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Ultra-Short Echo-Time MRI Detects Changes in Bone Mineralization and Water Content in OVX Rat Bone in Response to Alendronate Treatment

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Abstract

In this work we hypothesize that bisphosphonate treatment following ovariectomy manifests in increased phosphorus and decreased water concentration, both quantifiable nondestructively with ultra-short echo-time (UTE) ^{31}P and ^1H MRI techniques. We evaluated this hypothesis in ovariectomized (OVX) rats undergoing treatment with two regimens of alendronate. Sixty female four-month old rats divided into four groups of 15 animals each: ovariectomized (OVX), OVX treatment groups *ALN1* and *ALN2*, receiving $5\mu\text{g/kg/day}$ and $25\mu\text{g/kg/day}$ of alendronate, and a sham-operated group (*NO*) serving as control. Treatment, starting one week post surgery, lasted for 50 days at which time animals were sacrificed. Whole bones from the left and right femora were extracted from all the animals. ^{31}P and ^1H water concentration were measured by UTE MRI at 162 and 400 MHz in the femoral shaft and the results compared with other measures of mineral and matrix properties obtained by ^{31}P solution NMR, CT density, ash weight, and water measured by dehydration. Mechanical parameters (elastic modulus, EM, and ultimate strength, US) were obtained by three-point bending. The following quantities were lower in OVX relative to *NO*: phosphorus concentration measured by ^{31}P -MRI (−8%; 11.4 ± 0.9 vs 12.4 ± 0.8 %, $p<0.005$), ^{31}P -NMR (−4%; 12.8

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± 0.4 vs 13.3 ± 0.8 %, $p < 0.05$) and μ -CT density (-2.5 %; 1316 ± 34 vs 1349 ± 32 mg/cm³, $p = 0.005$). In contrast, water concentration by ¹H-MRI was elevated in OVX relative to NO ($+6$ %; 15.5 ± 1.7 vs 14.6 ± 1.4 %, $p < 0.05$). Alendronate treatment increased phosphorus concentration and decreased water concentration in a dose-dependent manner, the higher dose yielding significant changes relative to values found in OVX animals: ³¹P-MRI ($+14$ %; $p < 0.0001$), ³¹P-NMR ($+9$ %; $p < 0.0001$), ash content ($+1.5$ %; $p < 0.005$), μ -CT mineralization density ($+2.8$ %; $p < 0.05$), ¹H-MRI, (-19 %, $p < 0.0001$). The higher dose raised phosphorus concentration above and water concentration below NO levels: ³¹P-MRI ($+6$ %; $p < 0.05$), ³¹P-NMR ($+5$ %; $p = 0.01$), ash content ($+1.5$ %; $p = 0.005$), ¹H-MRI (-14 %; $p < 0.0001$), drying water (-10 %; $p < 0.0005$). Finally, the group means of phosphorus concentration were positively correlated with EM and US ($R^2 \geq 0.98$, $p < 0.001$ to $p < 0.05$) even though the pooled data from individual animals were not. The results highlight the implications of estrogen depletion and bisphosphonate treatment on mineral composition and mechanical properties and the potential of solid-state MR imaging to detect these changes *in situ* in an animal model of rat ovariectomy.

Introduction

Estrogen deficiency causes increased activation frequency for bone remodeling (13). The chief consequences are an increase in osteoclasts and resorption lacunae. There is also evidence that the reduced levels of estrogen decrease bone formation, albeit to a lesser extent than the increase in bone resorption (18). In cancellous bone excessive resorption results in net loss of bone volume along with structural deterioration of the trabecular network whereas in cortical bone a decrease in cortical thickness occurs, along with increased resorption cavities resulting in trabecularization of the endosteum (39).

Meunier et al (20) in ovariectomized baboons, provided compelling evidence that increased bone turnover results in decreased mineralization since the shortened remodeling cycle does not allow secondary mineralization to occur. They also showed that upon treatment with alendronate, mineralization increased beyond that in control animals. In studies in postmenopausal women with osteoporosis undergoing antiresorptive treatment, Boivin et al (6) and Roschger et al (28) noted increased mineralization density in both trabecular and compact bone. Effects of ovariectomy on mineralization density have also been reported in rodents, paralleling some of the observations in primates and humans. Huang et al found substantially reduced mineralization on the basis of chemical analysis of calcium and phosphorus in rat tibial cortex upon ovariectomy (17). Yao et al observed reduced bone mineralization upon ovariectomy in rat vertebrae and a partial reversal of this effect upon antiresorptive treatment (37). Since the majority of the bone's compressive strength, typically 60–80%, is attributed to its mineral content (29) reduced bone mineralization should have significant mechanical consequences independent of the bone's macrostructural deterioration. In fact, the degree of mineralization was found to correlate with the mechanical properties of trabecular bone, even after accounting for differences in bone volume fraction and architecture (15).

