SB^{3}C2016
Summer Biomechanics, Bioengineering and Biotransport Conference
June 29 –July 2, National Harbor,MD, USA

DESIGN OF FATIGUE TEST FOR EX-VIVO MOUSE VERTEBRA

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INTRODUCTION

Exposure to ionizing radiation increases degeneration, risk of fracture, and decreases long-term bone quality. The complications related to high levels of ionizing radiation impact both astronauts and radiotherapy (RT) patients. Astronauts embarking on deep-space missions outside the Earth's protective magnetosphere, such as lunar, Mars, or near-Earth asteroid missions, will be exposed to high-energy ionizing radiation from cosmic or solar sources. Bones neighboring radiotherapy treatment are exposed to high doses of gamma radiation.

Previous studies have demonstrated significant changes in bone mechanics and quantity with radiation. Women with cervical, rectal, or anal cancer and treated with RT were 1.7 - 3.2 times more likely to suffer a pelvic fracture than patients who did not receive RT [1]. Animal studies of low to high dose radiation (2 – 20 Gy) have shown decreased trabecular bone volume fraction [2], bone embrittlement [3], and altered tissue composition, such as collagen cross-linking [4].

Understanding changes in bone mechanics is important for identifying increased risk of fracture with radiation exposure. In particular, fatigue, or cyclic load testing provides insight into changes at the molecular level that are not apparent in a monotonic test. Changes to the organic matrix can decrease energy dissipation mechanisms intrinsic to bone and manifest in a decreased fatigue life.

The mouse model is the IACUC preferred and most common mammalian model for experimental testing. However, mechanical testing of mouse vertebrae is not trivial due to its geometry and small scale. Thus, the purpose of this work was to develop a protocol, including sample preparation and test methodologies, for testing mouse vertebrae in fatigue. Fatigue testing of human bone was used for validation of the methods presented here. This work can be widely applied to investigations on the effect of musculoskeletal disuse, drug treatment, or genetic modification on bone mechanics.

METHODS

Sample Collection and Preparation

Mice were euthanized and the lumbar spine was dissected using IACUC approved protocols. The vertebra (L5) was extracted from the spine via cuts through the adjacent intervertebral discs. Surrounding musculature and soft tissues were removed with kimwipes and rubber-tipped surgical tools to protect the bone during preparation.

The cranial endplate of the mouse lumbar vertebral body is at an angle with respect to the caudal endplate. Planoparallel surfaces are critical for reliable uniaxial compression testing; however, the small specimen size makes this a challenging and arduous task. To ensure parallel surfaces for uniaxial compression testing, we modified the methods reported by Tommasini *et al*. for mouse vertebrae to improve repeatability [5]. All tasks done by hand were automated to remove human error in endplate removal and as such decreased scatter in the data.

First, the vertebral posterior process was potted in polymethyl methacrylate (PMMA). The vertebra is housed in a 3D-printed, custom-made jig designed with a pin through the spinal canal to ensure alignment (Fig. 1) and to fit within a SP1600 Leica microtome (Leica Biosystems, Nussloch, Germany). PMMA is slowly applied to the posterior process via a syringe to avoid getting PMMA on the vertebral body. Then, the jig was placed into the microtome and two parallel cuts were made, under constant irrigation. The height of the microtome arm was adjusted with respect to the blade to make the second parallel cut (1 μm resolution), resulting in a final sample height of between 2.0 to 2.5 mm. The specimen was removed from the PMMA with a scalpel, leaving the posterior process embedded in the jig. Samples were wrapped in saline-soaked gauze and stored at -20˚C until testing.

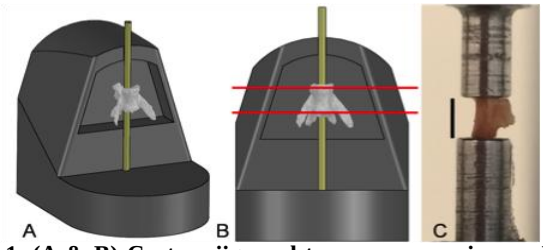

Figure 1. (A & B) Custom-jig used to prepare specimens. Yellow cylinder represents the pin used for alignment, and red lines represent parallel cuts. (C) Specimen in mechanical testing device (saline bath not shown). Bar = 2.25 mm.

Imaging & Mechanical Testing

Samples were imaged using microcomputed tomography (μCT, resolution = 10 μm/voxel; SCANCO Medical AG, Brüttisellen, Switzerland). Images from the caudal-end of the vertebral body were used to calculate minimum cross-sectional area (ImageJ; Fig. 2).

