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Raloxifene Enhances Material-Level Mechanical Properties of Femoral Cortical and Trabecular Bone

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We have previously documented that raloxifene enhances the mechanical properties of dog vertebrae independent of changes in bone mass, suggesting a positive effect of raloxifene on material-level mechanical properties. The goal of this study was to determine the separate effects of raloxifene on the material-level mechanical properties of trabecular and cortical bone from the femur of beagle dogs. Skeletally mature female beagles (n = 12 per group) were treated daily for 1 yr with oral doses of vehicle or raloxifene (0.50 mg/kg·d). Trabecular bone mechanical properties were measured at the femoral neck using reduced platen compression, a method that allows the trabecular bone to be tested without coring specimens. Cortical bone properties were assessed on prismatic beam specimens machined from the femoral diaphysis using both monotonic and dynamic (cyclic relaxation) four-point bending tests. Trabecular bone from raloxifene-treated animals had significantly higher ultimate stress (+130%), modulus (+89%), and toughness (+152%) compared with vehicle-treated animals. Cortical bone from raloxifene-treated animals had significantly greater toughness (+62%) compared with vehicle, primarily as a function of increased postyield displacement (+100%). There was no significant difference between groups in the percentage of stiffness loss during cortical bone cyclic relaxation tests. These results are consistent with previous data from the vertebrae of these same animals, showing raloxifene has positive effects on biomechanical properties independent of changes in bone volume/density. This may help explain how raloxifene reduces osteoporotic fractures despite modest changes in bone mass. (*Endocrinology* 148: 3908–3913, 2007)

FRACTURE RISK IS determined in part by the skeleton's biomechanical properties, most notably the load carrying capacity and the ability to absorb energy (1, 2). These whole bone properties (ultimate load and energy absorption) can be improved by increasing bone mass/density, by altering the geometry or architecture of the bone, and/or by enhancing the material-level mechanical properties of the bone. We have previously shown, using a beagle dog model, that raloxifene has beneficial effects on whole bone mechanical properties of lumbar vertebrae independent of changes in bone density (3). These findings are consistent with clinical trials of post menopausal women, in which raloxifene has a modest effect on vertebral bone density yet significantly reduces vertebral fracture risk (4–6). Furthermore, changes in vertebral BMD with raloxifene have been shown to explain only 4% of the reduction in vertebral fracture risk (7). These data suggest raloxifene reduces fracture risk in ways that do not involve large changes in BMD (8, 9).

As the vertebra is comprised of both trabecular and cortical bone, our previous studies were not able to address whether one or both bone types experienced altered material properties with raloxifene treatment (3). This is an important distinction, as it could help shed light on the mechanism for the antifracture efficacy of raloxifene, which is currently unclear (9). For instance, if beneficial effects exist predominately in trabecular bone, it may suggest alterations in trabecular architecture (10), or reductions in resorption cavity stress risers (11, 12) may explain enhanced fracture resistance with raloxifene. Positive changes in biomechanical properties of both cortical and trabecular bone would suggest raloxifene has an effect on the bone tissue itself, and that the effect is likely mediated independently of turnover suppression. It is well accepted that annual turnover rates of trabecular bone exceed those of cortical bone (13), making the effect of remodeling suppression more prominent at trabecular bone sites. If the positive effects of raloxifene are linked to remodeling suppression, it would be predicted that trabecular, but not cortical bone would show improved biomechanical properties.

The goal of this study was to use material-level biomechanical tests to separately evaluate the effects of raloxifene on cortical and trabecular bone. Our hypothesis was that raloxifene would improve the material properties of trabecular bone while having minimal effect on cortical bone compared with vehicle-treated animals.

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Abbreviations: BV/TV, Trabecular bone volume fraction; microCT, microcomputed tomography; RPC, reduced platen compression.

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Materials and Methods

Experimental design

All procedures were approved before the study by the Indiana University School of Medicine Animal Care and Use Committee. One-year-old female beagles were randomized into two groups (n = 12/group) and treated daily for 1 yr with oral doses of saline vehicle (1 ml/kg·d)

or raloxifene (0.5 mg/kg·d) as previously described (3). This dose of raloxifene was chosen to produce serum levels similar to those of the dose used to treat postmenopausal osteoporosis in humans (data on file; Eli Lilly Co., Indianapolis, IN). After 1 yr of treatment, bilateral femora were harvested and stored at −20 C until analyses.

