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Positive alterations of viscoelastic and geometric properties in ovariectomized rat femurs with concurrent administration of ibandronate and PTH

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ABSTRACT

Besides bone mineral density (BMD), structural and nano-level viscoelastic properties of bone are also crucial determinants of bone strength. However, treatment induced viscosity changes in osteoporotic bone have seldom been characterized. In this study, the effects of anabolic, antiresorptive and concurrent treatments on ovariectomized rat bones were thoroughly analyzed using multiple bone strength parameters. A total of 52 female Sprague–Dawley rats of 3 months age were divided into 5 groups and subjected to sham (SHM group) or ovariectomy surgery (OVX, PTH, IBN and COM groups). Weekly low-dose parathyroid hormone (PTH) and/or ibandronate or its vehicle was administered subcutaneously to the respective groups starting from 4th week post-surgery. Four rats per group were euthanized every 4 weeks and their femurs were harvested. The BMD, micro-architectural parameters, cortical bone geometry and viscoelastic parameters were measured at the distal femoral metaphysis. Our results showed that PTH, ibandronate or its concurrent treatment can effectively reverse ovariectomy induced deteriorations in both trabecular and cortical bone. Different drugs had selective effects especially in preserving geometric and viscoelastic properties of the bone. The concurrent administration of PTH and ibandronate was shown to offer an added advantage in preserving mean BMD and had a positive effect on cortical bone geometry, resulting from an increased periosteal formation and a decreased endocortical resorption. Viscosity (η) was prominently restored in combined treatment group. It is in accordance with an observed denser alignment of collagen fibers and hydroxyapatite crystal matrix with fewer pores, which may play an important role in hindering fracture propagation.

Introduction

Osteoporosis is characterized by low bone mass and micro-architectural deterioration of bone tissue, which leads to impaired skeletal strength and increased susceptibility to fractures [1]. The most common clinical index to assess the osteoporotic fracture risk is bone mineral density (BMD), measured using dual-emission X-ray absorptiometry (DXA). The measured apparent density, which is represented as T-score, is used to predict bone strength of patients [1].

Consequently, the efficacy of anabolic and anti-resorptive drug treatments on osteoporotic bone is often assessed by BMD and via biomarker analysis. Weekly parathyroid hormone (PTH) administration has been shown to stimulate bone formation and increase BMD [2]. On the other hand, ibandronate, a potent nitrogen-containing bisphosphonate inhibits bone resorption, leading to increment in BMD and reduction in fracture risk [3]. However, it was reported that the concurrent use of anabolic and antiresorptive drugs does not necessarily promote a synergistic effect in terms of total BMD or biochemical marker [4]. Although the ratio between drugs plays a role in combination therapy effect [5], it is also important to further prove or disprove the synergistic effect by other bone strength determinants besides BMD. Numerous critical determinants of bone strength have been largely investigated which may or may not be correlated with BMD measurement [6–9]. These determinants can be employed to evaluate the degree of osteoporosis and drug efficacy from different aspects which might be ignored in traditional assessments using BMD. Thus, these parameters can be considered along with BMD for a more accurate determination of various changes in osteoporotic bone quality.

As a biological material, bone exhibits a complex hierarchical structure with components dynamically undergoing remodeling,

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resulting in complicated mechanical properties [10,11]. These components are composed of macro scale bone cortex — trabeculae structure, micro scale osteon — lamellae structure and nano scale collagen fiber — hydroxyapatite crystal [12,13]. Viscoelasticity of bone, which has been of recent concern, arises from the void collapse, densification of trabeculae and the natural viscoelastic response of collagen fiber as a polymer [6,14–16]. During daily life, time-dependent viscoelastic deformation occurs in bone as the primary function of skeleton is to bear long-term load from bodyweight and muscular activity [14,17]. Certain viscoelastic parameters of cortical bone such as loss tangent have been demonstrated as an effective tool to test human bone strength [18,19]. Furthermore, Les et al. reported that long-term ovariectomy decreases ovine compact bone viscoelasticity [20]. These studies revealed a possibility that the development of osteoporosis is associated with the deterioration in viscoelasticity of bone. Furthermore, there is still a lack of characterization of various treatment effects on viscoelasticity of osteoporotic bone. For a better understanding of the pathogenesis and drug efficacy in osteoporosis, timely investigation into bone viscoelasticity is necessary.

Nanoindentation has been applied in recent years to measure elastic modulus (E) and contact hardness (H) of bone matrix at high resolution of load and displacement [21]. However, nano-indented creep viscosity (η) has not been rigorously studied. In one recent study, the precursory Voigt model was successfully used to describe the viscoelastic creep behavior of bone matrix during the holding period of indentation [17]. The results indicated that prolonged creep deformation (lower η) can play an important role in the long-term degradation of mechanical stability of older bone. In this study, nanoindentation tests were performed on individual trabeculae and cortex of the distal metaphyseal femur of ovariectomized Sprague–Dawley (SD) rats, treated with hPTH (1–34), ibandronate, or their combination. The main objective of this study was to assess the changes in nano-viscoelastic behavior (E, H, η) associated with ovariectomy and treatments. In comparison and explanation, possible concomitant alterations in macro bone geometry, micro-architecture and traditional BMD measurements will also be studied. It is hypothesized that osteoporotic bone properties would be influenced by mono or concurrent treatments in various aspects.

