MRI Assessment of Matrix Development in Ultrasound-treated Cartilage Constructs

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Introduction: Production of hyaline cartilage in scaffold-based constructs remains a key goal in the development of tissue engineering approaches to degenerative cartilage disease. Characterization of the developing tissue is an important component of attempts to optimize this process. Evaluation of cartilage development generally relies upon the gold standard invasive techniques of histology and biochemistry, which are not ideally suited for ongoing *in situ* evaluation. More recently, detailed correlations have been established between noninvasive MRI outcome measures and tissue biochemistry [1,2], including a description of matrix development from bovine chondrocytes seeded in a collagen I scaffold[3]. In addition to these analytic techniques, a number of approaches are under investigation to augment cartilage tissue growth. One of these approaches is pulsed low-intensity ultrasound (PLIUS), a form of low-amplitude mechanical stimulation. The pressure waveform is sensed by membrane-bound mechanoreceptors in chondrocytes, initiating a signaling cascade resulting in increased cartilage matrix production. PLIUS has been shown to improve cartilage repair in rabbits[4] and to increase aggrecan gene expression in cultured rat chondrocytes[5]. The objectives of this work are to use MRI to probe the effect of PLIUS treatment on matrix development collagen I scaffolds seeded with bovine chondrocytes. The MRI parameters used include: longitudinal (T1) and transverse (T2) relaxation times, which are sensitive to macromolecular concentration, and magnetization transfer rate which is sensitive to collagen content. Additionally, multiexponential T2 analysis was used to evaluate differences in water compartment size and mobility within the tissue construct.

Materials and Methods: Articular chondrocytes were isolated from 2-4 week old bovine stifle joints following an 18-hr collagenase digestion of articular cartilage tissue. Chondrocytes were then encapsulated in a collagen type I gel. Collagen type I was first neutralized using NaOH and subsequently mixed with chondrocytes suspended in media to yield a final collagen gel concentration of 2.7mg/ml (0.27%). The disc-shaped constructs were seeded at a density of 2.5 x10^6 cells/ml, gelled at 37°C, and incubated in 6-well plates in serum-supplemented culture media. PLIUS was applied to the constructs for 20 mins, once a day, 5 days a week, at an intensity of 30mW/cm^2 (spatial average-temporal average) and a frequency of 1.5 MHz, with a pulse burst frequency of 1 kHz and burst duration of 200µs. Constructs were maintained in culture and treated with ultrasound for a total of 3 weeks. The collagen-cell constructs were then analyzed biochemically, histologically and using MRI. *Biochemical quantification:* Sulfated glycosaminoglycan (sGAG) content, a measure of proteoglycan (PG) content, was determined using the 1,9-dimethylmethylene blue assay and normalized to sample wet weight. *Histology:* Collagen-cell constructs were fixed in 10% neutral buffered formalin for 6 hours. After fixation, the constructs were rinsed twice in PBS and then immersed in 35% ethanol. After dehydration using increasing concentrations of ethanol, samples were paraffin-embedded and sectioned into 4µm slices. Paraffin-embedded slices were stained with Alcian blue to visualize proteoglycan deposition.
Magnetic Resonance Imaging: Images were obtained using a 9.4T Bruker DMX spectrometer at 4°C. T2 relaxation was measured using a 64-echo Carr-Purcell-Meiboom-Gill (CPMG) sequence. Additional T2 data was acquired by using a spectroscopic CPMG pulse sequence with TE/TR=0.6ms/5s, 1024 echoes and NA=128. These results were used to define T2 distributions in the constructs using a non-negative least squares analysis. T1 was measured using a spin-echo sequence with TE=12.8ms and TR ranging from 100ms to 15s in 12 steps. MT-weighted images were obtained using a spin-echo sequence (TE/TR=12.8ms/5s) preceded by a 6kHz off-resonance saturation pulse with amplitude B1 = 12 micro, with saturation pulse length incremented from 0.1 to 4.6s in 8 steps. Apparent diffusion coefficient (ADC) was measured using a spin-echo sequence (TR = 5s), incorporating a pair of identical gradient pulses of δ = 5 ms duration placed on either side of the 180° refocusing pulse, retaining an interval of Δ =13.8 ms between the gradient pulse centers. The diffusion gradient strength was increased from 0 to 320 mT/m in 8 steps. *Statistics*: Statistically significant differences at the p<0.05 level are indicated by *

