Experimental Gerontology 55 (2014) 19–28

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Nano-structural, compositional and micro-architectural signs of cortical bone fragility at the superolateral femoral neck in elderly hip fracture patients vs. healthy aged controls

Petar Milovanovic [a,d], Zlatko Rakocevic [b], Danijela Djonic [a], Vladimir Zivkovic [c], Michael Hahn [d], Slobodan Nikolic [c], Michael Amling [d], Bjoern Busse [d], Marija Djuric [a,*]

[a] Laboratory for Anthropology, Institute of Anatomy, School of Medicine, University of Belgrade, 4/2 Dr Subotica, 11000 Belgrade, Serbia
[b] Laboratory for Atomic Physics, Institute of Nuclear Sciences Vinca, University of Belgrade, 11001 Belgrade, Serbia
[c] Institute of Forensic Medicine, School of Medicine, University of Belgrade, 31a Deligradska, 11000 Belgrade, Serbia
[d] Department of Osteology & Biomechanics, University Medical Center Hamburg-Eppendorf, 59 Lottestr., D-22529 Hamburg, Germany

ARTICLE INFO

Article history:
Received 2 September 2013
Received in revised form 18 February 2014
Accepted 2 March 2014
Available online 12 March 2014

Section Editor: Christiaan Leeuwenburgh

Keywords:
Bone fragility
Hip fracture
Aging
Hierarchical structure
Atomic force microscopy
Quantitative backscatter electron microscopy

ABSTRACT

To unravel the origins of decreased bone strength in the superolateral femoral neck, we assessed bone structural features across multiple length scales at this cortical fracture initiating region in postmenopausal women with hip fracture and in aged-matched controls. Our combined methodological approach encompassed atomic force microscopy (AFM) characterization of cortical bone nano-structure, assessment of mineral content/distribution via quantitative backscattered electron imaging (qBEI), measurement of bone material properties by reference point indentation, as well as evaluation of cortical micro-architecture and osteocyte lacunar density. Our findings revealed a wide range of differences between the fracture group and the controls, suggesting a number of detrimental changes at various levels of cortical bone hierarchical organization that may render bone fragile. Namely, mineral crystals at external cortical bone surfaces of the fracture group were larger (65.22 nm ± 41.21 nm vs. 36.75 nm ± 18.49 nm, $p < 0.001$), and a shift to a higher mineral content and more homogenous mineralization profile as revealed via qBEI were found in the bone matrix of the fracture group. Fracture cases showed nearly 35% higher cortical porosity and showed significantly reduced osteocyte lacunar density compared to controls (226 ± 27 vs. 247 ± 32 #/mm^2, $p = 0.05$). Along with increased crystal size, a shift towards higher mineralization and a tendency to increased cortical porosity and reduced osteocyte lacunar number delineate that cortical bone of the superolateral femoral neck bears distinct signs of fragility at various levels of its structural organization. These results contribute to the understanding of hierarchical bone structure changes in age-related fragility.

1. Introduction

Femoral neck fractures are one of the most common osteoporotic fractures worldwide, becoming particularly prevalent after the age of 70 (Ström et al., 2011). Women are at a higher fracture risk, and global data shows that their age-standardized incidence of hip fracture is two-fold higher than in men (Kanis et al., 2012). Moreover, whereas the 10-year risk of hip fracture in women is 0.3% at the age of 50, it increases by 30-fold at the age of 80 (van Staa et al., 2001). However, it is not clear yet what makes some people susceptible to fracture even after minimal to moderate trauma; therefore, investigation of bone intrinsic properties is essential to improve current understanding of the fracture resistance and risk in the elderly.

Bone is a natural material displaying a remarkable hierarchical organization (Busse et al., 2013; Currey, 2002, 2012; Fratzl and Weinkamer, 2007; Launey et al., 2010; Seeman, 2008), where its mechanical integrity certainly receives contributions from all levels of hierarchy, from nano-scale (mineral crystals and collagen) to micro- (bone micro-architecture, osteocyte lacunar network) and macro-scales (bone size and geometry) (Busse et al., 2013; Launey et al., 2010; Milovanovic et al., 2013a; Rho et al., 1998; Rodrigues et al., 2012). Therefore, studying bone at various length-scales in individuals who sustained an age-related fracture may provide a framework for gaining insights into structural and compositional determinants of bone strength.

