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Nanostructure and mineral composition of trabecular bone in the lateral femoral neck: Implications for bone fragility in elderly women

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ABSTRACT

Despite interest in investigating age-related hip fractures, the determinants of decreased bone strength in advanced age are not clear enough. Hitherto it has been obscure how the aging process affects the femoral neck nanostructure and composition, particularly in the lateral subregion of the femoral neck, which is considered as a fracture-initiating site. The femoral bone samples used in this study were obtained at autopsy in 10 women without skeletal disease (five younger: aged 20–40 years, and five elderly: aged 73–94 years). Atomic force microscopy (AFM) was applied to explore the mineral grain size in situ in young vs. old trabecular bone samples from the lateral femoral neck. The chemical compositions of the samples were determined using inductively coupled plasma optical emission spectroscopy and direct current argon arc plasma optical emission spectrometry. Our AFM study revealed differences in trabecular bone nanostructure between young and elderly women. The mineral grain size in the trabeculae of the old women was larger than that in the young (median: 95 vs. 59 nm), with a particular bimodal distribution: 45% were small grains (similar to the young) and the rest were larger. Since chemical analyses showed that levels of calcium and phosphorus were unchanged with age, our study suggests that during aging the existing bone mineral is reorganized and forms larger aggregates. Given the mechanical disadvantage of large-grained structures (decreased material strength), the observed nanostructural differences contribute to our understanding of the increased fragility of the lateral femoral neck in aged females. Moreover, increasing data on mineral grains in natural bone is essential for advancing calcium-phosphate ceramics for bone tissue replacement.

1. Introduction

Hip fractures represent one of the most important health problems in elderly population worldwide, particularly in females. Despite the significance of this problem and numerous studies addressing age-related bone fragility, the factors leading to decreased bone strength with advanced age are still not fully clear.

For a long time, bone mineral density (BMD) assessed by dual energy X-ray absorptiometry has been considered as the main predictor of bone strength and fragility [1]. BMD has also been analyzed by quantitative computed tomography (QCT), adding some architectural data to the densitometric values [2]. However, age-related decrease in BMD does not sufficiently explain the high increase in hip fracture risk with aging [3,4]. Namely, as demonstrated in the Rotterdam study, a 13-fold increase in hip fracture risk between 60 and 80 years was accompanied by less than 2-fold decrease in BMD [3]. Furthermore, the overall proportion of fractures attributable to a low BMD was modest in a large cohort of US elderly women [5]. In particular, there is a significant overlap in BMD values between hip fracture patients and controls [6]. Therefore, it is apparent that other parameters that also change with age contribute to the increased bone fragility in senescence. Considering the hierarchical organization of bone [7], age-related bone changes can be observed at the macroscopic, microscopic and nanoscopic levels. Namely, aging has been reported to be associated with deterioration in the femoral geometry [8], alteration of the femoral microarchitecture [9], a reduced number of osteocyte lacunae per bone area, with a higher proportion of mineralized lacunar occlusions [10], insufficient and/or delayed bone repair process [10,11] and microdamage accumulation [11], as well as changes at material level [12,13].

In order to improve our knowledge regarding bone structure at the nanolevel, atomic force microscopy (AFM) has been recently applied in various studies [7,14–18]. However, AFM analyses

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evaluating age-related differences at the human bone matrix level have been lacking so far.

The lateral region of the femoral neck is considered as an initiating site of an age-related fracture [19], and undergoes the greatest stress during a fall [20–21]. Previous micromorphometric and geometric studies showed preferential bone loss at the lateral femoral neck in elderly individuals [8,9,22]. However, to date, the effects of the aging process on that femoral subregion in terms of nanostructure and chemical composition have been completely obscure. In particular, AFM data about the mineral size in situ in young vs. old bone are insufficient.

The aim of our study was therefore to analyze the nanostructure and composition of trabecular bone samples from the lateral portion of the femoral neck in young and old women, in order to improve the picture about the determinants of age-related fragility of that particular femoral subregion.

2. Materials and methods

2.1. Specimen selection and preparation

Bone samples from proximal femora of five younger women (age: 20–40 years) and five elderly women (age: 73–94 years) were obtained at autopsy (Institute of Forensic Medicine, School of Medicine, University of Belgrade). The individuals included in the study neither displayed any sign of disease affecting the bones nor used any medications known to interfere with bone metabolism. Prior to analyses, all femora were subject to radiological evaluation in order to exclude the effects of other bone diseases. Study approval was granted by the institutional review board (Nr. 1600/1–3).

