

# Characterization of Disuse Mouse Bone Structural Changes by Nuclear Magnetic Resonance

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**Background**— 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 [Horch et al., 2012; 2010; Wilaon et al., 2006]. In addition, evidence shows that water [Yamashita et al., 2001] may also play a major role in viscous response of bone, most likely *via* the so-called sacrificial bonding mechanism [Yeni et al., 2006]. In fact, bound water has been considered as a biomarker for prediction of bone fragility fractures [Nicolella et al., 2012; Granke et al., 2015; Manhard et al., 2016]. 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 vitro. It is known that the total amplitude of T<sub>2</sub> relaxation envelopes, measured by the NMR spin echo train (CPMG) [Carr and Purcell, 1954; Meiboom and Gill, 1958], 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) [Ni et al., 2007]. Therefore, the components of total mobile 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. Furthermore, the bound water in solid phase (mineral

and organic constituents), especially, the dominated component of calcium hydroxyapatite (Ca<sub>10</sub>(OH)<sub>2</sub>(PO<sub>4</sub>)<sub>6</sub>) can be determined by using high-field (400 MHz) magic angle spinning NMR [Singh et al., 2013]. With MAS technique we can conduct further research into the <sup>1</sup>H and <sup>31</sup>P elements and environments of bone materials to identify the locations of bound water within minerals and bone architecture. The previous studies have indicated that coupling with water biglycan apparently plays a key role in sustaining the toughness of bone [Wadhwa et al., 2007].

**Materials and Methods**— A total of 14 mouse leg bone specimens with 7 in control and 7 in disuse (biglycan knockout) were obtained from 6 month-old male. It was used a biglycan, decorin, and biglycan/decorin deficient mouse models [Young et al.; 2002] to prepare the samples. Since biglycan (*Bgn*) gene is located at X chromosome, we crossed homozygous male (*Bgn*-/0) with heterozygous female (*Bgn*-/+ ) mice to generate homozygous (*Bgn*-/0) and WT (wild type) (*Bgn*+/0) littermate mice. We also used homozygous decorin (*Dcn*-/-) male mice for comparison. *Bgn*/*Dcn* double KO (knockout) mice was generated through two breeding steps; we first produced heterozygous *Bgn*/*Dcn* deficient mice by breeding a homozygous *Bgn* deficient female (*Bgn*-/- / *Dcn* +/+) with a *Dcn* heterozygous deficient male (*Bgn*+/0 / *Dcn*+/-); F2 *Bgn*/*Dcn* double deficient mice was obtained by interbreeding F1 heterozygous *Bgn*/*Dcn* deficient animals. To maximize the number of double KO mice, we were breed *Bgn* 0/- / *Dcn*+/- males with *Bgn* +/- / *Dcn*+/- females. The animals were sacrificed at the age of 6 months and the bone soft tissues were dissected and stored in -80°C freezer prior to experiments.

**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.  $^1\text{H}$  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  $6.5 \mu\text{s}$  wide  $90^\circ$  pulse,  $\tau$  of  $500 \mu\text{s}$  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. 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.

**High-Field NMR with Magic Angle Spinning (MAS) Measurement** High-Field proton NMR spectra were acquired on a Bruker 400 MHz superconducting solenoid magnet. For MAS experiments the rotor axis makes an angle of  $54^\circ 44'$ , the “magic angle”, with the static applied field. For single pulse experiment, a  $4.5 \mu\text{s}$  pulse produced a  $90^\circ$  tip angle in MAS probe with spinning rate at 8 kHz. In order to reach this high spinning rate, the mice bone was put into the rotor first, then added Teflon powders to fill the empty space of the rotor to make the spinning uniformly. Only 16 scans were accumulated with quadrature phase cycling. The acquisition time was 4.0 s with 8 kHz sweep width. The bone tissues were free dried on air for 48 hours to remove the mobile water, and used for measurements.

