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Relationships between tissue composition and viscoelastic properties in human trabecular bone

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ABSTRACT

Trabecular bone is a metabolically active tissue with a high surface to volume ratio. It exhibits viscoelastic properties that may change during aging. Changes in bone properties due to altered metabolism are sensitively revealed in trabecular bone. However, the relationships between material composition and viscoelastic properties of bone, and their changes during aging have not yet been elucidated. In this study, trabecular bone samples from the femoral neck of male cadavers ($n=21$) aged 17–82 years were collected and the tissue level composition and its associations with the tissue viscoelastic properties were evaluated by using Raman microspectroscopy and nanoindentation, respectively. For composition, collagen content, mineralization, carbonate substitution and mineral crystallinity were evaluated. The calculated mechanical properties included reduced modulus (E_r), hardness (H) and the creep parameters (E_1, E_2, η_1 *and* η_2), as obtained by fitting the experimental data to the Burgers model. The results indicated that the creep parameters, E_1, E_2, η_1 *and* η_2, were linearly correlated with mineral crystallinity ($r=0.769$–0.924, $p<0.001$). Creep time constant (η_2/E_2) tended to increase with crystallinity ($r=0.422$, $p=0.057$). With age, the mineralization decreased ($r=-0.587$, $p=0.005$) while the carbonate substitution increased ($r=0.728$, $p<0.001$). Age showed no significant associations with nanoindentation parameters. The present findings suggest that, at the tissue-level, the viscoelastic properties of trabecular bone are related to the changes in characteristics of bone mineral. This association may be independent of human age.

1. Introduction

Trabecular bone is a dynamic tissue that consists of calcified bone matrix and marrow. Remodeling occurs on the surfaces of trabecular bone with high surface to volume ratio (Seeman, 2009). The calcified matrix is mechanically viscoelastic (Isaksson et al., 2010b; Schoenfeld et al., 1974; Yamashita et al., 2002a). Thus, bone enables dissipation of energy when loaded mechanically (Yamashita et al., 2001; Yamashita et al., 2002a). This mechanism helps the bone to resist fractures under dynamic or impact loading. Viscoelasticity of the calcified matrix is believed to mostly relate to the collagen matrix, which also adds plasticity and ductility to the bone (Saito and Marumo, 2010; Yamashita et al., 2002b). During aging, changes in the collagen matrix may make the calcified matrix more brittle (Burr, 2002; Isaksson et al., 2010a; Knott and Bailey, 1998; Wang et al., 2002).

Raman spectroscopy has been used to assess molecular composition and crystallinity of the calcified matrix of bone (Ager et al., 2005; McCreadie et al., 2006; Turunen et al., 2011; Yerramshetty et al., 2006). Mineralization has been reported to increase with age in femoral cortex in men (Yerramshetty et al., 2006). Similar results, using Fourier transform infrared (FTIR) microspectroscopy, were reported for trabecular bone of proximal femur and calcaneus (Turunen et al., 2013). None of these studies found any age-related changes in mineral crystallinity (Ager et al., 2005; McCreadie et al., 2006; Turunen et al., 2011; Yerramshetty et al., 2006).

The hierarchical structure contributes to strong mechanical properties of the whole bone (Rho et al., 1998). Nanoindentation can be used to analyze the mechanical properties of bone at the tissue-level (Lewis and Nyman, 2008; Norman et al., 2008). Previous nanoindentation studies have mainly focused on measuring bone elasticity and hardness (Hoffler et al., 2000a; Hoffler et al., 2000b; Rho et al., 1997; Zysset et al., 1999). More recently, viscoelasticity of bone has been assessed (Bembey et al., 2006; Fan and Rho, 2003; Isaksson et al., 2010a; Shepherd et al., 2011; Wu et al., 2012), mostly using

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semi-dynamic or creep testing (Fischer-Cripps, 2004; Isaksson et al., 2010b). At present, studies addressing the associations between the tissue composition and viscoelastic properties in aging human trabecular bone are lacking.

In this study, we hypothesized that mineralization of human trabecular bone changes with age. Possibly, alterations in mineralization could also contribute to viscoelastic properties during aging. To evaluate this hypothesis, we quantified the viscoelastic properties and the molecular composition of hydrated human trabecular bone using nanoindentation and Raman microspectroscopy, respectively. Viscoelastic parameters were derived from Burger's creep model using a single constant loading rate (Fischer-Cripps, 2004; Isaksson et al., 2010a; Isaksson et al., 2010b; Shepherd et al., 2011; Wu et al., 2012). Age-dependent changes in composition and nanoscale mechanical properties were analyzed, and the associations between the composition and mechanical properties were assessed.

