Ex vivo Exposure to Calcitonin or Raloxifene Improves Mechanical Properties of Diseased Bone through Non-cell Mediated Mechanisms.

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Abstract

Raloxifene (RAL) reduces clinical fracture risk despite modest effects on bone mass and density. This reduction in fracture risk may be due to improved material level-mechanical properties through a non-cell mediated increase in bone hydration. Synthetic salmon calcitonin (CAL) has also demonstrated efficacy in reducing fracture risk with only modest bone mass and density improvements. This study aimed to determine if CAL could modify healthy and diseased bone through cell-independent mechanisms that alter hydration similar to RAL.

26-week-old male C57BL/6 mice induced with chronic kidney disease (CKD) beginning at 16 wks of age via 0.2% adenine-laced casein-based (0.9% P, 0.6% C) chow, and their non-CKD control littermates (Con), were utilized. Upon sacrifice, right femora were randomly assigned to the following ex vivo experimental groups: RAL (2 μM, n=10 CKD, n=10 Con), CAL (100 nM, n=10 CKD, n=10 Con), or Vehicle (VEH; n=9 CKD, n=9 Con). Bones were incubated in PBS + drug solution at 37°C for 14 days using an established ex vivo soaking methodology. Cortical geometry (μCT) was used to confirm a CKD bone phenotype, including porosity and cortical thinning, at sacrifice. Femora were assessed for mechanical properties (3-point bending) and bone hydration (via solid state nuclear magnetic resonance spectroscopy with magic angle spinning (ssNMR). Data were analyzed by two-tailed t-tests (μCT) or 2-way ANOVA for main effects of disease, treatment, and their interaction. Tukey's post hoc analyses followed a significant main effect of treatment to determine the source of the effect.

Imaging confirmed a cortical phenotype reflective of CKD, including lower cortical thickness ($p<0.0001$) and increased cortical porosity ($p=0.02$) compared to Con. In addition, CKD resulted in weaker, less deformable bones. In CKD bones, ex vivo exposure to RAL or CAL improved total work (+120% and +107%, respectively; $p<0.05$), post-yield work (+143% and +133%), total displacement (+197% and +229%), total strain (+225% and +243%), and toughness (+158% and +119%) vs. CKD VEH soaked bones. Ex vivo exposure to RAL or CAL did not impact any mechanical properties in Con bone. Matrix-bound water by ssNMR showed CAL treated bones had significantly higher bound water compared to VEH treated bones in both CKD and Con cohorts ($p=0.001$ and $p=0.01$, respectively). RAL positively modulated bound water in CKD bone compared to VEH ($p=0.002$) but not in Con bone. There were no significant differences between bones soaked with CAL vs. RAL for any outcomes measured.

RAL and CAL improve important post-yield properties and toughness in a non-cell mediated manner in CKD bone but not in Con bones. While RAL treated CKD bones had higher matrix-bound water content in line with previous reports, both Con and CKD bones exposed to CAL had higher matrix-bound water. Therapeutic modulation of water, specifically the bound water fraction, represents a novel approach to improving mechanical properties and potentially reducing fracture risk.

Keywords: Bone hydration, material properties, salmon calcitonin, Raloxifene, nuclear magnetic resonance

1. Introduction

Susceptibility to fracture in chronic kidney disease (CKD) appears well before the need for dialysis and cannot be fully explained by changes in bone mineral mass and density [1]. There is a growing body of pre-clinical literature documenting that negative alterations in bone matrix water and collagen, both linked to bone brittleness, are present in CKD [2-4]. Bone water, which can be free, bound, or structural, constitutes approximately a quarter of cortical bone by volume and substantially influences mechanical properties and tissue quality. Bound water, which is associated with mineral and/or collagen, plays an essential role in transferring loads between the collagen and mineral interfaces, reducing shear stresses and increasing overall tissue toughness. When bound water is removed, bone becomes less tough, illustrating its critical role in governing post-yield mechanical behavior [5-8]. As a result, therapeutic modulation of bound water, or the non-collagenous proteins involved in attracting and retaining bound water, represents a novel and potentially desirable approach to improve mechanical properties.

Several therapeutics have been identified for their ability to reduce fracture risk with little-to-no change in bone mineral (based on changes to bone density), suggesting a different mechanism of action to improve mechanical integrity. Raloxifene, a selective estrogen receptor modulator (SERM), has demonstrated significant decreases in vertebral fracture risk (~50%) while only modestly increasing bone mineral density (BMD) and suppressing bone remodeling [9-11]. Data suggests an additional mechanism beyond what can be captured using dual x-ray absorptiometry to measure BMD or by dynamic histomorphometry and serum markers of bone turnover. Our group has shown that raloxifene directly interacts with the bone matrix in a cell- and estrogen independent manner [12] to improve bone material properties including increased matrix bound water [13]. Pre-clinically, treatment with raloxifene can improve bone hydration [14], and in the presence of disease, can increase mechanical properties and decrease fracture [15]. When administered to rats with CKD, raloxifene beneficially impacted several skeletal consequences of disease [16], although bone water was not measured.

Salmon calcitonin, an FDA-approved analog of the 32 amino acid polypeptide hormone secreted by thyroid C-cells, has demonstrated efficacy in mildly inhibiting bone resorption and reducing recurrence of fracture in established osteoporosis in several studies [17, 18], but not others [19]. However, fracture reduction in patients taking calcitonin is observed with only modest improvements in bone mineral mass and density [17]. Further, early preclinical work with calcitonin showed higher bone toughness via three point bending test to failure in calcitonin treated animals versus controls [20]. These data are intriguing and may suggest calcitonin could modify the matrix in a manner similar to Raloxifene, but no data exist evaluating its impacts on the bone matrix or bone hydration following treatment. The goal of the current work was to test the hypotheses that both calcitonin and raloxifene improve CKD bone tissue through cell-independent mechanisms that modify hydration and positively impact estimated material-level mechanical properties in bone tissue exposed to either compound.