Trabecular bone biomechanical tests: reduced platen compression (RPC)

Femoral neck specimens were prepared for microcomputed tomography (microCT; right femur) or RPC (left femur). This bone site was chosen because it contained an appreciable amount of trabecular bone (Fig. 1). Bones were thawed to room temperature, and a 3-mm-thick specimen was cut perpendicular to the neck axis under constant irrigation using a band saw (right femur) or low speed Isomet saw (left femur), each with a diamond tipped blade.

The right femoral neck specimen was scanned using microCT (μCT-20; Scanco Medical, Bassersdorf, Switzerland). The scanning location corresponded to the mid-region of the isolated specimen (2 mm thickness; 119 slices, 17 μm isotropic resolution). Trabecular bone morphometric indices, assessed using three-dimensional image reconstructions, included trabecular bone volume fraction (BV/TV), trabecular thickness, trabecular number, and trabecular separation.

The left femoral neck was prepared for reduced platen compression (RPC), as previously described with slight modifications (14). Isolated specimens (~3 mm thick) were radiographed and digitized to obtain cross-sectional images (Fig. 1) for platen sizing. Compression platens were sized to correspond to 70% of the largest circle inscribed within the endocortical perimeter. Platen sizing was rounded to the nearest 0.1 mm diameter. Before testing each specimen, a visual inspection of the platen-bone interface was performed to ensure that the platens were not contacting cortical bone.

Specimens were loaded to failure under displacement control (0.5 mm/min), and data were collected at 10 Hz (Instron 1125; Instron, Norwood, MA). Trabecular bone apparent material properties were estimated assuming uniaxial compression of the cylindrical region directly between the platens. Specifically, ultimate stress (σ_{ult}), elastic modulus (E), and toughness (u) were calculated using the following equations: $\sigma_{ult} = (F/A)$, $E = (k \times L/A)$, and $u = U/(L \times A)$; where F is the maximum force, A is the cross-sectional area of the platens, L is the specimen thickness, k is the slope of the linear portion of the load-displacement curve, and U is the area under the load-displacement curve up to ultimate load. Considering only a fraction of the volume between the two compressive platens is comprised of bone, σ_{ult}, E, and u were further normalized by BV/TV measured on the contralateral femoral neck.

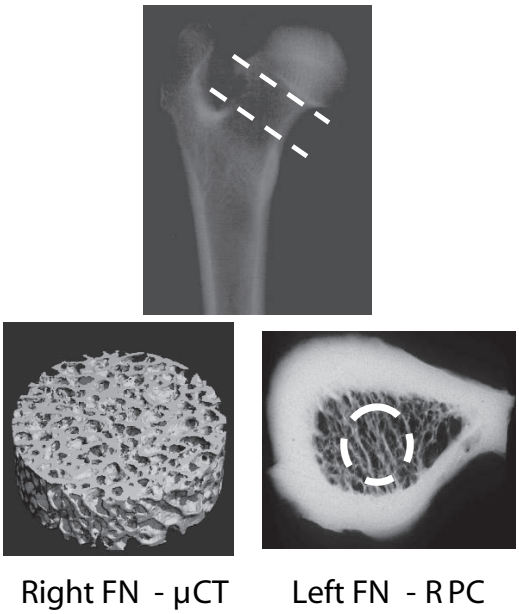

FIG. 1. Graphical depiction of femoral neck trabecular bone analyses. The central portion of the each femoral neck was isolated, with the right neck undergoing microCT analysis to determine trabecular architecture and the left femoral neck undergoing RPC to determine material properties. The *circle* inscribed on the radiographed image of the left femoral neck approximates the region of platen contact during the RPC test.

Cortical bone biomechanical tests

Cortical bone beam specimens were machined from the middiaphysis of the femur. Under constant irrigation, the midportion of the shaft (~30 mm length) was isolated using a band saw, and two prismatic beams were cut along the longitudinal axis of the anterior and posterior cortices using a wire saw (Histosaw; Delaware Diamond Knives, Wilmington, DE). Surfaces were sanded with 400-grit sandpaper until the beam was in ~0.2 mm of the final dimensions, with final polishing done using 600-grit sandpaper to achieve final dimensions of 25 mm (length), 1.8 mm (width), and 1.5 mm (height) for each of the two beams. Anterior and posterior cortex beams were randomized between monotonic and relaxation four-point bending tests.

