

Prednisolone treatment and restricted physical activity further compromise bone of *mdx* mice

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Abstract

Objectives: The purpose of this study was to determine the extent to which prednisolone treatment and restricted physical activity caused deleterious changes in inherently compromised *mdx* bone. **Methods:** Four week-old male *mdx* mice (n=36) were treated for 8-wk either with or without prednisolone (0.8-1.3 mg/kg/d) and were housed in traditional or small cages (restricted activity). Tibial bone strength, geometry, and intrinsic material properties were assessed at the mid-shaft by three-point bending and micro-computed tomography (μ CT). **Results:** Three-point bending results showed that both prednisolone and restricted activity reduced bone strength (7%), however stiffness was only reduced in restricted-activity mice. μ CT analyses showed that cortical bone area and cortical thickness were 13% smaller in restricted-activity mice, and may have accounted for their compromised bone strength. Intrinsic material properties, including volumetric bone mineral density (vBMD) and modulus of elasticity, were not impacted by either treatment, however, vBMD tended to be lower in restricted-activity mice (p=0.06). **Conclusions:** These data show that prednisolone treatment and restricted physical activity independently accentuate reductions in the strength and geometry of *mdx* bone, but do not influence intrinsic material properties.

Keywords: Prednisone, Corticosteroid, Duchenne Muscular Dystrophy, Bone Strength, Bone Geometry

Introduction

Bone health is compromised secondarily to many diseases. One example of this is Duchenne muscular dystrophy (DMD), an X chromosome-linked neuromuscular disease that has a prevalence of 1.3 to 1.8 per 10,000 males aged 5-24 years¹. DMD is characterized by progressive muscle weakness that leads to physical inactivity and inevitably causes patients to become non-ambulatory by their early teenage years^{2,3}. Consequently, as dystrophic muscle becomes weaker, not only are there less forceful contractions applying lower loads on bone, fewer muscle-induced loads to bone are applied. As a result,

bone is continually adjusting its material and geometric properties to its current loading environment⁴ and disuse-mediated bone loss can be initiated⁵. The bone loss that results may largely explain the declines in areal bone mineral density that has been shown in boys with DMD as they age^{6,7}.

Another insult to bone health is chronic usage of glucocorticoids such as prednisone, prednisolone and deflazacort, which are prescribed to improve muscle strength and performance in DMD patients⁸⁻¹¹. For example, prednisone has been shown to thwart muscle deterioration, improve physical endurance, decrease falls, improve stair climbing, and prolong independent ambulation in patients with DMD⁸⁻¹¹. In parallel with these benefits to muscle tissue, glucocorticoids induce osteoporosis and increase fracture risk by increasing osteocyte and osteoblast apoptosis as well as extending the lifespan of osteoclasts¹²⁻¹⁴. Specific to DMD, the use of glucocorticoids has been shown to accentuate reductions in bone mass and density^{7,15,16}, increase the incidence of long bone fractures by nearly three-fold¹⁵, and provoke the emergence of vertebral compression fractures which do not occur in patients with DMD whom are not treated with glucocorticoids^{15,17}. These results suggest that altered os-

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teoblast and osteoclast viability disrupt the homeostasis of bone remodeling and therefore may translate to compromised bone structure and strength. Considering that bone is inherently compromised in strength and has altered geometry in individuals with DMD¹⁸⁻²⁴, the addition of reduced physical activity and the use of a glucocorticoid may cause further decrements to the structural and material integrity of bone, but contributions of these combined insults have not been thoroughly investigated.

The *mdx* mouse is an animal model of DMD that can be utilized to investigate the musculoskeletal system because the mice display muscle weakness²⁵, reduced physical activities²⁶, and compromised bone structure and function compared to wildtype mice¹⁹⁻²¹. The deficits in muscle function and physical activity in *mdx* mice are less severe relative to patients with DMD and theoretically would have lesser impacts on bone loading. Therefore, an additional intervention was utilized in the current study to amplify reductions in mechanical loading of hindlimb bone in *mdx* mice. Traditional approaches for reducing mechanical loading in rodent models include denervation, hindlimb suspension and casting^{27,28}. These models however, drastically disrupt the ability of mice to ambulate, and therefore would not appropriately model reductions in physical activity in DMD patients. We used the approach of housing *mdx* mice in cages that were 80% smaller in volume than typical mouse cages to restrict physical activities while still permitting the mice some movement. We theorized that this approach would better mimic boys with DMD who are physically inactive yet retained some ambulation, standing, and activities of daily living.

