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Effect of suspension on mouse bone microhardness

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Antiorthostatic (hindlimb) suspension of mice results in a considerable reduction of bone formation at the femur mid-diaphysis. Comparisons with appropriate control groups indicate that this reduction is attributable to the unloading aspect of the model, and not to physiological stress or changes in feeding. Microhardness measurements of bone are used to provide information on site-specific mineralization and structural properties. The microhardness of femora formed during suspension is significantly less than that formed in the bone of control mice. These differences are observed both along the endocortical (11%) and periosteal (8%) perimeters. The microhardness of bone formed prior to the experimental period ("extant bone") is not different in comparing suspended and control mice, and increased microhardness values for these areas are observed in comparison to baseline controls. Mice used to control for the physiological stress and feeding portions of the suspension model do not demonstrate reduced microhardness. Thus, the limb unloading effects of suspension, not the induced stress or feeding changes, cause a reduction in microhardness. As microhardness is positively related to mineralization in these bones, it appears that the reduced mineralization accompanying suspension unloading may contribute to compromised structural properties of the bone formed.

1. Introduction

Reduced mineralization of newly formed bone is apparently observed in rats exposed to spaceflight [1]. However, direct determination of this hypothesis was not determined using site-specific measurement of structural properties. The site-specific structural properties of bone are highly dependent on the mineral content [2]. Altered mineralization during unloading conditions would also lead to differences in the material behaviour of newly formed bone. Spaceflight appears to affect the collagen phase of the newly formed bone in rats [1]. Since collagen fibre orientation is ranked highly as a predictor of bone tensile [3] and flexure [4] strength, changes in collagen may determine site-specific structural parameters in bone.

Because spaceflight opportunities are limited, a ground-based model for unloading provides researchers with the opportunity to study this aspect of spaceflight. Antiorthostatic tail suspension [5] was introduced as a ground-based model for the effects of spaceflight [6] on the hindlimb bones of rats. Because mice offer cost, size and transgenic advantages over rats, the suspension model has recently been extended to mice. The hindlimb bones of mice are indeed deleteriously affected by suspension [7]. These effects have been demonstrated in strains which respond with "high-stress" (Balb-C, DBA-2) and "low-stress" (C57BL-6) to the suspension protocol [8], indicating that stress itself does not produce the bone changes. The hindlimb unloading mechanics of the suspension protocol apparently cause the reduced bone formation this protocol produces in growing mice [9]. This finding appears to support the hypothesis that bone modelling, more so than remodelling, may be affected by loading mechanics [10].

In order to determine if unloading caused by tail-suspension alters bone composition and quality, a site-specific measurement of bone structural quality is required. Microhardness measurements indirectly determine mechanical behaviour at the microscopic level [11], and quantify the physical effects of small variations in bone composition [2], thereby providing a site-specific indicator of structural competence [12]. Furthermore, microhardness measurements are nearly linearly related to Young's modulus (E) and yield stress [2, 12].

The thickness of bone formed along the periosteal and endocortical surface of femora in mice between 40 and 54 days of age averages 30–80 μm, depending on the location of the measurement along the actively forming surface [9]. Microhardness indents caused by 50 gm loading can be readily accommodated in bone regions of 50 μm on a side, while avoiding edge and

† Now deceased, Dr. Luttges lasting contributions to the subject are acknowledged

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boundary effects [11]. Thus, microhardness measurements may be used to quantify the structural properties of bone formed under the unloaded conditions of tail-suspension.

The present set of experiments quantify the effects of suspension on bone microhardness. With the use of fluorochromes, sites of new bone formation at the femur mid-diaphysis are identified. The microhardness of bone formed before and after the beginning of the experiment is determined.

2. Materials and methods

Forty HS (heterogeneous strain, IBG, Boulder CO) male mice [13], 40 days in age, were assigned to one of four equally-sized groups. These groups include baseline controls and *ad libitum* fed (normal) controls, sacrificed at 40 and 54 days, respectively. Normal bone properties for mice aged 40 and 54 days were determined from these two groups. A separate control for feeding and the stress due to attaching the suspension gear (unloading controls) were used. These groups are described in more detail elsewhere [9]. The fourth group was suspended for the duration of the 2-week [14] study using the suspension protocol defined previously [7]. This protocol is derived from the rat suspension protocol [5]. The mice (excluding baseline controls) were given tetracycline injections (15 mg/kg, I.P.) on days 0 and 13 of the study to label sites of active bone formation.

