Journal of Biomechanics 42 (2009) 82–86

Contents lists available at ScienceDirect

Journal of Biomechanics

journal homepage: www.elsevier.com/locate/jbiomech
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Short communication

Effects of tissue preservation on murine bone mechanical properties

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ARTICLE INFO

Article history:
Accepted 23 September 2008

Keywords:
Murine
Bone
Fixation
Freezing
Elastic and viscoelastic properties

ABSTRACT

Murine bone specimens are used extensively in skeletal research to assess the effects of environmental, physiologic and pathologic factors on their mechanical properties. Given the destructive nature of mechanical testing, it is normally performed as a terminal procedure, where specimens must be preserved without affecting their mechanical properties. To this end, we aimed to study the effects of tissue preservation (freezing and formalin fixation) on the elastic and viscoelastic mechanical properties of murine femur and vertebrae. A total of 120 femurs and 180 vertebral bodies (L3–L5) underwent non-destructive cyclic loading to assess their viscoelastic properties followed by mono-cyclic loading to failure to assess their elastic properties. All specimens underwent re-hydration in 0.9% saline for 30 min prior to mechanical testing. Analysis indicated that stiffness, modulus of elasticity, yield load, yield strength, ultimate load and ultimate strength of frozen and formalin-fixed femurs and vertebrae were not different from fresh specimens. Cyclic loading of both femurs and vertebrae indicated that loss, storage and dynamic moduli were not affected by freezing. However, formalin fixation altered their viscoelastic properties. Our findings suggest that freezing and formalin fixation over a 2-week period do not alter the elastic mechanical properties of murine femurs and vertebrae, provided that specimens are re-hydrated for at least half an hour prior to testing. However, formalin fixation weakened the viscoelastic properties of murine bone by reducing its ability to dissipate viscous energy. Future studies should address the long-term effects of both formalin fixation and freezing on the mechanical properties of murine bone.

1. Introduction

Murine animal models are used extensively in musculoskeletal research, with femur, vertebrae and tibia being the most frequently studied sites. This is evidenced by a recent survey of the literature referencing over 360 publications involving assessment of bone strength in various murine models over the past 2 years (Lories et al., 2007; Ng et al., 2007; Nordstrom et al., 2007). Given the essential contribution of the skeleton to locomotion and protection of vital organs, and its susceptibility to changes due to environmental, physiologic and pathologic factors; maintenance of the strength and stiffness of the skeleton is of major concern. As a result, mechanical testing of bone has been used as an important assay to monitor the effects of various environmental, physiologic and pathologic manipulations on its structural and mechanical properties. Due to the destructive nature of mechanical testing (most specimens tested to failure), the order of such testing is traditionally reserved for the latter stages of most studies, where specimens have undergone other non-destructive analyses prior to mechanical testing. Tissue fixation with 10% formalin (4% formaldehyde) is widely used to preserve specimens without refrigeration, offering researchers the added benefit of protection from specimens with communicable diseases (Boskey et al., 1982; Nimni et al., 1987; Wilke et al., 1996; Nuccion et al., 2001; Moreno and Forriol, 2002; Randall et al., 2002; Wingerter et al., 2006; Zech et al., 2006). However, researchers refrain from using formalin to decontaminate and preserve bone tissue to be tested mechanically, since its effects on the mechanical properties of bone have been the subject of much debate. Chemical fixation through the use of aldehydes has been shown to cause a direct effect on bone mechanical properties by forming an increased number of inter- and intra-fibrillar cross-links of primary amine groups of polypeptide collagen chains (Currey et al., 1995). Boskey et al. (1982) have shown that while formalin fixation has no effect on the mineral composition of bone, it causes the collagen fibrils to be more closely packed. Other methods, such as freezing, is frequently used to preserve harvested specimens prior to mechanical testing (Evans, 1973). However, it involves overhead

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doi:10.1016/j.jbiomech.2008.09.037

in the purchase and maintenance of frozen storage space and does not offer any protection from existing biological pathogens within the specimens. Currey et al. (1995) state that the future of research on human bone mechanical properties will depend on the use of successful fixation protocols that will enable researchers to work safely yet sustain the inherent bone mechanical properties.

