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The influence of water removal on the strength and toughness of cortical bone

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Abstract

Although the effects of dehydration on the mechanical behavior of cortical bone are known, the underlying mechanisms for such effects are not clear. We hypothesize that the interactions of water with the collagen and mineral phases each have a unique influence on mechanical behavior. To study this, strength, toughness, and stiffness were measured with three-point bend specimens made from the mid-diaphysis of human cadaveric femurs and divided into six test groups: control (hydrated), drying in a vacuum oven at room temperature (21 °C) for 30 min and at 21, 50, 70, or 110 °C for 4 h. The experimental data indicated that water loss significantly increased with each increase in drying condition. Bone strength increased with a 5% loss of water by weight, which was caused by drying at 21 °C for 4 h. With water loss exceeding 9%, caused by higher drying temperatures (≥70 °C), strength actually decreased. Drying at 21 °C (irrespective of time in vacuum) significantly decreased bone toughness through a loss of plasticity. However, drying at 70 °C and above caused toughness to decrease through decreases in strength and fracture strain. Stiffness linearly increased with an increase in water loss. From an energy perspective, the water–mineral interaction is removed at higher temperatures than the water–collagen interaction. Therefore, we speculate that loss of water in the collagen phase decreases the toughness of bone, whereas loss of water associated with the mineral phase decreases both bone strength and toughness.

Keywords

Mechanical properties; Bone mineral; Collagen; Femur; Three-point bend

1. Introduction

Bone is a two-component composite material in which the mineral phase (mainly hydroxyapatite) confers the strength (Zioupos, 2001) and stiffness (Currey, 1988), and the organic matrix (mainly Type I collagen) primarily influences the toughness of bone (Wang et al., 2001,Zioupos, 2001,Zioupos et al., 1999). While mineral and collagen each contribute to the bone's competency, as do microarchitecture (e.g., porosity and trabecular connectivity), macrostructure (e.g., curvature of diaphysis and thickness of cortical shell), and in vivo microdamage (e.g., microcracks and diffuse cracks), their interaction with water is equally

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important to the mechanical behavior of bone. Thus, bone is also a fluidimbibed material in which the distribution of water affects the mechanical properties of bone.

Early studies demonstrated that the stiffness, tensile strength, and hardness increases, whereas the strain at fracture and energy to fracture decreases, following the dehydration of bone tissues (Dempster and Liddicoat, 1952,Evans, 1973,Evans and Lebow, 1951,Sedlin and Hirsch, 1966,Smith and Walmsley, 1959,Yamada and Evans, 1970). Reduced energy to fracture has also been observed for dehydrated dentine (Jameson et al., 1993). In addition, as trabecular bone loses water, its buckling behavior changes from ductile to brittle (Townsend et al., 1975). Lastly, dehydration affects the viscoelasticity of bone: compared with wet bone, dry bone has less anelastic deformation (i.e., less recoverable strain from creep) (Currey, 1965), lower loss factor tan δ (Yamashita et al., 2001,Yamashita et al., 2002), and much higher relaxation rate (Sasaki and Enyo, 1995). Despite the documented effects of drying on bone properties, little is actually known about the underlying mechanism of such changes.

Water is not only present in the microscopic pores, which increase in number and size with age, but also exists within the extracellular matrix of bone tissues. The distribution of water in bone appears to change throughout life. It has been reported that water in bone tissues decreases with skeletal growth (Jonsson et al., 1985) and with progressive mineralization (Robinson, 1979,Robinson, 1975). The observation that mineral content increases with age, tapering at 60 years (Mueller et al., 1966,Timmins and Wall, 1977), implies that the amount of water in the tissue would likely be reduced in the elderly skeleton. Furthermore, non-enzymatic, glycation-induced collagen cross-links increase with age (Wang et al., 2002) and may decrease water's interaction with collagen as seen in connective tissues (Kopp et al., 1989). Understanding the role of water distribution in the mechanical behavior of bone may provide another insight into the susceptibility of bone to fracture in the elderly population.

