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Daily Acute Bouts of Weight-bearing During Hindlimb Unloading Mitigate Disuse-Induced Deficits in Cancellous Bone

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ABSTRACT

International Space Station crewmembers experience microgravity, resulting in musculoskeletal losses. It remains unclear how much mechanical loading during disuse is sufficient to mitigate disuse-induced bone loss. We examined 75 minutes of weight-bearing per day on disuse-induced bone loss during hindlimb unloading (HU). Female C57BL/6J mice, 17 weeks (n=10/group), were exposed to HU for 28 days or were ambulatory controls (CC). Half of the HU animals were continuously unloaded while the remainder were removed from tail suspension for ~75 min/day for cage activity weight-bearing (HU+WB). HU and HU+WB led to total body mass and bone mineral density loss. HU+WB mitigated HU-induced losses in total body fat and lean mass and, in the distal femur, prevented losses in μCT measures of cancellous bone volume and microarchitecture. These findings support the robust impact of short durations of normal loading on preventing or mitigating HU-induced bone loss.

Key words: Microgravity; Rodent; Body Composition

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INTRODUCTION

International Space Station (ISS) crewmembers experience loss of musculoskeletal mass and strength due to months of exposure to zero gravity (Sibonga, 2013; Sibonga et al., 2007; Smith et al., 2012). Long-duration spaceflight (~6 months) results in approximately 10% bone mineral density loss at the hip and spine (Sibonga, 2013). Without preventive action, this loss would be expected to increase astronauts' risk of fracture upon return to gravity. An assessment of non-weight-bearing bone sites in cosmonauts following spaceflight revealed no recovery in cortical porosity nor in trabecular bone, suggesting a continued need for protective measures in spaceflight (Vico et al., 2017). Seventeen weeks of bed rest, a human spaceflight and disuse analog, causes bone loss in the total body as well as specific regions including the lumbar spine, hip, tibia, forearm, and calcaneus (Leblanc et al., 1990). Although most of these sites recovered bone mass after 6 months of re-ambulation, only the calcaneus recovered to 100% of pre-bed rest values (Leblanc et al., 1990). Previous studies have extensively shown that hindlimb unloading (HU), a ground-based animal model analog of spaceflight in which the hindlimbs of an animal experience disuse, leads to bone loss in the hindlimbs (Globus and Morey-Holton, 2016; Lang et al., 2004; Metzger et al., 2017; Morey-Holton and Globus, 2002; Vico et al., 2000).

Intermittent weight-bearing during hindlimb unloading has been shown to mitigate HU effects on muscle mass (Alley and Thompson, 1997; Sandmann et al., 1998; Widrick et al., 1996; Yamazaki, 2003). One study found that 40 minutes per day of intermittent weight-bearing, if applied in four 10 minute sessions or one 40 minute session, mitigated HU-induced losses in muscle mass (Yamazaki, 2003). With regards to bone, classic studies with isolated turkey ulnas demonstrated that a small number of loading cycles was sufficient to prevent disuse-induced intracortical resorption and that additional loading cycles provided no added changes to the bone (Lanyon and Rubin, 1984). Bone formation rate is normalized to control levels with 10 minutes per day of low-level mechanical intervention in HU rats (Rubin et al., 2001). However, no differences were found in bone loss with 20 minutes per day of intermittent weight-bearing 5 days per week in male Sprague-Dawley rats exposed to hindlimb unloading (Leung et al., 2015). Together these studies indicate that intermittent weight-bearing/applied loading may have the potential to mitigate disuse-induced bone loss; however, the length of time needed to produce a benefit remains unknown. Therefore, this study was designed to investigate whether a 3½ fold increase in weight-bearing per day (75 minutes) during HU verses that used by Leung et al. (2015) would mitigate HU-induced alterations in hindlimb cancellous microarchitecture. We hypothesized that animals experiencing 75 minutes of normal weight-bearing activity during HU would have attenuated bone loss compared to animals with continuous HU.