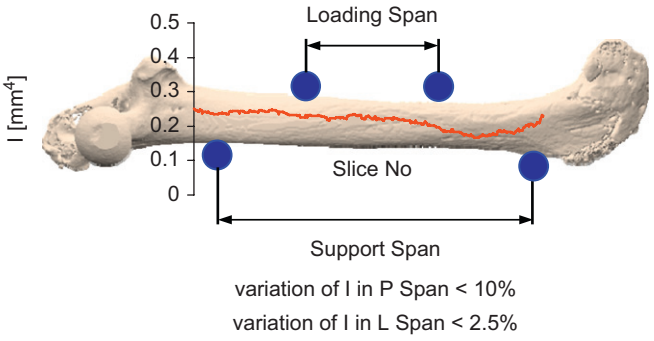

Fig. 1. Variation of moment of inertia across the length of a mouse femur, including support and loading spans, with anterior aspect facing down in 4-point bending position.

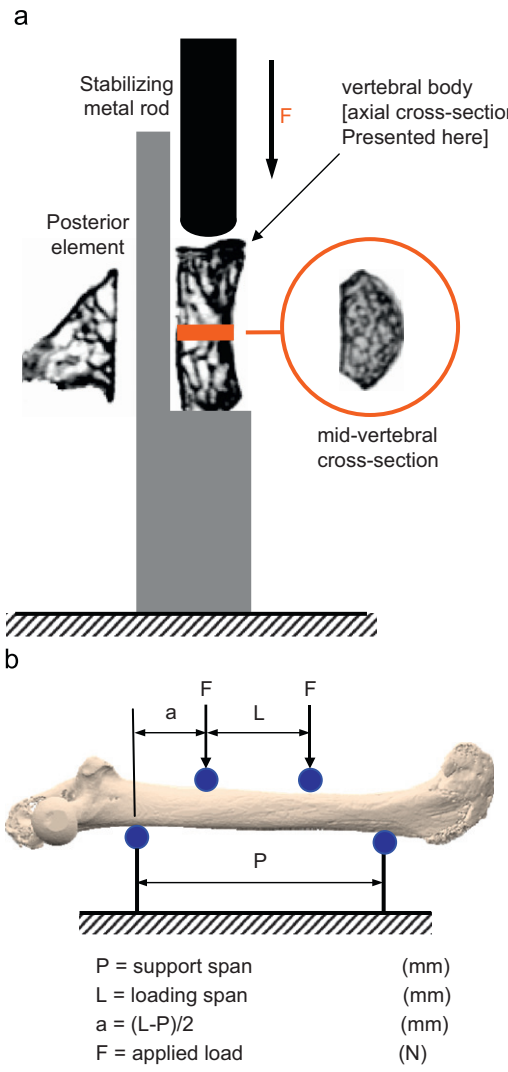

Fig. 2. (a) A schematic overview of murine vertebral compression testing. The mid vertebral cross-section is highlighted in the diagram and (b) a schematic overview of murine femoral 4-point bending testing.

However, to the best of our knowledge, no studies have been carried out to investigate the effects of tissue preservation methods on the mechanical properties of murine bone specimens, despite the prevalence of use of such specimens within the musculoskeletal research community. Additionally, previous works studying the effects of tissue fixation on human, bovine, feline and ovine specimens have generated mixed results; where changes in different mechanical properties have been observed, in part due to differences, in species, testing modalities, testing rate and hierarchy (whole bone vs. tissue testing) (McElhaney et al., 1964; Sedlin, 1965; Goh et al., 1989; Currey et al., 1995; Beardsley et al., 1997; Kikugawa et al., 2002; Tan et al., 2002; Kikugawa and Asaka, 2005).

To that end, we hypothesize that formalin fixation and freezing will not adversely affect the viscoelastic and elastic mechanical properties of murine bone. Therefore, we aim to study the effects of tissue preservation via freezing and formalin fixation on the mechanical properties of murine femur and vertebrae (L3, L4 and L5) for two commonly used testing methods of vertebral compression and femoral 4-point bending. Cyclic loading will be performed to examine the effects of tissue preservation on collagen cross-linking and bone viscoelastic properties, whereas mono-cyclic failure testing will be performed to study the effects of preservation modes on bone elastic properties (Figs. 1 and 2).

