Stony Brook University

Integration of refractory periods in the administration of low-magnitude mechanical signals increases mesenchymal stem cell numbers in the bone marrow

A Thesis Presented

by

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to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Master of Science

in

Biomedical Engineering

Stony Brook University

May 2011

Stony Brook University

The Graduate School

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Abstract of the Thesis

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High-frequency, low-magnitude mechanical signals (LMMS) applied exogenously as low-intensity vibrations (LIV) have been shown to bias mesenchymal stem cell (MSC) differentiation towards osteoblastogenesis at the expense of adipogenesis. Recent in-vitro work has indicated that refractory periods allow additional benefit from these mechanical signals when scheduled into the loading routine. Work is presented here from three distinct 6-week experiments conducted to investigate the role of refractory periods allowed in the administration of LMMS in an in-vivo animal model. Results from the first experiment using 7-week old male C57BL/6J mice suggested that multiple animal handling had an adverse effect on bone morphology, with more loading periods, and thus more handling, suppressing the relative anabolic response. A second

experiment also using 7-week old male C57BL/6J mice employed a timer-controlled semi-automated vibration platform that minimized animal handling by allowing in-cage administration of LMMS. Data from this experiment showed no obvious phenotypic changes in bone morphology between LMMS-treated animals and untreated controls. However, animals that received either 30 minutes or 10 minutes three times daily bouts of LMMS showed a significant increase in MSC numbers ($p<0.05$) compared those that were not vibrated or only vibrated for 10 minutes daily. Importantly, animals were observed to have remained in a recumbent position when vibrated in their cages; unlike their standing, alert, position when moved to and vibrated in ventilated boxes, raising the issue that the automated system allowed the animals to sleep through the day portion of the diurnal cycle. In a third experiment that attempted to build on observations made from the previous two, 8-week old female BALB/cyJ mice that were vibrated twice daily for 15 minutes, separated by 6 hours, had 1.7 times the number of cells that stained positively for MSC antibodies compared to animals vibrated once daily for 30 minutes ($p=0.004$). Separating LMMS into discrete loading bouts separated by 6 hours therefore appeared to improve the ability of LMMS to proliferate MSCs. These results provide insight not only into the importance of recognizing the complications that may arise with handling animals, but also into optimizing a potential clinical intervention for increasing MSCs in conditions like aging, obesity, and osteoporosis characterized by a compromised bone marrow cell population.

Dedication Page

To my mother, Janet, for her innumerable selfless sacrifices.

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List of Abbreviations

BMI:	body mass index
BMD:	bone mineral density
BV/TV:	bone volume fraction
Conn-Dens.:	connective density
IGF-1:	insulin-like growth factor 1
LeanV:	lean tissue volume
LIV:	low-intensity vibrations
LMMS:	low-magnitude mechanical signals
MSC(s):	mesenchymal stem cell(s)
μCT:	micro-computed tomography
PTH:	parathyroid hormone
PPARγ:	peroxisome proliferator-activated receptor gamma
SD:	standard deviation
SE:	standard error
TAT:	total adipose tissue
Tb.N:	trabecular number
Tb.Th:	trabecular thickness
Tb.Sp:	trabecular separation

Acknowledgments

I would like to thank my advisor, Dr. Clinton Rubin for his unwavering support, guidance and unique way of inspiring the best out of me.

Thank you to Drs. Michael Hadjiargyrou and Yi-Xian Qin for serving on my thesis committee.

A warm gratitude to Dr. Mei Lin Chan for her thorough training and insistence that I make my lab notebook my closest companion.

Thank you to my labmates and my lab manager, Alyssa Tuthill, for their help with everything, especially my animal sacrifices and preparation for this thesis defense.

Thank you to my family for their firm support, even if it came from a few thousand miles away.

And finally, thank you to all other faculty of the Biomedical Engineering department, staff and colleague students for creating a distinctively congenial learning atmosphere.

Chapter 1

INTRODUCTION

Osteoporosis

Osteoporosis and its related fractures present a major public health problem (1). The disease is “characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk” (2). This trend usually continues silently in the individual until fracture occurs in such areas of the skeleton as the spine, hip, vertebra, pelvis and humerus (3, 4). In the United States alone, about 10 million people are estimated to have osteoporosis with an additional 34 million suspected to be at risk for the disease due to low bone density. In 2005, fractures resulting from osteoporosis cost a total of $19 billion in the U.S. and this figure is expected to rise to around $25 billion by 2025 (5). Risk factors for osteoporosis include genetic or constitutional ones such as female gender, Caucasian and Asian ethnicities, premature menopause and maternal history of fractures. In addition, lifestyle and nutritional habits like smoking, excessive alcohol intake and inactivity can contribute to the disease, so can medical disorders including anorexia nervosa, primary hyperparathyroidism and rheumatoid arthritis (6). Osteoporosis can also be caused by drug therapies involving chemotherapy for thyroid therapy and the chronic use of corticosteroids (7). All of the above risk factors are compounded by a natural decline in bone mass that begins in the third decade of life and accelerates with hypogonadism in males and menopause in females as an individual ages (8).

Obesity

The terms overweight and obese are broadly defined as ranges of weight that exceed what is generally accepted as healthy for a specific height. The term body mass index (BMI), defined as mass/height^2, with mass in kilograms and height in meters, serves as a general indication of an individual being overweight or obese (9). That said, factors such as age, race, skinfold thickness, diet and physical activity should be considered alongside BMI to assess obesity and predict possible weight problems (10, 11). For adults, a BMI between 25 to 30 kg/m^2 is considered overweight while anything above 30 kg/m^2 is regarded as obese. This evaluation provides essential information that can help diagnose obesity and bring attention to associated conditions like hypertension, type II diabetes, coronary heart disease, and stroke (12). For instance, moderately obese men (BMI of 32.5) between the ages of 45 to 54 have a 30.1% chance of having hypertension compared to a 17.7% chance in their non-obese peers. In children and adolescents, obesity presents great concern since obese children are likely to enter adulthood with the condition, increasing their likelihood of developing the above-mentioned conditions later in life. Moreover, significant psychological and cardiovascular morbidities have been observed in children and adolescents who are obese (13). In 13 to 14 year-old adolescents, 34% of obese Caucasian girls had lower self esteem compared to 8% of non-obese counterparts (14). On the cardiovascular front, obese 5-10 year old children were found to be between 2.4 to 12.6 times more likely to develop higher diastolic and systolic blood pressure, LDL cholesterol level, triglycerides, and fasting insulin concentration than their non-obese peers (15).

Economically, numbers from the National Health Account estimate that as much as $78.5 billion was spent on conditions directly related to being overweight or obese in

the United States. This sum represents 9.1% of total national medical spending in that year with average annual medical expenses being 37.4% higher for obese individuals (16). These high human, social, and economic costs make it necessary to explore effective preventative and treatment options.

Current prevention and treatment approaches

Osteoporosis

Current strategies for the prevention and reversal of osteoporosis centers on the use of parathyroid hormone as an anabolic therapy or antiresorptive bisphosphonates to decrease bone resorption (17). Parathyroid hormone (PTH) works by suppressing apoptosis in osteoblasts which leads to an extension of the life-span of these bone-forming cells (18). High cost and a risk of osteosarcoma in rats, however, present significant challenges to PTH therapy and use of the drug is not recommended beyond two years (19, 20). Bisphosphonates, the most widely-used class of osteoporosis drugs, work by selectively adsorbing to mineral surfaces where they are internalized by bone-resorbing osteoclasts. Once adsorbed by the cells, they reduce their activity by disrupting key biochemical processes leading to increased apoptosis (21, 22). Depending on the chemical composition of the bisphosphonate, frequency of dosage can be once daily, once monthly, or once yearly These drugs are administered either orally or by injection (23). Despite their effectiveness in reducing osteoporosis-related fractures, severe side effects have been identified for those using bisphosphonates for five years or longer (24). These side effects include osteonecrosis of the jaw, atrial fibrillation, esophageal cancer, ocular inflammation, severe musculoskeletal pain, and an elevated risk of atypical fractures of the femur (25, 26). While at first counterintuitive,

the occurrence of atypical fractures has been shown to be caused by the accumulation of bone microdamage and increased skeletal fragility due to a highly suppressed bone turnover, which is – somewhat ironically – the very goal of the therapy (27, 28). As such, more comprehensive system-based pharmacologic or non-pharmacologic preventative and treatment approaches are essential.

Obesity

Obesity treatment focuses primarily on weight loss and weight loss maintenance, reducing abdominal fat, improving obesity-related health risks, and reducing mortality rate. Successful treatment also impacts on reduced health care costs and aims to improve the general quality of life of patients (29, 30). Specific steps taken include the recommendation of low-caloric diets, increased physical activity, behavioral modification of lifestyle, and – towards the extreme – the use of drugs and bariatric surgery (30). Recommended low-caloric diets are usually high in carbohydrate, protein and fiber but low in fat (29). Physical activity should form an integral part of a comprehensive obesity management program, but should take into consideration factors like age, degree of obesity, and co-morbidities in a patient (31). When deemed necessary by the healthcare provider, anti-obesity drugs can be prescribed to be used along with diet, exercise and lifestyle changes. These drugs target either the central nervous system or peripheral tissues and aim to control regulatory and metabolic disturbances pathogenic to obesity. Here too, serious side effects have been seen such as the development of valvular heart disease by patients treated with the anoretic drug combination, fenfluramine-phentermine (30, 32). Because physical activity in the form of exercise not only leads to reduced fat mass, but can also prevent loss of lean body mass and bone

mass, it presents a viable multiple-pronged approach to overall well-being. The link between bone and fat because of their precursor, mesenchymal stem cells, makes such a multiple-pronged approach even more favorable (33).

