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Black bear parathyroid hormone has greater anabolic effects on trabecular bone in dystrophin-deficient mice than in wild type mice

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1.1 Introduction

Boys with Duchenne muscular dystrophy (DMD) progressively lose muscle strength and between the ages of 12–15 years are usually confined to a wheelchair [1]. The condition is typically fatal by 20–25 years due to respiratory or cardiac muscle failure [2]. DMD induced muscular necrosis and fibrosis are treated with glucocorticoids (e.g., prednisone or deflazacort) [3, 4]. DMD patients treated with glucocorticoids demonstrate a 30–50% decrease in bone mineral content and bone mineral density in the lumbar spine, hip, and long bones of the lower limbs as compared with healthy age-matched controls [3, 5–8]. Greater losses occur in trabecular bone than cortical bone due to its greater surface area for remodeling activity [3]. Fracture risk increases as a result. One study found that 44% of 71 boys with DMD sustained at least one fracture; of boys over the age of 16 years, 67% sustained at least one fracture [6]. The majority of fractures occur in the proximal and distal femur in DMD patients [9–11].

Like boys with DMD, *mdx* mice lack dystrophin [12–14]. These animals exhibit myopathic lesions characteristic of those seen in DMD patients [15, 16]. *Mdx* mice display larger body sizes and limb muscles, though these muscles are heavily fibrosed and less capable of high muscular forces [17]. At 3 weeks of age, *mdx* femurs display ~40% reductions in trabecular area, trabecular area fraction, and trabecular thickness as compared to age-matched wild type mice. They show decreases in osteoblast surface and increases in osteoclast surface area, leading to increased resorption and decreased formation [18]. In 4 week old male *mdx*

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mice, the metaphysis of the tibia showed decreases in bone volume fraction of 35%, with a decrease in trabecular number and increase in trabecular spacing. One study found no difference between *mdx* and wild type cortical thickness in the femur at 4 months of age [17]. However, at 6 months, *mdx* mice have decreased cortical bone properties relative to wild type mice and increased osteoclast number [18]. Whole bone breaking force and deformation at fracture are reduced in *mdx* mice regardless of age [19]. At 24 months of age, *mdx* mice show greater declines (relative to wild type mice) in trabecular and cortical properties in the tibia than at 7 weeks of age [20].

Treatments for DMD-related osteoporosis have not been widely explored. Treating DMD patients with the bisphosphonate alendronate prevented further decreases in bone mineral density over a two year period [21]. The impaired osteoblast function seen in both DMD patients and *mdx* mice suggests that an anabolic treatment for osteoporosis (i.e., PTH therapy) would be an effective therapeutic for increasing bone mass in these patients. Indeed, PTH treatment leads to greater reductions in fracture risk than alendronate in patients with glucocorticoid-induced osteoporosis [22]. Our laboratory has been investigating a novel PTH analog linked to preservation of bone during disuse. Black bears (*Ursus americanus*) hibernate for 6 months of the year, and do not experience disuse-related osteoporosis [23–25]. Black bear parathyroid hormone (bbPTH) has been positively correlated to bone formation markers during hibernation and is implicated in the mechanism of bone preservation during disuse [23, 25, 26]. Furthermore, bbPTH 1–34 causes greater reductions in serum starved induced caspase activity in osteoblasts than hPTH 1–34 [27]. Thus, bbPTH may be well suited to improve bone mass in cases of dystrophin deficiency that impair mobility. We hypothesized that bbPTH treatment in *mdx* mice would demonstrate improved bone properties compared to vehicle treated mice, and that bbPTH treatment would restore *mdx* bone properties to wild type levels.

