

Musculoskeletal response of dystrophic mice to short term, low intensity, high frequency vibration

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Abstract

Objectives: We aimed to identify parameters of low-intensity vibration that initiate the greatest osteogenic response in dystrophin-deficient mice and determine vibration safety for diseased muscle in three separate studies. **Methods:** Study1: *Mdx* mice were randomized into seven vibration treatments and 14 d later, plasma osteocalcin and tibial osteogenic gene expression were compared among treatments. Study2: Three days of vibration was compared to other modalities known to elicit muscle injury in *mdx* mice. Study3: Dystrophic mice with more severe phenotypes due to altered utrophin were subjected to 7 d vibration to determine if muscle injury was induced. Muscle torque and genes associated with inflammation and myogenesis were assessed in Studies 2-3. **Results:** Two sets of parameters (45 Hz 0.6 g and 90 Hz 0.6 g) evoked osteogenic responses. 45 Hz upregulated alkaline phosphatase and tended to upregulate osteoprotegerin without altering RANKL, and 90 Hz simultaneously upregulated osteoprotegerin and RANKL. Thus, subsequent muscle studies utilized 45 Hz. Vibration for 3 or 7 d was not injurious to dystrophic muscle as shown by the lack of differences between vibrated and non-vibrated mice in torque and gene expression. **Conclusions:** Results indicate that vibration at 45 Hz and 0.6 g is safe for dystrophic muscle and may be a therapeutic modality to improve musculoskeletal health in DMD.

Keywords: Bone, Duchenne Muscular Dystrophy, *Mdx* Mice, *Dko* Mice, Osteogenesis

Introduction

Bone and muscle are biomechanically and biochemically linked on many levels. As such, bone strength, geometry, and mass are largely determined by the mechanical influences of its associated muscles. In muscle diseases, such as Duchenne muscular dystrophy (DMD), decrements in bone mass are apparent in various skeletal regions as a result of muscle weakness and the consequential reductions in mechanical loading¹⁻⁴. The loss of bone mass appears to compromise the strength of the bone, as indicated by the prevalence of fragility fractures, which occur after falling from standing or sitting heights^{1,5-8}. The primary determinant of bone strength is geometry and this is altered in several skeletal regions of both patients and mouse

models of DMD^{3,4,9,10}. Exercise regimens such as running and jumping are typically prescribed to improve bone strength and geometry in healthy populations, however these activities are ill advised in patients with DMD due to their high susceptibility to fragility fractures in bone^{1,5,6,8} and concern for exercise-induced muscle damage¹¹. Therefore, an alternative bone-sparing strategy is needed for DMD, one which is affective at improving bone strength and geometry, but also safe for muscle.

Low intensity vibration (i.e., less than 1 g of acceleration, where 1 g is equivalent to gravity) has been shown to have an anabolic effect on bone as well as prevent disuse-mediated bone loss¹²⁻¹⁵. However, some studies have failed to replicate these findings when utilizing similar acceleration and frequency parameters of vibration¹⁶⁻²⁰. The conflicting results suggest that these parameters may need to be customized to the population of interest. That is, the optimal mechanical signal delivered by vibration to stimulate bone formation in effort to improve geometry and strength may differ depending on the underlying status of the bone and condition(s) that precipitated its decline.

Thus, prior to considering vibration as a bone-sparing modality for muscular dystrophy, our first study was designed to identify parameters of vibration that initiated the greatest osteogenic response in *mdx* mice. *Mdx* mice were selected be-

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cause they have a bone phenotype across the lifespan similar to patients with DMD^{9,10,21}. We have previously shown that cortical bone geometry and strength in *mdx* mice is altered 6 to 57% and that trabecular architecture is also affected by as much as 78%⁹. We chose circulating osteocalcin and CTX, and specific genes along the osteoblastic lineage as well as genes associated with the inhibition and activation of osteoclasts based on previous work^{19,22-24}. In Study 1, we tested the hypothesis that *mdx* mice exposed to 14 daily bouts of low intensity vibration would have increase osteogenic gene expression and elevated circulating levels of osteocalcin compared to non-vibrated *mdx* mice.

After identifying the parameters of vibration that appeared to be most anabolic for bone of *mdx* mice, our next objective was to assess short-term responses of dystrophic skeletal muscle to those specific vibration-induced mechanical signals. Our previous work on wildtype mice showed that low intensity vibration training improved muscle contractility. Specifically, up to 20% improvements in strength and maximal rate of relaxation occurred following 6 wk of training, with no indication of adverse effects to muscle function²⁵. However, the efficacy and impact of vibration training on dystrophic muscle is unknown and is critical to determine because the lack of dystrophin renders skeletal muscle vulnerable to mechanical stress²⁶. Consequently, we performed Studies 2 and 3 to determine the extent to which low intensity vibration impacted skeletal muscle contractility and altered the expression of genes associated with inflammation and myogenesis. Study 2 tested the general hypothesis that three daily bouts of vibration would not be injurious to *mdx* muscle. The study was designed so that vibration-induced responses of hindlimb muscles could be directly compared with those from muscles of *mdx* mice that completed 3 d of voluntary wheel running or were subjected to a bout of injurious eccentric contractions. This was done in attempt to place vibration training on a continuum with other physical interventions known to elicit relatively minor and major muscle injury, that is, acute response to wheel running²⁷ and eccentric contractions, respectively^{26,28}. The specific hypothesis tested in Study 2 was that contractility and gene expression of muscles from vibrated *mdx* mice would not be different than those from control *mdx* mice, but would be different than those from *mdx* mice that wheel ran or were subjected to eccentric contraction-induced injury.

