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Effect of sequential treatments with alendronate, parathyroid hormone (1–34) and raloxifene on cortical bone mass and strength in ovariectomized rats☆

Sarah K. Amugongo [a,1], Wei Yao [a,1], Junjing Jia [a], Weiwei Dai [a], Yu-An E. Lay [a], Li Jiang [a], Danielle Harvey [b], Elizabeth A. Zimmermann [c], Eric Schaible [d], Neil Dave [c], Robert O. Ritchie [a,e], Donald B. Kimmel [f], Nancy E. Lane [a,*]

[a] Musculoskeletal Research Unit, Department of Medicine, University of California Davis Medical Center, Sacramento, CA 95817, USA
[b] Division of Biostatistics, Department of Public Health Sciences, University of California, Davis, CA 95616, USA
[c] Materials Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA
[d] Experimental Systems Group, Advanced Light Source, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA
[e] Department of Materials Science and Engineering, University of California, Berkeley, CA 94720, USA
[f] Osteoporosis Research Center, School of Medicine, Creighton University, Omaha, NE 68131, USA

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ABSTRACT

Anti-resorptive and anabolic agents are often prescribed for the treatment of osteoporosis continuously or sequentially for many years. However their impact on cortical bone quality and bone strength is not clear.
Methods: Six-month old female rats were either sham operated or ovariectomized (OVX). OVX rats were left untreated for two months and then were treated with vehicle (Veh), hPTH (1–34) (PTH), alendronate (Aln), or raloxifene (Ral) sequentially for three month intervals, for a total of three periods. Mid-tibial cortical bone architecture, mass, mineralization, and strength were measured on necropsy samples obtained after each period. Bone indentation properties were measured on proximal femur necropsy samples.
Results: Eight or more months of estrogen deficiency in rats resulted in decreased cortical bone area and thickness. Treatment with PTH for 3 months caused the deposition of endocortical lamellar bone that increased cortical bone area, thickness, and strength. These improvements were lost when PTH was withdrawn without followup treatment, but were maintained for the maximum times tested, six months with Ral and three months with Aln. Pre-treatment with anti-resorptives was also somewhat successful in ultimately preserving the additional endocortical lamellar bone formed under PTH treatment. These treatments did not affect bone indentation properties.
Summary: Sequential therapy that involved both PTH and anti-resorptive agents was required to achieve lasting improvements in cortical area, thickness, and strength in OVX rats. Anti-resorptive therapy, either prior to or following PTH, was required to preserve gains attributable to an anabolic agent.

Introduction

Musculoskeletal diseases including osteoporosis are the second greatest cause of disability worldwide. Their overall impact on death and disability has increased 45% over the past 20 years [1,2]. Treatments for osteoporosis now focus on two major medication classes, anti-resorptive and anabolic agents. All the approved anti-resorptive agents for the treatment of osteoporosis, that include selective estrogen receptor modulators (SERMs), an inhibitor of RANKL, and bisphosphonates, preserve bone mass and strength by suppressing bone turnover. Most preclinical studies with these bone active agents only evaluate their effects on trabecular bone [3–5,75]. All preserve trabecular bone mass, microarchitecture and bone strength. Numerous clinical trials have demonstrated that these agents reduce the risk of vertebral fractures in

Abbreviations: AED, average energy dissipation (J); Aln, alendronate (Sigma, Cat# A-4978, St. Louis, MO, USA); BMU, basic multicellular unit; DBM, degree of bone mineralization (microCT); IDI, first cycle indentation distance (IDI) (μm); PTH, parathyroid hormone [hPTH (1–34) (human) acetate (Bachem Biosciences Inc., Cat# H-4835, King of Prussia, PA USA)]; Ral, raloxifene (Sigma, Cat# R-1402, St. Louis, MO, USA); Veh, normal saline (Life Technologies, Cat# 10010, Grand Island, NY, USA).

