

Characterization of Microstructural Changes (Part 2) on Biglycan/Decorin Induced Mice Bone by Low-field NMR

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Abstract:

Biglycan predominant role is known as in maintaining bone structure, water retention, and fracture toughness. Our previous pilot study has showed the bone microstructural changes on only the biglycan knockout mice. Here, this new experiment will further show the comparison of the microstructural changes on biglycan, decorin, single knockout, and biglycan and decorin double knockout mice bone studies. An NMR spin-spin (T_2) relaxation technique has been described for determining the porosity, and the bound water distribution changes in biglycan/decorin induced mice bone tissues and the results can be used to correlate their mechanical properties. The technique of low-field proton NMR involves spin-spin relaxation and free induction decay (FID) measurements, and the computational inversion methodology for decay data analysis. The CPMG T_2 relaxation data can be inverted to T_2 relaxation distribution, and this distribution can then be transformed to a pore size distribution with the longer relaxation times corresponding to larger pores. The FID T_2 relaxation data from dried bone (the most mobile water removed) can be inverted to T_2 relaxation distribution, and this distribution then can be transformed to bound and solid-like water distribution with the longest relaxation time corresponding to bound water component. These techniques are applied to quantify apparent changes in porosity and bound water in controlled, and biglycan/decorin single and double knockout mice bone tissues. It is known that hydration status significantly affects the toughness of bone, and bound water has been considered as a biomarker for prediction of bone fragility fractures. In addition to the collagen phase, recent evidence shows that glycosaminoglycans (GAGs) of proteoglycans (PGs) in the extracellular matrix also play a pivotal role in regulating the tissue-level hydration status of bone, there by affecting the tissue-level toughness of bone. Furthermore, biglycan and decorin are two major types of PGs in bone reports. Biglycan/decorin single and double knockout induced changes in GAGs, bound water, as well as bone tissue toughness. Among all subtypes of PGs, biglycan and decorin are identified as a major subtype in the bone mineral matrix. In this study, we used a biglycan and decorin single and double knockout mice model and the obtained bone samples were measured by low-field NMR to determine the bone porosity and bound water changes, and used to predict if

single or double knockout of biglycan/decorin may affect the amount of bound water and subsequently lead to reduce toughness of bone.

Keywords:

NMR, Mouse Bone, Bound Water, Porosity, Biglycan/Decorin

1. Introduction

Previous study has revealed that water in bone is present in three different conformations; namely freely mobile water in pores; bound water at surfaces and/or within the mineral and collagen phases; and structural water as part of collagen and mineral molecules [1,2]. In addition, evidence shows that water may also play a major role in viscous response of bone [3], most likely via the so-called sacrificial bonding mechanism [4]. In fact, hydration status significantly affects the toughness of bone, and bound water has been considered as a biomarker for prediction of bone fragility fractures [5,6,7]. In addition to the collagen phase, recent evidence shows that glycosaminoglycans (GAGs) of proteoglycans (PGs) in the extracellular matrix also play a pivotal role in regulating the tissue-level hydration status of bone, thereby affecting the tissue-level toughness of bone. And biglycan and decorin are two major types of PGs in bone reports. Biglycan/decorin knockout induced changes in GAGs, bound water, as well as bone tissue toughness. Among all subtypes of PGs, biglycan and decorin are identified as a major subtype in the bone mineral matrix [8-10]. Our previous pilot study [31] has showed the bone microstructural changes on only the biglycan knockout mice. Here, this new experiment will further show the comparison of the microstructural changes on biglycan, decorin, single knockout, and biglycan and decorin double knockout mice bone studies. Therefore, in this study we used a biglycan/decorin knockout mouse model and the obtained bone samples were measured by low-field NMR to determine the bone porosity and bound water changes and used to predict if the knockout of biglycan/decorin may affect the amount of bound water and subsequently lead to reduce toughness of bone.