A subsequent study tested the general hypothesis that seven daily bouts of low intensity vibration would not be deleterious to muscle of dystrophic mice, even those with phenotypes substantially more severe than the *mdx* mouse. Mice lacking both dystrophin and utrophin (*dko* mice²⁹) and mice lacking dystrophin that are haploinsufficient for utrophin (*het* mice^{30,31}) as well as *mdx* mice, were subjected to seven daily bouts of vibration or a sham vibration intervention. The specific hypothesis tested in Study 3 was that contractility and gene expression of hindlimb muscles from vibrated *dko*, *het*, and *mdx* mice would not be different than those from littermates that were subjected to the sham vibration protocol. Support of hypotheses in Studies 2 and 3 would endorse the premise that

low intensity vibration is not harmful to dystrophic muscle and that vibration therapy could be considered as a bone-sparing strategy in patients with DMD.

Methods

Animals

Five wk-old male *mdx* mice (C57Bl/10ScSn-DMD^{*mdx*}) were obtained from Jackson Laboratories (Bar Harbor, ME) for Study 1. For Studies 2 and 3, dystrophic mice were obtained from our breeding colony at the University of Minnesota³². Mice in this dystrophic colony had recently been backcrossed to obtain a pure C57BL/10 background (>99%) as determined by The Jackson Laboratories Genome Scanning Service. Studies 1 and 2 were conducted with male *mdx* mice aged 6 and 8 wk, respectively, because peak muscle pathology occurs in the *mdx* mouse from about 5-10 wk of age. Study 3 utilized 3 wk-old *mdx*, *het* (i.e., *mdx* mice haploinsufficient for utrophin), and *dko* (i.e., mice lacking both dystrophin and utrophin) littermates of both sexes. Relatively younger mice were selected for this study in order to determine the impact of vibration at the onset of disease pathology. This earlier timepoint was also selected to accommodate the inclusion of *dko* mice which have a shortened lifespan (i.e., 8 wk). All mice were housed on a 12:12-h light-dark cycle at 20-23°C and were provided food and water *ad libitum*. Mice in Study 1 were killed by sodium pentobarbital overdose (200 mg/kg body mass) and exsanguination. Immediately following *in vivo* contractility testing, mice in Studies 2 and 3 were further anesthetized with sodium pentobarbital (75 mg/kg body mass), muscles were dissected, and then mice were euthanized by exsanguination. All animal care and use procedures were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Experimental design

The purpose of Study 1 was to determine the parameters of vibration (i.e., acceleration and frequency) that increase circulating osteocalcin levels and initiate the greatest osteogenic response in tibia of *mdx* mice. To do this, *mdx* mice were randomly assigned to one of seven vibration treatments as outlined in Table 1. At 6 wk of age, *mdx* mice began daily bouts of vibration (7 d/wk for 15 min/d) for 14 d. For this and all subsequent studies, non-vibrated control mice (i.e., 0 Hz at 0 g) were placed on a vibration platform while the system was turned off. As an indicator of stress, mice were weighed daily to ensure that they were eating normally and maintaining body mass. Approximately 1 hr following the last bout of vibration, mice were weighed, anesthetized and plasma was collected via cardiac puncture to assess the impact vibration training had on circulating markers of bone formation and resorption. Tibial bones were removed, flash frozen in liquid nitrogen, and then stored at -80°C until the time of RNA isolation.

The purpose of Study 2 was to determine if vibration was injurious to *mdx* muscle by placing it along a continuum with other physical interventions that are known to elicit minimal or major muscle injury in mice (i.e., acute wheel running and

	Study 1: Determine optimal parameters of vibration	Study 2: Compare vibration to other physical activity interventions known to elicit muscle injury	Study 3: Confirm that vibration is not injurious to muscle in more severely affected dystrophic mice
Mouse age at beginning of the study (wk)	6	8	3
Genotype	<i>mdx</i>	<i>mdx</i>	<i>mdx, het, dko</i>
Sex	males	males	males/females
Treatment groups (number of mice per group)	Non-vibrated control (n=8) 30 Hz at 0.3 g (n=6) 30 Hz at 0.6 g (n=6) 45 Hz at 0.3 g (n=8) 45 Hz at 0.6 g (n=8) 90 Hz at 0.3 g (n=6) 90 Hz at 0.6 g (n=6)	Non-vibrated control (n=10) vibration (n=11) wheel running (n=8) eccentric contractions (n=8)	Non-vibrated <i>mdx</i> (n=8) Vibrated <i>mdx</i> (n=9) Non-vibrated <i>het</i> (n=8) Vibrated <i>het</i> (n=12) Non-vibrated <i>dko</i> (n=7) Vibrated <i>dko</i> (n=7)
Duration of vibration treatment (days)	14	3	7
Outcome measures:			
Stress ^{33,37} :	Body mass	Cage activity	Body mass
<i>In vivo</i> muscle function:	N/A	Yes	Yes
Muscle inflammation and myogenesis:	N/A	Gastrocnemius muscle	Tibialis Anterior muscle
Muscle damage:	CK	CK	CK
Bone formation:	Circulating osteocalcin, RNA from Tibial Bone	N/A	N/A

wk: week. *N/A*: not applicable. *CK*: Creatine kinase activity.

Table 1. Objectives and designs of Studies 1-3.

eccentric contractions, respectively). To do this, *mdx* mice were randomly assigned to one of four groups; control, vibration, wheel running, or eccentric contractions (Table 1). Cage activities were monitored in control, vibration, and wheel running groups to determine whether vibration was immediately stressful to *mdx* mice such that alterations in behavior were apparent³³. Following the third daily bout of vibration (15 min/d, using 45 Hz and 0.6 g), 3 d of voluntary wheel running, or 3 d after a single bout of injurious eccentric contractions, mice were weighed, anesthetized, and plasma was collected from the retro-orbital cavity to assess creatine kinase (CK) activity and stored at -80°C. Contractility of the posterior crural muscle group (gastrocnemius, plantaris, soleus muscles) was then tested *in vivo*. Immediately following contractility testing, gastrocnemius muscles were excised, flash frozen in liquid nitrogen, and stored at -80°C. Subsequently, these muscles were used to determine the relative potential of low intensity vibration to increase expression of genes associated with muscle inflammation and myogenesis. The gastrocnemius muscle was selected for gene expression studies because it is the best muscle to compare across interventions. That is, the gastrocnemius muscle was the specific, major muscle that performed the eccentric contractions and it is also a highly recruited muscle during wheel running.

