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Long-term ovariectomy decreases ovine compact bone viscoelasticity

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Abstract

Changes in bone mineral density associated with estrogen depletion in humans do not account for all of the associated change in fracture risk, and it is possible that some of this variation may lie in changes of other aspects of bone quality. The purpose of this study was to investigate changes in viscoelastic behavior of compact bone that may be associated with estrogen depletion. Changes in compact bone viscoelastic properties associated with three years of ovariectomy were investigated with dynamic mechanical analysis (low-amplitude 3-point bending at frequencies of 1–20 Hz) using beams milled from the diaphysis of the ovine radius. The viscoelastic storage modulus was significantly (5.2%) lower at the higher frequencies for the ovariectomized animals. The general anatomic variation in storage modulus, in which cranial sectors had higher values than caudal sectors, did not change with ovariectomy. The loss tangent ($\tan\delta$, a measure of damping) was also greatly decreased (up to 83%) at high frequencies in the ovariectomized animals. Anatomic variation in $\tan\delta$ at low (6–12 Hz) frequencies (cranial and caudal sectors having higher values than lateral or medial sectors) was enhanced with ovariectomy. Changes in viscoelastic properties associated with long-term estrogen depletion could be responsible for a significant reduction in the toughness or strength of a bone without concomitant changes in screening modalities used to evaluate bone quality (e.g., DXA, QCT, QUA).

Keywords: Viscoelasticity; Compact bone; Dynamic mechanical analysis; Loss tangent; Storage modulus

Introduction

Compact bone is a viscoelastic material [8,15,27]. Using constant strain-rate experiments, Carter and Hayes [8] determined that the Young's modulus of trabecular bone is proportional to strain rate, raised to a small (0.06) power. (In that report, no difference in strain rate effects was found between cancellous and compact bone.) Whether this relationship changes significantly with age or disease in compact bone has not, to our knowledge, been addressed.

Bone loss associated with estrogen depletion is associated with increased fracture risk in humans [36]. While much of the bone loss seen in postmenopausal osteoporosis is from the cancellous envelope, significant losses are also seen in compact bone [1–3,35] which may contribute to the risk of fracture [31,34]. Standard screening methods (e.g., dual-energy X-ray absorptiometry (DXA), quantitative computed tomography (QCT), or quantitative ultrasound (QUS)) may not be effective at identifying the population at risk of fracture [6,10,16,28]. While some of the variability in the

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prediction of fracture risk must be attributed to a lack of knowledge of in vivo loads, it is also possible that changes in bone tissue not visible to X-ray or ultrasound methods are occurring that could alter the bone strength.

It has been shown [8,11,32,38] that the viscoelastic properties of cortical and cancellous bone correlate with the ultimate strength and toughness of the tissue. It is not yet clear whether viscoelastic parameters are a dominant player in the prediction of fracture risk. However, viscoelastic properties are known to be significant predictors of the impact strength of polymers [30], and the strain rate dependence of bone can account for up to a fifth of the strength of the human femur during high speed loading [9]. If changes in the viscoelastic properties of bone material accompany estrogen depletion, in the absence of a loss of the linkage between strength and viscoelastic properties, then fracture risk could be increased dramatically without changing bone mineral content—the property measured by X-ray based imaging.

We have shown [23] that short-term (1 year) estrogen depletion in the female sheep can result in subtle but structurally-significant changes in the distribution of bone density within the compact bone of the radial diaphysis, possibly altering the preferred direction of bending. The hypothesis that there is a preferred bending axis in long bones (that is, a direction in which the bone will bend regardless of the loading pattern) is well supported as both a matter of geometry [5] and of material property distribution [25]. Changes in the preferred mechanical bending axis would automatically change the safety factor of the bone, assuming that muscle loads are unchanged. In addition, changes in the distribution of viscoelastic mechanical properties could make the preferred bending axis dependent on loading rate and lead to the possibility that a bone's structural strength could be compromised for falls but not for the slower loading rates associated with walking or normal activities (again, assuming no change in the basic linkage between bone strength and viscoelastic properties).

The objective of this study was to evaluate the time-dependent mechanical properties of compact bone material as a function of long-term ovariectomy. A second objective of this study was to evaluate any alterations in the anatomic variation of these time-dependent material properties within the compact bone structure that may be associated with long-term ovariectomy.

