Modulation of Bone's Sensitivity to Low-Intensity Vibrations by Acceleration Magnitude, Vibration Duration, and Number of Bouts

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SUMMARY

Variables defining vibration based biomechanical treatments were tested by their ability to affect the musculo-skeleton in the growing mouse. Duration of a vibration bout, but not variations in vibration intensity or number of vibration bouts per day, was identified as modulator of trabecular bone formation rates.

INTRODUCTION

Low-intensity vibrations (LIV) may enhance musculoskeletal properties but little is known regarding the role that individual LIV variables play. We determined whether acceleration magnitude and/or the number and duration of daily loading bouts may modulate LIV efficacy.

METHODS

LIV was applied for 3wk to 8wk old mice at either 0.3g or 0.6g, the number of daily bouts was one, two, or four, and the duration of a single bout was 15, 30, or 60min. A frequency of 45Hz was used throughout.

RESULTS

LIV induced tibial cortical surface strains in 4mo old mice of approximately 10με at 0.3g and 30με at 0.6g. In trabecular bone of the proximal tibial metaphysis, all single daily bout signal combinations with the exception of a single 15min daily bout at 0.3g (i.e., single bouts of 30min and 60min at 0.3g and 15min and 30min at 0.6g) produced greater bone formation rates (BFR/BS) than in controls. Across all signal combinations, 30min and 60min bouts were significantly more effective than 15min bouts in raising BFR/BS above control levels. Increasing the number of daily bouts or partitioning a single daily bout into several shorter bouts did not potentiate efficacy and

in some instances led to BFR/BS that was not significantly different from those in controls. Bone chemical and muscle properties were similar across all groups.

CONCLUSIONS

These data may provide a basis towards optimization of LIV efficacy and indicate that in the growing mouse skeleton, increasing bout duration from 15min to 30min or 60min positively influences BFR/BS.

KEYWORDS

Low intensity vibrations; bone formation; bone resorption, trabecular bone; cortical bone; muscle mass; mechanical strain

Introduction

High levels of physical activity can confer substantial benefits to the growing and young adult skeleton, aiding in the accretion of tissue and attenuating the erosion of surfaces, ultimately reducing fracture rates [1-4]. However, strenuous activities should not necessarily be *prescribed* to young untrained individuals as fracture rates in children may be directly related to physical activities [5]. As an alternative to high loads associated with exercise, the application of low-intensity vibrations (LIV) to the musculoskeleton as a means of raising bone mass without exercising per se has been suggested. In animal models, exposure of the developing musculo-skeleton to low intensity (<1g with ***g*** referring to Earth's gravitational acceleration) vibrations enhanced bone formation and bone strength, attenuated resorption, and augmented muscle cross-sectional area [6-8].

No large-cohort randomized prospective study has been completed but smaller clinical studies have been largely consistent with results from animal models. For instance, LIV was able to raise bone mass in young adult women and men as well as in postmenopausal women [9-11], disabled children [12, 13], or prevent bone loss in post-menopausal women [14]. While LIV exposure has been primarily associated with a positive skeletal response, several investigations did not detect treatment effects in animal [15] or clinical studies [16]. Further, comparisons between the magnitudes of the treatment effect reveal a large range [12, 14]. Presumably, at least some differences among study outcomes are linked to variables including age, species, genetics, gender, experimental duration, or experimental techniques while others may reflect specific differences in the applied mechanical signal.

The waveform used for vibrations is typically sinusoidal in nature and can be specified by the frequency (number of oscillations per second, Hz) and the magnitude of the induced peak acceleration (expressed typically as multiples of ***g*** as defined above). In both animal and clinical vibration studies, the most commonly utilized frequencies range from 15Hz to 90Hz. Variability in the applied peak acceleration has even been greater with differences larger than two orders of magnitude (from 0.1g to about 30g). Other variables defining a loading protocol include the duration of a loading session [6, 9] and the number of loading sessions per day [17].

Manipulation of these variables may augment LIV efficacy but little is known about their individual roles in orchestrating the biologic response and even less is known about interactions between vibration variables. In contrast to bone adaptation experiments using lower-frequency mechanical signals (<10Hz), mechanotransduction of vibrations is not tuned to the magnitude of the induced matrix deformation or one of its byproducts like fluid shear [18, 19]. For instance, exposure to 0.3g vibrations can be less effective than 0.1g vibrations in increasing trabecular bone volume in a murine model [20]. Different vibration frequencies have rarely been directly compared to each other and at least in the rodent musculoskeleton may have a preference for 90Hz over 45Hz vibrations [21-23].

Because of the large number of vibration variables including their potential interactions that may play a role in modulating bone's response, an exhaustive in vivo optimization study using a full factorial design is impractical. Here, in an effort towards identifying variables that can enhance the response of the growing skeleton to LIV, we hypothesized that increasing the number of daily vibration bouts [17] and partitioning a single daily bout into multiple discrete

bouts interspersed with rest [24], but not bout duration [9] or vibration magnitude [20], will modulate the LIV induced increase in bone formation rates.