Results and Discussion: sGAG content increased significantly with ultrasound treatment (Fig. 1), with an average value of 5.3 ± 1.7µg/mg in control samples and of 7.7 ±1.7µg/mg in PLIUS-treated samples. Histologic analysis was consistent with this, with a more intense Alcian blue staining seen in the PLIUS treated samples. These results agree well with previous work which demonstrated the stimulatory effects of mechanical loading on chondrocyte matrix synthesis[6]. Results of MR measurements are shown in Figure 2. T2 decreased significantly with US treatment, from 418.4 ± 50.3ms in control samples to 355.1 ± 37.1ms in treated samples (Fig 2a), while T1 did not change with US treatment (Fig. 2b). T1 and T2 have been shown to decrease with increasing macromolecular tissue content, so that the observed decrease in T2 can be attributed to increased sGAG in PLIUS-treated samples. The MT rate increased significantly with PLIUS treatment, from 0.035± 0.016s^{-1} in control samples to 0.067 ± 0.019s^{-1} in treated samples (Fig. 2c), consistent with an increase in collagen content resulting from PLIUS treatment[5]. Finally, multiexponential T2 analysis indicated the presence of intermediate-rate and slowly-relaxing components within the constructs, with the latter previously assigned to water loosely associated with PG's. A decreased T2 value was found for this slowly relaxing T2 component in the PLIUS-treated samples (Fig. 2d), indicating increased matrix interactions within this water compartment[7].

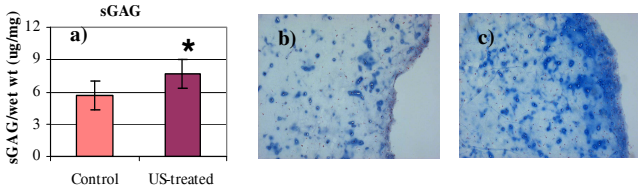

Figure 1 a) sGAG content of control and ultrasound treated 3 week old collagen constructs, *p<0.05; Representative micrographs of Alcian blue stained b) control and c) ultrasound treated collagen constructs at a 100X magnification

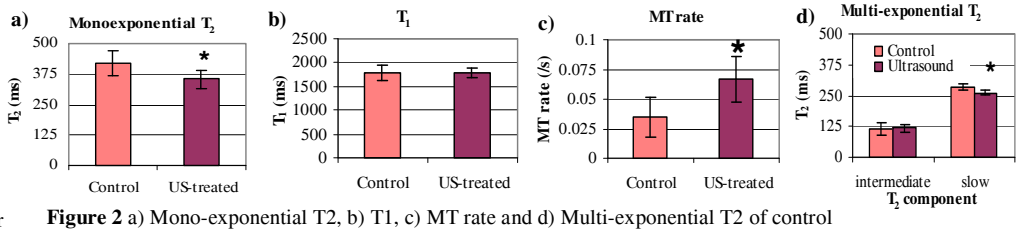

Figure 2 a) Mono-exponential T2, b) T1, c) MT rate and d) Multi-exponential T2 of control and PLIUS treated constructs. Intermediate-rate and slowly-relaxing components correspond to intermediate T2 and long T2 components. *p<0.05

Conclusion: In this work, we demonstrate that MRI can be used as a sensitive, non-invasive means to evaluate the effects of anabolic interventions on cartilage matrix within tissue engineered constructs. In addition, these results are supportive of the potential use of MRI for longitudinal *in situ* evaluation of matrix within tissue engineered cartilage implants.

References: 1. Chen et al., *Arthritis Rheum*. 2003, 48(4): 1047-56. 2. Potter et al., *Arthritis Rheum*. 2000, 43(7): 1580-90. 3. Reiter et al., *Trans. Orthop Res. Society* 2008, 33 (Paper No 0565). 4. Parvizi et al. *J Orthop Res* 1991, 17: 488-94. 5. Laurent et al, *Mag Res Imaging* 2001, 19(10):1279-1286. 6. Wang et al. *Biomed Mater Eng* 2008;18(1 Suppl):S47-54. 7. Reiter et al, *Trans. Orthop Res. Society* 2008, 33 (Paper No. 1648).