During bone formation, matrix is initially laid down by osteoblasts. Subsequently, under retention of whole-bone volume, mineral is deposited onto the osteoid while displacing the loosely bound bone water (12). The inverse relationship between mineral and water density has been shown in previous work in which hypomineralization induced in a rabbit model of osteomalacia was found to be paralleled by an increase in water concentration (14). In the present work we extend this idea and hypothesize that the expected reduced secondary mineralization following ovariectomy is associated with increased bone water concentration. Further, since stiffness has long been known to decrease with decreasing mineral content (see, for example, (10)) and water content is known to increase as mineral content decreases (38),

we expect both compositional parameters to be associated with the bone's elastic modulus and ultimate strength.

During the past two decades a number of nitrogen-containing bisphosphonates have emerged as leading effective treatments for postmenopausal osteoporosis (27). Depending on dosage and the particular agent used, treatment results in an increase in overall bone mass (3), which is in part due to increased bone mineralization attributed to the bone matrix undergoing secondary mineralization (7,20).

True bone mineralization density (also referred to as 'degree of mineralization of bone', (7)) is difficult to measure nondestructively since the clinical modalities for evaluation of bone density (dual X-ray absorptiometry (DXA) and quantitative computed tomography (CT)) measure only apparent density (except at micrometer resolution, such as in the case of synchrotron μ CT (19)). Thus, the latter methods cannot distinguish reduced volume of fully mineralized bone (as in osteoporosis) from a larger volume of hypomineralized bone (as in osteomalacia). Even though microradiography (20) and backscattered electron imaging (8) can measure true mineralization density at the tissue level, these techniques are destructive and thus require biopsies when used for drug response monitoring. Thus, a noninvasive technique to evaluate true bone mineralization would be desirable.

During the past 10 years magnetic resonance imaging techniques tailored for the detection of spins in solid biomaterials have emerged as potential nondestructive tools for the quantification of bone mineral constituents (2,35). The molecular motions for spins embedded in a solid matrix such as in the case of phosphorus or protons in cortical bone are too slow to fully average out the spin-spin dipolar interactions on the NMR time scale (absence of motional narrowing). This, in turn, leads to extremely short T_2 relaxation times (1). Conventional Cartesian imaging techniques using phase encoding after excitation, therefore, yield very little or no signal from bone water and mineral constituents.

One promising approach toward imaging short- T_2 spins is based on ultra-short echo-time (UTE) radial acquisition schemes with non-selective hard pulse excitation (35) or spatially selective half-pulse excitation (26) of k-space. Recent work from this laboratory on 3D solid-state MRI of ^{31}P and ^1H (1) in conjunction with hard pulse excitation followed by center-out radial acquisition has been shown to be able to distinguish hypomineralized from normal bone in an *ex vivo* rabbit model of osteomalacia.

We tested the hypothesis that ^{31}P and ^1H UTE MR imaging of cortical bone can identify changes in phosphorus and water concentration following ovariectomy as well as in response to bisphosphonate treatment in a rat model of postmenopausal osteoporosis, and further, that the expected changes are biomechanically significant. Toward these objectives, a three-dimensional radial projection imaging sequence described previously (1,2) was used to quantify bone mineral phosphorus and bone water. The water measurements were validated gravimetrically, the phosphorus measurements by high-resolution ^{31}P NMR after dissolution of the bone. Mineralization density was further measured by micro-computed tomography (μ -CT) and measurement of ash weight. Finally, the implications of ovariectomy and antiresorptive treatment were assessed by mechanical testing.

Materials and Methods

Animal model and specimen preparation

The OVX rat is often chosen as a model for postmenopausal osteoporosis because of the similarities in bone remodeling seen in humans (21,22). While most work has focused on cancellous bone much less is known about the effect of estrogen deficiency in cortical bone of

rodents but there is evidence that intracortical remodeling is enhanced following ovariectomy (4). Further, the *OVX* rat is one of two models recommended by the Food and Drug Administration (FDA) for the evaluation of the efficacy of drug treatment of osteoporosis and its prevention (31). There appears to be little consistency in alendronate dosing used in research studies of *OVX* rats (ranging from 2 µg/kg/day (25) to about 100 µg/kg/day (11)). Significant changes in bone mineralization were generally observed for doses above 20 µg/kg/day only. In the present study two different regimens were chosen, one above this value and one near the lower end of the above range in order to investigate the dose dependent sensitivity of UTE imaging technique.