The purpose of Study 3 was to extend the evaluation of short-term responses of dystrophic muscle to vibration by

lengthening the duration of vibration exposure to 7 d and by using dystrophic mouse models that have more severe phenotypes than *mdx* mice. To do this, *mdx, het* and *dko* mice were randomly assigned to vibration training or non-vibrated control groups at 3 wk of age (Table 1). Twenty-four hours after the seventh bout of vibration (15 min/d at 45 Hz and 0.6 g), mice were weighed, anesthetized, and plasma was collected from the retro-orbital cavity to assess CK, and then posterior crural muscle contractility was assessed *in vivo*. Immediately following testing, tibialis anterior (TA) muscles were excised, flash frozen in liquid nitrogen, and stored at -80°C until RNA was isolated to assess gene expression. The TA muscle was chosen, rather than the gastrocnemius muscle for gene analyses, to further confirm that vibration was not injurious to skeletal muscle, especially in more severe models of DMD.

Vibration training

Our low intensity vibration device was designed after the work of Fritton et al.³⁴ to produce vertical vibration stimuli with minor modifications that improve device performance and ensure fidelity^{25,35}. Briefly, our vibration device has a circular-shaped platform that is driven by a centrally-mounted linear actuator (Moog CSA Engineering, Mountain View, CA) on the underside of the platform. A custom-designed program created with LabVIEW software (National Instruments, Austin, TX) controls and modulates the linear actuator based on continual

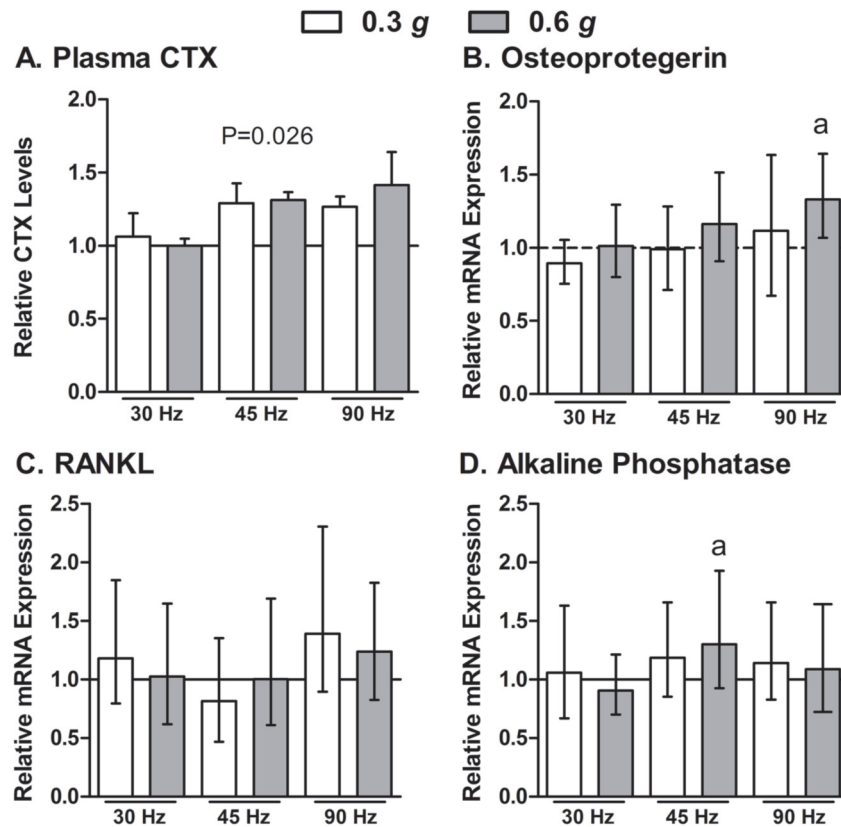


Figure 1. Six different permutations of low intensity vibration parameters were investigated in Study 1. **A:** Circulating plasma CTX values were different between groups and appeared to increase with frequency, however post-hoc tests did not detect differences between any groups. Data are mean, SE. P-value from one-way ANOVA's is indicated above the bars. **B-D:** The different parameters had minimal effects on tibial bone osteogenic mRNA expression following 14 days of daily vibration exposure. The height of the bars indicate the fold change mRNA expression (i.e., delta-delta CT) above non-vibrated *mdx* mice (represented by the horizontal gray line) and the error bars indicate the 95% confidence intervals. ^aSignificantly different from non-vibrated *mdx* mice as determined by REST, 2009 software ($P < 0.05$).

accelerometer feedback. The feedback ensured that the platform produced accelerations with less than 1% error at the 45 Hz and 0.6 g settings used in this study³⁵. Mice were placed into one of four individual compartments of the centrally-mounted cage (see ³⁵ Figure 1A in this issue). The height of the cage was set to 5 cm to limit rearing and jumping by the mice.

Wheel running

Mice were individually housed in cages containing voluntary running wheels (Single Activity Wheel Chambers, Model 80820, Lafayette Instruments Co., Lafayette, IL). One gram of resistance was applied to each wheel using resistance brakes (Model 86070-B1, Lafayette Instruments). Mice were allowed to run *ad lib* over the 3-d study duration, thus exceeding the 15 minute exposure to vibration. This duration of wheel running, however, is necessary to induce mild muscle injury²⁷, thus providing a benchmark for assessing the safety of vibration. Total distance run over the 3-d study was measured using the manufacturer's software (Model 86065, Lafayette Instruments). This 72-hr of distance recording excluded the 30 min during which cage activity was monitored.