Here, the techniques of low-field pulsed proton nuclear magnetic resonance (NMR) spin relaxation and in addition with high-field NMR are described for assessment of structural changes of normal and disuse (biglycan knockout) mice bone in situ. It is known that the total amplitude of T2 relaxation envelopes, measured by the NMR spin echo train (CPMG) [11,12], is a representation of the liquid phase inside the pores. Therefore, the NMR CPMG magnetization amplitude can be transferred to the volume of water after calibration with the NMR signal amplitude of the known volume of the selected water. In this study, the distribution of mobile water, porosity that can be determined by using low-field (20 MHz) CPMG relaxation technique, and the pore size distributions can be determined by a computational inversion relaxation method. It is also known that the total proton intensity of magnetization from the NMR free induction decay (FID) signal is due to the water present inside the pores (mobile water), the water that has undergone hydration with the bone (bound water), and the protons in the collagen and mineral matter (solid-like protons) [13]. Therefore, the components of total solid and bound water within bone that can be determined by low-field NMR free induction decay technique. This technique involves spin-spin relaxation measurement and inversion spin-spin relaxation spectral analysis methods.

2. Materials and Methods

2.1. Sample Preparation

A total of 24 mice leg bone specimens with 6 in wild (WT), 6 in biglycan knockout, 6 in decorin, knockout, and 6 in biglycan/decorin double knockout were obtained from 6-month-old males. It used biglycan, decorin, and biglycan/decorin deficient mice models [14] to prepare the samples. .

The biglycan knockout mice were generated as previously described by Xu, et al., [16]. The Decorin knockout mice were generated as previous described by Danieison, et al., [20]. All experiments were performed using age-matched WT. All the mice utilized in this study were of C57BL/6 background and were housed in a temperature-controlled room with a 12-hour light/dark cycle at the University of Texas Health Science Center at San Antonio (UTHSCSA) Institutional Lab Animal Research (LAR) facility. The mice had ad libitum access to food and water. All animal procedures were conducted in compliance with the National Institutes of Health guidelines for the care and utilization of laboratory animals. The animal protocols were approved by the UTHSCSA Institutional Animal Care and Use Committee.

The animals were sacrificed at the age of 6 months and the bone soft tissues were dissected; bone marrow was removed by flushing with cold PBS and stored in -80 °C. Prior to NMR measurements, the samples were completely thawed at room temperature.

2.2. Low-Field NMR Measurement

A low-field NMR spectrometer (Bruker 20 MHz) was set up at a proton frequency of 20 MHz for these measurements. ^1H spin-spin (T_2) relaxation profiles were obtained by using NMR CPMG $\{90^\circ [-\tau- 180^\circ - \tau- (\text{echo})]_n - T_R\}$ spin echo method with a 6.5 s wide 90° pulse, τ of 500 μs for mouse bone and T_R (sequences repetition rate) of 15 s. Each T_2 profile, one thousand echoes (one scan with $n = 1000$) were acquired and sixty-four scans were used. Thus, one scan will have repeated 1000 echoes in the window. For each FID profile, 1000 data points were acquired in one scan (an approximate 2 ms delay window). The data was measured on fresh frozen leg tissues after complete thawing in the room temperature ($21 \pm 1^\circ\text{C}$). The full saturated bone tissues were used for CPMG measurements, and free air dried (mobile water removed) bone tissues were used for FID measurements.

2.3. The Relationship Between NMR Data and Effective Pore Sizes

Based on the low field NMR principle the diffusion effect may be negligible. Here, we accept the Brownstein and Tarr [15] assumption that the relaxation rate $1/T_2$ is proportional to the surface-to-volume (S/V) ratio of the pore

$$1/T_2 = \rho(S/V)_{\text{pore}} \quad (1)$$

where ρ is the surface relaxivity, which is a measure of the effects of the pore surface enhancing the relaxation rate [16]. Equation (1) indicates that the NMR relaxation time is proportional to pore size with longer relaxations corresponding to larger pores. For a porous bone, the observed NMR magnetization will depend upon the T_2 of broad distributions of water in all pores. This implies that NMR transverse relaxation (T_2) data can be expressed as a sum of exponential functions:

$$M(t_i) = f(T_{2,j})\exp(-t_i/T_{2,j}) \quad (2)$$

where $f(T_{2,j})$ is proportional to the number of spins which relax with a time constant $T_{2,j}$. $M(t_i)$ is the NMR magnetization decay from fluid saturated bone. Equation (2) can be inverted into a T_2 relaxation time distribution. Thus, instead of estimating a single relaxation time from a magnetization decay, it is necessary to estimate an inversion T_2 spectrum or distribution of relaxation time $f(T_{2,j})$, and an inversion relaxation technique was applied [17, 13]. Since T_2 depends linearly upon pore size, the T_2 distribution corresponds to pore-size distribution with the longer relaxation times having the larger pores. The method for the inversion of FID ($T_{2\text{-Fid}}$) is similar to the inversion of CPMG data by using (echo) shorter as $1\mu\text{s}$ and 2 ms for delay time in the measurements. Three separate relaxation components from inversion $T_{2\text{-FID}}$ were detected corresponding to protons in solid, bound and mobile phases [13].

2.4. Bound Water Intensity Determined from NMR FID T_2 Inversion Spectrum

From FID measurement for full saturated bone sample, after T_2 inversion three peaks should be displayed that are (from left to right) [13] solid-like water component, bound water component, and liquid-like component. Since the liquid-like component intensity is too strong, and is not the important point at this moment (since the CPMG method already determine the detailed mobile water distributions), this time we used dried samples (the free water inside pore is removed by free air dry for 48 hours). Therefore, only two peaks (bound and solid-like) are displayed in inversion T_2 relaxation spectrum (Figure 2). We are cognizant of the sample size variation, but known (after test) the solid-like intensity is proportional to the sample mass, then we propose the bound water changes with the sample by using the ratio of bound water component to solid-like component from inversion T_2 relaxation spectrum.

3. Results and Discussions

In a Comparison of disuse mice leg bone group with control leg bone group, the significant differences are found. For example, Figure 1 shows the inversion NMR CPMG relaxation data spectra. The porosity, and the pore size differences for the sample of control WT and the disuse KO of biglycan are clearly observed. In Figure 1, the high intensity one is for KO ($\bullet$), and low intensity one is for WT ($\blacktriangle$). Since the longer relaxation times correspond to larger pores, and the higher intensity corresponding to higher porosity, therefore, the KO one has larger pores and porosity than the WT one. The comparison of porosities between the KO and WT groups is listed on Table 1. Where B, D and BD are for biglycan, decorin, and biglycan and decorin double knockout samples, respectively.

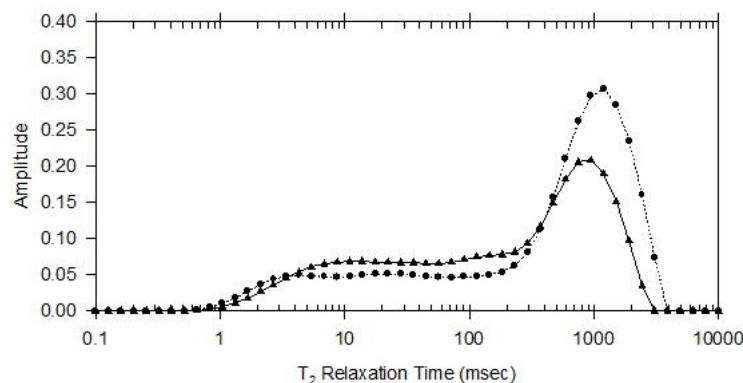


Figure 1. Inversion CPMG T_2 relaxation time spectra for mice WT and KO leg bones.

The high intensity is for KO (●), with larger porosity, and the low intensity (▲) is for WT, with lower porosity, respectively, measured by low-field NMR.

Table 1. Low-Field NMR Results for the % of porosity of mice.