Mesenchymal stem cells: link between bone and fat

Epidemiological observations made by Meunier and collaborators in the 1970s indicated an inverse relationship between adipocyte number and size and the degree of bone loss in marrow biopsies of aging and osteoporotic patients – with total marrow fat increasing as bone cells decreased (34, 35). More recently, Justesen and coworkers analyzed iliac bone marrow biopsies and showed that whereas adipose tissue volume as a fraction of total tissue volume increased from 40% to 68% from age 30 to 100, bone volume as a percentage of total tissue volume decreased from 26% to 12% over the same age range in non-osteoporotic individuals, with biopsies from aging osteoporosis sufferers showing even more pronounced changes (35). Such findings suggest that aging compromises the careful balance that exists between bone formation and resorption in early life, and this overall reduction in bone mass is, to a degree, due to a reduction in the activity, life span and differentiation of osteoblasts from their precursor, the mesenchymal stem cell. As it turns out, this is the same progenitor as an adipocyte and thus the increase in fat in the marrow and other sites could be associated with the decrease in skeletal mass. As we age, it would appear that the fat-bone “see-saw” swings in favor of fat formation at the expense of bone maintenance (36, 37). In addition, lipid is redistributed from fat depots to tissues such as muscle and bone marrow in the aging individual with a worsening of the trend by increased osteoclast activity and hormone deficiency at menopause (38).

On the other side of the coin, diet-induced obesity has been shown in animal models to correlate with a decrease in indicators of bone quality such as trabecular bone mass, trabecular number, trabecular width, spacing and connectivity (39, 40). Femoral strength, toughness and stiffness as well lamellar and osteocyte alignment were also inferior in both adolescent and adult mice fed a high-fat diet (40).

At the cellular level, seminal work by Beresford and colleagues used cultured adult rat marrow stromal cells to demonstrate an inverse relationship between the production of osteogenic and adipocytic cells. The investigators observed that the relatively equal inclination of bone marrow-derived mesenchymal stem cells towards either osteoblast or adipocyte differentiation was driven preferentially towards either pathway when culture conditions were modified. Specifically, the delayed addition of dexamethasome, a glucocorticoid, led to a predomination of adipocytic differentiation whereas the presence of vitamin D_3 led to a push towards osteogenic cell differentiation with an inhibition of adipogenesis (41). Taken together, the above observations suggest that targeting bone marrow-derived MSC population towards osteoblastogenesis can serve as an effective means of driving bone growth while suppressing fat formation. Interventions such as mechanical signals presented through exercise could therefore work through targeting the differentiation fate of MSCs towards bone endpoints (33).

Exercise and mechanical signals

Specific types of exercise have been shown to improve bone mass, density and mechanical strength (42). The work of Hert and colleagues, 40 years ago, showed in rabbits that bone is responsive to dynamic (not static loading) and strains generated by dynamic loading drive bone adaptation (43). Rubin and Lanyon subsequently confirmed

this finding in the isolated avian ulna model showing a direct proportionality between peak longitudinal strains and bone adaptation (44). Judex and Zenicke also showed that whereas treadmill running in growing roosters failed to promote bone growth, high-impact drop jumps increased bone formation in the mid-diaphysis of the tarsometatarsus, with strain rate correlating significantly with specific sites at the periosteal surface that showed increased formation rates (45). Apart from strain magnitude and rate, bone adaptation is also sensitive to number and frequency of loading cycles (44, 46, 47). These mechanical signals produced through loading or exercise are transduced into biochemical responses that may lead to changes in gene expression, function and morphology of cells through mechanotransduction (48).

Although bone mechanotransduction is still an area of continuous research, it is widely accepted that osteocytes sense mechanical signals and in turn instruct osteoblasts and osteoclasts to perform their respective functions of formation and resorption (49, 50). That said, osteocytes are not the only cell types in the bone/bone marrow environment that sense and mechanical signals; pluripotent mesenchymal stem cells that differentiate into various mesenchymal lineages such as adipocytes, chondrocytes and osteoblasts also reside in a stem cell “niche” in the bone marrow (48, 51, 52). As previously mentioned, because MSCs can differentiate into a number of cell types including adipocytes and osteoblasts, their commitment to one pathway inherently prohibits a simultaneous commitment to another (41). Recent evidence showed an increase in the fat marker, PPARγ in the absence of mechanical stretching but an increase in the osteogenic markers, Runx2 and osteocalcin with mechanical stretching in cultured bovine MSCs. It can be therefore be asserted that the absence of

mechanical signals permit the promotion of the fat phenotype, because MSC differentiation defaults towards adipogenesis, whereas an introduction of mechanical signals reverses that tendency (33). Thus, mechanicals signals presented in a safe manner can be an effective way to simultaneously control obesity, osteoporosis, and related ailments, all through targeting the fate of bone and fat cell progenitors, rather than the fully differentiated bone or fat cell.

Low-magnitude mechanical signals

Recent work has shown that in lieu of mechanical signals represented by large-strain events from sources such as exercise and high-impact loading, low-magnitude mechanical signals (LMMS), applied exogenously as low-intensity vibrations from a vibrating plate at 30 to 90Hz frequency and of 0.2*g* to 0.4*g* peak acceleration (where 1*g* = earth's gravitational field) can also promote osteogenesis and suppress adipogenesis (53). Administered for 15 to 20 minutes daily for 5 days a week, these LMMS induce strain levels three orders of magnitude below what is induced during exercise (54). Nonetheless, their effect on bone and fat formation has been seen in various animal models and preliminary clinical studies (55). Specifically, mature female sheep, after receiving LMMS treatment (30Hz frequency, 0.3*g* peak acceleration for 20 minutes per day) for a year showed a 34% increase in trabecular bone density and a 32% in trabecular bone volume fraction in the proximal femur compared with controls (54). Besides that, normal mice fed a regular diet attained 27% lower torso fat volume after 12 weeks of receiving LMMS (90 Hz frequency, 0.2*g* peak acceleration for 15 minutes per day) compared to non-treated controls (56). In addition, compared to non-treated controls, growing mice fed a high-fat diet (45% kcal fat) showed a 28% suppression of

visceral adipose tissue formation after 12 weeks with an 11% increase in trabecular bone volume fraction and a 46% increase in bone marrow-derived MSCs after 14 weeks of applying LMMS. Six weeks of treatment in a parallel experiment also showed upregulation of the Runx2 osteogenic transcription factor by 72.5% and a 27% downregulation of the adipogenesis promoting counterpart, PPARγ, in bone marrow extracted from animals (55, 57). At the human level, young women (aged 15 to 20 years) with low bone mineral density (BMD) who received LMMS (30Hz frequency, 0.3*g* peak acceleration, 10 minutes per day) for 12 months showed a 3.8±1.6 [SE] mg/cm^3 increase in cancellous BMD in the spine whereas women in the control group only had a non-significant 0.1±1.5 mg/cm^3 increase. Visceral fat was also significantly suppressed in the lumbosacral region in the LMMS-treated group compared to controls (55, 58). These studies point at a role of LMMS in stimulating osteogenesis and suppressing adipogenesis in the bone marrow space.

Major molecular players

The PPARγ transcription factor in particular plays an important role in the bone-fat balance. Data from one study showed an upregulation of osteoblastogenesis in both heterozygous PPARγ-deficient mice (40% higher trabecular bone mass compared to wildtype littermates) and homozygous mouse embryonic stem cells cultured in the absence osteogenic supplements (59). Direct association between PPARγ and adipogenesis was confirmed with the reintroduction of the PPARγ gene using a retrovirus vector (59). Intrinsic PPARγ signaling therefore seems to effectively suppress osteoblast differentiation in the absence of osteogenesis-promoting factors and a major pathway through which PPARγ affects this bone-fat relationship is though the canonical

Wnt-β-catenin signaling (59, 60).

In general, Wnt-β-catenin signaling contributes to bone mass increase through stem cell renewal, osteoblastogenesis induction, preosteoblast replication stimulation, and osteoblast apoptosis inhibition (61). The pathway also plays a role in maintaining preadipocytes in their undifferentiated state (60). On the other hand, PPARγ restrains Wnt signaling by inducing the proteasomal degradation of phosphorylated β-catenin (62). Suppression of PPARγ activity by LMMS therefore appears to increase osteoblastogenesis and restrain preadipocyte differentiation by promoting Wnt-β-catenin signaling. Coupled with the general increase in MSC numbers observed from LMMS administration, these signals present a promising clinical tool for maintaining skeletal tissue and preventing obesity especially with improved knowledge about the most beneficial bout or dose regimen.

Refractory periods in mechanical signals

Robling and colleagues observed that dividing 360 daily bending cycles into 4 bouts of 90 cycles each or 6 bouts of 60 cycles each both produced a greater osteogenic response compared to applying all 360 cycles at once (63). They proposed that mechanotransductive bone cells seem to develop an increasing “deafness” to a mechanical stimulus when loading cycles are administered uninterrupted. The incorporation of rest or refractory periods between loading bouts therefore may allow recovery of mechanosensitivity from the previous bout and increase the osteogenic benefit of subsequent bouts (63). Recent data have shown that both low and high intensity mechanical signals exhibit an enhanced suppression of adipogenesis in cultured MSCs when a refractory period of at least 1 hour is allowed between two 20

minute daily bouts (64). Analogous in-vivo data would help elucidate an appropriate scheduling approach to increase the effectiveness of LMMS in influencing MSC fate for potential clinical use.

HYPOTHESIS

We hypothesized that **incorporating rest periods with LMMS administered over the course of 24 hours in a mouse model would lead to an increase in the number of mesenchymal stem cells in the bone marrow cavity**. As a secondary outcome, **indicators of bone quality would be greater with a parallel suppression of fat formation**. This hypothesis was tested by measuring indices of bone morphology as well as fat and lean muscle volume using high-resolution microcomputed tomography and quantifying the mesenchymal stem cell pool in the femoral bone marrow with flow cytometry.

