Micro-cantilever Bending for Elastic Modulus Measurements of a Single Trabecula in Cancellous Bone

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Abstract

Mechanical tests performed on small bone specimens such a single trabecula remain challenging because their isolation, fixation, and precise loading are complicated. Hence, we describe a novel experimental method to measure the elastic properties of a single trabecula using micro-cantilever bending (MCB) testing. The method does not require specimens to be completely separated from the cancellous bone, and the specimen can be easily fixed during the test. In total, 10 trabecular specimens taken from the proximal epiphysis of an adult bovine femur were used in the present study. Measurements were conducted using a small testing device comprising a 1-axial stage, load cell, optical microscope, and small plate with a taper bore for applying load at the edge of the specimen. Each specimen was positioned at the edge of the bore and was deformed by displacing the stage. The deflection of the specimen was observed by optical microscopy. The elastic modulus of the specimen was calculated on the basis of the force–deflection relationship, assuming that the shape of the specimen was a vertical circular cylinder. As a result, an average elastic modulus of 9.1 ± 5.4 GPa was obtained for a single trabecula, including the values in literature. Thus, the MCB test is a novel simple method for

biomechanical analysis of a single trabecula. (214 words)

1. Introduction

The cancellous bone is organized into a three-dimensional network of single trabeculae, and the apparent elastic modulus depends on this network. The mechanical properties and nanostructure of each trabecula are important factors in determining the mechanical properties of the cancellous bone. Accordingly, small bone specimens such as a single trabecula must be investigated to understand the impact of aging, osteoporosis, and/or medicines on the risk of cancellous bone fractures. However, few studies have performed mechanical tests on such specimens (Carretta et al., 2013a; Lucchinetti et al., 2000) because such studies remain technically challenging.

Tensile tests were performed on a single trabecula in previous studies (Yamada et al., 2014; Carretta et al., 2013b, 2013c; McNamara et al., 2006; Hernandez et al., 2005; Bini et al., 2002; Rho et al., 1993). Although tensile tests present some advantages, it is difficult to completely isolate, fix, and examine small bone specimens. We recently examined trabecular specimens (at least 3 mm in length) by tensile testing; however, the specimens were larger than the standard size and existed in the edges of the cancellous bone (Yamada et al., 2014). Three-point bending tests (Carretta et al., 2013b, 2013c;

Hambli and Thurner, 2013; Szabó et al., 2011; Jungmann et al., 2011; Busse et al., 2009) were also performed on a single trabecula. However, such tests generally require complete isolation of the specimen, deflection measurements at high resolution, and very precise loading, which are usually difficult to obtain. Therefore, a simpler and more reliable experimental method is required for biomechanical analysis of a single trabecula.

Here we demonstrate a novel experimental method to investigate the elastic properties of a single trabecula on the basis of cantilever bending, as shown in Fig. 1. The micro-cantilever bending (MCB) test does not require the specimen to be isolated completely from the cancellous bone, and the specimen can be fixed easily during the test.

2. Materials and Methods

Specimen preparation

A total of 10 trabeculae were dissected from the proximal epiphysis of an adult bovine femur (two years old) as shown in Fig. 2. First, the proximal epiphysis was sliced vertically to the longitudinal axis of the femur (bone axis; Fig. 2a), and the bone marrow was removed by brief water jetting (Fig. 2b). Second, plate-like cancellous bone samples were cut out from the slices using a low-speed diamond wheel saw (model 650, South Bay Technology Inc., USA). Third, a specific single trabecula of about 1 mm in length aligned in the plane of each plate-like sample was randomly selected and isolated from the cancellous bone, while keeping one extremity connected to the other trabeculae (Fig. 2c). Fourth, the remaining attached cancellous bone portion was shaped into a small rectangle such that its height corresponded to the depth of a specimen holder (Fig. 2d). The specimen was placed into the holder almost vertically and fixed by embedding the cancellous bone portion in epoxy resin (Fig. 2e).

Trabecular axis and morphology

The longitudinal orientation of each trabecula within the femoral epiphysis was visualized by scanning the plate-like samples (Fig. 2c) using a microfocus X-ray CT instrument (inspeXio SMX-90CT, Shimadzu Corporation, Japan) at a tube voltage of 90 kV, tube current of 110 μA, and voxel size of 0.092 mm/voxel. The trabecular orientation, α, was defined as the angle between the longitudinal direction of the trabecula and bone axis (Fig. 3).