It is generally taken that cortical bone plays a crucial role in bone strength of the femoral neck (Holzer et al., 2009), and – although the trabecular bone is essential for transfer of stresses imposed on cortical shell – the thick cortical bone is considered to be the major load-

* Corresponding author. Tel./fax: +381 11 2686 172.
E-mail addresses: drpmilovanovic@gmail.com (P. Milovanovic), zlatkora@vinca.rs (Z. Rakocevic), ddjonic@yahoo.com (D. Djonic), vladinmejl@yahoo.com (V. Zivkovic), hahn@uke.de (M. Hahn), bobanvladislav@yahoo.com (S. Nikolic), amling@uke.de (M. Amling), b.busse@uke.uni-hamburg.de (B. Busse), marijadjuric5@gmail.com (M. Djuric).

http://dx.doi.org/10.1016/j.exger.2014.03.001
0531-5565/

bearing compartment (Verhulp et al., 2008). Various characteristics of the femoral neck cortical bone in humans were assessed in previous studies to detect structural features attributable to increased fracture risk (Bell et al., 1999a,b; Bergot et al., 2002; Bousson et al., 2004; Kaptoge et al., 2008; LaCroix et al., 2010). However, apart from geometric and architectural factors, determinants of fracture risk at the mineralized bone matrix level also deserve the growing attention of bone researchers (Bousson et al., 2011).

In this context, atomic force microscopy (AFM) has been recently established as a promising tool for the characterization of bone matrix changes. AFM provides an excellent imaging resolution even without complex preparation procedures that are necessary for other high resolution microscopies (Hassenkam et al., 2004, 2005, 2006; Milovanovic et al., 2011, 2012a). In cortical bone, AFM was applied in several qualitative studies to visualize the bone matrix and to determine the spatial relationship between mineral and collagen (Sasaki et al., 2002; Thurner et al., 2007). Quantitative structural studies using AFM on cortical bone are scarce, but have contributed valuable data on the morphology of lacuno-canalicular system in bovine and sheep bone (Lin and Xu, 2011; Reilly et al., 2001). Moreover, characteristics of collagen fibrils in rats (Wallace et al., 2010) have been assessed through AFM techniques focussing on banding patterns. Our recent study analyzed the size distribution of mineral crystals in young vs. aged women at the external cortical surface of the femoral neck, suggesting nano-structural signs of periosteal apposition during aging (Milovanovic et al., 2013b). However, cortical bone from women with hip fractures vs. healthy controls was not yet quantitatively characterized using AFM. We hypothesized that the cortical bone surface in fracture cases would also express unfavorable nano-structural features that may contribute to increased fragility. Moreover, we aim to unravel cortical bone features across multiple length scales in the same individuals to observe how various structural and compositional parameters differ between the individuals who had sustained a hip fracture and control cases.

Therefore, in order to assess the origin of decreased cortical bone strength, we applied a combined approach encompassing AFM characterization of cortical bone nano-structure, assessment of matrix mineral composition and distribution, mechanical testing of the bone material, as well as evaluation of cortical micro-architecture and osteocyte lacunar density in the region of the superolateral femoral neck (i.e. the fracture initiating region) in postmenopausal women suffering from hip fracture and in a control group of elderly women.

2. Material and methods

The study sample comprised cortical bone specimens from elderly women who sustained hip fractures ($n = 5$, age: 82 ± 4.6 years) and age-matched control cases of women ($n = 4$, age: 82 ± 9.8 years). Both groups of donors were Caucasian females from Serbia that were subject to autopsy at the Institute of Forensic Medicine, University of Belgrade — School of Medicine. Based on patients' records and autopsy findings, the donors in the fracture group were devoid of local cystic, neoplastic or inflammatory bone lesions, and they did not show signs of systemic diseases that could affect bone integrity. The control group was part of the AFM study reported previously (Milovanovic et al., 2013b). These cases did not present with history of musculoskeletal diseases or fragility fractures. Both groups were not subject to anti-resorptive medication or other medications with recognized influence on bone quality (hormonal therapy, osteoporosis therapy, antiepileptics). The Ethics Committee of the University of Belgrade — School of Medicine approved the specimen collection and all procedures.

