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Positive effects of bisphosphonates on bone and muscle in a mouse model of Duchenne muscular dystrophy

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Abstract

Patients with Duchenne muscular dystrophy are at increased risk of decreased bone mineral density and bone fracture as a result of inactivity. To determine if antiresorptive bisphosphonates could improve bone quality and their effects on muscle we studied the Mdx mouse, treated with pamidronate during peak bone growth at 5 and 6 weeks of age, and examined the outcome at 13 weeks of age. Pamidronate increased cortical bone architecture and strength in femurs with increased resistance to fracture. While overall long bone growth was not affected by pamidronate, there was significant inhibition of remodeling in metaphyseal trabecular bone with evidence of residual calcified cartilage. Pamidronate treatment had positive effects on skeletal muscle in the Mdx mice with decreased serum and muscle creatine kinase and evidence of improved muscle histology and grip strength.

Keywords: Duchenne muscular dystrophy; Bisphosphonates; Bone mineral density; Bone strength; Creatine kinase

1. Introduction

Duchenne muscular dystrophy (DMD) is one of the most frequently occurring genetic diseases, affecting approximately 1 in 3500 boys [1]. This X-linked disease, caused by mutations of the gene encoding dystrophin, results in loss of dystrophin, a protein required to stabilize the link between actin fibers and the sarcolemmal membrane [2,3]. DMD patients suffer from chronic muscle inflammation resulting in muscle weakness, necrosis and wasting. The only current therapy for DMD is long-term glucocorticoid treatment to decrease muscle inflammation, improve dystrophic muscle strength, and prolong ambulation and lifespan [4,5].

DMD patients also have chronic skeletal problems with low bone mineral density and greater fracture prevalence [6–10]. Many factors contribute to this poor bone health including decreased physical activity, low vitamin D levels and long-term glucocorticoid therapy. It has also been shown that chronic muscle inflammation releases cytokines that increase osteoclast activity, possibly contributing to bone loss through excessive bone resorption [11,12].

The C57BL/10ScSn-Dmd$^{mdx/J}$ (Mdx) mouse is the most widely used animal model of DMD [13–15]. The Mdx mice have a spontaneous single nucleotide mutation on the dystrophin-encoding gene, resulting in loss of functional dystrophin, muscle wasting and inflammation from 3 weeks of age [13]. Many studies have examined dystrophic muscle in Mdx mice showing inflammation, weakness and elevated muscle fiber regeneration [13–15]. A few studies have examined skeletal health in the Mdx mice showing reduced bone density and strength [16–19]. One study suggested that inflammatory cytokines, particularly IL-6, are present in the serum from Mdx mice as well as patients with DMD and may stimulate osteoclastogenesis [11].

Antiresorptive agents such as bisphosphonates (BPs) have been used extensively to increase bone mineral density and prevent fractures, both in adults and children with a variety of bone diseases [20–23]. The pharmacokinetics of bisphosphonates are unique, with very low oral bioavailability (less than 1% for most bisphosphonates) [23]. All bisphosphonates bind very selectively to calcium and are therefore rapidly sequestered into bone mineral (20–80%), while the rest of the drug is removed from circulation within 4–5 hours, and excreted through the kidneys within 48

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hours. However, bisphosphonates have half-lives of more than 10 years in bone [24]. Bisphosphonates mediate their effects on bone through inhibition of osteoclast activity and numbers. Nitrogen-containing bisphosphonates, including pamidronate, inhibit farnesyl pyrophosphate synthase (FPPS) and other enzymes of the mevalonate/cholesterol biosynthetic pathway, resulting in interruption in isoprenylation of proteins. Some of these proteins play crucial roles in intracellular signaling pathways controlling cell morphology, cytoskeletal arrangement, membrane ruffling, trafficking of vesicles and apoptosis [25–28]. It has been suggested that the uptake of bisphosphonates by osteoclasts during bone turnover results in inhibition of activity and even initiation of apoptosis resulting in a net decrease in bone resorption [26].

Bisphosphonates have been used in DMD patients who were previously treated or currently being treated with glucocorticoids. When DMD patients are treated with bisphosphonates, positive effects on bone mineral density z-scores and bone pain were shown, and the effect was the greatest when given early in the course of disease [20]. Also, in a study of glucocorticoid-treated DMD patients who were treated with bisphosphonates for a minimum of 2 years, the patients showed delayed loss of ambulation, prolonged lifespan, and better overall health in their 30s [22]. This study suggests that there may be positive effects of bisphosphonates that extend beyond bone health, and there are some reports of bisphosphonates having positive effects on muscle strength in children with *osteogenesis imperfecta* [29].

