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Constrained Tibial Vibration Does Not Produce an Anabolic Bone Response in Adult Mice

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Abstract

Osteoporosis is characterized by low bone mass and increased fracture risk. High frequency, low-amplitude whole-body vibration (WBV) has been proposed as a treatment for osteoporosis because it can stimulate new bone formation and prevent trabecular bone loss. We developed constrained tibial vibration (CTV) as a method for controlled vibrational loading of the lower leg of a mouse. We first subjected mice to five weeks of daily CTV loading (0.5 G maximum acceleration) with loading parameters chosen to independently investigate the effects of strain magnitude, loading frequency, and cyclic acceleration on the adaptive response to vibration. We hypothesized that mice subjected to the highest magnitude of dynamic strain would have the largest bone formation response. We observed a slight, local benefit of CTV loading on trabecular bone, as BV/TV was 5.2% higher in the loaded vs. non-loaded tibia of mice loaded with the highest bone strain magnitude. However, despite these positive differences, we observed significantly lower measures of trabecular structure in both loaded and non-loaded tibias from CTV loaded mice compared to Sham and Baseline Control animals, indicating a negative systemic effect of CTV on trabecular bone. Based on this evidence, we conducted a follow-up study wherein mice were subjected to CTV or sham loading, and tibias were scanned at the beginning and end of the study period using *in vivo* microCT. Consistent with the findings of the first study, trabecular BV/TV in both tibias of CTV loaded and Sham mice was, on average, 36% and 31% lower on day 36 than day 0, respectively, compared to 20% lower in Age-Matched Controls over the same time period. Contrary to the first study, there were no differences between loaded and non-loaded tibias in CTV loaded mice, providing no evidence for a local benefit of CTV. In summary, 5 weeks of daily CTV loading of mice was, at best, weakly anabolic for trabecular bone in the proximal tibia, while daily handling and exposure to anesthesia was associated with significant loss of trabecular and cortical bone. We conclude that direct vibrational loading of bone in anesthetized, adult mice is not anabolic.

Keywords

Vibration; microCT; WBV; CTV; mechanical loading

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Introduction

Osteoporosis is characterized by low bone mass and increased fracture risk. High frequency, low-amplitude whole-body vibration (WBV) has been proposed as a treatment for osteoporosis, since reports have indicated that WBV can stimulate the formation of new trabecular bone [1–8] or prevent bone loss associated with disuse, ovariectomy, or menopause [9–14]. However, the mechanisms by which vibrational loading may stimulate bone formation are not known.

It is well established that mechanical stimulation of bone formation is largely influenced by the magnitude of applied bone strain [15–22]. For example, 100 daily cycles of 1 Hz loading with peak strains below 1000 $\mu\epsilon$ resulted in net bone loss in the avian ulna, whereas the same loading regimen with peak strains above 1000 $\mu\epsilon$ caused substantial new bone formation [16]. This concept has been supported in only one WBV study, wherein a dose-dependent increase in labeled trabecular bone surface was observed in turkeys subjected to increasing magnitudes of WBV from 0.1–0.9 G ($G = 9.8 \text{ m/s}^2$) [11]. On the other hand, rats subjected to 45 Hz WBV, which caused peak-to-peak tibial strains of 2 $\mu\epsilon$, had a significantly lower rate of trabecular bone formation than rats loaded at 90 Hz, which produced peak-to-peak strains of less than 1 $\mu\epsilon$ [7]. This latter result suggests that strain magnitude may not be the most important factor in the osteogenic response to vibration.

Similarly, it is unclear if weight bearing is required for the anabolic effects of WBV or if the effects can be produced by cyclic acceleration without weight bearing. A recent study in mice suggests that accelerations < 1.0 G in the absence of weight bearing can stimulate increased bone formation [23]. More work is needed to confirm this finding and determine whether or not cyclic acceleration *per se* can increase bone density.

Studies that have investigated the osteogenic effect of vibrational loading have primarily focused on WBV [24]. In small quadrupeds such as mice, one limitation of WBV is that the vibration experienced at a particular skeletal site is difficult to control since the animal can move and change posture during loading. To address this concern, we developed constrained tibial vibration (CTV) as a new method for controlled vibrational loading of bones in mice [25]. In CTV, the lower leg of the mouse is subjected to vibrational loading in a fixed orientation (Fig. 1), and factors such as vibration magnitude, frequency, and peak bone strain can be adjusted in a controlled way.

Our objective was to use CTV loading as an experimental model for examining how bone responds to controlled vibrational loading. We subjected mice to daily CTV loading; loading parameters were varied to independently investigate the effects of dynamic strain magnitude, loading frequency, and cyclic acceleration on the osteogenic response to vibration. We hypothesized that the loading group subjected to the largest magnitude of dynamic strain would have the largest bone formation response.

Materials and Methods

Constrained Tibial Vibration Loading System

The system for CTV loading has been previously described [25]. Briefly, the loading stage is driven by an electromagnetic actuator with a sine wave at the desired frequency and amplitude. The right lower leg of the mouse is positioned vertically, the knee contacting the loading stage and the foot supporting the weight of the top platform. The left leg is not loaded and serves as a contralateral control.

Overview of Studies

Prior to *in vivo* studies, seven mice were used for *post mortem* strain analysis. We then conducted two *in vivo* studies. First, to evaluate the effect of loading parameters on bone structure and formation (“Loading Parameter Study”), the right legs of 60 adult mice (male C57Bl/6, 5–6 mo. old, Harlan Sprague Dawley, Indianapolis, IN) were loaded *in vivo* with CTV. An additional ten mice were killed without loading and used as baseline controls. Evaluations were done *post mortem*. Second, to assess temporal changes (“Temporal Change Study”), *in vivo* microCT was used to scan 36 mice (male C57Bl/6, 6–7 months) before and after the 5-week loading period. All animals were housed in a licensed animal facility with unrestricted access to standard mouse chow and water. Animal weights were recorded weekly. Euthanasia was by CO₂ asphyxiation. All methods were consistent with NIH guidelines for the care and use of laboratory animals, and were approved by the Washington University Animal Studies Committee.

