure 4).

It remains unknown, however, what the actual effect of reduced total vBMD has with respect to overall functional bone strength at this site. Total vBMD assessed with pQCT is an integration of bone mineral content and total cross-sectional area. Due to the presence of marrow at the proximal tibia, vBMD underestimates "true" density of the bone mineral. In the current study, difference in total vBMD between HU/R and age-matched controls are completely accounted for by differences in bone mineral content, as total cross-sectional area did not differ among groups. We therefore chose total vBMD as our index of bone loss and recovery due to its integration of changes in both bone mineral content and geometry and its high clinical relevance. However, it is clear that future work should focus on directly assessing the strength of the bone/muscle complex, including the myotendinous and osteotendinous junctions, during the period of highest mismatch to directly measure the susceptibility of compromised bone to muscle contraction-induced trauma.

Conclusion

These data demonstrate the differential recovery rate of unloading-induced bone and muscle adaptations in skeletally mature rats. Bone mineral density remains suppressed for two to three times the duration of unloading while muscle strength recovers within a period equal to one-half of the duration of unloading. The implications of these results relate to rehabilitation programs for those returning to normal weight-bearing activities following periods of disuse (limb immobilization, bed rest, spaceflight). If rehabilitation results in a rapid return of muscle strength without concurrent strategies that will speed normalization of bone structure and strength, the risk of avulsion fracture at muscle insertion sites appears heightened. It is important to determine if this mismatch of bone density and muscle strength during recovery truly does heighten risk of bony injury, and whether specific rehabilitation regimens or short-term pharmacological therapy might effectively minimize this functional mismatch.

Acknowledgements

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