The Significance of ATF-4 and TGF-β to Bound Water and Bone Fracture Resistance

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KEYWORDS. ATF-4, TGF-β, bound water, NMR, fracture resistance

BRIEF. This study explores the effectiveness of bound water as an indicator of bone fracture resistance through NMR analysis.

ABSTRACT. Bone is a composite material made from mineral and soft material such as water and collagen. Disease or age can cause fluctuations of such components in bone. Currently, doctors can only assess the mineral of a patient's bone in vivo because clinical tests rely on X-rays which cannot measure the water or collagen in bone. This study sets out to test the significance of the water in the bone as a function of strength with the use of NMR scanning. Specifically, bones with different protein expressions that potentially affect the water in bone were analyzed by NMR, X-ray CT imaging, and biomechanical tests. We found that water was a good indicator and a potential surrogate of bone strength. In other words, collagen bound water could be useful in future evaluations of patients' fracture risk.

INTRODUCTION.

Bone is a composite of organic and non-organic material that forms a resilient substance capable of withstanding every day physical stress imposed by exercise and daily activities. Unfortunately, as bones age, they are more likely to deteriorate. New drugs and diagnostic tests for bone attempt to prevent adverse effects such as bone loss. There are many factors that contribute to bone's strength including 1) mineral density which determines how hard bone is, 2) flexible collagen fibers which allows the bone to absorb deformation, and 3) cells that control bone breakdown and buildup. The cells responsible for building bone are called osteoblasts, while cells responsible for breaking bone down are called osteoclasts. These cells are regulated by various signaling molecules that are expressed in bone such as transforming growth factor beta (TGF-β), activation transcription factor four (ATF4), osteoclast differentiation factor (ODF), and other protein signaling molecules [1]. Imbalances in the expression of these signaling proteins can cause bones to degenerate since bone buildup and removal become unbalanced. Although extensive research has been done regarding the effects of signaling on the physical characteristics of bone [1], very few studies have attempted to reveal the effects of these biological factors on collagen bound water. The effects of collagen bound water (also referred to as bound water) on bone strength are not fully understood; however, water content may serve as a potentially important marker of bone fracture resistance and strength.

Currently, to evaluate bone for such structural changes in a noninvasive manner, physicians utilize CT (computed tomography) technology. CT relies on X-rays which results in radiation exposure as well as limits the scope of analysis to the mineral portion of bone but not the collagen and water content [2]. As a result, this does not provide a complete representation of the overall integrity of bone since collagen and water content could significantly impact bone strength. New studies have shown that the volume of collagen and water found in bone can be quantified by nuclear magnetic resonance (NMR), a noninvasive and radiation free imaging technique [2]. The use of NMR to evaluate bone is relatively new and rarely used in conventional studies.

There were two main goals in this study: to analyze the effects of cell signaling on water content, and to examine the effectiveness of NMR as an evaluator of bone integrity. Since suppression of signaling proteins such as TGF-β promote osteoblast differentiation and cause an increase in bone strength, it was hypothesized that there should be an increase in bound water in mice with such proteins down regulated [1]. Additionally, since suppression of signaling proteins that regulate osteoblasts such as ATF4 weakens the bones of mice, there should be a decrease in collagen bound water in mice with those proteins turned off [3]. It was also expected that NMR can be as effective as CT when it comes to the non-invasive evaluation of bone for fracture risk. Overall, this study aims to provide a more representative and accurate evaluation of bone strength in both clinical and research settings.

MATERIALS AND METHODS.

Bone Model Preparation.

Fourteen week old ATF4 knockout (-/-) and wild type FVB mice (+/+) of both genders were treated in two separate groups each with either TGF-ß suppression antibody 2G7 or control antibody 12CA5 (Vanderbilt Antibody CORE, Nashville, TN, USA) at a dose of 15mg/kg for four weeks by a bone biology lab (n=10). Four weeks after initial treatment, the mice were sacrificed. Extracted L6 vertebrae and left femurs were made available for the study. All animal procedures were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) protocols.

Bone Imaging.