In this study we tested the effects of early bisphosphonate therapy in young Mdx mice on both their muscle and bone health. The mice were treated with pamidronate for 2 weeks, then followed over 6 weeks during their rapid bone growth phase. We demonstrate here that this brief exposure to pamidronate in growing mice resulted in increased bone mineral density and strength but also had positive effects on muscle with small but significant improvement in grip strength and an attenuation in muscle damage as evidenced by decreases in serum and muscle creatine kinase (CK) levels.

2. Materials and methods

2.1. Animals and experimental design

To address the effects of early treatment with bisphosphonates on bone and muscle during a period of rapid growth, five-week old C57BL/10ScSn-*Dmd* $^{mdx/J}$ (Mdx) mice (Jackson Laboratory, Bar Harbor, ME, USA) were randomly assigned into bisphosphonate (BP) and untreated control (Con) groups with 10 mice in each group. At 5 and 6 weeks of age, BP mice received pamidronate (Sigma-Aldrich, Oakville, ON, Canada) dissolved in saline and injected subcutaneously at a dose of 2 mg/kg body weight twice per week (to avoid hypocalcemia), with a total dose of 8 mg/kg body weight. The dose of pamidronate is consistent with the 9 mg/kg used in steroid-treated boys with DMD to treat vertebral fractures [21]. Control and BP mice were housed under standard conditions with food and water *ad libitum*. Mice were sacrificed at 13 weeks of age to assess bone and muscle pathology. All animal care procedures were reviewed and approved by the University of Toronto Animal Care Committee.

2.2. Tissue harvest and storage

Blood samples drawn at the time of sacrifice were allowed to clot for 1–2 hours and serum was isolated and frozen at −20 °C for creatine kinase analysis. Excised left femurs, L5 and L6 vertebrae were dissected free of surrounding tissue, immediately wrapped in saline-soaked gauze and frozen at −20 °C for bone mineral density and microarchitecture analyses, and biomechanical tests. Right femurs and left tibiae were fixed in 70% ethanol or 10% formalin, respectively, and kept at 4 °C for histological analyses. Right tibiae were placed in pre-warmed αMEM media with antibiotic-antimycotic mixture immediately following harvest. Bone marrow stromal cells were collected by flushing the marrow cavities with the medium. Quadriceps were rapidly frozen in liquid nitrogen immediately following excision and stored at −80 °C until RNA extraction was performed.

2.3. Measurement of femur length

To assess the normal longitudinal growth of bones, the left femur from each mouse were fully thawed and measured for length using digital calipers (VWR International, Mississauga, ON, Canada).

2.4. Bone mineral density and morphometry

The left femur, L5 and L6 vertebral bodies from each mouse were used to determine areal bone mineral density (aBMD, g/cm^2) by dual-energy X-ray absorptiometry (DEXA) using a PIXImus bone densitometer (GE Medical Systems, Madison, WI, USA) [30]. For cortical and trabecular bone microarchitecture analyses of femurs and L6 vertebrae, micro-computed tomography (MicroCT; Skyscan 1174, Skyscan, Kontich, Belgium) was also performed. Transverse images were acquired at 50 kV and 800 μA. Mid-diaphyses of femurs were scanned at a voxel size of 11.6 μm^3 resolution for cortical bone assessment, while distal femurs and vertebral bodies were scanned at 6.1 μm^3 resolution for trabecular bone assessment. Three-dimensional images were analyzed using the Skyscan CT-Analyzer software (version 1.5.0). Volumes of interest (VOI) were established as follows: an 86 section VOI at the mid-shaft of the femoral cortical shaft, a 100 section VOI measured 1.2 mm from formation of the first cartilage bridge for the trabecular compartment of the femur, and VOI in between the growth plates for L6 vertebrae. Trabecular and cortical morphometry measurements were performed with global threshold selected automatically to delineate bone from soft tissue (cortical bone was considered as pixels with a threshold of 1.1 g hydroxyapatite (HA)/cm^3 (gray value 110)), and trabecular bone with a threshold of 0.9 g HA/cm^3 (gray value 65). At the mid-diaphysis of the femur, cortical bone area (B.Ar, mm^2), cortical thickness (mm), anteroposterior and mediolateral (AP, ML; mm) diameters were measured. In distal femurs, trabecular morphometry measurements included bone volume fraction (BV/TV, %), trabecular thickness (Tb.Th, μm), trabecular number (Tb.N, mm^{-1}), trabecular separation (Tb.Sp,