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Partial Removal of Pore and Loosely Bound Water by Low-Energy Drying Decreases Cortical Bone Toughness in Young and Old Donors

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Abstract

With an ability to quantify matrix-bound and pore water in bone, ¹H nuclear magnetic resonance (NMR) relaxometry can potentially be implemented in clinical imaging to assess the fracture resistance of bone in a way that is independent of current X-ray techniques, which assess bone mineral density as a correlate of bone strength. Working towards that goal, we quantified the effect of partial dehydration in air on the mechanical and NMR properties of human cortical bone in order to understand whether NMR is sensitive to water-bone interactions at low energy and whether such interactions contribute to the age-related difference in the toughness of bone. Cadaveric femurs were collected from male and female donors falling into two age groups: 21 to 60 years of age (young) and 74 to 99 years of age (old). After extracting two samples from the medial cortex of the mid-shaft, tensile tests were conducted on Wet specimens and paired, Partially Dry (PtID) specimens (prepared by low-energy drying in air to remove ~3% of original mass before testing). Prior analysis by micro-computed tomography found that there were no differences in intra-cortical porosity between the Wet and PtID specimens nor did an age-related difference in porosity exist. PtID specimens from young and old donors had significantly less toughness than Wet specimens, primarily due to a dehydration-related decrease in post-yield strain. The low-energy drying protocol did not affect the modulus and yield strength of bone. Subsequent dehydration of the PtID specimens in a vacuum oven at 62 °C and then 103 °C, with quantification of water loss at each temperature, revealed an age-related shift from more loosely bound water to more tightly bound water. NMR detected a change in both bound and pore water pools with low-energy air-drying, and both pools were effectively removed when bone was oven-

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dried at 62 °C, irrespective of donor age. Although not strictly significant due to variability in the drying and testing conditions, the absolute difference in toughness between Wet and PtID tended to be greater for the younger donors that had higher bone toughness and more bound water for the wet condition than did the older donors. With sensitivity to low-energy bone-water interactions, NMR, which underpins magnetic resonance imaging, has potential to assess fracture resistance of bone as it relates to bone toughness.

Keywords

Fracture Resistance; Age; Nuclear Magnetic Resonance; Magnetic Resonance Imaging; Micro-Computed Tomography; Water; Collagen

Introduction

To improve the clinical assessment of fracture risk, ^1H nuclear magnetic resonance (NMR) methods are being developed to quantify water within bone (Anumula et al., 2010; Bae et al., 2012; Horch et al., 2010). Furthermore, NMR signal sizes correlate strongly with cortical bone material properties, provided the signal source is delineated as being matrix-bound water or pore water protons (Horch et al., 2011). Moreover, such methods can conceivably be translated to clinical assessment of fracture risk using selective radiofrequency pulses in conjunction with ultrashort-echo time magnetic resonance imaging (uTE-MRI) (Horch et al., 2012). When derived from a NMR-acquired transverse relaxation (T_2) spectrum (Fig. 1), pore water is a measure of intra-cortical porosity (Ni et al., 2007; Wang and Ni, 2003); and of course, high porosity is indicative of weak bone (McCalden et al., 1993). This was recently illustrated in patients by finite element analyses of distal radius models that were generated from high resolution, peripheral computed-tomography (HR-pQCT) scans (Burghardt et al., 2010). On the other hand, the significance of bound water to the mechanical properties of bone is less clear.

Various observations support the concept that interactions between water and the bone tissue contribute to fracture resistance: 1) water bound to collagen stabilizes the collagen matrix thereby maintaining the mechanical integrity of the fibrils (Bella et al., 1995; Luescher et al., 1974; Palfi and Perczel, 2009), 2) equatorial spacing of collagen molecules in bone decrease (i.e. pack more closely together) with a decrease in water content (Lees and Mook, 1986; Lees and Page, 1992), 3) there is an inverse relationship between this spacing and the amount of bone mineral (Bonar et al., 1985; Lees, 1987), 4) hydration of bone, not necessarily the structural integrity of collagen (i.e., intact vs. denatured fibrils), affects viscous energy dissipation (Yamashita et al., 2002), 5) dehydration of bone reduces the distance between the collagen and mineral phases (Rai and Sinha, 2011), 6) hydrogen bonding between water and collagen can exist at different energy levels (Nomura et al., 1977; Pineri et al., 1978), and 7) dehydration of bone causes brittleness (Dempster and Liddicoat, 1952; Evans and Lebow, 1951; Nyman et al., 2006; Sedlin and Hirsch, 1966; Yamada and Evans, 1970; Yan et al., 2008). Thus, a reduction of water bound to the matrix could lead to a reduction in the fracture resistance of bone.

