Reduction of Water Diffusion Coefficient with Increased Engineered Cartilage Matrix Growth Observed using MRI

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Abstract— **Non-destructive monitoring of tissue-engineered cartilage growth is needed to optimize growth conditions, but extracting quantitative biomarkers of extracellular matrix development remains a technical challenge. MRI provides a non-invasive way to obtain a three dimensional map of growing tissue where the image contrast is based on tissue water relaxation times and the apparent diffusion coefficient (ADC). In this study, bovine chondrocytes were seeded in alginate beads (0, 1, 2, and 4 million cells/ml) and the ADC was measured weekly using diffusion-weighted MRI at 14.1 T over a one-month incubation period. Two groups of tissue-engineering constructs were created: one with ascorbic acid (vitamin C) added as a vitamin cofactor to increase collagen synthesis, and another with no added ascorbic acid. When normalized to the control beads without chondrocytes, the ADC was found to monotonically fall with incubation time (decreasing by up to 40% at 4 weeks), and with the administration of vitamin C. These results reflect the expected development of the extracellular matrix in the tissue-engineered constructs. We conclude that the normalized ADC is a potential biomarker for characterizing engineered cartilage tissue growth.**

I. Introduction

Tissue engineering and regenerative medicine (TE/RM) is an evolving interdisciplinary field that integrates engineering with biology and medicine for the development of functional tissues and organs. It is considered to be the foremost future treatment option for cartilage damage caused by injury, trauma or aging [1]. However, many challenges remain unaddressed in realizing the full potential of this treatment path. Foremost among the technical challenges is the lack of noninvasive monitoring of tissue growth at all stages, from cell seeding to post-implantation [2]. This is important in order (a) to assess the efficacy of tissue engineering approaches at an early growth stage, (b) for providing an accurate assessment of engineered tissue growth post-implantation, and (c) for monitoring the healing of cartilage after treatment. Magnetic resonance imaging (MRI) is a leading non-invasive characterization technique for monitoring growth in cartilage tissue engineering [2-7].

Research was partially supported by NIH/NIBIB R01 grant EB007537.

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Cartilage tissue in its native state contains chondrocytes (~1%) imbedded in an extracellular matrix (ECM). The ECM is important structural integrity and load-bearing functional properties of cartilage. The ECM of cartilage is composed of tissue fluid (~ 70 - 80 %) and structural macromolecules that include proteoglycans (~ 5%), collagen type-II (~ 20%), and non-collagenous proteins and glycoproteins [8]. Proteoglycans (PGs) are composed of a protein core attached to one or more glycosaminoglycan chains (GAG). These GAG chains are negatively charged and attract positive ions, such as sodium, in tissue fluid. This high concentration of cations is responsible for 50% of cartilage tissue stiffness and is one of the indicators of cartilage health. Collagen type-II, a positively charged protein at neutral pH, forms a cross-banded fibril network inside the tissue with its orientation changing along the depth of the tissue [9-11]. Collagen fibrillar meshwork gives cartilage its form and tensile strength [9]. Cartilage tissue engineering aims to recreate these bulk mechanical and surface lubricant properties using a combination of cells, scaffold and growth conditions.

Cartilage is 70-80% water; therefore, the dynamics of water protons provides a window into the biomechanical properties of engineered tissues. The diffusion-weighted MRI (dMRI) and particularly, the water apparent diffusion coefficient (ADC) has been used to probe the growth in cartilage tissue engineering [2]. Miyata et al. [12] found a strong negative correlation of relative ADC (rADC, where $rADC = ADC_{sample}/ADC_{PBS}$, PBS = phosphate buffer saline) with an increasing amount of GAG as a function of the growth period in chondrocyte/agarose disks. Yin et al [13] showed a similar strong negative correlation of ADC with increasing amounts of proteoglycans and collagen in a scaffold-free chondrocyte pellet culture. The current study is designed to take these findings further by testing the efficiency of ADC in identifying different levels of tissue-growth in tissue-engineered (TE) cartilage constructs. For the purpose of this study, a well-tested cartilage tissue-engineering model, bovine chondrocytes seeded in alginate beads, was chosen.

