Ash Content Modulation of Torsionally Derived Effective Material Properties in Cortical Mouse Bone

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The purpose of this study was to evaluate the effects of isolated alterations in mineral content on mouse bone torsional properties. The femora and tibiae from 25 eight-week-old male A/J strain mice were divided into five groups and selectively decalcified from 5% to 20%. The right femora were then tested to failure in torsion while the tibiae were ashed to determine final mineral content of the decalcified bones. Contralateral femora were serially cross-sectioned to determine geometric properties, and effective material properties were then calculated from the geometric and structural properties of each femoral pair. We found that the relationship between ash content and effective shear modulus or maximum effective shear stress could best be characterized through a power law, with an exponential factor of 6.79 ($R^2=0.85$) and 4.04 ($R^2=0.67$), respectively. This indicates that in a murine model, as with other species, small changes in ash content significantly influence effective material properties. Furthermore, it appears that (in adolescent A/J strain mice) effective shear modulus is more heavily affected by changes in mineralization than is maximum effective shear stress when these properties are derived from whole bone torsional tests to failure. [DOI: 10.1115/1.1611513]

Introduction

The factors contributing to long bone strength are incompletely understood. Bone is composed of a mineral phase (hydroxyapatite), an organic phase (Type I collagen with smaller amounts of other proteins), and water; the relative quantities, microscopic architecture of each, and the macroscopic shape of the whole bone determine mechanical strength [1–3]. Numerous transgenic and knockout mouse models have been created to examine the influence of specific molecules and matrix constituents on bone quality [4–6]. A recent paper examining the effects of Growth/differentiation factor-5 (GDF-5) deficiency on murine femoral properties noted a 6% lower ash content in mutant bones, with a 36% reduction in effective shear modulus when compared with control femora [6]. Interestingly, there was no difference in maximum effective shear stress between groups. Little literature exists, however, relating ash content to torsion-derived effective material properties to aid with the interpretation of such mutant mouse data. The contribution of mineral quantity in the determination of bone torsional properties is not clear, and it is difficult to determine whether the observed differences in ash content fully explain the differences in biomechanical behavior.

The influence of ash content and related parameters such as bone mineral content (BMC) on other mechanical properties of cortical bone has been widely studied. In early papers, Currey demonstrated a high correlation between ash fraction and breaking strength during three-point loading, with an extremely rapid rise in strength found over a small range of ash values [7–9]. Later, he also demonstrated a strong correlation and roughly cubic relationship between calcium content and modulus of elasticity in bones from mammals, birds, and reptiles [10]. By contrast, Brodt et al. [11] found significant increases in bone material properties with aging in female C57B1/6 mice (regardless of whether properties were derived from torsional or bending structural tests), but no significant changes in ash content with age. While BMC clearly plays a critical role in influencing bone material properties, other factors, including the water content, collagen content, and collagen cross-linking are also important [10,12–15].

Although it is well established that mineral content strongly correlates with elastic modulus and ultimate strength, this relationship has not been well studied in murine models. Many studies investigating the relationship between ash content and bone strength have relied on naturally occurring variation in mineral content in different bones within or even across widely diverse species [7–10,16]. Unfortunately, it is not certain that mineral content is all that differs among these bones. A more controlled approach would involve alteration of only BMC in otherwise equivalent bones. This has been achieved *in vivo* using a variety of methods, including surgical [17,18], pharmaceutical [19–21], or nutritional means [22–25], but these methods likely affect important hormonal and metabolic processes, thus making it difficult to isolate the effects of BMC alone. A recent study involving *ex vivo* chemical decalcification of cat femora found a correlation coefficient of 0.970 between percentage decalcification and bending strength, in which a 20% loss of ash content led to a 35% loss of bending strength [26]. To our knowledge, no such studies on murine bones have been reported in the literature. Due to the increasing use of transgenic mouse models in studying bone mechanics, as well as the use of torsional testing as a common methodology [6,26,28,30], establishing the relationship between ash content and effective torsionally derived material properties in murine bones would provide valuable information to assist in the interpretation of data from a variety of mouse models.