#### **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 (1979) 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  $\rho$  is the surface relaxivity, which is a measure of the effects of the pore surface enhancing the relaxation rate. 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:

$$M(t_i) = \sum_{j=1}^m f(T_{2,j}) \exp(-t_i/T_{2,j}) \quad (2)$$

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 [Ni et al., 2004, 2007]. 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  $\tau$  (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 [Ni et al., 2007].

**Results and Discussions**— Comparison of disuse mouse leg bone group with control 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 sample #5 between the control WT and the disuse KO are clearly observed. In Figure 1, the high intensity one is for KO (red), and low intensity one is for WT (black). Since the longer relaxation times are corresponding to larger pores, therefore, KO one has larger pores and porosity than the WT one.

Figure 2 shows  $^1\text{H}$  NMR spectra of the intact mouse bone tissue obtained from solid-state MAS measurement that shows two peaks as indicated by Singh et al., 2013, one peak at 5.2 ppm comes from water (bound) in bone matrix and second one at 1.4 ppm is assigned to hydroxyl ion ( $\text{OH}^-$ ) attached to  $\text{Ca}^{++}$  surface. Kafalak-Hachuska et al., [2003] assigned this peak as hydroxide ion in a unique hydrogen-bonded state in carbonated apatite. Other studies by Wilson et al. [2005; 2006] suggested that this peak might come from organic matrix [Wilson et al., 2005; 2006]. But due to insufficient resolution and heterogeneous environment in their studies of bone, this signal was not clearly identified. So we cannot say conclusively that  $\text{OH}^-$  at 1.4 ppm is coming from organic matrix or attached to  $\text{Ca}^{2+}$  [Singh et al., 2013].

But all studies confirm that it was coming from hydroxide ion (OH<sup>-</sup>). It is observed that WT spectrum has better peak resolution at 1.4 ppm than KO one.

In study it is found that the average bone porosity is increased 40.6% for disuse bone, the average ratio of bound water to mobile water is reduced to 82.4%, and the average mineral component of calcium hydroxyapatite (Ca<sub>10</sub>(OH)<sub>2</sub>(PO<sub>4</sub>)<sub>6</sub>) is reduced to 82.9% compared with the control group.

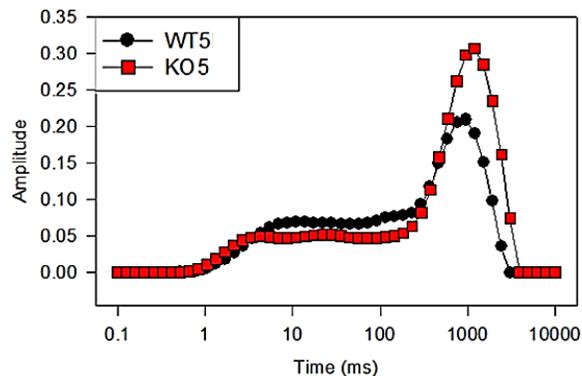


Figure 1. Inversion CPMG T<sub>2</sub> relaxation time spectra for mouse WT and KO leg bones. The high intensity is for KO (red), with porosity 37.7%, and the low intensity (black) is for WT, with porosity 21.33%, respectively, measured by low-field NMR.

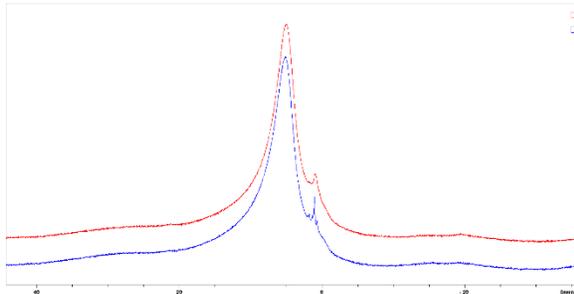


Figure 2. <sup>1</sup>H spectra of mouse leg bones for the same samples as in Figure 1, WT and KO. The top one (red), and the bottom one (blue) are the KO and WT, respectively, measured by high-field (400 MHz) proton NMR with MAS probe.

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