The present study involved sixty female rats (Sprague Dawley; four months old) divided into four groups of fifteen each. The first group was ovariectomized to induce osteoporosis (*OVX*). The second and third groups (denoted *ALN1* and *ALN2*, respectively) also were ovariectomized but received two different daily doses of alendronate subcutaneously as treatment (5µg/kg/day and 25 µg/kg/day respectively). The fourth group was sham operated (*NO*) to serve as a control. Both *OVX* and *NO* groups were administered saline. The animals were either ovariectomized or sham operated by the vendor and arrived one week after surgery. Treatment with alendronate or saline started immediately thereafter. All animals received standard chow and water *ad libitum* for 50 days at which time they were euthanized by CO₂ asphyxiation. Whole bones from the left and right femora were extracted from the animals after cleaning the bones of external soft tissue. The specimens were then wrapped in aluminum foil and kept frozen until the time of experiments. For the experiments 2 cm long pieces centered at the midpoint of the femur were sectioned from both the left and right femurs and the marrow was removed by repeated rinsing with a high-pressure water jet. Different sections of these bones were used for the various experiments detailed below and summarized in Figure 1.

Tubular pieces (1 cm in length) from the right femoral shaft were sectioned with a circular electric saw to measure mineral phosphorus and matrix water using solid-state UTE MRI as described previously (1,2). For comparison with established techniques, water and mineralization metrics were also quantified destructively. Plate-like cortical bone specimens with dimensions 10 mm × 3 mm × cortical thickness were sectioned from the posterior sides of the right femoral shaft to estimate water and ash content by gravimetry. The residue after incineration of the specimens was used to quantify phosphorus concentration by ³¹P high resolution NMR spectroscopy. Specimens from the left femur were used for assessing mineralization density by high-resolution µ-CT imaging. From the cross-sectional moment of inertia obtained from these high-resolution images the specimens' material properties were obtained via mechanical testing by three-point bending as described before (14). All specimens were soaked in saline to allow rehydration (to recover water that might have been lost during specimen preparation) until the time of the experiments.

The research was performed in compliance with federal regulations and the guidelines of the Institution's Animal Care and Use Committee (IACUC).

Magnetic Resonance Imaging and Spectroscopy

Both the solid-state imaging and spectroscopy pulse sequences were implemented on a vertical-bore 9.4 T micro-imaging system (Bruker Avance DMX-400, Bruker Instruments, Billerica, Massachusetts) equipped with gradients of 100 G/cm maximum strength and 100 µs ramp time. Operating frequencies were 162 MHz for phosphorus and 400 MHz for proton, respectively. The imaging experiments were performed with an NMR probe in conjunction with a bird-cage type radiofrequency (RF) coil for ¹H experiments and a home-built solenoidal RF coil of length 2.7 cm and diameter 1 cm with 8 turns for ³¹P similar to the coil used in (2).

³¹P and ¹H image acquisition and processing—Details of the solid-state imaging sequence used have been given in previous papers from this laboratory (1,2). In brief, the specimens were scanned by collecting a series of radial readouts after excitation by a nonselective RF pulse, which was followed by radial sampling of angular projections. In this manner signal loss due to short T₂ relaxation times of ³¹P and ¹H is minimized. The magnetic field gradients were modulated simultaneously in all three spatial directions so as to sample k-space uniformly on sixty-five coaxial cones in equally spaced angular increments, resulting in a total of 2626 views on the surface of a unit sphere. Ramp sampling was incorporated to map the center of k-space. In order to quantify bone phosphorus/water, the bone specimens were co-imaged with reference capillaries having known concentrations of ³¹P and ¹H. The reference capillaries were calibrated with a series of samples of varying H₂O/D₂O isotopic ratio (for ¹H MRI) and K₂HPO₄ (for ³¹P) yielding calibration curves from which the exact volume fraction of water and phosphorus in the reference solution was obtained. An overview of the imaging and analysis protocol used for both the nuclei is given in Figure 2a. The images were reconstructed after regridding, yielding a nominal isotropic resolution of 157×157×157 μm³ for ³¹P and 139×139×139 μm³ for ¹H (after taking into account the number of points on the linear gradient ramp).

3D bone volume rendering was performed with Image J (NIH, Bethesda, Maryland, USA). Bone phosphorus and water concentration were quantified as weight percent from the mean signal intensity obtained in bone and the reference capillary (from center 20 slices) after appropriate adjustments for the differences in relaxation times between bone and reference, as detailed in (1).

The mean signal intensity of the bone was computed from the intensity histogram after segmenting the images to include the pixels with intensities above the mode of the histogram (Figure 2a). Finally from the measurement of the weight and volume of the specimens, and the known concentration of phosphorus and water in the reference samples, bone phosphorus and water concentration were computed as weight percent. Specimen volume was obtained from high-resolution 3D gradient-echo images after their immersion in doped water.

