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Short communication

# Effects of freeze–thaw and micro-computed tomography irradiation on structure–property relations of porcine trabecular bone

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ABSTRACT

We study the effects of freeze–thaw and irradiation on structure–property relations of trabecular bone. We measure the porosity, apparent density, mineral content, trabecular orientation, trabecular thickness, fractal dimension, surface area, and connectivity of trabecular bone using micro-computed tomography (micro-CT) and relate them to Young's modulus and ultimate strength measured by uniaxial compression testing. The analysis is done on six-month porcine trabecular bone from femoral heads. The effects of freeze–thaw are studied by using bones from three different groups: fresh bone and bones frozen for one and five years. We find that the porosity and apparent density have most dominant influence on the elastic modulus and strength of fresh bone. Also, five years of freezing lowers both Young's modulus and ultimate strength of trabecular bone. Additionally, the effects of radiation are investigated by comparing Young's modulus before and after micro-CT exposure. We find that the micro-CT irradiation has a negligible effect on the Young's modulus of trabecular bone. These findings provide insights on the effects of tissue preservation and imaging on properties of trabecular bone.



## 1. Introduction

Testing of freshly harvested bone is generally impractical (Huss et al., 1995). Previous research has shown that freeze–thaw does not affect mechanical properties of bone and thus is a popular method for storing bone (Panjabi et al., 1985). However, little is known about the effects of long term freezing on the properties of bone.

Knowledge of structure–property relations of trabecular bone is of importance for diagnosis and assessment of osteoporosis. Morphological measures of trabecular bone microarchitecture have been used, in addition to porosity and apparent density, to correlate with mechanical properties of trabecular bone (Hodgskinson and Currey, 1990a, 1990b; Goulet et al., 1994). Micro-computed tomography (micro-CT) has been employed to obtain such data (Muller, 2009; Burghardt et al., 2011). Effects of radiation on bone properties have been studied but mainly for sterilized bone (Barth et al., 2010, 2011; Singhal et al., 2011).

In this paper we study the effects of freeze–thaw and micro-CT irradiation on structure–property relations of porcine trabecular bone. We conduct this analysis using fresh bones, bones frozen for one and five years, and bones before and after micro-CT exposure. We measure the porosity, apparent density, mineral density, fractal dimension, surface area, orientation, thickness, and connectivity of trabeculae using micro-CT and relate them to Young's modulus and ultimate compressive strength obtained by compression testing. This study contributes to better understanding of the effects of tissue preservation and imaging on properties of trabecular bone.

## 2. Materials and methods

### 2.1. Sample preparation

Femurs from six-month old pigs (*Sus scrofa domestica*) were obtained from the Meat Science Lab at the University of Illinois at Urbana-Champaign. Porcine bone was selected because its biology is similar to human bone (Pearce et al., 2007). All animals were healthy and raised under diets satisfying nutrient levels recommended by the Nutrient Requirements of Swine (2012). After harvesting, femurs were either tested fresh (group A) or stored in the freezer at −20 °C for one year (group B) or five years (group C). Three femurs from each group were used. Prior to freezing, the bones were wrapped in gauze saturated with 0.1 M Phosphate Buffered Saline (PBS) and sealed in zip lock bags to prevent drying (Kang et al., 1997). Prior to preparing samples, each frozen femur was thawed for approximately 24 h at 4 °C. All samples were cut into a cylindrical shape; six to eight specimens were made from each femoral head (Fig. A1 in Appendix-online). We used an aspect ratio 2:1 (height 8 mm × diameter 4 mm) for uniaxial compression test samples (Keaveny et al., 1993b). Each sample was stored in PBS at 4 °C. All tests were completed within 48 h from thawing.

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### 2.2. Micro-CT and image post-processing

Samples were placed in an Xradia micro-CT sample holder for imaging after being dried from the PBS using tissue paper. An Xradia micro-CT (MicroXCT-200, Pleasanton, CA) with a 2 × magnification was used to obtain images while rotating each sample 190 degrees. Such rotation was selected to minimize length of time of imaging. Images were taken every 0.5 degrees and there was no special filter used except one converting x-ray to digital images. The voltage and power for the x-ray were 40 keV and 10 W, respectively, and the camera exposure time was 5 s. The voxel size of the image file was approximately $10 \times 10 \times 10\ \mu m^3$ which is adequate for accurately imaging the trabecular bone microarchitecture (Peyrin et al., 1998). Standard Xradia software was used for scanning, reconstruction and exporting image files for post-processing. The XM3DViewer program converted the files to. ooc files which were compatible with the image post-processing software, AMIRA (Version 5.4.2, Visage Imaging, Inc., Berlin., Germany). Measured parameters, selected following Odgaard (2001) and Muller (2009), are listed in Table 1.

### 2.3. Mineral density measurements

High mineral density composite calibration phantoms were used to measure the hydroxyapatite (HA) volume density (Deuerling et al., 2010). Seven different phantoms in the range of 0–1860 mg HA/cm$^3$ with 0, 10, 20, 30, 40, 50, and 60% HA by volume were used. Each phantom was scanned with micro-CT (MicroXCT-200, Pleasanton, CA) while immersed in PBS using the same settings: magnification (2 ×), voltage (40 keV), power (10 W) and beam hardening coefficient (2). Standard water phantoms (1.16 HU scale) were then scanned using the same settings and all the intensity factors from the HA phantoms were calibrated to the Hounsfield scale. As a result, a quadratic curve was created which is the HU scale versus HA volume fraction. The trabecular bone samples were scanned with the same settings and, using this quadratic curve, the average mineral density of each sample was measured.