After sacrifice (90 mg/kg sodium pentobarbital followed by cervical dislocation), the right and left femora were removed and cleaned of all non-osseous tissue. The right femora were analysed whole for dry mass (Dry-M, 105 °C drying for 24 h), mineral content (Min-M, 800 °C drying for 24 h) and percentage mineral (%Min = Min − M/Dry − M × 100%). The left femora were air dried (25 °C evaporation for 48 h), then embedded whole in Spurr resin (Polysciences), cured (70 °C, 18 h) and sectioned using a low-speed diamond bone saw (Buehler, 300 μm diamond blade) at the mid-diaphysis for fluorescent microscope evaluation. Care was taken to ensure that the distal ends of the 1 mm sections were evaluated. These ends correspond to the mid-diaphysis where the diaphyseal cross-sectional area is minimal [15]. Moreover, uniform geometric (within 5%) measurements are obtained throughout this region. Preliminary studies [9] demonstrated similar modelling properties in this region. Thus, the mid-diaphysis was an ideal location for obtaining characteristic bone formation (modelling) measurements. The cross-sectional surfaces (thick sections to allow later microhardness measurements to be obtained) from the mid-diaphysis were wheel-polished to a flat and smooth surface with systematically finer grades of wet/dry silicon carbide paper, ending with 600-grit, followed by polishing with a napped cloth impregnated with 6-μm diamond paste. The cross–sections were cooled with distilled water throughout the preparation process. The polished cross-sections were illuminated under a fluorescent microscope (Carl Zeiss, Inc., Axioskop with MC80 camera mount) and photomicographs were taken at 40 × under far blue light, generating colour prints in which the cross-sections averaged approximately 9 cm by 5 cm along the axes.

The microhardness measurements were obtained from the same mid-diaphyseal sections using a Tukon model MO microhardness tester (Wilson, Bridgeport, CT) with a 136° pyramid–shaped (Vicker's) diamond indenter. An average microhardness value was calculated from the three microhardness measurements obtained in each of six cross-sectional regions (Fig. 1): newly-formed endocortical and periosteal bone, and anterior, lateral, medial and posterior preexisting ("extant") bone. The three measurements per region produced a range of values that, on average, was only 34% of the range of values among all femora from one group. A 50 g load, selected to ensure uniform measurements that are less prone to variance due to bone heterogeneity [16], was applied for at least 10 s to ensure complete indent deformation [11]. A separation distance of at least one indent diameter was maintained between the indent sites, sample edges and visible osteocytes and lacunae in order to minimize undesired edge effects [11]. Lengths of the pyramid diagonals were measured at 250 × and the Vicker's hardness number (V_{HN}) was calculated using $V_{HN} = (2P \sin(x/2))/d^2 = 1854.4P/d^2$, where P is the applied load (g), x is the pyramid angle (136°), and d is the average length of the two indent diagonals (μm). The mineral formation rate divided by (i.e. referred to) the bone area of the mid-diaphyseal section (MFR/B.Ar, calculated from MAR*aM.Pm/B.Ar, or mineral apposition rate *active mineralizing perimeter/bone area, using standardized nomenclature [17]) was evaluated using the fluorescent labels to permit the calculation of the weighted average microhardness value for each cross-section. This value was the weighted average of microhardness values for each of the six cross-sectional regions. Clearly, for baseline controls, there were no regions of newly formed bone between double fluorescent labels. However, measurements along the endocortical and periosteal surfaces where new bone was observed to form in *ad libitum* controls were obtained, and a weighted average

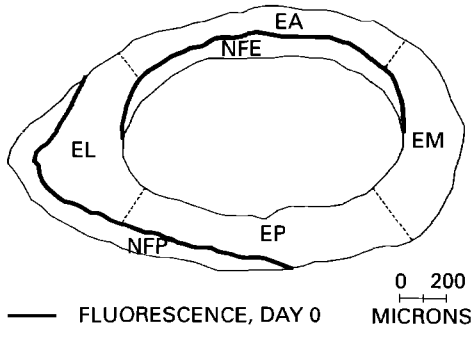


Figure 1 Regions of femur cross-section. Newly formed endocortical (NFE) and periosteal (NFP), and pre-experimentally formed ("extant") bone in the anterior (EA), lateral (EL), medial (EM) and posterior (EP) aspects are indicated. Three microhardness measurements were taken in each of these regions for each femur cross-section.

microhardness value calculated, for comparative purposes.