We hypothesized that the dependence of the material stiffness and damping efficiency on stress frequency would be altered with long-term ovariectomy. We further hypothesized that there would be a significant change in the distribution of these material properties associated with long-term ovariectomy.

Materials and methods

One means of non-destructively evaluating the viscoelastic properties of a material uses subyield oscillatory tests at a range of frequencies [13,37]. If an oscillating stress σ is applied to a linear viscoelastic material at an angular frequency ω:

$$\sigma = \sigma_0 \cos \omega t \quad (1)$$

then the resulting strain ε can be defined as

$$\varepsilon = \varepsilon_0 \cos(\omega t - \delta) \quad (2)$$

where δ is the phase angle between σ and ε. The complex modulus of the material under these conditions, E^*, can be defined as

$$E^* = E_1 + iE_2 \quad (3)$$

where E_1 is the real or storage modulus (roughly equivalent to the Young's modulus in non-oscillatory tests):

$$E_1 = (\sigma_0/\varepsilon_0) \cos \delta \quad (4)$$

and E_2 is the imaginary, dynamic, or loss modulus:

$$E_2 = (\sigma_0/\varepsilon_0) \sin \delta \quad (5)$$

The loss tangent, or $\tan \delta$, is defined as

$$\tan \delta = E_2/E_1 \quad (6)$$

and is a measure of how effectively the material can damp an oscillatory stress. While we recognize that bone is not likely to be a perfectly linearly elastic material at any stress or strain rate [20], we chose to use this approach to viscoelastic properties due to its ease of use and theoretical rigor.

Under local IACUC approval, 5-year-old Warhill female sheep were anesthetized and ovariectomized (OVX, $N = 6$) or subjected to a sham surgery (Control, $N = 6$), as part of other studies. The sheep were kept outdoors under dry lot conditions at an elevation of approximately 1600 m, and were fed a mixture of alfalfa and grass hay. After three years, the animals were sacrificed with an intravenous overdose of a barbiturate, and the left radius/ulna (the two bones functionally fuse early in life) were harvested and stored at 20 °C. Cortical thickness (Cranial, Caudal, Lateral, and Medial) at mid-diaphysis of the radius was measured from radiographs taken postmortem, after all soft tissue had been removed.

Multiple 2 × 2 × 19 mm (craniocaudal × lateromedial × proximodistal) beams were cut from each of six radial diaphyseal sectors (craniomedial, cranial, craniolateral, caudomedial, caudal, and caudolateral, Fig. 1) under cold water irrigation, and stored in 0.9% saline solution at −20 °C.

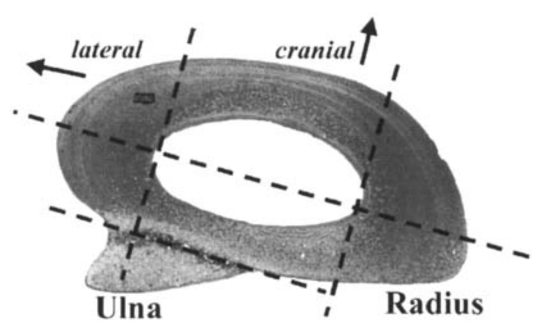

Fig. 1. Location of specimens, section of ovine left radius and ulna at mid-diaphysis. Cranial is to the top, lateral is to the left. The radius and ulna are fused before adulthood in the sheep. To make the beams, the ulna was removed, and the remaining radius was cut in half along the lateromedial axis. Beams were then cut, oriented with two faces parallel to the lateromedial cut, and two faces perpendicular to the lateromedial cut. Up to 6–8 beams could be made from each of the six anatomical sectors, and one was chosen at random from each sector for testing.

One beam from each of the six sectors was chosen at random for mechanical testing. The beams were thawed and tested in 3-point bending (outer supports 15 mm apart), in 0.9% saline solution at 37 °C in a dynamic mechanical analyser (DMA7e, PerkinElmer). The test was conducted under force control. Each beam was tested in a craniocaudal orientation, with the cranial side of the beam placed on the tension side of the test. (Beams were also subsequently tested in lateromedial bending; those data are not shown here.) A static load of 550 mN and dynamic load of 500 mN were applied in a frequency scan from 1 to 20 Hz at 0.2 Hz intervals. Each test was run in triplicate, with at least 40 min between tests for an individual specimen. E_1 and $\tan\delta$ were calculated at each frequency.