### 2.4. Density measurements

Samples were placed in a centrifuge 5415d (Eppendorf, Hauppauge, NY) with a speed of 11,000 rpm for 15 min to remove fluid from bone's interior. The samples were wrapped in tissue paper while in the centrifuge to prevent damage and then weighed using an electrical scale. The measured weight and the volume obtained from the micro-CT images were used for calculating the density and apparent density (Carter and Hayes, 1977; Galante et al., 1970; Zioupos et al., 2008).

### 2.5. Compression testing

An MTS Insight electromechanical testing system with a 2000 N load cell (MTS systems Corp., Eden Prairie, MN) was used for uniaxial compression testing. No preload was applied for the compression test and platen speed was 0.005 mm/s.

#### 2.5.1. Freeze–thaw effect

Effect of freeze–thaw was studied using bones from three groups: group A ($n=24$) fresh bone, group B ($n=23$) bones frozen for one year, and group C ($n=20$) bones frozen for five years. The compression test was stopped after the load passed its maximum and started decreasing. The slope of the "linear portion" of the stress–strain curve was used to evaluate Young's Modulus and the maximum stress gave the ultimate strength (Morgan et al., 2001).

#### 2.5.2. Radiation effect

To study the radiation effect uniaxial compression tests were done on trabecular bone samples ($n=4$) within the elastic limit. Then, the samples were scanned with Xradia for approximately 2 h. The settings were identical to experiments used to image trabecular bone samples except for the camera exposure time (6 s). Lastly, the same platen compression test was conducted on these irradiated samples to measure Young's modulus. Young's moduli before and after irradiation were compared.

### 2.6. Statistical analysis

First, a normality test was done for the compression test results to see if they had a normal distribution. Then, one way ANOVA test was used by OriginPro 9 (OriginLab Corporation., Northampton, MA., USA) to test significant differences of mean and variance between the groups.

## 3. Results

Effects of freeze–thaw on Young's modulus and strength, studied using bone samples from all three groups (fresh bone, and bones frozen for one or five years), are summarized in Fig. 1

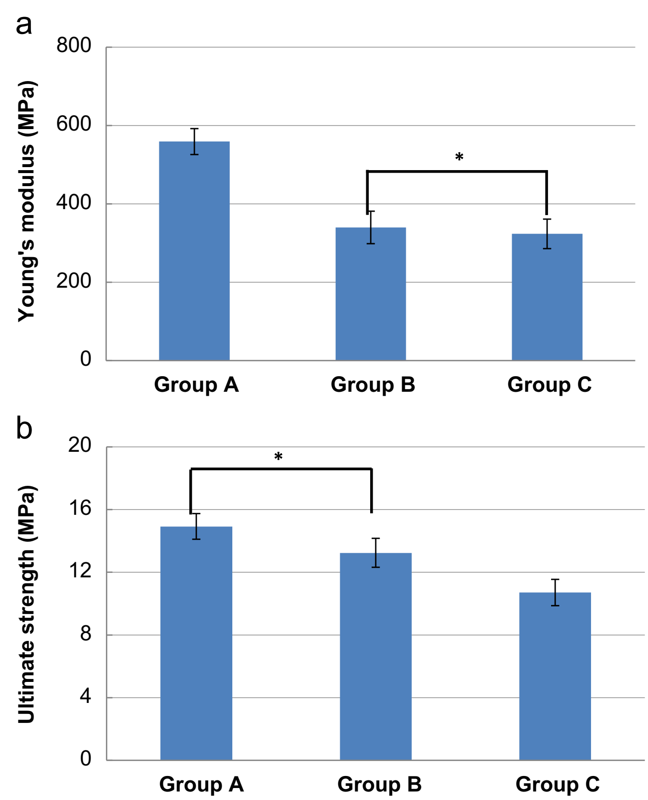


**Fig. 1.** Average (a) Young's modulus and (b) ultimate strength of groups A (fresh bone), B (bone frozen one year) and C (bone frozen five years). The bars represent standard deviation. '*' mean no significant difference ($p > 0.05$).

**Table 1**
Morphological and material parameters measured.

| Parameter | Definition | Unit |
|---|---|---|
| Porosity | The ratio of the volume of voids to the total volume | % |
| Area 3D | The area of the object surface | $\mu m^2$ |
| Euler characteristic | An indicator of the connectedness of a 3D structure | N/A |
| Fractal dimension | A measure of a self-similar roughness of the surface | N/A |
| Orientation, Orientation 2 | The angle between the orientation, or the second orientation of the particle and the longitudinal axis | Degree |
| Fragmentation | An indicator of connectivity measured by image dilatation | N/A |
| Thickness | The average thickness of a trabecula | $\mu m$ |
| Mineral density | Hydroxyapatite density in bone | mg/cm$^3$ |
| Apparent density | Weight divided by the volume including the pores | g/mm$^3$ |
| Density | Weight divided by the volume excluding the pores | g/mm$^3$ |