Bone 97 (2017) 49–53

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Full Length Article

Even with rehydration, preservation in ethanol influences the mechanical properties of bone and how bone responds to experimental manipulation☆

Evan O. Vesper [a], Max A. Hammond [a], Matthew R. Allen [a,b,c,d], Joseph M. Wallace [a,d,*]

[a] Department of Biomedical Engineering, Indiana University–Purdue University Indianapolis, IN, United States
[b] Department of Anatomy and Cell Biology, Indiana University School of Medicine, IN, United States
[c] Roudebush Veterans Administration Medical Center, Indianapolis, IN, United States
[d] Department of Orthopaedic Surgery, Indiana University School of Medicine, IN, United States

ARTICLE INFO

Article history:
Received 4 October 2016
Revised 16 December 2016
Accepted 1 January 2017
Available online 03 January 2017

Keywords:
Collagen
Advanced glycation end products
Mechanical testing
Ribosylation
Micro-computed tomography
Cortical geometry

ABSTRACT

Typically, bones are harvested at the time of animal euthanasia and stored until mechanical testing. However, storage methods are not standardized, and differential effects on mechanical properties are possible between methods. The goal of this study was to investigate the effects that two common preservation methods (freezing wrapped in saline-soaked gauze and refrigerating ethanol fixed samples) have on bone mechanical properties in the context of an in vitro ribosylation treatment designed to modify mechanical integrity. It was hypothesized that there would be an interactive effect between ribose treatment and preservation method. Tibiae from twenty five 11 week old female C57BL/6 mice were separated into 2 preservation groups. Micro-CT scans of contralateral pairs assessed differences in geometry prior to storage. After 7 weeks of storage, bones in each pair of tibiae were soaked in a solution containing either 0 M or 0.6 M ribose for 1 week prior to 4 point bending tests. There were no differences in any cortical geometric parameters between contralateral tibiae. There was a significant main effect of ethanol fixation on displacement to yield (−16.3%), stiffness (+24.5%), strain to yield (−13.9%), and elastic modulus (+18.5%) relative to frozen specimens. There was a significant main effect of ribose treatment for yield force (+13.9%), ultimate force (+9.2%), work to yield (+22.2%), yield stress (+14.1%), and resilience (+21.9%) relative to control-soaked bones. Postyield displacement, total displacement, postyield work, total work, total strain, and toughness were analyzed separately within each preservation method due to significant interactions. For samples stored frozen, all six properties were lower in the ribose-soaked group (49%–68%) while no significant effects of ribose were observed in ethanol fixed bones. Storage in ethanol likely caused changes to the collagen matrix which prevented or masked the embrittling effects of ribosylation that were seen in samples stored frozen wrapped in saline-soaked gauze. These data illustrate the clear importance of maintaining hydration if the eventual goal is to use bones for mechanical assessments and further show that storage in ethanol can alter potential to detect effects of experimental manipulation (in this case ribosylation).

1. Introduction

Characterizing functional properties of bone is a critical part of studies associated with disease states, pharmacological treatment, mechanical intervention, etc. There is growing recognition in the field of the importance of these mechanical measurements, as demonstrated by two recent review articles focused on understanding commonly measured mechanical properties in bone [1] and how to properly execute mechanical tests in small mouse bones to extract the most useful data [2]. This increased use of mechanical testing, particularly in genetic mouse models, is exciting but it comes with potential problems.

Due to practical time constraints associated with animal experiments, bones are typically harvested at the time of animal euthanasia and stored (often for extended periods of time) until needed for mechanical testing. Most commonly, bone storage involves freezing or placing in a fixative to preserve the bone, but these methods of bone preservation are not standardized. For those labs that freeze their bones, freezing protocols are inconsistent but the most common tends to be wrapping the bone in saline-soaked gauze and storing at −20 °C [3,4]. Some labs store bones in ethanol at 4 °C and then rehydrate prior to testing [5]. Studies in the literature suggest that rehydrating the bone prior to testing allows its mechanical properties to recover to normal values [6–8], but rigorous testing of this question has not been undertaken. It is important to understand what effects, if any, different

☆ Supporting grants:JMW: K25-AR067221Supplemental information:No supplemental information was submitted with this manuscript
* Corresponding author at: Department of Biomedical Engineering, Indiana University–Purdue University Indianapolis, IN, United States.
E-mail address: jmwalla@iupui.edu (J.M. Wallace).

http://dx.doi.org/10.1016/j.bone.2017.01.001
8756-3282/

storage methods have on bone properties including stiffness, strength, and ductility in order to increase reproducibility across labs.

In addition, the argument is often made that the storage method is inconsequential, given that the relative differences between groups within a study stored in the same manner is most critical. However, it is unclear whether the preservation method modifies the bone in such a way as to affect its response to experimental manipulation. The goal of this study was to investigate the effects of two common preservation methods on bone mechanical properties from a commonly used murine model in the context of an in vitro bone treatment designed to modify mechanical integrity. For seven total weeks, pairs of bones were stored either wrapped in saline-soaked gauze at −20 °C or submerged in 70% ethanol at 4 °C. Right bones from each pair were then incubated in a ribose solution (to induce the formation of advanced glycation end products (AGEs)), [9], while the contralateral bones were incubated in a control solution. It was hypothesized that there would be an interactive effect on mechanical properties between ribose treatment and the way a bone was stored prior to treatment. Specifically, it was believed that storage in ethanol (with rehydration prior to testing) would stiffen the bone matrix versus freezing, potentially masking the embrittling effects of AGE formation.