MATERIALS AND METHODS

Experimental Design and Animals

Female C57BL/6J mice (17 week old) were group housed and allowed *ad libitum* access to food and water in an animal facility with 12-hour light/dark cycles. Upon arrival, animals were switched from standard rodent chow (Teklad 2018) fed by the vendor to the purified AIN93-G diet (Research Diets, Brunswick, NJ) and allowed to acclimate for four weeks. Refer to Figure 1A for a study timeline. Animals were randomly assigned to three treatment groups: ambulatory cage control (CC, n=10), continuously hindlimb unloaded (HU, n=11), and hindlimb unloaded with intermittent weight bearing (HU+WB, n=10). HU, used to simulate disuse of the hindlimbs as experienced in zero gravity, suspends animals by their tails so that their hind feet no longer touch the ground. Suspension was achieved by the surgical insertion of a tail ring, comprised of surgical steel wire, between the 4th and 6th caudal vertebra. A 25-gauge needle was inserted in the intervertebral space between the 4th and 6th caudal vertebra. Using this hole, a 10 cm surgical steel wire was inserted into the hole and tied into one large loop with a small top loop where the tail

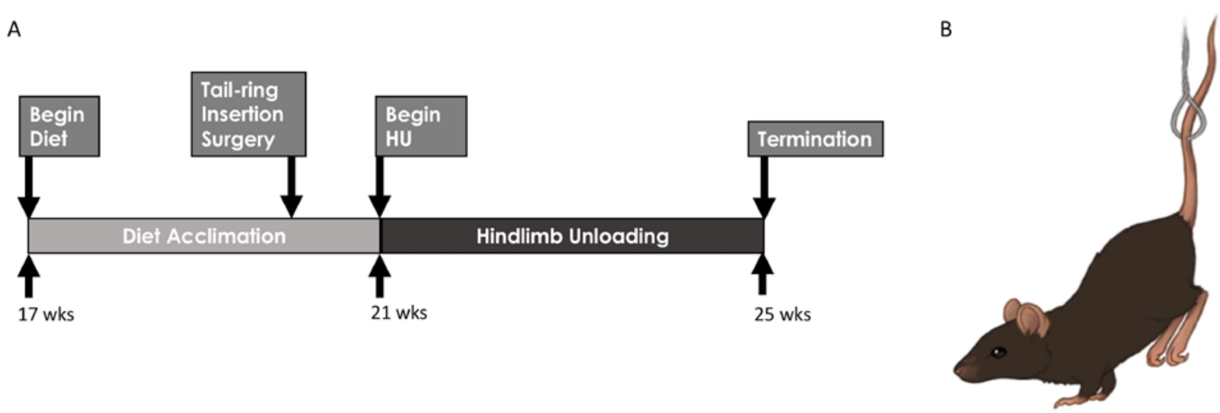

Figure 1. Study information. A) Study timeline: Animals acclimated to AIN-93G diet starting at 17 weeks of age. HU animals began HU four weeks later, while HU+WB animals were removed from tail suspension and allowed weight-bearing cage activity for 75 minutes per day. B) HU mouse suspended via tail ring.

suspension mechanism was inserted. Figure 1B illustrates a HU mouse. Animals were anesthetized using isoflurane gas for 10 minutes during the procedure. No analgesics were required as there were no signs of pain, distress, or mutilation of the affected area following this minor surgery (Ferreira et al., 2011). After a 10 day period of recovery, tail rings were connected to a fish-hook suspension mechanism attached to a metal bar positioned over a 50x40x20 cm cage. Animals' tails were wrapped in gauze so that the tail pointed upward, allowing for improved blood flow to the tail. HU animals were allowed to swivel but could not move laterally. Though HU animals were housed two per cage, the cage configuration disallowed any physical contact between co-housed HU mice, so CC animals were housed singly.

To achieve intermittent weight bearing, animals were unhooked from the fish hook hanging apparatus and allowed normal ambulation within their cage. Animals were removed from the hanging apparatus for approximately 75 minutes during their dark cycle for 3 days in a row, then remained continuously hindlimb unloaded for one full day. This cycle was repeated for the 28 days of HU. This schedule was chosen to parallel the weight-bearing time incurred during an exercise protocol used in a different experiment. During this period of weight-bearing, activity was not measured; however, we observed that animals spent the majority of their time grooming. Following a four-week experimental period with HU, animals were anesthetized using isoflurane gas and sacrificed by both cervical dislocation and thoracotomy. All animal protocols were approved by the Texas A&M University Institutional Animal Use and Care Committee.

