Bone Strength, Toughness, and Crack Initiation Toughness Decline with Age in Female C57BL/6N Mice

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INTRODUCTION: The C57BL/6N mouse strain is widely used in aging research because aging colonies are maintained by the National Institute on Aging (NIA). With advanced aging, these mice lose trabecular bone [1] while bending strength of the femur mid-diaphysis decreases between 6- and 24-months of age [2]. Less clear is whether toughness and fracture toughness of cortical bone also decrease with age in this strain without a secondary disease [3]. To validate a mouse model of aging for treatment strategies that target the bone matrix to prevent age-related declines in the fracture resistance of bone (i.e., beyond bone mass and strength), we hypothesized that both post-yield displacement, a marker of brittleness, and fracture toughness of cortical bone are lower in aged female mice than in adult female mice because of age-related declines in bone matrix hydration (i.e., bound water).

METHODS: Fifteen, female, 5-month and 23-month-old C57BL/6N mice from the NIA-supported colony at Charles River were kept under standard housing and monitored for 1-month prior to euthanasia, when left and right femurs were harvested and stored fresh in phosphate buffered solution at -80 °C until analyzed. Using ^{1}H nuclear magnetic resonance relaxometry (4.7-T horizontal-bore magnet, Varian Medical Systems), we measured bound water volume per bone volume (V.BW/VB) of each femur [4]. The posterior surface of these right femurs was then micro-notched, and the notched region was scanned by micro-computed tomography (μCT) at an isotropic voxel size of 6 μm (Scanco Medical) to determine the notch angle and bone geometry for calculating crack initiation toughness ($K_{Ic,ult}$) based on the ultimate force and linear fracture mechanics [5]. This force was measured by loading the femur (posterior side in tension) to failure at 0.05 mm/min in three-point bending such that the span was ~4 times the anterior-posterior width (Instron Dynamight 8841). For each left femur, the mid-diaphysis was scanned and evaluated by μCT at a voxel size of 6 μm (Scanco Medical) to determine mineral density (mgHA/cm^3) and structural parameters like cortical thickness, cortical porosity (Ct.Po = 1-BV/TV), polar moment of inert ($J = (I_{min} + I_{max})/2$) and distance between the centroid and outer bone surface at the point of loading (c_{min}). Subsequently, these hydrated long bones were loaded to failure at 3 mm/min in three-point bending such that the span was ~5.5 times the anterior-posterior width. Defining the yield point by the 0.2% offset method in each moment (force x span / 4) vs. normalized displacement (12 x displacement / span^2) curve, we measured ultimate moment (M_u), span-adjusted post-yield displacement (PYD), and work-to-fracture (W_f) per cross-sectional bone area (Ct.Ar from μCT). Depending on checks of normality and homoscedasticity of the data by D'Agostino-Pearson test and the F test, respectively, significant differences in properties between age groups were determined based on p-values ($p<0.05$) from unpaired t tests, Welch's t test, or Mann-Whitney U test (GraphPad Prism 10). Analysis of covariance (ANCOVA) was used to identify a significant relationship ($p<0.05$) between ultimate moment and resistance to bending. The use of animals for this study followed an IACUC-approved protocol.

RESULTS: Volume fraction of the femur bound water (V.BW/VB) was lower in 24-month-old mice compared to 6-month-old mice (n=15/age group) (Table). The femur mid-diaphysis of the aged mice (n=15) had higher cortical tissue mineral density (Ct.TMD), lower cortical thickness (Ct.Th) and greater polar moment of inertia (J) than young mice (n=12), but the cortical porosity (Ct.Po) was not significantly different between the age groups (Table). The ultimate stress ($\sigma_S = M_u \times c_{min} / I_{min}$), post-yield displacement (PYD), and W_f/Ct.Ar, an estimate of toughness, experienced by the un-notched femur were lower in aged than young mice, while ultimate moment did not significantly differ between the age groups. For a given section modulus (I_{min}/c_{min} from μCT), the ultimate moment was lower with age (Fig). For the notched femur, crack initiation toughness significantly decreased with age (n=15/age group) (Table).