Cortical Strain During CTV Loading

Mice ($n = 7$) were killed and strain gages (Micron Instruments Model #SS-080-050-500P-S1, Simi Valley, CA) were immediately attached with cyanoacrylate to the anterior-medial surface of the tibia at the predicted location of maximum tensile strain [25]. Vibration was applied at frequencies from 20–150 Hz (5 Hz increments) at 0.5 G maximum input acceleration. The right leg of each mouse was loaded using both a “high” (125 g) and “low” (40 g) mass upper platform. Additionally, each mouse was loaded in a “fixed mass” orientation using the 125 g mass in which the upper platform was lowered onto the right leg of the mouse, and then locked into place to prevent vertical movement independent of the bottom platform (Fig. 1). In this configuration, the movement of the lower leg should approximate rigid body translation. Soft tissue of the mouse lower leg was left intact as much as possible, and was rehydrated as needed during loading. Strain signals were processed using a signal conditioner and amplifier (National Instruments SCXI-1321 and SCXI-1121, Austin, TX) and data were collected at 1200 Hz (Labview, National Instruments). Peak-to-peak strain was quantified at each loading frequency. Two data sets were collected consecutively for each mouse in each of the three configurations. Strain rate was determined by three-point numerical differentiation.

Loading Parameter Study

In Vivo Loading Groups—Mice were assigned randomly to six groups ($n = 10$ –12/group) – a Baseline Control group and five loading groups (Table 1). The right leg of each mouse was loaded under isofluorane anesthesia (1.5–2.0% in air, 1.5 L/min, delivered via nosecone) for 15 minutes/day, 5 days/week for 5 weeks, with an input magnitude of 0.5 G maximum acceleration. Mice were injected with calcein green (7.5 mg/kg body weight, Sigma-Aldrich, St. Louis, MO) on days 5 and 25 of loading, and alizarin complexone (30 mg/kg body weight, Sigma-Aldrich, St. Louis, MO) on day 15. The High Strain group was loaded at the resonant frequency of the CTV system for the high mass, weight-bearing configuration (70 Hz). Under these conditions, dynamic (peak-to-peak) strain magnitude was found to be $330 \pm 82 \mu\epsilon$. The High Frequency group was loaded at 140 Hz and supported the high mass, producing $79 \pm 27 \mu\epsilon$. The Low Mass group was loaded at 70 Hz, but supported the low mass, resulting in $59 \pm 31 \mu\epsilon$. The Fixed Mass group was loaded with the upper platform locked into place (Fig. 1) after being preloaded with the high mass, resulting in $17 \pm 5 \mu\epsilon$. The Sham group was statically loaded with the high mass for 15 min/day, but was not subjected to vibrational loading. Thus, the High Strain group experienced the largest peak-to-peak dynamic strain during loading. Dynamic strain magnitude was reduced in the other loading groups due to a change in one of the loading parameters: first, driving the system at frequencies away from its natural frequency reduces dynamic strain (High Frequency group); second, reducing the magnitude of the mass supported by the lower leg reduces dynamic strain (Low Mass group); and third, applying

cyclic acceleration with minimal deformation of the mouse leg reduces dynamic strain (Fixed Mass group).

MicroCT—Bilateral tibias were removed *post mortem* and placed in 70% alcohol. Bones were scanned using micro-computed tomography (SCANCO, Model μ CT 40, Bassersdorf, Switzerland: Energy = 55 kVp, Intensity = 145 mA, Resolution = High (~8 μ m), Diameter = 16.4 mm, Integration Time = 300 ms). A 480 μ m thick volume of trabecular bone was analyzed at the proximal tibial metaphysis (60 slices, 8 μ m/slice) distal to the metaphyseal growth plate. The trabecular region was designated using contours inside the cortical shell on two-dimensional slices. Trabecular bone volume per total volume (BV/TV), trabecular thickness (Tb.Th*), trabecular separation (Tb.Sp*), trabecular number (Tb.N*), connectivity density (Conn.Dens) and structure model index (SMI) as well as tissue mineral density of trabecular bone (mg HA/cm³ BV) and apparent bone mineral density (mg HA/cm³ TV) were determined using the manufacturer's 3-D analysis tools, where * denotes the direct method of calculation (not based on stereological models) [26]. A threshold linear attenuation coefficient of 2.4 cm⁻¹ was used to differentiate bone from non-bone for all post mortem scans.

Dynamic Histomorphometry—After scanning, tibias were embedded in methylmethacrylate (Sigma-Aldrich, St. Louis, MO) using standard techniques for undecalcified bone [27]. Thick sections (~100 μ m) were cut on a saw-microtome (Leica 1600SP) in the transverse plane at the mid-diaphysis (Fig. 2, top), at the approximate location of maximum tibial strain [25]. Thick sections were also cut from the proximal tibia in the frontal plane (Fig. 2, bottom) in order to analyze tibial trabecular bone of the proximal tibial metaphysis. Slides were mounted on glass and left unstained for epifluorescence imaging of calcein/alizarin labels. Two-color fluorescent images were obtained using a confocal microscope (LSM 510, Axiovert 200M, Plan-Neofluar 10 $\times$ /0.30 NA Objective, Carl Zeiss, Jena, Germany). Dynamic histomorphometric analysis was performed on duplicate slides from each location of each bone using commercial software (Bioquant, Nashville, TN) and results were averaged. Because fluorochrome injections were given at three timepoints, we analyzed indices of dynamic bone formation over two time periods. Double-labeled surfaces were designated as either day 5–15 or day 15–25 based on the relative locations of the red (alizarin) and green (calcein) labels. Green single labels were assumed to have equal contribution from days 5 and 25. Mineralizing surface was calculated as follows