Quantifying the functional capacity of bone is a good predictor of fracture risk. This requires *ex vivo* analyses of bone and thus is not possible to directly study in boys with DMD following glucocorticoid treatment and in response to reduced physical activity. Therefore, the primary objective of this study was to use the *mdx* mouse model of DMD to determine the extent to which a glucocorticoid and restricted physical activity alter the functional capacity of the tibia as well as determine if alterations in functional capacity are explained by changes in geometric and/or intrinsic material properties. Based on glucocorticoids' resorptive effects on bone, we hypothesized that tibial bones from prednisolone-treated *mdx* mice would have diminished bone strength and altered bone geometry compared to placebo-treated *mdx* mice. We also sought to determine if restrictions on cage activities (i.e., to mimic limited physical activity in boys with DMD) exacerbated the deleterious effects of disease and prednisolone treatment on tibial bone in *mdx* mice. We hypothesized that imposing restrictions on cage activity of *mdx* mice would be detrimental to tibial bone functional capacity and cortical bone geometry and further that reduced physical activity coupled with prednisolone treatment would be the most detrimental to bone structure and function.

Methods

Animals

Male *mdx* mice aged 4 week (n=36) were purchased from Jackson Laboratories. Mice were housed in groups of four on a 12:12 hour light-dark cycle at 20-23°C and were provided

food and water *ad libitum*. At 5 weeks of age, mice were anesthetized for pellet implantation using fentanyl citrate (10 mg/kg body weight (BW)), droperidol (0.2 mg/kg BW) and diazepam (5 mg/kg BW). At the age of 12-13 weeks, mice were sacrificed with an overdose of sodium pentobarbital (200 mg/kg BW). Animal care and use procedures were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Experimental design

At 5 weeks of age, *mdx* mice were randomly assigned to one of four conditions, either without (placebo) or with prednisolone treatment and either housed in normal sized cages (placebo: n=8; prednisolone: n=10) or small cages (i.e., placebo restricted activity, n=8 and prednisolone restricted activity, n=10). At this same age, placebo or prednisolone treatment was initiated and continually administered over a period of 60 days via implanted time-released pellet as previously described²⁹. Mice that were assigned to restricted activity groups were placed individually in small cages (plastic inserts sized length x width x height: 11.8 cm x 9.0 cm x 10.7 cm) to limit horizontal physical activities, namely ambulation.

Cage physical activity of each mouse was measured over a 24-hour period, approximately 35 days after pellet implantation. At 12-13 weeks of age, mice were sacrificed and tibial bones were excised. Bones were stored in phosphate-buffered saline at -20°C until the time of analysis. A subset of the physical activity data have been published previously. Specifically, cage activity monitoring for the *mdx* placebo and prednisolone groups were reported in Study 2 of Baltgalvis et al.²⁹. All data pertaining to mice with restricted activity, as well as all bone data have not been previously published.

Prednisolone pellet implantation and dosage

As previously described, mice were anesthetized using fentanyl citrate, droperidol and diazepam and the pellets were implanted on the dorsal side of the neck using a trochar²⁹. The 1.5 mg, 60-day slow-release pellet (Innovative Research of America) is designed to release a constant daily dosage of drug (approximately 0.8-1.3 mg/kg/day of prednisolone). This dosage approximates the most effective dose of prednisone (0.75 mg/kg/day) prescribed to DMD patients (reviewed in¹¹) and has also been shown to improve muscle performance in *mdx* mice³⁰⁻³⁴. We chose to treat mice with the glucocorticoid, prednisolone, as it eliminates the need for hepatic conversion of prednisone into its active form prednisolone and also because deflazacort was not available to us. Placebo pellets contained the same matrix-driven delivery system as the prednisolone pellets, but did not contain the glucocorticoid.