To determine resorptive activity in the femora of all mouse groups, eroded perimeter referenced to bone perimeter (E.Pm/B.Pm, or eroded perimeter/bone perimeter), and porosity (Po = Vd.Ar/Ct.Ar, or void area/cortical area) were calculated for the cross-sections. Histomorphometric analysis was performed using a SigmaScan digitizing tablet and accompanying analysis software with 40× (for MFR/B.Ar) and 250× (For E.Pm/B.Pm and Po) photomicrographs. Porosity calculations were computed using porosities with at least one dimension of 10 μm or greater to avoid inclusion of cellular or lacunar spaces.

Statistical comparisons of the data obtained for the mouse groups were performed using an analysis of variance (ANOVA). If the F-value obtained indicated significant between-group variance, the ANOVA was followed by Duncan's multiple range test. A 95% level of confidence (type I error) was utilized for each of these tests. Linear regression lines, where obtained, were tested at the same ($P < 0.05$) level of significance.

3. Results

The non-suspended 2-week experimental groups had similar bone %Min (the means ranging from 60.9–62.7% in the femur), which were significantly different from the %Min mean (58.1%) for the femora of suspended mice (Table I).

The contralateral bones were readily tested for microhardness. All microhardness indents had diagonals in the range of 27–36 μm, which corresponds to square dimensions in the range 19 μm × 19 μm to 25 μm × 25 μm. Indents were readily localized to areas of both new and extant bone formation (Figs. 1 and 2). Microhardness values in extant bone were typically lower for baseline controls than for all other groups (Fig. 3). For extant bone, the microhardness values in the femora of suspended mice were not different from those of either control group.

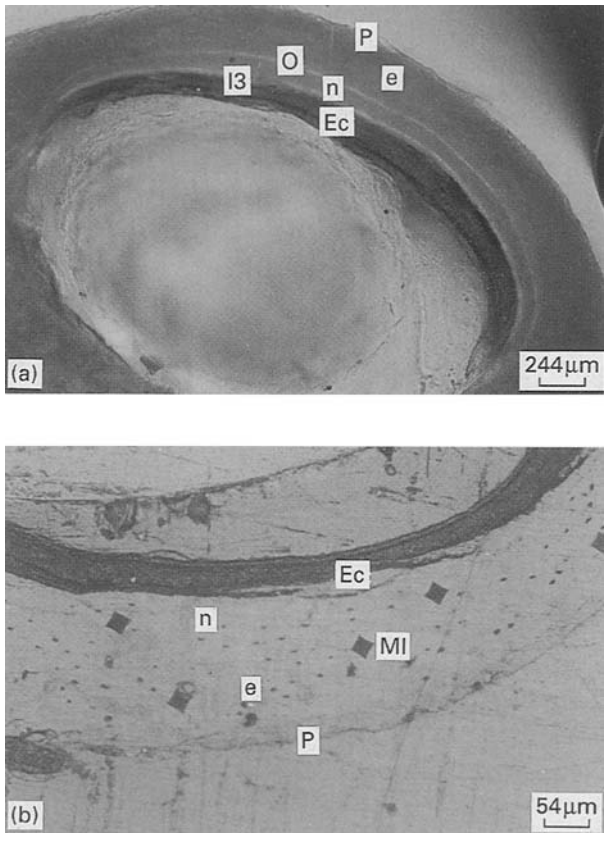


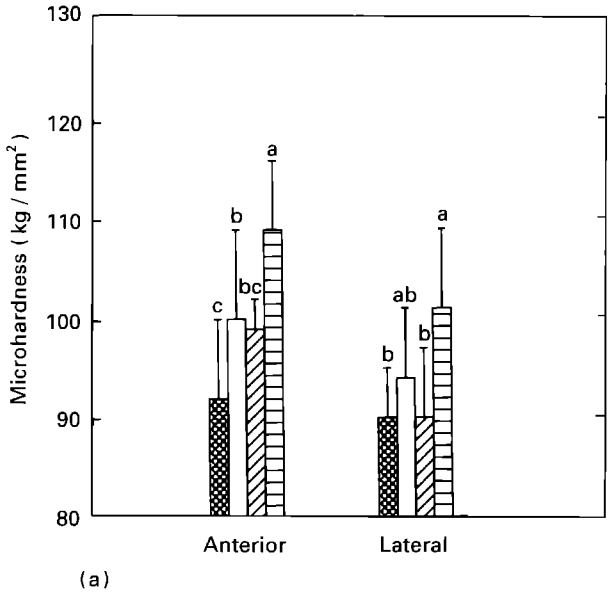
Figure 2 Photomicrographs illustrating (a) tetracycline label at day 0 (0) and day 13 (13). Newly formed bone (n) is along the endocortical perimeter (Ec), and extant bone (e) is along the periosteal perimeter (P); (b) microhardness indents (MI) in newly-formed bone (n) along the endocortical perimeter (Ec) and in extant bone (e) along the periosteal perimeter (P). Because of depth of focus differences for the label (surface) and the indents (below surface), both photomicrographs are required to demonstrate the combined technique.