While there is evidence that the amount of bound water in bone and the toughness of bone decrease with aging (Nyman et al., 2008), little is known about whether aging affects loosely or tightly bound water-matrix interactions. Also not entirely clear is the relative sensitivity of NMR – and by extension MRI – to these types of interactions. Our previous NMR work indicates that bound water interacts with solid protons of the bone matrix, as evident by magnetization exchange between these two proton pools (Horch et al., 2010), but we do not know whether these matrix-water interactions are mainly low energy bonding, mainly high

energy bonding, or both equally. To establish the relevance of loosely bound (low energy) water to the fracture resistance of bone, we hypothesized that removal of loosely bound water via air drying affects the toughness of young human bone to a greater extent than elderly bone because elderly bone has less low-energy, matrix-bound water contributing to post-yield deformation. We used micro-computed tomography (ICT) to assess potential differences in intra-cortical porosity and ^1H NMR relaxometry to assess water states as a function of drying. In addition, water removal was quantified by mass changes after oven-drying at 62 °C and again at 103 °C to determine whether matrix-water bonding interactions shift from a lower energy to a higher energy state with aging.

Materials and Methods

Sample preparation

Fresh-frozen cadaveric femurs were acquired from the Musculoskeletal Transplant Foundation (Edison, NJ), an allograft bank, and the Vanderbilt Donor Program (Nashville, TN). The bones were divided into 2 age groups: *young* included 3 female and 11 male donors between 21 years and 60 years of age with a median age of 48 years and *old* included 6 female and 8 male donors between 74 years and 99 years of age with a median age of 84.5 years. Using a band saw, each frozen mid-shaft was cut into 2 halves approximately 70 mm in length. All subsequent cutting included constant irrigation. A circular diamond-embedded saw (660, South Bay Technologies, Inc., Torrance, CA) was then used to extract two cortical bone strips (~2 mm in thickness) along the longitudinal axis of the mid-shaft from the medial side of each proximal half. The periosteal and endosteal edges of each bone strip were milled away using a custom-made clamp that produced “dogbone” tensile specimens having a gauge region (nominal dimensions of 10 mm × 2 mm × 2 mm) connected to the grip regions (5 mm × 5 mm × 2 mm) with a transition fillet radius of 20 mm spanning 5 mm. All of the paired bone specimens were wrapped in gauze that was soaked in phosphate buffered saline (PBS), and stored in a freezer at (−20 °C) until further analysis.

Micro Computer-Tomography analysis

To quantify intra-cortical porosity of each tensile specimen, the mid-section of the gauge region (1.24 mm in length) was imaged at an isotropic voxel size of 6 μm while the specimen was immersed in PBS and its long axis was aligned with the long axis of the scanning tube. To minimize noise, the micro computed-tomography (μCT) scanner ($\mu\text{CT}40$, Scanco Medical, Brüttisellen, Switzerland) acquired 1000 projections per 180° with an integration time of 300 ms at energy settings of 70 kVp and 145 mA from all specimens. In addition, for all scans we used the standard filter for the scanner, the manufacturer’s beam hardening correction, and the phantom calibration of attenuation to equivalent density of hydroxyapatite (HA). To define the volume of interest, a contour was placed at the edge of the bone for each cross-section (Fig 2a) within the image stack using the auto-contouring feature provided in the Scanco software. All contoured bone scans were evaluated with two different schemes: 1) calculated the mean apparent volumetric bone mineral density (avBMD) among voxels within TV as well as the mean tissue mineral density (TMD) among voxels with equivalent density of 700 mgHA/cm^3 or higher and 2) calculated porosity and the architecture of the pores using the Scanco evaluation script for trabecular bone on a segmented image stack of void spaces (Fig 2b). This latter scheme involved including all voxels with a density of −500 mgHA/cm^3 to 775 mgHA/cm^3 and a Gaussian noise filter with a support of 1 and a variance of 0.5.

