Low-magnitude mechanical signals that stimulate bone formation in the ovariectomized rat are dependent on the applied frequency but not on the strain magnitude

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Abstract

There is growing evidence that extremely small mechanical signals, if applied at a sufficiently high frequency, can serve as anabolic signals to bone tissue. To determine if the responsiveness of bone to low-magnitude, high-frequency parameters is modulated by endocrine imbalance, ovariectomized (OVX) Sprague–Dawley rats were subjected to whole body vibrations (WBV, 0.15 g) at 45 Hz ($n = 6$) or 90 Hz ($n = 6$) for 10 min/day, and compared to OVX age-matched controls ($n = 6$). Five additional rats were used, in vivo, to establish the induced bone surface strain magnitudes (and strain rates). Following a 28 d protocol, bone formation rates in the metaphysis of the proximal tibia were 159% greater in 90 Hz rats when compared to age-matched controls, but 45 Hz rats were not significantly different from controls. Bone morphology of 90 Hz rats indicated significantly greater trabecular bone volume (22% and 25%) and thicker trabeculae (11% and 12%) over either controls or 45 Hz rats in the epiphysis of the distal femur, respectively. Despite the enhanced sensitivity of the skeleton towards the 90 Hz signal, the strain magnitudes and strain rates induced by this frequency were significantly lower than during 45 Hz vibration, suggesting that factors other than matrix strain are driving the anabolic response. Ideally, such mechanical signals represent a non-pharmacologic means of controlling bone mass and morphology in spite of systemic pressures for bone resorption.
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1. Introduction

Extremely low-magnitude (< 10 microstrain ($\mu\varepsilon$)), high frequency (10–100 Hz) mechanical signals, introduced to the skeleton using whole body vibration (WBV) can be anabolic to bone tissue, contributing to a skeletal structure that is less prone to fracture (Judex et al., 2003; Rubin et al., 2001a; Ward et al., 2004; Jankovich, 1972; Flieger et al., 1998; Tanaka et al., 2003). These mechanical signals can be orders of magnitude below those more typically considered in

conjunction with exercise or applied loading regimens (Rubin and Lanyon, 1985; Turner et al., 1994), and thus may present a non-pharmacologic means of preventing/reversing osteoporosis without putting the skeleton at risk of damage. Despite this promising potential, few studies have investigated whether vibrations can prevent the changes in bone formation/resorption and the deterioration of bone morphology induced by a catabolic stimulus.

In postmenopausal women, WBV applied at magnitudes exceeding 5 g were able to increase hip bone mineral density (BMD) (Verschueren et al., 2004) while a similar WBV intervention, but with peak (vibration) accelerations reduced by an order of magnitude, prevented the decline in BMD in regions of the femoral

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neck and spine (Rubin et al., 2004a). Children suffering from cerebral palsy (Ward et al., 2004) and rodents subjected to disuse may also benefit from this extremely low-level mechanical countermeasure (Rubin et al., 2001b).

The degree by which variations in the parameters defining a WBV intervention, such as acceleration magnitude, frequency, or duration, alter the efficacy of the low-level mechanical signals is largely unknown. In the ovariectomized (OVX) rat, vibrations applied at either 17 Hz (0.5 g), 30 Hz (1.5 g), or 45 Hz (3 g) were all sensed in cortical bone but the signal at the highest frequency (and acceleration magnitude) was most effective in enhancing cellular activity and was the only one that prevented the loss of cortical bone strength (Oxlund et al., 2003). If bone indeed has a preference towards certain vibration frequencies and/or accelerations, the specific mechanical parameters modulating this different sensitivity have not been identified.

Considering the distinction between the relatively infrequent, but large magnitude locomotory strain signals and the omnipresent, but low-level mechanical signals that persist through actions such as standing, it is possible that the manner of the adaptive response to low-magnitude mechanical regimens does not follow the adaptive rules defined by factors such as longitudinal normal strain (Rubin and Lanyon, 1985; Turner et al., 1994), strain rate (O'Connor et al., 1982; Lamothe et al., 2005), or strain gradients (Judex et al., 1997; Gross et al., 1997), and that other loading characteristics, including the frequency of the signal or the number of loading cycles, play a more important role at these smaller magnitudes.