Accordingly, we chose to evaluate the effects of alterations in mineral content on mouse bone torsional properties. The right femora and tibiae from 25 A/J wild-type mice were divided into five groups and selectively decalcified with ethylene diamine tetraacetic acid (EDTA) for 0, 1, 2, 3, or 4 hours. The femora were then tested to failure in torsion while the tibiae were ashed to determine final mineral content of the decalcified bones. Con-

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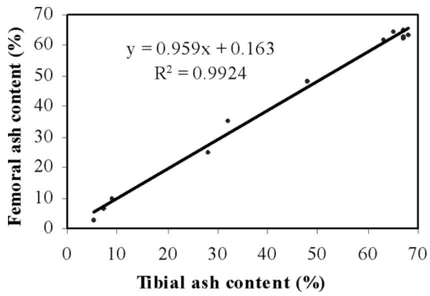

Fig. 1 Tibial versus femoral ash contents. Twelve tibial and femoral pairs were either placed in phosphate buffered saline or 0.5 M EDTA, pH 7.4 for 4, 12, 24, 48, 72, or 144 hours to obtain a relationship between femoral ash content and tibial ash content. This relationship was then used to convert tibial ash contents from the primary study to estimated femoral ash contents for the femora that were used for torsional testing.

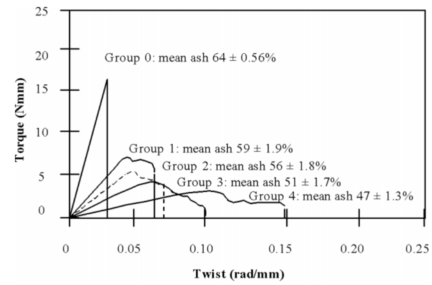

Fig. 2 Composite torque versus twist curves for each decalcification group

tralateral femora were serially cross-sectioned to determine geometric properties, and effective material properties were then calculated from the geometric and structural properties of each femoral pair.

We found that the relationship between ash content and effective shear modulus (G_{eff}) or maximum effective shear stress (τ_{max}) could best be characterized through a power law, with an exponential factor of 6.79 for G_{eff} ($R^2=0.85$) and 4.04 for τ_{max} ($R^2=0.67$). This indicates that in a murine model, as expected, small changes in ash content significantly influence effective material properties. Furthermore, it appears that effective shear modulus is more heavily affected by changes in ash content than is maximum effective shear stress when these properties are derived from whole bone torsional tests to failure. If a murine mutation affecting ash content results in significant differences in one, but not both of these properties, it is likely that other compositional and/or microstructural characteristics have likely been affected as well.

Methods

Overview. Twenty-five eight-week-old male A/J strain mice were sacrificed using CO_2 inhalation in accordance with appropriate institutional guidelines. From each animal, the intact right and left femora and right tibiae were harvested and cleaned of all soft tissue. Right femora were decalcified varying amounts, then tested to failure in torsion to determine structural properties. Left femora were not decalcified and were serially sectioned to determine cross-sectional geometric properties. Geometric and structural properties from each femoral pair were then combined to determine effective material properties. To determine the exact extent of decalcification of each mechanically tested (right) femur, the right tibia was decalcified in an identical manner. The ash content of this tibia was then converted to an estimated ash content for the femur. (The relationship between tibial and femoral ash contents was predetermined in a preliminary study, using 12 matched tibial-femoral pairs subjected to a broad decalcification regimen. This preliminary study demonstrated a linear relationship between tibial and femoral ash content and decalcification rates [$R^2=0.99$; $femoral\,ash\%=([0.959\,x\,tibial\,ash\%]+0.163)$; Fig. 1]. This result was then used in the primary study to convert tibial ash content to femoral ash content.) In summary, bones from each animal were treated as follows: right femur—decalcification then torsional analysis; left femur—cross-sectioning and determination of geometric properties; right tibiae—decalcification, then determination of ash content.

