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Multi-level femoral morphology and mechanical properties of rats of different ages

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ABSTRACT

A macro–micro–nano–multi-level study was conducted to explore age-related structural and mechanical properties of bone, as well as the effects of aging on bone properties. A total of 70 male Wistar rats were used, ranging in the ages of 1, 3, 5, 7, 9, 11, 14, 15, 16, and 17 months ($n = 7$/age group). After micro-computed tomography (CT) scanning, longitudinal cortical bone specimens with a length of 5 mm were cut along the femoral shaft axis from left femur shafts for mechanical testing, and the cross-sectional areas were measured. The macro-mechanical properties obtained in mechanical testing and microarchitecture parameters measured by micro-CT were significantly correlated with the animal age ($r^2 = 0.96$, $p < 0.001$). Scanning electron microscopy was used for detecting the microarchitecture features of the fractured surfaces, which exhibited age-related plate-fibrous-mixed fibrous-plate texture, resulting in changes in macro-mechanical properties ($r^2 > 0.90$, $p < 0.001$). The mineral phase of the left femoral shaft and head was analyzed by atomic force microscopy. Longitudinal and transverse trabecular bone tissues, as well as longitudinal cortical bone tissue, were used for nanoindentation test, and the chemical composition was evaluated by quantitative chemical analyses. The correlations between mineral content and bone material properties (i.e., elastic properties of the bone tissue and size and roughness of bone mineral grains) were highly significant ($r > 0.95$, $p < 0.001$). Multi-level femur morphology, mechanical property, and mineral content were significantly correlated with the animal age. The correlations between bone mineral content and bone material morphological and mechanical properties may partly explain the increase in bone fragility with aging, which will provide a theoretical basis for the investigation of age-related bone properties in clinics.

Introduction

Advanced aging is a natural process that negatively affects bone integrity, which is associated with increased bone fragility that leads to high risks of hip fracture even after minimal trauma [1–3]. Hip fractures are among the most common age-related fractures, and age-related bone loss is a primary cause of osteoporotic fractures in the elderly [4]. Type II osteopenia begins at almost 40 years of age and progresses linearly at a rate of 0.5%–1% per year, accounting for nearly 40% of total bone loss accrued by 70 years of age [5,6]. The cortical area at the femoral midshaft increases until the seventh decade, when it begins to decline in both males and females. The medullary area triples in females and doubles in males from 21 to 97 years of age [7]. Such bone loss results in decreased mineralization in regions of trabecular and cortical bones, and increased fracture risk [5,7].

The variations in bone properties with aging need to be characterized and related to their mechanical behaviors for a better understanding of the etiology of age-related skeletal fragility. Studies of cadaveric proximal femora showed that deterioration in femoral geometry and microarchitecture and changes at the material level are recognized as important age-related distinct features [8–10]. Bone quality is a key component for assessing fracture risk. It is determined by complex features, such as bone mineral density (BMD), bone mechanical properties, and bone microarchitecture. BMD measurements are commonly used in clinical settings to estimate the degree of bone fragility and subsequent risk of age-related hip fracture [11]. However, several studies of bone density in men and women showed that BMD alone has limited efficiency for a thorough understanding of the decreased bone strength in the elderly, and age-related decrease in BMD does not sufficiently explain the high increase in hip fracture risk with aging [12,13]. A study in Rotterdam showed a 13-fold increase in hip fracture risk between 60 and 80 years, which was accompanied with a less than two fold decrease in BMD [12]. Furthermore, the overall proportion of fractures attributable to a low BMD was modest in a

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large cohort of elderly women. In particular, a significant overlap in BMD values was observed between hip fracture patients and controls [14]. Therefore, other bone properties, such as microarchitecture and mechanical parameters that also change with aging, may contribute to the increased bone fragility in senescence.

Given that the changes in trabecular bone microarchitecture may affect bone strength independently of bone mass, quantifying the characteristics of local trabecular bone architecture is essential to obtain a more complete estimation of the contribution of trabecular structure to mechanical competence, as well as its pathophysiology [15]. Through the advancement in spatial resolution of 3D imaging techniques and computational methodology, micro-computed tomography (CT) scanning has become a frequently used method for unbiased quantities of trabecular microarchitecture parameters [16,17]. However, the detailed morphology of bone surfaces cannot be fully identified by this method. Scanning electron microscopy (SEM) is a powerful technique for investigating bone architectural integrity and bone quality. It is effective in investigating the active states of bone surfaces [18]. Olszta et al. [19] and Benezra-Rosen et al. [20] examined the skeletal ultrastructure of mammals using SEM, and suggested that bone is a nanocomposite material composed of continuous mineral phase, organic material, and water. To improve our knowledge regarding bone structure at the nanolevel, atomic force microscopy (AFM) has been recently applied in some investigations [21–24]. Hassenkam et al. presented detailed AFM images from the edge, the front end, and the bottom of a resorption pit in a human trabecular bone sample that showed signs of incomplete remodeling and a scalloped surface left behind by the osteoclasts and the surface morphology of preexisting bone tissue and new bone tissue [22]. Milovanovic et al. observed that the mineral grain size in lateral femoral trabecular bone of old women is larger than that in young women, thereby decreasing elasticity of the lateral femoral neck in aged females. The mineral grain size can be considered as one of the determinants of its particular fragility in advanced age [3,23]. However, little is known about the age-related bone mineral grain size and the effects of grain size on mechanical properties of bone material. As of this writing, the effects of aging on the femoral subregions in terms of nanostructure and mechanical properties of bone material have not been fully understood. Nanoindentation, a technique first used in material science, was introduced to musculoskeletal investigations to determine the mechanical properties of bone material [25–29]. Compared with other indentation methods, this technique can be used to obtain the elastic modulus and hardness of a material [27]. Previous nanoindentation studies focused mainly on comparing the mechanical characteristics of bone tissue within various individuals [29–31], as well as bone mechanical properties in patients with particular bone diseases [25,32]. However, studies about the influence of aging on the mechanical properties of bone material are scarce [29,33].