One beam was subjected to monotonic four-point bending under displacement control (3 mm/min) and data was collected at 10 Hz (EnduraTEC; Bose, Eden Prairie, MN). Structural properties of strength, stiffness, displacement, and energy absorption were obtained from the load/deformation curve. Material properties of σ_{ult}, E, and u were estimated using standard equations $\sigma_{ult} = F \times (3a/wt^2)$, $E = (k/wt^3) \times (6La^2) - 8a^3)$, $u = 9U/(wt(3L - 4a))$, where L is the bottom support span (21 mm), a is the span between the top support (6 mm), t is specimen thickness, w is specimen width, F is the maximum force, k is the slope of the linear portion of the load-displacement curve, and U is the area under the load-displacement curve up to fracture. Yield displacement, for use in the relaxation test, was calculated using a 0.2-mm offset (15).

The second beam was used for cyclic relaxation tests following a previously published protocol (16) (Fig. 2). The goal of this test was to

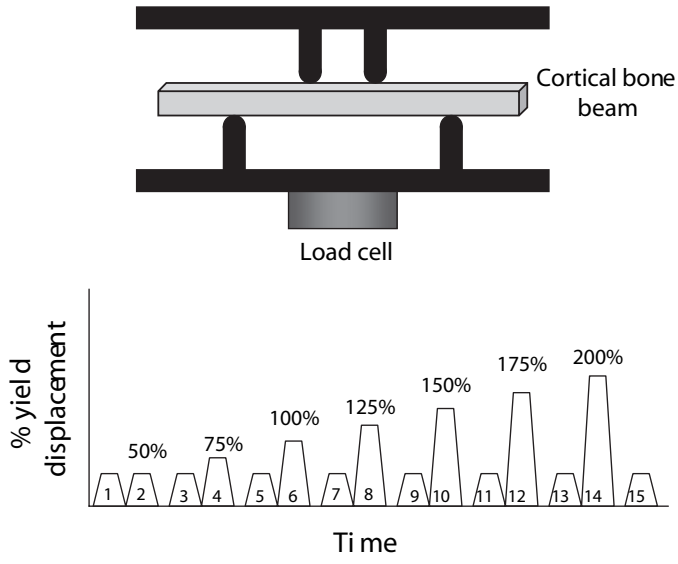

FIG. 2. *Top*, Schematic of four-point bending test setup for testing of cortical bone beam specimens. This same testing setup was used for monotonic compression and relaxation tests. *Bottom*, Schematic of the relaxation test protocol used to assess cortical bone properties under cyclic loading conditions. In this 15-cycle test, specimens undergo an alternating series of diagnostic (cycles 1, 3, 5, 7, 9, 11, 13, 15) and damage (cycles 2, 4, 6, 8, 10, 12, 14) cycles. For diagnostic cycles, specimens were loaded to 50% of yield displacement (determined from monotonic tests), held for 1 min, and then unloaded. Damage cycles were loaded to 50, 75, 100, 125, 150, 175, and 200% of yield displacement, held for 1 min, and then unloaded. After each damage cycle, specimens were allowed a 5-min rest period. All loading and unloading occurred at a rate of 3 mm/min.

TABLE 1. Properties of femoral neck trabecular bone and femoral shaft cortical bone

	Vehicle	Raloxifene	*P* value
Trabecular bone (n)	10	12	
BV/TV (%)	33.1 ± 1.2	31.3 ± 1.7	0.402
Tb.N (1/mm)	3.23 ± 0.16	2.99 ± 0.15	0.275
Tb.Th (μm)	119 ± 3.6	117 ± 3.8	0.663
Tb.Sp (μm)	324 ± 11.2	334 ± 11.0	0.524
Cortical bone (n)	12	12	
BMD (g/cm^2)	0.121 ± 0.001	0.122 ± 0.001	0.517
Ultimate load (N)	56.5 ± 2.5	60.8 ± 0.92	0.124
Yield load (N)	44.4 ± 1.5	45.6 ± 0.95	0.489
Stiffness (N/mm)	77.3 ± 2.7	83.9 ± 2.4	0.080
Ultimate stress (MPa)	238 ± 9	254 ± 5	0.129
Modulus (GPa)	16.7 ± 0.5	17.8 ± 0.6	0.152

Tb.Th, Trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular separation; BMD, bone mineral density; MPa, megapascal; GPa, gigapascal.