In the newly formed bone, however, the femora of suspended mice demonstrated significantly lower microhardness than measured in all other similarly aged groups (Fig. 4). The value for suspended mice was comparable to that of the 2-week younger

TABLE I Mineralization (right femora), microhardness (left femora) and histomorphometric (left femora) data. The mouse group names and histomorphometric terms are described in the text. Data is presented as mean ± standard deviation (n = 10/group). Histomorphometric values of E.Pm/B.Pm and MFR/B.Ar were first reported elsewhere [23].

Parameter	Baseline control	*Ad libitum* control	Suspended	Unloading control
Mineral percentage in contralateral femur				
%Min (%)	59.7 ± 2.5^{bc}	60.9 ± 1.4^{ab}	58.1 ± 4.5^{c}	62.7 ± 1.8^{a}
Weighted average microhardness of mid-diaphyseal section				
VHN (kg/mm^2)	91 ± 4^{c}	100 ± 5^{ab}	97 ± 3^{b}	105 ± 5^{a}
Histomorphometry				
E.Pm/B.Pm (%)	12.5 ± 5.0^{b}	14.3 ± 3.3^{b}	15.3 ± 3.3^{b}	20.0 ± 2.4^{a}
MFR/B.Ar (%)	NA	20.4 ± 1.9^{b}	14.1 ± 1.8^{c}	23.5 ± 3.6^{a}
Vd.Ar/Ct.Ar (%)	0.7 ± 0.6^{a}	1.0 ± 1.2^{a}	1.0 ± 0.9^{a}	11.1 ± 0.9^{a}

Statistics: The superscripted letters are used to indicate groups that are significantly different. If two groups share any letter in common, then they are not significantly different ($p < 0.05$) using Duncan's multiple range test for pair-wise comparisons. If two groups do not have any letter in common, then they are different ($p < 0.05$) from each other. For example, microhardness of newly formed endocortical bone differs for *ad libitum* control and suspended mice, since the former group has the a and b superscripts, and the latter group the c superscript (Fig. 3). This statistical comparison protocol is also used in Figs. 3 and 4.



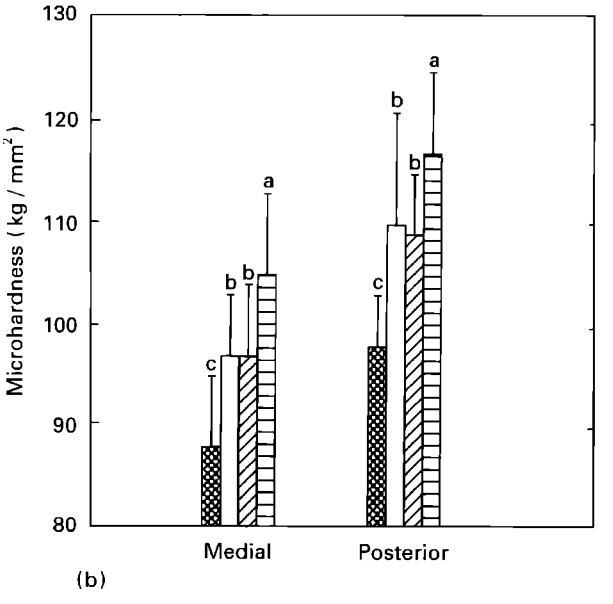


Figure 3 Microhardness in previously deposited ("extant") bone in anterior and lateral (a) and medial and posterior (b) quadrants of the femur mid-diaphyseal cross-sections. Error bars indicate one standard deviation (n = 10/group). Statistical comparisons among groups are presented in Table I (▩ baseline controls; □ *ad libitum* controls; ▨ suspended; ▤ unloading controls).

baseline controls. The "newly formed" bone of baseline controls was formed prior to 40 days of age, while the newly formed bone of suspended mice was deposited between 40 and 54 days of age. For newly formed femoral bone, the microhardness of *ad libitum* (99 $\pm$ 8 and 96 $\pm$ 4 kg/mm^2 in the endocortical and periosteal regions, respectively) and unloading (100 $\pm$ 4 and 105 $\pm$ 5 kg/mm^2 in the endocortical and periosteal regions, respectively) controls had greater values than the femora of both suspended (88 $\pm$ 7 and 88 $\pm$ 4 kg/mm^2, respectively) and baseline control (90 $\pm$ 9 and 83 $\pm$ 8 kg/mm^2, respectively) groups. The trend for weighted average microhardness values was the same as for newly formed bone and for %Min in the contralateral femur (Table I).