DISCUSSION: Postmenopausal women are ~4 times more likely to suffer a fragility fracture than men of the same age [6]. Ovariectomy of young adult mice is a common pre-clinical model to investigate treatment strategies, but it does not capture the age-related changes in the quality of the bone *matrix* that contribute to bone fragility. The present study found that the cortical bone of C57BL/6N female mice becomes more brittle, loses both toughness and crack initiation toughness with advanced aging. Thus, this common pre-clinical model of aging can be used to test therapeutic strategies that target the material properties of bone, not just bone mass and structural-dependent bone strength. One target could be bound water that declined with age (Table), which phenocopies what happens in humans [7]. Several limitations affect the interpretation of the findings. While male rodents have widely been used in studies of age-related changes in bone, the observed changes in fracture resistance of female cortical bone cannot necessarily be extended to males. Our previous study found though that the age-related declines (6-month vs. 20-month) in material properties like crack initiation toughness and bound water similarly occurred in male and female BALB/c mice [8]. The matrix-related factor(s) causing the decline in bound water remains to be elucidated. It could be due to the observed increase in mineralization of cortical bone (Ct.TMD in Table), but it could also be due to age-related declines in glycosaminoglycans or accumulation of non-enzymatic post-translational modifications, which we have not yet quantified. The effect of aging on the mechanical properties of cortical bone in C57BL/6N female is more than just a decline in strength; it involves a loss in the ability to resist post-yield crack formation.

REFERENCES: [1] Glatt et al. J Bone Miner Res. 2007, [2] Almedia et al. J Biol Chem. 2007, [3] Heveran et al. Bone. 2019, [4] Creecy et al. Bone. 2020, [5] Ritchie et al. Bone. 2010, [6] Eastell et al. Nat Rev Dis Primers. 2016, [7] Granke et al. J Bone Mineral Res. 2015, and [8] Creecy et al. Bone. 2020.

SIGNIFICANCE/CLINICAL RELEVANCE: The C57BL/6N mouse strain exhibits an age-related loss of bone *matrix* quality like that experienced by humans, validating its use in further understanding how to enhance bone toughness by targeting properties of the bone matrix.

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Table. Age-related differences in selected properties of C57BL/6N mouse cortical bone.

		6-month-old		24-month-old		6 vs. 24
Property	Units	Median	(1st, 3rd quartile)	Median	(1st, 3rd quartile)	p-value
V.BW/VB	%	7.96	(7.66, 8.53)	6.18	(5.81, 6.63)	<0.0001*
Ct.TMD	mg·HA/cm^3	1294	(1282, 1298)	1306	(1299, 1314)	0.0077*
Ct.Th	μm	0.172	(0.170, 0.183)	0.136	(0.131, 0.145)	<0.0001*
J	mm^4	0.290	(0.250, 0.322)	0.348	(0.331, 0.394)	0.0029*
Ct.Po	%	3.75	(3.57, 4.11)	4.11	(3.73, 4.43)	0.2284^
σ_S	MPa	203	(196, 220)	155	(140, 177)	<0.0001*
M_u	mm^4	32.57	(30.08, 33.44)	29.90	(25.02, 33.38)	0.1297#
PYD	1/mm	0.117	(0.091, 0.168)	0.037	(0.027, 0.041)	<0.0001^
W_f/Ct.Ar	MJ/m^3	5.10	(4.21, 6.68)	1.64	(1.21, 1.97)	<0.0001^
$K_{Ic,ult}$	MPa·m$^{0.5}$	4.36	(4.28, 4.94)	3.92	(3.61, 4.07)	0.0109^

*Unpaired t-test; ^Mann-Whitney U; #Welch's t-test

Figure. Regressions of ultimate moment vs. resistance to bending.