Dual X-ray Absorptiometry (DXA)

Bone density and body composition were assessed using a small animal Lunar PIXImus densitometer (General Electric, Madison, WI) 1 day prior to the start of HU and 2 days prior to termination. Animals were anesthetized using isoflurane (Henry Schein Animal Health, Dublin, OH) during this procedure (≤10 minutes total). All mice were placed in the same prone position. Whole body values (excluding the head and tail) for lean mass, fat mass, and bone mineral content were assessed. Bone mineral density (BMD) was calculated by the PIXImus software (GE Lunar version 1.42) based on the active bone area in the region of interest (ROI).

Micro-computed Tomography (μCT)

To determine cancellous architecture, the left femur from each animal was subjected to micro-computed tomography (μCT) analyses, using a Skyscan 1172 system (Bruker) with the following settings: 60 kV, 167 μA, 2K scanning mode, 0.7 degree rotation step, 6 μm isotropic voxel size, and 0.5 mm aluminum filter. Structural parameters of trabecular bone region of interest (a 1 mm thick region starting ~0.5 mm from the proximal most point of the growth plate) were measured using standard manufacturer software. The trabecular region was manually drawn to exclude the cortical bone. Key outcomes reported in both the femur and the spine are cancellous bone volume (%BV/TV), trabecular thickness (Tb. Th), trabecular number (Tb. N), and trabecular separation (Tb. Sp). Nomenclature conformed to standard recommendations (Bouxsein et al., 2010). The representative images depicted in Figure 2 were determined using the image of the sample closest to the mean.

Statistical Analysis

All data are presented as mean ± standard deviation of the mean (SD) and were evaluated for differences using SPSS (IBM, version 23). Outliers were detected and removed using SPSS program, which identifies outliers first by calculating the interquartile range, multiplying it by 1.5, adding this value to the top of the interquartile range, and subtracting this value from the bottom of the interquartile range to obtain the boundaries of acceptable values. Any value outside this range was deemed an outlier. A Shapiro-Wilk test for normality of data was run on all data sets. All data was normally distributed except total body bone mineral density and trabecular number. Normally distributed data were analyzed via a one-way ANOVA. If ANOVA were significant, a Duncan post hoc analysis was performed to determine differences among groups, denoted by different letters. A non-parametric Kruskal-Wallis ANOVA test was performed on any data that were not normally distributed. If the ANOVA were significant, pairwise comparisons between groups were performed to determine the differences between groups as post hoc analysis is not possible with non-parametric analysis. Statistical significance was accepted at a $p<0.05$.

RESULTS

As summarized in Table 1, at the start of the study body mass was not different among groups. At the end of 4 weeks of HU, body mass of CC animals was 9% greater than HU and 7% greater than HU+WB ($p=0.004$; Figure 2). There was no difference between the two HU groups. Animals exhibited minimal health issues, with normal grooming and eating behaviors and no overt signs of distress (e.g., porphyrin staining at eyes or nose).

Total body bone mineral density (BMD) was not different between groups at the start of HU. Total body PIXImus (DXA) scans revealed that total body BMD was significantly lower in both HU (-7%; $p=0.001$) and HU+WB (-7%; $p=0.030$) compared to CC mice, as illustrated in Figure 3. Total body lean mass was significantly lower (-5%) in HU ($p=0.039$), but not in HU+WB, compared to CC. Total body fat mass in HU mice was 21% lower than CC and HU+WB ($p=0.0001$); there was no difference between CC and HU+WB.

As depicted in Figure 4, μCT of distal femurs revealed that cancellous bone volume (%BV/TV) was 22% and 35% lower in HU compared to CC and HU+WB, respectively ($p<0.0001$). Trabecular thickness (Tb. Th) was 9% lower in HU compared to CC and 20% lower compared to HU+WB ($p<0.0001$). Trabecular separation (Tb. Sp) was not significantly different among groups. Trabecular number (Tb. N) was 13% lower in HU compared to CC ($p=0.034$) and 21% lower in HU compared to HU+WB ($p=0.024$).

Table 1. Total body bone mineral density at the start of hindlimb unloading (HU). There were no differences in intermittent weight-bearing (HU+WB) or HU compared to cage controls (CC).

