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RALOXIFENE NEUTRALIZES BONE BRITTLENESS INDUCED BY ANTI-REMODELING TREATMENT AND INCREASES FATIGUE LIFE THROUGH NON-CELL MEDIATED MECHANISMS

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Abstract

Pre-clinical data have shown that tissue level effects stemming from bisphosphonate-induced suppression of bone remodeling can result in bone that is stronger yet more brittle. Raloxifene has been shown to reduce bone brittleness through non-cellular mechanisms. The goal of this work was to test the hypothesis that raloxifene can reverse the bone brittleness resulting from bisphosphonate treatment. Dog and mouse bone from multiple bisphosphonate dosing experiments were soaked in raloxifene and then assessed for mechanical properties. Mice treated with zoledronate in vivo had lower post-yield mechanical properties compared to controls. Raloxifene soaking had significant positive effects on select mechanical properties of bones from both vehicle and zoledronate treated mice. Although the effects were blunted in zoledronate bones relative to vehicle, the soaking was sufficient to normalize properties to control levels. Additional studies showed that raloxifene-soaked bones had a significant positive effect on cycles to failure (+114%) compared to control-soaked mouse bone. Finally, raloxifene soaking significantly improved select properties of ribs from dogs treated for 3 years with alendronate. These data show that ex vivo soaking in raloxifene can act through non-cellular mechanisms to enhance mechanical properties of bone previously treated with bisphosphonate. We also document that the positive effects of raloxifene soaking extend to enhancing fatigue properties of bone.

Keywords: bisphosphonate, toughness, mechanical properties, zoledronate, alendronate.

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Resumen

EL RALOXIFENO INVIERTE FRAGILIDAD ÓSEA INDUCIDA POR EL TRATAMIENTO ANTI-REMODELACIÓN Y AUMENTA LA RESISTENCIA A LA FATIGA A TRAVÉS DE MECANISMOS MEDIADOS NO CELULARES.

Los datos preclínicos han demostrado que los efectos a nivel de tejido que se derivan de la supresión del remodelado óseo inducida por bifosfonatos puede dar como resultado un hueso que es más fuerte pero más frágil. Está comprobado que el raloxifeno reduce la fragilidad ósea a través de mecanismos no celulares. El objetivo de este trabajo fue probar la hipótesis de que el raloxifeno puede revertir la fragilidad ósea resultante del tratamiento con bifosfonatos. Se emplearon huesos de perro y ratón de múltiples experimentos con diferentes dosis de bifosfonatos los cuales fueron sumergidos en raloxifeno y luego se evaluaron sus propiedades mecánicas. Ratones tratados con zoledronato in vivo mostraron propiedades mecánicas post-rendimiento más bajas en comparación con los controles. Luego de sumergirlos en raloxifeno se observaron efectos positivos significativos en algunas propiedades biomecánicas tanto en los huesos de ratones tratados con vehículo como con zoledronato. Aunque los efectos se atenuaron en los huesos tratados con zoledronato en relación con los tratados con vehículo, el raloxifeno fue suficiente para normalizar las propiedades a niveles basales. Estudios adicionales mostraron que los huesos sumergidos en raloxifeno tuvieron un efecto positivo significativo en los ciclos de fractura (+ 114%) en comparación con los huesos de ratón sumergido en vehículo. Finalmente, el raloxifeno mejoró significativamente las propiedades de costillas de perros tratados durante 3 años con alendronato. Estos datos muestran que la inclusión ex vivo en raloxifeno puede actuar a través de mecanismos no celulares para mejorar las propiedades mecánicas de huesos previamente tratado con bifosfonatos. También documentamos que los efectos positivos del raloxifeno mejoran las propiedades de fatiga del hueso.

Palabras clave: bifosfonato, dureza, propiedades mecánicas, zoledronato, alendronato.