Statistical analysis

The plot of the storage modulus E_1 as a function of test frequency for each test was fit to an exponential model ($E_1 = E_1^* f^b$), where f is the applied stress frequency in Hz. The coefficient E_1^*, and exponent b were used as dependent variables in a two-way repeated-measures analysis of variance (ANOVA), using treatment (OVX or control) and anatomic location as categorical variables. Alpha for main effects was set at $p = 0.05$, at which time post hoc Fisher's LSD tests were used to distinguish between levels of the main effect. Alpha for interactions was set at $p = 0.10$, at which time post hoc Fisher's LSD tests were used at $p = 0.05$ to distinguish between levels. Similarly, $\tan\delta_f$ (where $f = 1, 3, 6, 9, 12, 15, 18$, and 20 Hz) was used as dependent variables in a series of two-way repeated-measures ANOVAs, examining the effect of treatment and anatomic location. Finally, the effect of treatment and anatomic location (cranial, caudal, lateral and medial) on mid-diaphyseal cortical thickness was tested using a 2-way repeated-measures ANOVA.

All output variables were tested for normality (Kolmogorov–Smirnov test with Lilliefor's correction, $\alpha = 0.05$) and for equal variance between groups (Levene median test, $\alpha = 0.05$). Where variables were found to not meet these normality and equal-variance criteria, the two-way repeated-measures ANOVAs were run a second time with rank-transformed data. In no cases were the results of these tests qualitatively different from those using the original data; we report here only the results of the original tests.

Results

There was a very small but statistically-significant difference ($p = 0.016$) in the 1-Hz storage modulus (coefficient E_1^*) between the bone material from OVX and control animals (The mean value for OVX bone material was 0.5% greater than that for control bone material, Table 1). There was a significant anatomic variation ($p = 0.012$) in this parameter (numerically equal to Young's modulus in a monotonic test), with material from the cranial half of the bone having higher values than material from the caudal half. This distribution did not demonstrably change with long-term estrogen depletion (interaction $p = 0.649$).

There was a significant decrease in the stress-rate sensitivity (as represented by the exponent b) that was associated with long-term estrogen depletion ($p = 0.009$, Table 1). No overall anatomic variation in this parameter could be demonstrated ($p = 0.787$), nor could a change in the distribution of this parameter be demonstrated with long-term estrogen depletion (interaction $p = 0.659$).

When these two parameters are taken together (Fig. 2), there is a profound loss of storage modulus that is associated with long-term estrogen depletion, a loss that is especially evident at higher frequencies.

At frequencies of greater than 3 Hz, the bone material also showed a significant decrease in $\tan\delta$ (a measure of the damping efficiency of the material) that was associated with long-term ovariectomy ($p < 0.004$, Fig. 3).

At frequencies of 12 Hz or less, the bone material showed a significant ($p < 0.032$) anatomic variation in the distribution of damping efficiency ($\tan\delta$), with sectors in the cranial and caudal aspects of the radius demonstrating significantly higher values for this parameter than did material from sectors on the lateral and medial aspects (Fig. 4). This pattern of $\tan\delta$ distribution was demonstrably altered with long-term ovariectomy (interaction $p < 0.074$, post hoc tests $p < 0.05$, Fig. 5) in the frequency range 6–12 Hz, becoming less homogeneous with ovariectomy. This change in anatomic variation

Table 1
Comparison of values (mean ± standard deviation) of the coefficient E_1^* and the exponent b of the $E_1 = E_1^* f^b$ equation, as a function of treatment (there were significant differences between treatment groups for both parameters. The effect of these parameters on the frequency-dependent behavior of the material is shown in Fig. 2)

Parameter	Control	OVX	p (Effect of treatment, from 2-way repeated-measures ANOVA)
E_1^* (GPa)	10.466 ± 0.080	10.521 ± 0.082	0.016
b	0.043 ± 0.024	0.026 ± 0.026	0.009