2. Methods

2.1. Sample preparation

Twenty-one proximal femurs were extracted from human male cadavers with no pre-existing metabolic bone diseases ($n=21$, age$=50\pm18$ years, range from 17 to 82 years) (Table 1). After soft tissue removal, the proximal femurs were stored in a freezer (-20 °C) until further preparation. The study design was approved by the National Authority for Medicolegal Affairs (5783/04/044/07). A transverse cross-section of ~10–15 mm in thickness was cut from the femoral neck of each cadaver with a band saw. From the cross-section, a cylindrical trabecular bone plug (diameter $=10$ mm) was drilled. The bone plugs were cut to a length of 8 mm with a slow speed saw (Buehler Isomet, Buehler Ltd., Lake Bluff, IL). The bone marrow was removed using an ultrasound cleaner in Phosphate Buffered saline (PBS). The samples were sonicated for 10 min. The procedure was repeated twice. The flat ends of the cylindrical samples were polished (FORCIPOL 1 V, Metkon Instruments Ltd., Bursa, Turkey) stepwise using progressively finer SiC papers (P1000, P2000 and P4000). After polishing, the samples were rinsed with water and stored in PBS (-20 °C) until analyses.

Table 1
Anthropometric data of the male cadavers. (Mean ± SD).

Age (years)	Height (cm)	Weight (kg)
17	178	74
22	186	106
26	175	60
32	171	69
35	187	102
37	177	74
39	185	84
43	171	98
44	179	96
46	185	85
49	178	85
50	185	108
52	180	136
54	176	73
59	175	73
59	169	96
64	170	72
74	166	64
77	171	78
78	177	72
82	165	53
50 ± 18	**176 ± 7**	**84 ± 19**

The samples were thawed before measurements. Nanoindentation testing was conducted first followed by Raman microspectroscopy of the same trabeculae. To localize the nanoindentation and Raman measurement points, a reference mark was made with a marker pen on the edge of the polished sample surface and a stereomicroscopic image of the entire polished surface was taken (Leica MZ75, Leica Microsystems Ltd., Heerbrugg, Switzerland). Same person processed all the samples under identical conditions to minimize the influence of variations in measurement environment on the results.

2.2. Nanoindentation

To characterize the roughness of the polished surface, two random samples were taken from the sample pool. For both samples, two 50 μm x 50 μm areas within one trabeculae were scanned with an atomic force microscope (XE-100, Park Systems Corp., Suwon, Korea). The surface roughness was measured from the cross-sectional height profile of the scanned area using the device's software (XEI 1.8.0., Park Systems Corp., Suwon, Korea). The measured surface roughness ($R_a=64$ nm) was defined as the arithmetic average of the absolute values of the scanned profile.

For nanoindentation, a diamond Berkovich tip with a diameter of 50 nm was used (NanoTest, Micro Materials Ltd, Wrexham, United Kingdom). The tests were performed with the samples submerged in PBS. A load of 30 mN was applied with a loading rate of 5 mN/s, and the maximum load was maintained for 60 s. In each sample, two trabeculae were characterized using five indentations 40 μm apart. All indentations were made in the longitudinal direction of the trabeculae and at *x-y* points along the axial direction of the cylindrical samples (Fig. 1).

The Oliver and Pharr method (Oliver and Pharr, 1992) was used to determine the reduced modulus E_r and hardness H from the unloading curve according to Eqs.1 and 2 (Fig. 2A).

$$E_r = \frac{dP}{dh}\frac{1}{2}\frac{\sqrt{\pi}}{\sqrt{A}} \quad (1)$$

and

$$H = \frac{P_{max}}{A}, \quad (2)$$

where dP/dh is the experimental stiffness derived from the elastic portion of the unloading curve with a linear fit, A is the contact

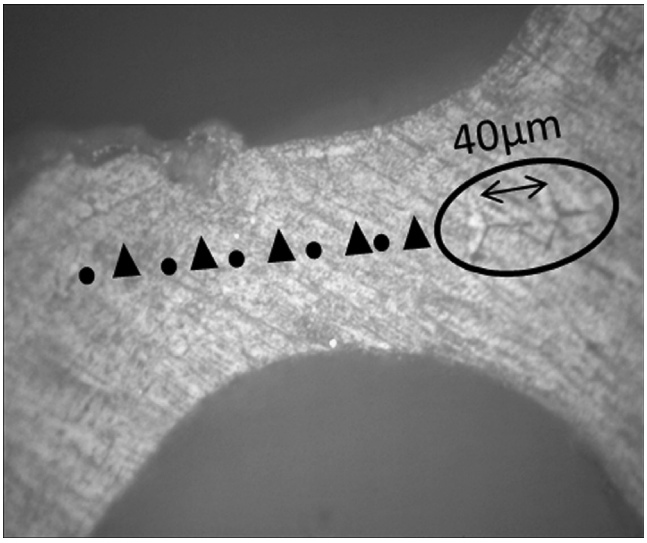

Fig. 1. Microscopic image of the reference points made using nanoindentation with 250 mN force. The triangles and dots indicate the measurement sites for nanoindentation and Raman microspectroscopy, respectively.