$$MS_{5-15} = dL.Pm_{5-15} + 1/2 [sL.Pm_{15} + 1/2 (sL.Pm_{5/25}) + dL.Pm_{15-25} + dL.Pm_{5-25}] \quad (1)$$

$$MS_{15-25} = dL.Pm_{15-25} + 1/2 [sL.Pm_{15} + 1/2 (sL.Pm_{5/25}) + dL.Pm_{5-15} + dL.Pm_{5-25}] \quad (2)$$

where MS is mineralizing surface (subscripted to indicate time period), dL.Pm is double-labeled perimeter, and sL.Pm is single-labeled perimeter. Mineral apposition rate (MAR) and bone formation rate (BFR) were calculated as usual [28]. Samples that did not have double label in both the loaded and non-loaded limbs for a particular time span (day 5–15 or day 15–25) were not considered in the loaded vs. non-loaded analysis of MAR or BFR for that time span.

Temporal Change Study

Following completion of the Loading Parameter Study we performed a follow-up study using *in vivo* microCT. Mice were randomly assigned to three groups (n = 12/group): High Strain, Sham, and Age-Matched Control. The right legs of mice from the Sham and High Strain groups were subjected to loading as described above. Mice from the Age-Matched Control group

remained in their cages except for day 0 and day 36 microCT scans. Under isofluorane anesthesia (as described above), the right and left tibia of each mouse were scanned prior to the loading (day 0) and at the end of 5-week loading period (day 36). Scans of the proximal metaphysis and the mid-diaphysis were obtained using microCT (vivaCT 40, SCANCO; Energy = 70 kVp, Intensity = 57 mA, Resolution = Medium (~21 μ m), Diameter = 21.5 mm, Integration Time = 300 ms). A threshold linear attenuation coefficient of 1.2 cm^{-1} was used to differentiate bone from non-bone. Analysis of trabecular bone from a 600 μ m thick region beneath the growth plate was done by the same procedure as described above for the Loading Parameter Study. Cortical bone parameters were assessed over a 500 μ m thick region at the mid-diaphysis; cortical bone area, medullary area and tissue mineral density were determined.

Statistical Analysis

Animal weights were analyzed by Repeated Measures ANOVA (Statview 5.0; SAS Institute). Post hoc comparisons were made using Fisher's Protected Least Squares Difference test. MicroCT and histomorphometry data were analyzed using a paired Student's t-test of the loaded (right) vs. non-loaded (left) limb. In addition, one-way ANOVA was used to compare between groups. Data were tested for normality using an Anderson-Darling test. Data were found to be normally distributed in nearly all cases, but the few parameters found to not be normally distributed for all groups were re-analyzed with non-parametric tests (a Wilcoxon Signed Rank test to compare loaded vs. non-loaded tibiae, and a ranked one-way ANOVA for between group comparisons). Data are reported as mean $\pm$ standard deviation. Significance was defined as $p < 0.05$.

Results

Tibial Strain during CTV Loading

For all loading conditions, the peak-to-peak cortical strain magnitude depended on the input frequency (Fig. 3). For example, with the high mass, weight-bearing configuration, the maximum peak-to-peak tibial strain was $330 \pm 82 \mu\epsilon$ and occurred at 60–70 Hz. Difference in peak strain and resonant frequency between the different configurations allowed us to select loading parameters to produce a range of dynamic strains for the *in vivo* studies (Table 1). The dynamic strains for each loading group used for the *in vivo* studies were significantly different from one another, except for the High Frequency and Low Mass groups (one-way ANOVA).

Loading Parameter Study

Body Weight—Mild weight loss was observed in three CTV groups during the loading period, although all but the High Strain group recovered by the end of the study (data not shown). Animals in the High Strain loading group lost an average of 1.3 g (4.1% of original body weight) during the 5-week loading period; weight on day 1 was significantly greater than at all other time points. Animals in the High Frequency and Fixed Mass groups also had significant weight loss of less than 1.0 g between day 1 of loading and some intermediate time point, but by the end of loading they did not differ significantly from their original weight. The Low Mass and Sham groups had no significant changes in body weight during the study.

MicroCT—MicroCT provided evidence of a slight, local beneficial effect of CTV loading on trabecular bone. In the High Strain group, trabecular BV/TV was 5.2% higher in the loaded (right) tibia compared to the non-loaded (left) tibia (Fig. 4; Table 2). Likewise, Tb.N*, Tb.Th* and apparent BMD were 2.6%, 4.4% and 3.1% higher, respectively, in loaded versus non-loaded limbs from the High Strain group. In the Low Mass group Tb.Sp* was a 2.7% lower, and in the Fixed Mass group Tb.Th* was a 3.9% higher in the loaded limbs than in the non-loaded limbs. There were no significant differences in the loaded limb compared to the non-loaded limb in the High Frequency and Sham groups.

Despite these positive right vs. left differences, we observed significantly lower measures of trabecular structure in both loaded and non-loaded tibias from some groups compared to the Sham and Baseline Control groups, indicating a negative systemic effect of CTV on trabecular bone. For example, in the non-loaded limb of the High Strain group BV/TV was 29% lower than in the non-loaded limb of the Sham group, while Conn.Dens, Tb.Th*, and apparent BMD were 43%, 10%, and 18% lower, respectively. Similarly, in the loaded limb of the High Strain group Conn.Dens was 36% lower than in the loaded limb of the Sham group. Results from the Sham group did not differ from those of the Baseline Control group.