2.1 Materials and Methods

2.1.1 PCR cloning and sequencing of bbPTH

Genomic DNA was extracted from black bear whole blood samples using the GenomicPrep Blood DNA Isolation Kit (Amersham Biosciences, Piscataway, NJ). The genomic DNA was used for PCR amplification of PTH using consensus primers designed based on alignment of eight full-length mammalian PTH sequences available in GenBank including bovine (*Bos taurus*, AAA30749), cat (*Felis catus*, Q9GL67), dog (*Canis familiaris*, P52212), human (*Homo sapiens*, NP_000306), macaque (*Macaca fascicularis*, Q9XT35), mouse (*Mus musculus*, NP_065648), pig (*Sus scrofa*, NP_999566), and rat (*Rattus norvegicus*, NP_058740). PCR amplification was performed using 10–15 ng genomic DNA, 100 μ M dNTPs, 0.2 μ M each primer, and 1 unit REDTaq (Sigma, St. Louis, MO) in 20 μ L reaction volume. PCR products were gel-purified using the UltraClean GelSpin Kit (MoBio Carlsbad, CA) and cloned into the pCRII vector using the TA cloning kit (Invitrogen, Carlsbad, CA). DNA sequencing was performed using the DTCS Quick Start kit and the CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Nucleotide sequences were searched against the GenBank protein database using BlastX [28] to confirm their putative identity as PTH. Multiple sequence alignment was performed by ClustalW version 1.82 [29]. The sequence obtained for mature black bear PTH (bbPTH; GenBank #GU563375) is shown in Figure 1.

Proteos (Kalamazoo, MI) recombinantly produced bbPTH 1–84 in *E. coli*. The purified protein were characterized by analytical HPLC and mass spectroscopy and subjected to quantitative amino acid analysis to determine exact protein concentration. Lyophilized protein aliquots were stored at -80 °C.

2.1.2 cAMP Assay

The PKA/cAMP pathway is primarily responsible for PTH's anabolic effects on osteoblasts, and previous work suggests that small changes in the amino acid sequence of human PTH can produce a peptide that induces greater cyclic adenosine monophosphate (cAMP) production than the hormone's native version [30]. Thus, bbPTH peptide bioactivity was investigated via quantification of cAMP production. PTH receptor expression and PTH-stimulated cAMP production are temporally regulated during osteoblast differentiation [31, 32], where maximal PTH-stimulated cAMP production in MC3T3 cells occurs in cells incubated for 5–10 days in osteogenic medium; therefore, this time frame was chosen for our studies. MC3T3-subclone 4 cells were seeded at 10,000 cells/cm² in 6 well plates in basal medium (α -MEM, 10% FBS, 1% penicillin-streptomycin). Cells were allowed to attach overnight, before medium was changed to osteogenic medium (basal medium supplemented with 50 μ g/ml ascorbic acid and 10 mM β -glycerophosphate) (Day 0). The medium was replaced with fresh osteogenic medium on Day 3, and the cAMP assay was performed on Day 5.

For the cAMP assay, the medium was aspirated and confluent cells were washed with PBS. The cells were stimulated with vehicle (1 mM acetic acid) or bbPTH (1–84) (10, 30, or 100 nM) in PBS containing 1 mM isobutylmethylxanthine (IBMX) for 10 min at 37°C with N = 3 wells per treatment. At the end of the treatment period, the PBS was collected and samples were prepared and assayed in triplicate without acetylation as per the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). Cell culture supernatants were assayed directly; samples were diluted as necessary in cAMP EIA buffer.

2.1.3 Caspase-3/7 Activity Assay

We performed a caspase-3/7 assay because human PTH has been shown to have an anti-apoptotic effect on osteoblasts [31]. MC3T3-S4 cells were seeded at 20,000 cells/well in 96 well, tissue culture-treated white-walled plates. The cells were seeded in basal medium and allowed to attach overnight. The next day, the medium was aspirated, cells were washed with PBS, and then pretreated with vehicle (1 mM acetic acid) or bbPTH (1–84) (30 or 100 nM) in basal medium for 1 hr with N = 6 wells per treatment. At the end of the pretreatment period, the medium was aspirated, the cells were washed with PBS, and either basal medium (unstarved cells) or α -MEM alone (serum-starved cells) was added to each well. After 6 hours, the medium was aspirated, the cells were washed with PBS, and 50 μ l of both PBS and a luminogenic caspase-3/7 substrate (Caspase-Glo 3/7 Assay, Promega, Madison, WI) were added to each well. After 1 hour, luminescence was quantified using a Synergy HT Multi-Detection Microplate Reader (Bio-Tek, Winooski, VT). Luminescence was converted to international units (U) of caspase-3/7 activity using a standard curve generated with human recombinant caspase-3 (Enzo Life Sciences, Farmingdale, NY).