Physical activity monitoring

Physical activity of individual mice was monitored for 24 hours using an open-field activity chamber and Activity Monitor software, version 5 (Med Associates Inc.) as previously described³⁵. Briefly, the chamber contains an array of infrared beams, in which the software monitors 'activity counts' every

time an infrared beam is interrupted, along with how long the beam is interrupted. The methods used to discern the different types of physical activity are defined in Greising et al., and include active time, resting time, ambulatory distance, rearing counts, and stereotypic (i.e., eating or grooming) counts³⁵. *Mdx* mice randomized to the restricted activity groups remained housed in small-sized cages (11.8 cm x 9.0 cm) within the activity chamber during physical activity monitoring, and *mdx* mice randomized to full activity groups had access to the entire area of the activity chamber (27.1cm x 27.9 cm).

Mechanical testing of tibial mid-diaphysis

The procedures used to assess the functional capacities of the mouse tibia have been described in detail previously^{21,36,37}. Briefly, tibial bones were placed on their lateral side in a Mecmesin MultiTest 1-D test machine and underwent three-point bending at the mid-diaphysis using a Mecmesin AFG-25 load cell (Mecmesin). Functional measures of the tibial bone were quantified by ultimate load, stiffness, and deflection and energy absorbed to ultimate load using custom designed TestPoint software (TestPoint version 7; Measurement Computing Corp.) as previously described²¹. Upon completion of mechanical testing, bones were refrozen in PBS and stored at -80°C until the time they underwent micro computed tomography (μ CT) scanning. Intrinsic material properties for each tibia (i.e., ultimate stress and modulus of elasticity) were derived from three-point bending and μ CT outcome measures as previously described^{21,36,37}.

μ CT of the tibial diaphysis

Following mechanical testing, a region near the mid-diaphysis of the tibia was scanned with a μ CT system (Scanco Medical μ CT 40) to quantify cortical bone geometry and volumetric bone density (vBMD). The μ CT was set to a voltage of 55 kVp and a current of 145 μ A, and bones were scanned using an isotropic 12 μ m voxel size with a 250-ms integration time. For diaphyseal scanning, the distal half of the tibiae was positioned vertically in a scan tube and its length measured on the scout view image. Start position was set at 85% of the total length in the proximal direction from the distal-most point of the tibia (i.e., slightly distal to where the bone broke during three-point bending), and 66 slices (~0.8 mm) distal to this point were scanned. Outcome measures for cortical bone at the tibial diaphysis include cortical bone cross-sectional area, cortical thickness, periosteal diameter, cross-sectional moment of inertia (CSMI) and vBMD. Each of these outcomes measures were assessed independently at each of the 66 slices, and the average of all 66 slices was calculated and used for statistical analyses. As previously noted, the minimum principal CSMI (CSMI_{min}) was utilized in the present study due to its correspondence with the CSMI about the bone bending axis during three-point bending testing²¹.

Statistical Analyses

To analyze the effects of drug (placebo vs. prednisolone) and physical activity (“normal cage size” activity vs. “small