	CC	HU	HU+WB
Total Body Bone Mineral Density (g/cm^2) One Day Prior to Hindlimb Unloading	0.060 ± 0.001	0.055 ± 0.001	0.057 ± 0.004

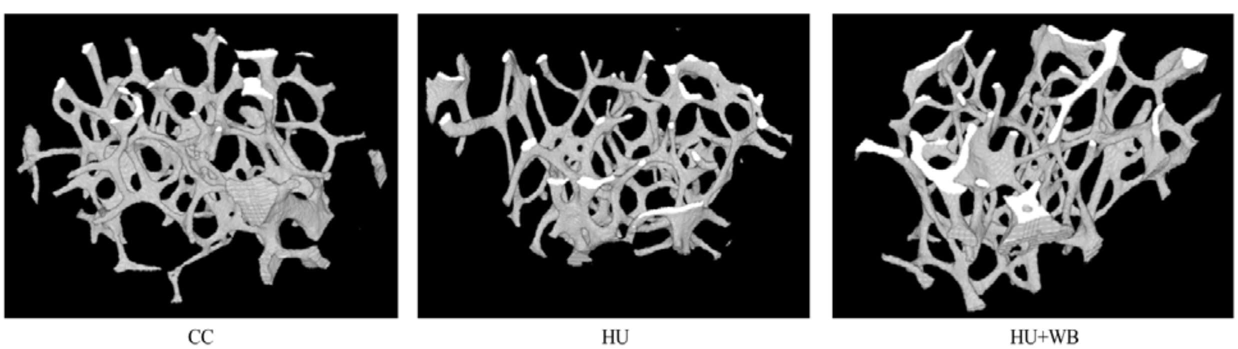

Figure 2. Representative images of cancellous bone volume and microarchitecture at the distal femur after 4 weeks for cage control (CC), hindlimb unloaded (HU), and hindlimb unloaded + weight-bearing (HU+WB) mice.