Materials and methods

Overall study design

Fifty-two female Sprague–Dawley (SD) rats (Laboratory Animal Centre, National University of Singapore) at the age of 3 months were housed at 25 °C with 12:12-hour light–dark cycle. They were given standard rodent diet (Harland, Model T.2018S) and water *ad libitum*. The animals were subjected to ovariectomy or sham surgery after two weeks of acclimatization at the Animal Holding Unit. All animal experiments were conducted in accordance with an approved protocol from the Institutional Animal Care and Use Committee (IACUC) at National University of Singapore. The rats were divided into the following 5 groups: (1) vehicle-treated sham operated group (SHM, n = 16); (2) vehicle-treated ovariectomized group (OVX, n = 12); (3) hPTH (1–34) treated ovariectomized group (PTH, n = 8); (4) ibandronate-treated ovariectomized group (IBN, n = 8) and (5) combined hPTH (1–34) and ibandronate treated ovariectomized group (COM, n = 8). Human parathyroid hormone (hPTH (1–34), Sigma-Aldrich, Singapore) and ibandronic acid (Roche Diagnostics GmbH, Mannheim, Germany) were diluted in 0.9% saline. Starting from week 4 post-surgery, 10 μg/kg body weight of hPTH (1–34) [22] and/or 7 μg/kg body weight ibandronate [23] or its vehicle (0.9% saline) was administered subcutaneously once a week until week 12 to the respective groups.

At week 0, four SHM rats were euthanized by carbon-dioxide asphyxiation for baseline value. At week 4, four rats each from SHM and OVX group were euthanized to measure the development of osteoporosis at the starting point of treatment. At weeks 8 and 12 respectively, four rats from each group were euthanized. Both right and left femur bones were harvested, wrapped in 0.9% saline soaked gauze and stored at −20 °C until they were used for the experiments.

Micro-computed tomography (μCT)

Majority of osteoporotic bone fractures in rat models occur at the trabeculae-rich metaphysis of the long bones [24]. Hence, metaphyseal femur was chosen to determine the degree of osteoporosis in the ovariectomized SD rat model. The metaphysis region of the distal femur was scanned ex-vivo in an upright position with a source to object distance (SOD) of 121 mm and a source to camera distance (SID) of 161 mm using a Skyscan 1076 micro-CT scanner (Skyscan, Belgium). A 3.6 mm-thick volume of interest (VOI, 200 CT slices) was selected 1 mm above distal growth plate (Fig. 1) [25]. The resultant grayscale images obtained had an isotropic voxel size of 17.75 μm from cone-beam reconstruction (100 kV, 100 μA, using a 0.5 mm Al filter and averaged 3 times). An automated method employing dual thresholds [26] was used to subtract the trabecular region from cortical bone using erosion and dilation kernels of size 3 to remove cortical porosities and reconnect marrow cavities. The grayscale images were segmented using a global threshold of 15% of the maximal grayscale value [27]. Direct 3D measurement methods [28] were used to assess trabecular bone volume ratio (BV/TV), trabecular bone surface to volume ratio (BS/BV), trabecular separation (Tb.Sp), trabecular number (Tb.N), structural model index (SMI), trabecular thickness (Tb.Th) and cortical porosity (Ct.Po) for the same VOI using the CT Analyser program (Phil Salmon, Skyscan). 3D visualization of the VOI using adaptive rendering algorithm was done with the ANT program (Skyscan, Belgium).

Peripheral quantitative computed tomography (pQCT)

The pQCT scans were carried out with pixel size of 0.1 mm and slice thickness of 0.5 mm using StraTEC's XCT (Research SA+, StraTEC Medizintechnik, GmbH, Pforzheim, Germany). A scout view scanning was performed prior to the actual scan to enable exact positioning of the bone specimens. The VOI selected was identical to that used for μCT where the effect of osteoporosis was expected to be significant. The threshold used for separating soft tissue from bone and sub-cortical from trabecular bones was set at 280 mg/cm^3 and 550 mg/cm^3 respectively. The mineral density measurements from pQCT were taken from 5 adjacent slices (inter slice distance: 0.75 mm) at the VOI for each femur sample (Fig. 2). Three types of volumetric bone mineral density (vBMD) were measured: mean BMD, trabecular density (Tb.BMD) and cortical density (Ct.BMD). Besides the vBMDs, cortical bone area (Ct.Ar), cortical bone thickness (Ct.Th), periosteal perimeter (Ps.Pm), endocortical perimeter (Ec.Pm), cross-sectional moment of inertia (CSMI) and Y axis Strength-Strain Indices (SSIy) were also determined at the VOI. SSI is related to both geometrical properties and cortical density [29]. The axial SSIy predicts the bending strength in the anterior–posterior direction, with respect to the y-axis, the longitudinal axis of rat femur.

Nanoindentation testing

The harvested rat femurs were kept with its longitudinal axis positioned vertically to the ground and with its distal condyles touching the base, to be embedded in epoxy resin. Over-night hardened samples were then metallographically cut to create a smooth surface 1 mm above growth plate, perpendicular to the long axis of the bone. Silicon carbide paper of grit sizes 320, 500, 1200, and 4000 was consequently used to grind the sample surface. Further polishing was done by microcloths with alumina powder of grit sizes 3 μm and