Introduction

A bone's mechanical properties, specifically those related to displacement and energy absorption, can be described as being brittle or ductile.[1-3] A ductile bone is able to undergo significant displacement and absorb significant energy following the manifestation of permanent damage. Conversely, a brittle bone fails soon after the initiation of permanent damage. Classic clinical examples of these extremes are developing bone and osteopetrotic bone, respectively.[4] In the laboratory, decalcification of a bone makes it extremely ductile,[5] while removal of the organic material (using heat) makes it extremely brittle.[6] In general, increasing the ductility of bone is advantageous for improving its resistance to fracture.[1]

The goal of anti-osteoporotic interventions is to reduce fracture.[7] Whether or not a bone fractures depends on several factors, including bone mass, propensity to fall, and the mechanical properties of the bone tissue.[1] Interventions such as bisphosphonates primarily reduce fracture risk by increasing bone mass which leads to improvements in whole bone mechanical properties.[8] In many cases though, improving bone mass and bone strength comes at the expense of changes to the tissue which are not completely positive. Pre-clinical data in dogs[9-14] and mice (C57BL/6),[15] have shown that suppression of bone remodeling by bisphosphonate treatment can result in bone that has higher ultimate force yet lower toughness. It has been hypothesized that this reduction in tissue toughness, brought about by deleterious changes to the tissue level properties

(altered mineral heterogeneity,[16,17] properties of mineral crystals,[16] collagen cross-linking,[18,19] microdamage),[9,20] is linked to atypical femoral fractures.[21,22]

Enhancing the ductility of bone at the tissue level has been shown to occur with anabolic treatment due to its remodeling away older tissue and replacing it with new matrix. Raloxifene (RAL), an FDA approved selective estrogen receptor modulator, also reduces brittleness of bone,[23,24] but through an alternative mechanism involving non-cellular mediated modification of tissue hydration.[25,26] The goal of this work was to test the hypothesis that in vitro exposure to raloxifene is sufficient to neutralize thebone brittleness that occurs following bisphosphonate treatment.

Methods

Animal experiments. The bones utilized in this report come from three different experiments. All sample sizes can be found in the data tables and figures. In experiment one, designed to determine if zoledronate produced effects on mechanical properties, male C57BL/6 mice were treated saline or zoledronate (ZOL) for 8 weeks, from 16 to 24 weeks of age.[15] At 24 weeks of age, bilateral femora were removed, wrapped in saline-soaked gauze, and frozen at -20 ºC until analysis. Mechanical testing of the right femora was performed and these data have been previously reported.[15] Left limbs, used in this current work, were thawed, soaked in RAL for 7 days and then subjected to mechanical testing. These results were compared to those from the contralateral femora that was tested without soaking.

In experiment two, bilateral femora from untreated 16 week old male C57BL/6 mice were collected to study the fatigue properties of mouse bone. A subset of these bones were used in the current work.

In experiment three, skeletally mature female beagles were treated for three years with daily oral saline (10 ml) or alendronate (ALN, 0.2 mg/kg/day in 10 ml).[10] After three years of treatment, ribs were dissected free, wrapped in saline-soaked gauze, and frozen at -20 ºC until analysis. All animal experiments were approved by the Indiana University School of Medicine IACUC prior to the live animal experiments.

Raloxifene soaking. RAL was purchased from Sigma and dissolved in DMSO following previously published protocols.[25] Bones were soaked in 1% penicillin-streptomycin/phosphate buffered saline solution, with either 2 μM DMSO or 2 μM RAL at 37 ºC for 14 (experiment 3), 7 (experiments one) or 2 (experiment two) days, changing the solution every 2-3 days.

Peripheral quantitative and microcomputed tomography (pQCT, microCT). To normalize mechanical properties, one femur from each mouse in all experiments was scanned to determine cortical bone geometry at 50% of bone length. MicroCT scans were obtained using a Skyscan 1176 scanner at 9 micron resolution. Scan reconstruction and analysis at the mid-diaphysis were conducted using manufacturer software combined with a custom MATLAB program.[27] All ribs from experiment 3 were scanned using pQCT (Norland Stratec XCT Research SA+) at the spot of greatest curvature (approximately midrib).[28] A single slice was imaged at this spot using a scanning resolution of 0.07 x 0.07 x 0.50 mm. Anterior–posterior diameter (APdia, mm) and cross-sectional moment of inertia (CSMI, mm^4) were obtained using standard scanner software for estimation of material properties.

