**Spatial Distribution and Remodeling of Elastic Modulus of Bone in Micro
Regime as Prediction of Early Stage Osteoporosis**

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Abstract

We assessed the local distribution of bone mechanical properties on a micro-nano scale and its correlation to strain distribution. Left tibia samples were obtained from 5-month old female Sprague Dawley rats, including baseline control (n=9) and hindlimb suspended (n=9) groups. Elastic modulus was measured by nanoindentation at the dedicated locations. Three additional tibias from control rats were loaded axially to measure bone strain, with 6-10N at 1Hz on a Bose machine for strain measurements. In the control group, the difference of the elastic modulus between periosteum and endosteum was much higher at the anterior and posterior regions (2.6GPa), where higher strain differences were observed (45 $\mu\epsilon$). Minimal elastic modulus difference between periosteum and endosteum was observed at the medial region (0.2GPa), where neutral axis of the strain distribution was oriented with lower strain difference (5 $\mu\epsilon$). In the disuse group, however, the elastic modulus differences in the anterior posterior regions reduced to 1.2GPa from 2.6GPa in the control group, and increased in the medial region to 2.7GPa from 0.2GPa. It is suggested that the remodeling rate in a region of bone is possibly influenced by the strain gradient from periosteum to endosteum. Such pattern of moduli gradients was compromised in disuse osteopenia, suggesting that the remodeling in distribution of micro-nano elastic moduli among different regions may serve as a predictor for early stage of osteoporosis.

KEYWORDS: Remodeling, bone mechanical property, disuse, nanoindentation, strain, elastic modulus

Introduction

Bones, the load bearing organ, bear the weight of the human body and also of the loads applied on it during locomotion (Pekka, 2007). As a living tissue, the skeleton has the capacity to model and remodel itself (Burger and Klein-Nulend, 1999). It is demonstrated that loading increases the mechanical properties of bone, while the absence of loading causes loss of its mechanical strength (Burger and Klein-Nulend, 1999; Huang and Ogawa, 2013; Qin et al., 1998; Wolf, 1995). Bones of astronauts on long-term space flights, patients of prolonged bed rest or of paralysis and traumatic brain injury experience long states of functional disuse (Lau and Guo, 2011). Functional disuse under such conditions causes loss of overall bone strength, decrease in mineralization and a structural deterioration of bone architecture. It moreover leads to bone fragility and increased tendency of bone to fracture (Faibish et al., 2006). It is proposed that shear forces induced by bone fluid, due to the loading conditions, on the osteocytes leads to more bone formation (Burger and Klein-Nulend, 1999; Knothe Tate et al., 1998; Qin et al., 2010; Reich et al., 1990; Rubin et al., 1997; Weinbaum et al., 1994). The racquet holding arm of a tennis player being stronger than the contralateral is an example of this phenomenon (Taylor et al., 2009).

Work conducted in the past showed correlation between bone tissue strain and mechanical properties of bone (Carter, 1982; Frost, 1983; Lanyon, 1987). On a micro scale also remodeling has shown to be dependent upon the loading the bone experiences. Meulen in 2006 showed that for cancellous bone in mice, the bone volume fraction, direct trabecular thickness and mineral apposition rate were higher for limbs exposed to periods of in vivo cyclical loading, when compared to their respective contralateral limbs which served as controls (van der Meulen et al., 2006). For cortical bone, it has also been shown that the regional microstructural properties are significantly influenced by the mechanical strains experienced. For example, using an avian ulnae model, there were significant differences between different bone regions in the cross sections, in which mean laminarity indices (LI) were varied between sub adult ($40.0\% \pm 10.7\%$) and adult ($50.9\% \pm 10.4\%$) bones, and their related bone strain (Skedros and Hunt, 2004). Regional variation in collagen orientation and volume, as well as in mineralization in cortical bone, were found to be

dependent upon the regional variation in bone strain. Skedros et al. (2013) showed that both osteon size and the collagen organization in the osteons are strongly influenced by the local strain magnitude (Skedros et al., 2013). Regional collagen fiber orientation and mineralization affect the regional bone mechanical properties. Manjubala et al. (2009) showed that with an increase in mineralization in embedded callus, there was also an increase in indentation modulus from 6GPa at early stages to an average of 14GPa at the later stages of mineralization (Manjubala et al., 2009). The possible effects of collagen fibers orientation on the mechanical properties of bones were shown to use bovine and equine samples (Martin and Boardman, 1993; Martin and Ishida, 1989; Skedros et al., 2006). Martin and Ishida in their study determined the importance of collagen fibers orientation on tensile strength of cortical bone, whereas the Martin and Boardman's 1993 study showed that the fibers orientation was the most significant predictor, using stepwise multiple regression, of cortical bone bending in bovine tibias. Skedros et al. (2006) showed that in strain-mode-specific testing fiber orientation had a significant effect on post yield behavior and total energy in the testing.

