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Bone cell-independent benefits of raloxifene on the skeleton: A novel mechanism for improving bone material properties

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ABSTRACT

Raloxifene is an FDA approved agent used to treat bone loss and decrease fracture risk. In clinical trials and animal studies, raloxifene reduces fracture risk and improves bone mechanical properties, but the mechanisms of action remain unclear because these benefits occur largely independent of changes to bone mass. Using a novel experimental approach, machined bone beams, both from mature male canine and human male donors, were depleted of living cells and then exposed to raloxifene ex vivo. Our data show that ex vivo exposure of non-viable bone to raloxifene improves intrinsic toughness, both in canine and human cortical bone beams tested by 4-point bending. These effects are cell-independent and appear to be mediated by an increase in matrix bound water, assessed using basic gravimetric weighing and sophisticated ultrashort echo time magnetic resonance imaging. The hydroxyl groups (–OH) on raloxifene were shown to be important in both the water and toughness increases. Wide and small angle X-ray scattering patterns during 4-pt bending show that raloxifene alters the transfer of load between the collagen matrix and the mineral crystals, placing lower strains on the mineral, and allowing greater overall deformation prior to failure. Collectively, these findings provide a possible mechanistic explanation for the therapeutic effect of raloxifene and more importantly identify a cell-independent mechanism that can be utilized for novel pharmacological approaches for enhancing bone strength.

Introduction

Skeletal fractures can occur when the loads imparted to the bone exceed its mechanical resistance. A bone's mechanical properties are determined by both its structure (mass, geometry, architecture) and the material properties of the tissue itself, such as mineral and collagen matrix composition, microdamage accumulation, collagen cross-linking, and tissue hydration [1]. Clinical strategies to reduce fracture risk have focused almost exclusively on improving bone mass, often assessed by bone mineral density (BMD). FDA approved anti-resorptive agents like bisphosphonates and denosumab significantly reduce fracture risk mainly by reducing osteoclast activity and bone turnover, thereby maintaining or elevating bone density by increasing mineralization [2]. Although increasing bone mass certainly improves bone's structural mechanical properties, changes in properties of the tissue itself can also significantly enhance bone's mechanical properties.

Raloxifene is a SERM (Selective Estrogen Receptor Modulator) used clinically in post-menopausal women to slow bone loss and decrease fracture risk [3]. Raloxifene suppresses osteoclast activity and bone remodeling in a manner similar to estrogen through high affinity interactions with ERα [4]. Compared to other anti-remodeling agents, such as bisphosphonates, raloxifene only modestly suppresses bone remodeling and induces little or no change in bone mineral density [5]. Despite small improvements in BMD, raloxifene significantly reduces vertebral fracture risk nearly as much as the bisphosphonates [6].

The mechanism for raloxifene's beneficial effects on bone has not been clearly elucidated, but our group has shown that raloxifene improves material-level mechanical (intrinsic) properties that are independent of bone mass and architecture [7–9]. These changes were most dramatic for bone toughness, a measure of the ability of the tissue to absorb energy prior to fracture. Following one year of treatment with clinically relevant doses of raloxifene in dogs, trabecular and cortical bone toughness in vertebrae, femoral neck and femoral diaphysis were twice those of vehicle-treated animals without a significant effect

Abbreviations: RAL, raloxifene; ALN, alendronate; RAL-4-Glu, raloxifene-4′-glucuronide; RAL bis-Me, raloxifene bismethyl ether.

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on bone volume or density [7,8]. Despite these effects, both clinically and in the laboratory, the mechanisms responsible for enhancement of mechanical properties are unclear. The current work investigates the mechanisms involved in raloxifene's enhancement of bone toughness. We hypothesize that raloxifene acts directly on the bone matrix to improve material properties, specifically the modulus of toughness.

Material and methods

Tissue, specimen processing and in vitro experiment

Canine bone samples from treatment naïve animals were obtained through tissue sharing at Indiana University School of Medicine. Femora from skeletally mature (15–24 mo/old) female beagles (1 dog) and male hounds (8 dogs) were used. Animals were part of Institutional Animal Care and Use Committee approved protocols. Human bone samples (unembalmed tibial diaphysis; male, 87 and 51 years old, donor 1 and 2, respectively) were obtained through the Indiana University School of Medicine anatomical donation program. Prismatic beams ($N = 8$–12 beams per experimental group) were machined following the bone longitudinal axis using a low-speed saw fitted with a diamond-coated circular blade, and hand-sanded to $1.37 \times 2 \times 25$ mm (Fig. 1a). Appropriate beam size was obtained using digital calipers (± 0.01 mm) and measured at 5%, 33%, 66% and 95% of beam length. Beams were sonicated (30 s) to remove debris and kept frozen in saline-soaked gauze until tested. All beams were subjected to freeze–thaw cycles (4–5 cycles) and a cell viability assay using lactase dehydrogenase (Suppl. Methods) showed no cellular survival after 1 freeze–thaw cycle (Fig. 1b). All incubations were performed in a 37 °C humidified incubator in PBS (1×, 0.22 μm filtered) supplemented with 1% penicillin–streptomycin. Because serum proteins can bind raloxifene, decreasing its relative binding affinity to ERα in vivo [10], fetal bovine serum (FBS) was used in one experiment to rule out this effect. Beams were incubated with specified compounds dissolved in dimethyl sulfoxide (DMSO) for 2 weeks at 2 μM unless otherwise noted. DMSO is one of the best organic solvents and is required for raloxifene to enter into solution. Vehicle (DMSO) was kept constant in all groups at 0.04% vol/vol. The high (2 μM) and low (5 nM) doses of raloxifene were chosen from the literature on the anti-oxidant effect of raloxifene, which spans from the low micromolar to the millimolar range [11–14], and its activation of the estrogen receptor, usually accomplished with low nanomolar concentration, respectively [15,16]. The low dose is also in the same range as the reported C_{max} (maximum effective concentration) of raloxifene (EVISTA product label, Eli Lilly). The alendronate dose used was equal on a molar basis to the high RAL dose (2 μM), while 17β-estradiol was used at 0.5 μM, a dose shown to exert anti-oxidant effects [11,17].