Dynamic Histomorphometry—There were few significant differences in trabecular bone formation indices for loaded vs. non-loaded limbs, with no evidence of a positive effect of CTV (Fig. 5; Table 3). In the High Strain group, trabecular MS/BS for day 5–15 was 12% lower, MS/BS for day 15–25 was 14% lower, and BFR/BS for day 5–15 was 13% lower in loaded limbs compared to non-loaded controls. No other right vs. left differences were observed.

Likewise, few differences were observed between loaded and non-loaded tibias at the cortical diaphysis. On the endocortical surface MAR for day 5–15 was 39% higher in the loaded limb of the High Strain group compared to the non-loaded limb (Fig. 6; Table 4). On the other hand, in the Fixed Mass group MAR and BFR/BS for days 5–15 were ~20 % lower in the loaded limb vs. non-loaded limb. No significant differences between any groups were observed for MAR or BFR/BS for days 15–25, and no significant differences in MS/BS were observed between any groups for either time period. On the periosteal surface, there were few samples with double-labeled surface and no consistent differences were noted between loaded and non-loaded limbs (data not shown).

Similar to the results of the microCT analysis, there were some bone formation indices that were lower in loaded groups than in the Sham group, suggesting a negative systemic effect of CTV. For example, at the proximal tibial metaphysis, trabecular MAR for day 15–25 in the non-loaded limb of the High Strain group was 14% lower than in the Sham group. Likewise, on the endocortical surface of the tibial diaphysis, MAR for day 5–15 was 31% lower in the non-loaded limb of the High Strain group than in the Sham group.

Temporal Change Study

Based on evidence of a negative systemic effect of CTV loading (especially in the High Strain group) from the Loading Parameter Study, we conducted a follow-up study wherein tibias were scanned at the beginning and end of the study period using *in vivo* microCT. Consistent with the findings of the first study, significant bone loss was observed in both tibias from each of the three experimental groups, with the magnitude of loss in the High Strain and the Sham groups being greater than in the Age-Matched Control group (Fig. 7; Table 5). Trabecular BV/TV at the proximal tibia was, on average, 36% lower on day 36 than day 0 in the High Strain group, and 31% lower in the Sham group. These differences were partly attributed to age-related declines, as BV/TV was reduced by 20% in Age-Matched Controls over the same time period. Similar changes were observed in Tb.Th* and Tb.N*. Contrary to the findings of the Loading Parameter Study, in the High Strain group there were no differences between loaded and non-loaded control tibias, and thus no evidence of a local effect of CTV.

Similar findings were observed at the tibial diaphysis, with evidence of cortical bone loss and medullary expansion in loaded mice. Cortical bone area was 13% lower on day 36 than day 0 in the High Strain group, and 8.3% lower in the Sham group. These were significantly greater changes than observed in the Age-Matched Control group over the same time period (1.9% loss). Again, there was no evidence of a local (loaded vs. non-loaded control) effect of CTV loading.

Discussion

We subjected mice to constrained tibial vibration (CTV) using a range of loading parameters designed to test the dependence of the adaptive response on bone strain, loading frequency, and cyclic acceleration. We hypothesized that strain magnitude is the most important factor affecting the bone formation response to vibrational loading, and therefore that the loading group subjected to the highest magnitude of dynamic strain would have the largest bone formation response. This hypothesis was partly supported by results from the microCT analysis of proximal tibia in the Loading Parameter Study, which showed significantly more trabecular bone in the loaded limb of the High Strain group compared to the contralateral control, while the lower strain groups showed negligible loading effects. However, in the follow-up Temporal Change Study we did not observe relative differences between loaded and non-loaded limbs. Apart from any possible local effects of CTV loading, we also observed in the Loading Parameter Study significantly less trabecular bone in some loading groups (particularly the High Strain group) compared to the Sham and/or Baseline Control groups, suggesting a negative systemic effect of CTV loading. These negative systemic effects were confirmed in the Temporal Change Study, wherein mice from bone High Strain and Sham groups lost more trabecular bone than Age-Matched Control mice.

The CTV loading method allowed us to examine the effect of vibration-induced bone strain magnitude in a more controlled way than possible with WBV. In particular, use of a different moving mass (125 vs. 40 g) produced average peak-to-peak tibial strains of 330 $\mu\epsilon$ in the High Strain group versus 59 $\mu\epsilon$ in the Low Mass group for the same input acceleration (0.5 G) and frequency (70 Hz). The High Strain loading group of the Loading Parameter Study provided evidence of a local loading effect, with significantly greater trabecular BV/TV and other measures of trabecular structure in loaded versus non-loaded limbs after 5 weeks of daily loading. In contrast, there were no consistent differences between loaded and non-loaded limbs in other (lower strain) loading groups, suggesting that the loading response depended on strain magnitude. However, the magnitude of the loading effect in the High Strain group was small ($\leq 5\%$) and, importantly, was not reproduced in the Temporal Change Study. Thus, although previous studies using a variety of loading methods have attributed the degree of bone formation to the magnitude of dynamic bone strain during loading [15,16,19–22] we do not have strong evidence for a magnitude-dependent response in the strain range we examined.