Tensile testing wet and partially dry bone

The tensile tests were performed at ~24 °C (30%–40% humidity) using servo-hydraulic testing system (DynaMight 8841, Instron, Norwood, MA). Secured within wedge-action

grips (M10-82716-16, Instron, Norwood, MA) with a tension coupler connecting one grip to the actuator, all specimens were pulled-to-failure at 5 mm/min while recording force from a 1000 N load cell (Dynacell, Instron, Norwood, MA) and displacement from a dynamic extensometer (Model 2620–603, Instron, Norwood, MA). After measuring the cross-sectional area of each specimen with digital calipers, the mechanical properties were calculated from the resulting engineering stress vs. strain curve with the yield point being identified by the 0.2% offset method (Fig 3) and the toughness parameters being derived from the area-under-the-curve. Water was dripped on those specimens assigned to the wet group. To facilitate removal of loosely bound and pore water, the paired specimens were air-dried until they lost 1.0–3.5% of their original mass and then monotonically tested in tension without irrigation (an additional 3–10 min. of air drying giving 1.4–5.7% loss of mass). Because of technical problems (e.g., zeroing pin was not pulled prior to loading, force on specimen tripped the limit of the load cell after yielding), several tests were not included in the analysis. Nonetheless, there were mechanical properties from 13 paired young bone specimens plus 1 wet and 1 partially dry (PtID) specimen from different donors. Likewise, there were mechanical properties from 13 paired old bone specimens with 1 wet specimen without a paired PtID specimen.

Gravimetric analysis

The wet mass, submerged mass, and post-testing mass were measured with an electronic balance (SI-215D, Denver Instrument, Bohemia, NY). After tensile testing, the PtID specimens were further dehydrated in a vacuum oven (Model 5831, NAPCO) at 60 °C to 64 °C for 48 h and then at 99 °C to 104 °C for another 48 h, and mass was recorded after each step. Thus, we estimated the loss of water by partial drying in air and by vacuum-oven drying at two temperatures as 1) percent loss by weight (%wt = (wet mass – dry mass)/wet mass) and as percent loss by volume (%vol = (wet mass – dry mass)/(wet mass – submerged mass)) in which the density of water is assumed to be 1.

¹H nuclear magnetic resonance analysis

As described in detail elsewhere (Horch et al., 2011; Horch et al., 2010a; 2010b), the ¹H NMR measurements (relaxometry protocol) involved a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with the bone sealed by a Teflon support structure inside a loop-gap style radiofrequency (RF) coil, all of which resided inside 4.7T horizontal bore magnet (Agilent, Santa Clara, CA) during acquisition. To assess whether NMR is sensitive to the drying protocol, a grip region of each broken tensile specimen (wet group only) was cut (nominal 5 mm × 5 mm × 2 mm) and analyzed by the ¹H NMR relaxometry protocol, which quantifies bound water and pore water pools based on the transverse relaxation time (T₂) domain (see Fig 1). With the inclusion of a microsphere of water as a reference volume and a measurement of apparent specimen volume by calipers, the volume fraction of total, pore and bound water within each cut sample was sequentially quantified for 3 conditions: 1) fully hydrated, 2) partially hydrated (air-dried to ~3% water loss by weight), and then oven-dried at ~62 °C for 48 hr. Because lipids and pore water signals reside in the same T₂ domain, the pore water signal was defined as the signal difference in this long >10 ms domain (Fig 1) before and after oven-drying.

Statistical analysis

Since differences in porosity and mineral density could confound the effect of partial drying (PtID) on the mechanical properties of bone, a Wilcoxon signed-rank test was used to determine whether differences existed in the μ CT properties between the specimens assigned to the Wet group and the specimens assigned to the PtID group within each age group. The Mann-Whitney test was then used to determine whether differences existed between age groups within each test condition. Mann-Whitney was also used to determine whether water

loss due to the various drying conditions differed between Young and Old bone. Since a Wet specimen and a paired PtID specimen came from each donor (i.e., inherently clustered), donor was included as a random effect in the general least squares (GLS) models that determined whether age group (categorical with Young = 0 and Old = 1) and test condition (categorical with Wet = 0 and Dry = 1) explained the variance in each mechanical property of bone. In addition, to avoid violating model assumptions such as normality, the data was bootstrapped using the default replicates of 50 (Stata v11.0, StataCorp, College Station, TX). When the GLS indicated that Age or Condition was a significant factor, a post-hoc assessment of age- and condition-related differences was conducted using Mann-Whitney and Wilcoxon signed-rank test, respectively. Lastly, differences in NMR-derived volume fractions among wet and dry conditions were assessed with Wilcoxon signed-rank test (i.e., paired), and age-related differences were with Mann-Whitney. Statistical significance was considered at p -value < 0.05 .