assess material properties under cyclic loading conditions. This protocol was chosen, rather than a true fatigue test, due to the relatively short nature of the relaxation test (~1 h). Using a 15-cycle test, specimens undergo an alternating series of "diagnostic" and "damage" cycles under four-point bending. For diagnostic cycles (cycles 1, 3, 5, 7, 9, 11, 13, 15), specimens were loaded to 50% of yield displacement (determined from monotonic tests), held for 1 min, and then unloaded. Displacement to 50% yield has been shown to provide adequate evaluation of stiffness without producing additional damage to the specimen (16). Damage cycles (cycles 2, 4, 6, 8, 10, 12, 14) were loaded to 50, 75, 100, 125, 150, 175, and 200% of yield displacement, held for 1 min, and then unloaded. Between each damage cycle, and the subsequent diagnostic cycles, specimens were allowed a 5-min rest period. All loading and unloading occurred at a rate of 3 mm/min. Specimens were kept moist with physiological saline solution throughout the test.

The magnitude of stiffness degradation was used to evaluate changes in specimens throughout the test. Stiffness was calculated as the slope of the linear portion of the load/displacement curve from each of the diagnostic cycles. Baseline stiffness was determined by the average of the first diagnostic cycle (cycle 1), the first damage cycle (50% yield displacement; cycle 2), and the second diagnostic cycle (cycle 3). The percentage of stiffness loss for each of the damage cycles was calculated as: (baseline stiffness − new stiffness)/baseline stiffness × 100.

Statistics

All statistical tests were performed using SAS (SAS Institute, Inc., Cary, NC). Data were compared between groups using two-tailed Wilcoxon-Mann-Whitney (percentage of stiffness loss from relaxation tests) or Student's *t* tests (all other data) with $P < 0.05$ considered significant. All data are presented as mean ± SE.

Results

Two specimens from vehicle-treated animals were damaged during machining, leaving n = 10 specimens for data collection. There was no significant difference in femoral neck trabecular bone volume, or measures of trabecular architecture, between vehicle- and raloxifene-treated animals (Table 1). Despite these similarities, specimens from raloxifene-treated animals had significantly higher ultimate stress (+130%), modulus (+89%), and toughness (+152%) compared with vehicle-treated animals (Fig. 3).

Raloxifene-treatment significantly increased cortical bone total energy to failure, a structural parameter, compared with vehicle (+61%; $P = 0.016$) (Fig. 4A). The material level equivalent of energy, toughness, was also significantly higher with raloxifene (+62%, $P = 0.014$) compared with vehicle treatment (Fig. 4B). These changes were due primarily to a significant prolonging of the postyield displacement (+97%; $P = 0.01$) compared with vehicle (Fig. 4D), with no change in preyield displacement between the two groups (Fig. 4C). There was no significant difference in any of the other structural properties between the two groups (Table 1). The two other material-level properties—ultimate stress (+7%; $P = 0.129$) and modulus (+7%; $P = 0.152$)—were not significantly altered with raloxifene.

There was no significant difference in the percentage of stiffness loss between cortical bone specimens from raloxifene- or vehicle-treated animals at any stage of the relaxation tests (Fig. 5). Stiffness degradation was nonsignificantly greater with vehicle treatment compared with raloxifene at yield displacements of 100% (+8%; $P = 0.10$), 125% (+7%;