³¹P Solution NMR spectroscopy (³¹P-NMR)—For comparison with results from ³¹P-MRI, phosphorus concentration also was measured by high-resolution ³¹P-NMR spectroscopy using the NMR system described previously. Details of the methodology are given in (14). The specimens were dissolved in 2 ml of 1.2 M HCl overnight and the resulting solution was diluted four times. ³¹P NMR spectra were acquired in the presence of a reference capillary containing methylene diphosphonate of known concentration and the phosphorus concentration in the specimens quantified from the peak integrals of the ³¹P spectrum and the reference capillary, again after correction for differences in T₁ relaxation times of the reference and bone solutions (Figure 2b).

Gravimetric Analysis

The gravimetric measurements were performed using standard procedures on cortical bone specimens 1 cm × 0.4 cm × cortical thickness in size (obtained from the posterior site of each femur). The initial wet weight of the specimens was obtained after blotting. The specimens were then dried at 100° C for 48 hrs to expel pore and collagen-bound water. The difference between these two weights yielded the weight of bone water, which was calculated as percent wet weight. Finally, the dried specimens were ashed in a furnace for 24 hrs at 600° C yielding mineral content as the ratio of ash weight over wet weight.

Micro-Computed Tomography (μ -CT)

Mineralization density was quantified in specimens of 20 mm length sectioned from left femoral diaphysis by micro-computed tomography (μ -CT) for comparison with the MRI-derived phosphorus concentration as described in (2). High-resolution 3D images ($29 \times 29 \times 29 \mu\text{m}^3$) were acquired on an eXplore Locus SP micro-CT specimen scanner (GE Healthcare, London, Ontario, Canada) with a peak voltage of 80 kV_p. In brief, 440 views were collected at 0.5 degree angular increments in a total scan time of 1 h 14 mins (4 averages per view). Images were collected in the presence of vials containing aqueous K₂HPO₄ ranging in concentration from 100 to 1067 mg/cm³ serving for density quantification. A calibration curve was obtained with the known K₂HPO₄ densities. The images were then thresholded to include pixel values with densities above 800 mg/cm³ and used in conjunction with the calibration curve to obtain the mineralization density of the specimens. The quantification process is illustrated in Figure 2c. The images were also used to obtain the cross-sectional area and moment of inertia of the specimens in order to evaluate material properties, details of which are given in the following section.

Biomechanics

Stiffness and static strength were obtained on the same femur specimens previously used for the assessment of mineral density. Three-point bending (32) was performed on a material-testing machine (Instron Universal, model 8500) as described in (14). The specimens were positioned with the posterior surface on a custom-made anvil with two supports separated by 15 mm. The load was applied at the center of the anterior surface of the femoral shaft with a strain rate of 4.57 mm/min. Elastic modulus and ultimate strength were computed from the load deformation curves by invoking the formulae from bending beam theory. Moment of inertia and location of the neutral axis were computed from the cross-sectional μ CT images (14).

Statistical Analyses

Water and mineral concentrations estimated by the various methods described above were examined to evaluate group differences by one-way ANOVA followed by post-hoc, unpaired, two-sided, student's t-tests for inter-group comparisons using JMP (SAS Institute, Cary, NC) statistical package.

Results

Mean values of magnetic resonance derived phosphorus and bone water concentration, ash weight, mineral density and mechanical parameters for the four groups are summarized in Table 1. Cortical bone phosphorus concentration obtained by ³¹P-MRI was found to be within the range of that found in rabbit tibia in previous work from this laboratory (1). The OVX group had significantly lower values compared to NO animals (11.4 ± 0.9 vs. 12.4 ± 0.8 %; $p=0.002$) suggesting ovariectomy to result in decreased mineralization. A slightly lower difference was found in the solution ³¹P-NMR derived values (12.8 ± 0.4 and 13.3 ± 0.8 %; $p=0.02$).

Treatment with alendronate increased phosphorus concentration in a dose-dependent manner relative to values found in OVX animals. The lower-dose treatment (ALN1) led to an increase in phosphorus concentration of 4.4% measured by ³¹P-MRI ($p=0.05$), and 3.8% measured by ³¹P-NMR ($p=0.02$) relative to values found in untreated OVX animals. In the high-dose group (ALN2) the increases in phosphorus concentration were 14% by ³¹P-MRI ($p<0.0001$) and 9% by ³¹P-NMR ($p<0.0001$). The relative increase in phosphorus concentration in ALN2 relative to NO animals was about 6% for ³¹P-MRI (13.0 ± 0.8 vs 12.3 ± 0.7 %; $p<0.0001$) and 5% for ³¹P-NMR (13.9 ± 0.8 vs 13.3 ± 0.8 %; $p=0.03$), respectively. The effects of ovariectomy

and the treatment effects in response to the two doses of alendronate on the bone phosphorus measured by the two methods are summarized in Figures 3a and 3b.