Mechanical testing. Bones from experiment one were tested in four-point bending.[27] Bones were placed anterior surface down on a bottom support span of 9 mm; the upper support span was 3 mm wide centered at the mid-diaphysis. Testing occurred at a displacement rate of 2 mm/min and load/displacement

data collected. Analysis of mechanical data test curves was done using a custom MATLAB program that integrates the CT data with the load/displacement data to produce both structural (yield/ultimate load, stiffness, pre-yield/post-yield/total displacement, pre-yield/post-yield/total energy) and material (yield/ultimate stress, modulus, strain, toughness) properties. The geometric properties used for normalization of both right and left bones was based on CT scanning of only one bone. Based on unpublished data from our laboratory, as well as published studies,[29] there is minimal right/left difference in geometry within an animal, thus supporting our use of CT data from one bone within an animal for normalization ofthe contralateral bone.

Mouse femora in experiment two were subjected to fatigue loading in four-point bending. Ten paired femurs were soaked in either PBS (left) or RAL (right) and then tested in fatigue using a sinusoidal waveform (loading between the force corresponding to 15% and 85% of the ultimate stress (determined from monotonic test on another set of bones) with a frequency of 0.5 Hz for the first ten preconditioning cycles and 4 Hz for the rest of the test. Femurs were hydrated throughout the test with the use of a heated saline bath (37°C) that contained 2% Pen-Strep. Any tests that reached 300,000 were terminated without failure.

Dog ribs were tested in three-point bending.[28] After thawing to room temperature, specimens were placed on a three-point bending fixture (bottom support span = 25 mm) with the convex surface of the rib facing up. The upper support contact point was at the midpoint of the specimen, matching the site of pQCT analyses. Specimens were loaded to failure at a displacement rate of 20 mm/minute, and load vs. displacement data were collected. Structural mechanical properties were determined and material property estimations were calculated as outlined above.

Statistics. All statistical tests were performed using SAS software. Data from experiments one and three were compared using two-way ANOVA with repeated measures (to account for right/left limbs). Statistically significant effects of in vivo treatment, soaking, and interactions (followed by analysis of simple main effects) between those two variables were determined using a $p<0.05$. Data from experiment two were compared using paired t-tests. All data are presented as mean and standard deviations.

Results

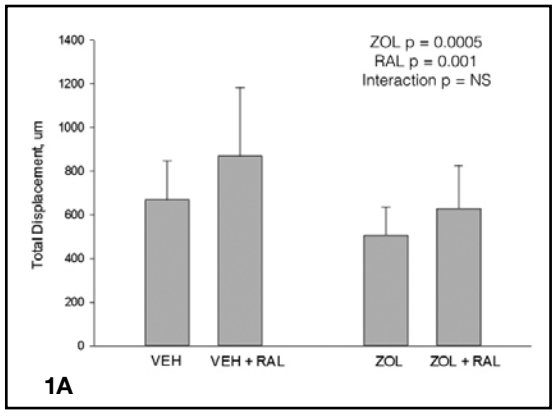
Experiment 1. There was a significant main effect of in vivo ZOL treatment for pre-yield (+27%), post-yield (-37%), and total displacement (-27%) and all estimates of material properties relative to animals treated with vehicle (VEH) (Table 1, Figure 1). There was a significant main effect of RAL-soaking on post-yield displacement (+10%), total displacement (+10%), and total strain (+26%) relative to the contralateral limbs that were not soaked. Significant interactions existed for ultimate load, post-yield energy and total energy where, in all three cases, the effect of RAL-soaking was significantly greater in bones from animals treated with VEH in vivo compared to those treated with ZOL. In summary, the effects of raloxifene soaking were less effective in ZOL-treated animals, yet sufficient to return select mechanical properties to those of normal untreated animals.

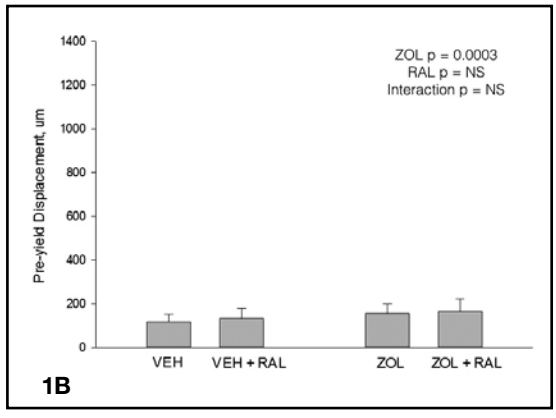
Experiment 2. PBS-soaked control bones subjected to fatigue loading failed at 116,005$\pm$90,767 cycles. RAL-soaked bones had 1.7-fold longer fatigue life ($p=0.019$), 202,894$\pm$125,607 cycles (Figure 2A). One of ten PBS-soaked bones (10%) and five of ten RAL-soaked bones (50%) were stopped at 300,000 cycles without failure. The majority of paired bones followed the trend of RAL being higher than PBS yet there were three sets that were either unchanged (both reached 300,000 cycles) or showed modest reductions

