Journal of Biomechanics 49 (2016) 1233–1237

Contents lists available at ScienceDirect

Journal of Biomechanics

journal homepage: www.elsevier.com/locate/jbiomech
www.JBiomech.com

Short communication

Micro-computed tomography assisted distal femur metaphyseal blunt punch compression for determining trabecular bone strength in mice

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ARTICLE INFO

Article history:
Accepted 21 February 2016

Keywords:
Metaphyseal cancellous bone strength
Distal femur
Blunt punch compression
Micro-CT
Mouse models

ABSTRACT

Shorter generation time and the power of genetic manipulation make mice an ideal model system to study bone biology as well as bone diseases. However their small size presents a challenge to perform strength measurements, particularly of the weight-bearing cancellous bone in the murine long bones. We recently developed an improved method to measure the axial compressive strength of the cancellous bone in the distal femur metaphysis in mice. Transverse micro-computed tomography image slices that are 7 μm thick were used to locate the position where the epiphysis–metaphysis transition occurs. This enabled the removal of the distal femur epiphysis at the exact transition point exposing the full extent of metaphyseal trabecular bone, allowing more accurate and consistent measurement of its strength. When applied to a murine model system consisting of five month old male wild-type (WT) and Ca^{2+}/calmodulin dependent protein kinase kinase 2 (CaMKK2) knockout (KO) *Camkk2*$^{-/-}$ mice that possess recorded differences in trabecular bone volume, data collected using this method showed good correlation between bone volume fraction and strength of trabecular bone. In combination with micro-computed tomography and histology, this method will provide a comprehensive and consistent assessment of the microarchitecture and tissue strength of the cancellous bone in murine mouse models.
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1. Introduction

With the popularity of mice as models for the study of bone related disease and treatment, biomechanical test methods must be developed to measure the strength of mouse cancellous and cortical bone. It is also desirable to measure the properties of bone from various anatomic sites such as the spine, skull and long bones. Due to their small size, mice present a difficult problem for mechanical testing using traditional test methods. Whereas three-point bending can still be used to study cortical bone in mouse femurs, methods such as direct compression that are commonly used to measure cancellous bone mechanical properties are either less effective or are restricted to the spine (Turner, 2006; Turner and Burr, 1993).

Cancellous bone is of particular interest in the study of bone diseases such as osteoporosis. Therefore better test methods specific to cancellous bone are needed. Micro, nano, and reference-point indentation tests can be used on both cortical and cancellous bone, but those methods measure tissue level properties (Diez-Perez et al., 2010; Turner et al., 1999; Zysset et al., 1999). Because many conditions affect bone morphology more than bone tissue material properties, it is still useful to measure cancellous bone at the apparent level.

We herein present a test method to more consistently measure the axial compressive strength of metaphyseal cancellous bone of the distal femur, as a modification of previously described techniques (An et al., 1997; Dubrow et al., 2007). This improved method was intended to complement three-dimensional imaging by micro-computed tomography (micro-CT) and the histological techniques used to document the cancellous bone morphology in the same region. We used a murine genetic model with known phenotype differences in distal femoral cancellous micro-architecture. Whereas 5 month-old WT mice have sparse cancellous bone architecture in the metaphyseal regions, KO mice of this age have robust cancellous bone in the metaphysis (Cary et al., 2013; Pritchard et al., 2015). It was our hypothesis that including

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http://dx.doi.org/10.1016/j.jbiomech.2016.02.040
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the epiphyseal/metaphyseal transition could better determine the strength differences of the metaphyseal trabecular bone in animals with different developmental cancellous bone growth phenotypes.

2. Methods

2.1. Mice

Five month old male wild-type C57BL/6 (WT, $n=10$) and *Camkk2*$^{-/-}$ (KO, $n=14$) mice of the C57BL/6 background were housed under a 12 h light and dark cycle, with food and water provided ad libitum. All care and procedures were performed according to Institutional Animal Care and Use Committee (IACUC) protocols and in compliance with NIH guidelines on the use and care of laboratory and experimental animals.

2.2. Micro-CT

Imaging was performed using a high resolution CT scanner (Actis HR225-150; BIR, Lincolnshire, IL, USA). Distal femurs were imaged at a nominal isotropic voxel resolution of 7 μm. An approximate length of 5.25 mm was scanned starting from the distal-most aspect of the condyles and progressing proximally to include the entire metaphysis and some diaphysis. Transverse images were processed using both two-dimensional (ImageJ, NIH Image, Bethesda, MD) and three-dimensional software (VG Studio Max, Volume Graphics, Heidelberg, DE) (Fig. 1). A previously described technique (Cary et al., 2013) was used to determine the metaphyseal trabecular bone volume fraction (BV/TV).

2.3. Epiphysis removal

For the first group of WT ($n=5$) and KO ($n=9$) femurs tested, a constant length from the distal surface of the femoral condyles was used to cut each femur to expose the cancellous bone of the metaphysis for mechanical blunt punch compression testing. This length was determined by averaging the approximate distance to the growth plate from the micro-CT images for the entire group in each genotype and adding 350 μm to ensure each cut would be proximal to the physis and within the metaphysis. This resulted in trim lengths of approximately 1.60 mm (WT) and 1.53 mm (KO) (Table 1). Exact measurements for this first group of femurs were not recorded as they were for the second group.