The shape of the trabecula was determined by high-resolution scanning of the specimens fixed to the jigs (Fig. 2e) using a voxel size of 0.009 mm/voxel. The area (A), circularity, and aspect ratio of the cross-sections were analyzed using ImageJ software.

MCB

The MCB tests were conducted using a small testing device (Fig. 4), comprising an acrylic plate to apply the displacement and load on the specimen, a 1-axial stage (ALS-4011-G1M, Chuo Precision Industrial Co., Ltd., Japan), and a load cell (LVS-1KA, Kyowa Electronic Instruments Co., Ltd., Japan). The tests were conducted under optical microscopic observation at a 3-μm resolution (VH-5000, Keyence Corporation, Japan). Each specimen was positioned at the edge of a taper bore drilled into the acrylic plate, with the contact position in close proximity with the free end. The plate was displaced horizontally in a stepwise manner using the stage, and microscopic images were captured vertically (Fig. 4a). The deflection, d, was defined as the horizontal displacement of the contact position in the direction of plate displacement and the load, F, in the same direction was measured by the load cell connected to the plate. The maximum *d* and *F* values were 178 ± 16 μm and 0.68 ± 0.75 N, respectively. A drop of water-based ink was applied to the edge of the bore, and the contact position at the surface of the specimen was observed by optical microscopy after the test. Then, the distance, *l*, between the fixed end of the specimen and contact position was measured for the calculation of the elastic modulus. Specimens were loaded and then unloaded three times under air-dried conditions.

The shape of the specimen was assumed to be a vertical circular cylinder of orthotropic material and the shear stress was considered to be negligible during loading. Then, the elastic modulus, *E*, in the longitudinal direction of the single trabecula was calculated as in Eq. (1) from the plane curve of the bending axis (e.g., Lekhnitskii, 1963).

$$E = \frac{l^3}{3I}\frac{F}{d} \qquad (1)$$

, where *I* is a second moment of area and is expressed by Eq. (2) (e.g., Timoshenko and Young, 1968).

$$I = \frac{\pi D^4}{64} \quad (2)$$

, where D is the diameter of cylinder. D value was calculated from the average cross-sectional area of each specimen as $2\sqrt{A/\pi}$.

3. Results

The morphological characteristics and orientation of the trabecular specimens are shown in Table 1. The specimens appeared to be rod-like in shape according to microscopic and X-ray CT observations. The longitudinal orientation of the trabecular specimens varied widely from 14° to 87° with respect to the bone axis.

The specimens deflected in the direction of the plate displacement and torsional deformation was not observed. Figure 5 demonstrates the high repeatability of the F–d relationships obtained for a specimen. The linear region of the relationships was used to calculate the average F/d (N/mm) value of the three measurements, which was then used to determine the elastic modulus. According to the Wilcoxon signed-rank test, the ratios of force–deflection trend (F/d) in the second and third measurements compared to the first measurements had no significant difference with the first measurements ($P > 0.05$). In the cancellous bone of a bovine femur, the average elastic modulus of the 10 trabecular specimens was 9.1 ± 5.4 GPa.

In the present study, the elastic modulus had no significant correlation with the orientation, size, or shape of the trabeculae. Furthermore, the trabecular orientation had no significant correlation with the size and shape.

4. Discussion

The present study found that the elastic modulus of trabecular specimens from a bovine femur was 9.1 ± 5.4 GPa. These values fall within the range reported for a single trabecula under dry conditions (2–16 GPa; Yamada et al., 2014; Carretta et al., 2013b, 2013c; Busse et al., 2009; Bini et al., 2002; Rho et al., 1993), including different species, ages, locations, size, and types of mechanical test. There was also no significant difference with the elastic modulus values obtained in our previous study using the same age bovine femurs (Yamada et al., 2014). These findings indicate that the new MCB protocol proposed herein can be used to determine the elastic modulus of a single trabecula.

Tensile and three-point bending tests have been performed on a single trabecula. Tensile tests are among the simplest mechanical tests, and are suited for the observation of the deformation behavior of mineral crystals within a single trabecula by X-ray diffraction (Yamada et al., 2014). However, it is rather difficult to completely isolate, fix, and examine small bone specimens, such as those used in the present study. On the other hand, three-point bending tests do not require fixation of the specimen to a jig with a resin or glue, while complete isolation of the specimens from the cancellous bone is generally required. Furthermore, the rod-shape specimen may move and roll on the support during loading; precise loading therefore needs to be taken into account. High-resolution measurements for the displacement are also required; in fact, the deflection generated by the same force is 16 times smaller by three-point bending than by cantilever bending of a uniform beam. These tests may not be suited for a single trabecula due to technical difficulty. Lorenzetti et al. (2011) conducted fixed-fixed beam tests on a single trabecula in a cancellous bone sample by applying force with a nylon wire. Although this method is quite convenient, it may generate displacement artifacts at the trabecular ends that must be removed from the calculations, and controlling the loading position and direction may be technically challenging. In contrast, the MCB test allows the trabecular specimens to remain attached to the cancellous bone at one extremity and allows for better control of the position during the test, which is a simple and useful experimental method for biomechanical analysis of a single trabecula.