In an effort towards the development and optimization of WBV-based regimes that can effectively prevent and counteract bone loss, here, we tested the hypothesis that under hormonal challenges, a 90 Hz mechanical signal can be more effective in stimulating bone's anabolic activity than a signal half its frequency and that this differential sensitivity is independent of the induced strain magnitude.

2. Methods

2.1. Experimental design

All experimental procedures were approved by Stony Brook's Institutional Animal Care and Use Committee. OVX retired breeders (Sprague–Dawley) were purchased (Charles River Laboratories Inc., Wilmington, MA) and subsequently subjected to low amplitude (0.15 g peak acceleration) WBV at either 45 Hz ($n = 6$) or 90 Hz ($n = 6$) for 10 min/day (5 d/wk), or served as age-matched (long-term) OVX controls ($n = 6$). All rats were received in a single shipment and were 6–8 mo old (female rats are

retired according to the number of pregnancies and performance). Rats were maintained on a regular rodent chow with a calcium and vitamin D content of 1.0% and 2.4 IU/gm (LabDiet Prolab RMH 3000, Purina Mills LLC, St. Louis, MO) and commenced their 28 d experimental protocol 2 wk upon arrival (∼5 wk post OVX). Demeclocycline (25 mg/kg, i.p.) and calcein (20 mg/kg, i.p.) were administered to all rats on days 1 and 18 to monitor static and dynamic indices of trabecular and cortical bone formation in the metaphyseal region of the proximal tibia. Body mass and femoral length of each rat were recorded. Trabecular bone and cortical bone morphology was assessed by micro-CT in the distal femur. An additional five age-matched rats from the same genetic strain were used for in vivo strain gaging.

2.2. Strain gage recordings

Under isoflurane anesthesia, single-element strain gages (UFLK-1-11-1L, 1 mm gage length, 120 Ω, TML Gages, Texas Measurements, College Station, TX) were attached (cyanoacrylate) to the anterior-medial surface of the proximal tibia of rats from the strain gage group. Upon recovery from surgery (1–3 h), strain data were collected for 5 s while the animals were standing on a plate vibrating (0.15 g) at 45 or 90 Hz. Strain gage signals were conditioned by a strain gage amplifier (Syminex Inc., Beacon Dynamics, Dover, NJ) with an excitation of 4 V and a 2000× gain. To minimize noise, all cables connecting the components of the data acquisition system were carefully shielded and grounded and a Faraday cage isolated the vibration plate from the measurement devices. Strain signals were acquired at a sampling rate of 2000 Hz and 16-bit resolution. In this configuration (Fritton et al., 2000), the nominal strain resolution is below 0.1 με. For data analysis, all strain data were digitally filtered through a low-pass Fast Fourier Transform (FFT) filter with a cutoff frequency of 100 Hz. Five trials were collected per animal and frequency. FFT were also used to confirm the dominant frequency of the recorded signal. For each trial, the dynamic strain range was calculated as the mean difference in peak-to-peak strain magnitude across the strain oscillations. Average peak strain rates were determined as the first derivate of the strain signal. For both strain magnitude and strain rates, the average of the five trials was used for further analysis.

2.3. Histomorphometry and morphology

Histomorphometric analyses focused on trabecular and cortical bone of the proximal tibia because previous studies in rodents had found the largest effect of WBV on measures of bone formation at this site (Rubin et al., 2001b; Judex et al., 2002). It further allowed the direct relation of bone's response to the two mechanical regimes

to strain data collected from the same anatomical region. The proximal tibia was embedded in methyl methacrylate and 50 µm-thick frontal sections were cut on a diamond wire saw (Well Wire Saws, Model 3241, Germany). Under an epifluorescent microscope ($\times 10$), trabecular bone of the proximal tibial metaphysis was evaluated over an area enclosed by two lines 800 and 2000 µm distal of the growth plate. Twenty-four adjacent squares, each displaying 1.6 mm^2, were captured by a video camera interfaced with a digitizing pad and a PC. For cortical bone, the endocortical surface surrounding the trabecular area of interest was analyzed (the periosteal surface did not display consistent labels). Fluorescent labels and bone surfaces were traced and histomorphometric indices were determined with morphometric software (Osteomeasure, OsteoMetrics Inc., Atlanta, GA). Trabecular bone formation rates, with bone surface as referent (BFR/BS), mineralizing surface (MS/BS), and mineral apposition rate (MAR) were determined (Parfitt et al., 1987). High-resolution micro-computed tomography (10 µm resolution) was used to describe trabecular bone morphology in the epiphysis and metaphysis of the distal femur. In the epiphysis, trabecular bone volume (BV) within the boundaries of the cortical shell and the physis was computed. In the metaphysis, because of a lack of similar boundaries, BV was normalized to the volume of interest (TV) that matched the volume of tissue used for the histomorphometric analyses. For both regions, connectivity density (Conn.D.), trabecular thickness (Tb.Th.), trabecular number (Tb.N.), and bone volume fraction (BV/TV) were calculated (Odgaard, 1997; Judex et al., 2004). All evaluations were performed without knowledge of the experimental identity of the sample.