Results

μ CT assessment of bone specimens

There were no differences in intra-cortical porosity (Por), pore number (Por.N), pore diameter (Por.Th), the connectivity density of pores (Conn.D), apparent volumetric BMD (avBMD), and tissue mineral density (TMD) between the specimens assigned to the Wet group and those assigned to the PtID group (Table 1). In addition, there were no differences in these properties between the young and old groups. Among all the μ CT properties, the only statistically significant difference was a greater TMD in the old bone specimens than in the young bone specimens assigned to the PtID group.

Loss of water from bone as a function of drying conditions

As intended, the degree of water loss caused by air-drying at room temperature was not different between the young and old bone specimens (Table 2). Surprisingly, oven-drying at 62 °C removed more water from the young bone tensile specimens than from the old bone tensile specimens. This was the case whether calculated as the percent loss of water by mass (%wt) or as the percent loss of water by volume (%vol). Since the removal of water at 62 °C can include both the pore and the bound types, we also compared the water loss by %vol minus percent porosity, as determined by μ CT, between the two age groups. This estimate of the loss of bound water was still higher for the young bone than for the old bone specimens. Water loss due to oven-drying at 103 °C was not different between the age groups (Table 2) suggesting that with aging there is a shift from loosely bound water to more tightly bound water.

Effect of partial dehydration on tensile properties of bone

As determined by GLS random effects models, the age group of donor (young vs. old) and the condition of testing (Wet vs. PtID) explained the variance in toughness and post-yield toughness, but these factors did not explain the variance in the elastic properties, namely Young's modulus and yield strength (Table 3). In other words, air-drying at room temperature to remove a modicum of water (1.5%–3.2% by wt) decreased the toughness of bone for both the young and old groups, but did not affect modulus or yield strength (Fig. 4). Moreover, the age-related decrease in toughness existed for both Wet and PtID bone specimens, and these differences were primarily due to differences in post-yield strain, which was considerably decreased by air-drying (Fig 4). For the PtID testing condition, the old bone samples yielded at lower strains (Table 4). Peak tensile strength was less for the old than for the young cortical bone, regardless of testing conditions, though this difference was not strictly significant ($p < 0.1$; Table 4). With increasing age, there was ~67% decrease in post-yield toughness, but only a ~10% decrease in elastic toughness (Table 4).

The absolute difference in toughness and post-yield toughness between the paired Wet and Ptd bone specimens tended to be greater for the Young group than for the Old group (Fig. 5) suggesting low energy dehydration could affect toughness of younger bone to a greater extent than elderly bone. Analyzing at the relationship (Fig. 5) between the toughness of the partially dried bone vs. the toughness of the paired wet bone, we observed that the slope ($m = 0.330$ with a 95% CI of 0.132, 0.528) significantly deviates from unity and the y-intercept ($b = 0.377$ with a 95% CI of -0.0137 , 0.767) is not significantly different than zero. Thus, the wet specimens with higher values of toughness tended to have a paired specimen in which partial dehydration apparently caused a greater loss in toughness than specimens with already low toughness. However, there was considerable scatter in the data (Fig 5a), especially for bone specimens in the young group, with 3 pairs of specimens per age group having similar toughness values whether tested wet or partially dried.

NMR sensitivity to dehydration protocol

In addition to removing the pore water, the air-drying protocol decreased the volume fraction of bound water (Fig. 6). As intended, there were no differences in the loss of water (% wt) due to air-drying between young and old samples ($p=0.297$), and this degree of water loss was not different from that of the tensile specimens (Young: $p=0.391$ and Old: $p=0.388$). Oven drying at 62 °C practically removed the entire bound water pool (Fig. 6). Interestingly, an age-related difference in bound water of wet specimens persisted after partial dehydration in air (Fig. 6).

Discussion

As a complementary tool for fracture risk assessment, MRI can potentially provide measurements that are independent of bone mineral density since it can quantify water residing in the pores of bone as well as water interacting with the matrix. Using ^1H NMR, which underpins MRI, the present study found that T_2 discrimination of these water pools is sensitive to low-energy interactions between water and bone. Moreover, these interactions (e.g. loosely bound water) primarily contributed to the toughness of bone, not the strength. With aging, there was a decrease in toughness, a decrease in bound water, and a shift from loosely bound to more tightly bound water. Thus, NMR and by extension MRI appear to be sensitive to the water-matrix interactions that are important to the toughness of bone.