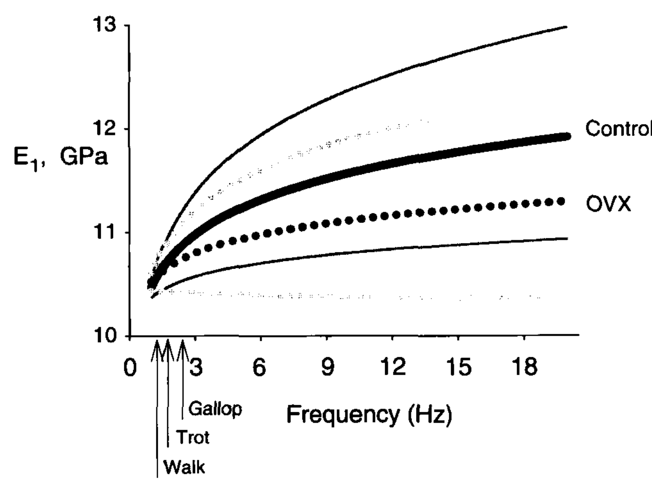

Fig. 2. Effect of long-term ovariectomy on the storage modulus E_1, mean ± one standard deviation (grey lines). Data from individual tests were fit to the equation $E_1 = E_1^* f^b$; this graph shows the overall effect of changes in the parameters seen in Table 1 on the storage modulus as a function of test frequency. Arrows indicate stride frequencies of the sheep at the walk, trot, and gallop [12]. While there is virtually no difference in this parameter between bone from OVX and control animals at low frequencies, at higher frequencies there is a significant effect of the changes in the coefficient E_1^* and the exponent b.

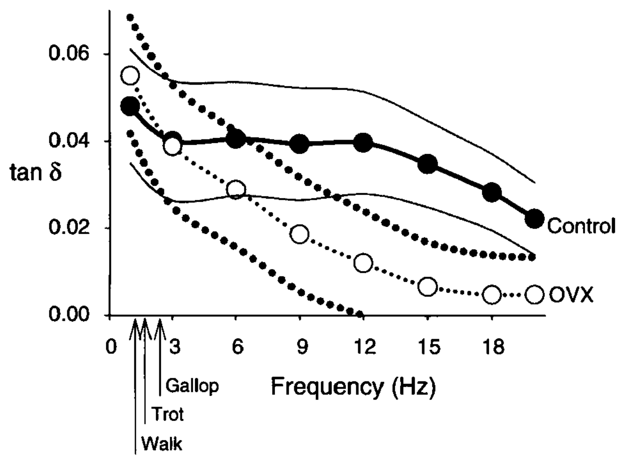

Fig. 3. Effect of long-term ovariectomy on $\tan\delta$, a measure of the damping characteristics of the material, mean ± one standard deviation (grey lines). Arrows mark stride frequencies at the walk, trot, and gallop for sheep [12]. At frequencies greater than 3 Hz, there was a significant decrease in $\tan\delta$ associated with ovariectomy, implying that the bone material had become less efficient at damping oscillatory stresses.