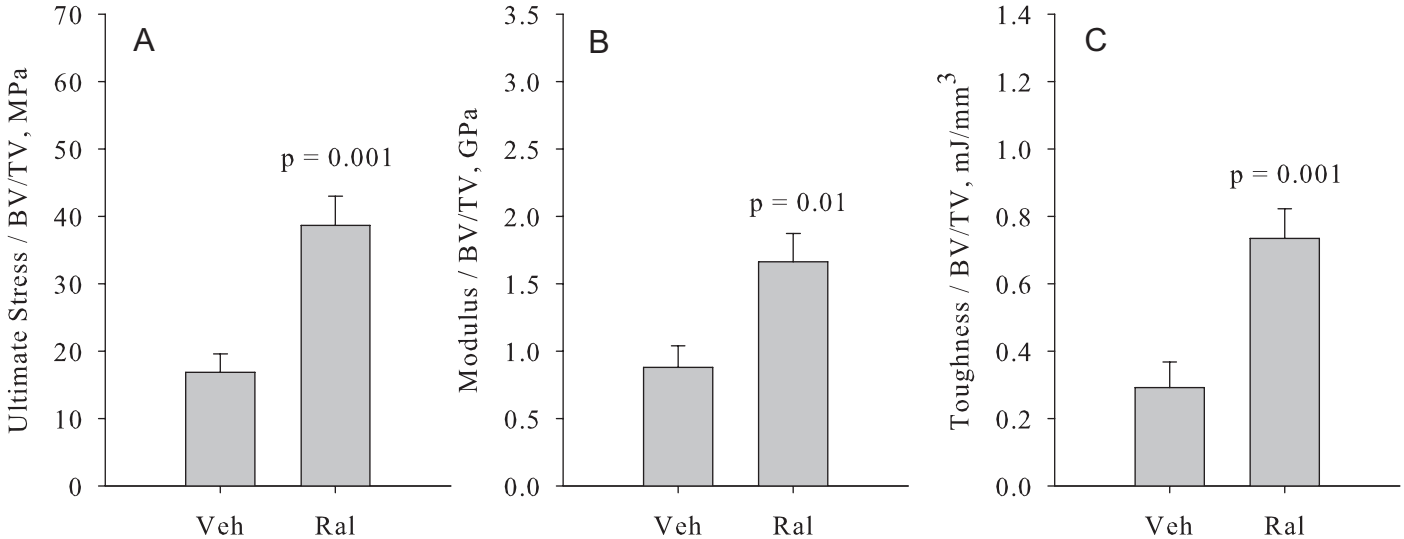

FIG. 3. Significant alterations of trabecular bone material-level biomechanical properties with raloxifene. After RPC testing, properties of ultimate stress (A), modulus (B), and toughness (C) were calculated. As only a portion of the region between the two RPC platens contains bone, values were normalized by BV/TV (assessed on contralateral femoral neck), thereby more accurately estimating the properties of the material. Raloxifene significantly increased all three parameters, compared with vehicle. Data are presented as mean ± SE.