Table 1. Mechanical properties of mouse femora in 4-point bending.

	Vehicle-treatment		Zoledronate-treatment		In vivo treatment (VEH vs ZOL)	Soaking (None or RAL)	Interaction
	Control (n=20)	RAL – soaking (n=20)	Control (n=17)	RAL – soaking (n=17)			
Ultimate Load, N	21.3± 3.7	23.4±3.8 *	27.8±4.1	25.8±6.4	**0.001**	0.500	**0.0001**
Stiffness, N/mm	115±36	133±47 *	153±45	162±60	0.189	0.917	**0.025**
Yield energy, mJ	0.72±0.38	1.06±0.64	1.48±0.63	1.63±1.1	**0.0004**	0.170	0.622
Post-yield energy, mJ	9.57±3.5	13.2±4.5 *	8.3±3.6	9.4±3.9	0.021	**0.0004**	**0.047**
Energy to failure, mJ	10.3±3.4	14.2±4.5 *	9.8±3.3	11.0±3.6	0.073	**0.0002**	**0.038**
US, MPa	252±25	266±33	286±31	286±45	**0.001**	0.119	0.551
Strain to failure, µE	94,084±26,788	123,289±43,715	73,113±18,473	87,851±25,203	**0.002**	0.286	0.595
Modulus, MPa	9.07±1.6	9.67±2.5	9.22±1.9	9.23±1.8	**0.0005**	**0.002**	0.267
Toughness, , MJ/m^3	17.2±5.2	22.9±6.3	14.9±5.3	17.7±5.3	**0.001**	0.159	0.880

* vs control within treatment in post-hoc test following significant interaction in two-way ANOVA. Data presented as mean and standard deviation. RAL – raloxifene; VEH – vehicle; ZOL – zoledronate.





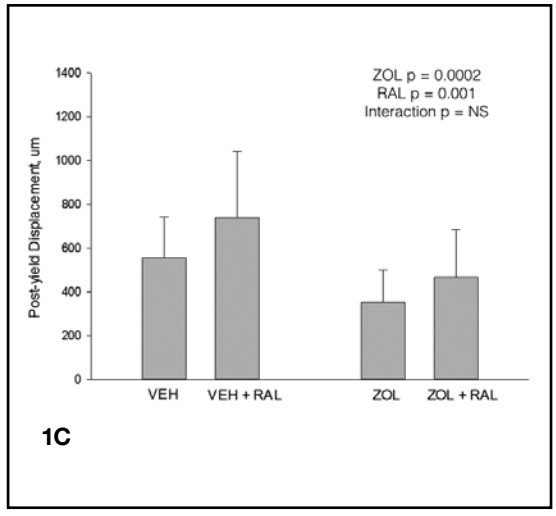


Figure 1. Ex vivo soaking in raloxifene significantly affects displacement properties of mouse bone previously vehicle (VEH) and zoledronate (ZOL). **A.** Total displacement during four-point bending was significantly affected by in vivo treatment with ZOL. Ex vivo soaking in raloxifene (RAL) significantly improved displacement in both VEH and ZOL treated animals with no interaction between the two variables (p=0.379). **B.** There was a significant effect of in vivo ZOL treatment on pre-yield displacement (p=0.003) with no effect of RAL-soaking or an interaction between variables (p=0.234 and 0.705, respectively). **C.** Post-yield displacement showed a similar pattern as total displacement.