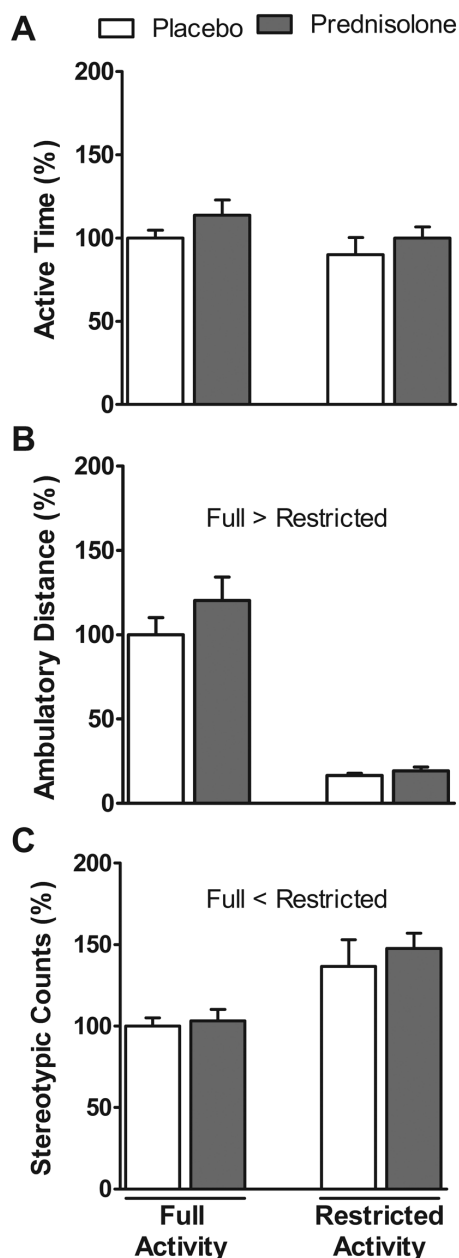


Figure 1. Effects of prednisolone and restricted activity on 24-hour physical activity measures including (A) active time, (B) ambulatory distance and (C) stereotypic counts. Values are mean \pm SE. Data are normalized to the values of placebo-treated *mdx* mice housed in normal-sized cages that permitted full activity. Restricted Activity refers to *mdx* mice housed in 80% smaller cage by volume. Significant main effects of two-way ANOVA ($p < 0.05$) are indicated above the bars.

cage size” activity), 2-way ANOVAs were utilized. When interactions were significant ($p < 0.05$), Holm-Sidak post-hoc tests were performed to determine which combinations of conditions were different from each other. Pearson correlations were also calculated between tibial bone length and other geometric properties to provide insight into the influence of lon-

itudinal growth on the bone cross-sectional geometry. All statistical analyses were carried out using SigmaStat version 3.5 (Systat Software Inc.).

Results

Physical activity levels and body masses

Twenty-four-hour activity monitoring was performed to confirm that housing *mdx* mice in small cages decreased physical activity, thus mirroring the reductions seen in ambulatory distance in DMD patients. There was no effect of prednisolone on any parameter of cage activity monitored ($p \geq 0.154$; Figure 1). There was also no effect of cage size on active time (Figure 1A). All groups of *mdx* mice spent ~4 hours per day being active; however, the types of activities performed during this time were different between activity groups. *Mdx* mice housed in small cages ambulated 84% less distance than traditionally housed *mdx* mice (76 ± 27 vs. 463 ± 27 m/24 hr, respectively; $p < 0.001$; Figure 1B), which equated to the mice in small cages spending 87 minutes less per day ambulating. Subsequently, those restricted-activity mice spent more time grooming, as indicated by 40% more stereotypic counts ($p < 0.001$; Figure 1C).

Body masses were not different among the four groups of *mdx* mice at 5 weeks of age (21.0 ± 0.2 g; $p > 0.061$). However at 12 weeks of age, prednisolone-treated mice weighed 4% less than placebo-treated mice (29.9 ± 0.3 vs. 31.1 ± 0.4 g, respectively; $p = 0.018$) and restricted-activity mice weighed 7% less than those housed in normal-sized cages (29.3 ± 0.3 vs. 31.6 ± 0.5 g, respectively; $p < 0.001$).

Effects of prednisolone and restricted activity on tibial bone mechanical functional capacity

In general, the functional capacity of the tibial bone was negatively affected by both prednisolone and restricted activity. Ultimate loads for the tibia of prednisolone-treated and restricted activity *mdx* mice were low (Figure 2A and Table 1), demonstrating that both treatments independently compromise bone strength. Restricted activity also affected tibial bone stiffness (Figure 2B and Table 1), indicating the bone's ability to resist bending was reduced. The combination of reductions in tibial bone ultimate load and stiffness in restricted activity mice translated to the 15% increase in deflection to ultimate load (Table 1). Energy absorbed to ultimate load, however, was not impacted by either prednisolone or physical activity level ($p \geq 0.081$). Collectively, these results indicate that restrictions on physical activity cause further decrements in the functional capacities of the *mdx* tibial bone compared to disease progression alone, while prednisolone appears to negatively impact only the bone's bending strength.