Methods

Experimental Design and Mice

Experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Stony Brook University. One hundred and thirty 6-week-old male BALB/cByJ (BALB) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). At 8wk of age, mice were randomly divided into 12 groups comprising nine experimental groups, one baseline control group and two age-matched (sham) control groups. At 8wk of age, mice are reproductively mature but bone elongation is still occurring and cortical bone in particular is still expanding [7]. The signal frequency of the vertical whole body vibration that each of the nine experimental groups was exposed to was identical (45Hz) while the peak acceleration magnitude of the LIV signal (either 0.3g or 0.6g), the number of bouts per day (one, two, or four), and the duration of each bout (15min, 30min, or 60min) was varied. The levels of each LIV variable (# bouts, bout duration, g) were selected within a relatively narrow range that reflected use of these variables in previous clinical and animal studies, practicality, and potential safety issues. Experimental duration was 3wk for all groups except baseline controls (BC, n=5) which were sacrificed at 8wk of age. Sample size for BC mice was relatively small because its purpose was to provide basic information on trabecular, cortical, and muscle variables at baseline and was not intended for direct comparisons with experimental groups. It was confirmed that average body mass was similar between all groups at start of the study.

The nine experimental groups comprised: **(1-3)** 0.3g, 15min/bout for either 1 bout/d (0.3x15x1, n=11), 2 bouts/day (0.3x15x2, n=11), or 4 bouts/day, (0.3x15x4, n=12), **(4-5)** 0.3g, 30min/bout, for either 1 bout/d (0.3x30x1, n=12) or 2 bouts/day (0.3x30x2, n=11), **(6)** 0.3g, 60min/bout, 1 bout/d (0.3x60x1, n=12), **(7-8)** 0.6g, 15min/bout, for either 1 bout/d (0.6x15x1, n=11) or 2 bouts/day (0.6x15x2, n=11), and **(9)** 0.6g, 30min/bout, 1 bout/d (0.6x30x1, n=11) (**Table 1**). For regimes with 2 bouts/d, bouts were separated by 6h; 4 bouts/d were separated by 3h each. All regimes were applied 5d/wk with two consecutive rest days. Age-matched control mice were handled similarly to experimental mice and exposed to an inactive vibrating plate either once a day for 15min (0x15x1, n=12) or twice a day for 30min (0x30x2, n=12) (**Table 1**). All mice in a given group received LIV (or sham LIV for controls) at the same time with up to four plastic containers placed on the vertically oscillating plate, each container housing up to five mice (LxWxH, 35x24x16 cm). During treatment, mice were allowed to freely roam the container.

When not engaged in the mechanical intervention, all mice were single-housed in regular mouse cages (22°C) and exposed to 12h light/dark cycles with ad libitum access to water and standard rodent chow (LabDiet RMH 3000, LabDiet, St. Louis, MO). To enable measurement of dynamic indices of bone formation, mice were injected (i.p., 0.5ml) with calcein (15mg/kg) dissolved in phosphate buffered saline on days 15 and 20 of the experimental protocol with the last injection 48h prior to sacrifice. Mice were euthanized by carbon dioxide inhalation.

Tissue Preparation

After the 3wk experimental duration, the right tibia was harvested and submerged in 70% ethanol for micro-computed tomography (μCT) and histomorphometry. Length of the right tibia

was measured with digital calipers. The left tibia was transected at the proximal and distal diaphysis (Buehler Isomet Slow Speed Saw) and fixed overnight in 10% neutral buffered formalin for staining of osteoclastic resorption via tartrate resistant acid phosphatase (TRAP) [6]. The right soleus was harvested and pinned to a wooden rod in an attempt to retain the original in vivo muscle length. The pinned muscle specimens were embedded in TBS tissue freezing medium (Triangle Biomedical Sciences, Durham, NC), snap frozen in cooled isopentane with liquid nitrogen, and stored in an isopentane-filled Eppendorf tube at -80°C until sectioning [25]. All analyses described below were performed by a single operator who was blinded to the identity of the samples.

Bone Strain Measurements

To estimate differences in the strain environment of the tibia between the 0.3g and 0.6g mechanical signal, cortical surface bone strains generated in the proximal tibia were measured in two additional BALB mice. Because of the small size of the mouse tibia at 8wk of age, young adult (4mo old) mice were used. It is entirely possible that the strains recorded in these older mice do not reflect the absolute values of peak strains induced in 8-10wk old mice but the main purpose of strain gaging these mice was to get an approximate estimate for the relative change in strain magnitude when the magnitude of the LIV acceleration is raised from 0.3g to 0.6g (and to relate this change in strain magnitude to the difference in bone formation between mice subjected to 0.3g and 0.6g LIV signals). Under isoflurane anesthesia, a miniature single-element strain gage (1mm gage length, 120Ω, TML Gages, Kenkyujo, Japan) was glued (cyanoacrylate) on the antero-medial surface of the proximal tibia [6]. Upon recovery from surgery (1-2h), and with

the animal standing on the vibrating plate, strain data were collected over two 10sec trials. Strain gage signals were amplified (SX500, Syminex Inc, Mt. Arlington, NJ) with an excitation of 4V and a 1000x gain, and acquired at a sampling rate of 1000Hz. This setup collected in vivo strain data at a resolution of approximately 0.5 microstrain [6, 26]. Strain data were plotted and the amplitude of the oscillatory strain reading was determined.

Microcomputed Tomography

We scanned 5mm of the right proximal tibia of all mice at a voxel size of 6μm (55kV, 145mA, 250ms integration time, 1000 projections) on a μCT 40 (Scanco Medical, SUI). The diaphysis was scanned at 12μm voxel size using the same scan parameters. The trabecular VOI in the proximal metaphysis was 600μm in length starting 500μm distal of the μCT slice in which primary spongiosa (non-resolvable as separate struts) separates at the center of a tibial cross-section (most distal point of the growth plate). Trabecular bone was manually separated from cortical bone with contour lines drawn about 50μm from the endocortical surface, marking the outside boundary of the VOI. Cortical bone was analyzed from the metaphysis (surrounding the trabecular VOI) as well as the middiaphysis (defined at 50% of tibial length comprising 10 slices). Global thresholds were determined, one each for trabecular and cortical bone, by visual comparisons between segmented and raw grey-scale images from at least two mice per group until the segmented images matched the morphology of the grey-scale images.