2. Materials and Methods

2.1. Study Design

All experiments were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee and the Indiana University Purdue University School of Science Institutional Animal Care and Use Committee prior to their initiation. This study utilized a total n=58 right femora from 26-week-old male C57BL/6J control (Con) mice (n=29) and mice induced with CKD (n=29). Fifteen-week-old male C57BL/6J mice (JAX #000664; n=29 per group) were ordered from Jackson Laboratories (Bar Harbor, ME, USA) and allowed to acclimate to the facility for one week. At 16-weeks of age, all mice were switched to a purified casein-based diet with adjusted calcium and phosphorous (0.9% P, 0.6% C) and were randomly assigned to a Con or CKD group. Animals in the CKD group were fed the same diet with the addition of 0.2% adenine (Envigo Teklad Diets) for 6 weeks to initiate disease before being switched back to the control casein-based diet for four weeks of maintenance as previously described [21, 22]. At 26 weeks of age, mice were anesthetized via vaporized isoflurane and euthanized via exsanguination. Right femora were resected, cleaned of soft tissue, wrapped in phosphate-buffered saline (PBS)-soaked gauze, and stored at -20°C.

Bones were thawed to room temperature while remaining wrapped in PBS-soaked gauze and prepared by removing proximal and distal ends and flushing marrow leaving only the shaft (average length (mm): Con = 11.10 ± 0.85, CKD = 10.22 ± 0.74).

Prepared femora were randomly assigned to the following treatment groups:

1. Calcitonin (CAL): 100 nM concentration (05-23-2401, Sigma-Aldrich), n=10 femora per group (Con, CKD)
2. Raloxifiene (RAL): 2 µM concentration (R1402, Sigma-Aldrich), n=10 femora per group
3. Vehicle (VEH): equimolar dimethyl sulfoxide (DMSO), 0.04% vol/vol, (J66650.AE, ThermoFisher), n=9 femora per group

The CAL dose was chosen based on previous unpublished data that demonstrated a positive increase in bone toughness, following ex vivo soaking, via monotonic mechanical testing. The RAL dose was chosen based on previous published work by our group that demonstrates effectiveness in improving mechanical properties [13]. Femora were incubated in PBS (1X, 0.22 µm filtered) supplemented with 1% penicillin-streptomycin (15140122, Gibco) and RAL, CAL, or DMSO at the respective concentration using an established *ex vivo* soaking methodology [13]. Treating the bones by exposing the non-viable tissue to RAL or CAL ex vivo will allow us to elucidate whether the compound possesses the ability to improve matrix properties in a non-bone cell and non-hormone mediated manner. Bones were placed one bone per well in a 24 well plate with 1 mL of solution per well. All incubations were performed in a 37°C humidified incubator for two weeks with full solution changes every other day. On the last day, femora were removed, wrapped in PBS-soaked gauze, and stored at -20°C until further testing.

2.2. Micro-Computed Tomography (µCT)

A subset of femora (CKD: n=4 VEH, n=5 CAL, n=5 RAL; Con: n=5 VEH, n=5 CAL, n=5 RAL) were scanned after the 14-day soaking period. Scans used a nominal isotropic voxel size of 7.9 µm through a 0.5 mm aluminum filter (V= 59 kV, *I* = 167 µA) with a 0.7-degree angle increment and two frames averaged (SkyScan 1172, Bruker). Two manufacturer-supplied cylindrical hydroxyapatite phantoms (0.25 and 0.75

g/cm^3 Ca-HA) were scanned daily using the same parameters as quality control. Scans were reconstructed (NRecon, Bruker), rotated (DataViewer, Bruker), and calibrated (CTAn, Bruker) to the hydroxyapatite-mimicking phantoms prior to analysis. Cortical geometry and microstructure parameters were measured at 50% of the femoral shaft length (following removal of the proximal and distal ends) using a 0.1 mm region of interest (ROI). Cortical bone was automatically segmented and analyzed for cortical thickness (Ct.Th), cortical area (Ct.Ar), marrow area (Ma.Ar), bone area fraction (BA/TA), and tissue mineral density (TMD) using a custom MATLAB program. Cortical porosity was calculated as the void area between the periosteal and endosteal surfaces and presented as a percentage of the overall cortical volume.

2.3. Three-point (3-pt) Bending Monotonic Mechanical Test to Failure

Femora that underwent µCT were next subjected to 3-pt bend using a TA Instruments ElectroForce 3200 system equipped with a 45 N load cell. While fully hydrated with PBS, each bone was placed on a 6 mm support span with the mid-diaphysis directly below the loading point. With the anterior surface in tension, a preload of 0.5N was applied, then each bone was tested to failure with a displacement control rate of 0.025 mm/s. Cross-sectional cortical properties were obtained from 10 micro-CT slices nearest the failure point to calculate stress-strain data from load-displacement data using a custom MATLAB script [23]. Structural-level and estimated material-level properties are reported following standard nomenclature [24].

2.4. Solid State Nuclear Magnetic Resonance (ssNMR) spectroscopy

Solid state nuclear magnetic resonance (ssNMR) spectroscopy was performed on the remaining femora that had not undergone 3-pt bending to quantify free and bound water volume fractions. Femora were prepared by finely cutting into solid cortical fragments (< 1 mm^3) and loaded into a 3.2 mm zirconium rotor. Any void space was covered with Teflon tape for stable spinning. All ssNMR spectra were recorded on a 400 MHz NMR spectrometer (Avance HD, Bruker Biospin, Switzerland) with a Bruker 3.2mm DVT probe. The magic angle spin (MAS) frequency was 10.0 kHz for all experiments. The MAS speed was controlled using Bruker's MAS pneumatic unit with an accuracy of ± 2 Hz. For total water, a 1D one pulse ^{1}H NMR was recorded with 1k data points for a total acquisition time of 12 ms [25]. The ratio of total water with respect to OH was calculated from the ^{1}H spectra by integrating the water peak (5.05 – 5.4 ppm) with respect to the OH resonance (1.4 ppm) according to peak assignments as previously described [26]. The OH resonance was chosen as our internal reference because previous studies have demonstrated that the 1.4 ppm peak shows little variance following dehydration of intact bone [27]. Bound water was determined using a 2D ^{1}H-^{31}P Heteronuclear Correlation (HetCor) experiments [25, 28]. HetCor is based on ^{1}H-^{31}P dipolar coupling and selectively excites ^{1}H signal associated with water and organic component of bone [18]. For ^{1}H–^{31}P HetCor experiments, the contact time was 1.0 ms and the maximum t_1 evolution time was 2.6 ms. The effective field during ^{1}H homonuclear decoupling period Phase Modulated Lee – Goldburg (PMLG) was 110 kHz and high power ^{1}H decoupling (100 kHz) was applied during t_2 period. A total of 32 transients per increment and recycle delay of 4 seconds were utilized. The spectra were zero filled, and sine bell apodization was used in both dimensions prior to Fourier transformation. To determine the relative OH content, rectangular method of integration was used in each HetCor experiment centered at the ^{1}H chemical shift at 0.4 ppm (OH) and 4.8 ppm (bound water) respectively. All spectra were processed and analyzed using Bruker Topspin (V. 4.1.1., Bruker).