Effects of prednisolone and restricted activity on tibial bone geometry

μ CT was utilized to determine if tibial bone geometry near the mid-shaft was impacted by prednisolone or restricted activity, which may have contributed to the alterations in mechanical functional capacity. For prednisolone-treated *mdx*

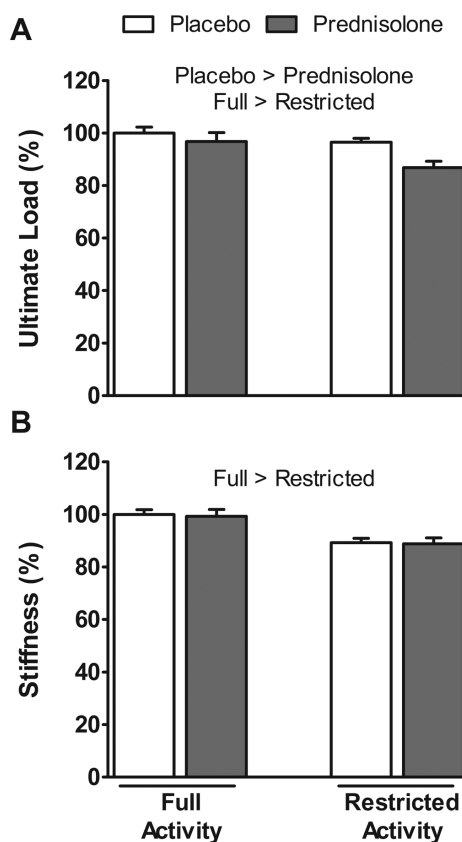


Figure 2. Effects of prednisolone and restricted activity on tibial bone (A) ultimate load and (B) stiffness. Values are mean \pm SE. Data are normalized to the values of placebo-treated *mdx* mice housed in normal-sized cages to permit full activity. Restricted Activity refers to *mdx* mice housed in 80% smaller cage by volume. Significant main effects of two-way ANOVA ($p < 0.05$) are indicated above the bars.

mice, the area of the cortical bone was 8% smaller, which resulted from 6% smaller cortical thickness (Table 1). These deficits likely contributed to the reduction in ultimate load seen in prednisolone-treated mice. Additional measures of cortical bone geometry, including periosteal diameter and CSMI, were not affected by prednisolone treatment ($p > 0.26$). Restricted activity consistently showed a detrimental influence on bone geometry. Specifically, *mdx* mice that were restricted had 13% smaller cortical cross-sectional area, 13% smaller cortical thickness and 3% smaller periosteal diameter, and 17% lower CSMI (i.e., reflection of the moment of inertia during three-point bending), compared to *mdx* mice with non-restricted activity (Table 1). Tibial bone length (Table 1), however was positively correlated with cortical cross-sectional area, cortical thickness and CSMI ($r = 0.37$ to 0.48 ; $p < 0.03$) but not periosteal diameter ($p = 0.21$). Combined, these reductions in cortical bone geometry in *mdx* mice with restricted activity indicate that reductions in physical activity resulted in smaller sized tibial bones and thus may have contributed to the differences in tibial bone mechanical properties.