Eccentric contractions

In effort to compare vibration training to a relatively major muscle injury intervention, the posterior crural muscles of anesthetized *mdx* mice underwent a single bout of 100 eccentric contractions on day 1 as previously described³⁶. Briefly, electrodes were percutaneously inserted on either side of the sciatic nerve following the peroneal nerve being transected. Eccentric contractions were performed by stretching the posterior crural muscles from 19 degrees of ankle plantarflexion to 19 degrees of ankle dorsiflexion, simultaneous to stimulating the sciatic nerve with a voltage between 3.0-9.0 V for a duration of 150 ms. Subsequent eccentric contractions were separated by a duration of 45 s.

Cage activity monitoring

As a more precise and immediate means of determining if vibration was stressful, as shown to occur with mild exercise in *mdx* mice³⁷, cage activities were monitored in vibrated mice and compared to non-vibrated control and wheel-running mice in Study 2. Cage activity was not measured in the mice that

performed the eccentric contractions in Study 2 because of the lingering effects of anesthesia. Briefly, immediately following vibration or sham vibration, mice were placed in a cage containing two parallel sets of infrared beams oriented in the transverse plane separated by a height of ~2.5 cm. As previously described³⁸, the movement of a mouse is spatially and temporally tracked as the infrared beams are broken due the presence of the mouse. Outcome measures of interest included: active time, ambulatory distance, and stereotypic time (i.e., eating or grooming) and were measured for 30 min immediately after a bout of vibration (Med Associates Inc., St. Albans, VT)³⁸.

Plasma osteocalcin, CTX and CK activity

Circulating levels of osteocalcin and CTX (fragments of collagen type 1) were assessed in plasma by ELISA (Biomedical Technologies Inc., Stoughton, MA and Immuno Disagnotic Systems Inc., Fountain Hills, AZ). The assays were performed in duplicate using plasma (1:10 dilution for the osteocalcin ELISA) and following manufacturers specifications. Data for the standards for each assay were fit using a 4-parameter logistic curve fit.

Plasma CK activity was determined in duplicate with plasma diluted 1:2 in PBS. Activity was measured using a kinetic assay (Creatine Kinase, C7512-300, Pointe Scientific, Inc., Canton, MI) and a Spectramax Plus 384 spectrophotometer with Softmax Pro v5 (Molecular Devices, Sunnyvale, CA)³⁹.

In vivo posterior crural muscle function

Contractile function of the posterior crural muscles was tested as previously reported⁴⁰. After severing the peroneal nerve, the sciatic nerve was stimulated at 250 Hz for 150 ms to elicit maximal isometric torque. Torque production as a function of stimulation frequency was measured as described previously³², with the addition of measurements at 200 and 250 Hz.

RNA isolation and real-time RT-PCR

RNA was isolated from the left tibial bones of mice in Study 1 by homogenizing in liquid nitrogen using a mortar and pestle and then transferring the powdered bone to TRI reagent (Applied Biosystems, Austin, TX). Phases were separated by centrifugation and RNA was precipitated by ethanol addition and then applied to RNA clean-up spin columns (Qiagen, Valencia, CA). DNA contaminants were removed by RNase-Free DNase treatment (Promega, Madison, WI) and total RNA concentration was quantified on a Nanodrop spectrophotometer (Nanodrop, Wilmington, DE). One microgram of cDNA was then diluted, synthesized and reverse transcribed using High Capacity cDNA Reverse Transcription (Applied Biosystems). cDNA samples were stored at -20°C until RT-PCR was performed. cDNA at 1:100 dilutions were then combined with TaqMan Universal PCR Master mix (Applied Biosystems, Carlsbad, CA) and TaqMan gene expression assays (Applied Biosystems), and were then run on a Biorad MyiQ thermocycler (Hercules, CA). Genes of interest associated with osteoblast cell activation, differentiation, signaling and bone formation included: alkaline phosphatase (ALP), runt-related

transcription factor 2 (Runx2), bone morphogenetic protein 2 (BMP2), and collagen type-1 α -1. Gene expression associated with the inhibition and activation of osteoclasts (i.e., Osteoprotegerin (OPG) and activator for nuclear factor κ B ligand (RANKL), respectively) were also quantified.

The effects of short term vibration on gene expression associated with muscle inflammation (macrophage-1 antigen, (MAC-1) and the chemokine ligand 2 (MCP-1)) and myogenesis (Paired box gene-7 (Pax-7), Myogenic differentiation-1 (MyoD), and Myogenin) were assessed in gastrocnemius and TA muscles from mice in Studies 2 and 3, respectively. RNA was isolated by homogenizing muscles in TRI reagent. One mg of RNA was reverse transcribed and used for RT-PCR at a dilution of 1:100. For all analyses, triplicates were run for each mouse and the average crossing threshold for each gene of interest and the housekeeping gene (i.e., GAPDH or 18s) were used to make comparisons against non-vibrated control mice using Rest 2009 software, described below.

Statistical analyses

To examine if vibration was stressful for dystrophic mice or altered circulating osteocalcin levels in Study 1, one-way ANOVAs were used to compare body mass as well as CTX and osteocalcin levels across groups. Kruskal-Wallis ANOVA on Ranks were used when normality failed, with Dunn's post-hoc testing. To determine optimal parameters of vibration on bone in Study 1, the combined effects of acceleration (0.3 and 0.6 g) and frequency (30, 45 and 90 Hz) were analyzed for tibial bone gene expression data using two-way ANOVAs (i.e., main effects of acceleration and frequency).