METHODS

To assess the MSC population in the bone marrow environment and potential bone and fat phenotypic changes as may be stimulated by LMMS, three 6-week experiments were performed using growing mice. Animals were distributed into groups that were subjected to one or several bouts of high-frequency low-magnitude mechanical signals over the course of the day, sham-handled, or served as age-matched controls. Indices of fat and lean mass were measured prior to sacrifice and trabecular bone morphology of the proximal tibia was determined after sacrifice with high-resolution microcomputed tomography. Flow cytometry was performed on cells flushed from the femoral bone marrow for analysis of the mesenchymal stem cell population.

Animal Groups

In the first of three animal studies, 48 seven-week old C57BL/6J male mice were obtained from The Jackson Laboratory (Bar Harbor, MN) and distributed into 6 groups (n=8) (Table 1). LMMS were administered 5 days per week for 6 weeks at 90Hz frequency and 0.2*g* peak acceleration by removing animals from cages and placing them in clear partitioned plastic boxes with ventilation holes. These low-intensity vibration parameters had been shown in a previous studies to elicit osteogenic and fat suppression responses both phenotypically and in the bone marrow space (55, 56). Animals were allowed free access to regular rodent chow (Purina LabDiet 5001, Richmond, IN) and their mass and weekly food consumption were monitored for the duration of the experiment. At sacrifice, the left tibia was fixed for 48 hours in 10% neutral buffered formalin and transferred to 70% ethanol at 4°C for long-term storage.

Bone marrow was flushed from the right femur with complete cell culture media for flow cytometry analysis.

Table 1: Animal groups and loading regimen for first animal study

Group name	**Daily loading/sham-handling regimen**
BC	Baseline Control, sacrificed at start
AC	Age-matched Control
10x3ShC	Sham Control: sham-handled for 10 minutes at 9am, 1pm and 5pm.
10x1V	10 minutes LMMS once at 9am
10x3V	10 minutes LMMS 3 times at 9am, 1pm and 5pm
30x1V	30 minutes LMMS once at 9am

To investigate whether animal handling had a negative effect on bone morphology, a second experiment was conducted. In this second study, 48 seven-week old C57BL/6J male mice were obtained from The Jackson Laboratory and put into three control and three experimental groups (n=8) (Table 2). LMMS were administered 5 days per week for 6 weeks using a timer-controlled semi-automated platform to deliver signals at 0.2*g* and 90 Hz (Figure 1). Animals were allowed free access to regular rodent chow and weekly measurements of their body mass and food consumption were made. At sacrifice, the left tibia was removed fixed in 10% neutral buffered formalin for 48 hours and transferred to 70% ethanol for long-term storage at 4°C. For flow cytometry analysis, bone marrow was flushed from the right femur with complete cell culture media.

Table 2: Animal groups and loading regimen for second animal study

Group name	Daily loading/sham-handling regimen
BC	Baseline Control, sacrificed at start
AC	Age-matched Control
10x1ShC	Sham Control, animals in cages were moved to mimic movement of LMMS groups onto platform. This group received no vibration
10x1V	10 minutes LMMS once in cages on vibration platform at 9am
10x3V	10 minutes LMMS in cages at 9am, 1pm and 5pm
30x1V	30 minutes LMMS once in cages on vibration platform at 9am

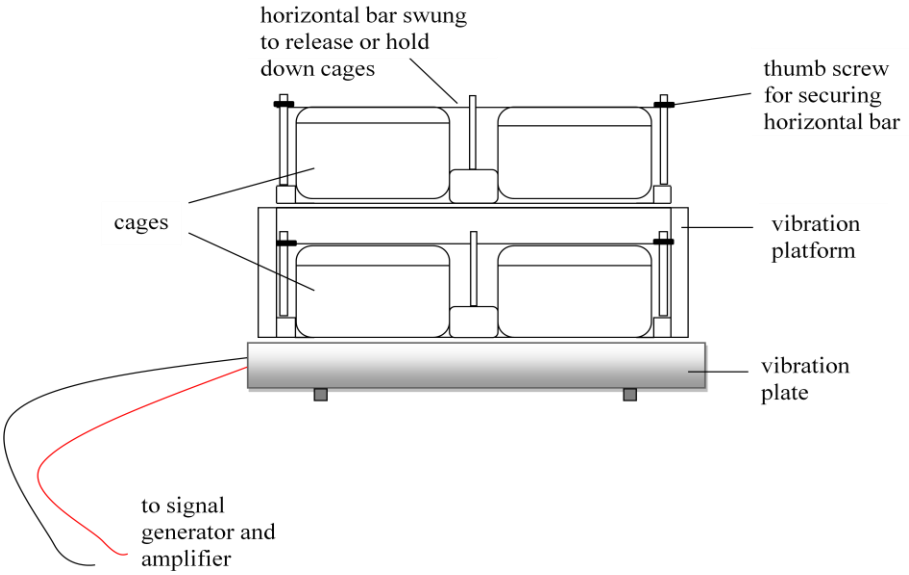


Figure 1: Front-view vibration platform with 8 slots developed to allow in-cage administration of LMMS from and minimize frequent handling.

Animals remained recumbent during in-cage vibration because of their sleep cycle instead of their alert, standing position when taken out of cages to be vibrated in conditions where one loading protocol was performed once per day. Thus, a third animal study was conducted to address observations from the first two.

Thirty-two eight-week old female BALB/cBJ mice obtained from The Jackson Laboratory were put into 3 groups that were either sham-handled or received LMMS under two different daily schedules (n=8) (Table 3). The switch to female BALB/cBJ mice was made because of previous data that showed a strong bone phenotypic response when LMMS were administered for 6 weeks in this gender and strain (65). LMMS were administered 5 days per week at 90Hz frequency and 0.2*g* peak acceleration (where 1.0*g* is the earth's gravitational field). Animals were taken from their cages and placed in clear partitioned plastic boxes for vibration. Animal mass and weekly food consumption (standard rodent chow) were monitored for the 6-week duration. The left tibia was removed at sacrifice and fixed for 48 hours in 10% neutral buffered formalin and then transferred to 70% ethanol at 4°C for long-term storage. Bone marrow was also flushed from the right femur at sacrifice into complete cell culture media for flow cytometry analysis.

Table 3: Animal groups and loading regimen for third animal study

Group name	**Daily loading/sham-handling regimen**
30x2Sh	Sham-handled twice for 30 minutes each time at 10am and 4pm. Received no LMMS.
15x2V	15 minutes LMMS plus additional 15 minutes in plastic box at 10am and at 4pm
30x1V	30 minutes LMMS at 10am, sham-handled for 30 minutes at 4pm

In-vivo micro-computed tomography

Animals were scanned using in-vivo micro-computed tomography (Scanco, Switzerland) to establish fat and lean muscle phenotype prior to sacrifice. Scanning was

performed at a resolution (minimum voxel size) of 76um, x-ray energy of 45 kVp, and tube current of 131uA. A highly precise automated algorithm described elsewhere in detail was used to separate and quantify lean and fat abdominal tissue at the mid-section from L1 to L5 lumbar vertebrae (66).

Ex-vivo micro-computed tomography

After sacrifice, micro-computed tomography (µCT) was used to examine possible changes in trabecular bone microarchitecture. Scanning was done at an isotropic voxel size of 12µm with x-ray energy and current at 45kVp and 131µA respectively. The region analyzed had a height of 960µm, taken 240µm distal to the growth plate at the proximal end of the left tibia. The cortical shell was separated from the trabecular core of the bone using an automated algorithm described elsewhere in detail (67). Trabecular parameters of bone microarchitecture computed and analyzed include bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), connectivity density (Conn-Dens.), and bone mineral density (BMD). These measurements were made from three-dimensional reconstructions of the trabecular bone separated from the cortical shell (Figure 2).

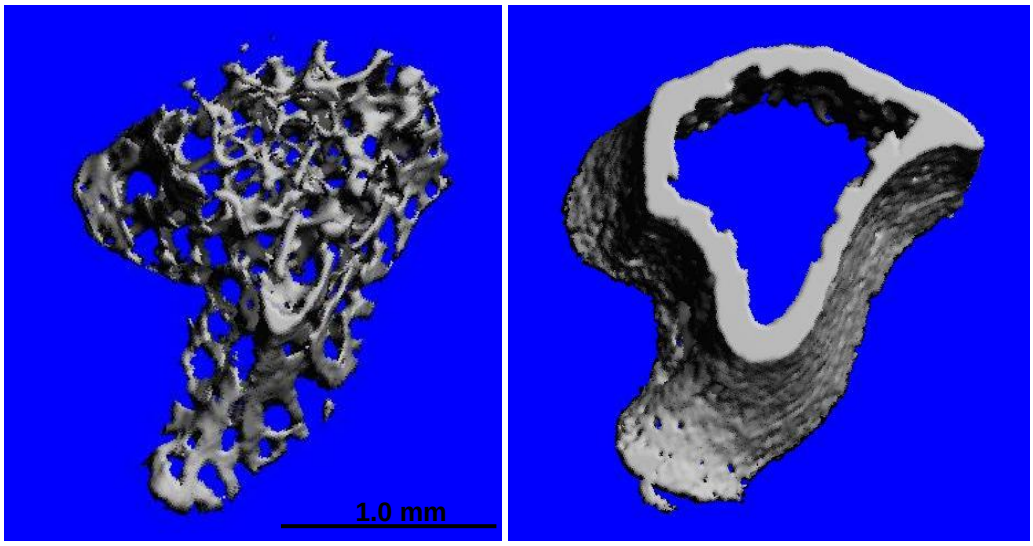


Figure 2: Representative images of 3D reconstructed images of the ex-vivo µCT region of interest in the proximal tibia. Trabecular bone (LEFT) was separated from the cortical shell (RIGHT) using an automated algorithm.

Flow cytometry

The right femur was removed at sacrifice, the ends snipped, and bone marrow flushed out. Red blood cells were lysed with Pharmlyse (BD Biosciences) after which the mesenchymal stem cell population was assessed by flow cytometry (FACSAria Cell Sorter, BD Biosciences, CA). Staining was done with Sca-1 and c-Kit markers at a 1:100 volume ratio (51, 55, 68, 69). These markers were conjugated to phycoerythrin (PE) and peridinin-chlorophyll fluorochromes respectively.