2.5. Statistical Analysis

Cortical geometry measures were analyzed using a two-tailed Student t test. Mechanical properties and ssNMR outcomes were evaluated via a non-repeated measures 2x3 factorial ANOVA for main effects of disease (control, CKD), treatment (VEH, CAL, RAL), and their interaction (disease x treatment). When a significant main effect of treatment was observed in the absence of an interaction effect, a Tukey post-hoc test was used to examine differences between treatments. When the interaction term was significant, simple main effects were investigated using an appropriate model. All data are represented as mean ± SD and all analyses were performed in GraphPad Prism (v.9) with a statistical significance level of $\alpha = 0.05$.

3. Results

3.1. Micro-Computed Tomography

Micro-computed tomography analysis confirmed a CKD cortical phenotype at the time of soaking treatment (**Table 1**). Cortical bone area was significantly lower ($p<0.0001$) and marrow area significantly larger ($p<0.001$) in CKD bones compared to Con, resulting in decreased cortical thickness and lower BA/TA ($p<0.0001$). CKD bones also had lower BMD ($p<0.001$) and higher cortical porosity ($p=0.012$) compared to Con femora. Representative cortical cross sections from the 50% ROI demonstrate the increased porosity due to CKD (**Fig. 1A**) and average geometry profiles of the cortical bone highlight the CKD-driven geometric changes (**Fig. 1B**).

Table 1. Micro-computed tomography (µCT) outcomes.

	Control (Con)	Chronic Kidney Disease (CKD)	Unpaired t-test P-Values
Cortical µCT outcomes			
Total area, T.Ar (mm^2)	2.33 ± 0.17	2.23 ± 0.16	0.949
Marrow area, Ma.Ar (mm^2)	1.25 ± 0.14	1.47 ± 0.17	**<0.001**
Cortical area, Ct.Ar (mm^2)	0.98 ± 0.07	0.75 ± 0.04	**<0.0001**
Bone area fraction, BA/TA (%)	44.09 ± 2.85	34.03 ± 3.33	**<0.0001**
Cortical thickness, Ct.Th (mm)	0.22 ± 0.01	0.16 ± 0.01	**<0.0001**
Bone mineral density, BMD (g/cm^3)	1.26 ± 0.03	1.20 ± 0.04	**<0.001**
Cortical porosity, Ct.Po (%)	0.96 ± 0.66	2.71 ± 2.56	**0.012**

Values are presented as mean ± SD. Bolded p-values indicate a significant difference between Con vs. CKD via unpaired two-tailed t-tests ($p<0.05$).

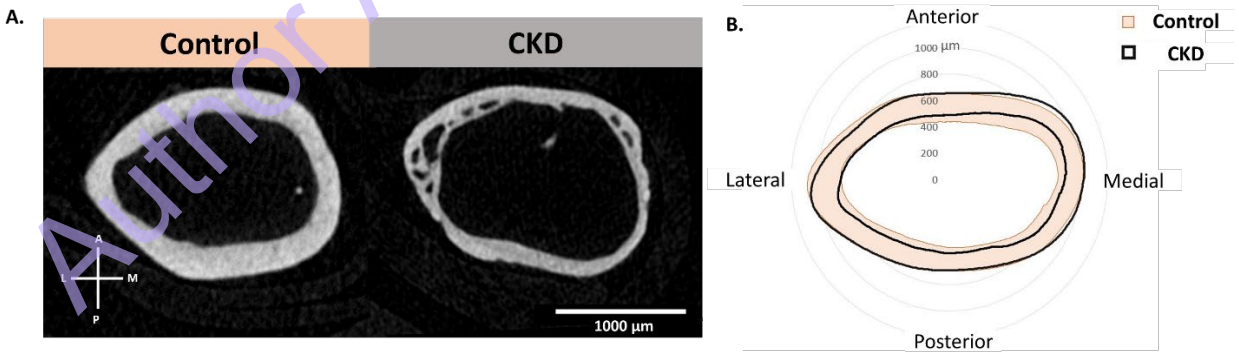

Figure 1. Micro-computed tomography of Control and CKD femora. A) Representative µCT images of cortical sections from C57BL/6 control (left) and CKD (right) femora used for cortical geometry and microstructural analysis. CKD femora had significantly higher cortical porosity compared to controls ($p=0.0166$). B) Average profiles of the femoral cortical ROIs from all bones in the control (orange) and CKD

(black) group, demonstrating that cortical bone area and thickness are reduced in C57BL/6 mice induced with CKD.

3.2. Three-point Bending

Compared to Con, animals with CKD had weaker, less deformable bones with a significant main effect of disease observed for ultimate force ($p < 0.0001$), yield force ($p < 0.0001$), work to yield ($p < 0.0001$), stiffness ($p < 0.0001$), resilience ($p < 0.0001$), yield stress ($p < 0.0001$), ultimate stress ($p < 0.0001$), and modulus ($p<0.001$). Interestingly, bones soaked in CAL or RAL were tougher with enhanced post-yield properties; these effects were more pronounced in CKD bone (**Figure 2A-H**). There were main effects of disease and treatment in post-yield work ($p<0.0001$, $p<0.001$), total work ($p<0.0001$, $p=0.001$), and toughness ($p<0.001$, $p<0.001$). Treatment (either CAL or RAL) was a significant factor for changes in total displacement ($p<0.001$), post-yield displacement ($p<0.001$), and total strain ($p<0.001$).

CAL and RAL soaking significantly improved post-yield displacement (+260% and +307%, respectively, **Fig 2A**), total displacement (+197% and +229%. **Fig 2B**), post-yield work (+143% and +133%, **Fig 2C**), total work (+120% and +107%, **Fig 2D**), total strain (+225% and +243%, **Fig 2E**), and toughness (+158% and +119%, **Fig 2F**) vs. VEH in CKD bones (p-values from follow-up post-hoc testing can be found on plots). CAL vs. RAL was not significantly different for any outcome. Further, post hoc testing following a significant main effect of treatment showed that there was no significant effect of either agent (CAL or RAL) in the Con bones. Detailed results from 3-pt bending test, including 2-way ANOVA analysis and follow-up post-hoc testing, for all structural mechanical properties and estimated tissue level properties can be found in **Supplementary Table 1**.

