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Even With Rehydration, Preservation in Ethanol Influences the Mechanical Properties of Bone and How Bone Responds to Experimental Manipulation

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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

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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).

Keywords

Collagen; advanced glycation end products; mechanical testing; ribosylation; micro-computed tomography; cortical geometry

1.1 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 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 less than 2% for all properties. The schematic cortical profiles shown in Figure 1 highlight how similar the two limbs were in terms of cortical geometry.

3.2 Mechanical Properties

Figure 2A and Figure 2B show schematic force-displacement and stress-strain curves of the four groups, respectively. When performing the mechanical tests, data from 4 bones were deemed unusable due to technical issues during the test (1 PBS-soaked gauze + ribose, 1 PBS-soaked gauze + no ribose, 2 ethanol-soaked + no ribose), and neither those tests nor

their contralateral pair were included in the analysis. Compared with bones stored in PBS-soaked gauze and frozen, ethanol soaked bones had a 16.3% lower displacement to yield ($p = 0.002$), a 24.5% higher stiffness ($p = 0.002$), a 13.9% lower strain to yield ($p = 0.009$), and an 18.5% higher elastic modulus ($p = 0.025$) (Table 2). Relative to control bones soaked in ribose-free solutions, ribose-soaked bones had a 13.9% higher yield force ($p = 0.001$), a 9.2% higher ultimate force ($p = 0.013$), a 22.2% higher work to yield ($p = 0.003$), a 14.1% higher yield stress ($p = 0.007$), and a 21.9% higher resilience ($p = 0.003$). Postyield displacement, total displacement, postyield work, total work, total strain, and toughness (all of which are primarily reflective of postyield behavior) had significant interaction effects, which necessitated a separate paired analysis within each preservation method. For bones stored frozen and wrapped in PBS-soaked gauze, all six properties were lower by 49% to 68% due to ribosylation (Figure 3, $p < 0.001$ for all) while for bones stored in ethanol, there were no significant effects of ribosylation ($p > 0.29$ for all) indicating that storage in ethanol abolished the impacts of ribose soaking in these bones.

4. DISCUSSION

The main goal of this study was to investigate if the method used to store a bone can impact how that bone responds to an in vitro treatment designed to modify mechanical properties. As a way to decrease variability and increase statistical power, this study was designed as a paired comparison between right and left limbs from the same animal. Contralateral bones are often used as controls for one another, especially in rodent mechanical loading/unloading studies or investigations of ex vivo manipulation (e.g., drug treatment). Although some studies have investigated bilateral symmetry in mouse bones [14, 15], most are limited in the properties they report, especially when considering cortical geometry. In this study, the contralateral tibiae of 11 week old female B6 mice lacked differences in any cortical parameter (Table 1 and Figure 1). These mice were only 11 weeks old and still rapidly growing, but it is expected that skeletally mature mice would be even more similar as rapid growth ceases. These findings are consistent with a study in cortical bone from the B6 femur at 13 weeks of age [14], and suggest that from the perspective of cortical geometry, contralateral limbs are appropriate internal controls.

A major finding here is that ethanol fixation causes irreversible changes to several key mechanical properties in mouse tibiae (Table 2). Even with 24 hours of rehydration in PBS prior to testing, the bones stored in ethanol still showed significant mechanical differences compared with the control storage condition (wrapped in PBS-soaked gauze and stored at −20 °C). Significant main effects from the mixed-model ANOVA show that the bone was made stiffer at both the structural and tissue level leading to decreased elastic deformation and elastic strain. Schematic force-displacement and stress-strain curves also showed a loss of postyield behavior in ethanol-fixed bones (Figure 2). However, these postyield properties demonstrated significant interaction effects which necessitated separate analyses within each preservation group. Previous studies have indicated that the negative effects of soaking in ethanol can be reversed with rehydration prior to testing in compression [6], tension [7], and beam bending [8]. However, one of these three studies only reported preyield parameters [6], and the postyield properties reported in the other two were limited to ultimate stress [8] or ultimate stress and strain to failure [7]. Although the one study reporting strain to failure did