	Placebo	Placebo + Restricted Activity	Prednisolone	Prednisolone + Restricted Activity	P-values for Two-Way ANOVA		
					Main effect of Prednisolone	Main effect of Restricted Activity	Interaction (Prednisolone x Restricted Activity)
Mechanical Function Properties							
Ultimate Load (N)	13.72 (0.32)	13.24 (0.21)	13.29 (0.46)	11.91 (0.34)	0.022	0.016	0.227
Stiffness (N/mm)	41.66 (0.78)	37.20 (0.71)	41.39 (1.08)	37.02 (0.94)	0.810	<0.001	0.963
Deflection to Ultimate Load (mm)	0.41 (0.02)	0.51 (0.04)	0.41 (0.01)	0.44 (0.03)	0.196	0.017	0.184
Energy Absorbed to Ultimate Load (mJ)	3.12 (0.20)	3.42 (0.32)	3.01 (0.10)	2.74 (0.23)	0.081	0.938	0.193
Geometric Properties							
Tibial Length (mm)	18.51 (0.08)	17.74 ^a (0.18)	18.04 ^a (0.07)	18.14 ^b (0.12)	-	-	<0.001
Cortical Cross-sectional Area (mm ²)	0.74 (0.02)	0.67 (0.02)	0.71 (0.02)	0.60 (0.02)	0.015	<0.001	0.381
Cortical Thickness (mm)	0.25 (0.005)	0.22 (0.004)	0.23 (0.004)	0.20 (0.003)	<0.001	<0.001	0.878
Periosteal Diameter (mm)	1.08 (0.01)	1.05 (0.01)	1.08 (0.02)	1.05 (0.01)	0.880	0.041	0.980
CSMI (mm ⁴)	0.070 (0.003)	0.053 (0.002)	0.061 (0.003)	0.052 (0.002)	0.255	<0.001	0.551
Intrinsic Material Properties							
Ultimate Stress (MPa)	284.8 (9.5)	327.2 ^a (5.5)	294.8 (8.0)	302.1 ^b (8.5)	-	-	0.039
Modulus of Elasticity (GPa)	13.8 (0.5)	14.7 (0.5)	14.3 (0.4)	15.0 (0.5)	0.396	0.112	0.871
vBMD (mg/cm ³)	1317.0 (6.1)	1312.1 (4.7)	1325.5 (5.4)	1309.6 (4.9)	0.582	0.061	0.311
<i>Values are means (SE). CSMI, cross-sectional moment of inertia. vBMD, volumetric bone mineral density. ^a Significantly different from Placebo. ^b Significantly different from Placebo + Restricted Activity.</i>							

Table 1. Effects of prednisolone and restricted activity on tibial bone mechanical function, geometric properties and intrinsic material properties in *mdx* mice.

Effects of prednisolone and restricted activity on tibial bone intrinsic material properties

Intrinsic material properties of the tibia were quantified by ultimate stress, modulus of elasticity and vBMD. Ultimate stress values were 15% higher in placebo-treated mice with restricted activity compared to placebo mice (i.e., significant interaction; Table 1). The combination of prednisolone and restricted activity blunted this increase, such that ultimate stress values were similar to placebo mice but 8% lower than restricted-activity placebo mice (Table 1). Differences among groups were not apparent for modulus of elasticity and vBMD,

although vBMD tended to be lower in restricted activity mice (i.e., $p=0.06$ main effect of activity).

Discussion

We had two primary findings from our study. First, mechanical testing of tibial bones from *mdx* mice showed that both prednisolone treatment and restricted physical activity independently reduced the ultimate load (Figure 2A and Table 1). Secondly, reductions in tibial bone strength and stiffness were primarily attributed to having smaller bone geometry at the

tibial midshaft, rather than alterations in the intrinsic material properties, such as vBMD (Table 1). Combined, these data indicate that prednisolone treatment and restricted physical activity independently augment the deleterious effects associated with DMD disease progression on the bone's functional capacity and geometry, and may therefore further predispose bone to fracture.

Our previous work confirmed that *mdx* mice have inherently weak tibial bones (e.g., 20-50% lower ultimate loads) relative to wildtype mice²¹, and the present study extends these findings using clinically-relevant conditions. Specifically, prednisolone treatment and restricted physical activity each resulted in a 7% reduction in ultimate load (Figure 2A). Similar 9% declines in bone strength have been realized in rats after 8 weeks of prednisone treatment³⁸; however up to 50% reductions in mouse femur bone strength have been realized with high doses of prednisone (i.e., 1.4 mg/kg/day)³⁹. Additional evidence implicating glucocorticoids' role in altering bone strength arises from the occurrence of vertebral compression fractures that are present in DMD patients only after taking prednisone in excess of three years^{15,17}. Compression tests performed on vertebrae have confirmed these clinical observations suggesting bone strength is indeed compromised following the use of glucocorticoids such as prednisone^{12,40,41}. Combined, these data highlight the deleterious effect of this class of drugs on the strength of various bones. The declines in mechanical function following restricted physical activity are in accordance with other forms of severe immobilization (i.e., denervation, fixation, or hindlimb suspension), in which bone strength is reduced 10-20%^{27,28}. Reductions in tibial bone stiffness, however, were only apparent in mice with restricted activity (-11%, Figure 2B), highlighting the importance of dynamic loading (e.g. ambulation) rather than static loads (i.e., grooming) for the preservation of bone's mechanical strength and stiffness in dystrophic mice.