2.1.4 Animals

Twenty 4-week old male C57BL/10ScSn/DMD-*mdx* and 10 wild type control C57BL/10Sn mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were co-housed six per cage in a 12-h dark, 12-h light environment at 20°C. Mice were fed a standard rodent diet containing 0.95% calcium (Purina LabDiet Autoclavable Rodent Diet #5010) and given water ad libitum. All mice were euthanized at 10 weeks of age, after 6 weeks of PTH treatment, using carbon dioxide asphyxiation. Three mice (1 *mdx* bbPTH, 1 *mdx* vehicle and 1 wild type vehicle) died during the study and were not included in subsequent data analysis. This study was approved by the Michigan Tech Animal Care and Use Committee.

2.1.5 PTH Treatment

Mice were given daily subcutaneous injections of bbPTH 1-84 or an acidic vehicle solution. Vehicle injections were prepared with 0.15 M NaCl and 0.001 N HCl. bbPTH solutions were prepared by dissolving PTH in the acidic saline solution. Mice were injected once daily, 5 times per week with 28 nmol/kg bbPTH 1-84. After six weeks of daily injection, mice were euthanized and their long bones were removed. At 1 and 4 days prior to sacrifice, mice were given calcein injections to label bone formation surfaces (10 mg/kg).

2.1.6 Isolation, Culture, and Mineralization of Bone Marrow Stromal Cells (BMSCs)

Bone marrow was flushed from the humeri of vehicle- and bbPTH-treated wild type and *mdx* mice. Cells were pooled by treatment group. Cells were seeded into 6 well plates (10^7 cells/well) in osteogenic culture medium (α -MEM, 20% FBS, 1% antibiotic-antimycotic, 1% non-essential amino acids, 50 μ g/ml ascorbic acid and 10 mM β -glycerophosphate). BMSCs were identified by adherence; after 72 hrs, non-adherent cells were removed. Media changes continued every 3 days. BMSCs were cultured in osteogenic medium for 21 days to promote calcified matrix production. At the end of this period, BMSCs were fixed (10% neutral-buffered formalin) and stained (2% alizarin red). Calcified matrix was quantified as the percentage of alizarin red-positive area over total well area using image analysis software (Bioquant Osteo, Nashville, TN).

2.1.7 Trabecular Bone Properties

Proximal tibial and distal femoral metaphyses were scanned using micro-computed tomography (μ CT) (SCANCO 35, SCANCO Medical, Switzerland) to determine trabecular properties. The scan region started 0.5 mm from the physis and was 100 slices (0.7 mm) thick. From these data, trabecular number (Tb.n), trabecular spacing (Tb.sp), trabecular thickness (Tb.th), and bone volume/tissue volume (BV/TV) were calculated. Dynamic histomorphometry was evaluated in the same region of the proximal tibia by determining the distance between calcein labels on the trabecular surface and the lengths of single- and double-labeled surfaces using Bioquant Osteo software (Nashville, TN). Static histomorphometry was evaluated by measuring osteoid surface, osteoid thickness, and eroded surface in slides stained in von Kossa with MacNeal's tetrachrome stain. Osteoblast and osteoclast surfaces were measured in decalcified proximal tibia segments stained in toluidine blue and tartrate-resistant acid phosphatase (TRAP).

2.1.8 Cortical Bone Properties

The right femoral diaphysis was embedded in methylmethacrylate. A cross-section was cut at the midpoint using an Isomet diamond wafering blade (Beuhler, Lake Bluff, IL), ground to a thickness of $<90 \mu$ m and mounted on a microscope slide. Cross-sections were digitized at 40 $\times$ magnification using a SPOT Insight camera (Diagnostic Instruments, Sterling Heights, MI). A custom macro and Scion Image software (Scion Corp., Frederick, ME) were used to quantify cross-sectional properties including medio-lateral (I_{ml}), antero-posterior (I_{ap}), and maximum (I_{max}) moments of inertia. Mineral apposition rate was determined by measuring the distance between calcein labels using Bioquant software. Mineralizing surface was determined by measuring the ratio of single- and double-labeled surfaces to the total cortical surface. Cortical thickness was measured as the average distance between the endosteal and periosteal surfaces.

2.1.9 Mechanical Properties

Bending properties of the left femur were determined by a 3-point bend test at a rate of 1 mm/min. The testing fixtures had a span of 10 mm and a radius of 1 mm. Mechanical testing was performed using an Instron test machine (Norwood, MA). Stress-strain plots were

determined using asymmetric beam theory [33], and used to calculate ultimate stress and modulus of toughness. Load-displacement plots were used to determine ultimate load and energy to failure for each sample.