2.4. Statistics

Paired t-tests were used to contrast differences in bone surface strain magnitude and strain rates induced by the 45 and 90 Hz signals. The same test was used to detect changes in bone mass over the 28 d protocol. Analysis of variance tested for differences in initial body mass, final body mass, and bone length between the experimental groups. Dunnet tests probed whether indices of bone formation or morphology were greater in the 90 Hz groups when compared to control and 45 Hz groups. Data analysis was performed with SPSS for Windows 13.0. The significance level was 0.05 and all data are presented as mean $\pm$ SD.

3. Results

3.1. Induced strain environment

In vivo strain data collected from the antero-medial surface of the proximal tibia showed that dynamic strain

magnitudes induced at 90 Hz averaged 0.74 ± 0.11 µε, 65% smaller ($p = 0.002$) than those induced at 45 Hz (2.12 ± 0.42 µε) (Fig. 1). Similarly, peak strain rates produced by the 90 Hz signal (194 ± 38 µε/s) were 38% ($p = 0.02$) smaller than during 45 Hz vibrations (312 ± 53 µε/s). The average intra-trial CV was 21% for 45 Hz trials and 48% for 90 Hz trials. The average intra-animal CV, across the five trials, was 9% and 7% for 45 and 90 Hz trials, respectively. Strains induced by standing on an inactive plate or next to an active plate (noise control) were substantially smaller than those induced by either vibration frequency (Fig. 1). Transforming these data into the frequency domain confirmed that the dominant peak in the frequency domain coincided with the vibration frequency. FFT also suggested the presence of a small 60 Hz noise component but the amplitude of this peak was less than 0.2 µε and equally visible in the 45 and 90 Hz vibration trials.

3.2. Differences in bone formation and morphology

All three groups gained between 6% and 9% ($p < 0.05$ each) body mass over the 28 d-experimental period, but there were no significant differences in either the initial (429 ± 68 vs. 406 ± 41 vs. 442 ± 41 g) or final (469 ± 90 vs. 433 ± 46 vs. 475 ± 29 g) body mass between groups. Similarly, post-sacrifice, no differences in bone length were detected between groups (data not shown). Trabecular bone formation rates in the metaphysis of OVX rats stimulated at 90 Hz were 159% ($p = 0.02$) greater than in age-matched OVX controls and 206% ($p = 0.01$) greater than in 45 Hz stimulated rats (Fig. 2). Greater bone formation rates in 90 Hz rats were accounted for both by greater MAR and a greater percentage of bone surfaces that were mineralizing, even though not all of these differences reached statistical significance (Table 1).

The osteogenic potential of the mechanical signal, as dependent on frequency, was also suggestive in cortical
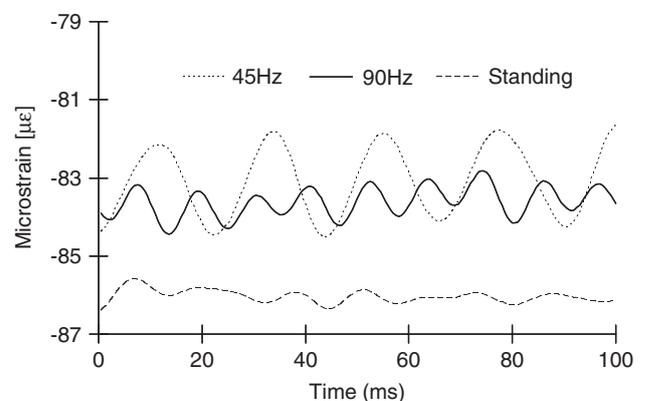