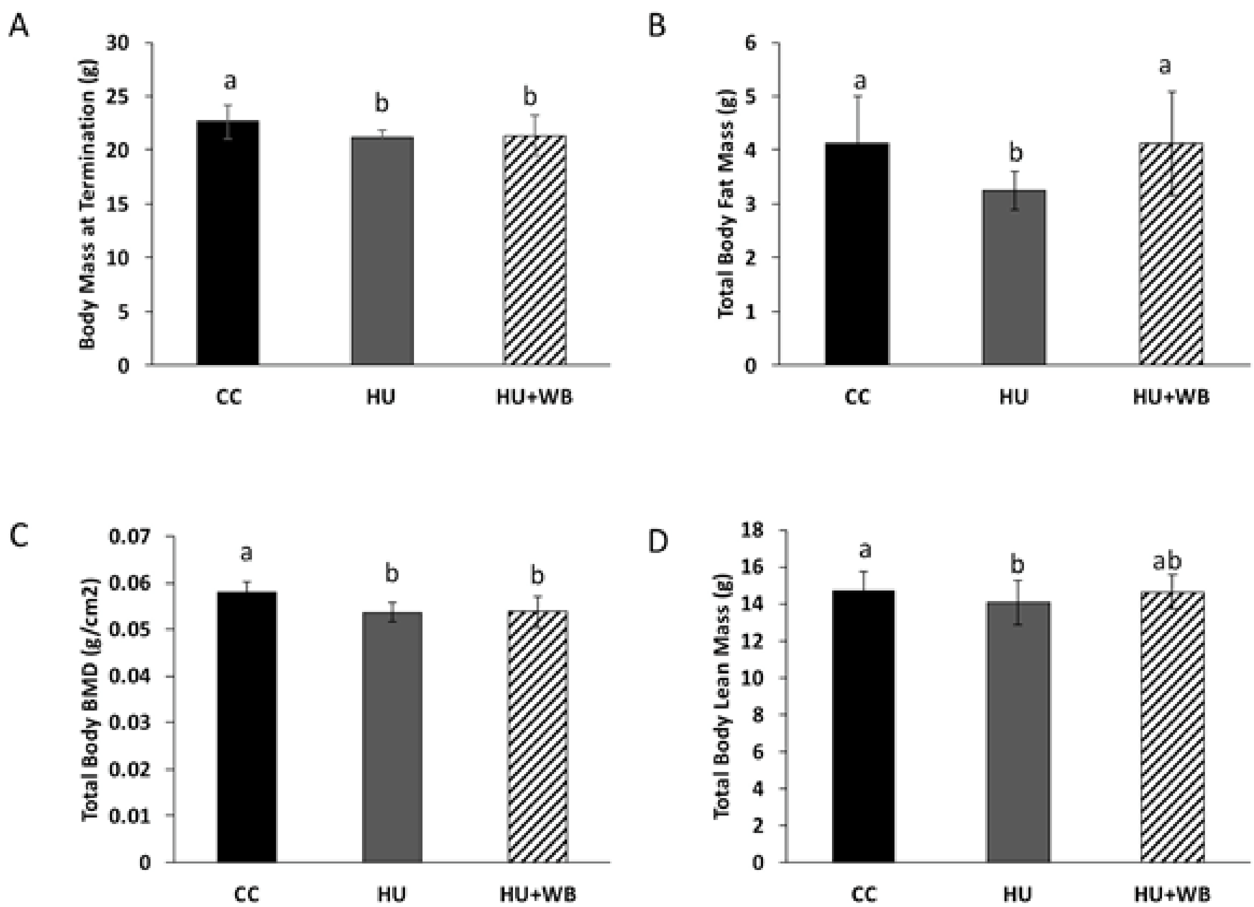

Figure 3. Body mass and body composition after 4 weeks for cage control (CC), hindlimb unloaded (HU), and hindlimb unloaded + weight-bearing (HU+WB) mice. A) Body mass at the end of HU was lower in HU (-9%) and HU+WB (-7%) mice compared to cage controls (CC). B) Total body BMD was lower in HU (-7%), and HU+WB (-7%) vs. CC. C) Fat mass was lower in HU (-21%) compared to CC and HU+WB. D) Lean mass was lower in HU compared to CC (-5%) with no difference from HU+WB. Groups sharing a letter are not different from one another by one-way ANOVA (p<0.05).

DISCUSSION

The primary finding of this study is that intermittent weight-bearing for only 75 minutes per day on 21 days out of 28 days of hindlimb unloading, mitigated losses in distal femur cancellous microarchitecture and total body lean and fat mass seen during continuous HU. Even with intermittent weight-bearing, however, the typical declines in bodyweight and total skeletal mass (total body BMD) were observed.

Previously, studies have shown a reduction in bone mineral density, lean mass, and fat mass with hindlimb unloading (Fluckey et al., 2002; Smith et al., 2005; Wade et al., 2013). Therefore, our body composition results are consistent with other published studies. Bone loss, particularly in the cancellous region, has also been well reported in actual spaceflight or HU, with pronounced deficits in trabecular BMD and microarchitecture (Gerbaix et al., 2017; Globus and Morey-Holton, 2016; Li et al., 2012; Morey-Holton and Globus, 2002; Shirazi-Fard et al., 2013; Vico et al., 2000). In this study HU animals had lower cancellous bone volume, trabecular thickness, and trabecular number compared to ambulatory controls. It is possible that cortical bone would also be lower with HU, though we were unable to measure it in this study.