2.1.10 Mineral Content

Mineral content was determined through ashing. After mechanical testing, femoral diaphyses were cleaned of marrow, placed in a furnace at 100°C for 24 hours to remove all water, and weighed to obtain dry mass. Samples were returned to the furnace for 48 hours at 600°C to burn off all organic material. Bones were again weighed to determine relative mineral content, defined as the ratio of ash mass to dry mass.

2.1.11 Statistics

Bone properties and cell parameters were compared between bbPTH- and vehicle-treated animals using one-way ANOVA and Tukey's *post hoc* analysis.

3.1 Results

3.1.1 Cell Studies

All three concentrations of bbPTH increased cAMP production in MC3T3 cells compared to vehicle controls ($p < 0.0001$). No difference was observed between 10 nM bbPTH and 30 nM bbPTH, but 100 nM bbPTH increased cAMP production more than the lower doses ($p < 0.0001$) (Figure 2). Caspase 3/7 activity was reduced ($p < 0.0001$) in serum starved MC3T3 cells treated with both concentrations of bbPTH, with 100 nM bbPTH decreasing caspase 3/7 to unstarved control values (Figure 3).

3.1.2 Bone Marrow Stromal Cells

Alizarin red staining showed greater ($p = 0.0021$) mineralized matrix in BMSC cultures from vehicle-treated *mdx* mice than in cultures from bbPTH-treated *mdx* mice (Figure 4A). In wild type mice, the difference ($p = 0.09$) between vehicle and bbPTH treatments was not as large (Figure 4B).

3.1.3 Trabecular Bone

Trabecular bone volume fraction was lower in the distal femoral and proximal tibial metaphyses of vehicle-treated *mdx* mice compared to wild type mice ($p = 0.0024$) (Figure 5A). Treatment with bbPTH improved trabecular bone volume in both the distal femur and proximal tibia in both *mdx* and wild type mice, but to a much greater degree in *mdx* mice (Figure 6). The increase in femoral bone volume fraction in *mdx* mice with bbPTH treatment was 7-fold, compared to a 2-fold change in wild type mice (Figure 5A), with similar magnitude responses in the tibia (Table 1).

Vehicle-treated wild type mice demonstrated greater trabecular thickness in the distal femur and proximal tibia than *mdx* mice ($p > 0.0212$). Trabecular spacing ($p > 0.7780$), trabecular number ($p > 0.9813$), and apparent mineral density ($p > 0.7733$) were not different between vehicle-treated *mdx* and wild type mice.

Both trabecular number (Figure 5B) and trabecular thickness (Table 1) increased ($p < 0.0001$) with bbPTH treatment in *mdx* distal femurs and proximal tibias, but not in wild type mice. Trabecular spacing decreased ($p < 0.0001$) in *mdx* femurs, but not in wild type mice.

In the proximal tibia there was no difference in mineralizing surface between any groups ($p = 0.7437$). Trabecular mineral apposition rate increased with bbPTH in wild type mice ($p = 0.0202$), but there was no difference in mineral apposition rate between vehicle- and bbPTH-

treatment in *mdx* mice ($p = 0.6628$). There were no differences in mineral apposition rate treatments between *mdx* and wild type mice in either treatment group ($p > 0.245$) (Table 1).

Despite the observed anabolic effect of bbPTH on trabecular bone volume fraction, no differences were found between groups in osteoid thickness ($p = 0.4047$) or osteoid surface ($p = 0.966$). However, bbPTH-treated *mdx* mice had increased osteoblast surface ($p = 0.0435$) and decreased osteoclast surface ($p = 0.0336$), compared to vehicle treatment. These changes likely contributed to the increased trabecular bone volume fraction that occurred with bbPTH treatment in *mdx* mice. Similar changes were seen in wild type mice treated with bbPTH, but the differences were not significant for osteoblast surface ($p = 0.0711$) or osteoclast surface ($p = 0.1242$) (Table 1). Normally, PTH treatment increases both osteoblast and osteoclast surfaces [34]. Increased osteoblast surface and decreased osteoclast surface, as seen in the bbPTH treated *mdx* mice, may be a feature unique to bbPTH.