It is presumable that the distribution of water within the tissue of bone—the amount of water bound to collagen, to mineral, and the mobile water in the vascular–lacunar–canalicular cavities—may certainly dictate the bone's mechanical behavior. To address this issue, the present study investigated the effect of water loss on the mechanical behavior of bone. Specifically, mechanical properties were obtained from three-point bend tests of cortical bone specimens that were dehydrated at varying temperatures. Based on the results, we put forth a model for how the distribution of water in the collagen matrix and the mineral phase affects the strength, toughness, and stiffness of bone.

2. Materials and methods

2.1. Specimen preparation

Six human cadaveric femurs (42–49 year old males) were collected from the Musculoskeletal Transplant Foundation (Edison, NJ). One cross-sectional segment (≅35 mm in length) was dissected from the middiaphysis of each femur with a band saw. Using a circular diamond saw, six bone strips (≅2.1 mm in thickness) were extracted along the longitudinal axis from the medial side of each cortex segment. With a bench top end-mill (Model 5000, Sherline, San Marcos, CA), we machined the bone strips into rectangular specimens (nominal dimensions of 30 mm × 4.2 mm × 2.1 mm). One bone specimen from each donor was included in each of the following test groups: control (no drying), drying in a vacuum oven at room temperature (21 °C) for an approximate time of 30 min, at 21, 50, 70, and 110 °C for 4 h. The highest temperature was chosen because it was below the temperature (160 °C) at which heat-induced collagen denaturation affects the mechanical properties of bone (Wang et al., 2001). Finally, the specimens were stored in gauze soaked with phosphate buffered saline at −20 °C prior to measurements and treatments.

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2.2. Dehydration

After being thawed, each specimen was wiped free of surface water, weighed in air with an electronic balance (PB303-S, Mettler Toledo, Greifensee, Switzerland), and weighed again while submerged in water. Then, the specimens were dehydrated in a vacuum oven (Model 280A, Fischer Scientific, Pittsburgh, PA) with 25±2 in of Hg and weighed in air immediately after drying and before mechanical testing. It was assured that the mass of bone specimens did not change (by more than 0.03%) while being measured nor during the time of testing. Water loss then was calculated as the difference between the mass of bone specimen before drying (W_{wet}) and the mass after drying (W_{dry}), normalized by W_{wet} and expressed as percent loss by weight. In addition, water loss was expressed as the percent loss by volume following Archimedes's principle,

$$\text{Water loss}(\%\ \text{by volume}) = 100 \times \frac{W_{wet} - W_{dry}}{W_{wet} - W_{sub}}, \quad (1)$$

where W_{sub} is the mass of wet bone when submerged in water.

2.3. Mechanical testing

Immediately after weighing, bone specimens were loaded at a cross-head speed of 5 mm/min in three-point bending using an Endura TEC mechanical testing system (Elf 3300, Bose Corporation, Minnetonka, MN). The span across the support rollers for the three-point bending test was 16.5 mm. Mechanical properties were determined from each force (P) versus displacement (d) curve (Fig. 1). Thus, the modulus of elasticity (E) was determined by the slope of the linear portion ($\Delta P/\Delta d$) of the curve and the deflection equation of beams

$$E = \frac{\Delta P}{\Delta d}\frac{L}{48I}^3, \quad (2)$$

where I is the moment of inertia and L is the length of support span. In the case where the bone exhibited post-yield behavior (in most cases, control specimens), yield force was determined using the 0.2% offset method, the flexure formula, and Hooke's Law

$$P_{0.2\%} = \frac{8I}{LH}E\,0.002, \quad (3)$$

where H is the height of the specimen. Then, the displacement that corresponded to the force at 0.2% strain was determined from the force–displacement curve. Starting at this displacement, a line parallel to the stiffness of the specimen was drawn, and the yield force (P_y) occurred at the intersection of the parallel line and the force–displacement curve (Fig. 1). For brittle behavior (in most cases, the dehydrated bones), the yield force equaled the maximum force, where strength (S_y) was calculated with the flexure formula. Toughness was reported as work to fracture (i.e., the area under the force–displacement curve).