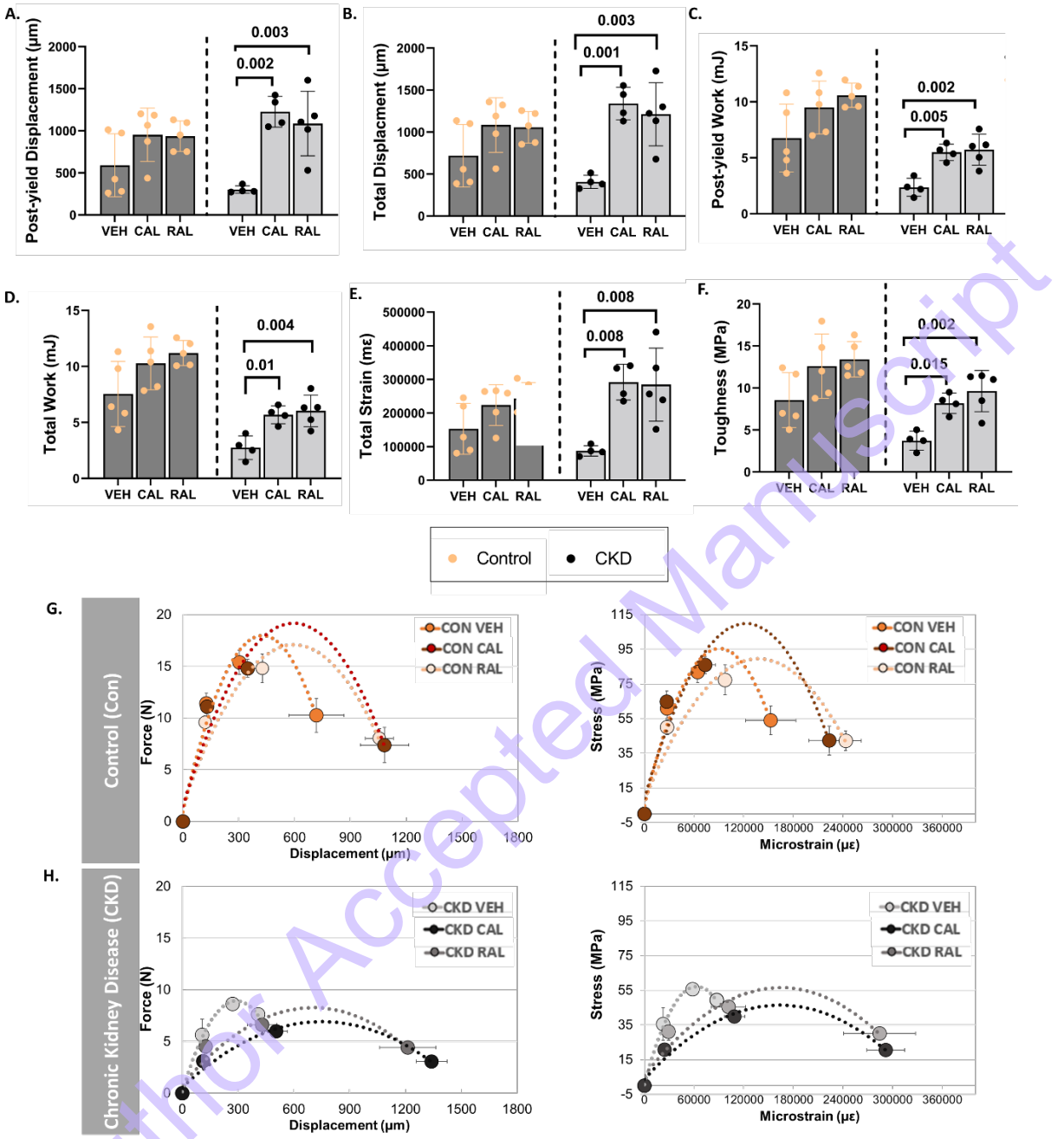

Figure 2. Three-point bending test to failure. CAL and RAL administered via *ex vivo* soaking significantly improved post-yield displacement (A), total displacement (B), post-yield work (C), total work (D), total strain (E), and toughness (F) compared to VEH in the CKD bones only. No significant impact of either agent was observed in the Con bones. G) CKD bones soaked with CAL had significantly lower yield force compared to VEH. No change was observed due to RAL in the CKD bones or for either CAL or RAL in the Con bones. P-values indicate a significant difference between groups (within disease) when Tukey post-hoc testing was conducted following a significant main effect of 'treatment' from the two-way ANOVA. Average force-displacement plot and stress-strain plot from three-point bend testing are depicted in H-I. Average force-displacement plots show CKD bones are weaker compared to Con bones regardless of

treatment. CKD bones treated with CAL or RAL increased post-yield displacement compared to CKD VEH treated bones. Stress-strain plots demonstrate that bones treated with CAL or RAL increase toughness and total strain with effects more pronounced in treated CKD bone. Data points in H) and I) are mean values ± standard error of the mean and lines were created using a second order polynomial function.

3.3. Solid State Nuclear Magnetic Resonance (ssNMR) spectroscopy

All femora exhibited six distinct peaks in the ^{1}H spectra which included water (5.05 ppm), hydroxide (OH-, 1.4 ppm), bone lipid protons (at 1.05 ppm and 1.5 ppm [29]), water occupying isolated OH- vacancies (2.2 ppm), and water molecules within the hydroxide ion channels (2.3 ppm) [29]. These observations are consistent with previous reports evaluating intact bone by ssNMR [26, 27]. The water spectra at 5.05 ppm and OH- at 1.4 ppm were the most distinct and used for analysis.

^{1}H spectra from the Con VEH group contained a sharp water peak at 5.05 ppm while the intensity of this peak was appreciably smaller for CKD VEH group. For the CKD group in particular, the water peak was noticeably altered in bones soaked with CAL and RAL (**Figure 3A**). Specifically, CKD CAL and RAL-soaked bones had ^{1}H spectra with a broader peak around 5.05 ppm, and in some cases a shoulder on the peak, suggesting additional water being brought into the cortical bone. Following integration of the water peak (5.05 – 5.4 ppm) to OH- (1.4 ppm), there was a significant main effect of treatment (p=0.003) and disease (p=0.001) but no significant interaction term (p=0.308) **(Figure 3B).** CKD VEH bones had lower total water relative to Con VEH. When CKD femora were soaked with CAL or RAL, total water relative to OH- was higher than VEH treated CKD femora. Within the CKD cohort, CAL-soaked bones had significantly higher total water vs. VEH (p=0.01). While Con femora treated with CAL or RAL had higher total water compared to Con VEH, this difference was not significant **(Figure 3B)**.