not show a significant difference [7], the sample size was low which necessitated non-parametric statistical analyses and a loss of power as indicated by the authors. Because of the variability associated with postyield behavior in bone, larger sample sizes are typically needed to detect differences between groups [16]. As an example, a sample size of n=13–14 would be needed to detect a 20% difference in total energy dissipation while fewer than 6 samples per group would be needed to detect the same 20% changes in stiffness or strength. Additionally, differences in testing modalities and testing beams instead of whole bones could also be partially responsible for the discrepancies between previous studies and the present data. Regardless, the data reported here clearly demonstrate that mechanical effects caused by ethanol fixation remain even after rehydration.

Studies of ribosylation in bone have repeatedly shown significant impacts on mechanical integrity through the accrual of AGEs [9, 11–13, 17]. However, to the best of our knowledge, ribosylation experiments have never been performed in whole rodent bones. As expected [9], 1 week of incubation in a ribose solution resulted in significant browning of the bone tissue, indicative of the Milliard reaction between protein and sugar which leads to AGE development. The result on mechanical properties was an increase in strength (resulting in an increase in preyield energy behavior) at both the tissue and structural levels (Table 2).

For the remaining mechanical properties, all of which were related to postyield behavior, there were significant interaction effects between storage method and ribosylation. In bones stored using the control method, all 6 properties were significantly decreased with ribosylation by between 49%–68%. This decrease in postyield behavior was expected, and has been suggested to be related to the stiffening of the collagen matrix through the induction of AGEs. However, these effects of ribose-soaking were not only muted in the ethanol-stored groups (Figure 2), they were completely abolished (Figure 3). Postyield behavior in bone is most commonly attributed to the state of collagen [18, 19]. This finding is particularly important as it indicates that ethanol fixation caused changes to the collagen matrix, preventing the effects of in vitro ribosylation from being realized. The direct effects of ethanol on bone collagen are not fully understood. FTIR and Raman-based analyses of bone have indicated a substantial change in collagen's secondary structure with ethanol fixation [20, 21], while circular dichroism has shown that collagen becomes thermally unstable with exposure to ethanol [22]. Regardless of the mechanism, these results demonstrate the clear importance that storage method can have on bone mechanical outcomes.

In many studies, an argument is made that the way a sample is treated prior to the performance of an assay or test is not important. As long as all samples are treated in the same way, relative differences between groups are of primary concern. This study demonstrates that this reasoning is potentially flawed. Soaking in ethanol caused changes to the bone matrix which modified the way in which that matrix then responded to in vitro ribosylation. A limitation of this study is that it was limited to an in vitro treatment. It is necessary to extend this work to understand if the results hold true for in vivo studies of disease or treatment, especially when changes in collagen and postyield mechanical behavior are investigated or expected.

In conclusion, this study demonstrated that cortical geometry in the contralateral limbs of growing female mice are statistical indistinguishable from each other and contralateral limbs are appropriate to use as internal controls. 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 PBS-soaked gauze. The use of ethanol fixation should be avoided for experiments where a primary endpoint is mechanical testing, and bones should instead be stored hydrated and frozen.

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HIGHLIGHTS

- There are no differences in cortical geometry between contralateral tibiae
- Storage in ethanol stiffens bone even with rehydration prior to testing
- Ethanol storage masks the embrittling effects of in vitro ribosylation
- Maintaining sample hydration is crucial for assessing mechanical properties