Fig. 1. Strain gage signals recorded from a rat tibia while the animal was subjected to either 45 or 90 Hz vibrations (0.15 g, each) or standing on an inactive plate.
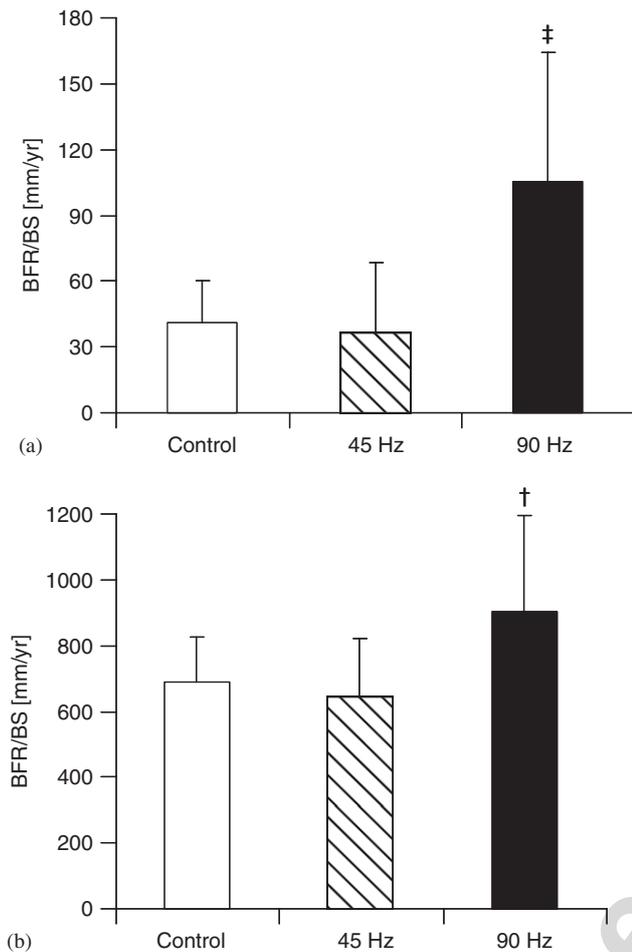
Fig. 2. (a) Trabecular and (b) cortical bone formation rates (mean + SD) in the metaphysis of OVX age-matched control rats and OVX rats subjected to short duration WBV at either 45 or 90 Hz. ‡: 90 Hz > 45 Hz and control; †: 90 Hz > 45 Hz.

Table 1
Mean (±SD) MAR and mineralizing surfaces (MS/BS) of trabecular (Tb) and endocortical (Ct) bone surfaces of the tibial metaphysis in OVX control and experimental rats

	Control	45 Hz	90 Hz	p-values
(Tb) MAR, μm/d	0.86±0.28	0.75±0.32	1.38±0.56	0.08, **0.04**
(Tb) MS/BS, %	13.5±6.2	12.9±7.9	20.1±6.3	0.11, 0.09
(Ct) MAR μm/d	2.56±0.36	2.46±0.55	3.59±2.05	0.17, 0.13
(Ct) MS/BS, %	73.6±11.0	71.6±9.6	74.9±19.7	>0.5, >0.5

The two p-values refer to the comparison between the 90 Hz signal to the control group and the 45 Hz signal, respectively. p-value of ⩽0.05 is highlighted.

bone of the metaphysis. In rats vibrated for 10 min/d at 90 Hz, endocortical bone formation rates were 31% ($p = 0.10$) and 40% ($p = 0.05$) greater than in control and 45 Hz rats, respectively (Fig. 2). These differences in bone formation rates were accompanied by MAR that were, yet not significantly, 40% and 46% greater than in control and 45 Hz rats (Table 1). Differences in

mineralizing surface between 90 Hz and the other two experimental groups amounted to only 2% and 5% (Table 1).