Decalcification. Each mouse in our study was randomly assigned to one of five experimental groups (N=5 for each group) subject to a different extent of decalcification. Group 0 bones were not decalcified and served as controls. The right tibiae and femora from groups 1, 2, 3, and 4 were decalcified for 1, 2, 3, and 4 hours, respectively, to produce approximately 5%, 10%, 15%, and 20% ash reduction, respectively (estimates based on the preliminary study). A 0.5M EDTA solution buffered to pH=7.4 with sodium hydroxide was used for decalcification [26,29]. Bones were placed in approximately 15 ml (50–75 ml/g) EDTA on an agitator tray at 4°C for the appropriate length of time (1, 2, 3, or 4 hours), then rinsed in 1x phosphate-buffered saline (PBS) for 48 hours. The right tibiae and femora from animals in group 0, although not decalcified, were similarly rinsed in PBS for 48 hours. After treatment, all bones were wrapped in PBS-soaked gauze and stored in sealed plastic bags at −70°C for a period not exceeding six months. All bones were then thawed at room temperature at the appropriate time for testing.

Structural Properties. The decalcified right femur from each animal was used for mechanical testing. The proximal and distal bone ends were potted in polymethylmethacrylate (GC America Inc., Alsip, IL) and the gage length measured. Shaft axis was used for alignment during potting using a custom-made jig that clamps that mid-diaphysis in place. Throughout mounting and testing, bones were kept moist with PBS solution. Mechanical testing was performed with angular displacement control at a rate of 1 deg per second using an electromechanically driven materials testing system (Instron Model 55MT1, Instron Corp., Canton, MA) with a 0.225 N-m torque transducer (Instron Corp., Canton, MA). All bones were loaded with the distal femur rotated externally with respect to the proximal femur. Torque and angular displacement were recorded throughout testing at a rate of 20 Hz. The following structural parameters were calculated: (1) maximum torque (T_{max}); (2) twist to failure, equal to angle to failure-per-gage length (θ/L); (3) torsional rigidity (defined as the slope of the initial linear region of the torque versus twist curve); and (4) energy to failure. In the non-decalcified bones, failure torque and maximum torque were easy to determine and coincided. As ash content decreased, however, torque versus twist curves became irregular (Fig. 2). For these later specimens, failure torque was defined as either the first value at which an obvious drop-off in structural strength occurred, or the final torque after it decreased to 50% of the maximum torque recorded. Energy to failure was then defined as the area under the torque versus twist curve until failure torque was reached.

Cross-Sectional Geometry. After bone length measurement, the left femur (which had not been decalcified) was embedded in polyester resin (GC America Inc.). Serial 100 μm-thick cross-sections were obtained through the bone diaphysis using a circular diamond-blade saw (11-1180 Low Speed Saw, Buehler Ltd., Evanston, IL). Sections were stained with a von Kossa stain and digitally imaged using a transmission light microscope (Eclipse E600, Nikon USA, Inc., Melville, NY) and digital camera (DKC 5000, Sony Co., Japan). Several (2–3) sections were chosen for analysis for each femur, consistently located immediately distal to the third trochanter in the mid-diaphysis of the bone. Each image

Table 1 Geometric, structural and material parameters of A/J mouse femora [mean (SD)]

	Decalcification Group				
	Group 0	Group 1	Group 2	Group 3	Group 4
Number of specimens	4	5	5	5	5
Average ash content (%)	66.0 (0.58)	60.9 (2.0)	58.3 (1.8)	53.5 (1.8)	48.9 (1.4)
Geometric parameters					
Torsional constant (mm^4)	0.168 (0.020)	0.168 (0.011)	0.164 (0.013)	0.151 (0.017)	0.161 (0.023)
Geometric strength index (mm^3)	0.209 (0.021)	0.218 (0.016)	0.213 (0.020)	0.196 (0.019)	0.218 (0.016)
Structural parameters					
Maximum torque (N-mm)	17.8 (2.8)	7.0 (2.0)	7.3 (1.4)	5.7 (1.7)	3.9 (0.4)
Twist to maximum torque (rad/mm)	0.033 (0.004)	0.064 (0.029)	0.079 (0.041)	0.283 (0.289)	0.239 (0.206)
Torsional rigidity (N-mm^2/rad)	581 (132)	285 (67)	211 (54)	140 (57)	61 (32)
Energy to failure (N-rad)	0.300 (0.05)	0.301 (0.13)	0.319 (0.12)	0.570 (0.48)	0.529 (0.33)
Effective material parameters					
Maximum effective shear stress (MPa)	86 (16)	32 (10)	34 (7)	29 (7)	18 (1)
Effective shear modulus (MPa)	3455 (695)	1692 (332)	1280 (291)	909 (290)	371 (141)

was then analyzed using the program VA-Twist [30] to determine relevant mid-diaphyseal geometric properties, including torsional constant (K) and geometric strength index. This software uses the digitized outlines of a cross-section to solve for torsional geometric properties. The geometric strength index is calculated as $(1/\tau_{norm})$, where τ_{norm} is the normalized geometric maximum shear stress and is dependent only on the geometry of the cross-section. The geometric strength index is analogous to the section modulus of a circular cross-section and is a geometric indicator of the overall strength of the cross-section.