Previous work has been conducted to measure micro-mechanical properties of the bone (Angker et al., 2005; Fan et al., 2002; Hengsberger et al., 2002; Hoffler et al., 2005). Donnelley et al showed that bone nano-mechanical modulus was higher in the periosteal region and correlated it with the bone tissue composition using Raman Spectroscopy (Donnelly et al., 2010). But a complete picture of how these properties spatially differ in various regions of a transverse bone section, from the periosteal to the endosteal side of bone and its dependence on the local mechanical strain values have not been investigated. Hence the first objective of this study was to assess the effect of regional strain distribution on spatial distribution of micro-mechanical modulus in a control bone. Our hypothesis was that the micro mechanical modulus distribution should be dependent upon the micro strains distribution in the different regions of the bone. Areas of higher micro-strains should also have higher micro-mechanical moduli as compared to areas of bone, which experience lower micro strains.

The second objective of the study was to assess remodeling in the spatial distribution of micro-mechanical moduli, due to functional disuse conditions. The hypothesis was that due to functional disuse conditions, the regional micro

mechanical moduli distribution would be significantly different from the control group for each investigated region.

To access the elastic moduli distribution in different regions, nanoindentation was used. Nanoindentation allows measurement of mechanical properties such as elastic moduli and hardness at a micro- and nano-scale resolution level (Angker et al., 2005; Ebenstein and Pruitt, 2004; Fan et al., 2002). Nanoindentation has been recently used in a lot of studies to measure bone mechanical properties (Pathak et al., 2011), lamellar level bone mechanical properties and sub micron mechanical properties (Pathak et al., 2012; Silva et al., 2004).

Materials and Methods

Bone samples from left tibia were obtained from 5-month old virgin female Sprague Dawley rats, including 1) baseline control (n=9), and 2) hindlimb suspended (HLS) (4 weeks, n=9) (Hu et al., 2012). The animal study was previously approved by the Stony Brook University IACUC.

Sample Preparation

After a cross-sectional cut and clearing of the bone marrow using a water jet, 2mm in longitudinal length segments at the midshaft were cut in the transverse plane using a diamond wheel saw (South Bay technology Inc., MODEL 650, Clemente, California, USA) under constant water cooling.

Embedding and Polishing

The bone samples were stored in ethanol of subsequent higher concentrations of 70%, 80%, 90% and then 100% for 2 days each for complete drying. Subsequently, they were embedded in epoxy resin and let cure for a day. To expose the surface of the bone, the embedded samples were first polished with silicon carbide papers (Buehler-Carbimet, Illinois, USA) of grit numbers 320, 600, 1200, 2400, 4000 mounted on a Buehler grinder Power pro 3000TM (Illinois, USA) in the respective sequential order. Finally the samples were polished by Polycrystalline Diamond Suspensions (Buehler MetaDiTM Supreme) of roughness 3 μ m, 1 μ m, 0.25 μ m and finally by 0.05 μ m in the given descending order.

Nanoindentation

The distribution of bone mechanical properties such as elastic modulus and hardness were measured on a micro scale in the z or axial directions by Nanoindentation (Hysitron Triboindenter TI-950, Minneapolis, Minnesota) at precise locations. A Berkovich tip was used for all of the indentations. For consistency in all the samples, indents were made near the periosteal side, near the middle, and also near the endosteal side of the bone in the anterior, posterior and medial regions of the bone as shown in Figure 1. The tip area function was calibrated from indentation analysis on fused quartz, and drift rates in the system were measured prior to each indentation using standard indentation testing procedures. A preload of $2\mu\text{N}$ was used during indentation. The indentation consisted of a 10 seconds loading period at a constant loading rate of $100\mu\text{N/s}$. A constant load segment at the peak load of $1000\mu\text{N}$ followed this for a time of 30 seconds. In the end, the tip was retracted in the unloading segment for another 10 seconds at a constant unloading rate of $100\mu\text{N/s}$. The total indentation time was 50 seconds. The elastic response was calculated from the 20–90% portion of the unloading curve. Elastic modulus was calculated assuming an elastic response during unloading and using Oliver Pharr Method (Hoc et al., 2006).