Statistical analysis

Data were presented as mean ± SD. One-way ANOVA with the Tukey post-hoc test was used to compare group means and determine statistical significance set at 5%. Statistical analysis was performed with version 17 of SPSS (IBM, Somers, NY).

RESULTS

Experiment 1: Impact of handling

Animal mass and food consumption

Animal groups showed a similar trend in mean weekly mass of food consumed and animal weights (Figure 3). There were no significant differences between groups in mass and food consumed.

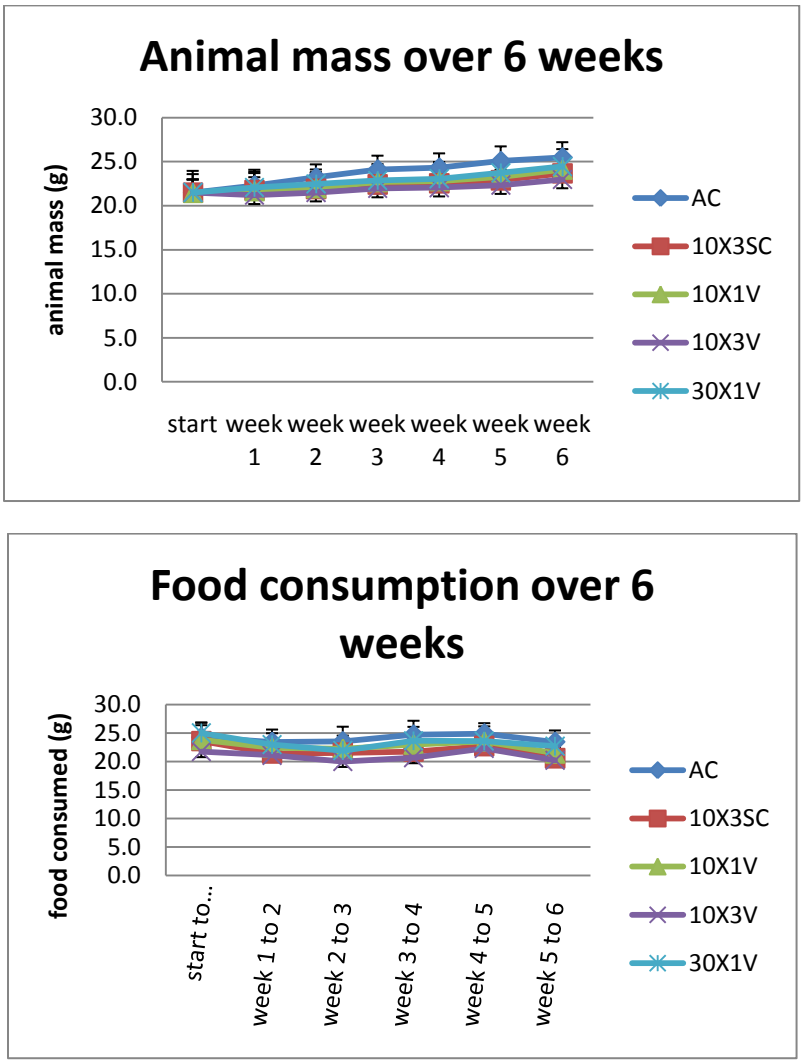
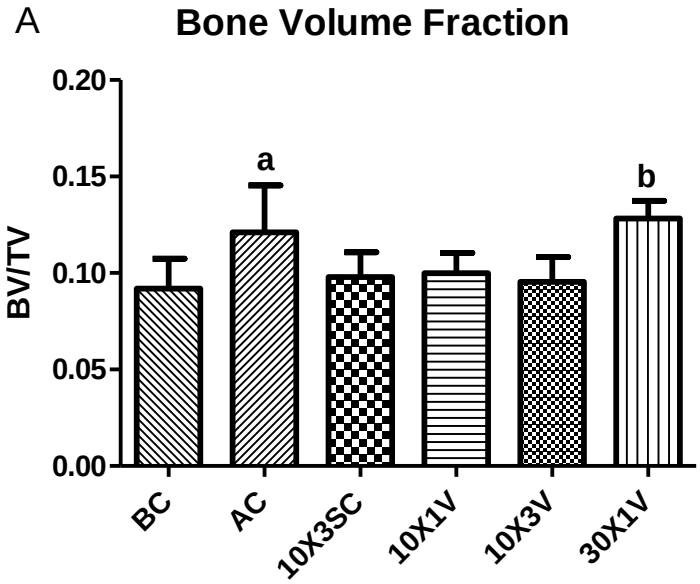


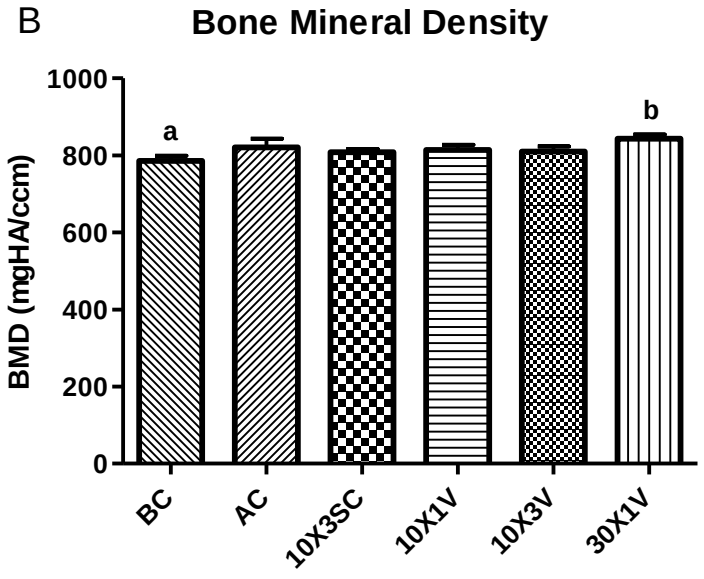
Figure 3: Animal weights and food consumed over 6 week experimental period in first animal study. Mean weights and food consumed across groups showed no significant difference.

Bone morphology

Animals in the 30x1V group showed significantly greater bone volume fraction (BV/TV), trabecular thickness (Tb.Th) and bone mineral density (BMD) than 10x3V, 10x3SC and BC groups ($p<0.05$)(Figure 4). There were no significant differences among non-baseline animal groups in connectivity density (Conn-Dens.), trabecular separation (Tb.Sp), and trabecular number (Tb.N) (not shown). Age-matched control animals (AC) also showed a higher BV/TV than groups handled or vibrated three times per day (Figure 4).



a: p<0.05 versus BC, 10X3SC and 10X3V
b: p<0.05 versus BC, 10X3SC, 10X1V and 10X3V



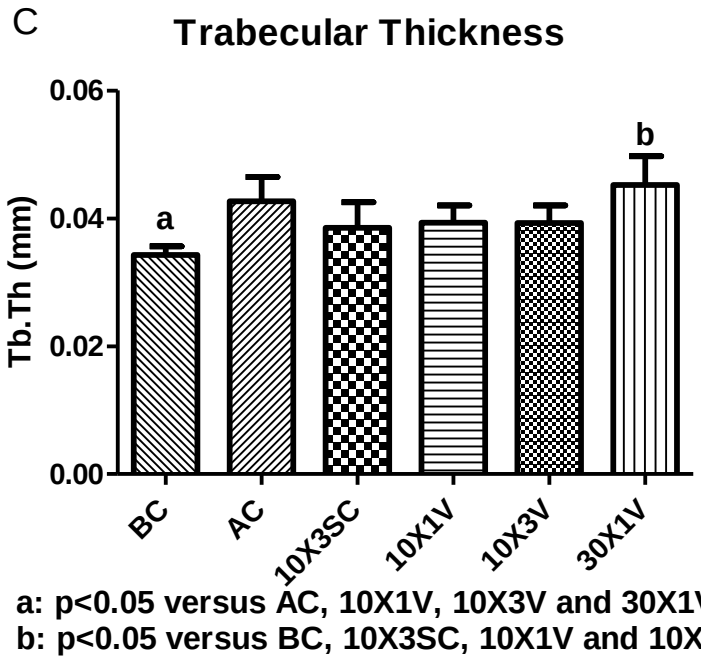


Figure 4: Mean indices of trabecular bone morphology: bone volume fraction (A), bone mineral density (B) and trabecular thickness (C). 30x1V showed significantly higher BV/TV, BMD, and Tb.Th than BC, 10x3SC, 10x1V and 10x3V ($p<0.05$).

Fat and lean tissue morphology

The AC group showed a significantly higher lean tissue volume compared to BC, 10x3SC, and 10x3V. There were no significant differences in volume of total adipose tissue among groups (Figure 5).