Increased endocortical resorptive perimeter [9] was observed in the unloading controls, but not in the

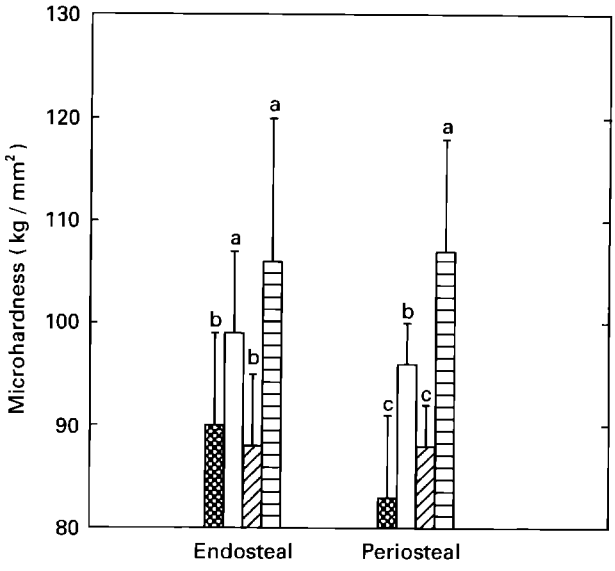


Figure 4 Microhardness in newly formed bone along endocortical and periosteal surfaces. Error bars indicate one standard deviation (n = 10/group). Statistical comparisons among groups are presented as in Table I (▩ baseline controls; □ *ad libitum* controls; ▨ suspended; ▤ unloading controls).

ad libitum controls. As a result, the overall percentage of bone perimeter exhibiting resorptive erosion (E.Pm/B.Pm) was significantly greater for the unloading controls than for the other three groups (Table I). Porosity was predominantly confined to the subendosteal space [18] contralateral to the regions where endocortical bone formation was observed, and all 54-day mice groups had similar mean values of approximately 1% for Vd.Ar/Ct.Ar. The baseline controls had a nonsignificantly lower 0.7% value of Vd.Ar/Ct.Ar.

Microhardness was positively related with mineral percentage for *ad libitum* controls (R = 0.39, n = 10, $p > 0.05$), and significantly correlated when all 54-day old mice were pooled (R = 0.37, n = 30, $p < 0.05$).

4. Discussion and conclusions

Suspension results in decreased MAR and MFR/B.Ar [9] without an increase in resorption (E.Pm/B.Pm and Vd.Ar/Ct.Ar). This is true for comparisons with either of the 54-day control groups. The reduced rate of formation was accompanied by reduced microhardness of the newly formed bone. However, the microhardness of bone formed prior to the suspension period was similar to that measured in the femora of either 54-day control group. Thus, the structural properties, and presumably the mineralization [2], of the previously formed bone, was not altered by suspension, in accordance with the lack of increased resorption. In newly formed bone, however, microhardness values were significantly reduced in suspended mice compared to age-matched mice. This reduction in microhardness of newly formed bone corresponds to a decreased %Min in the contralateral femur. Unfortunately, percentage mineralization was determined for the entire contralateral bone (and not subdivided for the mid-diaphysis) before the present combined histomorphometry and microhardness

protocol was developed. Despite this caveat, the two trends are likely related; that is, the decreased microhardness appears to reflect the decreased mineralization of newly formed bone. It is worth noting that the microhardness of newly formed periosteal bone was significantly greater in the unloading control femora than in the *ad libitum* control femora, in agreement with the trend for higher mineralization of the contralateral femora of the unloading control group. The observed alteration in microhardness of newly formed bone in the femora of suspended mice may be attributed to changes in the early stages of bone mineralization; that is, before extracellular matrix vesicle activity has been completed [19] ; however, such a hypothesis was not directly tested. It is not known why the unloading control had greater microhardness in the newly formed bone apposed to the periosteum.