Effective Material Properties. Assuming similar geometry for each femoral pair, we combined the geometric properties from the left femur with the structural data of the right femur to calculate two effective material properties: maximum effective shear stress (τ_{max}) and effective shear modulus (G_{eff}). For each pair, the geometric properties used were those from the section with the smallest geometric strength index (i.e., the presumed fracture site). Maximum effective shear stress was calculated as $T_{max}*\tau_{norm}$, where τ_{norm} was determined as previously described. Effective shear modulus (G) was calculated as torsional rigidity divided by the torsional constant (K) [30].

Ash Content. To determine the ash (mineral) content of the tibiae after decalcification, dry mass was obtained after defatting in acetone for 72 hours, air-drying for 24 hours, and drying for 6 hours at 60°C. The bone was then heated at 600°C for 18 hours, after which the ash mass was obtained. Ash content (%) was calculated as [(ash mass/dry mass)×100].

Data Analysis. A plot of ash content versus each structural property and effective material property was constructed. As expected, a power law formula ($y \propto a*x^b$), where y is the dependent variable, x is the ash content, and a and b are derived constants, provided the best fit for each relationship. The cutoff value for statistical significance of the coefficient of determination (R^2) for each individual relationship was chosen as $p<0.05$. Because the primary aim of this study was to quantify the relationship between ash fraction and structural and effective material properties, the only ANOVA performed was on the calculated geometric parameters to guarantee that each group was of comparable cross-sectional geometry. For these geometric comparisons, a one-factor ANOVA was performed with Group as the independent variable, and a cutoff value of $p<0.05$ was chosen for statistical significance. All statistical analyses were performed using StatView 5.0 (SAS Institute Inc., Cary, NC).

Results

One animal was eliminated from Group 0 after one femur was damaged during mechanical testing. All bones from the remaining 24 animals were fully tested and included in the analysis.

Geometric Properties. As expected, no significant difference in any of the examined geometric properties was found. All femora used had similar cross-sectional area, torsional constant, and geometric strength index (Table 1).

Structural Properties. The mean results of torsional tests to failure for each decalcification group are shown in Table 1. Coefficients of determination between ash content and the structural properties were all greater than 0.5, with the exception of ash content versus energy absorbed to failure (Fig. 3). Notably, coefficients of determination of 0.83 and 0.68 were observed between ash fraction versus torsional rigidity and maximum torque, respectively, with exponential constants of 7.03 and 4.05.

Effective Material Properties. Calculated material properties for each group are also shown in Table 1. The relationships for maximum effective shear stress and effective shear modulus fit a power curve with an exponential constant of b=4.04 ($R^2=0.67$; Fig. 4) and b=6.79 ($R^2=0.85$; Fig. 5), respectively.

Discussion

The objective of this study was to describe the relationship between ash content and the effective structural and material properties of murine femora derived from torsional tests to failure. We found that the relationships for torsional rigidity and maximum torque were best fit by a power curve, with respective exponential constants of 7.03 ($R^2=0.83$) and 4.05 ($R^2=0.68$). Similarly, the relationships for the examined effective material properties also were best fit by a power curve, with exponential constants of 4.04 ($R^2=0.67$) for maximum effective shear stress and 6.79 ($R^2=0.85$) for effective shear modulus.

It is well known that the mechanical properties of cortical bone are related to mineral fraction [6–9,13,15,31]. Many of these studies, however, involved bones from different species, thereby including bones of differing shapes and functionalities. There is significant heterogeneity in the structural properties of demineralized bone from different sites within the same animal and of bones from different species of animals [32]. A recent study used only chemically decalcified cat femora but tested the bones in bending tests [26], and, in fact, most studies of this relationship have evaluated the effects of mineralization on bending strength. These results are not necessarily applicable to the torsional tests used in ours and others' studies. Because transgenic murine models are increasingly used to examine bone mechanical properties, and because torsional tests are commonly used for assessing bone mechanical properties, a study investigating the relationship of ash content to torsionally derived murine bone mechanics is valuable for interpreting the results of various models.