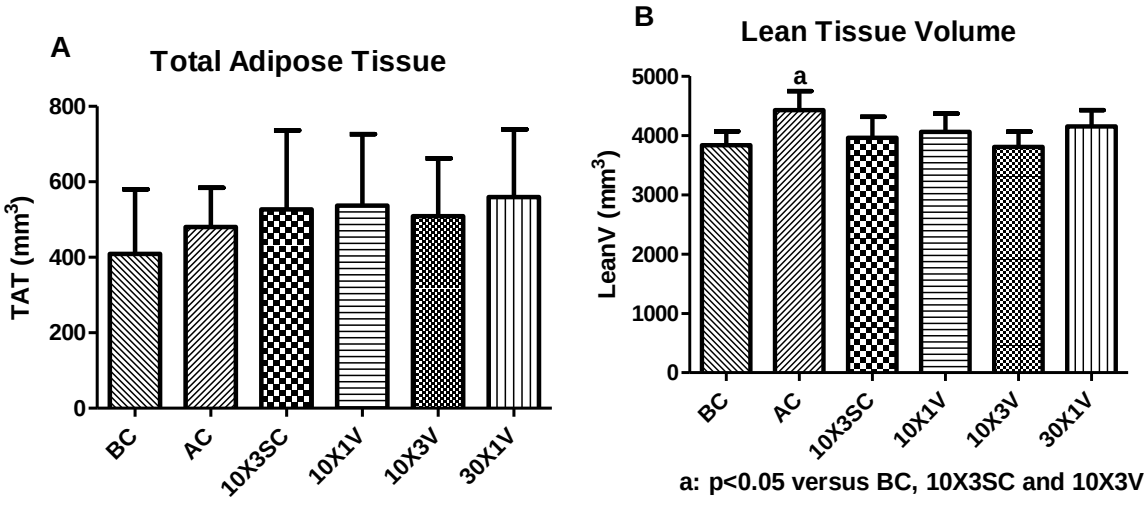
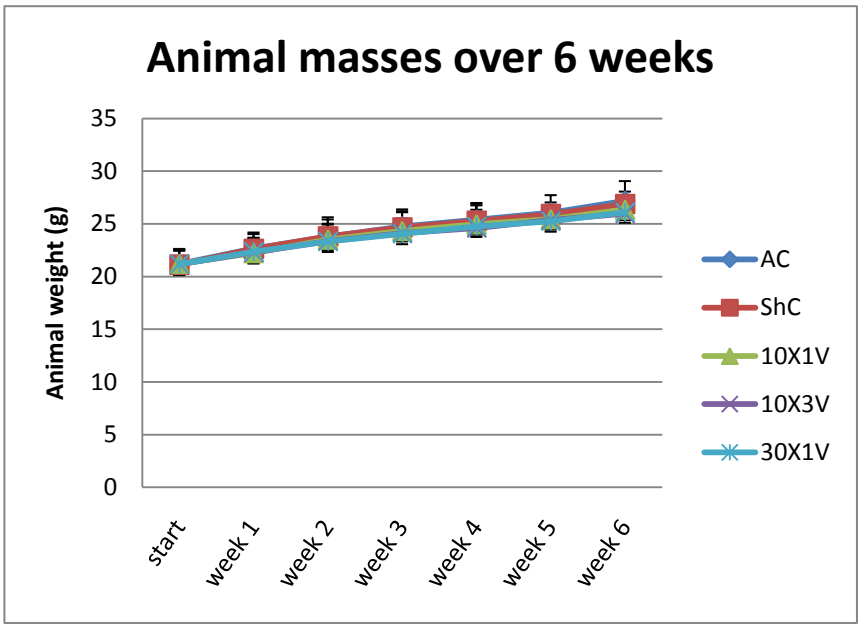


Figure 5: Total adipose tissue (A) and lean tissue volume (B). AC had a significantly higher LeanV compared to BC, 10x3SC and 10x3V ($p<0.05$). No significant differences were observed in TAT among groups.

Experiment 2: Impact of in-cage vibration during day cycle

Animal weights and food consumption

There was no significant difference in weekly animal mass and weekly weight of food consumed over the 6-week experimental period (Figure 6).



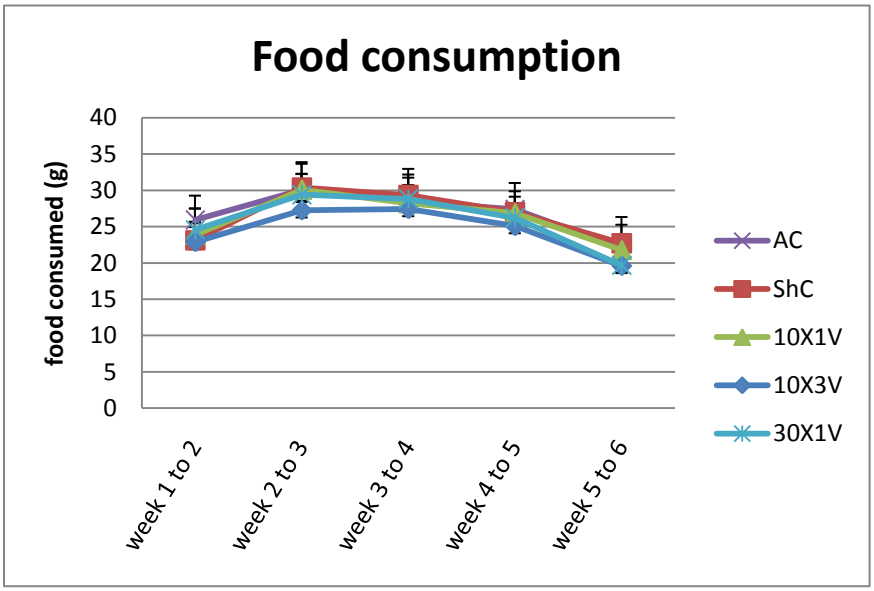


Figure 6: Animal mass and food consumed over 6-week experimental period in second animal study. No significant differences were observed in mass of animals or food consumed. Records of food consumed were kept beginning from the end of week 1.

Flow cytometry of bone marrow-derived cells

The population of cells that stained positively for Sca-1 and c-kit antibodies as a fraction of all bone-marrow derived cells were statistically higher in both 10x3V and

30x1V compared to all other groups (Figure 8). Gates were carefully drawn in flow cytometry profiles to include the subset of cells positive for both MSC markers (Figure 7).

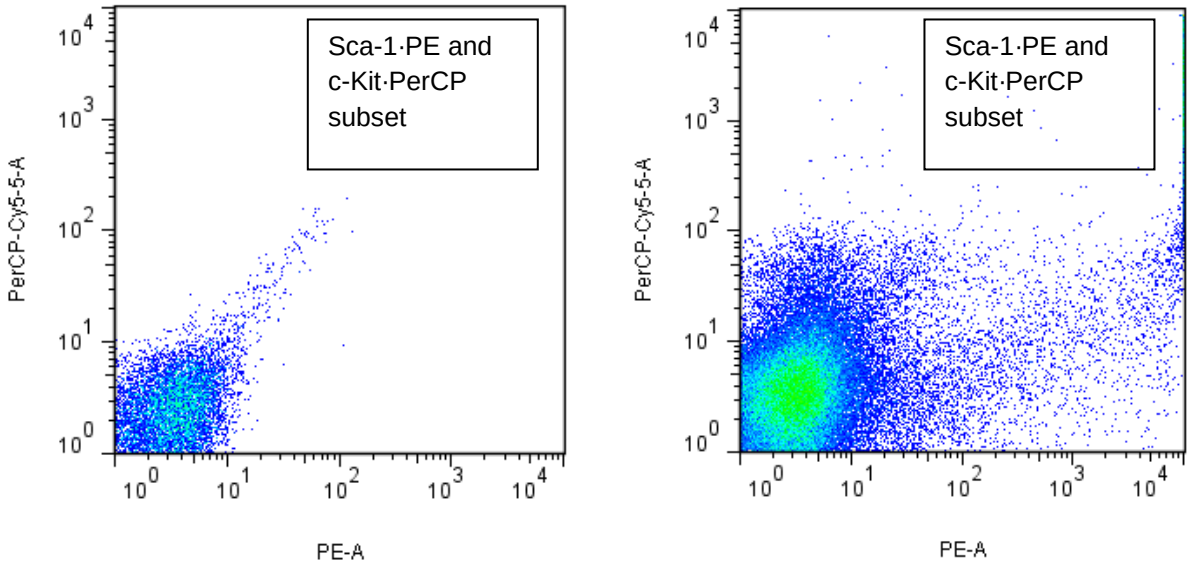


Figure 7: LEFT: Unstained control of bone marrow-derived cells sampled from all animals. This unstained control served as a guide for delineation of the subset of cells positive for Sca-1 and c-Kit. RIGHT: Cells stained for the Sca-1 and c-Kit markers. Inset rectangles show corresponding gates drawn to identify subset of cells positive for both markers.

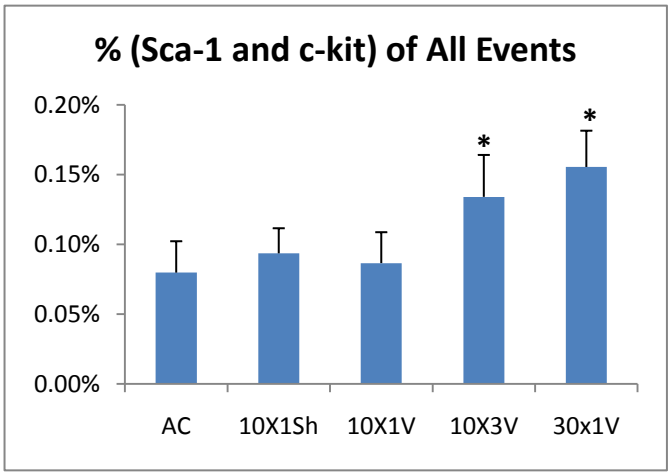


Figure 8: Mean percentages of cells positive for Sca-1 and c-kit MSC markers. 10x3V and 30x1V showed statistical significance compared to all other groups *=p<0.05 versus AC, 10x1Sh and 10x1V. Percentages are calculated as number of positive cells as proportion of100,000 sampled events.

Bone morphology

The Baseline Control group (BC) was statistically lower in three major indices of bone morphology: BV/TV, BMD and Tb.Th suggesting bone growth progressed from age 7 weeks to 13 weeks (Figure 9). AC also showed a significantly higher BMD compared to 10x1ShC (Figure 9). There were no significant differences among non-baseline groups in Conn-Dens., Tb.Sp and Tb.N (not shown).