2.4. Porosity measurement

The cross-sectional porosity of each specimen was measured near the fracture surface. A segment from one side of the fracture specimen (≈4.5 mm) was cut with a circular diamond saw and embedded in acrylic resin (Spurr low-viscosity embedding media, Polysciences, Inc., Warrington, PA). After curing overnight in a vacuum oven (25 in of Hg) at 70 °C, each cross-section was exposed by machining with a lathe (Model 4000, Sherline, San Marcos, CA), then hand grinding with successive grits of silicon carbide papers (600, 1200, 2400, and 4000), and lastly polishing with a suspension of 0.05 m Alumina. A microscope (ML5000, Meiji Techno, San Jose, CA), attached to a CCD camera (Spot Insight, Diagnostic Instruments, Inc., Sterling Heights, MI), was used to capture three digital images covering most of the cross-section of each specimen.

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An image-processing program written in Matlab (version 6.5, The Mathworks, Inc., Natick, MA) was used to calculate porosity. The pictures of the cross-sections were converted to binary form and filtered using user-defined threshold approaches. Specifically, a threshold gray value and a minimum area of target region were defined to identify the black pores (namely, resorption space and Haversian canals as shown in Fig. 2). The measured area of the black pores was then normalized by the total area of the image, and the values from the three measurements were averaged to determine the porosity of the specimen. Porosity was used to estimate the volume occupied by mobile water.

2.5. Statistical analysis

Given the difficulty in collecting human tissues, we minimized the sample size by utilizing the procedure known as MULTTEST for multiple testing of hypotheses (SAS/STAT version 8, SAS Institute Inc., Cary, NC). This procedure does not assume any parametric form of the data such as normality (Westfall et al., 1999). It employs a re-sampling technique called Bootstrap and obtains adjusted *p*-values from a family of hypothesis tests (Chernick, 1999). Having been widely applied in various disciplines (e.g., signal processing and automotive engineering), Bootstrap provides a substitute for the true unknown distribution and maintains correlations between multiple observations. For small sample sizes, MULTTEST with Bootstrap increases the power of the hypothesis tests.

An adjusted *p*-value was defined as the smallest significance level ($\alpha = 0.05$) for which the given hypothesis would be rejected when the entire family of tests (multiple comparisons) was considered. MULTTEST tested whether dehydration significantly affected water loss, and this was followed by Tukey's pair-wise comparisons to see if water loss increased with an increase in drying temperature. For each dehydration group, paired Student's *t*-test tested whether water loss by volume exceeded the estimated volume of mobile water in the bone specimen groups.

Our MULTTEST procedure also tested the null hypothesis that there were no linear trends between the mechanical properties and dehydration (i.e., provided an analysis of variance). Post hoc, multiple pairwise comparisons followed to determine statistically significant differences ($p<0.05$) between dehydration groups and control as well as among the dehydration groups. These comparisons of the multivariate data were one sided in which the alternative hypothesis was that the mechanical property at the higher temperature is less than the property at the lower temperature or control. To see whether water loss relates to the mechanical properties of bone, we applied a regression analysis (linear, quadratic, and cubic) on each property. Zero water loss was assumed for the control specimens. *P*-values and R^2 values were reported for the model in which all coefficients were statistically significant.

3. Results

3.1. Water loss in bone by heating

The percent loss of water significantly increased with an increase in the drying condition (Fig. 3). Porosity did not vary among the dehydration groups, and on average, equaled 9%. Based on a measurement of lacunae porosity for cortical bone from four of the same donors in a previous study (Wang and Ni, 2003), lacunae occupied only an additional 1.8% of the bone volume. It has been reported that the partial porosity of the canalicular network is 1% of bone volume (Martin, 1984). Therefore, the maximum average volume of free water (i.e., the water occupying the vascular–lacunar–canalicular void space) in the specimens of the present study could be approximated as 12%. Drying bone in a vacuum oven for 4 h at temperatures of 50 °C or above, significantly removed more water than the maximum possible volume of mobile water (Fig. 3). Hence, water was surely removed from the extracellular matrix of bone in addition to the pores.