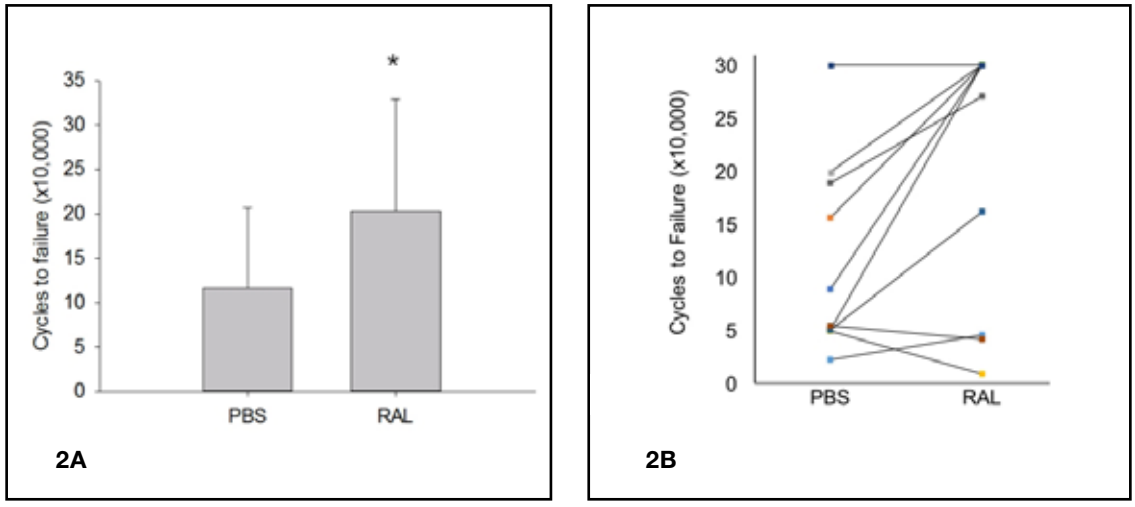


Figure 2. Ex vivo soaking in raloxifene (RAL) significantly improves fatigue properties of mouse bone. **A.** Cycles to failure were significantly higher in bones soaked in raloxifene compared to contralateral controls soaked in PBS. Data presented as mean and standard deviation. *p<0.05 in paired t-test versus PBS. **B.** Number of cycles to failure of individual sets of paired bones with each set representing the right and left bone of a given mouse. Note that bones not failing by 300,000 cycles were stopped.

with RAL (Figure 2B). The RAL-soaked bones had an ~114% average increase in cycles to failure versus their contralateral PBS-soaked control bone.

Experiment 3. There were no significant main effects of alendronate treatment (Table 2). There was a significant main effect of RAL soaking on energy to failure (+16%), post-yield energy (+21%), toughness (+38%) and post-yield toughness (+43%) (Figure 3).

Discussion

A bone made of brittle material is at an increased risk of fracture even if bone mass is increased. There are several illustrative examples, such as the clinical condition of osteopetrosis, where bone mass is high yet fractures are quite prevalent,[30] and pre-clinical models of osteogenesis imperfecta when drugs that increase bone mass are insufficient to normalize mechanical properties.[31,32] We and other have documented that bisphosphonates result in tissue brittleness, both in dogs and more recently in C57BL/6 mice.[13,15,28] Given that bisphosphonates have long-lasting effects even after treatment withdrawal,[33,34] finding active ways to neutralize/reverse the brittleness brought on by remodeling suppression necessitate new approaches. In this proof-of-concept study, we show that raloxifene can overcome the tissue brittleness caused by bisphosphonates through non-cellular mechanisms.

Raloxifene has a long history of having positive effects on bone.[35] It is FDA approved for the treatment and prevention of fracture in post-menopausal women.[36-38] Although the mechanism of action was originally thought to be related to suppressed osteoclast action, there remained a known disconnect between changes in bone mass and fracture risk reduction.[39] Recently, our lab has documented a potential explanation for this disconnect by showing that raloxifene can act through non-cellular mechanisms to increase tissue hydration.[25,26] This effect is associated with

Table 2. Mechanical properties of dog ribs in 3-point bending.