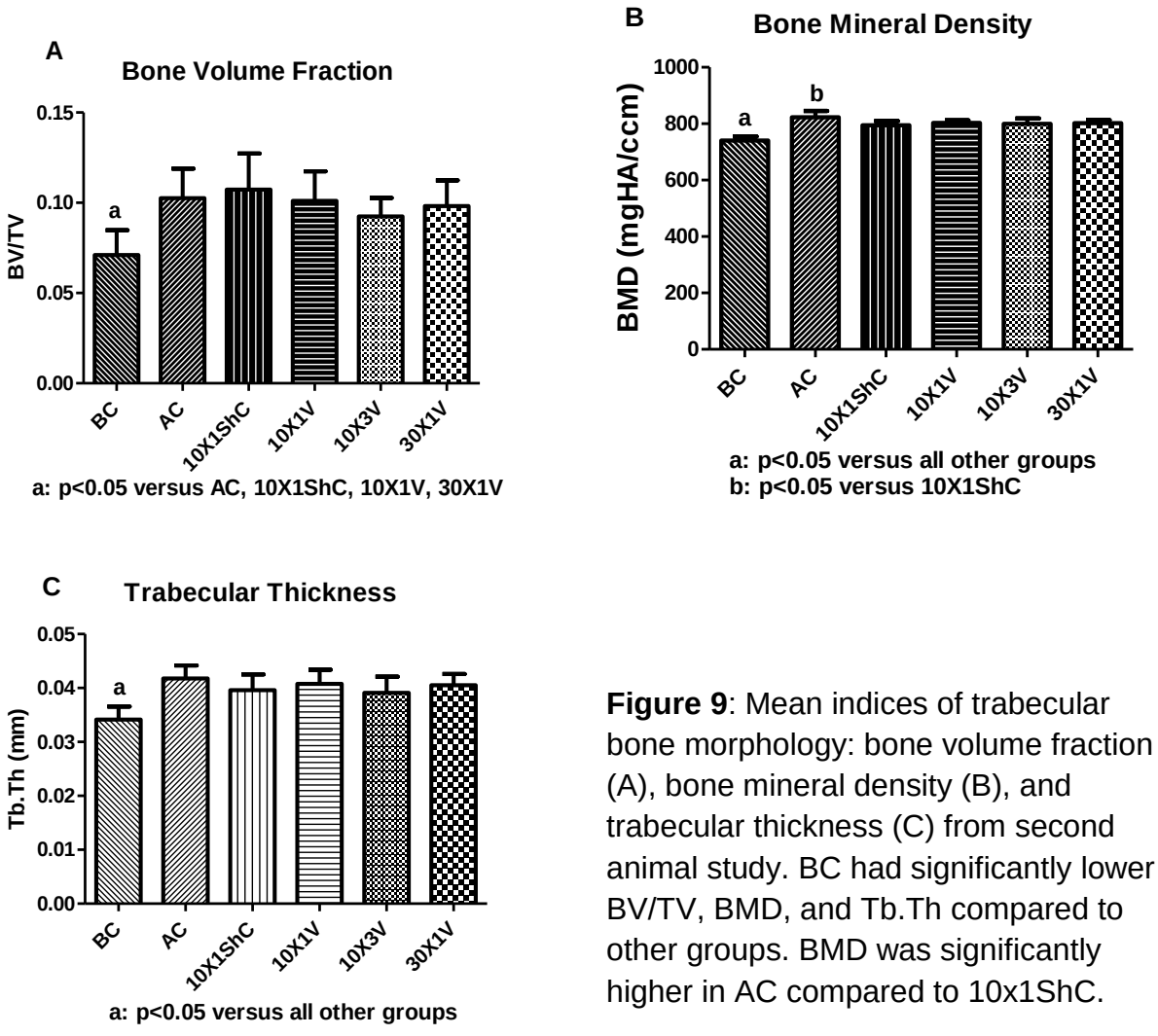


Figure 9: Mean indices of trabecular bone morphology: bone volume fraction (A), bone mineral density (B), and trabecular thickness (C) from second animal study. BC had significantly lower BV/TV, BMD, and Tb.Th compared to other groups. BMD was significantly higher in AC compared to 10x1ShC.

Fat and lean tissue volume

Here too, the BC group showed significantly lower total adipose tissue volume and lean tissue volume compared to all other groups (Figure 10). No other significant differences were observed.

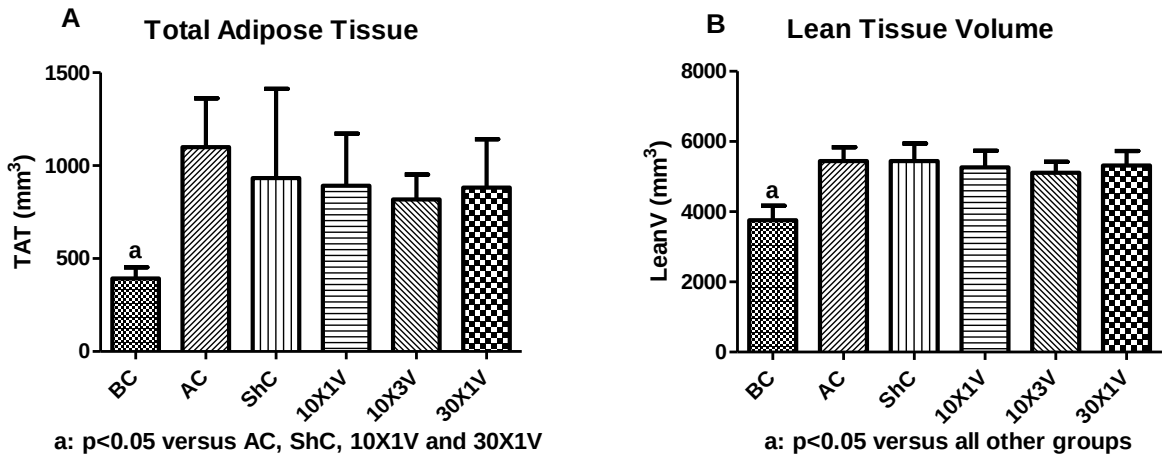
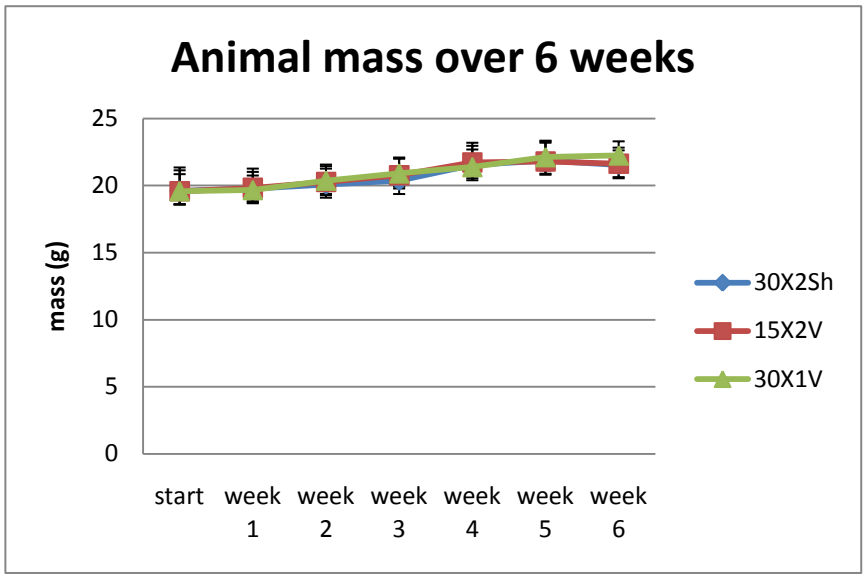


Figure 10: Total adipose tissue volume (TAT) and lean tissue volume quantified using in-vivo μCT. BC had a significantly lower TAT and LeanV compared to all other animal groups.

Experiment 3: Idealized LMMS regimen with refractory periods

Animal weights and food consumption

There was a similar rate of growth in animal weights and mass of food consumed over the 6 week period across all animal groups (Figure 11).



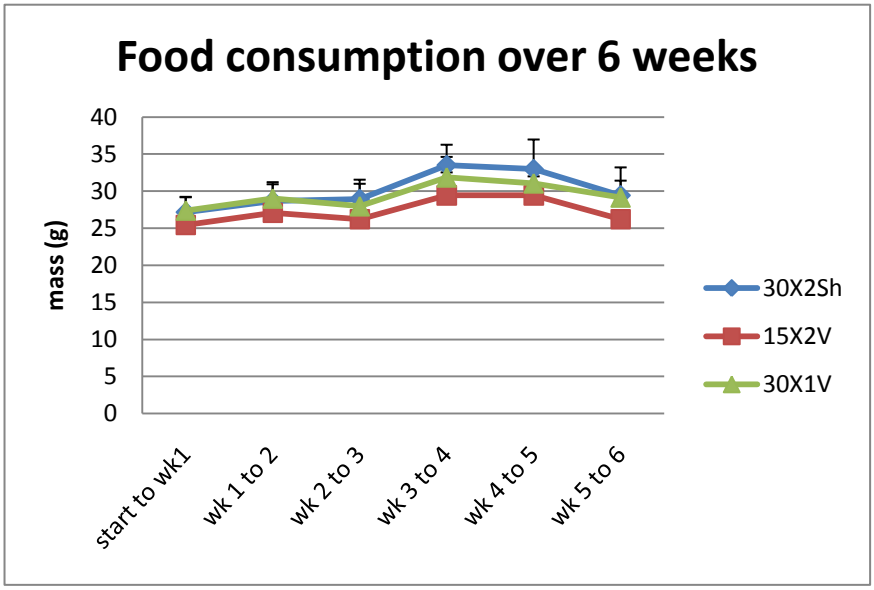


Figure 11: Animal weight and food consumed over 6 week experimental period in third animal study. There were no significant differences among groups in weekly measurements of animal weight and food consumed.

Flow cytometry of bone marrow-derived MSCs

15x2V showed a significantly higher proportion of bone marrow cells that stained positively for the MSC markers Sca-1 and c-Kit. As many as 1.7 times more 15x2V cells stained positively for the two markers compared the 30x1V group and 72.8% more cells

in the 15x2V group were positive for both markers compared to the 30x2Sh group (Figure 12). Flow cytometry gating was done in a similar manner as in Experiment 2 above (Figure 7).