Mechanical Loading and Strain Gauge Measurement

Three additional control rats were sacrificed and their left hindlimbs were removed from the hip joint. With the soft tissues and muscles still attached, the hindlimbs were axially loaded, from knee to the foot joint on the Bose ElectroForce machine (BOSE, Inc., Minneapolis, Minnesota). Strain gauges were attached to the mid-shaft tibia bone at the anterior, posterior and medial sides of the bone in a rosette pattern for measurement of strain induced by the loading for all the three rats. Axial loading by locking the hindlimb at the knee and foot joints was used in accordance with previous studies so as to analyze the compressive strains developed in the tibia midshaft (Patel et al., 2014a). Peak to peak strains at three strain gauge sites were measured around the mid-tibia (National Instruments Strain acquisition box). LabVIEW was used to control the operation of measurement of strains. For each rat, the hindlimbs were loaded with a sinusoidal load function with peak magnitudes ranging from 6-10N at 1Hz frequency in load control mode. The Bose Machine recorded displacement and

the load on the bone, while strains produced (peak to peak) were measured on the LabVIEW software via the NI Strain Acquisition Box.

μCT scanning

After strain measurements, the left tibias were later removed from the rest of the hindlimbs by dislocating the tibia at the knee and foot joint. All the soft tissues were removed and the bone was cleaned. The strain gauges were not disturbed at this step and still left attached to the bone. The whole bone was then scanned using μCT (SCANCO Inc.) with 36μm resolution. Image slices of the bones having the three strain gauges attached were chosen for further analysis of strain distribution.

MATLAB Image Processing

The bone transverse slice, with all the three strain gauges attached in a rosette pattern, was chosen for image processing. The edges of the bone slice were smoothened and some unnecessary external features were cropped out (such as wires) from the bone slice by ROI poly method in MATLAB (version 2013a). Coordinates of the image pixel representing the bone just next to the middle of the strain gauge attachment site was given the strain value (peak to peak) as measured by the strain gauge. Using linear beam theory MATLAB codes were built to find planar strain distribution across the whole bone slice (Serra-Hsu et al., 2011). Briefly in the linear beam theory, if strains at three different sites of known x, y and z coordinates, then the strain distribution in the plane containing all the three sites can be found. This method assumed that planar sections remain planar even after loading and a linear strain profile is present in the section. Strain value for each point in the planar section is acquired from the linear interpolation of the strain values at the three dedicated locations (Funk and Crandall, 2006). In this study, the coordination were found from the μCT images and the strain values from the methods described above.

Statistical Analysis

The Nanoindentation data from the scan software of Hysitron Triboindenter and the strain values from National Instruments Strain Acquisition Box were exported into Microsoft Excel (Microsoft Excel 2011, version 14.0.0). Two tailed Student's t-test was performed when the control and experimental groups were compared and when the periosteal elastic moduli values were compared with endosteal elastic moduli

values of a particular region. However one-way ANOVA test was performed for comparing the elastic moduli of all the three anterior, posterior and medial regions together in the control as well as in the experimental groups. IBM statistical software SPSS 22.0 was for the one-way ANOVA statistical analysis. The significance level was set at 0.05 for both the student t-test and one-way ANOVA tests.

Results

Nanoindentation

A total of 6 areas of interest were investigated for each segment. The mean of the periosteal, middle and endosteal elastic moduli was regarded as the mean indentation value of the region. The indentation values at the various regions are in Table1. The p values between the control and experimental group were: anterior, $p=0.04$; posterior, $p=0.28$; medial, $p=0.13$. It was observed that the elastic modulus difference from periosteum to endosteum was much higher in the anterior and posterior regions (2.6GPa) than the medial region (0.2GPa), in the control group (Fig. 2A). In the same group the periosteum and endosteum were significantly different (in terms of elastic moduli) in the anterior and posterior regions ($p<0.001$). In the medial region, the difference of elastic moduli between periosteal and endosteal side was not statistically significant ($p=0.41$).

In the disuse group, however, the elastic modulus difference in the anterior posterior direction was reduced to 1.2GPa, and increased in the medial direction, 2.7GPa (Fig. 2B). The periosteum and endosteum were not statistically different in the anterior region($p=0.4$). However in the posterior and medial regions the difference between the periosteal and endosteal side was statistically significant ($p=0.01$ and $p<0.001$ respectively).