While the present study did not quantify the contribution of tightly bound water to toughness, a previous dehydration study of human bone found that the dehydration-related decrease in work-to-fracture (bending) primarily occurred at drying temperatures of 21 °C and 50 °C with further decreases being modest as the temperatures increased to 70 °C and 110 °C (Nyman et al., 2006). Effects of extensive dehydration extend to mouse bone in which 48-h of drying can significantly decrease the ductility of the femur (Broz et al., 1993). It can also affect other properties characterizing brittleness. For example, by drying bovine bone at 60 °C for 24-h, Yan et al. observed a 31% decrease in fracture toughness as measured by the critical stress intensity factor as well as 84.5% decrease in work-to-fracture (Yan et al., 2008). Similarly, dehydration effects at room temperature (50% humidity for 7-d) have been observed for other mineralized tissues such as dentin (Kahler et al., 2003).

While dehydration has long been known to cause bone to behave in a brittle fashion (Sedlin and Hirsch, 1966), the relative effect of low-energy partial dehydration in bones from young and old donors has not been studied to the best of our knowledge. In doing so, we found that simple air-drying at room temperature for less than 15 min can reduce the toughness of both young (20–60 y.o.) and old (>70 y.o.) bone by primarily decreasing the post-yield strain (Fig. 2). The present findings do not however unequivocally support the central hypothesis that low-energy drying affects young bone to a greater extent than old bone since the paired

difference in toughness between wet and partially dry specimens was not strictly greater for the young than for the old bone (Fig. 5). Nonetheless, there was a trend in which tougher bones had a greater decrease in toughness following partial dehydration than less tough bone specimens. Moreover, the toughness of partially dry, young bone specimens was similar to the toughness of hydrated, old bone (Fig. 4), and bound water of hydrated bone was less for the old donors than for the young donors (Fig. 6). This suggests that with aging there is less matrix-bound water contributing to post-yield deformation, which is the primary determinant of the toughness of cortical bone. What remains to be determined is whether low-energy water interactions differentially contribute to the fracture toughness (i.e., resistance to crack propagation) of bone from young and old donors.

Variance in the effect of air-drying on the mechanical properties of bone, especially from young donors, is one explanation for why there was not a clearer difference in the Wet minus paired PtID toughness (Fig. 5) between young and old bone. This variance arises from a limitation of the present study: a lack of control of the drying kinetics. Although we attempted to reduce the mass of the specimens by ~3%, the actual loss of loosely bound water depended on several uncontrollable factors including porosity, the time to set-up the tensile test (i.e., actual mass loss varied), and humidity (varied between 30% and 40%). In effect, a bone with higher porosity contributes more pore water and less bound water to the mass loss than a specimen with lower porosity (i.e., greater matrix surface area). For example, there were two specimens from the young group for which the toughness values for Wet and paired PtID specimens (Fig. 5) were essentially the same, and they had the highest porosity (10.7% and 15.3% or 2.7 times higher than the group mean porosity). Also contributing to data scatter in Fig. 5 is that the paired PtID specimens would not necessarily have the same toughness if tested in the wet condition as the corresponding Wet specimens; but since the toughness measurement requires the destructive tensile test, we could not measure it before and after air-drying.

Another limitation of the study is that we could not independently control the loss of pore water and the loss of loosely bound water. Moreover, our NMR measurement of bound water could potentially contain some water residing in small pores such as the canaliculi (Horch et al., 2010). Despite this possibility, the air-drying protocol likely removed bound water since the surface area of the specimen is predominately matrix given that the estimated porosity including the lacunar-canalicular system is 8–12%. Thus, more bound water is exposed to air than pore water, the majority of which must diffuse from within bone out to the surface. Additionally, we used quasi-static loading conditions (5 mm/min), so any hydraulic effect from water movement during loading was likely to be minimal. Finally, previous studies using thermal gravimetric analysis (TGA) coupled with mass spectrometry (MS) have shown that adsorbed water (i.e., bound) can depart powdered bone at room temperature (Mkukuma et al., 2005; Mkukuma et al., 2004).