Following the harvesting of bone fragments, the specimens of the femoral neck were soaked in ethanol for at least 2 weeks and cleaned of soft tissues. It was noted that the control cases displayed a slightly wider femoral neck than the fracture cases (femoral neck diameter: $3.58 \text{ cm} \pm 0.18 \text{ cm}$ vs. $3.48 \text{ cm} \pm 0.27 \text{ cm}$; $p \geq 0.05$). To prepare bone specimens in the superolateral region of the femoral neck, a low-speed diamond wheel saw SYJ-160 (MTI Corp., USA) with water soluble coolant was used ensuring minimization of the thermal damage to the specimens during the specimen excision. Specifically, the cortical bone specimens were obtained by cutting parallel to the femoral neck axis at the superolateral region of the femoral neck. Following the sectioning procedure, the cortical bone specimens (each $4 \text{ mm} \times 4 \text{ mm} \times 1 \text{ mm}$ in size, with preserved periosteal cortical surface) were ultrasonically cleaned in alcohol for 5 min to remove any dirt or debris that arose from the sectioning. Subsequently, they were allowed to dry at room temperature without the use of a heating cabinet, so that any damage to the surface was kept to a minimum. In particular, all the specimens were prepared/analyzed in a consistent manner to ensure validity of inter-specimen comparisons.

2.1. Assessment of the nano-structure using atomic force microscopy

Given that intact specimens are preferred for AFM observation of the surface features, specimen preparation for AFM encompassed no subsequent embedding and polishing procedures of the surface that was going to be scanned (periosteal cortical surface). Therefore, directly following the sectioning and ultrasonication, each cortical bone specimen was glued horizontally onto the sample disk, and its external (periosteal) cortical surface was imaged by Multimode quadrex SPM with a Nanoscope IIIe controller (Veeco Instruments, Inc.) under ambient conditions. A commercial AFM probe (NanoScience Instruments, Inc.) with cantilever length of 125 μm, force constant 40 N/m, resonant frequency 275 kHz and the tip radius lower than 10 nm was used for probing the bone surface, acquiring topography and phase images simultaneously in standard AFM tapping mode. Images were recorded with 256 lines per scan. According to previous studies, height (topography) image displays the three-dimensional surface morphology (i.e., topography) of the sample, while the phase image depends on material properties of the sample (Bar et al., 1999; García et al., 2007; Jandt, 2001; Nenadović et al., 2012). More exactly, in phase-mode, it is the phase shift of the cantilever that is recorded, where this phase lag is directly related to a local change in the energy dissipation during the interaction between the AFM probe (tip) and the sample (Anczykowski et al., 1999; García et al., 2007; Strbac et al., 2010). Based on simultaneous acquisition of both height and phase images, it is possible to distinguish between the topographical elements of different material properties, as well as to enhance image contrast and improve detection of edges of the surface features (Jandt, 2001).

To account for inter-site variability and ensure representativeness of the observed features, a minimum of ten images were obtained from various locations in each specimen. Determination of the size of principal topographic elements of the surface (grains = mineral crystals) was performed in WSxM software (WSxM v5.0, developed by Horcas et al. (2007)), by measuring the maximum dimension (length) of individual crystals, in line with the previous studies (Milovanovic et al., 2011, 2012a,b, 2013b). For statistical analysis of inter-group differences, all grain sizes were pooled per study group and appropriate group-specific graphs of grain size distribution were obtained. In addition, surface roughness and textural complexity was evaluated on the topography images in accordance with the previous studies (Milovanovic et al., 2012a, 2013b).

2.2. Evaluation of bone matrix composition

2.2.1. Quantitative backscatter electron imaging (qBEI)

Following AFM imaging, the unembedded specimens were polished to achieve smooth coplanar surfaces using an automatic precision grinding system (Exakt, Germany) in accordance with well established protocols of quantitative backscatter electron imaging (Busse et al., 2009, 2010; Regelsberger et al., 2012). Subsequently, the polished external (outer, periosteal) surface of the cortical specimens was carbon coated and mounted on the scanning electron microscope (LEO 435