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3.2. Effect of water loss on mechanical properties of bone

Dehydration had a statistically significant effect on the strength ($p<0.0001$), toughness ($p<0.0001$), and stiffness ($p<0.0001$) of bone. Compared to the control, the strength of bone specimens were significantly weakened when dehydrated at 110 °C (Table 1). With a non-linear relationship between strength and water loss (Fig. 4A), drying at 50, 70, and 110 °C caused significantly lower yield strength than drying at room temperature for the same time of 4 hours. All drying conditions caused significantly lower bone toughness compared to the toughness of fully hydrated bone. Plasticity was virtually removed by dehydration at room temperature for either 30 min or 4 h, and further significant reductions in toughness by drying at 50 °C and above were due to decrease in strength and strain at fracture. The stiffness of hydrated bone was significantly less than that of bone dehydrated at temperatures of 70 °C and moderately less than bone dehydrated at 110 °C ($p = 0.0512$). Furthermore, stiffness of the bone specimens dried at room temperature and 50 °C were significantly lower than that of bone dried at 70 °C.

Non-linear statistical models best described the association between a loss in water (measured as percent loss of wet weight) and changes in strength and toughness of human cortical bone (Fig. 4). Yield strength increased and then decreased with increasing water removal (Fig. 4A). According to the model, the peak in strength occurred at a 5% loss of water by weight, and the strength fell below hydrated bone at a 9% loss. Toughness decreased with an increase in water loss, with the greater decline in toughness occurring at lower levels of water loss (4%) than at higher levels (10%). In other words, the decrease in toughness tapered as water loss exceeded 11% by weight (Fig. 4B). A general linear increase best described the relationship between stiffness and water loss (Table 2 and Fig. 4). However, beyond a water loss of 13% (drying at 110 °C), the increase was less pronounced. Water loss by dehydration was a better predictor of toughness than of strength and stiffness (Table 2).

4. Discussion

The present study investigated the relationships between the mechanical properties and water distribution in cortical bone. The results suggest that the removal of water from extracellular matrix, in addition to that removed from the void spaces within bone, affects the mechanical properties of bone. Water not only resides within the vascular canals, lacunae, and canaliculi, but also exists within the collagen matrix and the mineral apatite (i.e., extracellular matrix). The estimated maximum water content in the vascular–lacunar–canalicular space was 12% of the total volume of bone, a value higher than the 8% calculated by others (Zhang et al., 1998). Robinson (1960) reported that water could exist as two fractions, one driven off at 50 °C associated with marrow—vascular–osteoid and one driven at 100 °C associated with the calcified matrix. The research of Timmins and Wall (1977) indicated that removal of water by thermal dehydration is rather gradual. Our results found that drying at room temperature in a vacuum oven may even affect the mechanical properties of bone. Assuming that water in pores does not affect the mechanical properties (which is likely when measured with monotonic loading at a consistent strain rate), the present results suggest that water may be removed from certain phases of extra-cellular matrix even at room temperature.

In this study, we observed that: (1) for each drying temperature, the rate of water loss between the third and fourth hour in the vacuum oven was less than 2%; (2) an increase in the drying temperature always caused an increase in the loss of water; (3) there was a non-linear relationship between strength and water loss; and (4) water loss affected toughness, even when it did not exceed the amount that could exist in the vascular–lacunar–canalicular space. This implies that water distribution in bone not only exists as mobile water in pores but also has other forms, which interact with bone tissue at different energy levels.

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Further supporting evidence of this supposition is the observation that water interacts with the collagen and mineral phases of bone in several ways. First of all, the polarity of water facilitates its bonding with the hydrophilic groups of the collagen protein (glycine, hydroxyproline, carboxyl, and hydroxylysine) and the charged groups, PO_4^- or Ca^{2+}, of bone mineral. Secondly, studies on the hydration of collagenous tissue (human dura mater and rat-tail tendons) with dynamic mechanical spectroscopy indicate that water does bond with collagen at two levels (Nomura et al., 1977,Pineri et al., 1978). Thus, collagen has structural water and loosely bound water. The former results from hydrogen bonding within the triple helix of collagen molecules (due to the hydroxyl group of hydroxyproline) and requires more energy to remove than the latter, which results from hydrogen bonding with the polar side chains of collagen fibrils (Nomura et al., 1977,Pineri et al., 1978). Thirdly, there are two types of water interaction in the mineral phase, lattice water and surface bound water. X-ray diffraction and infrared (IR) spectroscopy of heat-treated synthetic, precipitated apatites found that water bound to surface of crystals is lost at a lower temperature (below 200 °C) than water inserted into the lattice structure (between 200 and 400 °C) (LeGeros et al., 1978). Nonetheless, the spectra intensities from nuclear magnetic resonance of both surface water and lattice water decreased when bone was dried at 120 °C, with more lattice water remaining at higher temperatures (Casciani, 1971). Based on the energy characteristics of water with collagen and mineral, it is conceivable that water removal from bone is related to energy level as follows: (a) mobile water molecules require less energy to evaporate than water molecules loosely bound to the outside of collagen fibrils or bone surfaces, (b) the removal of the loosely bound water (via hydrogen bonding) requires less energy than the water molecules trapped inside collagen molecules, which in turn requires similar or less energy than water molecules bound to the surface charges of mineral apatite (more ionic in nature), and (c) water that is imbedded in the lattice of hydroxyapatite (more covalent in nature) requires the highest energy to dislodge.