Sample	Sample weight (g)	Volume (sample)	Volume (Water in sample)	Porosity (%)
WT1	0.0275	0.0137	0.00434	31.7
WT2	0.0237	0.0137	0.00322	23.52
WT3	0.0300	0.0193	0.00439	22.76
WT4	0.0319	0.0171	0.00567	33.13
WT5	0.0285	0.0145	0.00366	25.24
WT6	0.0324	0.0112	0.00827	23.0
			Average	27.27±4.64
B1	0.0264	0.0114	0.00449	39.40
B2	0.0276	0.0155	0.00652	42.03
B3	0.0266	0.0107	0.00323	30.18
B4	0.0271	0.0147	0.00324	22.01
B5	0.0273	0.0157	0.00451	28.73
B6	0.0313	0.0209	0.00863	41.28
			Average	33.93±8.16
D1	0.0275	0.0133	0.00436	32.75
D2	0.0356	0.0191	0.01027	53.78
D3	0.0307	0.0176	0.00774	43.99
D4	0.0261	0.0131	0.00385	29.38
D5	0.0242	0.0152	0.00346	22.79
				36.6±12.3
BD1	0.0375	0.0205	0.00864	42.15
BD2	0.02490	0.0097	0.00315	32.45
BD3	0.02920	0.0173	0.00627	36.23
BD4	0.02240	0.0152	0.00549	36.09
BD5	0.02360	0.0142	0.00451	31.78
BD6	0.02650	0.0135	0.00374	27.74
			Average	34.46±10.6

Figure 2 shows the example of inversion FID spectra, after the mobile water was removed by free air dry. It is because in mice bone tissues the mobile water single is overwhelming than the bound water single, and the mobile water is not interesting at this moment. Therefore, only two water peaks are observed with the left first it is a solid-like component and second is a bound water component. From low-field NMR measurement, the relative amount of bound water is then estimated as the ratio of the total intensity of bound water signal with respect to the intensity of the solid-like from water signal (representative of bone mass because in these sample measurements, the solid form proton signal intensities were correlated with the sample mass weights) of each sample. In Figure 2, it is clearly to show in similar weight bone samples the solid-like water intensity will be almost the same and the only change is the bound water intensity. Therefore, it can be used the solid-like water intensity for the reference for bound water calculations (ratio of bound water to solid-like water). It is known that the T_2 relaxation distributions for liquid-like phase are from milliseconds to seconds (Figure 1), while the T_2 -Fid relaxation time distributions are only from microseconds to milliseconds even for three different phase components [13]. The major difference between the T_2 relaxation times obtained using the NMR FID and CPMG methods is that the T_2 relaxation time constant is significantly shorter in the FID technique than in the CPMG technique. This is because in FID measurement the signal decays in a time T_2 -Fid that often is determined by field inhomogeneity, while an ingenious

CPMG measurement is a practical method for overcoming the inhomogeneity problem [18].

The estimated results for ratio of the bound water to solid-like water were shown in Table 2. In addition, our previous results showed that the number of GAGs and bound water in bone matrix was significantly reduced in KO mice compared in WT mice [33]. These *in situ* results indicate that coupling with biglycan or decorin is apparently one of PGs in bone mineral matrix that plays a key role in sustaining the toughness of bone. Our recent study demonstrates that loss of GAGs may significantly reduce the tissue-level toughness of bone, and such effects are most likely induced by the associated loss of bound water in bone matrix [10]. The results show the similarity as that among the age-related ultrastructural changes [19-22], the loss of bound water in bone matrix with significant reduction of the toughness of bone [23-27]. Based on the evidence, it could be conjectured that changes in matrix GAGs may contribute to the deterioration of bone quality.