3.1.4 Cortical Bone

No differences were found in femur length for either treatment or mouse type ($p > 0.22$). *Mdx* mice appeared to have an altered femoral cross-section, with a more prominent linea aspera than wild type mice, regardless of bbPTH treatment (Figure 7). No differences were detected between *mdx* and wild type, or bbPTH and vehicle-treated mice for cortical thickness ($p = 0.2803$), $I_{\max}$ ($p = 0.4344$), or I_{ap} ($p = 0.4492$). However, moment of inertia about the bending axis (I_{ml}) was greater in bbPTH-treated *mdx* mice than vehicle-treated *mdx* mice ($p = 0.0097$) (Table 2).

No differences were detected in cortical bone mineralizing surface on the fluorescently labeled endosteal surface, either when analyzed by quadrant, or by total endosteal area ($p > 0.2579$). Mineral apposition rate was not different between any groups, though bbPTH-treatment tended to decrease endosteal mineral apposition rate ($p = 0.0776$). Similarly, no differences were found between any groups for periosteal mineralizing surface on the calcein labeled periosteal surface ($p > 0.1970$). bbPTH-treated *mdx* mice tended to have greater periosteal mineral apposition than vehicle-treated controls ($p = 0.0764$), unlike wild type mice ($p = 0.8890$). No other differences were detected in cortical bone mineral apposition rate between groups ($p > 0.1492$) (Table 2).

Three-point bend testing of the femur showed no differences ($p > 0.1314$) between ultimate load or energy to failure between bbPTH- and vehicle-treated mice or between *mdx* and wild type mice. Normalizing by body weight had no affect ($p > 0.1086$). Similarly, no differences were found in ultimate stress ($p = 0.9231$) or modulus of toughness ($p = 0.5541$) (Table 2).

Mineral content in the femoral diaphysis, as determined by ash fraction, was not different for *mdx* vs. wild type or PTH vs. vehicle-treated animals ($p = 0.8516$) (Table 2).

4.1 Discussion

We cloned the gene for black bear PTH and found nine amino acid residues were different from human PTH 1–84. We recombinantly produced bbPTH 1–84 and found it activates cAMP production as has been previously reported for human PTH [35]. The cAMP/protein kinase A pathway is believed to be responsible for the majority of PTH induced increases in histological and serum indices of bone formation [36–38]. PTH also produces osteoanabolic activity via anti-apoptosis mechanisms in osteoblasts [39]. We also found that treating osteoblasts with bbPTH prior to serum starvation reduces caspase 3/7 activity. Previously we found bbPTH 1–34 causes greater reductions in serum starved induced caspase activity

than hPTH 1–34 [27]. Here we demonstrate the ability of bbPTH 1–84 to have a greater osteoanabolic effect in dystrophin deficient mice than in wild type mice.

Though a strong anabolic response to bbPTH treatment (i.e., increased trabecular bone volume fraction) was observed in both *mdx* and wild type mice, the relative effect was much greater in *mdx* mouse bone. The lower mineralization in BMSC cultures from *mdx* mice treated with bbPTH raise the possibility that the majority of osteoblast precursors may have differentiated and been removed from the marrow to participate in bone formation during the course of treatment. Dystrophin deficiency alters calcium signaling and calcium reuptake in muscle cells, and calcium signaling is also an important biological mechanism in osteoblasts [40]. Thus, there is potential that the *mdx* mouse also features differences in calcium signaling in osteoblasts in response to PTH and mechanical loading [41]. This change, if it is occurring, may help explain the low bone volume in *mdx* mice and the differential response to PTH treatment between *mdx* and wild type mice. *Mdx* mice display a low bone mass phenotype at 3 weeks of age, which precedes the onset of muscle weakness [42]. Dystrophin deficiency may also affect primary spongiosa formation and the development of hypertrophic chondrocytes during development due to aberrant calcium signaling [43]. No connection between PTH and dystrophin has been established previously, but the marked difference in anabolic response to bbPTH in *mdx* as compared to wild type mouse trabecular bone raises the possibility that dystrophin or associated altered calcium signaling due to dystrophin deficiency could play a role in trabecular bone formation. However, surprisingly there is no difference in serum calcium concentrations between 2 month old *mdx* and wild type mice [44]. Because of the influence of PTH on calcium homeostasis, it will be important to quantify the effects of PTH on serum ionized calcium concentration in *mdx* mice in future studies. Bone losses attributed to the *mdx* phenotype may be due to decreased muscular force as well. Though mouse behavior was not markedly different by gross observation during this study, previous work has shown decreased muscle forces despite increased muscle size in *mdx* mice from ages 4 weeks to 24 months [17, 19, 20]. Notably, PTH has been shown to attenuate bone loss in disuse scenarios, such as hind limb suspension [45].