The water loss caused by drying at room temperature increased the strength of bone (Fig. 4B). The stiffness of collagen increases (Nomura et al., 1977,Pineri et al., 1978) and the molecular diameter of collagen decreases (Lees, 1981) with a decrease in hydration. Most likely then, there was enough energy in the vacuum oven at 21 °C to remove not only mobile water but also the water loosely bound to collagen. To illustrate, as the surfaces of vascular channels dried, water loosely bound in the nearby extracellular matrix could diffuse into the newly opened space. Subsequently, collagen fibrils would stiffen and contract longitudinally compressing the mineral phase, and thereby increasing the strength of bone (as observed). This is akin to the pre-stressed rebar that compresses the concrete struts used in construction. It has been suggested that increases in mineralization generates pre-strains in bone (Yeni et al., 2002). In this study, we propose that this mechanism may involve the reduction in the interaction between water and collagen. Under normal hydrated conditions, the collagen phase is thought to have a small influence on strength in comparison to mineral and porosity. Removing the water associated with collagen, however, would seem to impart a contribution to strength as described. Interestingly, strength decreased when bone was dehydrated at higher temperatures. Possibly, bone strength decreased when sufficient energy was applied to remove both the water–collagen interaction and the water–mineral interaction. How exactly the loss of water from the mineral phase affects strength is not clear, though the loss of lattice water could certainly have changed the size of the bone mineral crystal (i.e., distance between neighboring lattice sites decreased) as has been observed in dehydrated enamel and precipitated apatites (LeGeros et al., 1978).

A loss of plasticity (or post-yield toughness) occurred at room temperature drying in which only the water–collagen interaction was believed to be mainly affected. Collagen is known to influence toughness (Wang et al., 2002), and its interaction with water appears to be important in giving bone post-yield deformation behavior. At higher drying temperatures, there was also

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a decrease in work to fracture, suggesting that water bound to the mineral phase also affects the toughness of bone. Of course, this decrease was related to the decrease in strength and also to the decrease in strain at fracture. The decrease in the energy absorbing capacity of bone with an increase in mineral content, as observed by Currey et al. (1996), could likely be due to a decrease in the interaction between water and mineral. In deed, demineralization experiments have shown that there is less water in more mineralized bone (Broz et al., 1995,Lees, 1981).

Of the mechanical properties determined by the three-point bend tests, only modulus of elasticity had a linear association with water loss (Table 2). There was a general trend of an increase in stiffness with water loss (Fig. 4C). Thus, the strength and stiffness of bone has an inverse relationship when water is removed from the extracellular matrix by drying at 70 °C. As was found in early mechanical tests (Dempster and Liddicoat, 1952,Evans and Lebow, 1951,Smith and Walmsley, 1959,Yamada and Evans, 1970), dehydration increased the stiffness and decreased the toughness of bone. Also in agreement with earlier tests, it was observed in this study that dehydration decreased strain at fracture, correlating with the decrease in toughness.