2D ^{1}H-^{31}P HeTCor was used to determine bound water because the experiment can selectively excite ^{1}H signal associated with water and organic component of bone and provide spatial arrangements to nearby inorganic bone minerals. Because of this relationship, the ^{1}H-^{31}P HeTCor bound water peak intensity depends on the coupling with various ^{1}H resonances present in bone. HeTCor spectra showed two well-resolved peaks at 4.8 ppm (bound water) and 0.4 ppm (OH). This ratio thus serves to determine the amount of OH present nearby the inorganic surface [18, 26]. Although HetCor's cross-peak intensity is not quantitative in nature, primarily because of variations in cross-polarization transfer efficiency, it is still possible to determine the amount of bound water. This was conducted using a rectangular method of integration centered at the ^{1}H chemical shift at 0.4 ppm (OH) and 4.8 ppm (bound water) respectively.

There was a significant main effect of treatment (p<0.0001) and disease (p<0.001) on bound water intensity relative to OH- content (**Figure 3C).** As anticipated, CKD VEH had lower bound water vs. Con VEH. The treatment response in CKD was especially robust; CKD CAL and CKD RAL each had higher bound water than CKD VEH (a 2.12-fold and 1.99-fold increase over VEH for CAL and RAL, respectively). Post hoc analysis used to determine the source of the treatment effect within groups showed that in CKD, both RAL (p=0.002) and CAL (p=0.001) were significantly higher than VEH but not different from each other. While treated Con bones also increased bound water, the increase over VEH was less robust than in CKD (1.11 fold and 1.32 fold increase over VEH for CAL and RAL, respectively). Post hoc analysis showed that only Con bones soaked with CAL had significantly higher bound water content vs. VEH (p=0.02). A

representative 2D plot from the 2D ^{1}H-^{31}P HeTCor for CKD VEH, CKD CAL, and CKD RAL can be seen in **Figure 3D-F**.

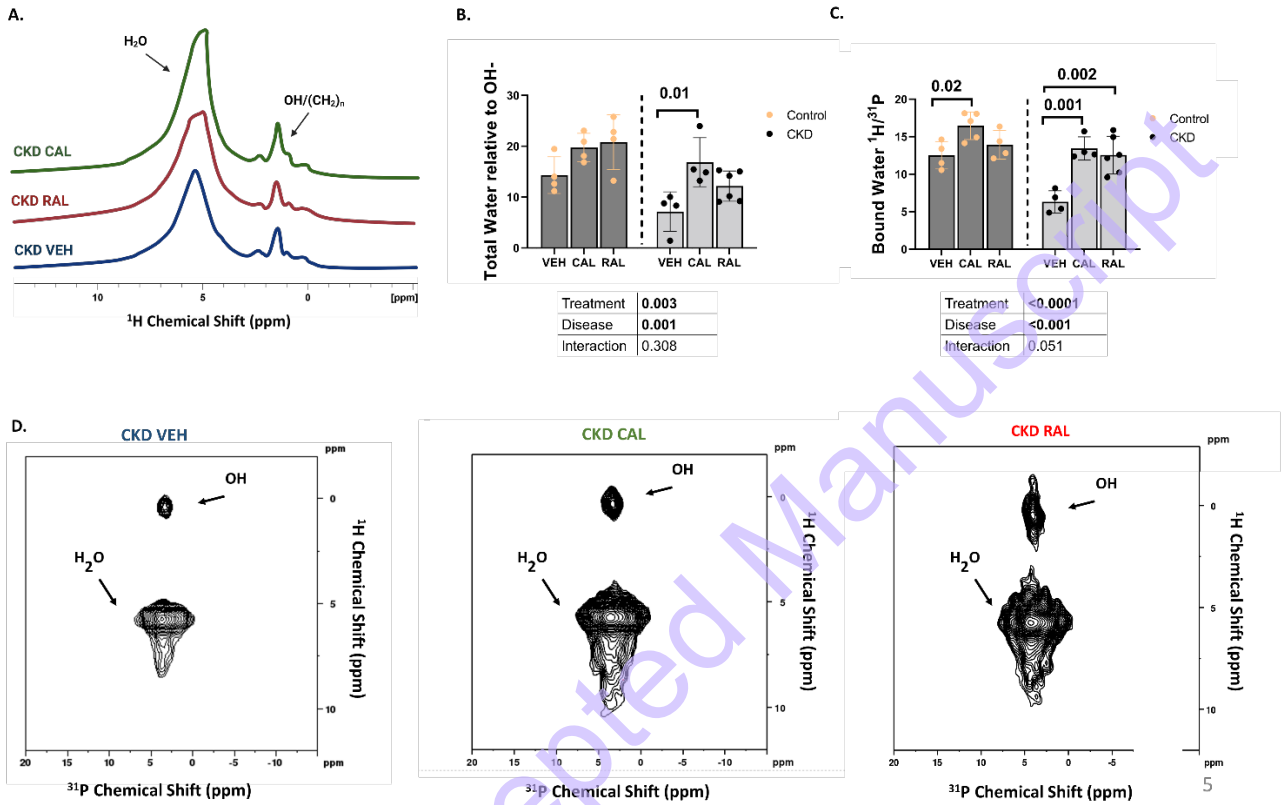

Figure 3. ssNMR results. A) Representative ^{1}H chemical shift spectra (ppm) from CKD femora treated with calcitonin (CAL, green), Raloxifene (RAL, red), or vehicle (VEH, blue). Femora treated with CAL and RAL demonstrate higher total water content with broader water peaks in the cortical bone following treatment. Peaks associated with water (H_2O), inorganic OH- are denoted. B) Two-way ANOVA revealed a significant effect of disease and treatment for total water quantified relative to OH- using ^{1}H ssNMR. CKD bones had lower total water vs. Control. Post-hoc analysis showed only CAL treated CKD bones had significantly higher total water vs. VEH. C) Bound water content relative to inorganic OH- variations quantified from the 2D ^{1}H-^{31}P Heteronuclear correlation experiment (HeTCoR) demonstrated significant main effects for treatment and disease and an interaction term of p=0.051. The figure shows CKD VEH bones have lower bound water vs. control VEH. Post-hoc analysis of treatment effects within groups demonstrated that both CAL and RAL significantly increased bound water above VEH levels in CKD. In Con bone, only CAL significantly increased bound water above VEH levels. Results from the Two-way ANOVA are found below the figure and bolded when significance was reached. P-values indicate a significant difference between groups (within disease) when Tukey post-hoc testing was conducted following a significant main effect of 'treatment' from the two-way ANOVA. 2D ^{1}H-^{31}P spectra from one CKD VEH treated bone (D), a CKD CAL treated bone (E), and a CKD RAL treated bone (F).