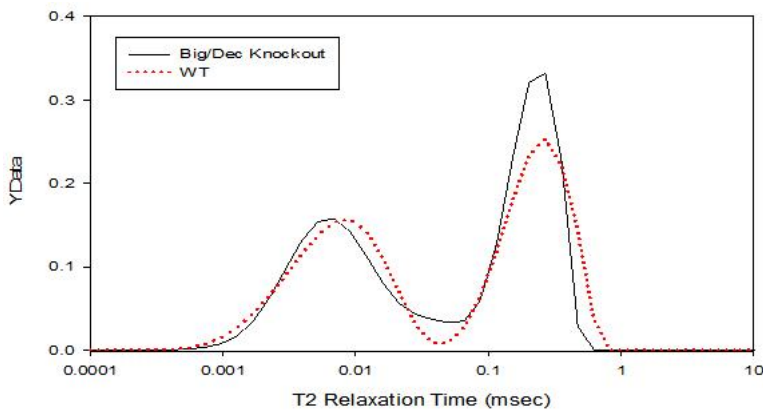


Figure 2. Example of inversion FID T2 relaxation time spectra from WT (solid line, black) and Big/Dcn double knockout mice (dotted line, red), respectively, measured by low-field NMR. The peaks from the left to the right are solid-like water and bound water intensities, respectively.

Table 2. Bound water of mice determined by NMR measurements.

Sample ID	gain	peak 1 (solid area)	peak 2 (bound area)	Ratio (bound/solid)
W1	106	0.0687	0.196	2.853
W2	106	0.107	0.230	2.153
W3	106	0.0575	0.201	3.491
W4	104	0.110	0.228	2.081
W5	105	0.0856	0.256	2.993
W6	104	0.102	0.190	2.398
Average			Average	2.662±0.548
B1	104	0.0730	0.153	1.596
B2	105	0.127	0.190	1.898
B3	105	0.0864	0.215	1.220
B4	105	0.100	0.198	2.258
B5	105	0.0951	0.241	2.718
B6	104	0.136	0.213	1.690
Average		0.103±0.0242	0.202±0.0298	1.897±0.528
D1	104	0.0586	0.173	2.819
D2	104	0.0844	0.182	2.153
D3	104	0.0912	0.2451	2.478

D4	106	0.129	0.207	1.734
D5	105	0.0922	0.181	1.566
D6	105	0.0833	0.203	1.978
Average		0.0867±0.0227	0.198±0.0265	2.121±0.468
BD1	106	0.0766	0.203	2.237
BD2	106	0.145	0.227	1.566
BD3	104	0.0882	0.158	1.796
BD4	104	0.0772	0.155	1.150
BD5	104	0.0775	0.162	2.109
BD6	104	0.0489	0.141	2.439
Average		0.0856±0.0319	0.175±0.0331	1.883±0.476

4. Conclusions

This is the further attempt that the porosity, and the bound water have been measured non-invasively and non-destructively in biglyan/decorin induced mice bone using an NMR approach. The porosity, and bound water pattern differences between WT and KO groups are observed. These differences can be used to predict and correlate with the bone mechanical properties.

Studying mice with targeted Bgn/Dcn disruption has revealed the involvement of Bgn/Dec in the regulation of postnatal skeletal growth. In general, Bgn/Dcn deficiency may lead to bone porosity increase, bone bound water decrease and bone biomechanical strength changes. Comparison among Bgn, Dcn and Bag/Dcn single and double KO samples, it is suggested Bgn deficiency may result in a more profound decrease in bound water than Dec KO mice bone. The porosity increases between Bgn and Dcn may be slightly higher in Dcn KO, however, the Bgn/Dcn double KO might not further decrease the bound water than Bgn KO mice and increase the porosity of single KO mice. This study has investigated the specific role of Bgn/Dcn in retaining bound water in bone matrix and maintaining bone toughness using a Bgn/Dcn-deficient mouse model. Biochemical, biophysical, and biomechanical experiments were performed to determine the number of total GAGs, and bound water, and their effects on the tissue-level toughness of bone. The coupling effect of proteoglycans and bound water in maintaining bone toughness has been shown in human bone during the aging process [28,29]. To examine the effects of Bgn/Dcn deficiency on the amount of bound water, cortical bone lysates of WT and Bgn/Dcn single and double KO mice were analyzed by low-field NMR measurements. Results showed that there was a significant reduction in bound water in bone matrix [30].

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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