For Study 2, one-way ANOVAs were used to compare body mass, plasma CK activity, cage activities and *in vivo* muscle function across treatments (i.e., controls, vibration, wheel running, and eccentric contractions). When assumptions of normality or equal variance were violated, Kruskal-Wallis One Way Analysis of Variance on Ranks was performed along with Dunn's post-hoc tests. For Study 3, two-way ANOVAs were used to analyze the effects of treatment (non-vibrated controls vs. vibrated) and genotype (*mdx*, *het* and *dko*) on body mass, CK activity, and *in vivo* muscle function. For all ANOVAs, when significant main effects or interactions were present (i.e., $P < 0.05$), Holm-Sidak post-hoc tests were performed to determine which conditions were different from each other. These statistical analyses were carried out using SigmaStat version 3.5 (Systat Software Inc; Point Richmond, CA).

To examine the effect of vibration on gene expression profiles compared to non-vibrated control mice, in all studies, real-time RT-PCR data were analyzed with REST 2009 Software (Qiagen). Data are expressed as relative expression compared to non-vibrated mice with 95% confidence intervals⁴¹. Note that the 95% confidence intervals represent data that has an exponential distribution, and therefore the data are more heavily distributed on the lower bound making the mean closer to the lower bound than the upper bound. As such, the length of the 95% confidence interval is smaller below the mean than above.

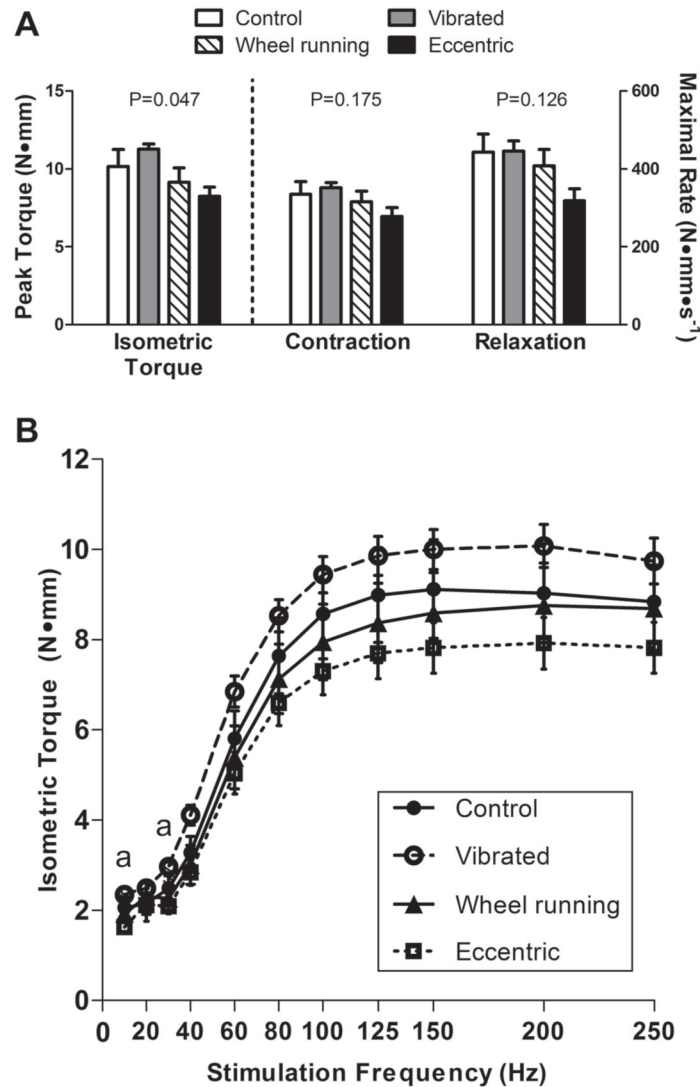


Figure 2. Three daily bouts of low intensity vibration on *mdx* mouse posterior crural muscle contractility were compared to other exercise modalities in Study 2. Data are mean, SE. **A:** Peak isometric torque and rates of contraction and relaxation were not detrimentally affected by vibration. Main effect P-values from one-way ANOVA's are indicated above each set of bars. Despite the significant main effect for peak isometric torque, post-hoc testing did not detect a significant difference among any of the four groups. **B:** Isometric torque as a function of stimulation frequency for non-vibrated Control, Vibrated, Wheel running and Eccentrically injured *mdx* mice. ^aSignificant difference determined from post-hoc testing with torque by Vibrated mice greater than that by Eccentric mice.

Results

Study 1, Osteogenic responses: In general, vibration treatment was well tolerated by *mdx* mice and did not appear to be stressful as indicated by the preservation of body mass (25.1 ± 0.4 g in vibrated vs. 24.4 ± 0.1 g in control mice at the end of the study, $P=0.978$). In addition, behavior of the mice did not appear to be affected during or immediately after a bout of vibration. Circulating plasma osteocalcin levels were not different among the seven groups of mice, suggesting that vibration did not alter osteoblast activity (59.2 ± 2.2 ng/ml, $P \geq 0.354$). Circulating plasma CTX levels were elevated 29-

41% in mice vibrated at 45 and 90 Hz, though post-hoc testing did not detect a significant difference between any of the groups (Figure 1A).

Results showed no main effects of acceleration ($P \geq 0.156$) or frequency ($P \geq 0.181$) on tibial bone mRNA expression data (data not shown), with the exception of osteoprotegerin, which had a trend toward upregulation at higher frequencies ($P=0.052$, Figure 1B). There were no significant differences in mRNA expression in Runx2, collagen type-I α -I, or BMP-2 between any of the six vibration groups compared to the non-vibrated group (range of fold change 0.837-1.44, $P \geq 0.156$, data not shown). Osteoprotegerin mRNA expression, however, was

	Control	Vibrated	Wheel Running	One Way ANOVA P-value
Active time (min)	17.1 (0.6)	18.3 (0.4)	16.0 (0.9)	0.054
Stereotypic time (min)	10.2 (0.5)	11.6 (0.6)	9.2 (1.0)	0.111
Ambulatory distance (m)	49.3 (2.5)	55.3 (2.3)	45.8 (5.0)	0.157

Values are means (SE).