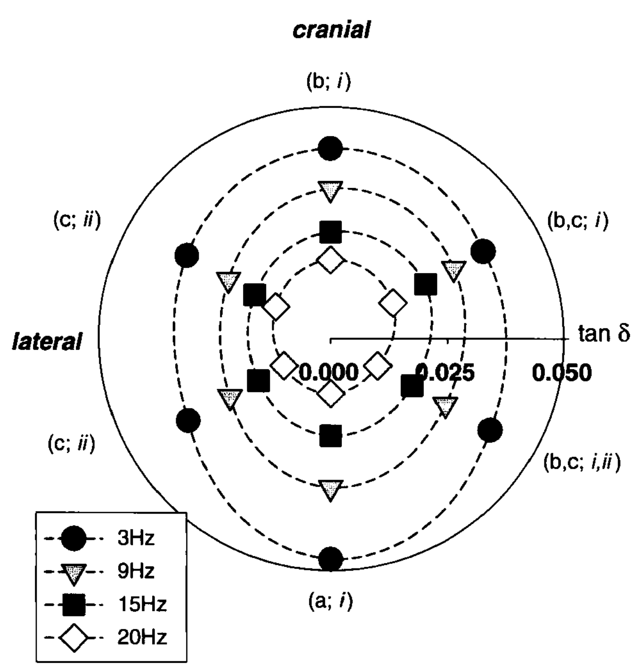

Fig. 4. Anatomic variation in $\tan\delta$, as a function of oscillatory frequency (Combined OVX and sham bones: See Fig. 5 for those frequencies in which there was a reasonable possibility of a different anatomic variation between bone material from overiectomized and sham-operated sheep). The anatomic orientation of this polar graph is roughly that of Fig. 1, with cranial sectors to the top, and lateral sectors to the left. The radial position of the data points is proportional to the value of the damping parameter $\tan\delta$. Letters in lower case, and Roman numerals, reflect the results of post hoc tests for 3 and 9 Hz data, respectively. Sectors that share a lower-case letter, for instance, are not significantly different from one another at 3 Hz. Above 12 Hz, there was no demonstrable anatomic variation in $\tan\delta$. At lower frequencies, there was a trend towards higher values of $\tan\delta$ in the cranial and caudal aspects, and of lower values of $\tan\delta$ in the lateral and medial aspects.

of $\tan\delta$ did not occur at other frequencies (interaction $p > 0.213$).

Treatment had no significant main effect ($p = 0.306$) on mid-diaphyseal cortical thickness. There was a significant effect ($p = 0.001$) of anatomic site on cortical thickness (lateral > medial > cranial and caudal), and no significant interaction between treatment and anatomic site (interaction $p = 0.935$).

Discussion

The viscoelastic properties of ovine compact bone are clearly affected by three years' ovariectomy. At low test frequencies (through the primary stride frequencies of the sheep through the gallop [12]), there was little effective difference between the bone material of the control and the OVX animals. At higher frequencies, however (e.g., 15 Hz, frequencies implicated in potentially osteogenic or osteoprotective cellular events [14,18,33]), the storage modulus in the ovariectomized animals is reduced by as much as 5%, and the loss tangent reduced by more than 80%. In general, with ovariectomy, the material became less sensitive to changes in applied stress rate (its stiffness increases less with increases in stress rate), and less efficient at damping oscillatory stresses.

Energy dissipation during an oscillatory stress ($\Delta W/W$) is related to δ [13]:

$$\Delta W/W = 2\pi \sin\delta \tag{7}$$

Thus, if bone is similar to other viscoelastic materials, at 20 Hz, the bone material from our control animals was theoretically able to absorb and subsequently dissipate four times as much energy in damping alone than was bone material from the ovariectomized animals. If, as in equine compact bone [22], the relationship between ash density ρ_a and the energy to yield U_Y is an exponential one:

$$U_Y = 1.05\rho_a^{1.83} \tag{8}$$

then this loss of energy absorptive capability at high frequencies might be equivalent to that seen in a 50% loss of ash density in a monotonic test. Such a loss in energy absorption could have serious implications for the strength of the material at high stress rates such as impact.

Though failure tests were not performed on these specimens, others have demonstrated [8,11,32,38] an association between viscoelastic properties, and bone strength and toughness. If the changes in viscoelastic properties are not accompanied by a change in the linkage between viscoelastic properties and the strength of the material, these results strongly suggest that ovariectomy (and presumably estrogen deficiency) can compromise cortical tissue strength and toughness. If such