MATLAB Image Processing and Strain Analysis

The planar strain distribution was found in MATLAB using linear beam theory as explained in the methods section. One such strain distribution is shown in Figure 3. The strain distribution clearly showed that the anterior side of the bone was under tension, while the posterior region was under compression. The average tension to compression ratio was approximately 1:1.25. This showed that the tibia mid-diaphysis is under higher compression than tension. This was in accordance with previous

studies done on rat tibia mid- diaphysis (Patel et al., 2014b). On even a more regional level, strain (peak to peak) distribution in mid tibia shaft further showed higher strain difference (from periosteum to endosteum) in the anterior-posterior ($45\mu\epsilon$) but reduced to $5\mu\epsilon$ in the lateral medial direction (again from periosteum to endosteum). The neutral axis obtained was roughly parallel to the posterior side of the bone in all the three sections investigated.

Discussion

Previous research showed that the fluid-flow in the canaliculi causes shear stresses on the osteocytes, which served as the beginning of the mechano-sensing pathway (Gross and Williams, 1982; Huang and Ogawa, 2013; Qin et al., 2010; Qin et al., 1998; Rubin and Lanyon, 1987; Zeng et al., 1994). The bone fluid-flow remodeling theory brought a high interest in the investigation of what causes the fluid-flow and what are its effects. Research has indicated that activities from daily life that cause bone strain, can induce bone fluid-flow (Burger and Klein-Nulend, 1999; Gross and Williams, 1982;; Hu et al., 2012; Qin et al., 2010; Reich et al., 1990; Serra-Hsu et al., 2011; Weinbaum et al., 1994; Wolf, 1995). Research has also been conducted to identify the nature of the bone fluid-flow and the effect of magnitude, frequency of flow on bone formation (Hu et al., 2012; Hu and Qin, 2014; Qin et al., 2003; Qin et al., 2010). Research has shown that bone remodeling depends upon the magnitude as well as on the cyclical nature of strain (Rubin et al., 1996).

Correlations have been shown between bone strains and mineralization density, as well as between strain and collagen fiber volume and orientation. The same parameters, mineralization and collagen fiber volume and orientations, have also been correlated with the regional mechanical modulus of bone.

Hence this study was focused on the analysis of possible correlation between the strain induced in different regions of bone and the micro-nano level mechanical properties in different regions of a bone section. In the present study the anterior, posterior and medial regions of the bone were investigated. For measurement of mechanical modulus in these regions the bones were indented using nanoindentation on transverse sections of embedded samples in epoxy from the cortical mid-diaphysis of rat tibia. Indents were made on the periosteal as well as on the endosteal side of the

bone. Frequency of 1Hz was chosen to mimic a normal rat's gait and low forces were investigated to produce strains in accordance with previous research (Hsieh et al., 1999; Torcasio et al., 2012).

The mean insignificant p values between all the three regions investigated in a healthy or control bone showed that the modeling and remodeling activity was similar in all the three regions and were not different from an elastic moduli point of view on a sub-millimeter or a macro level. However, on a micro level, in the control group, significant differences were observed between the periosteum and endosteum (in terms of elastic moduli) in the anterior and posterior regions and insignificant differences in the medial region. Wolff's law which was further improved upon in Frost's law, states that due to loading bone adapts itself to become stronger (Frost, 1994). However an adaptation to micro strain differences, from a mechanical perspective, in such small scales has not been investigated so far. Details of the mechanisms that mediate this process have not been elucidated so far and a potential future research can be investigating the mechanism in different other load bearing as well as non-load bearing bones. Such an anisotropic remodeling points out to the extreme sensitivity of the mechano-sensing and transduction mechanism. The study suggests that the remodeling mechanism inside the bone is not only dependent upon the total absolute strain value and its frequency but also on the differences in strain values between the periosteal and endosteal sides of bone. The strain gradients arising in different regions of the bone from periosteum to endosteum could also govern the remodeling rate of that region. A high modulus gradient from periosteum to endosteum suggests a possible high bone formation rate at the periosteal side or a high resorption rate at the endosteal side or a combination of both. A low modulus gradient from periosteum to endosteum suggests towards a low formation rate at the periosteal side or a low resorption rate at the endosteal side or a combination of both. Nevertheless, a high modulus gradient from periosteum to endosteum in a bone region suggests high remodeling in that region and a low modulus gradient could be an indicator of low bone remodeling in that region.

The turnover in the moduli gradients from periosteum to endosteum in different regions of bone suggested that remodeling rate of different regions changes during functional disuse. In the study conducted we observed that in the unloaded bones the

modulus gradient increased in the medial region and decreased in the anterior and posterior regions. In the posterior region (high strain gradient region), the modulus difference between periosteum and endosteum is highly significant ($p < 0.001$) in the control group, while such modulus difference is moderate in the disuse group ($p = 0.01$). This implies that strain difference between periosteum and endosteum in a region, induced by the loading, may significantly contribute to the remodeling rate within that region.