The ^{31}P data was found to parallel changes in the ash content, $\mu\text{-CT}$ density and material properties (Table 1) in that all measures were found to be reduced upon ovariectomy, although the difference was statistically significant only for mineral density. While treatment increased both ash content and $\mu\text{-CT}$ density above *OVX* values for both doses, the increase was significant only for the higher dose ($p=0.003$ for ash content and $p=0.01$ for $\mu\text{-CT}$ density respectively; Figure 3c). Analogous to the changes of phosphorus concentration, *ALN2* yielded a significantly greater increase in ash content, exceeding the levels of *NO* group (66.3 ± 1.0 vs 65.3 ± 1.0 %; $p=0.005$). This increased ash content at the higher dose is paralleled by a significantly increased CT density ($+2.8\%$, $p=0.01$) against *OVX* group, essentially matching values for the *NO* group (Figure 4a).

Figures 5a and 5b graphically show the bone water concentration, quantified by $^1\text{H-MRI}$ and gravimetry. $^1\text{H-MRI}$ -derived bone water was greater in *OVX* animals than in the sham group (15.5 ± 1.7 vs. 14.6 ± 1.4 %; $p=0.05$) corroborating our hypothesis that ovariectomy results in increased bone water (Figure 5a). Treatment with alendronate lowered bone water in accordance with a restoration of mineralization towards normal values. This change was again dose-dependent, similar to the findings for phosphorus concentration, in that bone from *ALN2* animals exhibited significantly lower water concentration than the *NO* group. Mean water concentration in *ALN1* animals was lower than in the *OVX* group but failed to reach the values in the *NO* group, suggesting only a partial recovery at the lower dose. The higher dose yielded 19% lower values than those in *OVX* animals (12.6 ± 0.9 vs $15.5\pm 1.7\%$; $p<0.0001$). These values are also significantly below *NO* values (12.6 ± 0.9 vs $14.6\pm 1.4\%$; $p=0.0001$). This result again is in line with our phosphorus measurements where *ALN2* bone enhanced phosphorus concentration beyond values observed for the *NO* group. Even though gravimetric data suggested higher bone water concentration in *OVX* than in *NO* animals, the difference was not significant.

Table 2 lists inter-parameter correlations for all material and mechanical parameters measured by pooling the data from all 60 animals. Of the 28 pairwise comparisons 12 were statistically significant ($p<0.0001$ to $p<0.05$). Positive correlations were obtained between MRI measures of phosphorus concentration and other measures of mineral content (ash weight fraction and μCT density). In contrast, measures of bone water were negatively correlated with those expressing mineral properties, as expected. There was no correlation between material properties and mechanical quantities when regressing data from individual animals, except for μCT density, which was weakly correlated with elastic modulus. However, the group means for phosphorus concentration, measured either by ^{31}P solution NMR or ^{31}P MRI were highly correlated with group means of the mechanical parameters ($R=0.99$ and 0.98 , $p=0.0007$ and 0.01 , respectively, for EM). Phosphorus concentrations were also predictive of US and both mechanical parameters were negatively correlated with bone water concentration, but, while CT density and ash weight indicated a trend, these associations were not significant. Correlation coefficients between material and mechanical properties are given in Table 3.

Discussion

Although $^1\text{H-MRI}$ and $^{31}\text{P-MRI}$ have previously been shown to be able to detect bone mineral and matrix constituents non-invasively (1,2,34,36), there is only one study showing the effect of intervention. Anumula et al (1) demonstrated the feasibility of the technique to detect changes in bone mineralization and bone water in a rabbit model of osteomalacia where hypomineralization was induced by subjecting the animals to a hypophosphatemic diet. The aim of the present work was to explore the potential of ^{31}P and ^1H UTE MRI to detect the

changes in bone mineralization and water secondary to estrogen deficiency induced by ovariectomy and to explore the method's sensitivity to detect the dose-dependent effects of intervention with alendronate on matrix and mineral properties and their mechanical consequences. The rationale for the choice of the two dosing regimens (5 µg/kg/day and 25µg/kg/day) has been given before. The results of this work demonstrate that ^{31}P -MRI can detect the subtle decreases in mineral phosphorus density following ovariectomy as well as the effect of antiresorptive treatment on bone phosphorus content, even for the lower dose. Mineral phosphorus concentration was found to increase by 4.4% and 14% over a seven-week treatment period with 5µg/kg/day and 25µg/kg/day of alendronate, respectively.