Table 2. Comparison of 30-minute cage activity levels between *mdx* mice in typical mouse cages (control), following vibration, or wheel running.

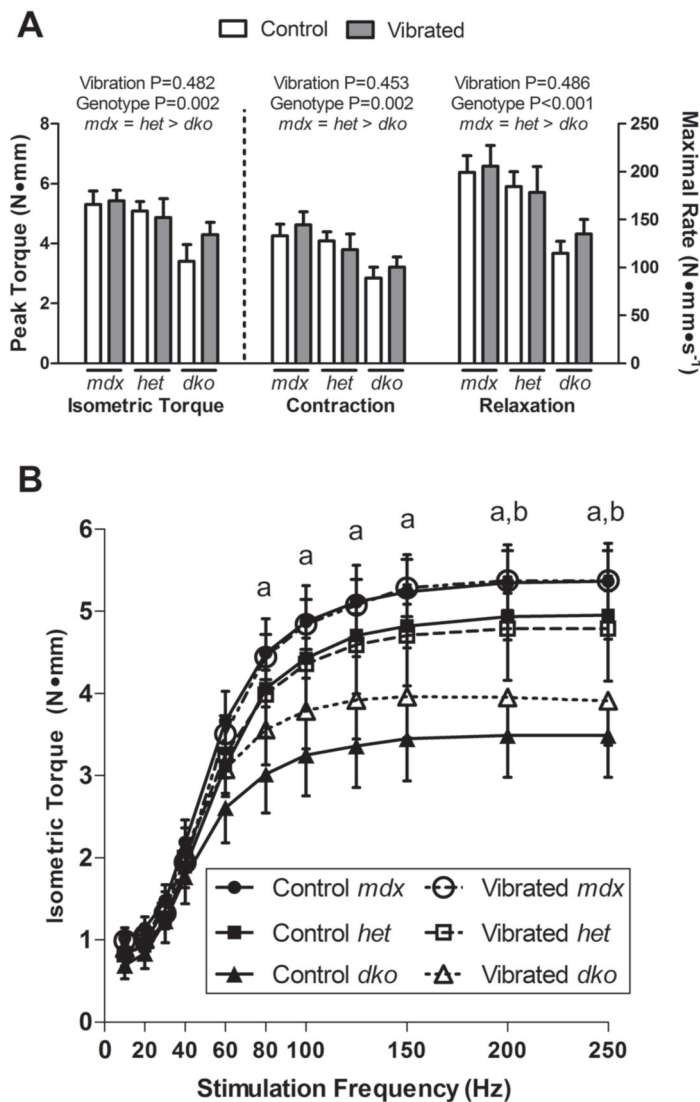


Figure 3. Seven daily bouts of low intensity vibration training on posterior crural muscle contractility in three mouse models of DMD which vary in their disease severity was investigated in Study 3. Data are mean, SE. **A:** Peak isometric torque and rates of contraction and relaxation were not different between Control and Vibrated mice. *Dko* mice consistently had lower contractility than both *mdx* and *het* mice. Main effect P-values from two-way ANOVA's are indicated above each set of bars with post-hoc results in words immediately below the genotype main effects. **B:** Isometric torque production as a function of stimulation frequency indicated that vibration did not impact torque production at any frequency; however *dko* mice had lower torque production at frequencies above 80 Hz. Only main effects of genotype were detected, and therefore only post-hoc testing results are displayed: ^aSignificant difference between *mdx* and *dko*; ^bSignificant difference between *het* and *dko*.

significantly upregulated in *mdx* mice vibrated at 90 Hz and 0.6 g, and trended at 45 Hz and 0.6 g ($P=0.109$) compared to non-vibrated *mdx* mice (Figure 1B). RANKL mRNA expression was 1.24 fold higher at 90 Hz and 0.6 g, and equivalent to control *mdx* mice at 45 Hz and 0.6 g (Figure 1C), however the ratio of osteoprotegerin and RANKL was not different from controls in either group (data not shown, $P\geq 0.476$). In addition, alkaline phosphatase mRNA expression at 45 Hz and 0.6 g was increased, but this increase was not apparent at 90 Hz at 0.6 g (Figure 1D). Due to the relative elevations of alkaline phosphatase and osteoprotegerin mRNA expression at 45 Hz at 0.6 g those parameters were used for subsequent muscle-specific studies in dystrophic mice.

Study 2, Contractility: *Mdx* mice in the wheel running group voluntarily ran 17.5 ± 2.0 km over the 3-d study and *mdx* mice in the eccentric group performed 100 eccentric contractions resulting in an immediate 79% loss in eccentric torque.

Following three days of normal cage activity (i.e., controls), vibration, wheel running, or a bout of eccentric contractions, peak isometric torque produced by the posterior crural muscles was significantly different between groups (Figure 2A). Vibrated mice were equivalent to both control and wheel running mice. As hypothesized, *mdx* mice that performed eccentric contractions had up to 37% lower isometric torque values, though post-hoc testing did not detect a significant difference between any of the groups (Figure 2A). CK activity levels were different among groups ($P=0.020$), with eccentric mice having 24% higher plasma CK activity levels than controls (2829.5 ± 95.2 U/L vs 2278.9 ± 122.1 U/L). There were no differences in CK activity levels between vibrated (2489.3 ± 115.6 U/L), wheel running (2648.9 ± 102.2) and control mice.

