Analysis of Mineral Distribution in the Trabecular Bone of Normal and Estrogen Deficient Rat Ulnae and Radii Using Micro CT and Nanoindentation

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Abstract
Recent studies have shown that bone tissue-level mineral distribution is altered in a sheep model of osteoporosis [1], and changes in mineralized crystal maturity, mineral-to-matrix ratio, and collagen cross-linking occur [2]. This study involved the use of µCT and nanoindentation to conduct an analysis of the effect of estrogen deficiency on the mineral distribution in trabecular bone from an ovariecomised (OVX) rat model. Micro CT (µCT) analysis was conducted at the Sub-Macro level (volumes of the trabecular network) to evaluate the extent of bone loss and overall mineral content changes due to estrogen deficiency (OVX and age-matched controls). Nanoindentation was carried out on individual trabeculae from these bone samples to obtain localised material properties at the tissue level. Micro CT was used for Tissue level analysis of these same individual trabeculae to evaluate the mineral distribution at precise locations in the trabecular network and to gain understanding of the material level mineral changes which occur in the distal forearm in estrogen deficiency.

1. Introduction
Osteoporosis results in severe bone loss, significantly reduced strength and altered bone tissue porosities. Recent studies have shown that bone tissue-level mineral distribution is altered in a sheep model of osteoporosis [1], and changes in mineralized crystal maturity, mineral-to-matrix ratio, and collagen cross-linking occur [2]. In particular quantitative backscattered imaging (qBEI) of trabeculae of ovine femurs was applied to analyse the mineral heterogeneity of bone tissue. This study showed that heterogeneity of mineral distribution in estrogen deficient animals compared to controls, was dependent on the anatomical region and the age of the animal [3]. Although fracture of the distal forearm is one of the most frequent osteoporotic fractures, the mineral changes in the trabecular bone of ulnae and radii during estrogen deficiency have not been investigated. The objective of this study was to use µCT and nanoindentation to conduct a comprehensive analysis on the effect of estrogen deficiency on the mineral distribution and mechanical integrity of trabecular bone in the distal forearm of an ovariecomised (OVX) rat model. Micro CT (µCT) analysis was conducted at the Sub-Macro level (volumes of the trabecular network) to evaluate the extent of bone loss and overall mineral content changes due to estrogen deficiency. Nanoindentation was carried out on individual trabeculae from these bone samples to obtain localised material properties at the tissue level. Micro CT was used for Tissue level analysis of these same individual trabeculae to evaluate the mineral distribution at precise locations in the trabecular network and to gain understanding of the material level mineral changes which occur in the distal forearm in estrogen deficiency.

2. Methods
The bone tissue used in this study originated from the skeletally mature rats of a European Research Council funded project, which were sacrificed 34 weeks post-ovariectomy (OVX). The distal end of the ulna and radius were studied in the OVX (n=4) and age-matched control (n=4) animals.

Micro-CT, Sub-Macro level: Trabecular regions of approximately 0.005mm³, were evaluated using µCT (μCT100, Scanco) at a resolution of 4.9µm. A threshold of 770 mgHA/ccm was determined based on calibration of the grey scale images from the first ulna and radius samples scanned. This thresholded scan data was used to evaluate the microarchitecture and mineral content, in particular the bone volume fraction (BV/TV), trabecular connectivity, trabecular number and bone mineral density distribution (BMD). The full width at half max (FWHM) was calculated for the BMDD histogram of each sample in order to evaluate the mineral heterogeneity, with a higher FWHM indicating a more heterogeneous mineral density distribution (see Fig. 1).