2. Materials and methods

2.1. Animals and sample preparation

With prior IACUC approval from the Indiana University School of Medicine (#10797), twenty five 11 week old female C57BL/6 mice (Envigo, Indianapolis, IN) were sacrificed via CO_2 inhalation in accordance with the National Institutes of Health guide for the care and use of Laboratory animals, at which time the left and right tibiae were harvested and stripped of soft tissue. The tibiae were randomly separated into 2 preservation groups (both tibiae from each animal were kept in the same group). Each tibia was stored individually in a microcentrifuge tube under one of the following conditions: wrapped in gauze soaked in phosphate buffered saline (Gibco PBS pH 7.4, Thermo Fisher Scientific, Waltham, MA; PBS) at −20 °C (n = 13) or submerged in 70% ethanol at 4 °C (n = 12). In total, the bones were stored for 7 weeks before beginning the ribosylation experiment.

2.2. Micro-computed tomography

Micro-computed tomography (μCT) scans were taken of each bone using a Skyscan 1172 μCT system (Bruker microCT, Kontich, Belgium). Scans were performed with a source voltage of 59 kV and a current of 167 μA through a 0.5 mm Al filter with an isotropic voxel size of 12.5 μm. NRecon (Bruker microCT) was used to reconstruct voxels with attenuation coefficients ranging from 0 to 0.11 mm^{-1}, apply a beam hardening correction of 40%, and apply a ring artifact correction of 5. Mineral density was calculated using daily scans of manufacturer supplied hydroxyapatite phantoms (0.25 and 0.75 g/cm^3). Reconstructed scans were rotated using Dataviewer (Bruker microCT) for consistent 3D alignment. Standard cortical regions of interest (ROIs) were taken from sites centered at a position proximal to the tibiofibular junction (TFJ) by 18% of the length from the TFJ to the start of the proximal growth plate. Each standard site ROI was a set of 7 slices, perpendicular to the proximal-distal axis. As previously described [3,10], a custom MATLAB (MathWorks, Natick, MA) program was used to calculate the following parameters: total bone area (B.Ar), marrow area (Ma.Ar), cortical area (Ct.Ar), average cortical width (Ct.Wi), periosteal bone perimeter (Ps.Pm), endocortical bone perimeter (Ec.Pm), maximum and minimum second moment of inertia (I_{max} and I_{min}, respectively), width of the anteroposterior axis (AP.Wi), width of the mediolateral axis (ML.Wi), and AP.Wi to ML.Wi ratio (AP.Wi/ML.Wi) according to standard guidelines.

2.3. In vitro ribosylation

After 7 weeks of storage, all left tibiae (n = 25) were soaked in Hanks Balanced Salt Solution (Sigma-Aldrich, St. Louis, MO) supplemented with 25 mM ε-amino-n-caproic acid (Sigma-Aldrich), 5 mM benzamidine (Sigma-Aldrich), 10 mM *N*-ethylmaleimide (Sigma-Aldrich), 30 mM HEPES (Sigma-Aldrich), 0.5 M $CaCl_2$ (Sigma-Aldrich), and 1× Pen-Strep (Sigma-Aldrich) [11–13]. All right tibiae (n = 25) were soaked in the same solution, with the addition of 0.6 M ribose (Sigma-Aldrich). The bones were soaked at 37 °C for 1 week. Stir bars were used to maintain constant circulation in the solutions, and the pH of these solutions was maintained between 7.2 and 7.4 with daily additions of HCl or NaOH as needed. After 1 week, the bones were removed from their soaking solutions and stored for 4 days in their original preservation methods until mechanical testing.

2.4. Mechanical testing

All bones were soaked in PBS at 4 °C overnight before testing to ensure that they were fully hydrated. Prior to testing, all samples were allowed to warm to room temperature. Bones were tested to failure in 4 point bending (upper loading span of 3 mm, lower support span of 9 mm) in displacement control at a rate of 0.25 mm/s while hydrated with PBS. The TFJ was placed just outside the loading span and the bones were tested in the mediolateral direction, with the medial surface in tension. The second moment of inertia about the anteroposterior axis and the extreme fiber in tension were obtained from μCT images using a 7 slice region centered on the fracture site and were used to map load-displacement to stress-strain curves. As previously described [3,10], pre- and postyield mechanical properties were calculated using a custom MATLAB program.

2.5. Statistical analysis

To evaluate if differences in cortical geometry between contralateral bones could influence the mechanical data, paired *t*-tests were performed between left and right bones (n = 25 per side) for cortical μCT parameters. To assess if the preservation methods differentially affected mechanical properties of bone or the mechanical changes expected due to ribosylation [9], a mixed-model ANOVA was used to evaluate the effect of ribosylation as the within subject effect, preservation method as the between subject effect, and the interaction between ribosylation and preservation method. For all tests, $p < 0.05$ was considered significant. In the presence of a significant interaction, paired *t*-tests were employed separately within each preservation group to test the effect of ribose with $p < 0.025$ considered significant due to a Bonferroni correction. All data are reported as mean ± standard deviation (SD) with the exception of the schematic mechanical curves where error bars indicate standard error of the mean (SEM).

3. Results

3.1. Cortical geometry

Prior to beginning the soaking experiments and performing mechanical measures, all bones were scanned using μCT to assess the suitability of the paired design in this study. A battery of cortical properties in the diaphysis was assessed. There were no significant differences in cortical geometry between right and left bones (Table 1), and the percent differences were <2% for all properties. The schematic cortical profiles shown in Fig. 1 highlight how similar the two limbs were in terms of cortical geometry.