Because of the short duration of the intervention, bone's anabolic response to WBV was primarily assessed by histomorphometric methods. In addition, trabecular bone morphology and micro-architecture of the three different groups was assessed by micro-CT in the epiphysis and metaphysis of the distal femur. In the epiphysis, OVX rats that were subjected to WBV at 90 Hz (0.15 g) had significantly more trabecular bone (22% and 25% compared to control and 45 Hz rats) with trabeculae that were thicker (11% and 12%) but less densely connected (−35% and −37%) (Fig. 3, Table 2). No differences in trabecular bone morphology between the three experimental groups were detected in the metaphysis of the distal femur (Table 2).

4. Discussion

The hypothesis that extremely low-magnitude WBV can stimulate bone formation in the OVX rat was tested. Similar to previous studies in which vibratory stimuli positively influenced bone mass in post-menopausal women (Rubin et al., 2004a), the current data suggest that WBV can serve as an anabolic signal to a skeleton even upon the withdrawal of estrogen. The efficacy of the signal, however, was strongly dependent on the frequency of the applied signal. While WBV applied for 10 min/d at a frequency of 90 Hz (0.15 g) resulted in larger trabecular and cortical bone formation rates as well as enhanced epiphyseal trabecular bone morphology, decreasing the vibration frequency to 45 Hz eradicated the anabolic affect. Cortical surface strain magnitudes and strain rates collected from the tibia in vivo while the animal was standing on an either 45 or 90 Hz vibrating plate, however, were significantly greater in 45 than in 90 Hz rats. These data establish bone's ability to discriminate between two vibration frequencies and indicate that the optimization of a WBV intervention is unlikely to focus on strain magnitude, but instead on cycle number or a direct, preferential sensitivity of bone cells to a specific frequency.

For low-frequency mechanical loading regimes requiring much larger loads to be placed on the skeleton before an anabolic response can be observed, both strain magnitude (Rubin and Lanyon, 1985; Turner et al., 1994; Kerr et al., 1996; Cullen et al., 2001) and strain rate (O'Connor et al., 1982; Mosley and Lanyon, 1998; Lamothe et al., 2005) have been suggested as critical modulators. Not only were strain magnitude and strain rates measured in this study three and two orders of magnitude, respectively, below those associated with vigorous physical activity (Judex and Zernicke, 2000; Rubin and Lanyon, 1984) but the anabolic regime
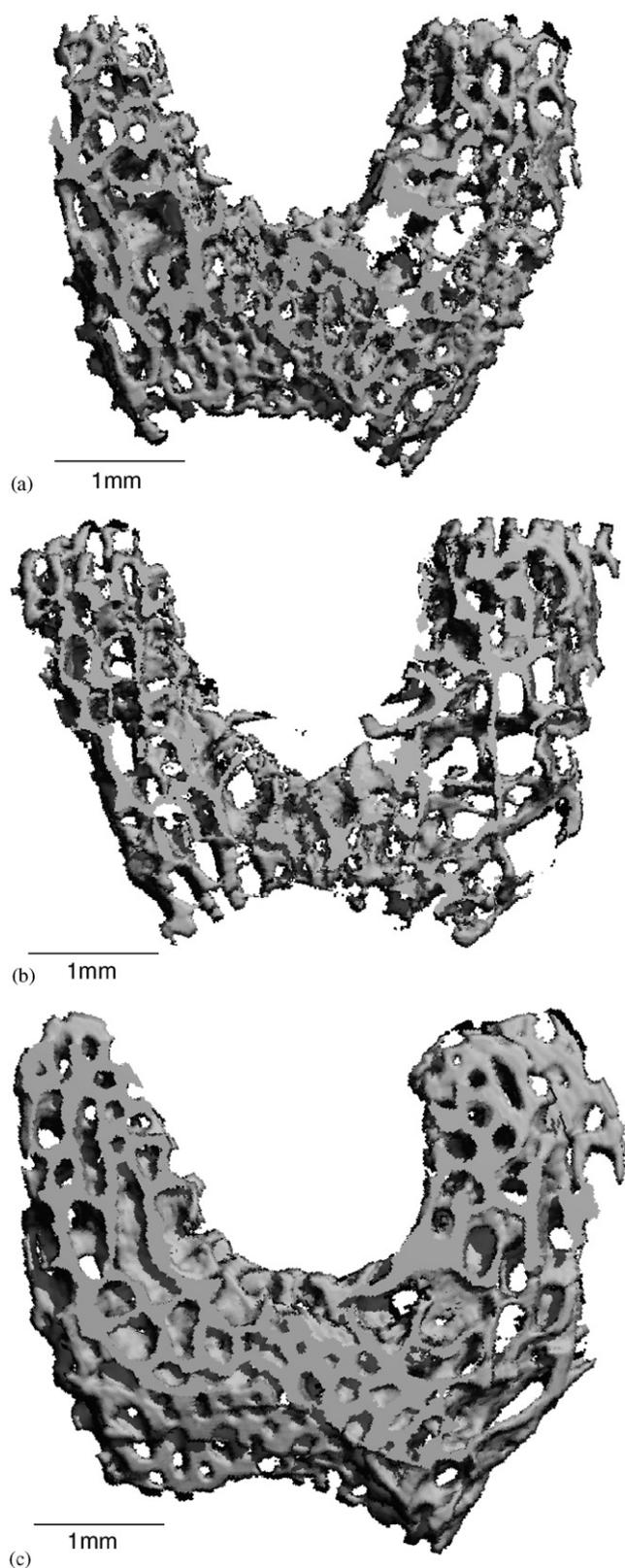
(a) 1mm