Additional measures of posterior crural muscle contractile function assessed in Study 2 including maximal rates of contraction and relaxation during isometric contractions and isometric torque as a function of stimulation frequency confirm that vibration was not injurious to *mdx* muscle (Figure 2). Muscles of vibrated mice generated equivalent torque compared to control mice and greater torque compared to eccentrically-injured muscles at both 10 and 30 Hz (Figure 2B). Combined, these data indicate that muscle contractile function was not compromised by vibration in *mdx* mice, as was shown with eccentrically-injured muscle of *mdx* mice.

In addition, cage activity levels were not reduced during the 30 min immediately following vibration, and in fact, these mice tended to be 7% more active than non-vibrated controls (Table 2). This increase in cage activity corresponded to ~70 sec, and was primarily spent ambulating (11.6 ± 0.5 min in vibrated mice and 10.2 ± 0.5 min in control mice, $P=0.107$). Despite the increase in active time, vibrated mice traveled equivalent total distances (Table 2).

Study 3, Contractility: Body mass did not significantly differ among the groups following 7 d of vibration exposure (14.5 ± 0.5 g, $P\geq 0.429$).

In vivo contractile testing of the posterior crural muscles showed that vibration had no deleterious consequences on muscle function, however, by design, genotypic differences in

contractile function were apparent. Peak isometric torque was not influenced by vibration, while *dko* mice had up to 38% lower torque and rates of contraction and relaxation compared to both *mdx* and *het* mice (Figure 3A). Similar patterns were seen for isometric torque as a function of stimulation frequency. Vibration had no effect on submaximal torques and *dko* mice had lower isometric torque values at all frequencies above 80 Hz (Figure 3B). CK activity was measured to assess if vibration was more injurious to models of DMD that are more susceptible to injury. There was no effect of genotype or 7 d of vibration on plasma CK activity (1354 ± 34 U/L for all groups; $P\geq 0.514$).

Studies 2 and 3, Gene Expression

mRNA expression profiles were assessed in two muscles to determine if early markers of muscle inflammation and myogenesis were affected by short-term vibration training. Vibration had no impact on any of the genes assessed in the gastrocnemius muscle following 3 d of exposure ($P\geq 0.126$), however, wheel running and eccentric injured muscles had a nearly 7-fold upregulation of MAC-1 and MCP-1 mRNA expression compared to control muscles (Figure 4A). Extending vibration training out to 7 d and using the most severe model of DMD (i.e., *dko* mice) did not evoke changes in tibialis anterior muscle mRNA expression levels in genes associated with muscle inflammation or myogenesis (Figure 4B, $P\geq 0.192$). *Het* mice, however did have a small but statistically significant 1.4 fold increase in Pax-7 mRNA when vibrated (Figure 4B, $P=0.044$). Combined, these data suggest that vibration is not injurious to dystrophic muscle in terms of eliciting an inflammatory response and also showed little evidence for initiating myogenesis.

Discussion

We had three primary findings from our project. First, low intensity vibration was well tolerated in each of the three dystrophic mouse models utilized (i.e., *mdx*, *het* and *dko* mice) and at two ages in the *mdx* mice. It also did not appear to be stressful based on preservation of cage activities and body mass. Second, in response to 14 d of low intensity vibration training, 45 Hz and 0.6 g was identified as the set of parameters that tended to be the most osteogenic in *mdx* mice. As such, subsequent studies on muscle responses to vibration were performed using these parameters of vibration. Third, vibration was consistently shown to be non-injurious to dystrophic muscle of various disease severity. Contractile function of the posterior crural muscles was not different between non-vibrated and vibrated dystrophic mice, and plasma CK activity and expression of genes associated with muscle inflammation also were not affected by vibration. The long term goal that this study begins to address is the potential of low intensity vibration as a therapeutic modality for DMD. The utility of vibration has received increasing attention in recent years due to its ability to improve musculoskeletal health⁴²⁻⁴⁴, especially