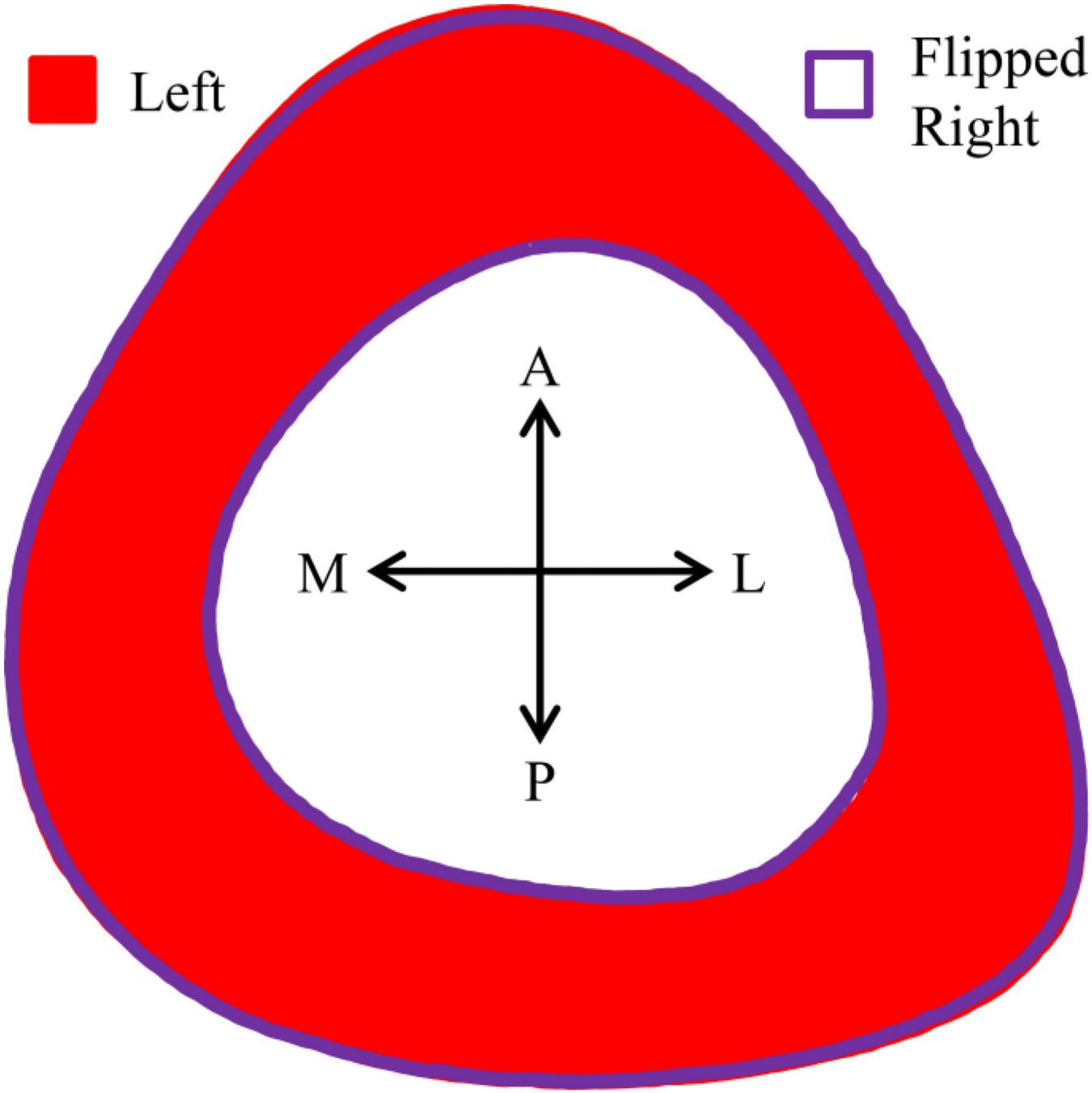

Figure 1.
Average cortical profiles of tibial mid-diaphysis from all 25 pairs. The right profile was flipped along the mediolateral axis to directly compare geometry with the left profile.