Strength, however, did not necessarily increase after dehydration but did so in uniaxial tension and compression tests reported in earlier studies (Dempster and Liddicoat, 1952,Evans, 1973,Evans and Lebow, 1951). On the other hand, the three-point bending tests of Sedlin and Hirsch (1966) found no significant change in maximum stress for mid-femoral cortical bone tested after 1 week of incubation when compared to wet bone of similar type. As an explanation for the discrepancies in the literature, those studies observing an increase in strength and stiffness did not heat the specimens; and therefore, applied energy was not sufficient to remove the water associated with the lattice. It seems that water removal by low-temperature drying increases strength (as collagen stiffens) while greater water removal at higher temperatures decreases strength (as mineral lattice changes). Regardless, the failure mechanism of bone is complex because of its hierarchical architecture and interacting constituents, but water certainly plays a role in the mechanical behavior of bone.

Use of heating as a means to determine the water distribution in bone was a limitation in the present study because this approach does not necessarily distinguish the exact distribution of water in pores and bone constituents. Emerging techniques with NMR (Fernandez-Seara et al., 2004,Fernandez-Seara et al., 2002,Wang and Ni, 2003) will likely provide a more accurate means to distinguish between water in pores and water in the bone matrix. Whether the pores in the control specimens were actually filled up with water was not clear, since the specimens were not tested in a water bath. Thus, the maximum volume of mobile water (12%) could have been over-estimated. Regardless, room temperature drying likely removes water at least from the collagen matrix, given its effect on strength (which increased). Had room temperature drying only caused a loss of mobile water, strength would have decreased because there would not have been any fluid pressure to resist deformation. The pressure generated by loading is time dependent because water moves. Therefore, the bone exhibits viscoelastic behavior and so water moving in the pores certainly influences viscoelasticity, as has been observed by others when collagen was even denatured (Yamashita et al., 2002). The present study does not provide insight into how water loss affects time-related properties of bone.

In summary, the loss of water from the extracellular matrix of bone tissue affected the mechanical properties of bone. Based on the relationships between these properties and water loss, we propose a model for the effects of water distribution on mechanical behavior of bone as follows. The water bound to the collagen fibrils provides post-yield toughness to bone. Removing this water increases strength and stiffness but decreases bone toughness. Whether water bound to the surface of mineral increases or decreases strength is not clear. Nonetheless, loss of lattice water within the apatite likely decreases strength and toughness. We anticipate

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then that age-related changes in bone strength and toughness (indicators of bone fragility) may reflect age-related changes in water distribution. With aging, it is likely that the amount of water in the tissue decreases while the amount in the pores increases. Therefore, a loss of toughness may reflect decreases in water bound to collagen due to mineralization, or increase in collagen cross-links that displaces water in the collagen matrix.

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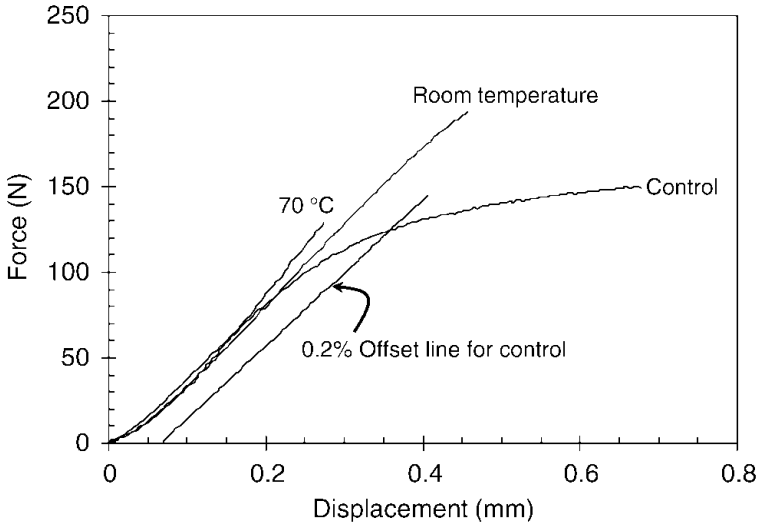

Fig 1.

Each force–displacement plot from the three-point bending tests of cortical bone provided the mechanical properties. As an example, a plot from a control specimen is compared to specimens dried at room temperature and at 70 °C showing the loss of ductility caused by low level of drying and the loss of strength at a high level of drying.