In this study, we decalcified murine bones using EDTA—a chelator that ''captures'' calcium and effectively removes bone calcium and phosphate [31]. Compared with other decalcification

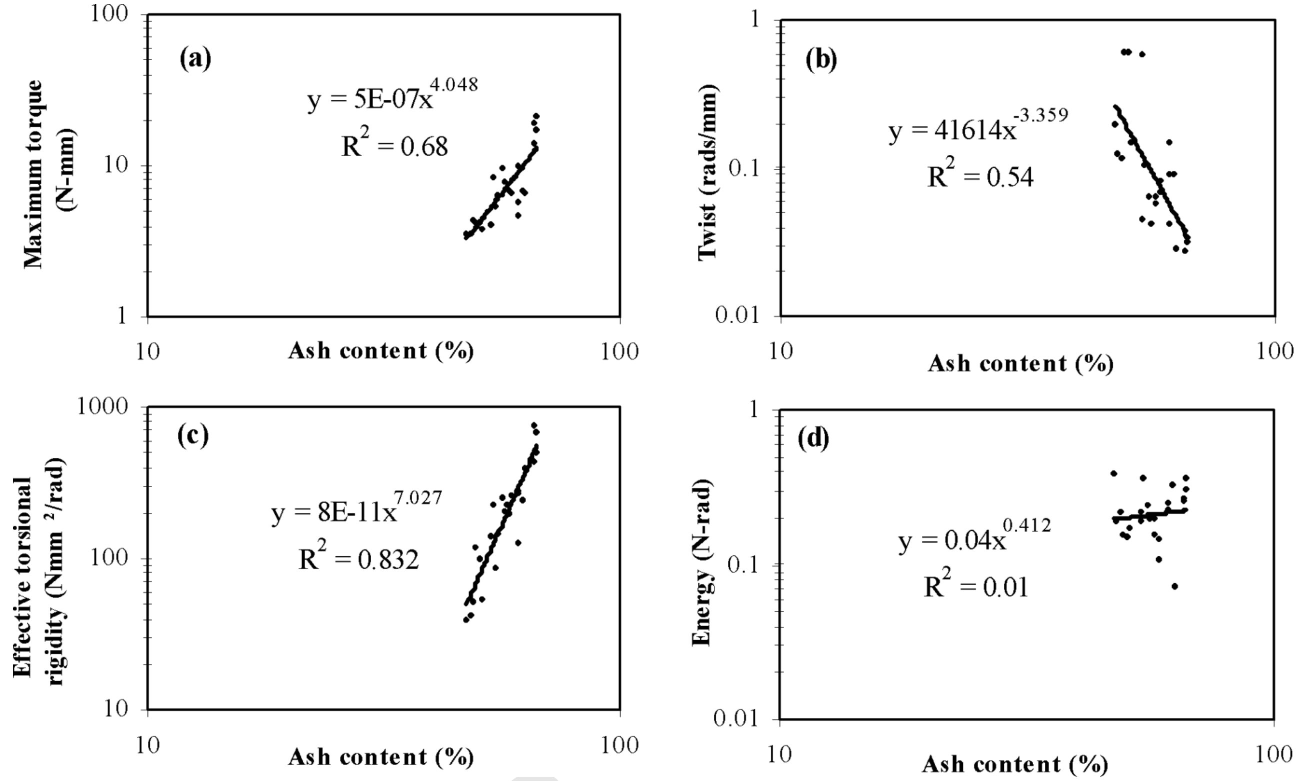

Fig. 3 Relationship of ash content to structural properties of mouse femora: (a) maximum torque; (b) twist to failure; (c) torsional rigidity; and (d) energy to failure

methods (e.g., hydrochloric or formic acids), there appears to be less tissue destruction with EDTA, thereby preserving mechanical properties of the collagenous phase [26,33–35]. In osteonal tissues, elastic modulus appears to be correlated with collagen fibril orientation as well as mineralization fraction [12]. Presumably, our method of decalcification affected only the desired changes in mineral content and did not disrupt collagen fiber architecture, as EDTA does not appear to disrupt organic matrices [26,34].