2. Materials and methods

2.1. Materials

Sixty female 16-week old C57BL/6J euthanized mice were obtained for this study (Charles River Laboratories, Charlestown, MA) and both femurs plus the L3, L4, and L5 vertebrae were excised from all animals. The overall length and midshaft diameter of each femur along with the vertebral height and mid-vertebral diameter were measured with a digital caliper using standard measurement protocols (5 measurements per site). The study was performed on fresh, frozen and formalin-fixed specimens undergoing common mechanical loading conditions. Specimens in the fresh group were mechanically tested immediately after excision on the same day of euthanasia; specimens from the fixed group were placed in a 1/10 volume ratio 10% formalin solution (4% formaldehyde) and left at 4 °C for 2 weeks (the formalin solution was replaced after 1 week); and specimens from the frozen group were wrapped in 0.9% NaCl physiologic saline-soaked gauze and stored at −20 °C for 2 weeks. A 2-week fixation period was used as an average time between when a specimen is harvested, fixed and processed for other non-invasive assays leading up to destructive mechanical testing. At the conclusion of week 2, the frozen and fixed specimens underwent the same mechanical testing protocol as did the fresh specimens. The femurs were randomly assigned to three equal groups of fresh, frozen and fixed specimens, with 40 femurs and 60 vertebrae per group (femurs from the same animal were not assigned to the same group). In a separate pilot study, 20 pairs of left and right femurs from identical specimens (animal strain and age) as those in this study were compared via 4-point bending mechanical

Table 1
DXA-based densitometric and mechanical properties of paired femurs and lumbar spinal units of mice employed in the pilot study (p values >0.05 for all cases).

Group	BMD (g cm^{-2})	Yield displ. (mm)	Yield load (N)	Stiffness (N/mm)
Left femur	0.053	0.47	19.13	40.51
Std. dev.	0.003	0.11	4.21	13.40
Right femur	0.052	0.48	19.52	41.34
Std. dev.	0.004	0.12	4.55	14.11
Vert.-L3	0.059	0.02	23.81	1432.12
Std. dev.	0.004	0.00	9.11	532.81
Vert.-L4	0.060	0.02	24.31	1481.72
Std. dev.	0.003	0.01	8.91	556.88
Vert.-L5	0.060	0.02	24.52	1512.61
Std. dev.	0.005	0.01	9.42	561.34

Table 2
Physical dimensions and elastic mechanical properties of fresh, frozen and fixed femurs.

Group	Length (mm)	Dia (mm)	I (mm_4)	Yield strain (−)	Yield stress (MPa)	Modulus (GPa)	Yield displ. (mm)	Yield load (N)	Stiffness (N/mm)	Ultimate displ. (mm)	Ultimate load (N)	Ultimate strain (−)	Ultimate stress (MPa)
Fresh	13.99	1.24	0.13	0.027	131.54	4.89	0.48	19.69	41.23	0.89	24.46	0.050	181.07
Std. dev.	0.70	0.07	0.02	0.007	24.99	1.98	0.08	3.26	11.05	0.16	4.32	0.02	33.42
Frozen	14.02	1.26	0.13	0.029	136.36	4.67	0.54	20.82	38.28	0.97	25.52	0.052	191.62
Std. dev.	0.67	0.08	0.02	0.005	23.18	1.25	0.10	3.93	11.73	0.18	4.82	0.0178	35.97
Fixed	14.17	1.27	0.13	0.026	125.24	4.78	0.49	19.43	39.50	0.93	23.48	0.050	188.27
Std. dev.	0.68	0.08	0.02	0.005	22.79	1.21	0.09	3.51	11.27	0.18	4.98	0.0154	38.05

Table 3
Physical dimensions and elastic mechanical properties of fresh, frozen and fixed vertebrae.

Group	Height (mm)	Dia (mm)	Yield strain (−)	Yield stress (MPa)	Modulus (MPa)	Yield displ. (mm)	Yield load (N)	Stiffness (N/mm)	Ultimate displ. (mm)	Ultimate load (N)	Ultimate strain (−)	Ultimate stress (MPa)
Fresh	3.00	1.25	0.13	23.03	174.63	0.02	25.82	1472.34	0.03	29.03	0.20	20.01
Std. dev.	0.20	0.10	0.03	5.64	43.85	0.00	8.01	452.98	0.01	6.98	0.05	5.41
Frozen	3.02	1.25	0.14	25.27	176.13	0.02	26.67	1513.34	0.03	29.83	0.22	21.65
Std. dev.	0.18	0.13	0.04	6.55	38.44	0.01	6.91	467.32	0.01	7.76	0.04	6.74
Fixed	3.07	1.25	0.14	24.34	172.23	0.02	25.38	1484.25	0.03	28.38	0.22	20.32
Std. dev.	0.21	0.14	0.04	6.40	41.15	0.00	7.15	396.24	0.01	6.30	0.05	6.40

properties and were observed not to be different from one another (Table 1). Paired Student's t-test was used to compare left and right femurs. Moreover, the vertebrae were assigned to three random and equal groups of fresh, frozen and fixed specimens, containing the same number of L3, L4 and L5 vertebral bodies per group. In the same pilot study using 20 murine spines, it was shown that a murine lumbar spinal unit (L3, L4 and L5) had similar BMD values and were mechanically (axial compression) no different from one another under physiologic compressive testing. As a result, L3, L4 and L5 vertebral bodies were randomized equally to each group (Table 1).