The bone strain magnitudes produced by CTV in the current study (approx. 60 to 300 $\mu\epsilon$; Table 1) are greater than the extremely low values produced by low-amplitude WBV but less than the peak values associated with vigorous physical activity or direct high-strain, low-frequency skeletal loading (e.g., tibial compression). Values of tibial surface strain (peak-to-peak) reported for WBV range from 1 or 2 $\mu\epsilon$ in rats (90 or 45 Hz, respectively, 0.15 G) [7] to 5 $\mu\epsilon$ in turkeys (30 Hz, 0.3 G) [11] to 11 $\mu\epsilon$ in mice (45 Hz, 0.3 G) [14], approximately an order of magnitude lower than the bone strains produced by CTV loading. On the other hand, vigorous physical activity can produce peak strain magnitudes in the range of 2000 to 3500 $\mu\epsilon$ across a wide range of species, bones and activities [29,30]. Peak ulnar surface strains during running in rats were approximately 1000 $\mu\epsilon$ [31]. Values of strain rate during CTV loading (0.007–0.079 s^{-1} , Table 1) are also intermediate to lower values reported for WBV [7] and higher values reported for high-strain, low-frequency loading [32–34]. Despite strain and strain rates substantially greater than those associated with WBV, our overall findings indicate that direct loading of bone at dynamic strains of approx. 100 $\mu\epsilon$ and at a frequency of 70 Hz is not anabolic for bone.

Serial *in vivo* microCT revealed age-related trabecular bone loss in the proximal tibia of the mice used in this study (~6 mo old, male C57BL/6), as trabecular BV/TV was reduced 15–20% in the Age-Matched control group of the Temporal Change Study from day 0 to day 36.

We note that this was not consistent with the lack of difference between Baseline and Sham Controls in the Loading Parameters Study. This inconsistency may be due to variability between mice from the two experiments or it may reflect the greater statistical power in a serial comparison versus a between-groups comparison. Nonetheless, age-related loss of trabecular bone is consistent with reports of declining BV/TV and other measures of trabecular structure in C57Bl/6 mice beginning at 4 to 6 months age (depending on the skeletal site) [35,36]. Most previous studies of vibrational skeletal loading in mice have focused on animals 4 months or younger [2,14,37,38]. The use of adult mice with a prevalent condition of age-related trabecular bone loss represents a challenging scenario for examining the anabolic effects of vibrational loading.

Serial *in vivo* microCT further revealed a negative systemic effect of the CTV loading protocol on both trabecular and cortical bone. In comparison to the Age-Matched Controls, mice in both Sham and High Strain groups in the Temporal Change Study lost significantly more trabecular bone in the proximal tibia, and cortical bone in the tibial diaphysis. This effect does not appear to be directly related to CTV loading, as there were no differences in bone loss between loaded and non-loaded control legs in the High Strain group, and moreover the magnitude of bone loss did not differ between Sham and High Strain groups. One possible explanation is a negative, stress response to daily handling and anesthesia. Isoflurane anesthesia has been shown to lower calcium ion concentration in serum and result in increased PTH and osteocalcin levels [39]. Although intermittent administration of PTH is anabolic, increased levels of PTH for more than 2 hours have detrimental effects on bone in rats [40]. A limitation of our study is that we did not evaluate serum levels of calcium, PTH or other markers related bone turnover or physiological stress (e.g., corticosterone). Although daily exposure to isoflurane anesthesia has been used in previous studies [23,41,42], usually comparisons are made based on *post mortem* assessment rather than serial assessment, and so negative systemic effects might be difficult to assess. Lastly, the age of the mice may also play a role in their response to daily handling and anesthesia. In a pilot study on 3-month old mice subjected to the same protocol as the High Strain group in the current study, increases in trabecular and cortical bone mass were observed during the 5-week period, suggesting that younger mice were more tolerant of the experimental procedures. Of note, one study of high-strain, low-frequency tibial compression in mice reported that a protocol that was anabolic for tibial trabecular bone in 2-month mice was catabolic in 5-month mice [43].

The effect of weight bearing on the bone formation response to vibrational loading remains unclear. Recently, it was reported that vibration with accelerations < 1.0 G in the absence of weight bearing can stimulate increased bone formation in mice [23]. In that study, 3 weeks of non-weight bearing vibrational loading (10 min/day, 0.3 G or 0.6 G, 45 Hz) that produced peak-to-peak bone strains of 1 $\mu\epsilon$ (0.3 G) or 2 $\mu\epsilon$ (0.6 G) resulted in increases of 88% and 66% in BFR/BS in loaded versus control limbs at 0.3 and 0.6 G, respectively. The Fixed Mass group in our study was subjected to cyclic acceleration with minimal deformation of the mouse leg, although the mouse leg was subjected to a 125 g preload, and $17 \pm 5 \mu\epsilon$ cyclic bone strain. The results of our study do not provide any support for the previous findings using non-weight bearing vibrational loading, as we observed no consistent loading effects in the Fixed Mass group as a result of CTV loading.

In summary, 5 weeks of daily constrained tibial vibration (CTV) loading of adult mice was, at best, weakly anabolic for trabecular bone in the proximal tibia. We did not observe a strain magnitude-dependent response in the strain range (20 – 300 $\mu\epsilon$) and frequency (70 Hz) we examined. Unexpectedly, we observed a negative systemic effect of the experimental protocol that was independent of vibrational loading. Daily handling and exposure to anesthesia was associated with significant loss of trabecular and cortical bone. We conclude that direct vibrational loading of bone in anesthetized, adult mice is not anabolic.

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Figure 1.

Weight bearing vs. fixed mass configurations. In the weight bearing configuration, the top platform of the CTV system (Mass) is supported by the lower leg of the anesthetized mouse. The top platform is free to move vertically, independent of the movement of the bottom platform (vibration source). This results in deformation of the mouse lower leg due to relative movement between the platforms. In the fixed mass configuration, the top platform is locked into place using shaft collars. In this case, relative motion between the top and bottom platforms is minimal, essentially resulting in rigid body translation of the mouse leg. Mice in the fixed mass configuration are subjected to cyclic acceleration of ± 0.5 G, with little bone deformation due to movement between the platforms.

Figure 2.