The changes in the mineral phosphorus values obtained from *in-situ* imaging are corroborated by ^{31}P -NMR measurements after acid dissolution of the bone where again the OVX bone results indicated partial recovery at the low dose (+3.1% above OVX values) and full recovery at the high dose (+9% above OVX values). The small effects observed at the lower dose are in agreement with other studies in which comparable doses did not significantly change either mineral constituents or material properties (16,25). Interestingly, ^{31}P -MRI was the strongest differentiator of OVX and sham groups ($p=0.002$) (Figure 3a). The decrease in phosphorus density following ovariectomy measured by ^{31}P -MRI was far greater than changes quantified by ^{31}P -NMR after dissolution of the bone (−8.1% versus −3.8%). A similar discrepancy between solid-state and solution magnetic resonance data was previously found by the authors in an experimental study in a rabbit model of osteomalacia where ^{31}P -MRI yielded greater group difference than solution ^{31}P NMR comparing hypophosphatemic to normophosphatemic animals (14.4 vs 9%) (2). On the other hand, the actual values of phosphorus concentration derived by ^{31}P -MRI were slightly lower than those obtained by high-resolution NMR (by −6.8% in NO group; $p=0.004$), similar to earlier observations in rabbit bone (1). A possible reason for this discrepancy could be the existence of a fraction of mineral with an even shorter effective T_2 which would not be captured by UTE MRI. The ^{31}P -NMR technique, on the other hand, detects all phosphorus constituents after dissolution of the bone in HCl.

Ash and µ-CT density were also reduced in OVX animals, albeit by a lower fraction. The µ-CT density results were suggestive of a decrease in these metrics in response to ovariectomy by 2.5%. The present ash content measurements could not differentiate OVX and NO groups. Giavaresi et al (16) previously did not see a significant difference in ash content in 10-month old OVX and sham-operated animals.

In the present work, treatment with alendronate increased ash content and CT density in a dose-dependent manner, again the higher dose yielding values exceeding those of the NO group. The higher dose caused relatively small increases in ash content and CT density (+1.7%, $p<0.005$ and +2.8%, $p<0.05$, respectively). It is of particular interest to note here that the changes in phosphorus concentration upon treatment were much larger than those in CT density (+14%, $p<0.0001$). This significant discrepancy between phosphorus concentration on the one hand, and CT density and ash weight, on the other hand, is unlikely due to some systematic error considering the prior validation and the fundamentally quantitative nature of NMR. Rather, the causes need to be sought in the non-stoichiometric nature of calcium apatite and transient phenomena during bone formation (see, for example, (34)).

Of note is that the magnetic resonance (both MRI and liquid-state NMR)-derived material properties were significantly correlated with most, albeit not all, of the destructively measured counterparts representative of mineral and matrix properties when pooling the data from all 60 animals (Table 2). For example, both ^{31}P MRI and ^{31}P NMR-derived phosphorus densities were positively correlated with ash weight fraction, µCT density and negatively with water fraction. The latter is again consistent with the notion of an inverse relationship between bone

water and mineral fraction, first established by chemical analysis by Elliott (12) and more recently shown by nondestructive techniques (1,14).

Ovariectomy did not significantly reduce the elastic moduli and ultimate strength obtained by three-point bending in the present work. The present data are consistent with those by Nazarian et al (23) who found that ovariectomy did not affect the mechanical properties of cortical bone material. Similarly, Giavaresi et al (16) did not detect a significant effect on the elastic modulus due to ovariectomy in 10-month old rats. Treatment with alendronate was suggestive of an increase in the mechanical parameters in a dose-dependent way in that the higher dose yielded elastic moduli that were 12% greater than those found for the *OVX* group but the effect did not reach statistical significance ($p=0.06$). Importantly, however, are the highly significant correlations observed in our work for group means between magnetic resonance-derived mineral properties as predictors for both EM and US, while the latter were inversely correlated with water concentration (Table 3) even though correlations of the pooled data of the individual animals generally were not significant. The strongest correlation of group means of mechanical parameters was between ^{31}P NMR-derived phosphorus concentration and elastic modulus ($R^2=0.99$, $p<0.001$) but significant correlations of similar strength were also obtained between mechanical parameters and other magnetic resonance measures as well as between μCT density and ultimate strength. These observations seem to be at odds with the absence of significant correlations of the collective data before group averaging (Table 2). One obvious explanation is that treatment has a similar effect on mechanical and compositional parameters despite the fact that the two types of parameters seem to be overall uncorrelated, presumably due to large variations within each group of rats (i.e. the within-group variation has a large variance and is uncorrelated between the two variables). The variations within each group may be dominated by measurement precision rather than being biological in origin. Therefore, whereas ^{31}P concentration may not by itself be a good predictor of absolute mechanical bone strength, it may be an accurate marker for relative changes in overall strength within a cohort of individuals following intervention.

The significantly greater water concentration measured by $^1\text{H-MRI}$ for the *OVX* relative to the sham group (+6%, $p<0.05$) supports our hypothesis that decreased mineralization secondary to estrogen loss is paralleled by an increase in water concentration. This effect has been shown to be particularly prominent in undermineralized bone such as in a rabbit model of hypophosphatemic osteomalacia where some of the present authors demonstrated first by ^{31}P solution NMR (14) and subsequently UTE MRI (1) that low mineral phosphorus density is associated with increased bone water.