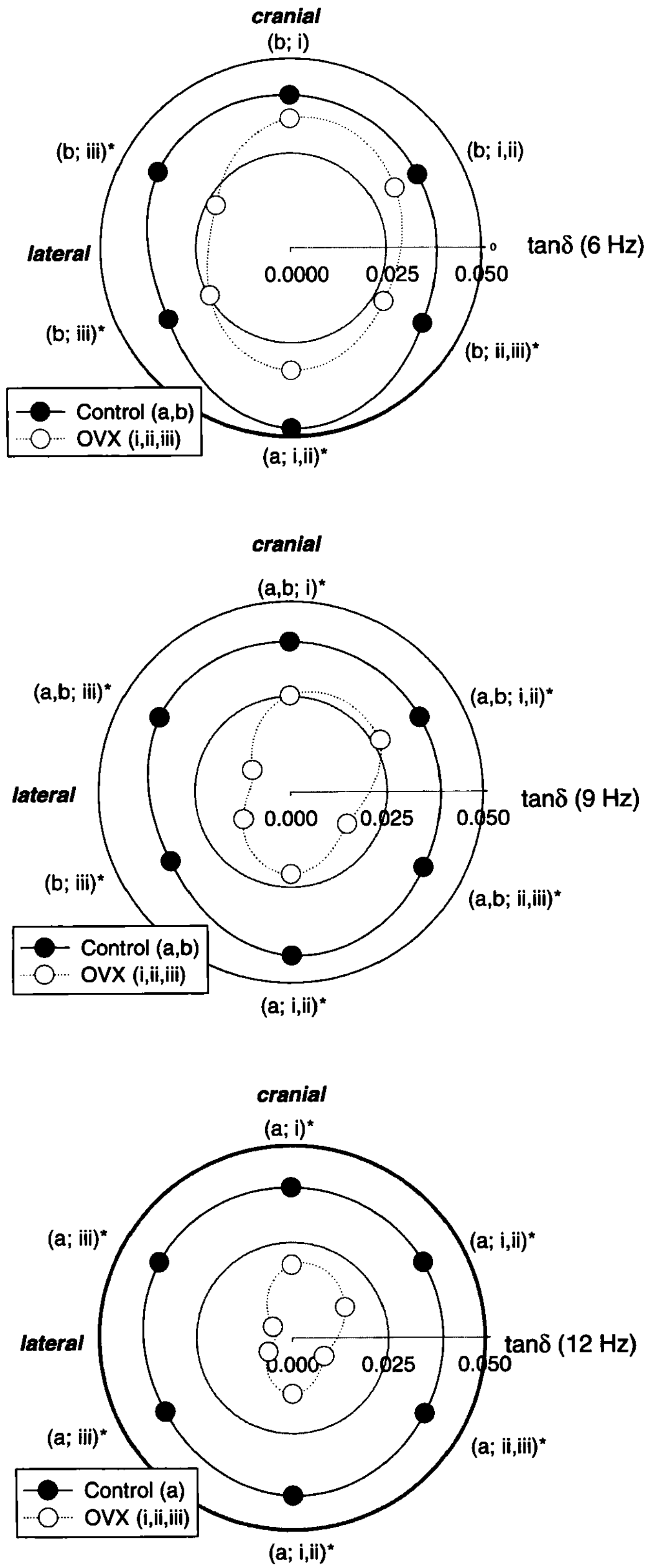

Fig. 5. The anatomic distribution of tan δ in the middle frequencies (6 Hz, top; 9 Hz, middle; 12 Hz, bottom) was altered with 3 years' estrogen depletion. Orientation and post hoc statistical coding is as in Fig. 4. Asterisked sectors indicate values that are significantly different between control and OVX animals. In general, tan δ in the middle frequencies had a homogeneous distribution in the control animals. In the OVX animals, however, there was a marked heterogeneity in these properties, with more efficient damping of stress oscillations found in the cranial and caudal sectors, and smaller values of tan δ in the lateral and medial sectors.

changes in strength and toughness, particularly at high stress- or strain rates, are independent of parameters that are measured in standard screening protocols, it could represent a serious compromise in material or structural integrity, one that would be both invisible prospectively to clinicians using current techniques, and most evident precisely in those situations (e.g., impact) in which the bone is most likely to fail.

We have also demonstrated that these changes in viscoelastic properties are largely independent of mineralization, porosity, or histologic evidence of remodeling [24]. We may cautiously speculate that these changes may occur at the level of the bone matrix. Obviously, this is a matter for further study.

This study made use of dynamic mechanical analysis, a non-destructive testing modality that allowed us to investigate the effect of multiple stress rates on a single piece of bone. We used a bone that was large enough for us to manufacture multiple beams from a single diaphysis, allowing us to also investigate regional variation in these material properties.

These sheep were taken out three years from their ovariectomies, relatively long for this animal model, and certainly long enough to account for the known circannual variation in cancellous bone mineral density that is characteristic of this seasonal breeder [17]. Like most models of estrogen-depletion-related bone loss, this is more a model of osteopenia and not of osteoporosis, and most of those changes that have been described are seen in trabecular bone [29].