The femurs and vertebrae were then assessed for structural integrity by μCT scanning (μCT40, Scanco Medical AG, Brüttisellen, Switzerland) at the femoral metaphysis, diaphysis, and vertebral body regions of the bone samples with a spatial voxel size of 12μm at 70keV. Furthermore, water content analysis was conducted on the femurs by NMR through Varian/Magnex 4.7T 31 cm bore magnet (Agilent Technologies, Santa Clara, CA, USA) scanning that revealed T2 spectra capable of accurately quantifying the amount of collagen, collagen-bond water, and pore water or lipids in the bone tissue [2]. T2 is calculated by how quickly the water molecules in each category return to their relaxed state after being excited by a series of radio waves. Before the bones were scanned, their submerged and dry masses were taken in order to allow for improved accuracy since the data will be normalized to volume using Archimedes' principle.

Mechanical Testing.

The mechanical strength and modulus of the femurs were additionally obtained through biomechanical testing using a 3 point bending protocol (ASTM D790, ISO 6872). Femurs were placed horizontally on two support rollers with a span of 8 mm while a vertical load was applied to the center of the diaphysis using a material testing apparatus (Dynamight 8841, Instron, Norwood, MA, USA) until failure; bones were kept hydrated at all times in order to acquire optimum bone conditions for the most accurate results. The load-displacement data were used to evaluate the force versus displacement curve and thus derive individual mechanical properties such as peak or maximum force and yield force.

Data Collection and Statistical Analysis.

The mean values from the data sets were then graphed and cross-correlated. In addition, the standard error of the mean was calculated and graphed alongside each parameter; the standard error of the mean quantifies the standard deviation of a sample divided by the square root of the sample size which indicates the confidence intervals of the mean. T-tests were also used to determine significance of a data set, and significant p-values were <0.05. Furthermore, linear correlations were calculated and r values were considered strongly correlated (>0.7), moderately correlated (0.5-0.7), and weakly correlated (0.3-0.5).

RESULTS.

The data analysis was centered on the diaphysis since cortical bone is a significant contributor to fracture risk. Although the metaphysis and vertebrae also contain cortical bone, they were excluded from the analysis since they are bet-

ter indicators of trabecular, or spongy, bone; this is because trabecular bone from mice is difficult to test for strength. In addition, all the analyses conducted on the TGF-β treated mouse groups were insignificant and excluded from the results.

μCT.

Measurements of interest in the μCT analysis include minimum moment of inertia and tissue mineral density (Table S1). These two variables are indicative of fracture risk and provide valuable predictions pertaining to how bone might react under stress without breaking the bone. Figure 1, with a p-value <0.01, indicates that the ATF4 wild type mice have a significantly higher minimum moment of inertia than the ATF4 knockouts.

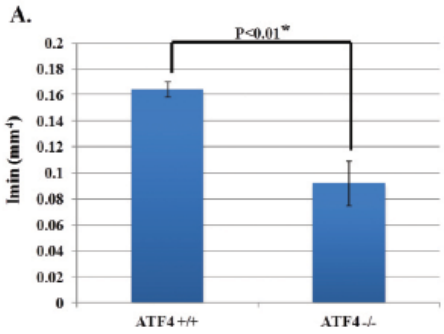

Figure 1. Figure 1 displays the mean data from the μCT scans with the error bars representing the standard error of the mean (*n*=10). Imin measures the distribution of mass around a central axis. The asterisk (*) signifies that the p-value of the difference between the two groups is statistically significant.

NMR.

NMR data was normalized to wet mass, Archimedes' principle, cortical bone volume, and bone volume (bone area at diaphysis*bone length). The graphs of the bulk bound water and bound water normalized to cortical bone volume are displayed since they provided the strongest correlations and statistical significance among all the NMR measurements (Table S2). Both bound water measurements are significantly greater in wild type than in knock-out mice, with p-values <0.01 (Figure 2).