Example histological sections from the tibial diaphysis (top) and the proximal tibial metaphysis (bottom). Mice were injected with calcein green on days 5 and 25 of loading, and alizarin complexone on day 15 of loading. Fluorescent labels from all three time points were easily distinguishable on the trabecular bone of the proximal tibial metaphysis as well as the endocortical surface of the diaphyseal cortical bone, and a majority of sections had double-labeled surface at these sites. In contrast, on the diaphyseal periosteal surface double-labels were observed on few sections and thus periosteal MAR and BFR were not calculated.

Figure 3.

Frequency response of peak-to-peak cortical strain on the tibia of a mouse leg subjected to CTV loading (0.5 G maximum acceleration input magnitude) with the “high” mass (125 g), “low” mass (40 g), and fixed mass configurations. Strain gages were placed on the anteriomedial surface of the tibia at the previously determined location of peak tensile strain. Each plot represents the average from seven mice (tested in duplicate). The loading frequency and average dynamic strain of the loading groups used for *in vivo* studies are indicated (“X”).

Figure 4.

Results from microCT analysis of trabecular bone from the proximal tibial metaphysis for the Loading Parameters Study. In the High Strain group there were significantly higher BV/TV, Tb.Th* and Apparent BMD in the loaded limb vs. the non-loaded limb, while loading groups subjected to lower magnitudes of strain had no consistent differences. However, in the High Strain group (as well as the High Frequency and Low Mass groups to a lesser extent) there were significantly lower values for some factors compared to the Sham and/or Baseline Control groups, indicating a systemic loss of trabecular bone in these groups.

Figure 5.

Results from 2-D histomorphometric analysis of trabecular bone from the proximal tibial metaphysis for the Loading Parameters Study. Few significant changes were observed in the loaded vs. non-loaded limbs, however in the High Strain group we observed significantly lower MS/BS and BFR/BS in the loaded limb.

Figure 6.

Results from dynamic histomorphometric analysis of the endocortical surface of diaphyseal cortical bone for the Loading Parameters Study. A significantly higher MAR was observed in the loaded limb of the High Strain group compared to the non-loaded limb for day 5–15, indicating an increase in bone formation in this group. In groups that were subjected to loading that produced lower strain magnitudes, either no response was observed, or a negative response to loading was observed (Fixed Mass group).

Figure 7. Percent change from day 0 to day 36 in tibial morphological parameters determined by *in vivo* microCT for the Temporal Change Study. Both loaded and non-loaded legs from the High Strain and Sham groups showed greater loss of trabecular BV/TV and trabecular thickness compared to corresponding Age-Matched controls. Systemic loss of cortical bone area and increased medullary area indicate endocortical bone resorption in Sham and High Strain groups.

Table 1

Loading parameters for CTV *in vivo* loading groups

Group	Loading Frequency	Loading Magnitude	Moving Mass	Peak-to-peak bone strain ^a	Maximum strain rate ^{ab}
High Strain	70 Hz	0.5 G	125 g	330 ± 82 µε	0.079 s ⁻¹
High Frequency	140 Hz	0.5 G	125 g	79 ± 27 µε	0.032 s ⁻¹
Low Mass	70 Hz	0.5 G	40 g	59 ± 31 µε	0.016 s ⁻¹
Fixed Mass	70 Hz	0.5 G	Fixed Mass	17 ± 5 µε	0.007 s ⁻¹
Sham	0 Hz	0 G	125 g	0 µε	0 s ⁻¹

^a Averaged from *post mortem* strain gage analysis of 7 mice (2 trials each)

^b Calculated from raw strain data using three point numerical differentiation

Table 2

MicroCT results for proximal tibial metaphyseal trabecular bone

Group	Limb	Bone Volume per Total Volume	Connectivity Density (1/mm ³)	Trabecular Number (1/mm)	Trabecular Thickness (mm)	Trabecular Separation (mm)	Apparent Density (mg HA/cm ³)	Min. Density (mg HA/cm ³)	Trab. Bone Density (mg HA/cm ³)
High Strain	Loaded	0.125 ± 0.058 ^{ac}	67.5 ± 43.5 ^{bc}	4.42 ± 0.57 ^{ac}	0.048 ± 0.004 ^d	0.226 ± 0.033 ^c	190.6 ± 47.8 ^{ac}	822.0 ± 17.8 ^d	
	Non-Loaded	0.119 ± 0.056 ^{bc}	62.9 ± 35.3 ^{bc}	4.31 ± 0.49 ^c	0.046 ± 0.005 ^b	0.230 ± 0.029 ^c	185.0 ± 46.7 ^{bc}	809.4 ± 12.2	
High Freq.	Loaded	0.123 ± 0.049 ^c	68.2 ± 39.1 ^{bc}	4.33 ± 0.40 ^{bc}	0.048 ± 0.005	0.228 ± 0.022 ^c	186.7 ± 40.0 ^{bc}	810.5 ± 19.4	
	Non-Loaded	0.124 ± 0.049	70.8 ± 37.2 ^{bc}	4.37 ± 0.35 ^c	0.047 ± 0.005	0.224 ± 0.021	189.1 ± 40.4	810.3 ± 18.3	
Low Mass	Loaded	0.135 ± 0.036	76.6 ± 29.1 ^c	4.46 ± 0.39	0.049 ± 0.005	0.221 ± 0.023 ^d	197.2 ± 32.0	815.7 ± 23.8	
	Non-Loaded	0.132 ± 0.039	78.4 ± 31.4	4.36 ± 0.38 ^c	0.048 ± 0.005	0.227 ± 0.025 ^c	192.3 ± 35.4	813.7 ± 25.7	
Fixed Mass	Loaded	0.152 ± 0.066	93.0 ± 49.3	4.58 ± 0.68	0.050 ± 0.005 ^d	0.216 ± 0.039	209.1 ± 56.1	816.8 ± 20.9	
	Non-Loaded	0.151 ± 0.068	92.8 ± 51.6	4.65 ± 0.62	0.049 ± 0.006	0.213 ± 0.034	210.8 ± 58.0	808.9 ± 24.4	
Sham	Loaded	0.169 ± 0.063	105.6 ± 51.1	4.74 ± 0.59	0.051 ± 0.005	0.208 ± 0.028	226.9 ± 52.0	817.3 ± 22.3	
	Non-Loaded	0.167 ± 0.063	110.4 ± 53.3	4.72 ± 0.64	0.051 ± 0.006	0.210 ± 0.028	224.8 ± 53.7	818.5 ± 19.6	
Baseline Control	Right	0.169 ± 0.044	119.2 ± 40.7	4.88 ± 0.44	0.051 ± 0.003	0.198 ± 0.020	228.4 ± 35.2	821.2 ± 13.8	
	Left	0.165 ± 0.043	111.3 ± 30.3	4.82 ± 0.48	0.050 ± 0.004	0.201 ± 0.024	224.1 ± 36.8	817.0 ± 14.0	