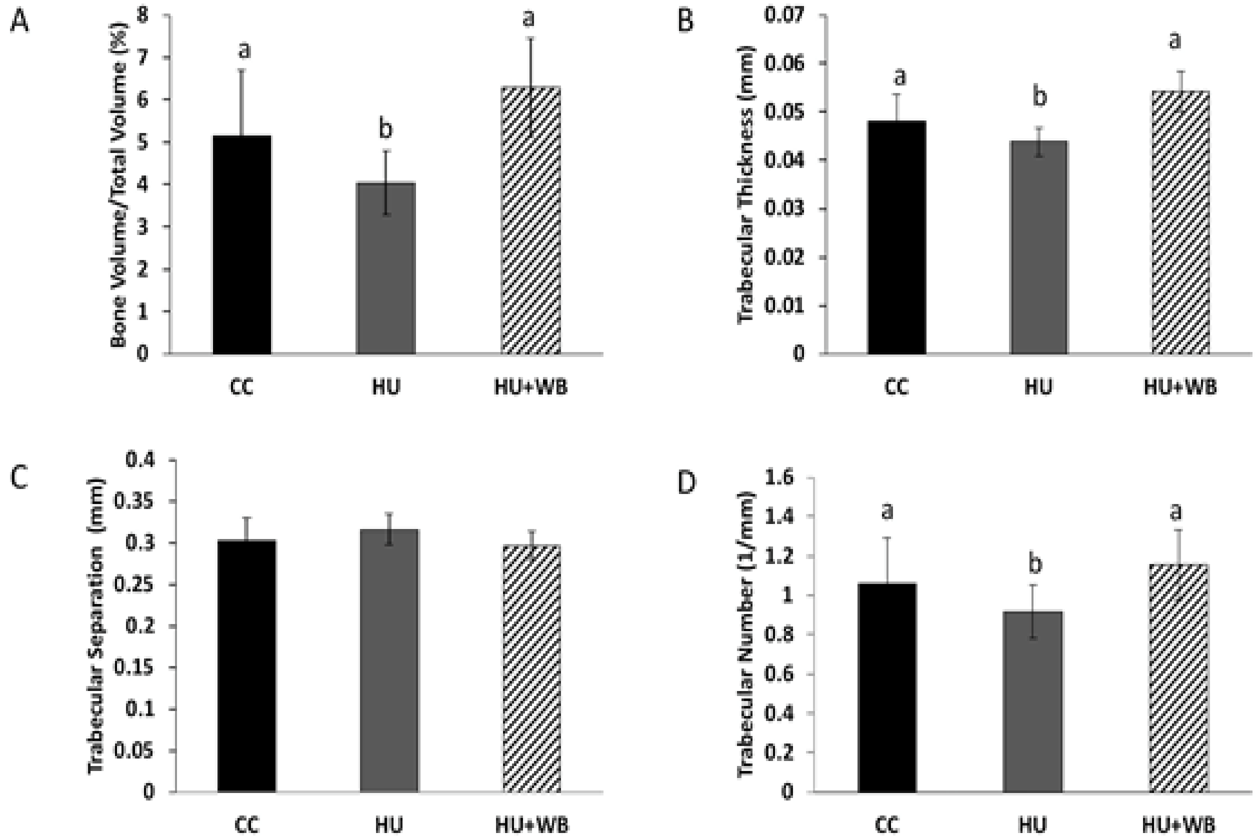

Figure 4. Cancellous bone volume and microarchitecture at the distal femur after 4 weeks for cage control (CC), hindlimb unloaded (HU), and hindlimb unloaded + weight-bearing (HU+WB) mice. A) % bone volume/total volume (%BV/TV) was 22% lower in HU compared to cage controls (CC), whereas this value in HU+WB was equivalent to CC mice. B) Trabecular thickness was 9% lower in HU compared to CC and HU+WB. C) Trabecular separation was not different among groups. D) Trabecular number was 13% lower in HU compared to CC, whereas HU+WB was not different than CC mice. Groups sharing a letter are not different from one another by one-way ANOVA ($p<0.05$)

Intermittent weight-bearing during hindlimb unloading mitigates HU effects on skeletal muscle mass, as measured in individual muscles of the unloaded hindlimb (Alley and Thompson, 1997; Sandmann et al., 1998; Widrick et al., 1996; Yamazaki, 2003). Our data demonstrated that intermittent weight-bearing during HU mitigated total body lean mass and fat mass compared to continuous HU mice, consistent with previous studies. We do not have data on site-specific muscle mass changes, but speculate that skeletal muscle mass in the hindlimb was positively impacted by intermittent weight-bearing. We did not find a mitigation of total body mass at termination in HU+WB compared to HU, though this is likely due to bone related outcomes.