4. Discussion

This study demonstrates that *ex vivo* exposure to calcitonin or raloxifene elicits non-cell-mediated improvements to tissue hydration and bone material-level mechanical properties. These effects were more robust in diseased bone tissue which is in line with previous reports which suggest that it may be difficult to make substantial improvements to otherwise 'good bone' [30, 31]. Three-point bending to failure showed that soaking in either calcitonin or raloxifene improved toughness and several important post-yield properties in CKD but not in Con bone. Importantly, both calcitonin and raloxifene increased matrix bound water in a non-cellular and non-hormone mediated manner. While raloxifene only significantly modulated matrix-bound water in the CKD cohort, calcitonin significantly improved matrix-bound water in both the Con and CKD bone. The mechanism by which calcitonin improves matrix-bound water following *ex vivo* exposure is unknown and should be elucidated. We have previously described how raloxifene interacts with bone collagen and the collagen/mineral interface, yet it does not impact the mineral alone, through its basic sidechain [12]. Therapeutic modulation of water represents a shift in current treatment paradigms aiming to reduce fracture risk by increasing bone formation or reducing the rate of bone loss. Raloxifene's ability to modulate bone hydration has gained recent interest, and this is the first study to demonstrate the feasibility of enhancing bone water through calcitonin.

Calcitonin is a 32-amino acid peptide released from the C-cells of the thyroid gland [32]. Endogenous calcitonin plays an essential role in bone remodeling [32, 33] and, when released during periods of hypercalcemia, it served to lower the serum calcium [34] even during renal failure [35], and can stimulate renal production of 1,25-dihydroxyvitamin D [36]. As a result, synthesized salmon calcitonin, an FDA-approved analog of the endogenous calcitonin polypeptide, gained interest as a treatment of bone disorders by inhibiting osteoclast resorption [37]. While the mechanism of endogenous calcitonin is understood [38], the mechanism of exogenous calcitonin treatment is less clear. Like the clinical experience with RAL in post-menopausal osteoporosis, calcitonin has been shown to reduce fracture risk in post-menopausal osteoporosis with only modest improvements in bone mineral mass and density [17, 39]. Perhaps because of the minimal impact on conventional measures of bone drug efficacy (BMD via DXA, serum marker of bone turnover), clinical calcitonin treatment has been neglected compared to other drugs such as bisphosphonates, which do inhibit bone resorption but with greater impacts on BMD. Despite this lack of attention, there has been a reemergence of interest in calcitonin treatment [40] in part for its analgesic effects and mild side effects making it particularly attractive to treat bone when pain management is also a concern (i.e. vertebral compression fracture) [41]. Finally, calcitonin's ability to positively modify non-diseased tissue as observed in this study is intriguing and may be valuable in various musculoskeletal settings.

Data evaluating calcitonin's impacts on factors that govern bone quality are limited. A 1992 study showed that six weeks of daily salmon calcitonin treatment in control rabbits and rabbits who had undergone an osteotomy resulted in significantly higher toughness in the ulna (via three-point bending) compared to untreated cohorts [20]. In our study, murine CKD bone exposed to calcitonin *ex vivo* for 14 days had significantly higher toughness compared to the untreated CKD cohort. Control bone tissue treated with calcitonin also had higher toughness vs. the untreated control bone tissue but like the previous study, this trend was not statistically significant. In a separate study, rats with moderate CKD via unilateral nephrectomy who were administered calcitonin had significantly reduced osteomalacia and PTH levels which were not different from control [42]. However, this study had no mechanical endpoints thus determining if similar improvements to estimated bone material properties was not possible.

Intriguingly, the Karachalios et al. study in rabbits noted a significant reduction in cortical porosity in the calcitonin treated cohort [20]. While we could not test the impact of calcitonin on cortical porosity in our present study, future work could utilize *in vivo* treatment to assess both material properties and cortical porosity especially in CKD. CKD is marked by a significant increase in pathological pores within the cortex which have clear negative impact on mechanical integrity [43-45]. Identification of therapeutics that can improve both composition (increase matrix-bound water) and microstructure (reduce porosity) is therefore highly attractive.

Clinical treatment with raloxifene, an FDA-approved selective estrogen receptor modulator, significantly decreases vertebral fracture risk (~50%), but with only modest changes in bone remodeling and minor increases in BMD. This clinical observation suggests bone quality changes beyond mineral are acting to improve mechanical properties [9-11]. Preclinical work by our group and others has shown that raloxifene treatment improves material-level (intrinsic) bone properties, especially toughness, and that these improvements are largely independent of bone mass and architecture [15, 46-48]. Ex vivo studies determined that raloxifene improves material-level properties through a non-bone cell and non-estrogen mediated mechanism by binding to collagen and the collagen/mineral interface and imbibing water [12, 13, 46]. Skeletally mature, non-viable beagle bone exposed to raloxifene *ex vivo* had improved intrinsic toughness and increased matrix-bound water measured with ultrashort echo time magnetic resonance imaging (UTE-MRI) compared to bone exposed to VEH treatment [14]. Like previous reports, bone exposed to RAL *ex vivo* had improved post-yield mechanical properties and bone toughness, but these mechanical improvements were only observed in diseased bone (CKD) and not controls. In this work, raloxifene treated CKD bones had higher matrix-bound water than CKD VEH, but this response was not observed in Con bone. This observation is in line with others who report that the response to raloxifene is much greater in diseased tissue compared to control bones [30, 31, 49-51]. These results suggest that raloxifene may not improve quality in otherwise healthy bone, but it can increase matrix hydration and improve mechanical and material-level properties in diseased tissue [30, 31, 49-51]. Further, while raloxifene reduced vertebral fracture risk in clinical trial, it should be noted that nearly 80% of all fractures are non-vertebral [52]; thus, future work must investigate the site-specific impacts of raloxifene on bone quality (including bone water), including evaluating the effects on trabecular and cortical bone.