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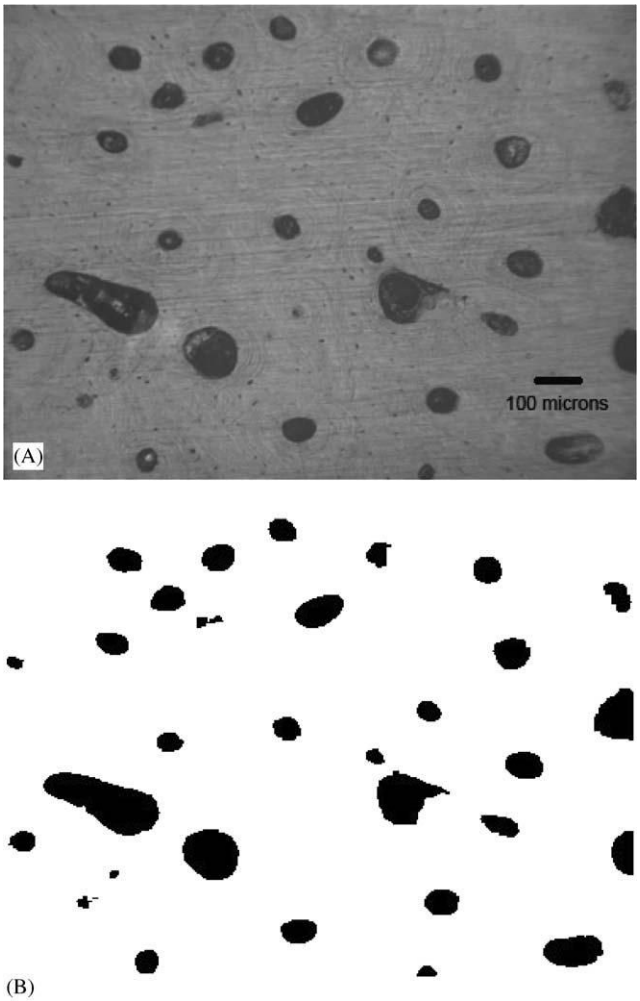

Fig 2.
Cross-sectional images of vascular pores within cortical bone were converted to binary form to calculate porosity (black area per total area).

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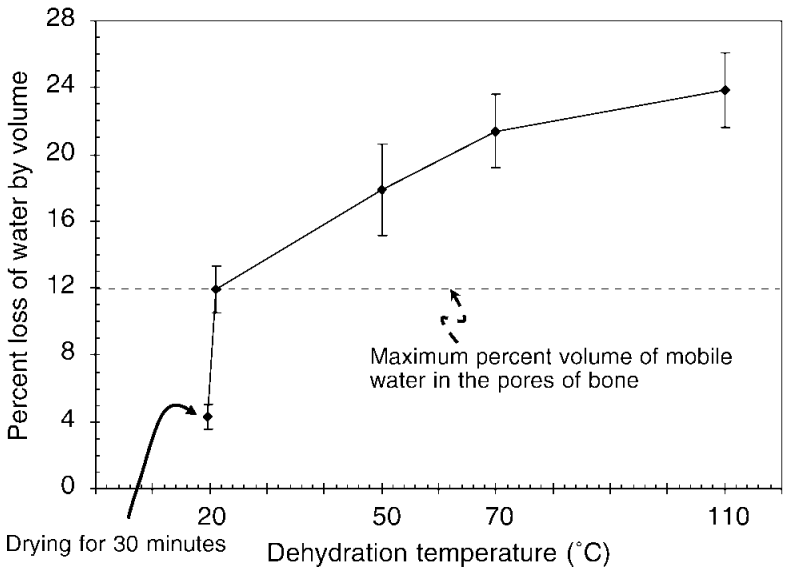

Fig 3.

There was a significant increase in water loss with each successive increase in temperature and drying time ($p \leq 0.0013$). Drying at 50 °C and above in a vacuum oven for 4 h removed more water than what would be expected to exist in the pores.