There has been less extensive study of intermittent weight-bearing effects during prolonged periods of unloading in bone; the work done thus far indicates a mixed story (Leung et al., 2015; Rubin et al., 2001). Twenty minutes per day of weight-bearing 5 days per week (less than 1/3 the weight-bearing time in the present study) provides no mitigation of losses in integral and trabecular bone mineral density and bone cross-sectional area in the tibia and femur of hindlimb unloaded rats (Leung et al., 2015). On the other hand, having hindlimb-unloaded rats stand on a platform oscillating at 90 Hz for only 10 minutes per day, adding the mechanical loading of vibration to the weight-bearing effect, prevents the declines in bone formation rate observed during uninterrupted HU (Rubin et al., 2001). In our study, we found no mitigation of HU-induced losses in total body bone mineral density with intermittent weight-bearing. However, with a specific focus on cancellous bone in the distal femur, our novel finding is that bone loss in this compartment is mitigated or even prevented by 75 minutes of weight-bearing on most days of the week. Given that the loss of total body BMD with HU was not mitigated by the intermittent weight-bearing, these results suggest that cortical bone, in all sites, may not be responsive to the intermittent weight-bearing in contrast to the metaphyseal cancellous bone of the distal femur. Multiple previous studies have demonstrated that cancellous-rich bone sites are most affected by disuse (Allen and Bloomfield, 2003; Bloomfield et al., 2002; Globus and Morey-Holton, 2016). Therefore, it is possible that with the increased surface area for osteoblast teams to act on, bone loss at this region can be more effectively mitigated by intermittent weight-bearing. The lack of data for cortical bone in this model is a limitation of this study; we did not have access to cortical bone samples for all three groups included in this study. This should be addressed in future studies. A previous study tracked recovery of bone mass in crew members for up to 12 months following ISS missions. Using high-resolution pQCT, that study demonstrated that cortical thickness and density at the distal tibia recovered, but cortical porosity and cancellous bone at the same site did not (Vico et al., 2017). It is clear that different bone compartments even within the same anatomical site can respond differently to changes in the loading environment. Our data demonstrate that even small amounts of weight-bearing during a period of disuse (but for a longer duration than previously tested) can mitigate or even prevent the detrimental effects of disuse on cancellous-rich bone sites, at least for a 28-day period of unloading.

There are several possible mechanisms that could explain the results described in this study, two of which are bone blood flow changes and altered osteocyte signaling. Bone blood flow has been shown to be reduced in the hindlimbs of rats within 10 minutes after the initiation of hindlimb suspension, which gradually worsens over a 28-day period of unloading. This lack of perfusion to the bone of the hindlimb could be a factor contributing to the reduced bone mass (Bloomfield, 2006; Colleran et al., 2000; Qin et al., 2003). It is possible that small amounts of weight-bearing per day restore bone blood flow for that period of time and that stimulus aids in diminishing bone loss. Secondly, osteocytes, the bone cells embedded within the bone matrix, are considered to be the primary mechanosensors of bone tissue. They form a vast communication network via dendrite-like cell processes connecting with other osteocytes and with surface cells. Osteocytes release signaling proteins to stimulate or suppress osteoblast and osteoclast differentiation and/or activity (Bonewald, 2011). In addition to undergoing cell death as a response to unloading, osteocytes upregulate proteins that halt osteoblastogenesis (sclerostin) and enhance osteoclastogenesis (RANKL) (Cabahug-Zuckerman et al., 2016; Gerbaix et al., 2017; Macias et al., 2016; Metzger et al., 2017; Spatz et al., 2015; Xiong et al., 2011). It is possible that small amounts of normal weight-bearing per day are capable of providing enough mechanical stimulus to osteocytes to diminish sclerostin signaling, leading to less suppression of osteoblastogenesis and/or decreased osteoclastogenesis. This in turn could be enough to mitigate the typical increase in osteoclast activity and decrease in osteoblast activity that contributes to bone loss (You et al., 2008). Future studies should measure osteocyte sclerostin and focus on determining a mechanism underlying the positive effect of relatively brief periods of weight-bearing during prolonged disuse.

In conclusion, this study demonstrates that just over one hour of weight-bearing cage activity per day mitigated HU-induced changes in body composition, prevented loss of cancellous bone mass, and prevented deficits in cancellous microarchitecture at the distal femur; however, it was unable to prevent declines in total body BMD observed with HU. Further studies including measures of bone turnover, site-specific changes, and exploration of the mechanisms underlying these findings are merited. That only 75 minutes/day of weight-bearing can effectively prevent cancellous bone decrements reinforces the notion that gravity is a robust regulator of bone cell function in support of the maintenance of bone mass. Future attempts to reduce bone loss in astronauts should include attempts to provide this duration of full gravitational loading during spaceflight.

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