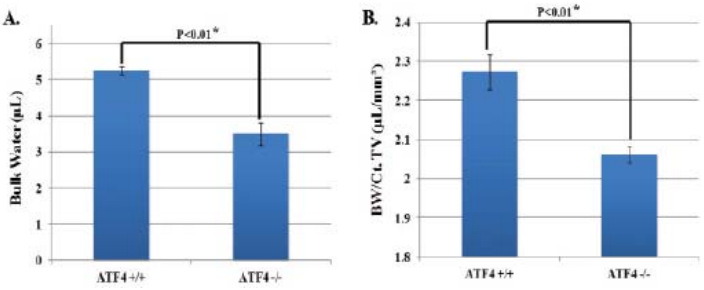

Figure 2. Figure 2 displays the mean values of the NMR scans with the error bars representing the standard error of the mean (*n*=10). Graph A measures total or bulk bound water in a sample while Graph B measures the amount of bound water/ cortical bone volume. The asterisk (*) signifies that the p-value of the differences seen in both graphs is statistically significant.

Mechanical Testing.

The 3 point bend test was used to measure the ability of the μCT and NMR analysis to assess fracture risk in terms of gold-standard properties such as strength and toughness (i.e., brittleness). Measurements of interest in the 3 point bend test data include: max force, post yield deflection, bending strength, and toughness (Table S1). Figure 3 displays the results from the 3 point bend tests done on wild type vs. ATF4 knockout mice. The tests show that wild type bones had superior toughness and withstood greater amounts of force than their knockout counterparts with p-values <0.01 (Figure 3). Nevertheless, no significant data was extracted from the tests conducted on the wild type mice treated with the 2G7 and 12CA5 antibodies.

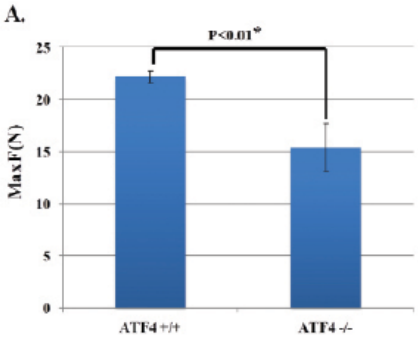

Figure 3. Figure 3 displays the mean data from the 3 point bend test with the error bars representing the standard error of the mean (*n*=10). MaxF is the peak force experienced by the bone before complete failure. The asterisk (*) signifies that the p-value of the difference between the two groups is statistically significant.

Correlation Analyses.

After the NMR and μCT measurements were graphed and analyzed for statistical significance, the data sets were then correlated using linear regressions with the 3 point bend test data, and the r values were placed in a table as seen in Table S2. By correlating the NMR or μCT data to the properties from the 3 point bend testing, it reveals which non-invasive method is more accurate at assessing fracture risk without inflicting any collateral damage on the bone. Strong correlations are marked in Table S2 with yellow, moderate correlations with blue and weak correlations with green. When Imin, Mean2, Ct.Ar, BW, and BW/Ct.TV were correlated against peak force between wild types and ATF4 knockouts, the Imin, Ct.Ar, and BW measurements all came out to be strongly directly correlated with peak force (r=0.899, 0.903, and 0.877, respectively); in addition, BW/Ct.TV came out to be moderately correlated to peak force (r=0.497). Meanwhile, for toughness, Imin, Ct.Ar, and BW were all moderately correlated (r=0.520, 0.491, and 0.547, respectively). As for the part of the study concerning the effects of 2G7 and 12CA5 on the wild type mice, correlations with Imin, Ct.Ar, BW, and BW/Ct.TV were found with peak force (r=0.878, 0.893, 0.896, and 0.495, respectively). The only significant correlation with toughness was made with BW/Ct.TV at an r-value of -0.304 which is inversely correlated. See Table S1 for an explanation of all the variable abbreviations.

DISCUSSION.

Based on the graphs and analysis of the NMR data, the effects of ATF4 activity in bone appears to have a significant influence on bound water (Figure 2). The trends between ATF4 regulation and bulk bound water and bound water normalized to cortical bone volume are statistically significant with p-values <0.01 (Figure 2). The higher bound water in the wild type mice supports the hypothesis that bound water is important to fracture risk and overall bone integrity. This is further supported by previous studies that state ATF4 knockouts develop brittle and weaker bones [3]; however, one critical element that has not been studied is the cause of such degeneration in bone. Fortunately, as a result of this study, it can now be said with more confidence that bound water is correlated to bone fracture resistance. This means that the predicted decrease in bound water within ATF4 knockouts explains, in part, why the bone becomes more brittle and weak when the transcription factor ATF4 is not present.