For trabecular regions, bone volume fraction (BV/TV), connectivity density (Conn.D), trabecular thickness (Tb.Th), and trabecular number (Tb.N) were determined. For cortical bone, total area (Tt.Ar), bone area (Ct.Ar), and bone marrow area (Ma.Ar) were calculated.

Histomorphometry

Following tomographic scanning, the right proximal and diaphyseal tibia were cut (Buehler Isomet Slow Speed Saw, Lake Bluff, IL) and embedded in methyl methacrylate resin (MMA) using a standard protocol [6]. The proximal specimens were sectioned longitudinally in the center to yield 5µm frontal sections (RM 2165 Microtome, Leica, Bensheim, Germany) while diaphyseal samples were sectioned at midpoint (40µm thick) with a diamond wire saw (Well Diamond Wire Saws, Norcross, GA). The evaluated regions in the metaphysis and diaphysis were similar to the regions scanned by µCT. The trabecular ROI spanned 800µm in the secondary spongiosa of the metaphysis starting 400µm distal from the border of the growth plate. Because of a lack of consistent double labels at the endocortical surface of the middiaphysis and the periosteal surface of the metaphysis, these surfaces were excluded from the analysis. Bone formation rates (BFR/BS, $\mu m^3/\mu m^2/yr$) were calculated as the product of mineral apposition rate (MAR, µm/day) and mineralizing surface (MS/BS, %, calculated as the sum of percent double label and ½ x percent single label) by histomorphometric software (Osteomeasure, OsteoMetrics Inc., Atlanta, GA). Standard nomenclature was adopted [27].

TRAP Staining

Tartrate resistant acid phosphatase (TRAP), an indicator of osteoclastic resorptive by-products, was used to estimate trabecular bone resorption *in situ* [28]. The left proximal tibia was fixed fresh in 10% neutral buffered formalin overnight and decalcified in 2.5% formic acid (pH 4.2) for 4d. Upon dehydration, samples were embedded in glycol methacrylate (GMA) according to the

manual of the JB-4 embedding kit (Polysciences, Warrington, PA). Frontal sections were cut (7µm) and stained for TRAP activity. Hexazotization was achieved by mixing equal amounts of 4% $NaNO_2$ and 4% pararosaniline solutions. Naphthol-ASTR-phosphate (Sigma, St. Louis, MO) was used as a substrate and the enzyme reaction was carried out in the presence of tartrate (10mM) to demonstrate TRAP activity (pH 5 in 0.1 M acetate buffer). Sections were counter-stained with methyl green to improve contrast. The ratio of trabecular osteoclast surface (Oc.S) to bone surface (BS) was determined (Osteomeasure) in a region that matched the histomorphometric analysis.

Chemical Composition

A critical requirement of any effective biochemical or mechanical intervention is that the bone which is formed as a result of the treatment is of high quality. To test whether LIV-induced bone formation was chemically different from bone forming in control mice, synchrotron infrared microspectroscopy was performed on newly formed bone enclosed by the double calcein labels as described previously [29]. To this end, chemical measurements were taken from five 12x12 µm spectra within two randomly selected cortical and trabecular regions each. Ratios of phosphate-to-protein, carbonate-to-protein, carbonate-to-phosphate, collagen cross-linking, acid phosphate-to-total phosphate, and crystallinity were compared between age-matched controls (n=8 randomly selected) and the 0.3x15x2 group (n=7 randomly selected), as a representative LIV group in which bone formation rates were significantly up-regulated by vibrations.

Muscle Histology

Prior to sectioning, each soleus was re-embedded and snap frozen in Tissue-Tek OCT [25]. Multiple frozen transverse sections were cut (8μm) from the mid-belly region with a cryostat at -20°C (CM3050S microtome, Leica, Bensheim, Germany). To evaluate potential changes in myofibers, myocyte myosin ATPase activity was stained histochemically by previously verified methods to classify slow- and fast-twitch fibers. Pre-incubation (pH 10.4) inactivated the myosin-ATPase enzyme in type I fibers (slow) and the remaining active ATPase enzyme in type II fibers (fast) produced a black insoluble compound. Cross-sectional images of the soleus were taken under a light microscope with a 4X objective (Axiovert 200M, Zeiss, Germany). Muscle cross-sectional areas and numbers of type I, type II, and total muscle fibers were evaluated with Image J (NIH, Bethesda, MD).