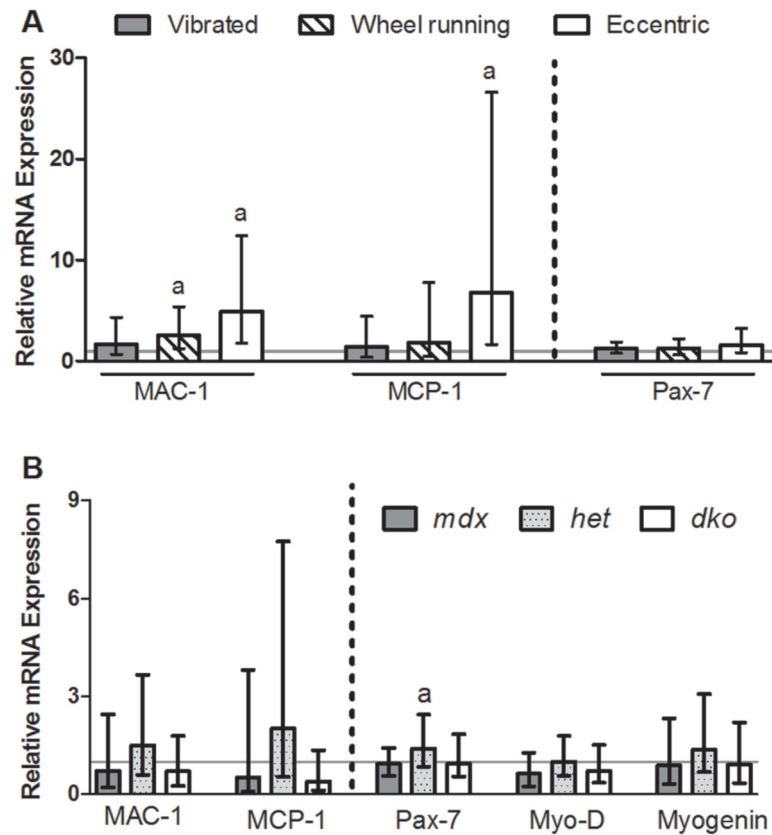


Figure 4. Low intensity vibration training for three or seven days (in Studies 2 and 3, respectively) did not affect muscle mRNA expression of genes associated with inflammation or myogenesis. RNA was isolated from **A:** gastrocnemius muscles of *mdx* mice three days following interventions of vibration, wheel running, or eccentric contractions and compared to those of control *mdx* mice (indicated by the solid gray line) and **B:** tibialis anterior muscle from *mdx*, *het* and *dko* mice that were vibrated for seven days and compared to genotype-matched, non-vibrated controls (indicated by the solid gray line). The height of the bars indicate the fold change in mRNA expression (i.e., delta-delta CT) above controls and the error bars indicate the 95% confidence intervals. *Significantly different from respective, non-vibrated *mdx* mice as determined by REST, 2009 software ($P < 0.05$).

in disabled children and women with low bone mass^{13-15,45}. Theoretically, vibration would mechanically load bone to maintain bone health yet would be safe for the adjacent diseased muscle, and results of our study support this theory.

Results from our first study identified two sets of vibration parameters (i.e., 90 Hz at 0.6 g and 45 Hz at 0.6 g) that evoked an osteogenic response (Figure 1). The 45 Hz and 0.6 g setting increased both osteoprotegerin and alkaline phosphatase mRNA levels up to 30%, but did not impact RANKL (Figure 1). Similar fold-change increases in alkaline phosphatase mRNA expression have been reported following 2-4 d of a known anabolic stimulus (i.e., four-point bending^{19,46}). The 90 Hz and 0.6 g setting up-regulated osteoprotegerin and RANKL to similar extents, and therefore the increase in RANKL could potentially have negated any osteoprotegerin-induced osteogenic effects. In fact, these mice had the highest levels of circulating bone resorption activity as indicated by plasma CTX levels (Figure 1A). Upregulation of RANKL has also been reported in mice vibrated for 21 d²², however in cells, vibration decreased RANKL mRNA expression up to 55% with no change in osteoprotegerin⁴⁷. Con-

sistent with our findings, others have shown no change in mRNA expression following vibration for Collagen type I²², BMP-2^{19,22}, and Runx-2¹⁹. Consequently, our subsequent studies were performed utilizing 45 Hz and 0.6 g because that pair of parameters tended to increase osteoprotegerin expression with no change in RANKL and also elicited a 1.4 fold increase of alkaline phosphatase mRNA expression, which is involved with matrix maturation and mineralization.

The results of our second and third studies consistently showed that vibration was not injurious to inherently fragile dystrophic muscle. The most crucial pieces of evidence to support this statement are the functional results. That is, there was no indication that strength (submaximal or maximal isometric torque) or contractility rates (contraction or relaxation) of the posterior crural muscles were affected by 3 or 7 d of vibration treatment in dystrophic mice. This was in contrast to other interventions, such as eccentric contractions, that did cause loss of strength (Figure 2). This was substantiated in the traditional *mdx* mouse at two critical ages, as well as in more severe dystrophic mouse models, *het* and *dko* mice (Figure 3).

The expression profiles of genes associated with muscle inflammation and recovery from injury further support our hypothesis that vibration is not injurious. MAC-1 and MCP-1 are genes that mediate the recruitment and activation of inflammatory cells following muscle damage and these genes were not upregulated after either 3 or 7 d of vibration, but were increased by wheel running or eccentric contractions (Figure 4). These data indicated that vibration is on the low end of the continuum of exercises that cause damage in dystrophic mice. Specifically, eccentrically-injured muscles had 5-7 fold increases in MAC-1 and MCP-1 similar to previous reports^{48,49}. Interestingly, *het* mice had trends toward increased MAC-1 and MCP-1 following 7 d of vibration, supporting the notion that *het* mice may be more sensitive to developing, and thus better models of, inflammation and fibrosis compared to *mdx* mice³⁰.

Genes associated with myogenesis were also not affected by vibration, with the exception of upregulated Pax-7 gene following 7 d of vibration in *het* mice (Figure 4). Vibration of cultured C2C12 myoblasts has previously shown a vibration dose-dependent increase in MyoD and myogenin expression⁵⁰. High intensity vibration training for two weeks in young mice induced muscle hypertrophy that was attributed to enhanced fusion and differentiation of satellite cells, and muscle fiber number and cross-sectional area^{51,52}. Vibration-induced muscle hypertrophy has also been associated with the inhibition of muscle atrophy pathways by downregulating myostatin gene expression⁵³. On the contrary, our previous work showed that eight weeks of low intensity vibration training in wildtype mice improved muscle functional capacity in the absence of hypertrophy²⁵. Specifically, muscle strength improved by 14% but muscle mass, protein content, and fiber cross-sectional area were not affected²⁵. Given these improvements in the muscle function of wildtype mice, a future goal is to determine if similar improvements are attainable in dystrophic mice and DMD patients. Reyes et al.⁴⁵ has applied vibration directly to the elbow of children with motor disabilities (including patients with DMD) and reported a 65% increase in muscle force compared to a 20% decline in control patients.

The present studies thoroughly characterized dystrophic muscle function following short-term vibration training, after determining the vibration parameters that evoked the greatest osteogenic response in mouse models of DMD. The results suggest that vibration at 45 Hz and 0.6 g has the potential to have anabolic impact on bone health, while not causing injury to inherently fragile dystrophic muscle. Additional long-term treatment studies are needed to determine the efficacy of vibration to improve both bone and muscle in dystrophic mice and to improve musculoskeletal health in DMD.

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