^a p < 0.05 vs. Non-Loaded limb (paired t-test)

^b p < 0.05 vs. ipsilateral Sham

^c p < 0.05 vs. ipsilateral Baseline Control

Table 3

Bone formation indices of trabecular bone of the proximal tibial metaphysis

Group	Limb	MAR Day 5-15 ($\mu\text{m}/\text{day}$)	MAR Day 15-25 ($\mu\text{m}/\text{day}$)	MS/BS Day 5-15	MS/BS Day 5-25	BFR/BS Day 5-15 ($\mu\text{m}^3/\mu\text{m}^2/\text{day}$)	BFR/BS Day 5-25 ($\mu\text{m}^3/\mu\text{m}^2/\text{day}$)
High Strain	Loaded	0.761 ± 0.067	0.735 ± 0.096	0.202 ± 0.044^a	0.211 ± 0.050^d	0.148 ± 0.036^d	0.155 ± 0.041
	Non-Loaded	0.726 ± 0.102	0.709 ± 0.098^b	0.230 ± 0.031	0.243 ± 0.038	0.169 ± 0.031	0.172 ± 0.209
High Freq.	Loaded	0.764 ± 0.089	0.761 ± 0.069	0.200 ± 0.067	0.205 ± 0.080	0.162 ± 0.043	0.177 ± 0.039
	Non-Loaded	0.816 ± 0.103	0.733 ± 0.112^c	0.198 ± 0.069	0.197 ± 0.073	0.183 ± 0.033	0.163 ± 0.040^c
Low Mass	Loaded	0.719 ± 0.114	0.730 ± 0.105	0.198 ± 0.049	0.213 ± 0.059^c	0.151 ± 0.037	0.174 ± 0.055
	Non-Loaded	0.808 ± 0.143	0.822 ± 0.158	0.207 ± 0.062	0.215 ± 0.065	0.169 ± 0.041	0.187 ± 0.063
Fixed Mass	Loaded	0.729 ± 0.100	0.756 ± 0.098	0.215 ± 0.039	0.232 ± 0.041^c	0.156 ± 0.034	0.176 ± 0.037
	Non-Loaded	0.751 ± 0.120	0.789 ± 0.108	0.206 ± 0.027	0.219 ± 0.035^c	0.154 ± 0.028^b	0.172 ± 0.028
Sham	Loaded	0.767 ± 0.086	0.763 ± 0.119	0.215 ± 0.030	0.221 ± 0.039	0.165 ± 0.032	0.171 ± 0.039
	Non-Loaded	0.830 ± 0.180	0.821 ± 0.129	0.224 ± 0.031	0.235 ± 0.033	0.186 ± 0.052	0.195 ± 0.035

^a $p < 0.05$ vs. Non-Loaded limb (paired t-test)

^b $p < 0.05$ vs. ipsilateral Sham

^c $p < 0.05$ vs. Day 5-15 (paired t-test)

Table 4

Bone formation indices on the endocortical surface of diaphyseal cortical bone

Group	Limb	Cortical Thickness (mm)	MAR Day 5-15 ($\mu\text{m}/\text{day}$)	MAR Day 5-25 ($\mu\text{m}/\text{day}$)	MS/BS Day 5-15	MS/BS Day 5-25	BFR/BS Day 5-15 ($\mu\text{m}^3/\mu\text{m}^2/\text{day}$)	BFR/BS Day 15-25 ($\mu\text{m}^3/\mu\text{m}^2/\text{day}$)
High Strain	Loaded	0.153 ± 0.013	$0.895 \pm 0.260^{a,b}$	0.933 ± 0.187	0.246 ± 0.064	0.273 ± 0.057^c	0.227 ± 0.089	0.264 ± 0.064
	Non-Loaded	0.151 ± 0.012	0.644 ± 0.143^b	0.903 ± 0.264	0.257 ± 0.060	0.276 ± 0.054	0.175 ± 0.052	0.264 ± 0.099^c
High Freq.	Loaded	0.156 ± 0.012	0.780 ± 0.296	0.927 ± 0.248	0.229 ± 0.073	0.258 ± 0.091^c	0.190 ± 0.095	0.244 ± 0.105
	Non-Loaded	0.155 ± 0.014	0.661 ± 0.141^b	0.903 ± 0.317^c	0.258 ± 0.072	0.263 ± 0.066	0.177 ± 0.060	0.250 ± 0.110^c
Low Mass	Loaded	0.153 ± 0.011	0.757 ± 0.199	0.873 ± 0.269	0.246 ± 0.094	0.268 ± 0.102	0.196 ± 0.073	0.247 ± 0.097
	Non-Loaded	0.153 ± 0.008	0.909 ± 0.336	0.818 ± 0.164	0.247 ± 0.074	0.267 ± 0.076	0.279 ± 0.116	0.236 ± 0.072
Fixed Mass	Loaded	0.156 ± 0.013	$0.665 \pm 0.125^{a,b}$	0.893 ± 0.098^c	0.264 ± 0.102	0.281 ± 0.101	$0.189 \pm 0.067^{a,d}$	0.260 ± 0.085^c
	Non-Loaded	0.157 ± 0.014	0.866 ± 0.266	0.854 ± 0.244	0.272 ± 0.084	0.288 ± 0.106	0.250 ± 0.095	0.236 ± 0.081
Sham	Loaded	0.162 ± 0.011	0.815 ± 0.254	0.966 ± 0.221	0.263 ± 0.069	0.279 ± 0.070	0.235 ± 0.110	0.272 ± 0.095
	Non-Loaded	0.160 ± 0.012	0.938 ± 0.430	0.919 ± 0.169	0.236 ± 0.088	0.274 ± 0.084^c	0.222 ± 0.107	0.249 ± 0.078