Statistical Analysis

All data were expressed as mean±SD. A total of 23 variables were tested but the principal outcome variables were trabecular BFR/BS and total muscle cross-sectional area. First, an unpaired t-test showed that there were no significant differences between the two sham-LIV groups, 0x15x1 and 0x30x2, for any outcome variable and these two groups were pooled into a single age-matched control group (AC) to which LIV groups were compared to. Second, the BC and AC groups were compared with an unpaired t-test to determine normal growth related changes in outcome measures occurring during the 3wk period. Third, three separate one-way ANOVAs were performed. The first ANOVA involved ten groups, the AC group and the nine LIV groups. When a one way ANOVA for a variable was significant, a Student-Newman-Keuls (SNK)

post-hoc test assessed differences between AC and any given LIV group. When a one way ANOVA for a variable was significant, two additional one-way ANOVA were performed to test for significant differences among control and 0.3g LIV groups (AC, 0.3x15x1, 0.3x15x2, 0.3x15x4, 0.3x30x1, 0.3x30x2, 0.3x60x1) and among control and 0.6g LIV groups (AC, 0.6x15x1, 0.6x15x2, 0.6x30x1). Finally, three-way ANOVA was used to test whether acceleration magnitude, bout duration, and number of bouts had main effects and interactions on BFR/BS, the principal outcome variable for bone in this study.

In exploratory studies like this that seek to generate new hypotheses, it is typical not to adjust for Type I errors that might occur due to use of multiple outcome variables [30]. Further, while we present a large number of outcome variables, we stress that the principal outcome variables relied upon were trabecular BFR/BS for bone and total cross-sectional area for muscle. Statistical significance was set at 0.05 throughout (SPSS 22.0, IBM, New York, NY).

Results

Strain Magnitudes at 0.3g versus 0.6g

The accelerometer attached to the vibration plate confirmed the sinusoidal LIV pattern of the vertically oscillating vibration plate (**Fig. 1A**). Concurrent *in vivo* recordings from a strain gage attached to the cortical tibial metaphysis demonstrated transmissibility of the mechanical signal into the tibia as indicated by sinusoidal strain patterns of the same frequency (**Fig. 1B**). Standing on an inactive plate induced a quasi-static compressive strain of approximately 70με in magnitude (**Fig. 1B**). Superimposed upon the strain level associated with weightbearing, LIV applied at a frequency of 45Hz and 0.3g induced dynamic peak bone strains of approximately

10με at the antero-medial surface of the proximal tibia (**Fig. 1B**). Doubling the peak acceleration to 0.6g raised dynamic peak strain magnitudes to approximately 30με (**Fig. 1B**).

Animals and Changes in Outcome measures during the 3wk Period in Control Mice

One mouse in the 0.3x15x2 group died of reasons unrelated to the protocol, reducing this group from n=11 to n=10. Changes occurring through normal development in the growing male BALB mouse were inferred by comparing 8wk-old baseline controls to 11wk-old age-matched controls. During the 3wk duration of the protocol, body mass increased by 19% ($p<0.05$) and the tibia extended its length by 5% ($p<0.05$) (**Table 2**). In the trabecular metaphysis, BFR/BS decreased by 57%, associated with a 37% drop in MAR and a 29% decrease in MS/BS (all $p<0.05$, **Table 2**). The prevalence of osteoclastic activity was not different between BC and AC mice (**Table 2**). Trabecular bone volume fraction (BV/TV) was 27% greater ($p<0.05$) at the end of the experimental period (**Fig. 2**), caused primarily by 17% thicker trabeculae ($p<0.05$, **Table 2**). At the endocortical surface of the metaphysis, BFR/BS declined by 50% ($p<0.05$, **Fig. 3)**, resulting from a 42% ($p<0.05$) decrease in mineral apposition rate and a 13% ($p<0.05$) decrease in mineralizing surfaces (data not shown). At the periosteal surface of the middiaphysis, similar reductions in BFR/BS (46%, $p<0.05$, **Fig. 3**) and MAR (31%, $p<0.05$, data not shown) but not MS/BS (data not shown), were observed. While growth did not significantly affect cortical μCT outcome variables in the metaphysis, cortical bone area increased by 5% in the middiaphysis ($p<0.05$, **Table 5**). There were also no differences in any histologic index of the soleus muscle between baseline and age-matched controls (**Table 4**).

Body Mass, Tibial Length, Chemical Properties, Muscle

There was no significant difference in body mass and tibial length between groups at 11wk of age (**Table 2**). There were also no differences in bone chemical properties when comparing newly formed trabecular and cortical bone between age-matched controls and a LIV group (0.3x15x2) in which the intervention significantly increased BFR/BS (**Table 3**). No differences in muscle histological properties, including fiber diameter, number, and type were detected between age-matched and experimental groups (**Table 4**).

Bone Formation (trabecular)

Every single mouse showed double labels in trabecular bone of the metaphysis. Using a three-way ANOVA on trabecular BFR/BS (**Table 2**), the principal outcome variable, bout duration but not acceleration magnitude or number of bouts had a significant main effect across all LIV groups with mice subject to 15min-bouts having lower trabecular BFR/BS than mice treated with 30min or 60min bouts. There was no difference between 30min and 60min bouts. There were also no significant interactions among the three factors. Trabecular BFR/BS results below were organized by hypotheses regarding the effects of individual LIV variables.

<u>Bout duration</u>

We first examined LIV duration for single bouts at 0.3g and 0.6g. At a LIV amplitude of 0.3g applied for 15min once a day (0.3x15x1), no difference in BFR/BS was detected when compared to the normal control group. However, increasing the duration of the bout to 30min at the same amplitude (0.3x30x1) led to 83% ($p<0.05$) greater BFR compared to controls (**Fig. 4A, Table 2**).

Extending the duration further to 60min (0.3x60x1) provided a similar benefit (88%, $p<0.05$) for BFR/BS to that provided by 30min (**Fig. 4A, Table 2**). At an acceleration amplitude of 0.6g and a bout duration of 15min (0.6x15x1), BFR/BS was 58% ($p<0.05$) greater than in controls (**Fig. 4A, Table 2**). Extending the duration to 30min per bout (0.6x30x1) yielded an 84% ($p<0.05$) difference to AC (**Fig. 4A, Table 2**). Differences between individual LIV regimes were not significant.