We recognize the complexity of treating skeletal deterioration associated with CKD due to the need to consider bone turnover status, density, mineralization defects, bone quality changes, and microarchitectural changes while balancing the mechanism of action of bone therapeutics with a medically fragile patient. While clinical data from calcitonin and raloxifene has come mainly from experience in osteoporosis patients, some data exists utilizing raloxifene in patients with CKD. Clinical trial experience in patients who had osteoporosis and mild CKD who were treated with raloxifene demonstrated a lower rate of vertebral fracture and a small improvement in BMD ([53]. Another study using raloxifene in CKD patients on dialysis showed that raloxifene improved BMD in the spine and not the hip [54] and increased lumbar BMD and decreased serum calcium levels [55]. While there is concern over venous thromboembolisms associated with raloxifene usage [56], a post-hoc analysis of CKD patients treated with raloxifene showed that risk of thromboembolism was no worse than the general population [57]. Literature regarding experience with calcitonin in CKD is even more sparse than raloxifene. In chronic hemodialysis patients with secondary hyperparathyroidism who were given salmon calcitonin in addition to 1-alpha-(OH)-D3 had reduced bone resorption (via serum markers) and increased BMD compared to either treatment alone[58]. In a prospective randomized trial evaluating the prevention of postrenal

transplantation bone loss in adult males, calcitonin prevented bone loss (compared to controls) during the first 12 months after renal transplantation[59]. However, long-term use of synthetic calcitonin treatment can result plateauing of the positive skeletal effects due to the formation of neutralizing antibodies against exogenous calcitonin. In a non-CKD study of postmenopausal osteoporosis, after 15 months of synthetic salmon calcitonin, 10 of the 19 patients developed antibodies neutralizing calcitonin. Further, the work presented in this study suggests that both raloxifene and calcitonin possess mechanisms of actions that improve bone beyond the mineral (by increasing bone water), thus conventional turnover and BMD outcomes may be inappropriate to fully capture positive changes elicited by these types of therapeutics. Important work is being conducted to evaluate efficacy of a new class of magnetic resonance imaging (MRI) techniques to help us understand how bone water can change in vivo [60].

Ex vivo exposure to raloxifene resulted in a bone that could undergo greater deformation because of increased bound water [13]. In contrast to the results of this work and previous work exposing mature canine cortical bone to raloxifene [13], Eby et al. did not observe a significant difference in toughness, energy to fracture, or post-yield energy between non-viable cancellous fetal bovine bone tissue exposed to raloxifene vs. vehicle [61]. While there was a trend for an increase in toughness due to raloxifene, the lack of robust treatment response could be due to the immaturity of the bone tissue at the time of treatment and the bone type (cancellous vs. cortical) and should be evaluated further. While exposure in our study and that of Eby et al. was ex vivo and helped us elucidate these compounds' acellular impact, we are limited in that we did not present in vivo data. Therefore, in vivo administration of raloxifene (in this CKD model) and calcitonin is necessary to determine whether these compounds elicit a similar mechanical and compositional (increasing bound water) in vivo. Raloxifene, a class of SERM, is a mild anti-resorptive and thus would cause biological responses beyond the acellular mechanism we studied in this current work. Similarly, synthetic calcitonin also acts as a mild anti-resorptive when administered in vivo, so it is plausible that it, too, would undergo biological responses in addition to the acellular increase in bound water we observed. Thus in vivo studies are underway and will serve as the crucial next step to determining if in vivo exposure to raloxifene and calcitonin can elicit a similar response as ex vivo or if they cause some biological response or produce molecular changes that may impact the ability to increase hydration of the matrix in the non-cellular mechanism we observed.

There remain exciting questions that the field needs to answer regarding the mechanism by which bone's bound water is decreased during aging and disease or how it can be therapeutically targeted to increase. Perhaps more mineralized bone tissue has less 'space' for additional water molecules to bind and plausibly less collagen with triple-helical-associated-tightly-bound water, and the mineral itself may act to 'push' out water as the bone becomes more mineralized. This reasoning may help explain the results from in vivo experience with raloxifene where healthy animals tend to have a less robust response than animals with skeletal disease. The literature shows that the mineralization process displaces some amount of water from the osteoid [62], while some water remains as 'structural water', helping give rise to crystal shape and orientation during the mineralization processes [63]. The degree of mineralization in rodents increases throughout life, and Granke, Does, and Nyman has demonstrated that bound water decreases per bone matrix volume while tissue mineral density (via μCT) increases (ρ= -0.89, $p<0.0001$) [64]. However, humans have osteonal remodeling throughout life and still show a significant decrease in bound water during aging, suggesting that increased mineralization may be part of the mechanism for decreased bound water (or perhaps why otherwise healthy bone does not respond as robustly to raloxifene) but are likely not the only factor and this process need to be explored further.

There is a growing body of literature documenting impaired bone matrix properties in CKD including alterations in collagen cross-linking and hydration [2, 4]. These tissue -level alterations can have significant and independent effects on whole bone fracture resistance [65]. In the current study, our analysis of CKD bone hydration by ssNMR demonstrated a main effect of disease for both total and bound water measures. Upon inspection, it was evident that CKD bone had lower total water and bound water compared to controls. This observation is in line with previous work where CKD rats with high bone turnover had lower bound water compared to healthy controls [66]. CKD rats with low bone turnover had higher bound water than healthy controls highlighting that CKD patients may also have differential bone water based on turnover status. While we utilized ssNMR to quantify total and matrix bound water in intact bone specimens in the current study using a protocol described first by Rai et al. [25, 67], ssNMR analysis of patient bone water would require an iliac crest bone biopsy. Although feasible in the CKD population, iliac crest biopsy is less common in other conditions [68]. Future ssNMR studies should be used to evaluate the impact of calcitonin and raloxifene treatment in pre-clinical models and should be done with additional ssNMR sequences to evaluate ^{31}P relaxation and studies to evaluate the distance between collagen and inorganic surface and collagen hydration effects due to treatment.