The reason for not using a more statistically powerful contralateral paired design is because the improved trimming method was developed after the tests on the first 5 WT and 9 KO mice revealed little correlation between mechanical testing and micro-CT determined BV/TV. Thus the Group 1 animals were no longer available when Group 2 testing was done.

For the second group of WT ($n=5$) and KO ($n=5$) femurs tested, transverse micro-CT image slices (each 7 μm thick) were used to locate the transition between the proximal aspect of the physis and the distal extent of the metaphysis (Table 1). By counting image slices, the distance to the nearest 7 μm from the distal end of the condyle to the transition was determined (Fig. 1). The epiphysis/metaphysis junction, appearing as a cross with four chambers on the micro-CT image (slice 177, Fig. 1), represents the region where the metaphyseal trabecular bone begins.

To prepare for removal of the bone distal to the predetermined cutting location, the entire distal end portion of each femur was mounted using a two part epoxy mix (Loctite® Epoxy Quick Set™, Westlake, OH, USA) atop a socket headed #10 cap screw (Fastenal, USA) (Fig. 2A). Care was taken to be sure the femoral axis was aligned with the axis of the screw. Samples were allowed to cure for 24 h while kept hydrated and refrigerated.

The femur was then cut to remove the epiphysis using a diamond sectioning saw (Isomet®, Lake Bluff, IL USA). The mounted femur was positioned on the micrometer arm of the diamond saw and advanced until the distal part of the condyle first contacted the blade. From this point, the micrometer was used to advance the femur to the calculated epiphysis position for cutting. The femur was kept wet with isotonic saline during the cutting process. The cut surface was perpendicular to the axis of the mounted femur.

2.4. Blunt punch compression

Immediately following the removal of the distal ephiphysis, the femur was centered on a servo-hydraulic load frame (Model 858 Bionix, MTS Corp., Eden Prairie, MN). Compression of distal femoral trabecular bone was performed via a blunt (flat-tipped) cylindrical punch measuring 3 mm in length and 1 mm in diameter (Fig. 2B) at a loading rate of 1 mm/min. Due to the curvature/slope of the posterior femur cortex (Fig. 1), it was possible for the punch to contact cortical bone after penetrating beyond 1.5 mm during testing. Therefore, alignment of the punch

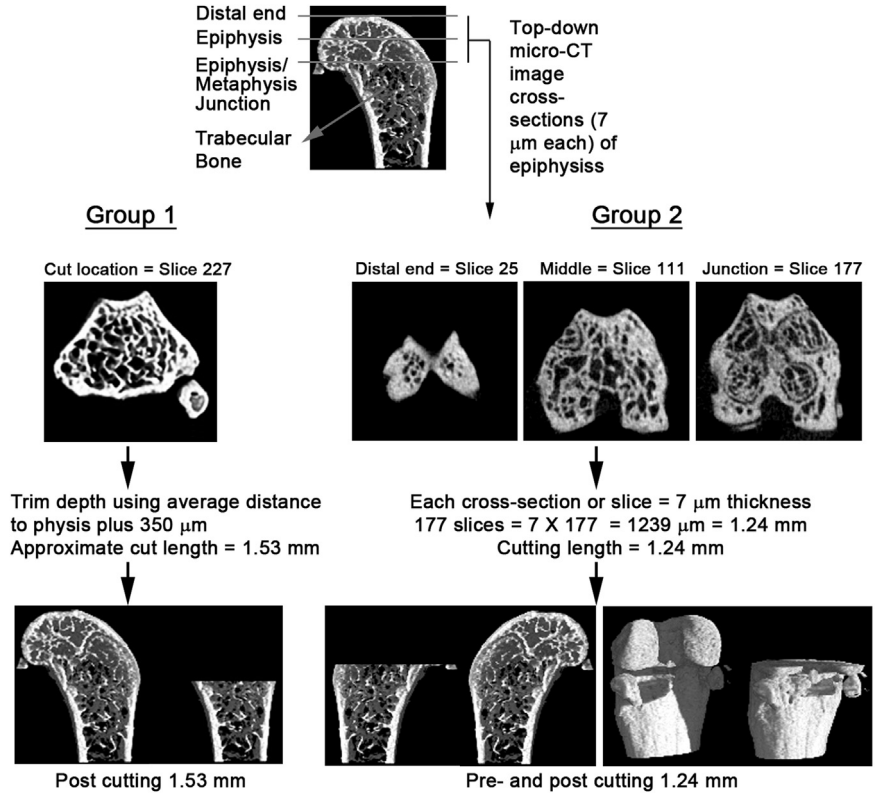

Fig. 1. Representative samples from Groups 1 and 2 to demonstrate the method for determining the cutting length for the removal of distal epiphysis to expose the metaphyseal cancellous bone in mouse femurs. Group 1: Trim depth was determined by averaging the approximate distance to the physis for the entire group in each genotype and adding 350 μm to ensure each cut would be proximal to the physis and within the metaphyseal cancellous bone. Group 2: The exact distance from the distal condyle to the epiphysis/metaphysis junction for each femur was calculated by counting the number of micro-CT image slices (each 7 μm thick). This cutting distance was utilized to trim the distal femurs to expose the metaphyseal cancellous bone.

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