Number of daily bouts

We then considered whether increasing the number of vibration bouts per day exerts a positive effect on BFR/BS in trabecular bone of the metaphysis at 0.3g and 0.6g. Using 15min per bout at 0.3g, a signal that did not raise BFR/BS when applied as a single bout per day (**Fig. 4A, Table 2**), doubling bout number to two bouts/d (0.3x15x2) produced 62% ($p<0.05$) greater BFR/BS compared to AC (**Fig. 4B, Table 2**). Further increasing the number of bouts to four per day (0.3gx15minx4) yielded BFR/BS that were not significantly different from AC (**Fig. 4B, Table 2**). A similar nonlinearity was observed for 0.6g vibrations applied for 15min; while one bout per day of this signal significantly raised BFR/BS over control levels, two bouts rendered this signal ineffective (**Fig. 4B, Table 2**). For the 30min per bout 0.3g signal, both one and two daily 30min bouts produced BFR/BS that were 85% greater ($p<0.05$) than in controls (**Fig. 4B, Table 2**). No significant differences between individual LIV groups were detected.

Partitioning a single daily bout into multiple daily bouts

Lastly, we investigated the effect of splitting a given LIV duration delivered as single bout into several bouts per day. At 0.3g, a daily vibration duration of 60min caused increased BFR/BS when

provided as a single bout (**Fig. 4A, Table 2**). Dividing this one bout of 60min into two daily bouts of 30min LIV (0.3x30x2) produced BFR/BS that were significantly different from that of controls (**Fig. 4C, Table 2**). Further dividing the 60min vibration bout into four daily bouts of 15min (0.3x15x4) produced BFR/BS that were not significantly different from controls (**Fig. 4C, Table 2**). The one-bout vibration regimes of 0.3g applied for 30min and 0.6g applied for 30min had similarly elevated BFR/BS as the 0.3g 60min regime described above. Partitioning the 30min 0.3g and 0.6g regimes into two discrete bouts of 15min yielded BFR/BS for 0.3x15x2 that was significantly greater than in AC, while BFR/BS for 0.6x15x2 was indistinguishable from AC (**Fig. 4C, Table 2**). BFR/BS in 0.6x30x1 mice was significantly greater than in 0.6x15x2 mice. All other LIV groups within the 0.3g and 0.6g series were not significantly different from each other.

Mechanically induced greater BFR/BS in LIV groups when compared to controls was a result of either osteoblasts producing tissue faster, as indicated by increased MAR, or a consequence of more surfaces with osteoblasts, as indicated by an increase in MS/BS (**Table 2**). Not a single LIV group showed concomitant increases in MAR *and* MS/BS.

Bone Resorption (trabecular)

None of the six regimes using 0.3g had a significant influence on trabecular Oc.S/BS (**Table 2**). Among the 0.6g regimes, one daily bout of 15min (0.6x15x1) caused 35% greater ($p<0.05$) Oc.S/BS compared to AC without significant differences between individual LIV groups (**Table 2**).

Bone Quantity and Architecture (trabecular)

Bone morphology in the trabecular metaphysis of mice exposed to 0.3g, one bout/d for 15min (0.3x15x1) was not different from age-matched controls (**Table 2**, **Fig. 2**). However, increasing bout number to two bouts per day while maintaining acceleration amplitude (0.3x15x2) was associated with greater trabecular BV/TV (16% over AC, $p<0.05$, **Fig. 2**) and greater Tb.N (9% over AC, $p<0.05$, **Table 2**). Increasing the number of bouts per day to four yielded a trabecular structure that was comparable to controls. Similarly, one daily bout of 30min (0.3x30x1) had no significant influence on bone morphology while increasing the number of daily bouts to two (0.3x30x2) raised BV/TV by 12% over controls ($p<0.05$, **Fig. 2**). At 0.6g, one LIV bout of 15min/d (0.6x15x1) gave rise to 16% ($p<0.0.5$) greater BV/TV over controls. Experimental groups within the 0.3g and 0.6 series were not significantly different from each other.

Bone Formation (cortical)

All mice displayed double labels at the endocortical surface of the metaphysis and the periosteal surface of the middiaphysis.

<u>BFR/BS in the cortical metaphysis</u>

At the endocortical surface of the metaphysis, one daily bout of 15 min at 0.3g (0.3x15x1) had no significant influence on endocortical bone formation rates (**Fig. 3A**), while two bouts per day (0.3x15x2) increased BFR/BS by 36% ($p<0.05$) over AC. Similar to trabecular bone, increasing bout number from two to four yielded BFR/BS level that were not different from those in AC. One daily (0.3x30x1) and two daily bouts (0.3x30x2) of 30min increased BFR/BS by 36% ($p<0.05$) each. One daily vibration bout of 15min at 0.6g (0.6x15x1) increased BFR/BS by 34% compared to controls

(p<0.0.5) (**Fig. 3A**). None of the other experimental groups displayed BFR/BS at the endocortical surface of the metaphysis that were either significantly different from controls or any given experimental group.