In Wolff's law the trabecular orientation plays an important role in the mechanical strength of the bone; however, the elastic modulus of single trabeculae had no significant correlation with the orientation in the present study. Our previous study also did not show this correlation, although the effects of the hydroxyapatite crystal strain ratio on the elastic modulus of single trabeculae were observed (Yamada et al., 2014). It suggests that the variation of the elastic modulus of single trabeculae may depend on its hierarchical structure. Furthermore, the variation may be relatively small locally since the single trabeculae were extracted from the same region, and the trabecular networks are important for the apparent strength of the cancellous bone in the region.

The present study has some limitations. First, we examined the 1-mm single trabeculae taken from a relatively close area to the diaphysis, which were easy to extract. Second, a stepwise deformation was applied to all specimens. Although the measured force did not show significant time dependence, a small decreasing force was observed immediately after the applied deflections. Accordingly, deflection was measured from optical images captured during the period of stable force. Third, the linear region of the force–deflection relationship was used to calculate the elastic modulus. In general, a non-linear relationship is observed with curved specimens, inhomogeneous shapes, large deformations, and non-linear elastic properties as well as after yielding. In the present study, the region used for the calculations had high linearity ($R^2 = 0.96 \pm 0.02$), and the specimens appeared as straight rods with linear elastic properties. Fourth, the specimen was assumed to be a vertical circular cylinder for the calculation of the elastic modulus to simplify the method. In general, asymmetrical shape and anisotropic material properties generate oblique deflection and rotation, which complicate the calculation; however, such phenomenon was not detected in the study. Furthermore, although the sample may slightly tilt with respect to the vertical axis, it has less impact on the results because the effects of the cosine of the tilt angle are negligibly small. Fifth, the measured elastic moduli are considerably affected by the elastic properties around the bottom region of the trabeculae in the bending test, and we assumed that the trabeculae had homogenous mechanical properties. Last, the specimens were assumed to be rigidly fixed to the holders. Although the fixation may represent a source of error, the embedded cancellous bone portion was larger than the single trabecula and the stiffness of the portion was considered to be sufficiently high to resist the applied load.

Acknowledgments

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Conflict of interest statement

There is no actual or potential conflict of interest associated with this project.

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Legends of the figures and table

Figure 1 Schematic of micro-cantilever bending for a single trabecula.

Figure 2 Specimen preparation: the proximal epiphysis of a bovine femur (a) was sliced vertically to the longitudinal axis of the femur (bone axis) and the bone marrow was removed using a water jet (b). Plate-like cancellous bone samples were cut out from the slices, and a specific single trabecula of about 1 mm in length aligned in the plane of each plate-like sample was randomly selected. The cancellous bone surrounding the target single trabecula was removed (c). The remaining cancellous bone portion was shaped into a small rectangle (d). The specimen was placed into a holder almost vertically and fixed by embedding the cancellous bone portion into epoxy resin (e). The trabecular specimen was observed by optical microscopy (f). White arrows indicate the bone axis.

Figure 3 Definition of the trabecular orientation α.

Figure 4 Experimental setup (a) and small testing device designed to conduct micro-cantilever bending tests on a single trabecula (b). The device comprised an acrylic plate (2.3-mm thickness), a 1-axial stage (ALS-4011-G1M, Chuo Precision Industrial Co., Ltd., Japan) equipped with a high-resolution (2 μm) and high-repeatability (0.3 μm) actuator, and a load cell (LVS-1KA, Kyowa Electronic Instruments Co., Ltd., Japan) with high repeatability (≤0.5%) and small hysteresis (<0.5%). The diameters of the taper bore drilled into the acrylic plate at the upper and bottom side were 6.2 and 4.3 mm, respectively, and the edge of the bore was sharp (approximately 20-μm radius).

Figure 5 Force–deflection relationships in a single trabecula. Tests of each specimen were conducted three times, with the same color dots indicating values from the same test.