(b) 1mm

(c) 1mm

Fig. 3. Trabecular bone from the epiphyseal region reconstructed by micro-computed tomography (10 μm) of (a) a OVX control rat, (b) a OVX rat that stood on a 45 Hz vibrating plate for 10 min/d over a 28 d period, and (c) a OVX rat that stood on a 90 Hz vibrating plate for 10 min/d over a 28 d period.

induced peak strains that were significantly smaller than those engendered by the regime that was ineffective. Based on our data, it is also unlikely that fluid flow magnitude, a byproduct of matrix strain and potential mediator of bone adaptation (Qin et al., 2003; Han et al., 2004) was mediating the differential effects of the two high-frequency regimes as fluid flow magnitudes would likely be similar between the two regimes (Weinbaum et al., 1994)—if any flow is generated at these high loading frequencies at all. Consequently, it is quite possible that the physical mechanism by which bone perceives high-frequency mechanical stimuli is substantially different from the sensing of low-frequency stimuli of much larger magnitude. An example for such a mechanism may involve the sensitivity of bone cells to the high-frequency oscillatory motion itself.

Mechanical regimes using sinusoidal oscillations are inherently limited by their ability to isolate specific mechanical parameters from each other. By changing the frequency of the stimulus and maintaining the same loading duration and acceleration magnitude, the number of loading cycles was twice as high in the 90 Hz regime (54,000) as compared to the 45 Hz regime (27,000). When using a limited number of large-magnitude loading cycles, bone's anabolic response quickly saturates after a few cycles, but the number of loading cycles may play a larger role with decreasing loads and an interrelation between the number of loading cycles and the magnitude of the induced strain has been proposed (Qin et al., 1998; Cullen et al., 2001). To alter the priority between strain magnitude and the number of loading cycles, the index m in the 'daily stress stimulus' (Beaupre et al., 1990) can either be increased to place greater emphasis on the magnitude of the induced strain or decreased to place greater emphasis on the number of loading cycles. This algorithm, $\Psi = (\sum_{Day} n_j \sigma_i^m)^{1/m}$, estimates the magnitude of an osteogenic stimulus by adding the number of mechanical loading events over a 24 h period. Reducing m from 4.5, a value suggested for low-frequency loading, (Beaupre et al., 1990), to $m = 1$, a value suggested for high-frequency loading (Qin et al., 1998), the strength of the 90 Hz stimulus would still have been 43% greater than the 45 Hz stimulus and m would have to drop below 0.66 before the relation reverses and the 90 Hz stimulus becomes more osteogenic. Whether a dynamically adaptive index m can be realized biologically or whether our results add to the experimental inconsistencies that cannot be explained with the daily stress stimulus hypothesis (Adams et al., 1997; Gross et al., 2004) remains to be determined.