BFR/BS in the middiaphysis

At the periosteal surface of the middiaphysis, exposure to 0.3g vibrations for one, two, three, or four daily 15min bouts failed to influence bone formation rates compared to AC (**Fig. 3B**). Similarly, 30min or 60min of single daily 0.3g bouts were ineffective. In contrast, partitioning the single 0.3g 60min bout into two (0.3x30x2) produced 73% greater (p<0.05) BFR/BS than in AC (**Fig. 3B**). For 0.6g LIV signals, two bouts of 15min each (0.6x15x2) significantly increased BFR/BS over controls (114%, p<0.05) as well as over 0.6x15x1 and 0.6x30x1 regimes (**Fig. 3B**).

Cortical Bone Area and Geometry

In the metaphysis, mice subjected to two bouts of 30min LIV at 0.3g (0.3x30x2) had a 7% greater (p<0.05) periosteal area and 13% greater (p<0.05) bone marrow area than controls (**Table 5**). In 0.3x30x2 mice, these two measures were also greater than in 0.3x30x1 and 0.3x60x1 mice. Two bouts of 15min at 0.6g (0.6x15x2) were associated with an 8% greater (p<0.05) cortical bone area, a LIV response that was significantly different from 0.6x30x1 mice (**Table 5**). In the middiaphysis, the only intervention that altered cortical bone properties with respect to controls were two bouts of 30min at 0.3g (0.3x30x2), resulting in 8% greater (p<0.05) total bone area (**Table 5**). No significant differences between individual groups were detected.

Discussion

We evaluated variables that may modulate the sensitivity of the growing musculoskeleton to LIV regimes and tested whether bone formation rates in the growing skeleton benefit from increasing the length of a bout, increasing the number of daily bouts of a given duration, partitioning one long daily bout into several discrete bouts, or doubling vibration magnitude. Three-way ANOVA across all LIV groups indicated that 30min and 60min LIV regimes produced trabecular bone formation rates in the proximal metaphysis of the tibia that were greater than for 15min regimes. In contrast, the number of LIV bouts per day (1, 2, or 4) or acceleration magnitude (0.3g or 0.6g) was not a modulator of metaphyseal trabecular bone formation rates. In metaphyseal and diaphyseal cortical bone, differences between individual regimes were more subtle but the longest duration applied over a single session was ineffective for both acceleration magnitudes. Soleus muscle morphology, bone's chemical properties, and bone resorption were predominantly unaffected by any of the LIV treatments.

Trabecular bone formation rates of most LIV schemes were not significantly different between each other even though some signals induced significantly greater BFR/BS than seen in controls while other signal combinations did not. Overall integration of results from varying LIV magnitude, duration, and number of bouts demonstrated a pattern that is distinct from the dose-response behavior commonly observed when skeletal tissues are exposed to low-frequency mechanical signals [31-33] extending results from previous in vivo [20, 34] and in vitro [19, 35] investigations that did not find linear associations between vibration acceleration/frequency and the cellular response. Identification of the differences in mechanotransduction between low-

and high-frequency mechanical signals may ultimately provide important clues towards a comprehensive optimization of LIV variables.

Because of the large number of variables defining a vibration regime [36], it is not practical to include all variables in a single study. For instance, the frequency of the signal was not varied here. This had the benefit of not introducing a potentially confounding variable through the intricate relation between signal frequency, bout duration, and cycle number but it needs to be considered that frequency (e.g., 45Hz vs 90Hz) can have powerful effects on the efficacy of low-intensity vibrations in the musculoskeletal system [21, 23]. We included two sham age-matched control groups but not six, the necessary number of groups to control for all combinations of number of bouts and bout durations. Therefore, we cannot exclude the possibility that a potential stress response to handling or interactions with other mice in the vibration container may have affected our results. Lastly, the 3wk experimental protocol was chosen as short enough to allow for effective quantification of changes in bone formation rates and long enough that changes in bone morphology could be detected if bone's response to the individual signals (and differences between them) was strongly anabolic. Our data suggest that, similar to previous LIV studies in the growing skeleton, protocol duration will need to be extended before greater structural changes can be observed [6, 7]. Thus, changes in bone quantity and geometry/architecture were considered only a secondary outcome measure here.

Dynamic bone strain magnitudes increased from approximately 10με to 30με when LIV acceleration was raised from 0.3g to 0.6g. While these data primarily provided an estimate of the relative difference in dynamic strain magnitudes as a function of acceleration magnitude and accurate tibial peak strains in a 8wk old mouse may be different [37], deformations were two

orders of magnitude below levels that may induce damage in the bone matrix and at least one order of magnitude below previously proposed anabolic strain thresholds [38]. The lack of differences between the higher strain 0.6g signals and the lower strain 0.3g signals emphasizes results from in vitro [18, 35] and in vivo [20, 21] studies that suggested the mechanism by which bone senses LIV is not primarily dependent on bone strain magnitude.

Splitting a single mechanical loading session into discrete bouts that are distributed over the event of a day has been shown to enhance the efficacy of high-magnitude mechanical signals applied at low (<10Hz) frequencies [39]. While cells subjected to LIV in culture may exhibit a similar phenomenon [17], we did not observe enhanced trabecular bone formation when bouts were partitioned. In contrast, a single session of 0.6gx30min provided greater trabecular bone formation than in controls but distributing this regime over two bouts engendered bone formations rates that were significantly smaller and similar to controls even though total duration and number of loading cycles were unchanged. The cause for these incongruent results may lie with a different mechanosensory mechanism by which low- and high-frequency mechanical signals are perceived in the skeleton and the different environment of cells in vitro versus in vivo. Using the same mouse model, we previously found that the inclusion of short rest periods (10-15sec) after every 1 or 15 seconds of LIV renders the stimulus less effective [6, 7] even though incorporation of short rest periods has been a successful strategy for potentiating the cellular response to low-frequency mechanical regimes in vivo [40]. Together, these data suggest that the efficacy of LIV is dependent on the temporal continuity of a relatively large number of loading cycles with 30-minute (81,000 cycles) and 60-minute bouts (162,000 cycles) being more efficacious than 15min bouts.