Further supporting the hypothesis is the finding that treatment with both bisphosphonate doses lowered water concentration significantly relative to the values found for the *OVX* group (−7%, $p=0.05$ and −19%, $p<0.0005$, for *ALN1* and *ALN2*, respectively). The water fractions obtained upon treatment with the lower dosing group were essentially at par with those of the *NO* group. In contrast, values obtained with the higher dosing group were 14% below *NO* values (12.6 ± 0.9 vs. 14.6 ± 1.4 %), $p<0.0001$). A hypothetical time course for the opposite changes in phosphorus and water concentration in response to ovariectomy and bisphosphonate treatment is illustrated in Figure 6. Lastly, $^1\text{H-MRI}$ derived absolute water concentration was somewhat lower than values obtained by drying (*NO*: 14.6 ± 1.4 vs. 16.9 ± 1.3 ; $p<0.0001$), presumably because some tightly bound water (e.g. water associated with the mineral phase) may elude MRI detection due to very short T_2 relaxation times but may be expelled by drying at 100°C .

Bone water represents the amount of water in all pore spaces, the fraction bound to the organic phase (collagen), and the small fraction of tightly bound crystal water that may not be detectable by the present UTE MRI technique. It is currently not possible to distinguish the various fractions, many of which are in exchange with each other. The increase in MRI-detectable

water following ovariectomy in the present work is associated with the decrease in mineral density by taking the space of mineral crystal locations, in keeping with the previously stated notion that matrix volume is preserved (5,12,24). Nevertheless, some of the increase in bone water following ovariectomy and subsequent decrease due to treatment as detected by UTE MRI could be attributed to changes in the resorption cavities and hence porosity (pore size and number (28)). Nevertheless, a significant portion of the water also resides in the lacuno-canalicular pore system, too small to be resolved *in situ* by imaging. This raises the question as to the extent to which changes in mineral and phosphorus density following ovariectomy (and conversely upon bisphosphonate treatment) represent true changes on a nanoscale as opposed to ‘apparent’ resulting, at least in part, from changes in microporosity. Support for the former interpretation is provided by Boivin et al (7) and Roschger et al (28) in work based upon microradiography and quantitative backscattered electron imaging, showing conclusively in osteoporotics an increase in mineral density and reduction in porosity upon alendronate treatment. While these findings relate to humans, similar observations have also been reported in rodents (17,37).

The quantification of water content measured by ultra-short echo-time ^1H -MRI provides new, albeit indirect, insight into the micro- and nanostructural changes that occur in metabolic disease. Notably, there is currently no technique other than magnetic resonance to quantify changes of the bone’s hydration state *in situ*. Recent work indicates that the majority of the proton signal observed in cortical bone is due to exchangeable water (as opposed to organic matrix) (1,30) even though matrix constituents can also contribute to the solid-state MRI signal (9).

In summary, our data highlight the potential of solid-state MR imaging methods to detect changes in mineral and matrix chemistry following hormone loss and in response to antiresorptive treatment *in situ*. The data also lend further support to the concept of inverse changes in bone water and mineral, both following estrogen depletion and bisphosphonate exposure. A limitation of the study is that it was conducted in specimens rather than *in vivo*. There are, however, no fundamental limitations to transferring the imaging technology to measurements *in vivo* in laboratory animals and humans. While studies in specimens allow for measurements with small, tightly coupled volume RF coils and operation at very high magnetic fields, the overall signal-to-noise ratio achievable *in vivo* in humans is much lower. Nevertheless, the feasibility of quantifying bone water in patients has recently been demonstrated (30). Measurement of phosphorus density by ^{31}P MRI is more difficult because of the much lower intrinsic detection sensitivity. Even though the feasibility of *in vivo* ^{31}P MRI in humans has been demonstrated, low intrinsic SNR severely limits resolution and requires very long scan times (26). Thus, proton UTE MRI offers an attractive alternative to study bone properties and to obtain information on matrix mineralization indirectly. Its detection sensitivity, considering the larger magnetic moment and the shorter T_1 relaxation times, is about four orders of magnitude greater than that of ^{31}P (33).

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Figure 1. Source of specimens used to quantify bone phosphorus, water, μ CT density, ash and mechanical properties.

Figure 2.

Summary of image and spectroscopy-based quantification bone mineral and matrix constituents: a) phosphorus and water by UTE ^{31}P and ^1H MRI; b) phosphorus by ^{31}P NMR; c) density by μ -CT. For details see text.

Figure 3.