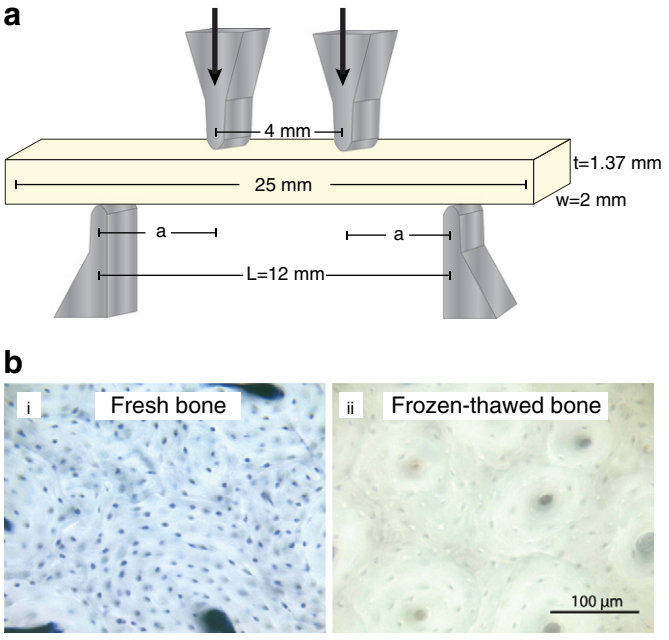

Fig. 1. (a) Schematic of the mechanical testing setup and beam dimensions. (b) Lactate dehydrogenase immunostaining of fresh (i) and frozen–thawed (ii) bone. Blue staining indicates living bone cells (osteocytes) in the bone matrix. Previously frozen–thawed bone (1 freeze–thaw cycle) shows no living cells.

Mechanical testing

Beams were subjected to 4-point bending on a 100P225 modular test machine (TestResources) with a 150 lb force transducer using a custom support with a lower span set at 12 mm and upper span at 4 mm (Fig. 1a). Beams were loaded to fracture at 2 mm/min, and displacement measured at 15 Hz from the actuator. We did not account for test frame compliance and although we recognize that this can affect the absolute measurements, it is not expected to alter the relative effects described in this paper. Structural variables recorded included ultimate load (F), stiffness (S), and energy to failure (U). Yield point was determined as 0.2% offset from the linear portion of the loading curve. Ultimate stress (σ_{ult}), modulus (E), and toughness (u) were estimated using standard equations for four-point bending of beam specimens: $\sigma_{ult} = F * (3\,L / 2wt^2)$, $E = (S / wt^3) \times (6La^2) - 8a^3$, $u = 9U / (wt(3\,L - 4a))$, where L is the span of the lower fixture, a is half of the difference between the lower and upper fixture span, and w and t are the specimen width and height (Fig. 1a) [7]. Following testing, the pieces of bone were wrapped in saline-soaked gauze and frozen.

Gravimetric analysis of water content

Pieces of previously broken beams were thawed and re-hydrated in PBS (or PBS + other compounds) for 2 days. Specimens were then patted dry, weighed (wet weight) and dried in a 100 °C oven. Weights were recorded every 24 h until stable for 2 consecutive days (3 to 4 days total). Bone density of PBS and RAL-treated samples (Suppl. Table 1) were obtained using wet weight and uCT-derived bone volume, and used to convert the lost water weight into volumetric percent of lost water. Water density was set at 1 mg/mm^3.

3D ultrashort echo time magnetic resonance imaging (UTE MRI)

The bone samples were stacked and placed in a 3 ml syringe filled with perfluorooctyl bromide (PFOB) solution to minimize susceptibility effects and enhance tissue–air contrast. A three-dimensional (3D) ultrashort echo time (UTE) sequence was implemented on a 3 T Signa TwinSpeed scanner (GE Healthcare Technologies, Milwaukee, WI) which had a maximum gradient strength of 40 mT/m and a maximum slew rate of 150 mT/m/ms. The 3D UTE sequence employed a short rectangular pulse (duration = 32 μs) for non-slice selective excitation followed by 3D radial ramp sampling with a nominal TE of 8 μs. The regular 3D UTE sequence was used to image both the short and long T_2 water [18,19]. The shorter T_2 water components were selectively imaged with 3D inversion recovery (IR) prepared UTE sequence, where a relatively long adiabatic inversion pulse (8.6 ms in duration) was employed to simultaneously invert and suppress long T_2 water signal [20]. A home-made 1-inch diameter birdcage transmit/receive (T/R) coil was used for signal excitation and reception. Typical imaging parameters included a TR of 300 ms, a flip angle of 10°, sampling bandwidth of 125 kHz, imaging field of view (FOV) of 8 cm, reconstruction matrix of $256 \times 256 \times 256$. For IR-UTE imaging, a TI of 90 ms was used for long T_2 free water suppression [18].