	Vehicle-treatment		Alendronate-treatment		In vivo treatment (VEH vs ALN)	Soaking (PBS or RAL)	Interaction
	PBS-soaking (n=9)	RAL-soaking (n=9)	PBS-soaking (n=10)	RAL-soaking (n=10)			
Ultimate Load, N	88±25	100±21	90±22	89±16	0.617	0.285	0.184
Stiffness, N/mm	165±53	200±35	176±51	174.5±42	0.687	0.209	0.131
Post yield displacement, mm	4.26±1.46	4.74±0.66	3.85±1.39	4.23±1.77	0.363	0.119	0.865
Total displacement, mm	4.59±1.43	5.16±0.74	4.19±1.34	4.56±1.19	0.325	0.087	0.697
Post-yield energy, mJ	312±91	404 ±134	286±119	319±84	0.234	**0.012**	0.188
US, MPa	114±53	148±49	128±85	124±70	0.859	0.196	0.085
Modulus, MPa	6049±2526	7868±2067 *	7240±4023	6790±3120	0.966	0.239	**0.041**
Post-yield toughness, MJ/m^3	13.6±4.4	22.6±10.8	12.3±7.6	14.7±8.1	0.175	**0.006**	0.073

* vs control within treatment in post-hoc test following significant interaction in two-way ANOVA. Data presented as mean and standard deviation. PBS – phosphate buffered saline; RAL – raloxifene; VEH – vehicle; ALN – alendronate.

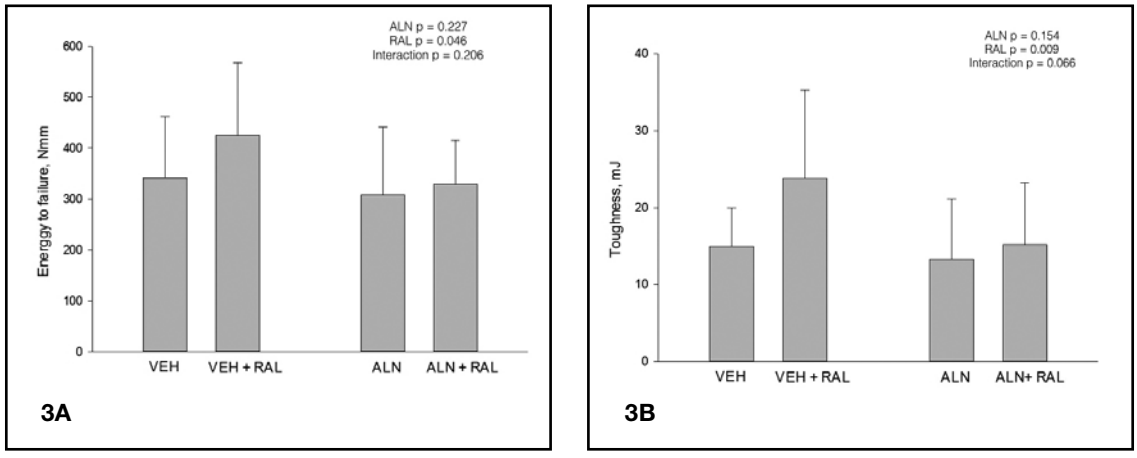


Figure 3. Ex vivo soaking in raloxifene restores displacement properties of ribs from vehicle (VEH) and alendronate (ALN)-treated dogs. Energy to failure (**A**) and toughness (**B**) were both was significantly higher in bones soaked in raloxifene (RAL) compared to those soaked in PBS.

improvements in mechanical properties, specifically post-yield properties.[25] Although the details regarding how raloxifene increases hydration remain to be clearly elucidated, the most recent findings point to binding of raloxifene at the mineral/collagen interface.[26]

In the experiments described herein, raloxifene soaking of the bones from animals treated with vehicle (thus normal animals) resulted in robust positive responses to properties that are influenced by post-yield behavior. This is consistent with previous work from both dog and human tissue soaked in raloxifene.[25] Raloxifene's significant positive effect on post-

yield and total displacement carried over to bones from animals treated with zoledronate in vivo. This effect resulted in RAL-soaked bones from zoledronate-treated animals having similar post-yield and total displacement values as normal bones. Simply stated, RAL-soaking normalized the mechanical phenotype of zoledronate bone.There were other mechanical properties where the positive effects of raloxifene soaking were significantly attenuated in bones from animals treated in vivo with zoledronate as evident by the significant interaction in ultimate load, post-yield and total energy.