![Figure 1: Representative Bone Mineral Density Distribution plot (BMDD)](image)

Nanoindentation, Tissue level: Ulnar bone specimens were sectioned along the longitudinal axis using a precision diamond blade saw (IsoMet™ LS Saw, Buehler) and then embedded in epoxy (Epo-thin low viscosity epoxy, Buehler) and polished to achieve a high quality test surface. Six indents were made using a Berkovich diamond tip indenter on a CSM Nano-Hardness Tester across the width of a trabecular strut on each sample. Each indent was made by driving the
indenter into the sample for 60 seconds to a max load of 20mN, holding for 120 seconds and unloading. The equations (1), (2) and (3), developed by Oliver and Pharr [4], were implemented to calculate the tissue elastic modulus and tissue hardness from the unloading segment of the indentation load-displacement curves (see Fig. 2).

\[
S = \frac{2}{\sqrt{\pi}} \frac{E_{\text{eff}}}{A} \sqrt{A} \\
\frac{1}{E_{\text{eff}}} = \frac{1}{E_i} + \frac{(1 - v_i^2)}{E_v} \\
H = \frac{P_{\text{max}}}{A}
\]

Where, \( S \) is the initial unloading stiffness, \( \beta \) is the indenter geometry constant, \( E_{\text{eff}} \) is the effective elastic modulus, \( A \) is the Indenter-Sample contact area, \( E \) and \( v \) are the Elastic constants of the sample, \( E_i \) and \( v_i \) are the elastic constants of the indenter, \( H \) is the sample hardness and \( P_{\text{max}} \) is the max indentation load applied.

**Figure 2: Force-displacement curves from nanoindentation of trabecular tissue.**

**Micro-CT, Tissue level:** A subset of the nanoindentented ulnar trabeculae were \( \mu \)CT scanned again at 6.6\( \mu \)m resolution (with previously mentioned scan parameters) to assess the mineral distribution at the tissue level. This thresholded scan data was used to evaluate the mineral content changes in estrogen deficiency through analysis of the bone mineral density distribution (BMDD).

Statistics: Student t-tests were used to analyse the statistical significance of the difference between the OVX and age-matched control groups, with significance of \( p<0.05 \)

**3. Results**

**Micro-CT, Sub-Macro level:** Significant differences were seen in the microarchitecture of the OVX ulna specimens compared to the controls; BV/TV (-40%, \( p=0.04 \)), trabecular connectivity density (-60%, \( p=0.03 \)) and trabecular number (-30%, \( p=0.026 \)), see Fig. 3B inset. Trabecular thickness was higher in OVX animals (+11%, \( p=0.09 \)). There was no significant difference in average mineral content (1195 mg Ha/ccm vs. 1192 mg Ha/ccm, \( p=0.85 \)). The mineral distribution in OVX bone (FWHM: 241.18 mg Ha/ccm) was significantly (\( p=0.04 \)) more homogeneous than controls (FWHM: 298.78 mg Ha/ccm).

**Micro-CT, Tissue level:** Preliminary results from analysis of individual ulnar trabeculae (OVX, \( n=2 \) and Control \( n=1 \)) showed increased Peak, Mean and FWHM values from the BMDD curves of OVX samples compared to controls.

**4. Discussion**

**Micro-CT, Sub-Macro level:** The results from analysis of trabecular volumes suggest that, although the bone mass is lower in OVX animals, there is a decrease in the mineral heterogeneity of trabecular tissue in the distal ulna and distal radius. However, there seems to be no difference between OVX and healthy tissue in the peak and average mineral content. This suggests that overall range of mineral content densities seen in OVX bone has reduced, but the peak and average mineral densities observed in the bone have not changed.

**Nanoindentation, Tissue level:** The preliminary nanoindentation results suggest that OVX ulnar trabecular tissue is stiffer than healthy tissue. These material properties did not match our observations in the sub-macro level bone mineral analysis. Therefore we sought to conduct a localized analysis of mineral distribution at the tissue level (individual trabeculae).

**Micro-CT, Tissue level:** This localized \( \mu \)CT analysis of mineral distribution indicated more heterogeneous mineral distribution and higher mineral content in the OVX ulnar trabeculae that had been indented. These findings suggest that the mineral distribution at the tissue level of estrogen deficient rat ulnae is similar to that seen previously in sheep femurs, but further study is required to confirm this observation.

The increased elastic properties and higher mineral content at the tissue level, might render the ulnar tissue more brittle and explain distal forearm fracture, but further research is required to explore this. All graphics should be centered and placed close to their point of reference.

**5. References**