Table 1 Mean values and standard deviations of each parameter measured on the trabecular specimens (n = 10): the distance between the fixed end of the specimen and contact position (l) observed by microscopy, which was almost the same as trabecular length; trabecular orientation (α), average cross-sectional area (A), circularity (*Cir*), and aspect ratio (*AR*) measured by micro CT scans; diameter of the specimen (D) measured by the approximation of a circular cylinder; elastic modulus (E) and R^2 value of force–deflection relationship from the micro-cantilever bending (MCB) test.

Figure and table

Figure 1

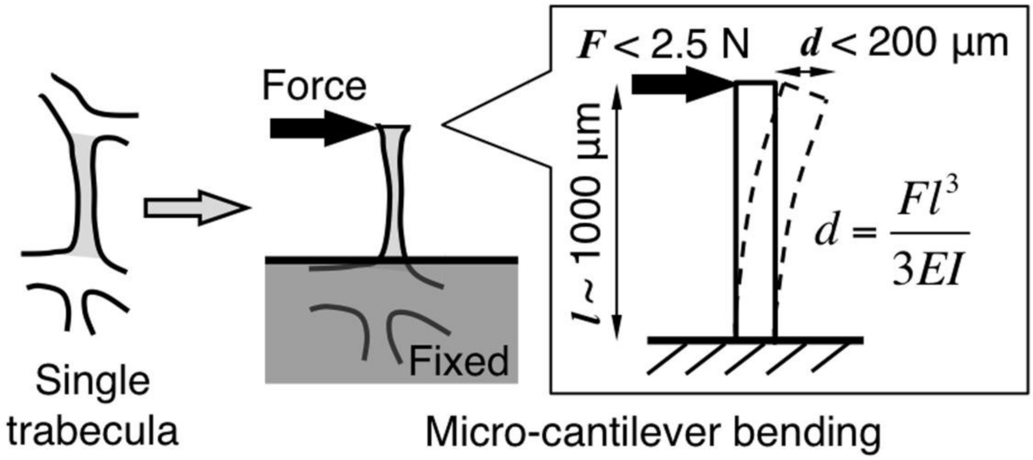

Figure 2

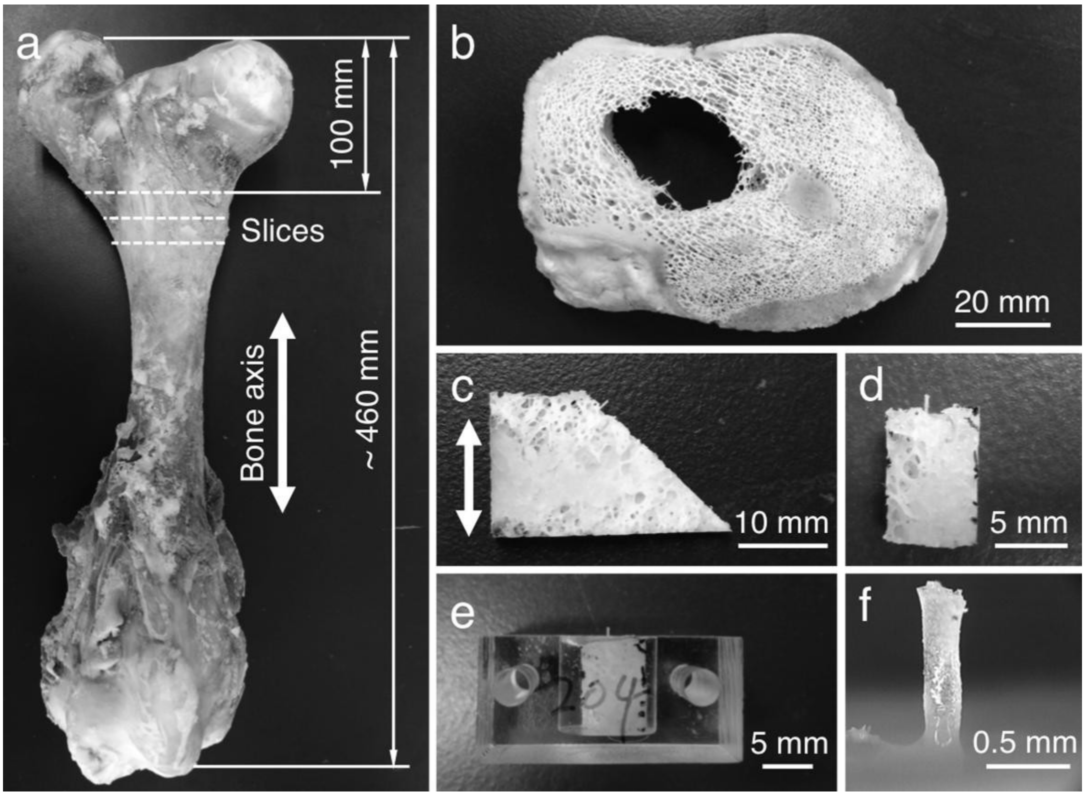