bbPTH increased bone volume fraction to a greater degree than has been observed in previous studies administering hPTH to mice, with a 91% increase in bone volume fraction in wild type mice in this study, compared to 14–38% increases in bone volume fraction in mice administered with high dose hPTH [46, 47]. Trabecular number was also increased in *mdx* bones, contributing to a large increase in bone volume fraction (Figure 5B). A possibility for the increase in trabecular number, since dystrophin deficiency leads to aberrant calcium signaling in muscle cells, and possibly other musculoskeletal cells, is that *mdx* mice have a greater number of hypertrophic chondrocytes or increased primary spongiosa since hypertrophic chondrocyte proliferation is regulated by a calcium signaling pathway [48]. Higher values of these parameters in *mdx* mice at the start of the PTH treatment period could lead to increases in trabecular number and bone volume fraction with PTH treatment [43, 49]. The substantial increase in bone surface in the bbPTH treated mice may explain the lack of change in mineralizing surface (MS/BS), which is normally seen with PTH treatment. Furthermore, *in vitro* data suggest that bbPTH has an anti-apoptotic effect in osteoblasts, which would increase osteoblast survival, consistent with the increased osteoblast surface we observed histologically in bbPTH treated *mdx* mice.

Cortical bone did not display the marked changes with bbPTH treatment that were observed in trabecular bone. This is similar to studies with PTH in other mouse models, which show approximately a 10% increase in cortical bone ultimate force with PTH [50, 51]. Our study showed a comparable increase in femoral ultimate force of approximately 8%, though the difference was not significant. PTH did not have the expected effect on endosteal

mineralizing surface, possibly because the majority of osteoblast precursors were mobilized in the heavily affected trabecular bone. However, bbPTH did significantly increase medio-lateral moment of inertia in *mdx* mice. This is important because the medio-lateral axis is the primary bending axis in the femur [52]. This raises the possibility that bbPTH may have a moderate effect on *mdx* cortical bone, which may improve with longer duration treatment.

The potent osteoanabolic response to bbPTH in *mdx* trabecular bone is clinically relevant to DMD patients, as the majority of fractures occur in the highly trabecularized regions of bone including the distal femur, proximal tibia, and vertebrae [9–11, 53]. An increase in trabecular bone volume, as occurs with parathyroid hormone treatment, could greatly reduce fracture risk by increasing trabecular bone density in DMD patients [54]. Additionally, a longer duration PTH treatment in DMD patients could have beneficial effects on cortical bone. However, the potential of PTH to increase the risk of osteosarcoma in children and adolescents is a concern that would need to be addressed before a recommendation to use PTH clinically in DMD patients could be made. Osteosarcoma develops in approximately 4 out of one million males less than 24 years of age with a peak incidence of approximately 10 per million for ages 15–19 years [55], and PTH has been shown to increase the incidence of osteosarcoma in rats that were given high PTH doses for the majority of their lifetime [56]. The use of PTH in children with hypoparathyroidism is considered safe and effective for maintaining calcium homeostasis and was found to promote normal skeletal development [57]. However, the potential for PTH to increase the risk of osteosarcoma in children and adolescents has not been studied. Decreasing fracture prevalence in DMD patients could improve overall quality of life by allowing greater mobility for a longer period of time, even with the use of glucocorticoids. Parathyroid hormone is effective in the treatment of glucocorticoid-induced osteoporosis [22, 58]. Therefore, treating DMD patients with parathyroid hormone could reduce the probability of fractures and prolong the amount of time these patients are independently mobile. Our data support the idea that bbPTH is an effective therapeutic to combat bone loss in cases of dystrophin deficiency. However, this study administered only high dose bbPTH. Future work comparing the dose responses of bbPTH and hPTH in *mdx* mice are needed to further elucidate the mechanisms by which PTH improves bone properties in the *mdx* model of Duchenne muscular dystrophy.

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Highlights

- Black bear PTH is a potent osteoanabolic agent in the dystrophin deficient *mdx* mouse
- Black bear PTH is more potent in *mdx* mice than in wild type mice
- Black bear PTH increases osteoblast surface and decreases osteoclast surface in *mdx* mice

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Figure 2. bbPTH increased cAMP production in a dose-responsive manner ($p < 0.0001$).