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* Corresponding author at: Endowed Professor of Medicine and Rheumatology, Director, Center for Musculoskeletal Health, 4625 2nd Avenue, Suite 1002, Sacramento, CA 95817, USA. Fax: +1 916 734 4773.
E-mail address: nelane@ucdavis.edu (N.E. Lane).
[1] These authors contributed equally to the study.

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women with established osteoporosis [6–14]. Alendronate, denosumab, and zoledronic acid also reduce incident hip fracture risk [15,16]. PTH (1–34), the sole approved anabolic agent, stimulates bone formation, increases bone mass and bone strength, and improves trabecular microarchitecture in preclinical studies [17–19]. It also decreases the risk of both vertebral and non-vertebral fragility fractures in osteoporotic humans [20–23].

The effects of anti-resorptive agents and PTH (1–34) on trabecular bone in animal models and osteoporotic patients are well-known [24]. One short duration study in intact rats not only reported that PTH had greater effects on cancellous bone than cortical bone, but also suggested that it might be more efficacious in intact rats than in rats with low bone mass [25]. This intriguing finding deserves longer-term followup. Preclinical data suggest that PTH may either decrease or increase the degree of bone mineralization (DBM) of cortical bone [22,26]. Results from clinical study samples found that PTH decreases DBM [27]. Similarly, raloxifene, a selective estrogen receptor modulator, reduces vertebral fracture risk in postmenopausal osteoporotic women despite very modest bone turnover suppression and gain in lumbar spine bone mineral density (BMD) [6,28]. On the other hand, bisphosphonates decrease fracture risk, increase BMD, reduce activation frequency, increase DBM [29–36], and may be associated with improved bone balance at the BMU level [37].

Cortical bone is important because it represents more than 80% of the bone mineral in the human body. It is also difficult to study, because either 3D imaging or histologic techniques must be employed to separate it from the trabecular bone that it surrounds. Moreover, both clinical and pre-clinical data suggest that osteoporosis treatment medications influence cortical bone. Bisphosphonates reduce endocortical bone formation [38,39], Haversian remodeling [39,40] and cortical porosity [35,41–46]; mildly increase cortical thickness [47,48] and increase cortical area [49]; improve cortical bone strength [47]; and have no effect on periosteal bone formation [50]. Bisphosphonates also reduce incident fractures in the proximal femur, a region composed primarily of cortical bone [16,29–36,38]. They may reduce cortical bone fracture risk by changing bone material properties independently of BMD or bone micro- or macroarchitecture [6,31,35,51–54]. Previously we reported that bisphosphonates increase DBM and reduce the heterogeneity of the trabecular bone matrix [54,55]. However, it is not known if the increase in DBM with bisphosphonates is associated with improved cortical bone strength. On the other hand, PTH increases endocortical bone formation [56–61] and cortical porosity [57,58,62,63]; increases cortical area and thickness [19,56,58,61,64–67]; decreases cortical bone strength [62]; increases the rate of Haversian remodeling [58,60,62,65]; and stimulates periosteal bone formation [55,58,59]. The opposite effects of bisphosphonates and PTH on cortical bone endpoints such as cortical porosity and endocortical bone formation rate suggest that combining them in strategic sequences could produce better therapeutic results than can be achieved by any monotherapy.

Osteoporosis patients now routinely cycle through bone active medications [68–72]. It is extremely difficult to do direct studies of fracture risk associated with such sequential treatments in humans, because of the large sample sizes required. Pre-clinical data addressing how these sequential osteoporosis therapies affect cortical bone strength and its surrogate measures could be very helpful. The goal of this study is to determine the effects of sequential treatments with currently approved osteoporosis medications that act through complementary tissue level mechanisms of action, on cortical bone strength and its surrogate measures. We evaluated cortical bone strength, architecture, indentation properties, and estimated strength, in adult ovariectomized (OVX) rats with low bone mass, given various sequences of anti-resorptive and anabolic therapy that have already been or could be applied clinically. We hypothesized that sequential treatment by traditional osteoporosis therapies with complementary tissue level mechanisms of action would improve cortical bone strength in OVX rats.