Bone is an extremely integrated system, and previous studies about the influence of aging on bone focused mainly on one or several features of bone. For example, the femurs and humeri of male C57BL/6J mice between four and 104 weeks were subjected to mechanical testing (three-point bending) and morphological analysis [34], and the results showed that mice experienced age-related decrease in the whole femur mass and deteriorated bone structure. Samuel et al. analyzed the bone mineral content and mechanical parameters of baboons between 0 and 32 years of age, and found that these physicochemical properties are correlated with the animal age [35]. Considering the hierarchical organization of bone, studies at the macro–micro–nano–multi-levels will be helpful for better understanding of the age-related changes in bone properties. Age-related bone loss has been documented in rats. Osteoid mineralization, after femur marrow ablation in male Wistar rats, significantly diminishes from six to 24 months of age. The reduced responsiveness of bone cells with aging is reflected in morphological and structural properties of bone [36]. Animal models of age-related bone loss may serve as useful tools for investigating bone loss. A thorough understanding of the effects of aging on bone properties in rodents will assist in clarifying the mechanism of age-related human osteoporosis. This study aimed to improve the understanding of age-related bone properties by characterizing the changes that occur in long bones from male Wistar rats with ages of 1, 3, 5, 7, 9, 11, 14, 15, 16, and 17 months. In this study, mechanical testing was used for analyzing femur macro-mechanical properties, and micro-CT scanning and SEM were used for imaging microarchitecture properties of bone. AFM, nanoindentation testing, as well as quantitative chemical analyses were used for quantifying the characteristics of bone material to explore the aging effects on the mechanical and architecture properties at nanolevels.

Materials and methods

Materials

This study was performed in strict accordance with the recommendations of the Laboratory Animal Standardization Committee. The protocol was approved by the Medical Ethics Committee of No. 1 Hospital of Jilin University (2013-145). All efforts were made to minimize suffering of animals.

A total of 70 male Wistar rats, ranging in the ages of 1, 3, 5, 7, 9, 11, 14, 15, 16, and 17 months (weighing approximately 105 ± 5 g, 120 ± 10 g, 200 ± 9 g, 360 ± 10 g, 400 ± 15 g, 475 ± 10 g, 570 ± 15 g, 620 ± 9 g, 610 ± 10 g, 625 ± 7 g, respectively) (n = 7/each age group) were purchased from the Experimental Animal Center of Jilin University. These rats were housed in the same condition, and provided with a standard rodent diet (autoclaved NIH-31 with 6% fat; 18% protein; Ca:P, 1:1; and fortified with vitamins and minerals) and tap water. The environmental temperature was 24 ± 2 °C in natural light condition. The animals included in this study had no diseases affecting the bone, and did not use medications known to alter bone metabolism. Under 10% chloral hydrate 40 mg/kg anesthesia, all the rats were sacrificed, and the femurs were harvested for tests (Fig. 1) following removal of skin, muscle, and tendons.

Measurement of microarchitecture parameters using micro-CT scanning

Because the same sample would be used for a variety of experimental study, considering the requirements for different experiments, 80% ethanol (EtOH) was used both as dehydrant and fixative, and left femurs were initially fixed with 80% EtOH. Then, quantitative analysis of femoral microarchitecture was performed with micro-CT scanning (Skyscan 1076, Skyscan, Belgium). The spatial resolution for specimen scanning was set to 18 μm. The microarchitecture parameters of trabecular bone in the femoral head, i.e. bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), BMD, and cortical porosity of femoral shaft were calculated by CTAn software (CTAn, Skyscan, Belgium) [37].

Analyses of macro-mechanical properties using compressive mechanical testing

Three-point bending and compressive mechanical testing were common methods used for macro-mechanical testing. In consideration of the differences in femur length of rats with different months in this study, samples with the same size were cut from the femurs for compressive mechanical testing [38]. Samples were taken out from 80% ethanol, and the location of mid-diaphysis was set as a benchmark, and longitudinal cortical bone specimens with a height of 5 mm were cut along the femoral shaft axis from left femur shafts with a low-speed diamond saw under constant deionized water irrigation to ensure the circumference and verticality. The height (H) of each sample was measured by a vernier caliper and was shaved manually, and micro-CT images of samples were used to assess the cross-sectional area (S). The specimen was placed on an electronic universal testing machine