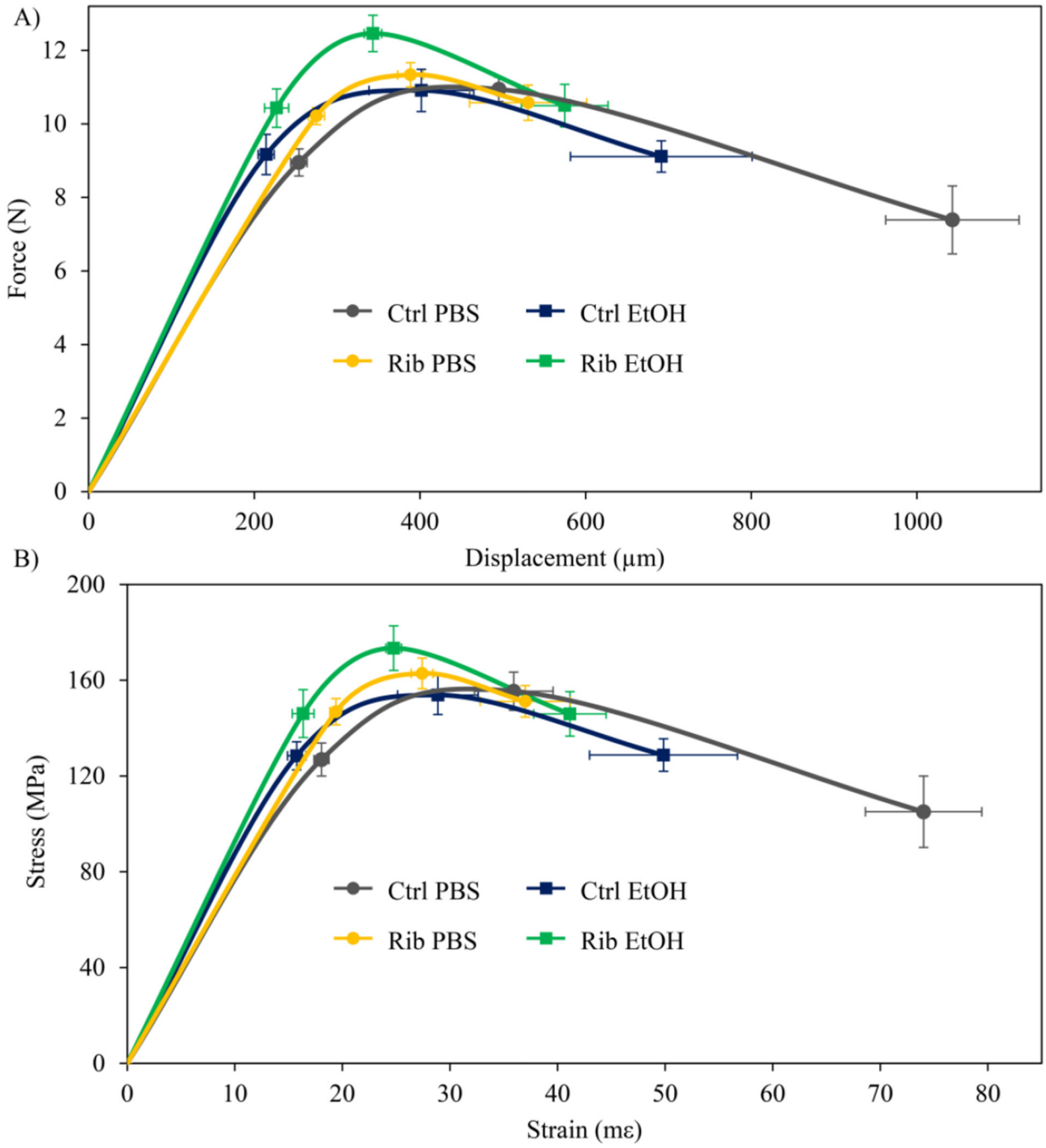

Figure 2.
Schematic A) force-displacement and B) stress-strain curves for all groups. Error bars represent standard error of the mean. Ctrl PBS (n = 11). Rib PBS (n = 11). Ctrl EtOH (n = 10). Rib EtOH (n = 10).