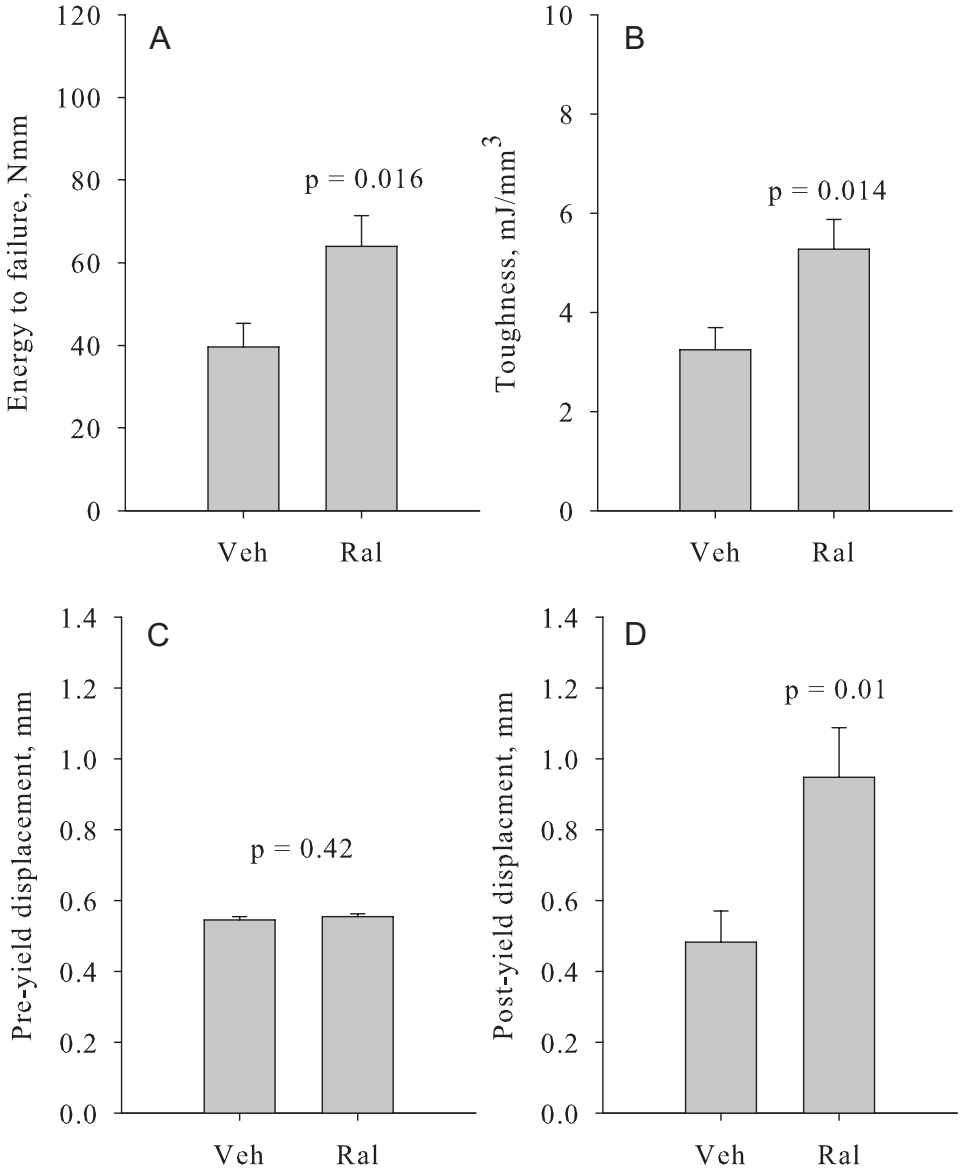

FIG. 4. Raloxifene alters energy to failure and toughness of cortical bone through changes in postyield displacement. Prismatic beams machined from the femoral diaphysis were subjected to four-point monotonic bending. Raloxifene significantly increased energy absorption (A) and toughness (B) compared with bone from vehicle-treated animals (A). These changes resulted from significant increases in postyield displacement (D) with no change in preyield displacement (C). Data are presented as mean ± SE.

$P = 0.52$), 150% (+14%; $P = 0.52$), and 175% (+11%; $P = 0.83$). Raloxifene specimens had nonsignificantly greater stiffness loss at 200% yield (+20%; $P = 0.31$) compared with vehicle.

Discussion

These results show that raloxifene has positive effects on biomechanical properties of both cortical and trabecular bone independent of changes in bone volume/density. This is consistent with results in these same animals at another bone site (vertebra) (3), as well as results from clinical trials where raloxifene significantly reduces vertebral fracture risk despite minimal changes in bone density (4–6). Although the material-level benefits of raloxifene are clear, the mechanism for such changes remains to be determined.

Raloxifene appears to alter the properties of preexisting bone tissue, as opposed to altering the composition of newly synthesized matrix. This is best illustrated by the significant changes in cortical bone properties such as energy to failure and toughness. Intracortical bone turnover in long bone diaphysis of this age beagle is relatively slow (17). Although this dose of raloxifene (0.50 mg/kg·day) was not found to significantly suppress intracortical bone turnover of the rib in these same animals (18), this degree of alteration in tissue toughness would seem rather large to be accounted for by a change in such a small percentage of the tissue. Consequently, the most likely scenario is that the material property changes caused by raloxifene treatment are occurring at least in part within the preexisting bone matrix.

Because there is no significant change in the trabecular bone volume or cortical bone mineral density after raloxifene treatment in this study, we hypothesize that raloxifene improves mechanical properties by altering properties of the organic matrix. Collagen is generally considered to contribute primarily to the postyield properties of bone (19) including such parameters as energy absorption and toughness (20–22). Analyses of vertebral bone of these same animals failed to find significant differences in collagen cross-links (pyridinoline, deoxypyridinoline), advanced glycation end