Since the initial studies characterizing water in bone (Robinson, 1960; 1975), water was understood to exist in an unbound and bound state. Over the years, different analytical tools including differential scanning calorimetry (Kronick and Cooke, 1996), TGA-MS (Mkukuma et al., 2005), solid-state NMR (Wilson et al., 2005), ^1H NMR spectroscopy (Horch et al., 2010; Turov et al., 2006), and wide-line NMR (Casciani, 1971) have characterized the nature of bound water. This type of water arises from 1) hydrogen-hydrogen bonding with the collagen residues (Lazarev et al., 1992), 2) electronic dipole-dipole interactions with surface charges of the mineral phase (Timmins and Wall, 1977), and 3) substitutions in the crystal lattice (LeGeros et al., 1978). These sources are associated with varying degrees of energy, and thus, water can exist as loosely, intermediately, or tightly bound to the matrix. In the aforementioned TGA-MS study (10 °C/min), the removal of adsorbed or bound water peaked at ~100 °C and removal of so-called structural water

began around 220 °C peaking at 340 °C (Mkukuma et al., 2005; Mkukuma et al., 2004). The effect of aging on the temperature (energy) of these 2 types of bound water is unknown, but the present study suggests a shift from lower energy interactions to higher energy interactions since vacuum oven drying at 62 °C removed on average 19% more water, as determined by mass loss, from the young bone than from the old bone (Table 2).

The cause of this moderate decrease in bound water with age is currently unknown. Since 1925 when Hammett published compositional properties of rat bones (Hammett, 1925), mineral accumulation has been thought to displace collagen-bound water. However, while this is the case for bone growth or maturation (Jonsson et al., 1985), mineral accumulation within the organic matrix or degree of mineralization does not necessarily increase beyond 60 years of age (Mueller et al., 1966). With respect to the collagen phase of bone, glycation-mediated, non-enzymatic collagen crosslinks or advanced glycation end-products (AGEs) accumulate in bone tissue with age (Nyman et al., 2007; Wang et al., 2002), even though bone continues to turn-over throughout life. While there is currently no direct proof that AGE accumulation decreases bound water in bone, this type of excessive crosslinking could conceivably reduce sites of hydrogen bonding. In a DSC study of connective tissue, Kopp et al. (Kopp et al., 1989) reported that collagen could bind more water when it had less crosslinks (enzymatic in this example). In another DSC study on non-calcified tissue (rat tail tendon), crosslinking by chemicals (e.g., glutaraldehyde) was reported to draw the collagen molecules together, displacing water between the molecules and creating a dehydration effect responsible for the crosslinking-associated increase in the temperature stability of the collagen (Miles et al., 2005). Lastly, dehydration without crosslinking or air-drying can be caused by immersion of the tissue in polar solvents with a lower Hansen solubility parameter (less hydrogen bonding) than water, such as 70% ethanol previously reported to lower the post-yield toughness of bone (Stefan et al., 2010).

Whether allowed to partially dry or immersed in alcohol, the dehydrated bone matrix has contracted collagen (Lees and Mook, 1986) within the much stiffer mineral phase. There is also likely a conformational change in the triple helical structure of the collagen during dehydration (Zhu et al., 2009). While moderate water loss does not provide sufficient contraction or shrinkage to increase bone strength in a pre-stressed manner, the reduction in ductility with contraction of the organic phase is sufficient to cause less energy dissipation as damage begins to form beyond the yield point. In effect, the plasticizing contribution of the organic phase to bone arises from hydrated, intact collagen. There is also the notion arising from the analysis of bone with solid-state NMR that a water layer bridges the mineral and collagen phases protecting collagen from off-axis deformation and stress that develops under uniaxial loading (Rai and Sinha, 2011; Wilson et al., 2006). If partial dehydration sufficiently reduces this sacrificial water layer, then collagen of partially dry bone is more likely to break under a given post-yield load than collagen of fully hydrated bone, thereby reducing apparent failure strain. One approach to explore this possibility is to conduct mechanical tests and solid-state NMR on equilibrated bone at different humidity levels (100% to 0%).

Conclusions

Air-drying to remove low-energy water interactions reduces the toughness of bone from young (20 to 60 yo) and old donors (>70 yo) by reducing the ability of the tissue to sustain deformation after yielding. Such dehydration has little effect on the modulus and the yield strength of bone. Moreover, there is an age-related shift from lower-energy to higher-energy interactions between water and bone, and NMR is sensitive to the low-energy interactions via its ability to quantify pore and loosely bound water. The age-related decrease in bone toughness may arise from changes that affect matrix hydration.

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- The partial removal of water by low-energy drying affects the post-yield toughness of bone from both young and old donors.
- With aging, there is a shift from more loosely bound water to more tightly bound water.
- ^1H nuclear magnetic resonance is sensitive to changes in both bound water and pore water pools with air-drying and oven-drying.

Figure 1.