Skeletal and muscular systems are interdependent and muscle-bone interactions have recently received considerable attention. Generally, morphologic adaptations of muscle to altered mechanical environments precede the adaptation of bone which often has been construed as evidence that bone responds to forces generated by muscle rather than those transferred as ground reaction forces [41]. Given that the mouse soleus can readily respond to 6wk of LIV treatment [7], the lack of changes in muscle histology in response to any of the 3wk LIV regimes may suggest that muscle forces are not necessary for bone to sense LIV [36]. Regardless, both muscle and bone can respond to LIV [9] and understanding the mechanisms and perhaps interaction by which these two tissues may benefit from LIV will aid in the development of integrated musculoskeletal mechanical countermeasures.

The principal aim of this study was to determine LIV variables that may augment bone accretion during growth, the most opportune period to increase skeletal strength with exercise [42]. Inherently, results from this study may not apply to adult bone with largely quiescent surfaces. We found bout duration to be the most robust modulator of trabecular bone formation rates while number of daily bouts and acceleration magnitude had no significant influence across LIV signals considered here. Bone resorption and muscle morphology were largely unaffected by the LIV regimes and new bone formed during LIV retained its chemical quality. Our data may provide a basis for designing LIV regimes with greater efficacy but a potential non-linearity of the biologic response to LIV duration, number of daily bouts, and magnitude may need to be considered.

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Tables

Table 1. Overview of experimental groups and daily exposure schedule.

Group		9am	10am	11am	12pm	1pm	2pm	3pm	4pm	5pm	6pm
0g	1x15min (n=12)										
AC	2x30min (n=12)										
0.3g	1x15min (n=11)										
	2x15min (n=11)										
	4x15min (n=12)										
	1x30min (n=12)										
	2x30min (n=11)										
	1x60min (n=12)										
0.6g	1x15min (n=11)										
	2x15min (n=11)										
	1x30min (n=11)										

The width of each black bar is proportional to the loading duration. The two sham age-matched-control groups (AC) were exposed to an inactive (0g) plate. Sham treatments are presented by grey bars.

Table 2. Body mass and trabecular histomorphometric/architectural endpoints (mean±SD).

	Body Mass (g)	*Length (mm)*	*Oc.S/BS (%)*	*MAR (μm/d)*	*MS/BS (%)*	*BFR/BS ($μm^3$/$μm^2$/yr)*	*Conn.D ($1/mm^3$)*	*Tb.N (1/mm)*	*Tb.Th (μm)*
BC	23.2±0.9*	15.9±0.6*	16.6±3.8	1.8±0.1*	17.6±3.0*	114±24*	309±54	6.4±0.3	37.1±1.1*
AC	27.5±1.2	16.8±0.5	17.1±3.9	1.1±0.2	12.4±5.4	49±19	301±65	6.3±0.5	43.2±2.1
0.3x15x1	27.7±1.3	16.6±0.5	20.4±4.0	1.5±0.3*	11.6±4.0	63±23	311±65	6.3±0.5^{-}	44.4±1.1
0.3x15x2	27.5±0.8	16.8±0.4	20.1±6.6	1.6±0.3*	13.4±3.0	79±21*	370±70	6.8±0.3*	43.3±3.0
0.3x15x4	26.7±1.2	16.9±0.3	19.8±6.0	1.1±0.2†	15.7±3.6	63±21	342±79	6.5±0.6	43.6±1.3
0.3x30x1	26.6±1.3	16.6±0.5	16.9±7.0	1.3±0.3†	18.9±4.9*†	90±38*	291±68	6.1±0.5^{-}	43.8±3.5
0.3x30x2	28.4±1.4	17.1±0.3	19.9±6.0	1.2±0.2†	18.8±5.6*†	90±34*	359±21	6.6±0.3*$^{\#}$	44.0±2.5
0.3x60x1	26.5±1.8	16.5±0.6	20.8±6.0	1.2±0.3†	20.7±6.6*†	92±38*	306±28	6.3±0.3^{-}	44.1±1.9
0.6x15x1	27.6±1.7	16.7±0.3	23.1±6.1*	1.6±0.4*$^{+}$	13.3±2.5^{+}	77±21*$^{+}$	343±40	6.6±0.3	44.3±1.7
0.6x15x2	28.0±1.3	16.6±0.5	17.3±6.5	1.6±0.4*$^{+}$	11.2±2.4^{+}	63±16^{+}	336±61	6.3±0.4	43.8±2.7
0.6x30x1	27.1±1.1	16.8±0.3	17.9±4.5	1.2±0.1	21.2±3.9*	96±21*	318±61	6.4±0.5	44.3±2.1

**: Different from AC*
$^{+}$: Different from 0.6x30x1
†: Different from 0.3x15x1 and 0.3x15x2
$^{-}$: Different from 0.3x15x2
$^{\#}$: Different from 0.3x30x1

Table 3. Chemical properties (mean±SD) of newly mineralized bone in trabecular and cortical bone of the tibial metaphysis in age-matched control and a vibrated group that showed significant increases in bone formation rates (0.3x15x2).