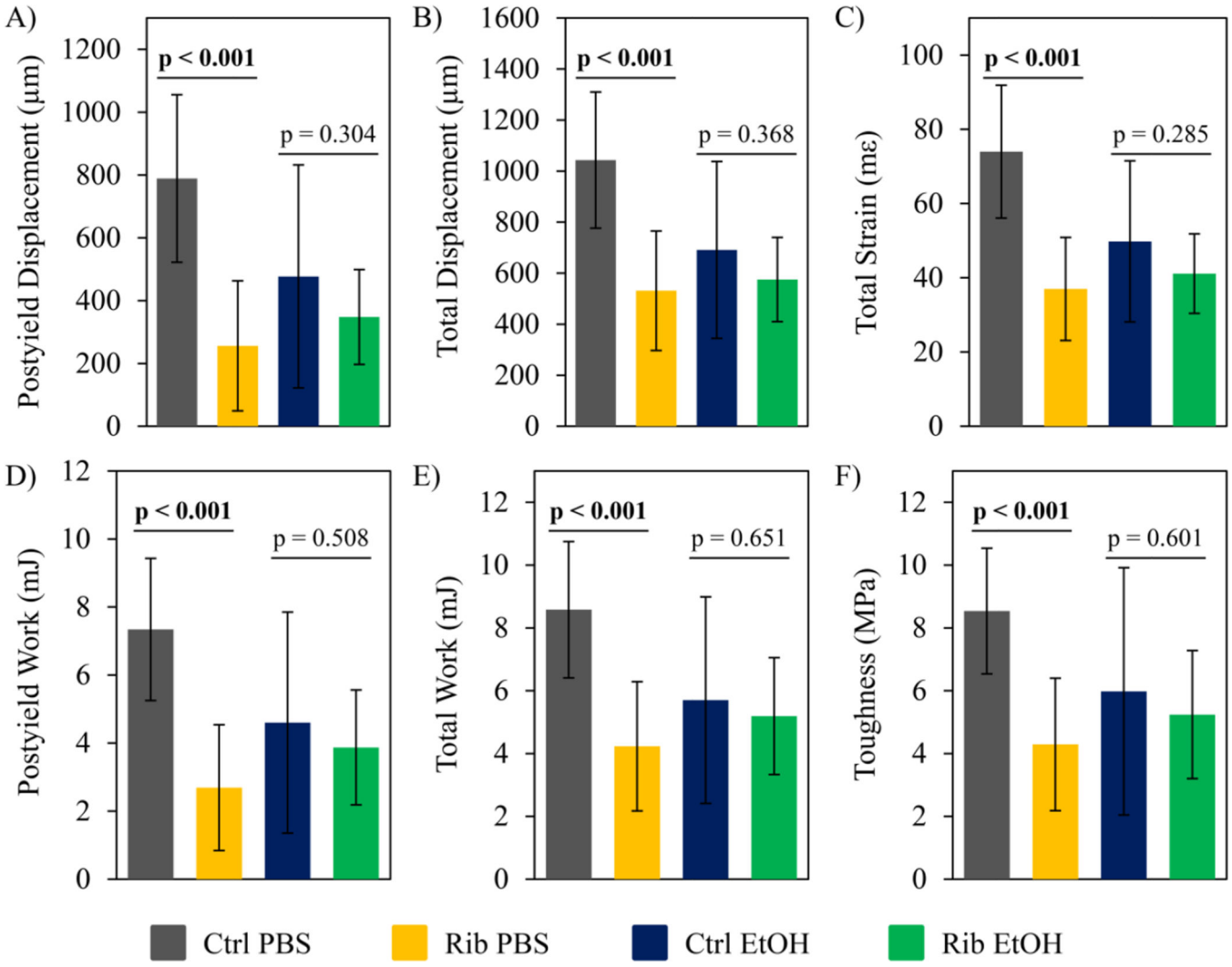

Figure 3.