We analyzed calcitonin and raloxifene in non-viable bone tissue as previously reported [13], where it was shown that cell viability is eliminated following one freeze-thaw cycle. Testing calcitonin in non-viable bone was essential to understand if calcitonin can improve bound water and mechanical properties in a bone-cell and hormone-independent manner. Future work must determine the impacts of hydration when calcitonin or raloxifene is administered to animals with CKD to determine if the *in vivo* impacts differ from our observations in the *ex vivo* experiment. While both calcitonin and raloxifene treatments are FDA-approved, neither treatment is a first-line intervention to treat bone and mineral disorder in CKD. Calcitonin has demonstrated 'calcium sparing' effects in renal tubules which may lead to a decrease in PTH in CKD, but its effects in moderate to severe CKD patients. Based on the results here and elsewhere, these compounds are worthy of study in any disease as they allow for the exploration of a paradigm-shifting way to enhance bone material properties through modulation of bone hydration. A limitation of this feasibility study is that sample sizes were small which likely contributed to the larger variability in mechanical outcomes in the control group. Evenso, a substantial mechanical response was observed in CKD bone exposed to RAL or CAL via ex vivo soaking. Similarly, we observed a significant main effect of treatment in ssNMR data evaluating total and bound water. Ex vivo treatment using the established treatment soaking method cannot recapitulate in vivo conditions; thus, future studies are needed to address whether the positive impact we observed is maintained when CAL or RAL is administered systemically to the CKD mice.

5. Conclusions

Ex vivo soaking with calcitonin or raloxifene improved post-yield properties and toughness in a non-cell mediated manner in bone from animals with chronic kidney disease. These effects were associated with positive changes in matrix-bound water content. Therapeutic modulation of water, specifically the bound water fraction, represents a novel approach to improving mechanical properties and potentially reducing fracture risk.

Declaration of Competing Interest

All authors declare that they have no competing interest related to the present study.

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Supplementary Table 1. Three-point bend testing outcomes.

	Control (Con)			Chronic Kidney Disease (CKD)			Two-Way ANOVA P-Values		
	VEH (n=5)	CAL (n=5)	RAL (n=5)	VEH (n=4)	CAL (n=4)	RAL (n=5)	Treatment	Disease	Interaction
Structural mechanical properties from three-point bending									
Yield Force (N)	11.4 ± 2.5	11.0 ± 2.2	9.6 ± 1.6	5.7 ± 3.5	3.1 ± 1.1	4.5 ± 1.8	0.286	**<0.0001**	0.343
Ultimate Force (N)	15.3 ± 2.9	14.7 ± 2.1	14.8 ± 3.5	8.6 ± 1.3	5.9 ± 0.5	6.6 ± 1.5	0.301	**<0.0001**	0.629
Displacement to Yield (µm)	126.6 ± 3.2	130.4 ± 17.9	119.7 ± 10	106.1 ± 44.7	112.8 ± 23.4	126.2 ± 12.3	0.783	0.206	0.33
Postyield Displacement (µm)	590.8 ± 372.8	953.2 ± 316.5	936.6 ± 181.3	301.5 ± 43.9	1227.8 ± 182.9	1086 ± 383.3	**<0.001**	0.682	0.112
Total Displacement (µm)	717.8 ± 371.8	1083.6 ± 326.1	1056.3 ± 187.6	407.6 ± 78.6 #,*	1340.5 ± 194.5 #	1212.2 ± 376 *	**<0.001**	0.756	0.107
Stiffness (N/mm)	97.4 ± 20.4	93.8 ± 22.6	88.3 ± 23.8	56.1 ± 17.7	29.9 ± 9.9	39.1 ± 16.9	0.233	**<0.0001**	0.48
Work to Yield (mJ)	0.8 ± 0.2	0.8 ± 0.2	0.6 ± 0.1	0.4 ± 0.3	0.2 ± 0.1	0.3 ± 0.1	0.288	**<0.0001**	0.188
Postyield Work (mJ)	6.8 ± 3	9.5 ± 2.4	10.5 ± 1.1	2.4 ± 0.8 #,*	5.5 ± 0.7 #	5.7 ± 1.4 *	**<0.001**	**<0.0001**	0.881
Total Work (mJ)	7.5 ± 2.9	10.3 ±2.4	11.1 ± 1.1	2.7 ± 1.1 #,*	5.7 ± 0.8 #	6 ± 1.4 *	**0.001**	**<0.0001**	0.942
Estimated tissue level mechanical properties from three-point bending									
Yield Stress (MPa)	60.8 ± 14.2	64.7 ± 15.2	50.0 ± 11.0	35.4 ± 21.4	20.6 ± 8	31.1 ± 12.6	0.504	**<0.0001**	0.166
Ultimate Stress (MPa)	81.7 ± 15.6	85.8 ± 11.9	77.2 ± 21.5	55.6 ± 4	39.9 ± 6.1	45.4 ± 9.8	0.475	**<0.0001**	0.295
Strain to Yield (µε)	27243.9 ± 2430.4	27080.87 ± 3053.56	27517.4 ± 2343.7	22716.7 ± 9302.5	24260.7 ± 4111.7	29241.2 ± 4366.3	0.257	0.297	0.33
Total Strain (µε)	152716.1 ± 75637.6	223412. 29 ± 60732.92	243234.8 ± 46951.3	87407.7 ± 15495 #,*	291599.2 ± 53254 #	284322.2 ± 108867.3 *	**<0.001**	0.579	0.116
Modulus (GPa)	2.4 ± 0.6	2.6 ± 0.7	2 ± 0.7	1.7 ± 0.5	0.9 ± 0.3	1.2 ± 0.7	0.315	**<0.001**	0.203
Resilience (MPa)	0.9 ± 0.2	0.9 ± 0.2	0.7 ± 0.1	0.5 ± 0.3	0.3 ± 0.1	0.5 ± 0.1	0.576	**<0.0001**	0.124
Toughness (MPa)	8.6 ± 3.3	12.5 ± 3.8	13.4 ± 2.1	3.7 ± 1.1 #,*	8.2 ± 1.2 #	9.6 ± 2.5 *	**<0.001**	**<0.001**	0.911

Values are presented as mean ± SD. Bolded p-values indicate a significant main effect from non-repeated measures two-way ANOVA ($p<0.05$). When there was a significant main effect of treatment, post-hoc Tukey tests examined pairwise comparisons between treatments within each disease group ('#' indicates a difference between VEH vs. CAL and '*' indicates a difference between VEH vs. RAL). VEH = Vehicle; RAL = Raloxifene, CAL = Calcitonin.