Methods

Animals and experimental procedures

Six-month-old virgin female Sprague–Dawley rats were purchased from Harlan Laboratories (Livermore, CA, USA). They were either ovariectomized (OVX) or sham-OVXd at the vendor and shipped to our laboratory two weeks post-surgery. They were individually-housed and maintained on rodent chow (Rodent Diet, Cat# 2918, Teklad; Madison, WI, USA) at 21 °C with a 12-hour light/dark cycle. Pair-feeding of OVX to Sham rats was initiated immediately upon arrival. A Sham–OVX ($n = 12$) and an OVX ($n = 10$) group were necropsied at two months post-surgery (Period 0) (Table 1). All remaining OVX rats were then randomized by body weight into ten groups (Table 1) that represented currently-applied and potential sequences of anti-osteoporosis medications.

The groups of OVX rats were treated for three months (Period 1) with Veh (1 ml/kg/dose, 3×/wk by subcutaneous (SC) injection); PTH (25 μg/kg/dose, 5×/wk SC); Aln (25 μg/kg/dose, 2×/wk SC); or Ral (5 mg/kg/dose 3×/wk by oral gavage (Table 1)). No Ral vehicle oral dosing was done. The PTH dose was based on previous publications [25,73,74]; the justification for doses of all drugs is discussed in more detail elsewhere [75]. Each rat was given dual fluorochrome labeling before necropsy by subcutaneous injection. The sequence was calcein (10 mg/kg) on Day 14 followed by alizarin red (20 mg/kg) on Day 4 before necropsy. The study protocol was approved by the University of California Davis Institutional Animal Care and Use Committee.

After 90 days (Period 1), 6–12 animals were randomly-selected from each group and necropsied (Table 1), while the remaining animals were switched to their Period 2 treatment regimen. After 180 days (Period 2), another 10–12 animals from each group were necropsied (Table 1), while the remaining animals were switched to their Period 3 treatment regimen. After 270 days (Period 3), all remaining rats were necropsied ($n = 7$–15/group) (Table 1). During the study, nine rats, randomly-disbursed over the ten groups, died, leaving 383 that reached necropsy as scheduled.

At necropsy, the rats were euthanized by CO_2 inhalation. The uterus was inspected visually to confirm OVX efficacy. Uteri with markedly shrunken horns, including decreased vascularity, yellow/beige color, and reduced diameter and length, were a sign of successful OVX. Both tibiae and femurs and lumbar vertebrae (LV) 5–6 were excised and cleaned. LV5 and LV6 were separated from one another. The right femur, right tibia, and LV5 were placed in 10% formalin for 24 h, then transferred to 70% ethanol for longer-term storage. LV6, the left femur, and the left tibia were wrapped in saline-soaked gauze and frozen at −20 °C until analysis. The data from LV5 and LV6 are reported elsewhere [75].

Biomechanical testing (left tibia)

Testing was performed after 5 mm of the end of each bone had been removed with a low speed saw with a wafering blade 60-20090 (Allied High Tech Products, Rancho Dominguez, CA), to decrease the possibility of buckling during the testing. The tibial test specimens were soaked in 37 °C HBSS (Hanks' Balanced Salt Solution; Sigma) for 12 h prior to testing. Each specimen was subjected to a three-point bending test, with a major loading span of 14.5 mm; the bone was loaded such that the posterior surface was under tension and the anterior surface was under compression, using an EnduraTEC Electro Force 3200 Testing System (Bose Corp., Eden Prairie, MN). Each tibia was loaded to failure at a displacement rate of 0.01 mm/s, and the load and displacement measured, the former using a calibrated 225 N load cell. After testing, a two-point average of the diameter and a six-point average of the cortical shell thickness were measured at the fracture site of each tibia using digital calipers with a 0.01 mm readout. The peak load (N) was recorded from the maximum load in each test. The corresponding yield and