T_2 spectra for wet, partially air-dried, and oven-dried bone. Intensities are normalized to a reference volume of water. Air-drying decreases the signal from the pore and bound water pools, while oven-drying at 62 °C almost removes both pools. The remaining signal for the pore water pool actually arises from lipids that are not removed by the aforementioned drying temperature.

Figure 2.

Micro-computed tomography images of human cortical bone. (a) A cross-sectional and longitudinal slice of bone depicts the directionality of porosity. The outer green contour defines the total volume. (b) Upon segmenting out the mineralized tissue, a three-dimensional rendering of the porosity can be generated such that the color-coding depicts the thickness of the pores (primarily Haversian canals).

Figure 3.

Engineering stress vs. engineering strain curves generated from tensile tests of wet and partially dry cortical bone. Modulus (E) is the slope of the initial linear portion of the curve. Toughness is calculated as the area under the curve, and post-yield strain is the failure strain minus the yield strain.

Figure 4.

Selected mechanical properties acquired from tensile tests (median $\pm$ IR). The elastic properties of modulus and yield strength (top panels) did not vary between wet and partially dry bone or between young and old bone. On the other hand, the toughness and post-yield strain (bottom panels) was lower for the partially dry bone than the wet bone, and the age-related difference in these properties persisted across testing conditions.

Figure 5.

Paired differences in bone toughness. The toughness of the wet specimen minus the toughness of the paired, partially dry (PtID) specimen (top panel; median $\pm$ IR) tended to be greater for the young bone than the old bone, but the variability in this absolute difference, especially for young bone, precluded a statistically significant difference. The linear relationship between wet and PtID toughness (bottom panel) deviates from unity suggesting tougher bone specimens are more vulnerable to the effect of partial dehydration on toughness.

Figure 6.

NMR-derived volume fractions of bound and pore water as percent of specimen volume (median $\pm$ IR). Acquired sequentially from the same specimens, the amount of bound (top panel) and pore (bottom panel) water decreased with drying. Air-drying primarily removed pore water (oven drying was assumed to remove all pore water), and oven-drying at 62 °C removed the rest of the bound water that air-drying did not remove.

Table 1
Median values (25%, 75% Quartiles) of selected μ CT properties of bone are provided for each experimental group.

Property	Young			Old		
	Wet	PtID	p-value ^a	Wet	PtID	p-value
Porosity(%)	4.25 (3.24, 5.04)	4.28 (3.75, 7.37)	0.753	5.55 (4.08, 6.54)	4.91 (3.52, 6.03)	0.345
Por.N(1/mm)	3.50 (3.27, 4.02)	3.63 (3.46, 3.72)	0.507	3.64 (3.38, 3.76)	3.51 (3.47, 3.82)	0.221
Por.Th(mm)	0.046 (0.042, 0.093)	0.058 (0.047, 0.078)	0.600	0.060 (0.049, 0.079)	0.055 (0.037, 0.070)	0.075
Conn.D(1/mm ³)	29.4 (10.4, 43.7)	28.7 (14.7, 51.5)	0.507	26.5 (16.3, 37.5)	18.6 (11.2, 23.6)	0.345
avBMD(mgHA/cm ³)	1284 (1227, 1289)	1266 (1198, 1280)	0.807	1266 (1253, 1286)	1264 (1247, 1310)	0.916
TMD(mgHA/cm ³) ^b	1323 (1316, 1334)	1315 (1281, 1320)	0.861	1333 (1316, 1357)	1330 (1321, 1352)	0.382

^a p-values were derived from a Wilcoxon signed-rank test for Wet vs. PtID within each age group.
^b Within the PtID group, TMD was different between Young and Old (p=0.0198). Otherwise, there were no other differences between age groups irrespective of whether the bone was assigned to the Wet or PtID group.

Median values (25%, 75% Quartiles) of water loss are provided for various drying conditions (in air at room temperature or in a vacuum oven at 1 of 2 temperatures).