Micro-computed tomographic imaging was performed on the specimens to assess their cross-sectional area and moment of inertia. For further details, please visit the Appendix page. The vertebrae were subjected to non-failure axial compressive cyclic loading followed by axial loading to failure. The femora were subjected to non-failure 4-point bending cyclic loading followed by 4-point bending loading to failure. Details of the mechanical testing protocols are provided in the Appendix. Extrinsic and intrinsic mechanical properties for both axial compression and 4-bending tests were assessed from the load–displacement data. Additionally, fast Fourier transform (FFT) was performed on the cyclic time, stress and the strain data for compression and bending tests in order to identify the viscoelastic properties of average storage (G_1) and loss (G_2) moduli. Mechanical data analysis is detailed in the Appendix.

3. Results

Results indicated that stiffness and modulus of elasticity of the frozen and formalin-fixed femoral and vertebral specimens were not different from the fresh femurs and vertebrae, respectively. Yield displacement, yield load, yield strain and yield strength were not different between the specimens in the two preservations modes in comparison to the fresh specimens ($p>0.05$ for all cases). Likewise, ultimate displacement, ultimate load, ultimate strain and ultimate strength of the formalin fixed and frozen femurs and vertebrae were not different from the fresh specimens ($p>0.05$ for all cases) (Tables 2 and 3).

Cyclic loading results indicated that femoral and vertebral storage moduli were unaffected by freezing and formalin fixation in comparison to fresh specimens ($p>0.05$ for both cases). Loss modulus was also unaffected by freezing ($p>0.05$ for both cases) but was significantly affected by formalin fixation for both femurs and vertebrae ($p<0.05$ for both cases). As a result, dynamic modulus was not different between the fresh and frozen groups ($p>0.05$ for both femurs and vertebrae) but was different between the fresh and formalin-fixed groups ($p<0.05$ for both cases) (Fig. 3a and b).

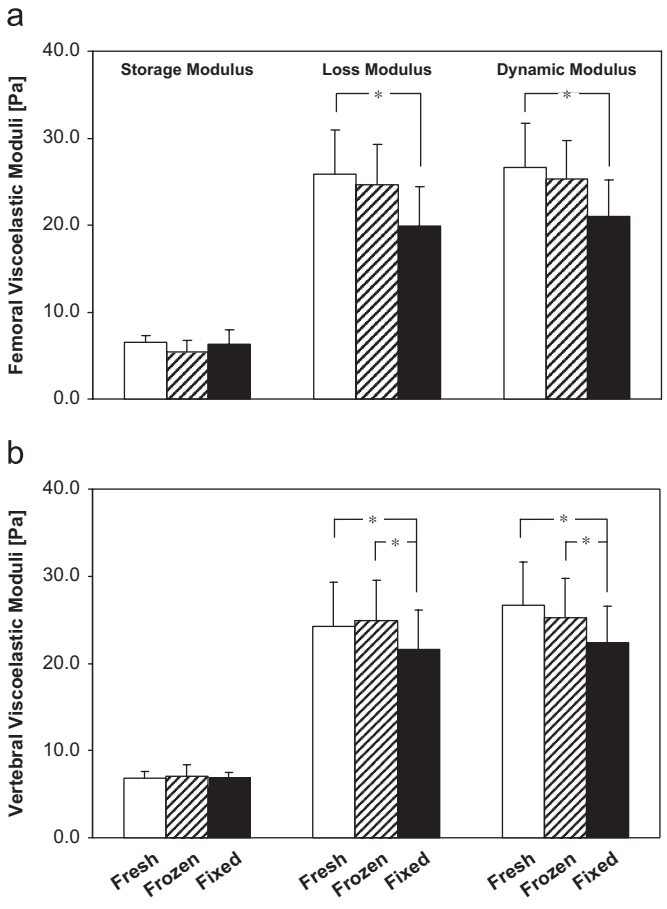

Fig. 3. (a) Storage, loss and dynamic moduli for fresh, frozen and formalin-fixed femurs and (b) storage, loss and dynamic moduli for fresh, frozen and formalin-fixed vertebrae. * denotes p-values <0.05.