Figure 3. bbPTH was antiapoptotic in serum starved MC3T3 cells ($p < 0.0001$). Groups with different letters are significantly different from one another.

Figure 4. Alizarin red stained mineralized matrix in 21 day BMSC cultures from the humeri of A) *mdx* and B) wild type mice. The percentage of well area stained for mineralized matrix was quantified.

Figure 5.

A) Bone volume fraction was lower in vehicle-treated *mdx* femurs compared to wild type femurs. *Mdx* femurs showed greater response to PTH treatment than wild type femurs. B) Vehicle-treated *mdx* mice had lower trabecular number in the femur compared to wild type mice ($p = 0.0028$). Trabecular number increased with PTH treatment in *mdx* mice ($p < 0.0001$), but not wild type ($p = 0.2047$). Mean values with standard error bars. Groups with different letters are significantly different ($p < 0.05$) from each other.

Figure 6. Micro-CT images of distal femoral metaphyses show compromised trabecular architecture in *mdx* mice compared to wild type mice and improved trabecular architecture with PTH treatment, particularly in *mdx* mice.

Figure 7.
mdx femoral cross-sections (left) had a more prominent linea aspera than wild type mice (right). Images are femoral cross-sections of vehicle-treated animals, but are representative of all samples treated or untreated.

Table 1

The distal femur and proximal tibia trabecular regions show 1) compromised trabecular architectural parameters in *mdx* mice vs. wild type; 2) an anabolic effect of bbPTH in *mdx* and wild type mice, with a stronger effect in *mdx*; 3) bbPTH treatment increased *mdx* trabecular microarchitecture beyond vehicle-treated wild type values. Average $\pm$ SE.

	<i>mdx</i> Vehicle	<i>mdx</i> bbPTH	Wild Type Vehicle	Wild Type bbPTH
Distal Femur μCT				
BV/TV (%)	6.6 $\pm$ 2.2 [†]	41.3 $\pm$ 2.2 ^{**††}	11.7 $\pm$ 3.2	22.3 $\pm$ 3.2 [*]
Tb.Th (mm)	0.03 $\pm$ 0.001 [†]	0.05 $\pm$ 0.001 ^{**}	0.04 $\pm$ 0.002	0.05 $\pm$ 0.002
Tb.Sp (mm)	0.22 $\pm$ 0.008	0.08 $\pm$ 0.008 ^{**††}	0.19 $\pm$ 0.001	0.18 $\pm$ 0.001
Tb.N (1/mm)	4.5 $\pm$ 0.4 [†]	11.1 $\pm$ 0.5 ^{**††}	5.1 $\pm$ 0.7	5.7 $\pm$ 0.7
Proximal Tibia μCT				
BV/TV (%)	5.7 $\pm$ 0.03	39.0 $\pm$ 0.03 ^{**††}	9.2 $\pm$ 0.04	20.6 $\pm$ 0.04 ^{**}
Tb.Th (mm)	0.032 $\pm$ 0.001 [†]	0.044 $\pm$ 0.001 ^{**†}	0.037 $\pm$ 0.001	0.044 $\pm$ 0.001
Tb.Sp (mm)	0.23 $\pm$ 0.02	0.11 $\pm$ 0.02 ^{**†}	0.20 $\pm$ 0.03	0.22 $\pm$ 0.02
Tb.N (1/mm)	4.3 $\pm$ 0.8	10.5 $\pm$ 1.8 ^{**††}	4.8 $\pm$ 1.17	5.1 $\pm$ 1.0
Proximal Tibia Dynamic Histomorphometry				
MS/BS	0.19 $\pm$ 0.03	0.19 $\pm$ 0.03	0.20 $\pm$ 0.04	0.15 $\pm$ 0.04
MAR (μ m/day)	2.66 $\pm$ 0.09	2.81 $\pm$ 0.09	2.31 $\pm$ 0.15	2.97 $\pm$ 0.15 [*]
Proximal Tibia Static Histomorphometry				
Osteoid Thickness (μ m)	2.51 $\pm$ 0.54	3.27 $\pm$ 0.54	1.59 $\pm$ 0.82	2.60 $\pm$ 0.82
Osteoid Surface (%)	22.39 $\pm$ 5.00	24.67 $\pm$ 5.00	31.09 $\pm$ 8.61	21.42 $\pm$ 6.67
Bone Surface (mm)	17.38 $\pm$ 3.86	48.48 $\pm$ 3.86 ^{**†}	23.29 $\pm$ 5.79	34.57 $\pm$ 5.79
Eroded Surface (%)	0.04 $\pm$ 0.014	0.05 $\pm$ 0.014	0.05 $\pm$ 0.022	0.04 $\pm$ 0.019
Osteoblast Surface/Bone Surface	0.10 $\pm$ 0.04	0.32 $\pm$ 0.05 [*]	0.13 $\pm$ 0.06	0.39 $\pm$ 0.06
Osteoclast Surface/Bone Surface	0.13 $\pm$ 0.01	0.04 $\pm$ 0.02 [†]	0.16 $\pm$ 0.02	0.09 $\pm$ 0.02