^a $p < 0.05$ vs. Non-Loaded limb (paired t-test)

^b $p < 0.05$ vs. ipsilateral Sham

^c $p < 0.05$ vs. Day 5-15 (paired t-test)

Table 5

Morphological parameter determined from microCT scans

Group and Day	Limb	Metaphyseal Section					Diaphyseal Section			
		Bone Volume per Total Volume	Trabecular Thickness (mm)	Trabecular Number (1/mm)	Trabecular Separation (mm)	Trab. Bone Min. Density (mg HA/cm ³)	Total Area (mm ²)	Cortical Bone Area (mm ²)	Medullary Area (mm ²)	Cort. Bone Min. Density (mg HA/cm ³)
High Strain Day 0	Loaded	0.207 ± 0.070 ^d	0.064 ± 0.008	5.30 ± 0.75 ^d	0.192 ± 0.032	667.9 ± 31.1 ^d	1,565 ± 0.193 ^e	0.739 ± 0.112 ^{ae}	0.826 ± 0.227 ^{ae}	958.5 ± 21.3
	Non-Loaded	0.222 ± 0.071	0.066 ± 0.007	5.53 ± 0.73	0.187 ± 0.031	635.3 ± 33.8	1,580 ± 0.153 ^e	0.767 ± 0.099	0.813 ± 0.203	957.4 ± 11.9
High Strain Day 35	Loaded	0.128 ± 0.033 ^{bc}	0.057 ± 0.004 ^{bc}	4.62 ± 0.45 ^{bc}	0.224 ± 0.028 ^b	667.4 ± 29.3 ^d	1,527 ± 0.167 ^{bc}	0.635 ± 0.126 ^{abc}	0.892 ± 0.272 ^{abc}	948.5 ± 24.4
	Non-Loaded	0.134 ± 0.033 ^{bc}	0.057 ± 0.004 ^{bcd}	4.74 ± 0.63 ^b	0.221 ± 0.035 ^b	628.6 ± 17.8	1,539 ± 0.128 ^{bc}	0.665 ± 0.119	0.874 ± 0.234	960.9 ± 22.0
Sham Day 0	Loaded	0.215 ± 0.035	0.065 ± 0.005 ^d	5.42 ± 0.45	0.186 ± 0.018	664.0 ± 46.8 ^d	1,437 ± 0.181	0.773 ± 0.073 ^d	0.664 ± 0.213 ^d	966.4 ± 20.7
	Non-Loaded	0.227 ± 0.044	0.067 ± 0.005	5.46 ± 0.59	0.186 ± 0.021	637.1 ± 27.9	1,461 ± 0.196	0.795 ± 0.060	0.665 ± 0.226	961.5 ± 24.6
Sham Day 35	Loaded	0.152 ± 0.029 ^b	0.060 ± 0.004 ^b	4.79 ± 0.38 ^d	0.213 ± 0.021 ^b	660.2 ± 38.6 ^d	1,452 ± 0.187	0.726 ± 0.088 ^{bc}	0.726 ± 0.252 ^{bc}	955.3 ± 23.5
	Non-Loaded	0.151 ± 0.030 ^b	0.060 ± 0.003 ^{bc}	4.79 ± 0.40 ^b	0.215 ± 0.021 ^b	626.4 ± 19.5	1,454 ± 0.203	0.732 ± 0.096 ^{bc}	0.722 ± 0.274 ^b	967.6 ± 18.8
Age Matched Day 0	Right	0.227 ± 0.069	0.065 ± 0.005 ^d	5.65 ± 0.66	0.176 ± 0.021	675.9 ± 23.0 ^d	1,379 ± 0.171	0.823 ± 0.080	0.556 ± 0.095	955.7 ± 19.6
	Left	0.236 ± 0.078	0.067 ± 0.006	5.79 ± 0.79	0.172 ± 0.024	649.4 ± 29.9	1,401 ± 0.184	0.830 ± 0.083	0.571 ± 0.107	965.1 ± 19.6
Age Matched Day 35	Right	0.183 ± 0.053 ^b	0.062 ± 0.006 ^d	5.01 ± 0.39 ^b	0.199 ± 0.018 ^b	678.2 ± 25.1 ^d	1,372 ± 0.161	0.806 ± 0.062	0.566 ± 0.117	946.4 ± 16.6
	Left	0.185 ± 0.063 ^b	0.065 ± 0.007	5.19 ± 0.65 ^b	0.196 ± 0.027 ^b	623.2 ± 35.3 ^b	1,383 ± 0.166	0.812 ± 0.066	0.571 ± 0.114	959.8 ± 15.8

^a p < 0.05 vs. contralateral limb

^b p < 0.05 vs. day 0 ipsilateral

^c p < 0.05 vs. day 35 ipsilateral age matched

^d p < 0.05 vs. day 35 ipsilateral sham

^e p < 0.05 vs. day 0 ipsilateral age matched