Alternative to combining strain magnitude and loading cycle number to estimate the strength of the mechanical stimulus, the extremely large number of loading cycles by itself may play a critical role in mediating high-frequency, low-magnitude vibratory loading. Given that the 45 Hz

Table 2
Mean ($\pm$SD) indices of bone morphology in the epiphysis (Ep) and metaphysis (Mp) of the distal femur in OVX controls and experimental rats

	Control	45 Hz	90 Hz	p-values
(Ep) BV, mm^3	2.58 ± 0.40	2.52 ± 0.49	3.14 ± 0.41	**0.03**, **0.02**
(Ep) BV/TV, %	31.1 ± 5.1	29.6 ± 5.2	34.3 ± 3.9	0.21, 0.09
(Ep) Tb.Th, µm	82.4 ± 6.5	81.5 ± 3.7	91.6 ± 6.9	**0.01**, 0.09
(Ep) Conn.D., 1/mm^3	130 ± 25	127 ± 48	82 ± 39	**0.04**, **0.05**
(Ep) Tb.N, 1/mm	5.53 ± 0.67	5.44 ± 0.79	5.35 ± 0.47	>0.5, >0.5
(Mp) BV/TV, %	0.17 ± 0.05	0.14 ± 0.07	0.17 ± 0.06	>0.5, 0.24
(Mp) Tb.Th, µm	82.1 ± 6.4	78.9 ± 8.0	78.9 ± 7.2	>0.5, >0.5
(Mp) Conn.D., 1/mm^3	61 ± 20	51 ± 33	65 ± 26	>0.5, 0.31
(Mp) Tb.N, 1/mm	2.66 ± 0.73	2.29 ± 1.02	2.87 ± 0.72	0.49, 0.20

The two p-values refer to the comparison between the 90 Hz signal to the control group and the 45 Hz signal, respectively. p-values of $\leqslant 0.05$ are highlighted.

regime with 27,000 loading cycles was ineffective in altering cellular activity, this stimulus had not reached an osteogenic threshold and even much less saturated bone's response. Despite the success of inserting rest periods into low-frequency high-magnitude loading schemes to overcome saturation (Gross et al., 2004), rest periods during extremely low-magnitude WBV do not seem to potentiate bone's response (Xie et al., 2006b), further suggesting that saturation to the number of loading cycles does not play a major role during low-level WBV. Consistent with this view, preliminary data indicate that doubling the number of loading cycles, for a given vibration frequency and acceleration amplitude, may significantly enhance bone morphology in the adolescent mouse (Xie et al., 2006a). Future studies probing the interdependence of mechanical parameters associated with WBV, including the number of loading cycles and the inherent frequency of the signal, will provide important clues towards identification of the physical mechanisms by which WBV perturbs bone's cellular activity.

The presented data are not to be interpreted that WBV below 90 Hz are ineffective in hormonally challenged skeletons of all species or that higher frequencies are necessarily more effective. Indeed, data from a small clinical trial demonstrate that a 30 Hz vibratory signal applied at identical acceleration magnitudes to those used in this study may prevent the loss of bone mineral density associated with menopause (Rubin et al., 2004a). Further, the same two WBV interventions as used in this study were previously tested in their ability to raise bone formation in retired Sprague–Dawley breeders with intact levels of estrogen. In contrast to this study, mechanical signals at both 45 and 90 Hz, despite the different number of loading cycles, were equally effective in raising the number and activity levels of osteoblasts in trabecular bone (Rubin et al., 2004b). Taken together, these studies suggest that hormonal levels, and the associated differences in bone's cellular activity and morphology, may influence the efficacy of bone to a specific vibration frequency (and

cycle number). The question as to whether acceleration magnitude and loading duration, age, body mass, nutritional status, or identity of the species, will also influence the optimization of a WBV regime requires further study.

In summary, WBV applied to the hormonally challenged rat skeleton was capable of stimulating bone's anabolic activity and perhaps presents a model for studying the molecular and cellular effects of this mechanical intervention in postmenopausal women. This study also demonstrates that changes in vibration frequency may influence the efficacy of a low-magnitude WBV regime to increase bone formation but future studies will have to test whether similar relations exist for bone's resorptive activity, for long-term changes in bone morphology, and for tissues and organs other than bone. The observed differential sensitivity is unlikely to be driven by the magnitude of the matrix strain (or strain rate) but rather by the increase in the number of loading cycles or an inherent preference of cells to specific frequencies. The identification of the precise physical mechanisms by which cells responds to WBV will ultimately enable the optimization of the non-pharmacologic means of controlling tissue mass and morphology in spite of detrimental systemic pressures toward the resorption of bone.

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