* p < 0.05 vs. vehicle-treated;

** p < 0.005 vs. vehicle-treated

† p < 0.05 vs. wild type vehicle;

†† p < 0.005 vs. wild type vehicle

Table 2

bbPTH did not change mechanical properties of cortical bone in mdx or wild type, or strongly affect geometric values. The only change in geometry was I_{ml} , the moment about the bending axis in PTH-treated mdx animals. Average $\pm$ SE.

	<i>mdx</i> Vehicle	<i>mdx</i> bbPTH	Wild Type Vehicle	Wild Type bbPTH
Cortical Geometry				
Cortical Thickness (μm)	440.2 $\pm$ 25.2	446.7 $\pm$ 25.2	363.2 $\pm$ 37.8	369.1 $\pm$ 37.8
Cortical Area (mm^2)	0.96 $\pm$ 0.06	1.06 $\pm$ 0.06	0.85 $\pm$ 0.10	0.93 $\pm$ 0.10
I_{ml} (mm^4)	0.17 $\pm$ 0.01	0.22 $\pm$ 0.01 ^{**}	0.19 $\pm$ 0.02	0.21 $\pm$ 0.02
I_{ap} (mm^4)	0.37 $\pm$ 0.03	0.44 $\pm$ 0.03	0.36 $\pm$ 0.06	0.44 $\pm$ 0.06
I_{max} (mm^4)	0.37 $\pm$ 0.04	0.45 $\pm$ 0.04	0.37 $\pm$ 0.06	0.44 $\pm$ 0.06
Endosteal Dynamic Histomorphometry				
MAR ($\mu\text{m}/\text{day}$)	1.36 $\pm$ 0.23	1.19 $\pm$ 0.23	1.59 $\pm$ 0.34	1.62 $\pm$ 0.34
MS/BS	0.61 $\pm$ 0.05	0.42 $\pm$ 0.05	0.59 $\pm$ 0.08	0.44 $\pm$ 0.08
Periosteal Dynamic Histomorphometry				
MAR ($\mu\text{m}/\text{day}$)	2.09 $\pm$ 0.17	2.31 $\pm$ 0.17	1.49 $\pm$ 0.26	1.76 $\pm$ 0.26
MS/BS	0.61 $\pm$ 0.04	0.61 $\pm$ 0.04	0.49 $\pm$ 0.06	0.48 $\pm$ 0.06
Ash Fraction				
Ash Fraction	0.65 $\pm$ 0.03	0.67 $\pm$ 0.02	0.66 $\pm$ 0.04	0.64 $\pm$ 0.03
Mechanical Testing				
Ultimate Force (N)	15.4 $\pm$ 0.82	16.7 $\pm$ 0.89	16.2 $\pm$ 0.23	17.1 $\pm$ 0.95
Energy to Failure (mJ)	15.0 $\pm$ 2.1	17.1 $\pm$ 2.3	10.0 $\pm$ 1.0	13.3 $\pm$ 2.6
Ultimate Stress (MPa)	179.7 $\pm$ 11.2	147.7 $\pm$ 11.3	166.3 $\pm$ 18.4	162.0 $\pm$ 18.4
Modulus of Toughness (mJ/mm^3)	16.9 $\pm$ 3.1	14.6 $\pm$ 3.1	9.1 $\pm$ 5.0	12.7 $\pm$ 5.0

^{**}
p = 0.0097 vs. vehicle-treated