Figure 3

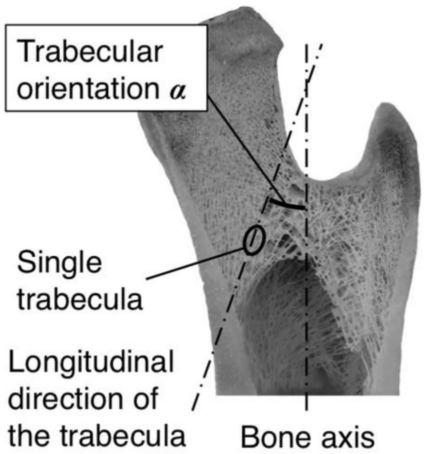
Trabecular
orientation α
Single
trabecula
Longitudinal
direction of
the trabecula
Bone axis

Figure 4

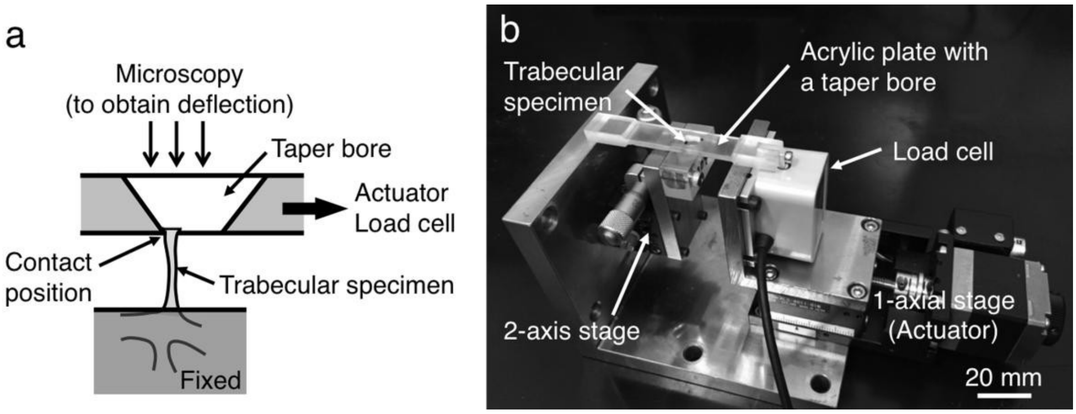
a
Microscopy
(to obtain deflection)
Taper bore
Actuator
Load cell
Contact
position
Trabecular specimen
Fixed
b
Trabecular
specimen
Acrylic plate with
a taper bore
Load cell
2-axis stage
1-axial stage
(Actuator)
20 mm

Figure 5

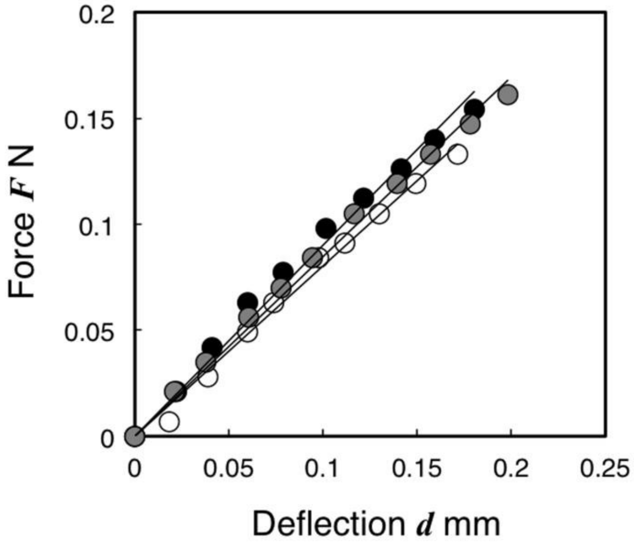

Table 1

Experiments	Microscopy	μ-CT					MCB	
Parameters	*l*	*α*	*A*	*Cir*	*AR*	*D*	*E*	R^2
Unit	mm	°	mm^2	-	-	mm	GPa	-
Mean	1.12	49	0.0575	0.67	1.4	0.25	9.1	0.96
S.D.	0.17	24	0.0515	0.10	0.1	0.10	5.4	0.02