Effect of ovariectomy and treatment with alendronate on a) phosphorus by $^{31}\text{P-MRI}$; b) phosphorus by $^{31}\text{P-NMR}$; c) ash weight by gravimetry. Data are shown as means $\pm$ S.D. ($n=15/\text{group}$). ^{a,b,c,d} versus *OVX* ($p\leq 0.0001$; $p\leq 0.005$; $p\leq 0.05$; $p>0.05$ respectively). ^{e,f,g} versus *NO* ($p\leq 0.0001$; $p\leq 0.005$; $p\leq 0.05$; respectively)

Figure 4.

Effect of ovariectomy and treatment with alendronate on a) femoral mineralization density by μ -CT b) Elastic modulus by mechanical testing. Data are shown as the means $\pm$ S.D. ($n = 15$). ^{b,c,d} versus OVX ($p \leq 0.005$; $p \leq 0.05$; $p > 0.05$ respectively). ^h versus NO ($p > 0.05$ respectively)

Figure 5.

Effect of ovariectomy and treatment with alendronate on a) bone water by $^1\text{H-MRI}$; b) bone water by gravimetry. Data are shown as the mean $\pm$ S.D. ($n=15$). ^{a,c,d} versus OVX ($p \leq 0.0001$; $p \leq 0.05$; $p > 0.05$ respectively). ^{e,f} versus NO ($p \leq 0.0001$; $p \leq 0.005$)

Figure 6.

Reciprocal relationship between bone phosphorus and water concentration following ovariectomy and alendronate treatment. The putative time course for the temporal evolution of mineral phosphorous (solid curves) is plotted together with the measured MRI data (squares), showing overshoot and undershoot relative to control sham-treated animals (dashed lines). After ovariectomy, loss in mineral ^{31}P is accompanied by increased water concentration, whereas with bisphosphonate treatment the behavior after ovariectomy is flipped yet still reciprocal.

Means and standard deviation for magnetic resonance, density and mechanical parameters in all four study groups (NO, OVX, ALN1 and ALN2, N=15 each).

Table 1

Group	³¹ P MRI (wt %)	³¹ P NMR (wt %)	¹ H MRI (wt %)	Drying Water (wt%)	Ash (wt %)	μCT Density (mg/cm ³)	EM (N/mm ²)	US (N/mm ²)
OVX	11.4±0.9	12.8±0.4	15.5±1.7	17.2±1.1	65.3±1.0	1316±34	7055±1420	185±19
NO	12.4±0.8 ^b	13.3±0.8 ^c	14.6±1.4 ^c	16.9±1.3	65.2±1.0	1349±32 ^b	7477±1094	190±19
ALN1	11.9±1.0	13.2±0.6 ^c	14.5±1.8 ^c	16.1±1.1 ^c	65.7±0.8	1325±31	7363±1488	188±24
ALN2	13.0±0.8 ^{a,e}	13.9±0.8 ^{a,e}	12.6±0.9 ^{a,d}	15.3±1.1 ^{a,d}	66.3±1.0 ^{b,d}	1353±44 ^c	7946±1533	192±31

a,b,c versus OVX :

^a p≤0.0001;

^b p≤0.005;

^c p≤0.05

d,e versus NO:

^d p≤0.005;

^e p≤0.05

Table 2

Inter-parameter correlations (N=60) pooling all strata. Statistically significant correlations are highlighted.

	³¹ P NMR	¹ H MRI	H ₂ O Drying	Ash	μCT Density	EM	US
³¹ P MRI (wt %)	0.465 **	-0.268	-0.325 ##	0.387 ***	0.375 ***	0.001	0.099
³¹ P NMR (wt %)		-0.406 ***	-0.451 ***	0.376 ***	0.331 #	0.088	0.108
² H MRI (wt %)			0.335 #	-0.224	-0.029	0.017	-0.169
H ₂ O Drying (wt %)				-0.691 *	-0.138	-0.073	-0.101
Ash (wt %)					0.146	0.051	0.158
μCT Density (mg/cc)						0.346 #	0.215
EM (MPa)							0.647 *

* p<0.0001,

** p<0.0005,

*** p<0.005,

p<0.01,

p<0.05

Table 3

Correlations between group means of MRI, CT density, ash weight and gravimetrically determined water fraction as predictors of elastic modulus and ultimate strength.

	EM (MPa)		US (MPa)	
	R ²	Slope	R ²	Slope
³¹ P MRI (%)	0.98 ^b	530	0.99 ^a	4.3
³¹ P NMR (%)	0.99 ^a	813	0.95 ^b	6.3
¹ H MRI (%)	-0.98 ^b	-297	-0.89	-2.2
CT-Density (mg/cm ³)	0.88	18	0.95 ^b	0.16
Ash Wt (%)	0.80	596	0.63	3.8
Water Conc. (%)	-0.86	-375	-0.74	-2.6

^a p<0.001;

^b p<0.05