However, when pooling data for all of the 54-day old mice, it is clear that microhardness and mineral percentage (%Min) are positively related. The correlation is limited by the fact that microhardness and whole bone mineral content were obtained from contralateral and not ipsilateral bones, as discussed above. The general trend observed here is in agreement with reported correlations between microhardness and mineral volume fraction [2].

The microhardness values obtained (range of 80–120 kg/mm^2) are higher than those obtained in fresh, unimbedded bone with similar %Min [2, 12, 20]. This was expected because of the resin infiltration and 70 °C curing temperature accompanying the preparation technique [11, 20]. The microhardness indents obtained in this study were squares with sides from 19–25 μm in length. These were formed from 50 g loading. Loading with lesser weight will result in smaller deformation "squares"; however, at the expense of uniform indent properties [16]. Even 25 g loading, however, would not have produced substantially smaller indents (14–18 μm squares). Because of the indent size, in some cases the indent was nearly half the width of the newly formed bone layer, which necessitated the placement of the indent further from the bone perimeter to assure a one-diagonal distance from the edge. This one-diameter distance was maintained throughout to avoid cracks formed by the indents [11] and to avoid placing the indents into non-mineralized osteoid which lines the perimeter at sites of active mineralization. Symmetry (within 5%) of indent diagonals was observed for all measurements, so that edge effects or effects of heterogeneity of the bone material (e.g. due to fluorescent label, osteoid, etc.) were not observed. Moreover, the variance of the three measurements in each region was considerably less (only 34% as much) than the variance obtained in comparing mean values among femora from identically-treated mice, indicating these three measurements accurately represent mean local microhardness.

These results further indicate that the unloading aspect of suspension results in significant bone changes. In a previous study, bone formation rate was shown to decrease in suspended mice, but not in unloading controls [9]. Matched feeding was shown to correspond to increased resorptive activity (E.Pm/B.Pm), in agreement (although with considerably less severity) with the effects of semi-starvation [21]. The present results demonstrate that subendosteal resorption (Vd.Ar/Ct.Ar) does not increase, and thus structural compromise due to the disproportionate weakening of the subendosteal cortical bone [18] is not expected. However, the short-term (2 weeks) nature of the experiment must be kept in mind. It is possible that subendosteal resorption was increasing, but had not progressed past the early stages of resorption, conditions under which collagen is solubilized by collagenase [22]. If so, the mineral percentage would be expected to rise. Indeed, a trend for increasing mineral percentage was observed in both of the 54-day control groups.

The increased microhardness corresponded to generally increased %Min in the contralateral femora of the unloading control group. Physiological stress results in increased levels of serum corticosteroids [23]. Attaching the suspension gear may therefore have been expected to increase corticosteroid production, an occurrence usually linked with an osteoporotic condition [24]. However, the response of rat bone to cortisol is dose dependent, with medium doses actually leading to increased Young's modulus and, for 48–64 mg/kg/day doses, increased calcium content [25]. Glucocorticoids do not appear to cause the resorption accompanying suspension in rats [26]. Moreover, tibial bone density has been observed to increase in rats treated with ≈25 mg/kg/day cortisol acetate [27], and tibial epiphyseal cartilage density to increase in rats treated with 40–50 mg/kg/day [28]. Thus, in rats, cortisol can act to increase bone density and mineralization. Since serum cortisone levels were not measured in the present set of experiments, the effect of attaching the suspension gear on cortisol levels remains unknown. Moreover, this interpretation assumes relatively similar effects of cortisol on mice and rats. It is likely, however, that regardless of the effects of equipping on cortisol and subsequently cortisol on bone, the increased microhardness seen in the femora of these mice is a result of increased mineralization and density. Direct measurement of %Min in the ipsilateral rather than contralateral limb should be incorporated in any future experiments which combine histomorphometry with microhardness measurements.

This study demonstrates that suspension, and in particular the unloading aspect of suspension, causes not only reduced bone formation rates, but reduced microhardness of newly formed bone. These results indicate that the structural properties of the newly forming bone are compromised in suspended mice. While this study does not elucidate the effects on bone properties of physiological stress or feeding changes accompanying suspension, it does demonstrate that control groups accounting for either of these two aspects of the model do not display reduced microhardness measurements. As such, the microhardness findings support the hypothesis that the unloading aspect of suspension causes significant alterations in

bone metabolism and integrity; specifically, compromised composition and quality of newly formed bone. The change in composition and quality of newly formed bone during suspension may be related to the compromised nature of bone formed during spaceflight [1] inasmuch as the overall results are due to similar causative factors.

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