Prediction of the risk of fracture from bone mechanical properties and density values has been the key focus of a lot of studies on osteoporosis. Dual Energy X-ray absorptiometry (DXA) uses low-level energy X-ray to determine the areal bone mineral density of bone, but does not provide the micro-mechanical properties (McPherson et al., 2014; Weryha et al., 1991). To assess bone mineral density through T and Z scores obtained from DXA is the main clinical measurement for osteopenia and osteoporosis (Gilsanz, 1998; Lata and Elliott, 2007; Ryan et al., 2011). But according to several recent studies bone mineral density is not an accurate representation of the fracture risk of the bone. Buxsein's group in their study concluded that therapeutic increases in bone mineral density are not proportional to reduction in fracture risk and the distribution and composition of bone also have high effects on the risk of fracture (Roberts et al., 2010). Efforts are being put in other methods of diagnosis of clinical fracture risk. Some the alternative methods include use of ultrasound (Hakulinen et al., 2004; Kotha et al., 2008; Lin et al., 2014; Qin et al., 2013) and quantitative computed tomography (QCT) and peripheral QCT (pQCT) techniques (Engelke et al., 2013; Xing et al., 2003) to determine bone strength and micro-architecture.

Conforming to the study, differences in the distribution of micro-mechanical modulus could also serve as a predictor for the onset of osteopenia. However, such change in mechanical modulus distributions within the bone will be completely unobservable in DXA as it assesses the mean mechanical properties on which no effect has taken place from one month of functional disuse. The studying of sub-micro mechanical differences will take into account mechanical properties as well as structural integrity or shape of the bone. Recent study has also demonstrated that microindentation on the bone surface, cause micro-fractures and the analysis of which may be able to correlate

with the overall mechanical property of bone tissue. In this method a reference point indentation instrument was used which loads the bone surface at a particular load and frequency and measures the surface mechanical properties (Diez-Perez et al., 2010; Gallant et al., 2013; Granke et al., 2014). Hence possibly use of reference point indentation methods can serve as a new tool for clinical diagnosis of early stages of osteoporotic conditions.

Limitations

The study conducted has some limitations. Firstly, indentation was performed on dehydrated samples, which can cause the indentation modulus to increase (Nyman et al., 2006). This is a major limitation to any nanoindentation study where bone samples are dehydrated before indentation. Before forming any conclusions, only two strain zones were investigated, the anterior- posterior direction and the later- medial. Investigations in the future should be in other directions so as to make a proper correlation between strain and moduli gradients.

Conclusion

While mechanical stimulation is essential for bone adaptation, the study showed that bone remodeling not only responded to the strain magnitude generated by the loading, but also significantly depended on the difference of local strain magnitude, such as its experienced value between periosteum and endosteum. Eventually, to assess the spatial distribution of micro-mechanical moduli of bone, i.e., at periosteum and endosteum regions, may lead to the prediction of bone health conditions.

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Figure caption

Fig. 1. Indents (black dots) distribution in the cross section of bone at the anterior, posterior and medial regions of a rat tibia.

Fig. 2A. Elastic moduli obtained at the periosteal and endosteal regions from arterial, medial, and posterial areas in the control group.

Fig. 2B. Elastic moduli at the periosteal and endosteal regions from arterial, medial, and posterial areas in the functional disuse group. * indicates $p<0.01$, ** indicates $p<0.001$.

Fig. 3. Longitudinal strain distribution in the cross section of rat tibia middle diaphysis generated by loading using beam theory. Red color region shows tensile strain values, whereas blue color region shows compressive strain values in bone.

Table 1

The table shows the average elastic moduli values from the indentation of the samples for both control and experimental groups at the periosteal and endosteal regions. The mean values and the standard deviations are in the unit of GPa.

Figure 1

Fig. 2A

Fig. 2B

Figure 3

Table

	ANTERIOR		MEDIAL		POSTERIOR	
	Periosteum (GPa)	Endosteum (GPa)	Periosteum (GPa)	Endosteum (GPa)	Periosteum (GPa)	Endosteum (GPa)
CONTROL	28.81±2.78	25.58±2.99	27.15±2.65	26.62±3.97	28.65±2.25	26.16±3.6
EXPERIMENTAL	27.13±3.15	26.52±4.41	29.13±3.23	25.17±4.3	28.45±3.35	27.15±3.21