The mechanical strength of bone is largely dependent upon the geometry, or shape, of the bone as well as its material properties. Therefore, to determine the extent to which cortical bone geometry was affected by prednisolone treatment and restricted activity, μ CT was performed. Our present work confirms previous studies suggesting that glucocorticoid treatment and reduced physical activity have the capacity to negatively alter bone geometry^{15,42,43}, as well as expands our previous work showing that the bone of dystrophic mice has altered bone geometry²¹. Specifically, prednisolone treatment and restricted physical activity independently caused up to 12% further decrements in tibial bone's cortical cross-sectional area and cortical thickness compared to disease progression alone (Table 1). These data are in agreement with preliminary pQCT findings in boys with DMD, which suggest that cross-sectional areas of the tibia and radius are reduced, and following the loss of ambulation, the areas become further reduced²²⁻²⁴. CSMI, which is the most precise determinant of bone's mechanical properties during three-point bending, was not affected by prednisolone treatment, but was lower in *mdx* mice with restricted activity (Table 1). Preliminary pQCT data show that boys with DMD have 32% lower strength-strain index

(i.e., a pQCT-derived surrogate for polar CSMI) compared to healthy boys²⁴. Further, those findings suggest that differences were accentuated in non-ambulatory boys, but congruent with the present study, were not influenced by the use of glucocorticoid. It is possible that in our study differences in the rates of longitudinal bone growth among groups of mice may partially or totally account for the observed differences in bone cross-sectional geometry. To probe this idea, we calculated correlations and found that cortical cross-sectional area, cortical thickness and CSMI (but not periosteal diameter) were modestly correlated with tibial bone length. Despite these positive associations, the among-group statistical comparison findings for cortical cross-sectional area and CSMI do not match those for tibial bone length (Table 1), suggesting that differential longitudinal bone growth rates do not completely explain the differences among groups in bone cross-sectional geometry. Combined, these results indicate that glucocorticoids and restricted physical activity both have deleterious effects on bone cross-sectional area; however, physical activity alone has a negative impact on CSMI.

Beyond investigating the role of prednisolone and restricted activity's capacity to alter bone geometry, we also investigated the impact that these factors have on the tibial bone's intrinsic material properties. Neither modulus of elasticity nor vBMD were impacted by prednisolone use or restricted activity; however, there was a trend toward *mdx* mice with restricted activity having lower vBMD ($p=0.06$, Table 1). Declines in bone mass following reductions in physical activity^{44,45}, as well as with DMD disease progression^{6,46,47} have been well documented. In the present study, the effect of restricted activity on tibia vBMD may have been minimized because mice engaged in some weight-bearing activities that loaded the hindlimb bones (i.e., rearing while doing stereotypic activities), which may have blunted loss of bone mass. Preliminary pQCT studies in boys with DMD show that vBMD is not affected by prednisone use²⁴ and cortical vBMD at the mid-diaphysis may actually be higher than age-matched controls^{22,23}, suggesting that bone's intrinsic material properties are also minimally affected by glucocorticoids.

In summary, the present study has established that prednisolone treatment and restrictions on physical activity negatively and independently accentuate reductions in the strength of cortical bone of *mdx* mice. Tibia from mice that were treated with prednisolone and were restricted in their physical activity had the lowest values of function and structure (e.g., ultimate load, cortical bone cross-sectional area, and cortical thickness). The reductions in bone strength are attributed to alterations in bone geometry rather than altered intrinsic material properties. These results indicate prednisolone and restrictions of physical activity have detrimental affects on skeletal health, and may possibly contribute to the development of bone fragility and heightened fracture risk in boys with DMD.

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