4. Discussion

Based on the results obtained in this study, freezing and formalin fixation preservation methods did have any effect on the extrinsic and intrinsic stiffness of both femoral and vertebral structures and materials. No preservation mode effects were detected on the yield displacement, yield load, yield strain and yield strength of the femurs and vertebrae either. Likewise, ultimate displacement, load, strain and strength were not different between the three groups for both anatomic locations. These findings suggest that freezing and formalin fixation of murine femurs and vertebrae over a 2-week period do not seem to alter the elastic mechanical properties of murine femurs and vertebrae undergoing 4-point bending and compression, provided that specimens are re-hydrated for at least half an hour prior to mechanical testing.

As expected, freezing had no effect on the viscoelastic properties of murine femurs and vertebrae as determined by cyclic loading. However, formalin fixation caused ~23% drop in the femurs' loss modulus (G_2), ~22% drop in their dynamic modulus (G^*), ~11% decrease in vertebral loss modulus and ~16% decrease in vertebral dynamic modulus. Storage modulus (G_1) was unaffected by formalin fixation for both femurs and vertebrae, as storage modulus reflects the ability of the material to store elastic energy, and this process seems to be largely unaffected in cortical bone by formalin-induced collagen cross-linking over a 2-week period. This finding confirms the results obtained from mono-cyclic failure tests. The drop in the loss modulus of the formalin-fixed specimens reflects their decreased ability to dissipate viscous energy. Findings from this study are expected to hold for bones from other strains of mice, unless significant differences in their matrix and mineral properties are evident.

Intrinsic material properties were assessed based on a few assumptions. The vertebrae were treated as short columns with the mid-vertebral cross-sectional area measured by μCT treated as the column cross-section. The femur was modeled as a thin-walled tube. As the cross-sectional area and moment of inertia variation between the support and loading spans were <10% and 2.5%, respectively, the mid-diaphyseal cross-sectional area and moment of inertia as measured by μCT were used to assess stress and strain. These assumptions are adequate for use in a comparative study, where the specimens are of very similar size and shape.

Results from this study are in agreement with previous work performed by Currey et al., as no changes in bending mechanical properties were reported under physiologic loading conditions. It is noteworthy that the work by Currey et al. (1995) was performed on bovine femur specimens. Additionally, Sedlin (1965) reported that freezing of human femoral cortical bone specimens had no effect on their mechanical properties, yet fixation increased tensile modulus significantly. Similar to this study, Goh et al. (1989) reported that freezing feline humeral and femoral bone

had no effect on their mechanical properties. Also, they found no changes in maximum loading capacity and stiffness of formalin-fixed specimens, however, energy absorption were reduced in torsional and bending tests. Kikugawa et al. (2002) and Kikugawa and Asaka (2005) demonstrated that long- and short-term formalin preservation of bovine cortical bone resulted in augmented bending stiffness and decreased bending strength and fracture toughness. In a partially related study, Edmonston et al. demonstrated no differences in the collagen, isodesmosine and desmosine content, and the extent of pyridinoline and deoxypyridinoline cross-links, between fresh and formalin fixed (25 weeks) human disk and ligament samples (Tan et al., 2002). While general consensus with previous works from the literature was reached in this study, it is noteworthy that in addition to the use of bones from different species and different mechanical testing methods, general mechanical testing errors and reporting of different parameters can result in significant divergence in results of studies from different laboratories.

The specimens in this study were preserved for a 2-week period, as studies in the literature have used a fixation period ranging from 3 h to 1 year without presenting a consensus on fixation time. The authors used the 2-week period as a reasonable estimate for the time when a sample is harvest from the animal, initially fixed (48 h), processed for other non-invasive assays, and finally tested destructively to assess its mechanical properties. Future studies should address the long-term effects of both formalin fixation and freezing on the mechanical properties of murine bone, as specimens at times stay in freezer or formalin for a long period prior to analysis. Also, the effects of multiple freeze-thaw cycles on mechanical properties of murine bone must be studied; since this is a common scenario in research laboratories, where specimens are thawed and frozen a number of times during the course of a study. Furthermore, cyclic loading with much greater loading cycles and variations in loading rates could be used to study the effects of preservation modes on viscoelastic properties of murine bone with further detail.

In conclusion, preservation of murine vertebrae and femurs by freezing and formalin fixation does not appear to change the elastic properties and storage moduli of these bone specimens; however, formalin fixation adversely affects their loss and dynamic moduli properties.

Conflict of interest

None.

Acknowledgements

The authors would like to thank Dr. Mary Bouxsein from the Orthopedic Biomechanics Laboratory at Beth Israel Deaconess Medical Center and Harvard Medical School for her insightful comments, and Ms. Vaida Glatt and Ms. Laurie Green for their help with animal dissections.

Appendix A. Supporting Information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jbiomech.2008.09.037.

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