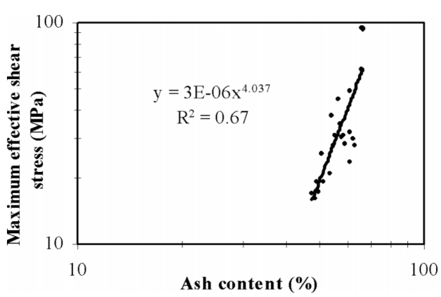

Fig. 4 Effect of ash content on maximum effective shear stress

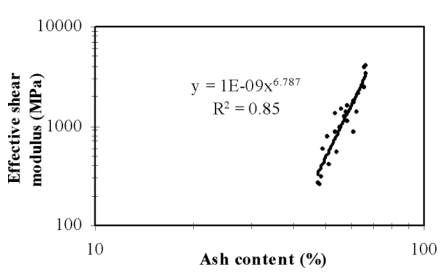

Fig. 5 Effect of ash content on effective shear modulus

Previous studies of EDTA decalcification also indicate that EDTA does not result in homogeneous decalcification throughout the cortex. Instead, EDTA treatment leaves a mineralized core of varying thickness (depending on immersion time) surrounded by a completely demineralized collagenous layer [31]. This heterogeneous mineral structure may result even when decalcifying samples as thin as 500 μm. Given the extremely small size of mouse femora (the average cortical thickness of sections used for analysis was slightly less than 200 μm), this effect may have been negligible. Still, removal of the bone ends from each specimen would have allowed demineralization to occur from both the periosteal and endosteal surfaces simultaneously. Given the small size of mouse femora, however, this would make subsequent mounting for torsional tests exceedingly difficult. It is also important to acknowledge that our decalcification procedure is a model of mineral loss whereas when such differences are observed in transgenic mice they can also be due to a failure to adequately gain mineral. These two processes may not be equivalent from the standpoint of mineral contributions to cortical bone material and structural behavior.

Early studies examining bone cross-sectional geometry often relied on the use of an ‘‘equivalent’’ hollow circular model, with equal polar moment and cortical area. With such a method, the derived values will differ somewhat from the directly measured true values. In this study, we used a program (VA-TWIST [30]) that calculates all geometric properties directly from the bone cross-section, thereby avoiding such approximations. Nevertheless, when calculating effective bone material properties with this model, one still uses only the geometric properties of the minimum diaphyseal cross-section and assumes no effect of lengthwise variation in geometry. This can potentially lead to significant underestimates of bone material properties [30]. However, in comparisons such as in this study involving bones of similar size and with relatively little variation in cross-sectional geometry along the length of the diaphysis, neglecting such variation may have little effect [36,37].

Despite these limitations, our results demonstrate the effect of small differences in ash fraction on the effective material properties of murine bone derived from whole bone torsional tests to failure. We chose to use a power law to describe our results, because if a linear relationship were used, murine bone would theoretically reach zero torsional strength at an ash content of approximately 45%. Our use of a power law is consistent with relationships obtained in previous studies [7,8,10,14]. We found strong correlations of ash content with all parameters except energy absorbed to failure. (Other investigators have also found that the relationship between ash content and energy to failure is less straightforward than the relationships between ash and the other biomechanical parameters examined here [7].) Specifically, in our study, a 5% decrease in ash content was associated with a 22% decrease in maximum torque, a 41% decrease in torsional rigidity, a 22% decrease in maximum effective shear stress, and a 39% decrease in effective shear modulus. These results are of a magnitude consistent with earlier studies examining bending strength, correlating a 2% decrease in ash content with up to a 20% decrease in elastic modulus and 20% decalcification with a 35% loss of bending strength [7,8,26]. More important, however, we demonstrate that in genetically altered mouse models in which significant differences in ash content are detected, both maximum effective shear stress and effective shear modulus should be altered, with larger effects expected for effective shear modulus. If significant differences are observed in one but not both of these properties, as in our examination of GDF-5 deficient femora [6], other compositional and/or microstructural characteristics are likely to have been affected as well.

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