Our fatigue methods are similar to those used previously for human and bovine trabecular bone [6-8]. The specimen was compressed between two concentric, stainless-steel plattens and tested at room temperature in a saline-water bath to maintain hydration (Fig. 3). The test was conducted in load control (ELF 3200, Bose Corp., Framingham, MA, USA). Fatigue loading was initiated by applying 0.35% strain. To determine the amount of force needed to apply 0.35% strain, the initial modulus, E_0, was determined by applying ten cycles of .1% strain at 2 Hz. E_0 was defined as the slope of the stress-strain response during the last cycle.

A cyclic uniaxial compressive load was applied in a sinusoidal waveform at 2 Hz. The load applied to each specimen (i.e. input amplitude) was calculated as $F = E_0 * \varepsilon_0 * A$, where the ε_0 was set to 0.35% and A represents the bone area measured from μCT images. Normalizing the applied load by the specimen modulus was important for minimizing scatter in the data due to differences in specimen porosity. Failure was defined as a ten-percent reduction in modulus (Eqn. 1).

Figure 2. Representative uCT image of mouse vertebrae (area = 0.74 mm^2). Bar = 1.20 mm.

$$\varepsilon_{\text{failure}} = \frac{\sigma}{(0.9)(E_0)} \qquad (1)$$

Force and displacement data were collected throughout the experiment (750 Hz). The maximum strain during a cycle was plotted with respect to cycle number to determine fatigue properties.

RESULTS

Maximum strain per cycle was plotted for the duration of the test resulting in a tertiary curve (Fig. 4; red). The primary phase shows a sharp increase in strain, followed by a secondary phase characterized by nearly constant strain that spans more than 85% of the total fatigue test. Finally, in the tertiary phase, there is a sharp increase in strain, as the specimen approaches failure. The same trend for strain versus cycles is reported in Lambers *et al*. for fatigue testing of cylindrical cores of cancellous bone from human vertebra (Fig.4; blue)[6]. The transition point between the secondary and tertiary phase was defined as the intersection of two lines that make a bi-linear fit for this region of data. The strain at the transition point between the secondary and tertiary phase was 0.98% for mouse vertebra and 1.12% for human vertebra. The percentage of cycles to failure (N/N_f) at the transition point between secondary and tertiary phase was 84% for mouse vertebra and 93% for human vertebra. The slope of the secondary phase for mouse was 0.0041 and for human was 0.0055.

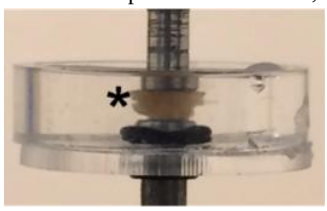

Figure 3. Mouse vertebrae (*) tested in saline bath.

DISCUSSION

Human vertebrae are characterized by a thin cortical shell surrounding a cancellous bone core with trabeculae of similar thickness to the shell. This geometry has been shown to affect load distribution between cortical and trabecular bone [10]. As such, it is important to use animal models with similar bone structure to compare to human vertebral mechanics. The geometry of mouse lumbar vertebra (Fig. 2) and intervertebral disc are comparable to the human vertebra and disc geometry, making the mouse an ideal animal model for understanding effects of degeneration, radiation, or injury on the lumbar spine [11].

In this study, we compared mouse fatigue behavior with human data available. We observed a tertiary response during fatigue, which has been previously reported for human, bovine, and rat cancellous and cortico-cancellous tissue [7-9]. The slope and length of the secondary phases have similar values. Also, the strain percentage for mouse and human at the inflection point of the secondary and tertiary phases show the values within 0.28%. These findings support the use of the mouse model for understanding the effect of radiation, degeneration or injury on human vertebra mechanics.

We have developed and validated testing procedures to use mouse vertebra for mechanical testing, which allows for reasonable inferences about human bone. On-going work is using this method to investigate dose and type of radiation. However, these methods can be applied for understanding the bone mechanics with respect to musculoskeletal disuse, drug treatment, or genetic modification.

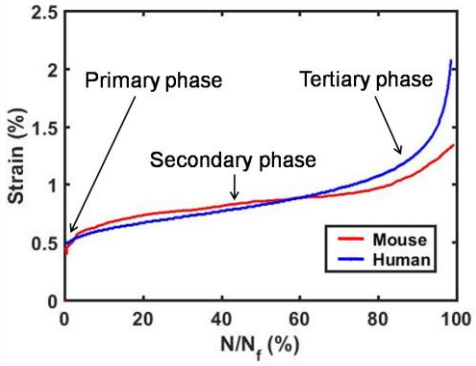

Figure 4. Strain versus number of cycles to failure (N_f) response for a representative mouse vertebra and human vertebrae (adapted from Lambers *et al.*)[6].

ACKNOWLEDGEMENTS

This work was supported by NASA Science and Technology Research Fellowship NNX14AM56H.

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