As for the data pertaining to TGF-ß inhibition, there is an unclear picture. None of the analyses conducted on the mice treated with the 2G7 antibody had a significant p-value <0.05. This is indicative that the treatment did not behave the way as predicted. This could be the result of insufficient treatment dosage and treatment time; more specifically, the mice might have needed a larger dose of 2G7 to start exhibiting changes in their bone phenotypes, or the treatment was not given enough time to manifest itself within the mice. In addition, the lack of the separation of the genders within the mouse groups could also have potential in skewing the significance of the scans since the individual variance within each group is higher than is normally expected. This would result in unnecessary outliers and high variance within the tests that would shroud underlying differences, if there were any. Nevertheless, this does not mean TGF-ß suppression does not affect bound water; instead, it means that there is not enough significant information to support such a claim. Nonetheless, this study

explores the possible linkages between a growth factor and the physical quantity of bound water, which has not been done in prior studies.

The final goal of this study was to explore the strengths of NMR vs. μCT with respect to how well they can predict fracture risk and bone integrity as assessed by 3 point bend testing. This is a novel step since few studies have proposed the use of NMR, which underpins clinical magnetic resonance imaging (MRI), to diagnose bone. In order to accomplish this, linear correlations between NMR and μCT were made with measurements from the 3 point bend tests. As seen in Table S2, there were correlations between the μCT and mechanical data between the wild type and ATF4 knockout mice as well as correlations in the wild type mice between 2G7 and 12CA5 antibody treatments. Correlations between NMR and mechanical tests were seen for both the ATF4 and TGF-β treatment groups as well (Table S2). Correlations, however, could not be made between all four 3 point bend test measurements since only the peak force measurements came out significant with p-values <0.01 (Figure 3).

In summary, NMR has shown the capability of complementing μCT analysis, since a) NMR exhibited many linear correlations to mechanical data that μCT didn't, b) NMR showed higher r values when correlated to peak force between 2G7 and 12CA5 treatment, and c) differences between experimental groups were more prominent with NMR-derived properties than μCT-derived properties (Figures 1, 2, 3, and Table S2). As a result, this study provides evidence that NMR can be used in conjunction with μCT for assessing bone biological and mechanical changes which not many studies recognize.

CONCLUSION.

Bound water was significantly greater in wild type mice when examined against ATF4 knockout mice (Figure 2). As a result, bulk bound water in bone, like moment of inertia, explained the lower peak force for the femur of ATF4 knockout mice. TGF-ß suppression, on the other hand, did not experience the same level of significance and confidence even though it was predicted that there should also be an increase of bound water since bone becomes stronger when TGF-ß is suppressed. This could be explained by a variety of reasons including an inadequate treatment dosage, inadvertent loss in effectiveness (e.g., denatured antibody), elevated group variance, and poor response of the FVB mouse line to the 2G7 antibody.

NMR has the capability of being a comparable indicator to μCT regarding the biomechanical properties of bone as determined by 3 point bend tests and provides complementary information, noninvasively (Table S2). This holds true since NMR achieved high, strongly correlated r values when compared to μCT vs. 3 point bend properties (Table S2). In addition, the NMR data had more linear correlations than μCT and in some cases had stronger correlations when the 2G7 and 12CA5 data set was correlated (Table S2).

Future experiments include NMR evaluation and vertebral body compression tests on the vertebrae extracted. Analysis done on the data derived from them can determine if the effects of bound water in bone is consistent throughout the skeletal system of the mouse. Additionally, some improvements to the experimental design of this study could include a larger mouse sample size, optimizing treatment dosage, separation of male and female mice, testing for the presence of the growth factors, and the use of a different mouse strain to see if any of the correlations and significances seen here are consistent or improve. Implementing these recommended improvements will further elucidate the effects of TGF-ß inhibition and ATF4 expression on bound water as well as the utility of NMR in bone characterization.

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SUPPORTING INFORMATION.

Table S1. Common parameters and their characteristics

Table S2. Correlations between NMR, μCT, and 3 Point Bend Test

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