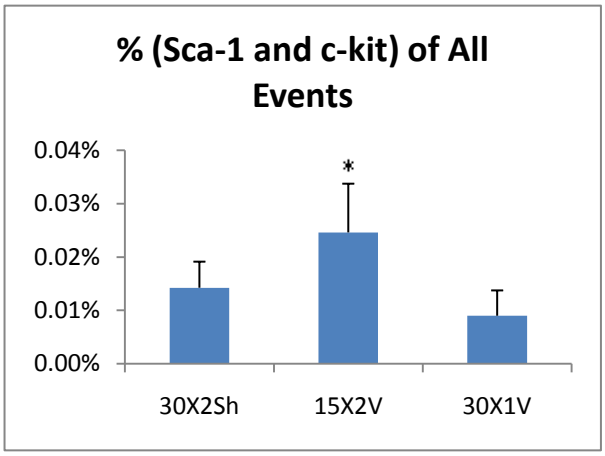
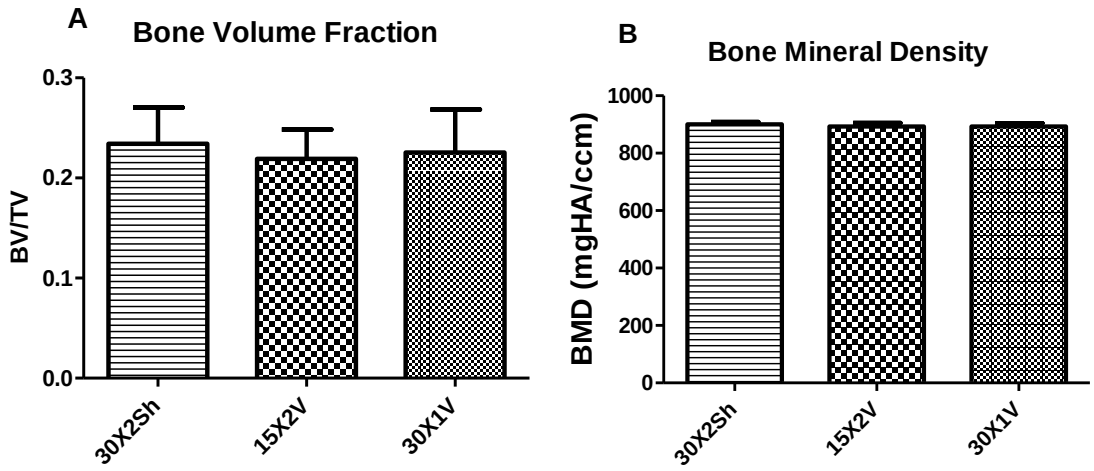


Figure 12: Mean percentages of cells that stained positively for Sca-1 and c-Kit MSC markers. *=p<0.05 versus 30x2Sh and 30x1V. Percentages are calculated as number of positive cells as proportion of100,000 sampled events.

Bone morphology

There were no significant differences in measured bone morphology indices among the three animal groups (Figure 12).



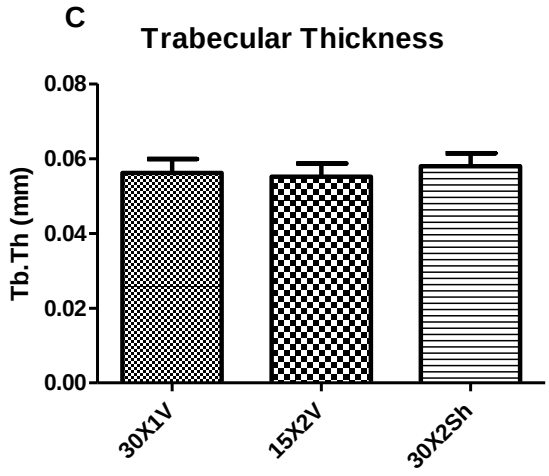


Figure 12: Mean bone volume fraction (A), bone mineral density (B) and trabecular thickness of animal groups in third study. No significant differences were observed.

Fat and lean tissue volume

Volume of total adipose tissue and lean tissue also showed no significant differences among groups (Figure 13).

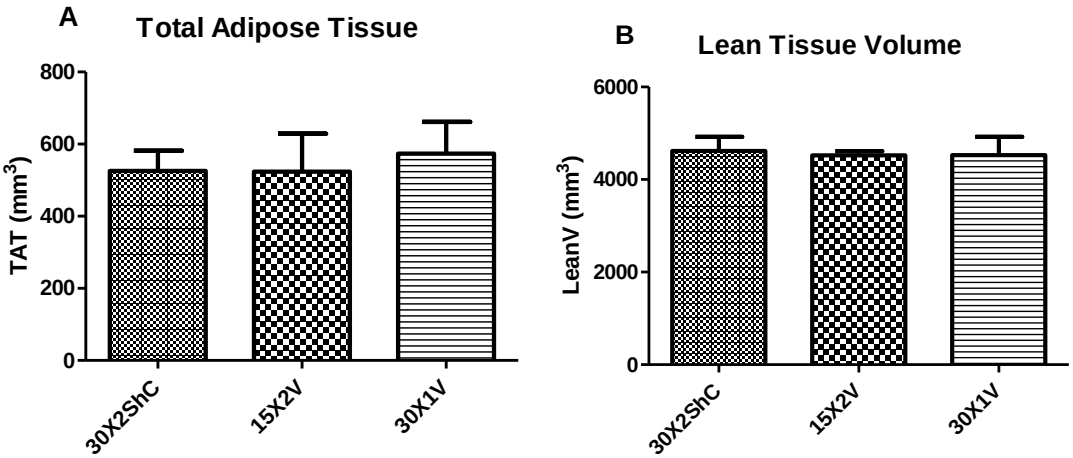


Figure 13: Total adipose tissue volume (A) and lean tissue volume (B) quantified using in-vivo μCT. No significant differences were observed.

DISCUSSION

Osteoporosis and obesity manifest as deficiencies in fat suppression and bone growth or maintenance and present considerable human and economic costs (5, 13, 16). Because of the numerous side effects that may accompany the use of pharmacological agents, interventions such as exercise present an effective means to increase bone mass and suppress fat build-up (31, 70). However, the optimal exercise regimen and exact molecular mechanism responsible for a mechanically-induced response are yet to be fully understood (71). In addition, exercise raises several issues of safety, while compliance among groups such as the elderly and the frail can be difficult (72). Alternatives to pharmacologic agents are essential, and biomechanical approaches, although attractive, must be approached with caution. Given that adipocytes and osteoblasts share a common progenitor, the mesenchymal stem cell, any intervention – physical or chemical – that promotes osteoblastogenesis can be expected to suppress adipogenesis and so may ultimately address bone loss and obesity simultaneously. This perspective is strengthened by the observed inverse relationship between the differentiation of fat and bone cells from mesenchymal stem cells in the bone marrow cavity, and one way of driving this relationship towards bone formation is through the application of mechanical signals (33). Bone responds to mechanical signals in a nonlinear fashion, as such, a few high-magnitude strain events like the kind generated by vigorous exercise or several thousand strain events of low-magnitude are both effective in eliciting an anabolic bone response (73). These low-magnitude strain events when induced exogenously at high frequencies mimic those produced from less strenuous but more frequent activities like muscle contraction to maintain posture (54). The bone formation and simultaneous fat suppression potential

of these relatively subtle mechanical signals has been well documented.

Using flow cytometry on transplanted GFP positive (GFP+) bone marrow cells, LMMS was shown to have lowered the ratio of GFP+ adipocytes to GFP+ marrow cells in the epididymal fat of treated animals fat by 19% compared to untreated controls (56). In addition, animals fed a high-fat diet showed an upregulation of the adipogenic transcription factor Runx2 by 72% and a downregulation of the adipogenic PPARγ by 27% together with a 46% increase in MSC numbers in the bone marrow when LMMS was administered for 6 weeks compared to similar animals that received no stimuli (55). Animals that received LMMS also showed significantly greater tibial bone volume fraction, less visceral fat and lower biochemical indices of obesity compared with controls (55). These promising results prompted an in-vitro investigation to determine the scheduling and bout parameters that would maximize the effect of LMMS on favorable MSC fate determination.

Over the course of 7 days, MSCs that received low-intensity vibrations twice a day for 20 minutes each time ,separated by an hour, showed a suppression of the fat markers PPARγ2, aP2 and adiponectin whereas daily single bouts of 20 and 40 minutes failed to suppress adipogenesis (64). When the duration between mechanical bouts was increased from an hour to 3 hours, repression of the adiponectin protein increased from 30±8% to 70±5% compared to untreated controls. No additional improvement in efficacy was seen when the refractory period was further increased to 6 hours. However, raising the number of daily LIV bouts from two to three with 3 hours separating them produced even greater inhibition of adipogenesis (64). These results show that scheduling of mechanical bouts has a significant effect on response as rest periods between bouts

appear to permit a “reset” of the sensitivity of cells to mechanical signals. Subsequently, the animal study presented here was conducted in an attempt to translate the in-vitro finding to an in-vivo system in an effort to “optimize” the mechanical input.