Table 2

Property	Young		Old		p-value ^a
H ₂ O loss air (%wt) ^b	2.86	(1.90, 3.18)	2.97	(2.36,3.58)	0.734
H ₂ O loss air (%vol)	5.91	(4.51, 7.51)	5.82	(4.63,7.14)	0.593
H ₂ O loss 62 °C (%wt)	9.84	(9.25, 11.13)	8.41	(7.99,8.88)	0.0001
H ₂ O loss 62 °C (%vol)	18.81	(18.07, 21.18)	16.36	(15.66,16.90)	0.0001
H ₂ O loss 62 °C - Por (%vol) ^c	13.91	(13.24, 16.05)	11.42	(10.92,12.77)	0.0049
H ₂ O loss 103 °C (%wt)	12.35	(11.65, 12.93)	11.98	(11.18,12.40)	0.1455
H ₂ O loss 103 °C (%vol)	23.72	(22.60, 24.64)	23.49	(21.74,23.98)	0.2071

^a p-values were derived from a two-sample Wilcoxon rank-sum (Mann-Whitney) test

^b Calculated as initial wet mass minus the mass after tensile testing normalized by either initial mass (%wt) or volume of the specimen (%vol)

^c To remove the contribution of pore water to water loss by oven-drying at 70 °C, μ CT-derived porosity (%) was subtracted from %vol giving an estimate of bound water loss.

Table 3

The coefficients (95% CI) of each predictor and the overall coefficient of determination of the GLS models are given for significant factors explaining the variance in selected mechanical properties of bone.

Property	Constant	Age ^a	Condition ^b	Age*Condition ^c	R ²
Modulus (GPa)	17.0 (16.4, 17.5)	N.S.	N.S.	N.S.	4.5
Yield strain (%)	0.747 (0.731, 0.764)	-0.0370 (-0.0615, -0.0125)	N.S.	N.S.	26.2
Yield stress (MPa)	93.4 (90.4, 96.5)	N.S.	N.S.	N.S.	7.5
Peak stress (MPa)	107 (102, 111)	-10.8 (-17.2, -4.5)	N.S.	N.S.	11.7
Post-yield strain (%)	1.94 (1.55, 2.32)	-1.27 (-1.65, -0.88)	-0.48 (-0.70, -0.26)	-0.67 (-1.27, -0.080)	64.6
E toughness (MJ/m ³)	0.445 (0.424, 0.466)	-0.0421 (-0.0748, -0.00949)	NS	NS	12.6
PY toughness (MJ/m ³)	1.96 (1.62, 2.29)	-1.30 (-1.66, -0.93)	-0.47 (-0.75, -0.20)	-0.70 (-1.36, -0.045)	61.3
Toughness	2.40 (2.05, 2.74)	-1.33 (-1.76, -0.91)	-0.44 (-0.75, -0.15)	-0.69 (-1.31, -0.06)	59.4

^aYoung is 0 and Old is 1;

^bWet is 0 and Dry is 1; and

^cCoefficient for the interaction is given for the case of Age = 0 (Young) and Condition = 1 (Dry). Therefore, interpretations of coefficients include: Irrespective of age or testing condition, the predicted modulus of bone is 17.0 GPa. In contrast, the predicted toughness for wet young bone is 2.40 MJ/m³, while it is 2.40 - 1.33 = 1.07 MJ/m³ for old wet bone. Moreover, in the case of the dry condition, predicted toughness is 2.40 - 1.33 - 0.44 = 0.63 MJ/m³ for old bone, but it is 2.40 - 0.44 - 0.69 = 1.27 MJ/m³ for young bone.

Table 4

Median values (25%, 75% Quartiles) of selected mechanical properties of bone are provided for each experimental group.

Property	Wet		Partially dehydrated		p-value ^a	Young	Old	p-value
	Young	Old	Young	Old				
Yield strain (%)	0.753 (0.715, 0.770)	0.719 (0.680, 0.749)	0.771 (0.768, 0.782)	0.723 (0.705, 0.740)	0.0808	0.771 (0.768, 0.782)	0.723 (0.705, 0.740)	0.0057
Peak strength (MPa)	108.2 (92.1, 116.1)	96.9 (88.3, 105.6)	108.1 (101.4, 111.8)	97.8 (91.0, 109.9)	0.0891	108.1 (101.4, 111.8)	97.8 (91.0, 109.9)	0.0990
Elastic toughness (MPa)	0.443 (0.395, 0.491)	0.404 (0.349, 0.461)	0.480 (0.437, 0.511)	0.422 (0.371, 0.511)	0.1827	0.480 (0.437, 0.511)	0.422 (0.371, 0.511)	0.1205
Post-yield toughness (MJ/m ³)	1.977 (1.559, 2.329)	0.635 (0.226, 0.953)	0.588 (0.341, 1.081)	0.199 (0.144, 0.274)	0.0001	0.588 (0.341, 1.081)	0.199 (0.144, 0.274)	0.0008

^a p-values were derived from a Wilcoxon rank (Mann-Whitney) test for Young vs. Old within each testing condition.