Contrary to previous work from these same animals,[12,13,28] the mechanical tests of dog ribs did not reveal significant effects of in vivo alendronate treatment. Properties most often noted as being negatively affected by alendronate, such as toughness, were non-significantly lower (-10%; p=0.15 main effect). Of note is that sample sizes here were lower than in previous reports (n=12/group) because specimens from some animals were no longer available, thus reducing the power in the statistical tests. It is also possible that soaking itself affected the ability to see effects of ALN as several of the parameters from PBS-soaked bones were qualitatively different compared to previous work[28] although it should be acknowledged that these were different ribs and thus different properties might not be unexpected. Despite the lack of significant differences brought about by ALN, there remained significant main effects of raloxifene soaking on post-yield and total displacement and energy absorption. Consistent with the mouse bones in experiment 1, there was a suggestion of an interaction in the effect of RAL-soaking, being mainly driven by the response of bones from VEH-treated animals. One plausible explanation is that changes to mineral and collagen brought about by bisphosphonate-treatment[16,18,19] alter the ability for raloxifene to modify hydration and this is more evident in a species that undergo intracortical remodeling (and thus suppressed intracortical remodeling). Alternatively, differences in bisphosphonate (alendronate vs zoledronate), duration of treatment (two months in mouse vs 3 years in dog), or bone (rib versus femur) could be the underlying reason for differences between the two experiments.

The precise mechanisms underlying tissue-level brittleness with bisphosphonates remains unclear. Altered mineral heterogeneity,[16,17] properties of mineral crystals,[16] collagen cross-linking,[18,19] microdamage[9,20] have all been documented in various model systems (including humans). Many of the changes in cortical bone are associated with the change in intracortical remodeling, yet data exist showing lower tissue mechanical properties independent of the degree of remodeling suppression in dogs. Furthermore, we and others have shown reductions in bone toughness with bisphosphonates in rodents, where intracortical remodeling does not take place under normal circumstances. The goal of the current work does not address the underlying mechanism for tissue brittleness with bisphosphonates, but rather focuses on the ability of raloxifene to neutralize whatever effect has occurred. Our results suggest modification of hydration (the presumed effect of raloxifene) is sufficient to overcome negative tissue-level changes with bisphosphonates.

Although monotonic mechanical tests provide valuable information regarding properties of the tissue, fatigue loading tests the tissue's ability to resist the initiation and propagation of damage leading to fracture.[40] The ability of in vitro raloxifene exposure to alter fatigue properties in normal C57/B6 femora was clear. Raloxifene-soaked bones had nearly 2x longer fatigue life than normal animals, and even this was likely an underestimate as half of the raloxifene bones were stopped at 300K cycles (compared to one untreated bone). Interestingly, 7 of the 10 matched pairs showed higher properties in the raloxifene limb while three showed nearly identicalor slightly values

across the two limbs. Previous work assessing fatigue properties of RAL bone is limited to experiments of cortical bones from in vivo treated dogs. Although monotonic tests from these same animals showed dramatic effects of in vivo treatment on mechanical properties, there were no differences when assessed using a cyclic relaxation test.[24] The cyclic relaxation test differs in several ways from a traditional fatigue test, most notably in that it loads to progressively higher loads with the goal of inducing damage and then testing the ability of the tissue to resist accumulation.[41] The link between altered hydration (the presumptive mechanism of effect in current soaking studies) and microdamage propagation remains unclear but it is possible that benefits of hydration are more apparent in traditional fatigue tests.

The data presented here should be considered in the context of various limitations. The original experiments (from which the bones were used) tested only males and only a single dose of zoledronate. Due to the matched design of experiment one, we did not have bones soaked for 7 days in control solution as is traditionally done in these experiments. We have previously shown that soaking in solution does not cause the tissue to decalcify (which if it occurred could cause improved ductility).[25] Although we have previously shown the main non-cellular effect of raloxifene is to increase hydration – measures of hydration in these bones was not possible. Finally, our fatigue data were conducted at a single stress level and cycle rate and cannot be assumed to be generalizable.

In conclusion we have shown that ex vivo soaking in raloxifene can act through non-cellular mechanisms to normalize the zoledronate-induced brittle behavior of mouse bone tissue. Less robust effects were noted in bones from alendronate-treated dogs and these differences need to be further explored. We also document the positive effects of raloxifene soaking on fatigue properties of bone.

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