The first animal experiment was designed to compare a 30-minute bout of LMMS given once a day to three 10-minute bouts given at 9am, 1pm, and 5pm daily to 7-week old C57BL/6J male mice. Ex-vivo µCT showed a significantly higher ($p<0.05$) bone volume fraction (BV/TV), trabecular thickness and bone mineral density (BMD) in the 30x1V group compared to the 10x3V and 10x3SC groups (Figure 4). Flow cytometry data from this experiment was not considered because of a shortage of consistent marker conjugates for animals in all groups at sacrifice. Since neither parameter showed a dependence on animal mass, we believe that the additional handling required by the 10x3V and 10x3SC groups may have caused stress and suppressed bone growth in these groups. Findings elsewhere have documented significant stress-related adverse changes in physiologic parameters, such as serum corticosterone or cortisol concentration that are rapid, pronounced and sustained, with routine handling of laboratory animals (74, 75). With repeated daily handling episodes, animals may have entered a state of heightened chronic stress. Chronic psychological and metabolic stress can promote “biological aging” leading to the early onset of diseases usually seen with advanced age. Stress may also lead to an increase in abdominal adiposity, leukocyte cellular aging from shortened telomere length, and oxidative stress in blood cells (76-79). These changes are accompanied by increased production of the hormone cortisol with decreased production of hormones anabolic to lean and skeletal mass, such as androgens and insulin-like growth factor 1 (IGF-1) (80). Correlations have also

been found between endogenous cortisol levels, used as a measure of chronic stress in animals and humans, and bone mineral density (81, 82). Specifically, although weak, a negative association was found between lumbar spine bone density and integrated cortisol concentration ($r=-0.37$; $p<0.05$) in healthy men between 61 and 72 years (81). Again, together with testosterone and body mass index, cortisol was found to be a key predictor of lumbar BMD in healthy men aged 43-73 years (-0.33, $p<0.05$).

Hence, although serum cortisol and androgen levels were not measured in animals during the course of the experiment as the possible impact of stress was only realized retrospectively, three times daily handling of the 10x3V and 10x3SC groups might have led to stress that suppressed bone growth. This suppression could have been caused by an increased cortisol level coupled by decreased levels of the anabolic androgen and IGF-1 hormones in groups handed three times daily. However, the longer single 30-minute bout received by the 30x1V group led to greater indices of bone morphology in that group. The prolonged daily bout in the 30x1V group may have therefore compensated for the possible negative impact of handling from this stress-related hormonal shift. Besides, daily handling in this group was only done once daily, as a result, any stress-related hormonal shift would have been relatively minimal to begin with. From these observations, a second experiment was designed to remove handling as a possible confounding factor by making it possible to vibrate animals in their cages (Figure 1).

This second experiment showed no significant differences in indices of bone morphology between vibrated groups and non-baseline control groups (Figure 9). Flow cytometry however showed a significantly greater ($p<0.05$) number of bone marrow-

derived cells from 10x3V and 30x1V animals that stained positively for the Sca-1 and c-kit MSC antibodies compared to cells from the AC, 10x1Sh, and 10x1V groups (Figure 8). It was also observed during this experiment that animals remained recumbent as LMMS through subtle low-intensity vibrations were administered in contrast to the standing position assumed when they are removed from their cages and vibrated in ventilated plastic boxes. This resting, recumbent posture was probably assumed by animals because in-cage vibration was conducted during the day cycle when the nocturnal mice are naturally dormant. In contrast, movement from cage to plastic boxes for vibration introduces them to a new environment which prompts them to be alert and standing for the duration of vibration. Thus, when vibrated in their cages, animals remained in a comfortable environment during a time of the day when they are naturally dormant. LMMS were therefore not transmitted through weight-bearing limbs of standing animals to the rest of their bodies, creating the possibility of a subdued response. That said, since animals in the 10x3V and 30X1V groups were vibrated for a total of 30 minutes daily, compared with animals in other groups that only received 10 minutes of vibration or no vibration at all, the greater number of daily cycles may have nonetheless promoted MSC proliferation in these two groups compared to the others. Thus, put together, data from the first two experiments suggest that frequent handling of animals may lead to a suppressed osteogenic response to LMMS whereas in-cage vibration may result in the animals sleeping through bouts. Consequently, a third experiment was performed to address both issues with “standardized” and less frequent handling and administration of signals to awake, alert animals.

This third experiment administered LMMS by moving animals to clear plastic

boxes to be vibrated. Handling in all experimental groups was “standardized” to twice daily, at 10am and 4pm, with animals spending 30 minutes in clear plastic boxes each time. Eight-week old BALB/cByJ female mice were used as that strain and gender had previously shown a phenotypic bone response to LMMS after 6 weeks of administration (65). Animals that received two 15-minute bouts (15x2V) of LMMS had 1.74 times the number of cells positive for Sca-1 and c-Kit as those that were vibrated once daily for 30 minutes (30x1V) ($p<0.05$) (Figure 12). In addition, the 15x2V group had a 72.8% greater number of bone marrow-derived cells that stained positively for the two MSC antibodies compared to the 30x2Sh sham-handled group. Thus dividing a daily 30-minute LMMS bout into two sessions with a 6-hour separation period seemed to have elicited a greater MSC proliferation response compared to administering the same number of cycles in one bout. The ability of LMMS to promote MSC numbers in the bone marrow cavity was therefore enhanced by incorporating a refractory period, suggesting that greater benefit could be obtained from LMMS through repeated bouts within a 24 hour period. Hence, it may not be necessary to wait a full day before administering a second bout of LMMS, as done in the 30x1V group, when greater benefit could be acquired with a repeated bout in the same day.

Because the only variable in the experiment was length and spacing of bouts, it is possible that signals received by the 30x1V group, which were also handled twice but only vibrated once, were overwhelmed with twice-daily handling without a corresponding divided bout of LMMS to mitigate any adverse effect of handling. This unequal matching of bouts with handling might have resulted in MSC proliferation not being significantly different between the 30x1V and the 30x2Sh groups. Despite these

differences in the progenitor population, there were no significant differences in phenotypic indices of bone morphology, fat or lean tissue volume as measured by microcomputed tomography (53). The reason for this could be two-fold: first, the use of healthy mice here may have prevented modeling of adverse changes in bone metabolism and architecture seen with diseased or perturbed animal models (83). Thus, to accelerate and facilitate the quantification of phenotypic differences in fat and bone, a perturbed animal model, particularly that of diet-induced obesity might be necessary (84). In addition, the experimental duration may also need prolongation to 14 weeks to allow adequate time for differences in fat and bone production to be assayed at the phenotypic level (55). Observations from the experiments presented here therefore teach important lessons about factors that may interfere with the effective delivery of LMMS and the importance of bout scheduling.

First, multiple daily handling may suppress the anabolic effect of LMMS on bone morphology. Given that this interference was not suspected during the course of first experiment, no measures of plasma cortisol concentration to indicate stress levels in animals were made (85). However, the unequal amount of handling received by the animals in the 10x3V and 10x3SC groups may have put them in a state of elevated chronic stress and shifted hormonal levels towards increased cortisol production. Increased number of cycles with reduced handling, as given in the 30x1V group was however able to supersede the catabolic impact of elevated stress on bone formation. It may be advisable to develop a protocol that includes regular assay of serum cortisol levels to investigate the effect of multiple daily handling on bone morphology. Such a study would inform investigators about the possible negative impact of increased

handling on bone formation and allow them to account for that factor in studies of bone disease and etiology. Second, the use of the timer-controlled semi-automatic vibration platform in the second experiment resulted in the animals sleeping through the stimulus and preventing its delivery to through weight-bearing skeleton. This recumbent posture during signal delivery was unlike the alert and standing position assumed by animals when manually removed from cages to ventilated boxes to be vibrated. As such, transmission of signals to the resident bone marrow cells may not have been done effectively even though the frequency and magnitude within cages were verified to be 90Hz and 0.2*g* respectively and validated on a daily basis. It might therefore not be advisable to impart such subtle mechanical signals on animals during their sleep cycle, as the signals might be received in a "passive" manner. The third and final experiment helped consolidate lessons learned from the first two.

In this third experiment, animal handling was standardized to twice daily for all groups at 10am and 4pm and animals received no vibration (30x2Sh), a 30-minute single bout (30x1V), or were subjected to two 15-minute bouts of LMMS (15x2V). Although phenotypic differences on bone and fat formation was not seen among groups from microcomputed tomography measurements, a cellular response was seen in the bone marrow MSC pool with the 15x2V group showing a significantly greater number of MSCs in the bone marrow compared to the other two groups. Expression levels of osteogenic and adipogenic genes were not measured but the boost in MSC number was consistent with previously reported data that showed an accompanying increase in the expression level of *Runx2* osteogenic gene with a decrease in the expression of the *PPARγ* adipogenic gene (55). Since the MSC population diminishes with increased

adiposity in the bone marrow during aging and osteoporosis, incorporating a refractory may offer a means to safely increase and encourage MSCs in the bone marrow cavity towards osteoblast differentiation (37, 86).

The use of young mice in all 3 animal studies presented here necessitates additional experimentation with older mice to explore the ability of LMMS to increase the number of MSCs in the bone marrow of aging animals. Furthermore, the use of healthy$_{\mu}$ animals may not have prevented the additional benefits of LMMS from being clearly observed. A diet-induced obesity model may be needed for future experimentation. Nonetheless, the significant increase in MSC numbers seen in the 15x2V group compared to the 30x1V and 30x2Sh groups suggests that with equal animal handling, allowing 6 hours between two daily bouts of LMMS may increase the ability of the signals to promote MSC proliferation in the bone marrow. Especially since the 30x1V and 30x2Sh groups were not statistically different, and given the equal handling received by all three groups, the refractory period applied in the 15x2V may have conferred that additional benefit that resulted in significantly higher MSC numbers in that group at the end of the 6 week experimental period. Transferred to the clinic, repeated daily bouts of these mechanical signals may provide a non-pharmacological treatment for boosting MSC numbers during aging and conditions like cerebral palsy and spinal cord injury characterized by a compromise in the bone marrow cell population. With this increased MSC number, more of these target cells would be available to differentiate towards the preferred osteogenic endpoint at the deprivation of the adipogenic endpoint. As such, the benefit of this potential treatment regimen can be seen over shorter treatment periods. In addition, other beneficial sources of mechanical

input, such as those introduced through exercise can be broken up into shorter sessions separated by a rest period to confer additional benefit to individuals. Data presented here also sheds new light on possible lifestyle effects on the pathogenesis of bone loss. Although, chronic glucocorticoid therapy is well-known and documented to increase bone loss, the role of its endogenous circulating equivalent, cortisol, is far less understood and studied (82). A greater effort on studying the relationship between endogenous cortisol and bone quality would shed more light on a possible lifestyle factor involved in the etiology of osteoporosis.

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