In vivo strain gauge data [21] suggest that the ovine radius/ulna is normally loaded during service in a combination of axial compression and sagittal bending, making our choice of craniocaudal bending for these materials tests a reasonable one. It is interesting that the highest values of $\tan\delta$ at low frequencies (Fig. 4) were found in the cranial and caudal sectors, which could result in more efficient damping of structural oscillations (possibly postural) in the sagittal plane. The effect of changes in the anatomic variation in damping characteristics with long-term ovariectomy (Fig. 5) on the structural properties of the whole bone remain unclear: it may well be that the effect of the overall decrease in damping characteristics outweighs any possible alteration in a supposed preferred direction of oscillation.

The beams were not tested in other modes (compression, tension, torsion, four-point bending; strain-rate rather then stress-rate control) or at higher frequencies (>20 Hz) than were available on this equipment. We have no data as to whether these changes occur in cancellous as well as in compact bone.

It is possible that the craniocaudal viscoelastic properties reported here correlate with the direction of bone lamellar orientation. However, these specimens were also tested in lateromedial bending (data not shown here), and while we did see ovariectomy-related alterations in viscoelastic anisotropy [26], there did not appear to be a clear correlation between any of our measurements, the anatomic location of the specimens, and the direction of testing. (That is, if lamellar orientation was a major determinant of viscoelastic behavior, one would expect a corresponding rotation of the polar graphs of Figs. 4 and 5 when the material was tested in lateromedial bending. Such rotations were not seen.). Similar tests, conducted with bone material from younger animals who have had less opportunity to secondarily remodel their originally-plexiform bone, could help to clarify this point.

We could not demonstrate in these sheep a change in the distribution of bone stiffness (specifically, E_1^*) with long-term ovariectomy. In contrast with our previous report [23], the distribution of ash density did not change, though the distribution of % mineralization was altered somewhat, becoming more mineralized in the caudal sectors with ovariectomy [24]. The earlier experiment used bone from animals that had been ovariectomized one year prior to sacrifice; the bone material from the current experiment reflects the effects of three years' ovariectomy. It may well be that the effects that we described previously are transient to the increased activation rate that accompanies estrogen depletion [4,7], but may take several remodeling cycles to resolve.

Whether these changes in material properties translate to real changes in the viscoelastic properties of the bone structure as a whole was not tested. Courtney et al. [9], in an in vitro study of human femora, were not able to demonstrate a significant interactive effect between age and displacement rate, or between gender and displacement rate, in the stiffness and fracture load of their constructs. Such significant interactive effects would be evidence of an age- or gender-related change in structural viscoelastic properties. However, the small number of specimens used in that study (10 older donors, 10 younger donors; 7 females; 13 males) may not have provided sufficient statistical power to conclusively address this issue.

Whether these changes occur in species other than *Ovis aries* has not, to our knowledge, been investigated. There are obvious public-health implications to this issue: the screening modalities that are commonly used to diagnose and evaluate osteoporosis in the human (e.g., DXA, QCT, QUA), in effect, measure the mineral density and/or porosity of the structure. These tests are currently neither sensitive nor specific for fracture risk assessment on an individual basis before quite large changes are evident [6,10,16,28]. Changes in viscoelastic properties that are unaccompanied by changes in mineral density or microarchitecture could potentially lead to either an increase or a decrease in the risk of fracture that would be unrelated to the findings of these clinical tests. Accounting for time-dependent bone material properties in screening protocols could improve these protocols' predictive value.

Finally, these data raise the intriguing issue of how the viscoelastic properties of the bone matrix affects the nature of the mechanical signal received by the cells embedded within that matrix. Jacobs et al. [19], using an in vitro system of osteoblast-like cells and relatively low frequencies (up to 2 Hz), showed a decreased responsiveness of cells to oscillating or pulsatile fluid flow with increased frequency, and related it to changes in fluid flow transport across cell membranes. Leaving aside for the moment the issue of how the regional variation in matrix damping characteristics is established and maintained, the effect of such regional variation in matrix damping characteristics could serve to modulate the physical signal that is received by the bone cells, to keep it within a range to which the cell is responsive.

In summary, three years' ovariectomy in the sheep is associated with significant deterioration in time-dependent bone material properties. Inasmuch as fracture risk of the bone structure may be dependent on viscoelastic properties of bone material, such changes, should they also occur in the postmenopausal human, would likely not be identified in screening modalities that measure, in effect, bone mineral density or porosity.

Acknowledgement

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