The effects of ribose on postyield-dominated mechanical properties were abolished with ethanol treatment. These properties had a significant interactive effect from the mixed-model ANOVA: A) postyield displacement ($p = 0.007$), B) total displacement ($p = 0.010$), C) total strain ($p = 0.003$), D) postyield work ($p = 0.003$), E) total work ($p = 0.005$), and F) toughness ($p = 0.024$). The effect of ribose was investigated separately within each preservation group using a paired t-test with $p < 0.025$ considered significant and the p-values from the t-tests are displayed in each panel. Error bars represent SD. Ctrl PBS ($n = 11$). Rib PBS ($n = 11$). Ctrl EtOH ($n = 10$). Rib EtOH ($n = 10$).

Table 1

No contralateral differences in cortical bone at tibial mid-diaphysis

	Left Tibia (n = 25)	Right Tibia (n = 25)	P-value
Tt.Ar (mm^2)	0.893 ± 0.062	0.895 ± 0.068	0.772
Ma.Ar (mm^2)	0.356 ± 0.035	0.352 ± 0.038	0.434
Ct.Ar (mm^2)	0.538 ± 0.032	0.543 ± 0.034	0.149
Ct.Wi (μm)	196 ± 6	199 ± 6	0.066
Ps.Pm (mm)	4.12 ± 0.14	4.11 ± 0.16	0.719
Ec.Pm (mm)	2.65 ± 0.14	2.64 ± 0.15	0.521
I_{max} (mm^4)	0.0624 ± 0.0088	0.0623 ± 0.0089	0.917
I_{min} (mm^4)	0.0481 ± 0.0062	0.0489 ± 0.0072	0.207
AP.Wi (mm)	1.10 ± 0.04	1.10 ± 0.04	0.388
ML.Wi (mm)	1.09 ± 0.05	1.10 ± 0.06	0.193
AP/ML	1.01 ± 0.03	1.00 ± 0.03	0.110

Data presented as mean ± SD. P-values are from a paired t-test.

Table 2

Ethanol storage and ribose treatment alter mechanical properties

	Ctrl PBS (n = 11)	Rib PBS (n = 11)	Ctrl EtOH (n = 10)	Rib EtOH (n = 10)	EtOH	Rib	EtOH × Rib
Yield Force (N)	8.95 ± 1.22	10.21 ± 0.77	9.17 ± 1.73	10.43 ± 1.65	0.683	**0.001**	1.000
Ultimate Force (N)	10.9 ± 1.1	11.3 ± 1.1	10.9 ± 1.8	12.5 ± 1.6	0.292	**0.013**	0.120
Displacement to Yield (μm)	254 ± 33	275 ± 32	214 ± 31	227 ± 46	**0.002**	0.099	0.665
Stiffness (N/mm)	40.8 ± 6.4	41.7 ± 4.8	48.9 ± 9.0	53.7 ± 8.8	**0.002**	0.084	0.226
Work to Yield (mJ)	1.24 ± 0.27	1.54 ± 0.24	1.10 ± 0.32	1.32 ± 0.38	0.126	**0.003**	0.589
Yield Stress (MPa)	7.34 ± 2.09	2.69 ± 1.85	4.60 ± 3.25	3.87 ± 1.69	0.961	**0.007**	0.854
Ultimate Stress (MPa)	8.58 ± 2.17	4.23 ± 2.06	5.70 ± 3.29	5.19 ± 1.86	0.589	0.097	0.443
Strain to Yield (mε)	127 ± 23	147 ± 18	128 ± 19	146 ± 32	**0.009**	0.140	0.559
Modulus (GPa)	155 ± 26	163 ± 21	154 ± 26	173 ± 29	**0.025**	0.249	0.587
Resilience (MPa)	18.1 ± 2.3	19.4 ± 1.9	15.8 ± 2.7	16.4 ± 3.2	0.156	**0.003**	0.458

Data presented as mean ± SD. P-values from mixed-model ANOVA. Bold p-values are significant.