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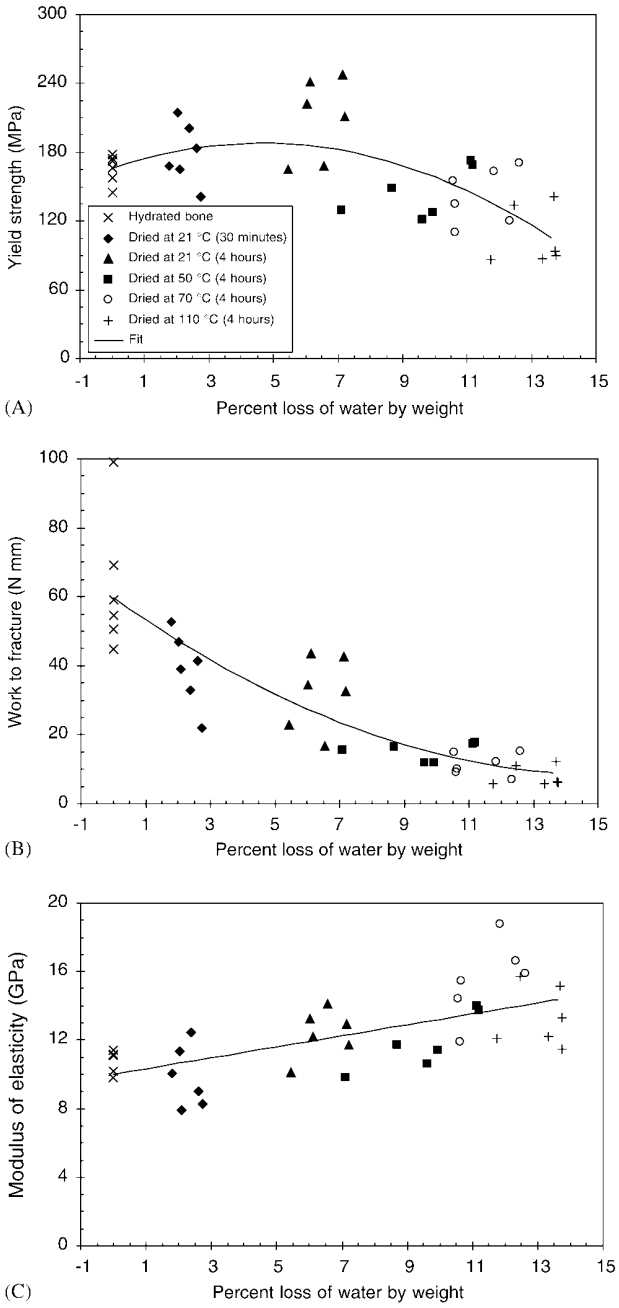

Fig 4.

Non-linear relationships existed between some mechanical properties of bone and water loss: (A) strength increased following vacuum drying at room temperature but decreased following vacuum drying at elevated temperatures, (B) a decrease in toughness occurred with increases in water loss, with the greatest change caused by drying at room temperature and (C) there was an increase in stiffness with an increase in water loss.

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Table 1

Mechanical property values (mean±standard deviation) for control (hydrated) and each dehydration group

Property	Control	21 °C (30)[a]	21 °C	50 °C	70 °C	110 °C
σ_y(Mpa)	166±12	179±26	209±35	145±22[†]	142±24[†]	105±25,[#*†‡]
W (N mm)	62.9±19.5	39.2±10.8[#]	32.1±10.6[#]	15.2±2.6,[#*†]	11.4±3.2,[#*†]	7.8±2.9,[#*†]
E (Gpa)	10.8±0.6[b]	9.9±1.8,[bc]	12.4±1.4[b]	11.9±1.7[b]	15.5±2.3	13.3±1.7

Significance from one-tail, pair-wise comparisons (p<0:05):

[#]less than control,

[*]less than set loss, and

[†]less than 21 °C,

[‡]less than 50 °C,

[b]bless than 70 °C, and

[c]less than 110 °C.

[a]Dried for 30 min, otherwise 4 h.

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Table 2

Statistically significant associations between selected bending properties and loss of water

	Strength		Toughness		Stiffness	
	R^2	*p*-value	R^2	*p*-value	R^2	*p*-value
Loss of water	0.4372	<0.0001	0.7671	<0.0001	0.4257	<0.0001