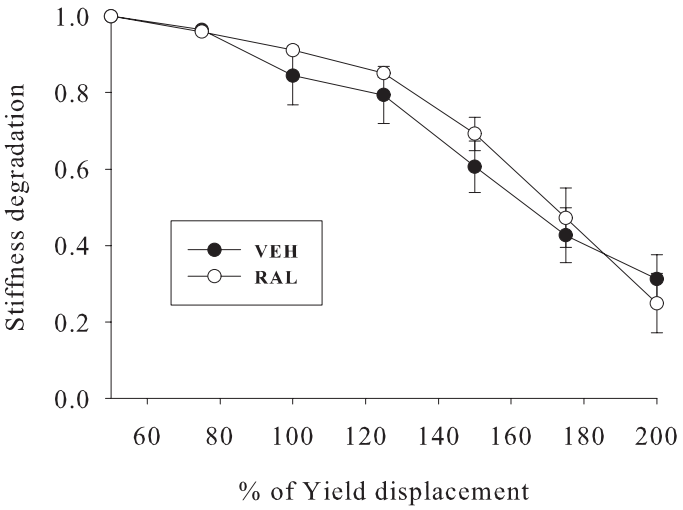

FIG. 5. Stiffness degradation of cortical bone specimens during relaxation testing. Specimens were progressively loaded to increasing percentages of preyield displacement, determined from monotonic tests, with stiffness measured after each loading cycle (see text for more details on the testing protocol). Each data point represents the change in stiffness from baseline after each of the six loading cycles. There was no significant difference in the percentage of stiffness loss between vehicle and raloxifene specimens at any stage of the test. Data are presented as mean ± SE.

products (pentosidine), or collagen isomerization (ratio of α/β C-telopeptide) between vehicle and raloxifene-treated animals (23). However, there remain several other potential changes to the collagen matrix that could manifest changes in biomechanical properties. For example, it is possible that raloxifene could alter the concentrations of divalent cross-links (hydroxylysinonorleucine or dihydroxylysinonorleucine) or pyrrole cross-links, changes that each could alter mechanical properties (21, 24). Raloxifene could also affect the interaction between the organic matrix and mineral crystals, known to influence biomechanical properties (22). Alternatively, these effects could be related to suppression of osteocyte apoptosis, through reduction of reactive oxygen species, by raloxifene (25).

The positive effects of raloxifene on material-level biomechanical properties were not as evident in the cyclic relaxation tests. At displacement levels corresponding to those achieved during the quasi-static tests (<150% yield displacement), the specimens from raloxifene-treated bones tended not to lose as much stiffness compared with vehicle. Although these changes were not statistically significant, the trends are consistent with the results from the quasi-static tests. It is important to note that although we used this relaxation test to assess cyclic loading properties of the material, the protocol was developed to assess differences in the propensity of the bone to form, accumulate and/or propagate microdamage (16, 26). Our results suggest that the propensity to initiate/accumulate damage under cyclic loading conditions is not significantly altered with raloxifene. Whether these results are indicative of an absence of alterations in the true fatigue properties of the material with raloxifene remain unclear.

Clinical trials with raloxifene have not demonstrated significant nonvertebral fracture risk reduction in postmenopausal women (5, 6). Use of the femoral neck in the current study to assess trabecular bone properties was not meant to address issues of hip fracture efficacy, but rather was chosen due to its general size and appreciable amount of trabecular bone. Nevertheless, these results may not be completely inconsistent with the results of the clinical trials. The Multiple Outcomes of Raloxifene Evaluation study had only 12% power to detect a 20% reduction in hip fractures (5, 6). This power was likely further compromised by the removal of a greater number of women from the placebo group (compared with raloxifene) because of rapid bone loss or multiple vertebral fractures during the study. Finally, in a subset analysis of those women with the lowest *t* scores (−2.5) at the femoral neck, a significant reduction in nonvertebral fractures was noted (27). Moreover, the material properties of the bone tissue are just one of many factors in the etiology of a hip fracture. It is also true that, although we detected a large and significant improvement in the material properties of both trabecular and cortical bone in the femur, strength and stiffness of the whole bone were not assessed.

These data should be considered within the context of various study limitations. Our study used intact female beagles; therefore the effects of raloxifene in the absence of estrogen, or in a model with established osteoporosis, could differ. In addition, we have assessed only one dose of raloxifene (chosen to produce serum levels within the range of those from postmenopausal women treated with the 60-mg dose) and, therefore, cannot be assured similar results would occur at other doses. As the trabecular bone tested during RPC remains connected to the cortical shell, some degree of load-sharing occurs during the test. Although this is assumed to be similar between treatment conditions (as bone volume was not changed), we cannot discount that the contribution of the cortical shell may play some role in differences in trabecular bone properties of the specimens from the femoral neck.

In conclusion, we show that raloxifene imparts significant improvements in the material-level properties of both cortical and trabecular bone. As these changes were found to occur in both trabecular and cortical bone, they appear to be independent of turnover suppression and therefore occur in preexisting bone. The changes in material-level properties observed with raloxifene are consistent with alterations in the organic matrix. This may help explain how raloxifene reduces osteoporotic fractures despite modest changes in bone mass.

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