After storage in 70% ethanol for a minimum of 2 weeks, bones were cleaned of adherent soft tissue. A water-cooled diamond saw (Exakt, Germany) was used to excise the femoral neck region (Fig. 1a and b). A low-speed diamond wheel saw 650 with water-soluble coolant and a 0.3 mm thick diamond wheel (South Bay Technology Inc., USA) was used to make bone sections in the lateral region of the femoral neck, providing trabecular bone samples of approximately 5 mm × 5 mm × 1 mm (Fig. 1c). The specimens were afterwards cleaned ultrasonically in alcohol for 5 min and dried naturally at room temperature. All the specimens were prepared and analyzed in the same manner in order to ensure the validity of inter-specimen comparisons.

2.2. AFM analyses

Each trabecular bone sample was placed horizontally and glued onto the sample disk, and imaged by a Multimode Quadrex SPM with a Nanoscope IIIe Controller (Veeco Instruments, Inc.) under ambient conditions. The topography and phase images were acquired simultaneously by standard AFM tapping mode using a commercial solid nitride cone AFM probe (NanoScience Instruments, Inc.) with a cantilever length of 125 μm, a force constant of 40 N m^{-1}, a resonance frequency of 275 kHz and the tip radius of less than 10 nm. The phase image is based on measuring the phase shift of the cantilever, which is directly related to a local change in the energy dissipated in the sample during the tip–sample interaction, reflecting specific material properties of the sample independent of the topography. The simultaneous acquisition of AFM topography and phase images allows matching between the topographical elements and the material properties. In each sample, a minimum of 10 typical images were obtained from various locations, to assure representativeness of the observed features. The surface roughness was estimated from topography images using the subprogram packages of the Veeco NanoScope III. As the length scale expands, the roughness increases, finally achieving a saturation value, which we determined in the bone specimens. The size of the grains was recorded on AFM images by measuring the maximum dimension of the grain using Veeco Nanoscope III software version 5.31r1. Origin Lab (version 8) was used for the data analysis and curve fitting.

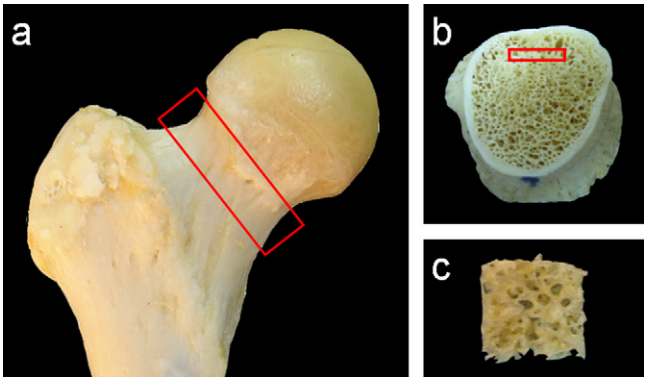

Fig. 1. Bone specimens: (a) proximal femur (the rectangle shows the neck region); (b) excised femoral neck (the rectangle in the lateral femoral neck depicts the site from which the trabecular bone sample was taken); (c) trabecular bone specimen.

2.3. Quantitative chemical analyses

Trabecular bone specimens were powdered using an electric grinder (Bosch Mkm 6000) to micron-sized particles (confirmed by AFM) and further prepared for chemical quantitative analysis. Inductively coupled plasma optical emission spectroscopy (Spectroflame, Spectro Analytical Instrument, Germany, 2.5 kW, 27 MHz) and direct current argon arc plasma optical emission spectrometry were used [23,24]. The user-friendly Quick Quant scan-based procedure was used to compare the intensities for measured elements in the samples with the intensities measured for standards with known concentrations. Calibration curves were calculated and the concentrations of the measured elements in the samples determined. A more detailed description of the procedures is available in the Supplementary data.

2.4. Statistical analysis

The Kolmogorov–Smirnov test was used to ascertain the normality of data distribution. In the case of normal distribution, Student's *t*-test for independent samples was applied; otherwise we used the Mann–Whitney *U*-test. Statistical analyses were performed with SPSS (Statistical Package for the Social Sciences) version 15, with a 0.05 significance level. The statistical power (0.8) for the given sample size, estimated sample variability, probability level and magnitude of differences intended to be detected was calculated using MedCALC software, version 9.1.0.1.

3. Results

Analysis of the AFM images revealed evident differences in nanostructure between the bone samples from the young and old females. Fig. 2 shows an AFM topography image of the bony trabecula. The images shown here are representative of those acquired from various areas of the sample and depict features observed consistently. In terms of materials, the observed bony surfaces represent a continuous phase, but with evident granular structure (AFM phase image, Fig. 3). The size of the grains differed between the young and old individuals in a specific manner (Figs. 4 and 5). In the elderly, the grains were on average larger (median: 95 vs. 59 nm; Mann–Whitney test, $p < 0.001$), but the variability

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