	Trabecular		***Cortical***	
	AC *(10^{-3})*	*0.3x15x2* *(10^{-3})*	*AC* *(10^{-3})*	*0.3x15x2* *(10^{-3})*
Phosphate/Protein	540±185	520±110	626±181	638±215
Carbonate·Protein	66±12	68±7	70±18	68±15
Carbonate·Phosphate	136±49	141±27	116±47	114±29
Acid Phosphate·Total Phosphate	3.7±0.9	3.7±0.8	3.9±0.8	3.7±1.0
Crystallinity	985±76	1044±105	815±100	818±129
Collagen Cross-Linking ratio	1548±184	1604±158	1655±128	1705±172

Table 4. Muscle histologic properties in the soleus (mean±SD).

	Total area (mm^2)	*Total fiber number*	*Type I number*	*Type II number*	Type-I/Type-II (%)
BC	0.99±0.18	627±102	165±35	441±70	35.8±3.8
AC	0.99±0.22	641±125	171±37	470±101	36.9±7.7
0.3x15x1	0.91±0.23	616±160	157±39	457±116	36.5±7.0
0.3x15x2	0.96±0.23	690±97	182±30	508±80	36.3±5.6
0.3x15x4	1.15±0.27	700±121	181±24	518±100	35.6±4.4
0.3x30x1	1.12±0.26	682±62	190±36	492±53	39.1±8.6
0.3x30x2	1.01±0.23	683±100	174±28	508±83	34.8±6.0
0.3x60x1	0.98±0.20	685±117	181±32	507±93	36.8±6.1
0.6x15x1	0.96±0.23	630±160	172±52	458±114	37.3±7.3
0.6x15x2	0.88±0.19	638±74	165±30	473±65	35.4±7.6
0.6x30x1	0.96±0.13	711±85	181±34	520±71	37.2±4.5

Table 5. Cortical bone properties of the metaphysis and diaphysis (mean±SD).

	Metaphysis			*Diaphysis*		
	Tt.Ar (mm^2)	Ct.Ar (mm^2)	Ma.Ar (mm^2)	Tt.Ar (mm^2)	Ct.Ar (mm^2)	Ma.Ar (mm^2)
BC	3.36±0.35	1.05±0.05	2.31±0.30	0.84±0.08	0.56±0.04*	0.28±0.04
AC	3.28±0.28	1.07±0.11	2.21±0.26	0.88±0.05	0.63±0.03	0.26±0.03
0.3x15x1	3.40±0.18	1.08±0.07	2.31±0.17	0.88±0.06	0.62±0.03	0.26±0.03
0.3x15x2	3.49±0.18	1.14±0.08	2.35±0.17	0.88±0.07	0.62±0.04	0.27±0.03
0.3x15x4	3.36±0.20	1.04±0.08	2.32±0.19	0.92±0.06	0.64±0.04	0.28±0.03
0.3x30x1	3.20±0.24^{+}	1.00±0.12	2.20±0.16^{+}	0.89±0.07	0.63±0.04	0.26±0.04
0.3x30x2	3.51±0.25*	1.02±0.14	2.49±0.13*	0.95±0.06*	0.67±0.04	0.29±0.02
0.3x60x1	3.23±0.24^{+}	1.03±0.07	2.20±0.22^{+}	0.89±0.06	0.62±0.03	0.27±0.04
0.6x15x1	3.40±0.27	1.12±0.12	2.28±0.16	0.88±0.07	0.63±0.04	0.26±0.03
0.6x15x2	3.46±0.18	1.15±0.07*	2.31±0.16	0.92±0.07	0.65±0.04	0.26±0.03
0.6x30x1	3.36±0.19	1.02±0.06†	2.34±0.16	0.90±0.05	0.61±0.04	0.28±0.02

**: Different from AC*

$^{+}$: Different from 0.3x30x2

†: Different from 0.6x15x2

Figures

Figure 1. (A) Accelerometer recording of the vertically oscillating vibrating plate producing peak accelerations of 0.3g or 0.6g at 45 Hz. (B) Simultaneous recording from a longitudinal strain gage attached to the antero-medial surface of the tibia while the mouse was subjected to 0.3g and 0.6g LIV or sitting on an inactive plate (0g). A compressive strain of approximately -70με was produced by the weight of the mice (0g). While the 0.3g signal recorded from the accelerometer was not perfectly sinusoidal, it was sufficiently robust to generate a sinuoidal signal from the strain gage.

Figure 2. Trabecular bone volume fraction of the proximal tibial metaphysis in control and LIV groups (mean+SD). The dashed line represents the value of the baseline control group (BC, mean±SD). *: different from AC.

Figure 3. Bone formation rates in control and experimental groups (mean+SD) measured at the (A) endocortical surface of the proximal metaphasis and (B) periosteal surface of the middiaphysis. The dashed line represents the value of the baseline control group (BC, mean±SD). *: different from AC. #: different from 0.6x15x2.

Figure 4. Relative differences in metaphyseal BFR/BS between LIV groups and age-matched controls (mean+SD). Presented are data from the nine LIV groups stratified to illustrate the effect of (A) duration per bout, (B) number of daily bouts, and (C) splitting a given daily treatment duration (30min or 60min) into discrete multiple bouts. Individual groups may appear multiple times across the three figures. The age-matched control group is identical in figures A-C. *: different from AC. ^: different from 0.6x15x2.