The culture of articular chondrocytes suspended in alginate beads has often been used as a cell entrapment bioreactor in the field of cartilage tissue engineering [14-18]. Alginate beads provide a gel like microenvironment and it has been shown that chondrocytes in this environment can maintain their phenotype for more than a month [18, 19]. We observed the growth of the cartilage extracellular matrix in this system for four weeks using diffusion MRI. By seeding chondrocytes in alginate beads, two separate groups of tissue engineering cartilage constructs were created. In one group,

ascorbic acid (vitamin C) was added in the growth culture medium for increased collagen synthesis, whereas another group of chondrocytes were grown without the addition of ascorbic acid using the same growth culture medium. The cell densities (0, 1, 2 and 4 million cells/ml) were varied within each group. The acellular sample group was treated as a control. This resulted in a total of 7 sample groups with varying ECM growth. The objective of this study was to test the sensitivity of the water ADC in differentiating the growth of ECM among these groups.

II. Theory

Water diffusion weighted magnetic resonance imaging (dMRI) is an established technique to probe tissue microstructure [20]. In tissues, water motion is not entirely random (Brownian) and is restricted by cellular membranes, intracellular organelles and macromolecules. This provides an estimate of tissue microstructure using dMRI. In MRI experiments, diffusion coefficient is measured by using a pair of gradient pulses in the standard spin echo experiment. The signal strength in this case is given by

$$S = S_0 e^{-bD}, where\ b = \gamma^2 G^2 \delta^2 \left(\Delta - \frac{\delta}{3}\right) \qquad (1)$$

where γ is the gyromagnetic ratio of water protons, G is the gradient strength, δ is the gradient duration and Δ is the delay between the pair of gradients. Fitting the echo intensity with the exponential decay curve as a function of the b-value gives an estimation of the diffusion coefficient. The obtained parameter is called the apparent diffusion coefficient (ADC) and is related to the true self-diffusion coefficient of water with a calibration coefficient λ (ADC = D/λ), where the coefficient λ depends on other motions due to the presence of gradients and tissue complexity. In the current study, normalized ADC (nADC = $ADC_{TE\_beads}/ADC_{control\_beads}$) was used to probe the tissue growth.

III. Materials and Methods

A. Cartilage tissue engineering constructs

Bovine chondrocytes were cultured in alginate beads according to the published protocol [14, 19]. The schematic of the sample preparation is presented in Figure 1. Briefly, chondrocytes were isolated from a full depth of articular cartilage from the metacarpophalangeal joints of a 1-2 year old steer by collagenase/dispase digestion and mixed with a solution of 1.25% alginate acid at 37 °C. The cell density was varied (0 (control), 1, 2 4 and 8 million cells/ml) to obtain the desired value. The beads were created by dropping the solution through a needle in a calcium chloride solution. The bead constructs were divided into two groups. One group of constructs were cultured with chondrogenic growth media (Cellgro medium (DMEM/F12 1:1 mix) from Mediatech, Inc.) only, whereas in another group, 50 μg/ml of ascorbic acid was added to the culture medium for additional collagen synthesis.

B. MRI measurements

The samples were fixed in 10% formalin after designated growth time and kept in a refrigerator at -4 C until the MRI

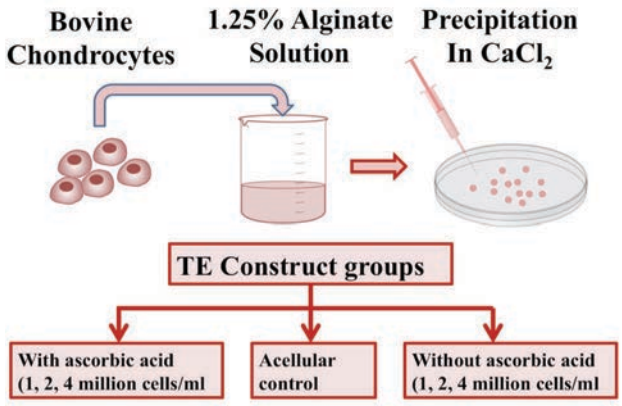

Figure 1: Schematic of tissue engineered (TE) construct preparation.

measurements were performed on them. Before the MRI experiments, the beads were washed with the growth culture medium and placed in a 5 mm (ID = 4.2 mm) microimaging tube as shown in Figure 2. Each tube contained 5 beads set at the bottom of the tube. Beads were approx. 2-4 mm in diameter so they fit within the tube well. The tubes were arranged so that all measurements for the single time point could be performed during one MRI session as shown in Figure 2. Each measurement group had one set of acellular beads designated as the control samples.

All MRI measurements were performed at the Beckman Institute of the University of Illinois at Urbana-Champaign on a vertical bore imaging scanner (Oxford Instruments, Abington, UK) equipped with a Unity/Inova console (Varian, Palo Alto, CA), operating at 14.1 T and dedicated to microimaging studies. The 14.1T Varian microimager

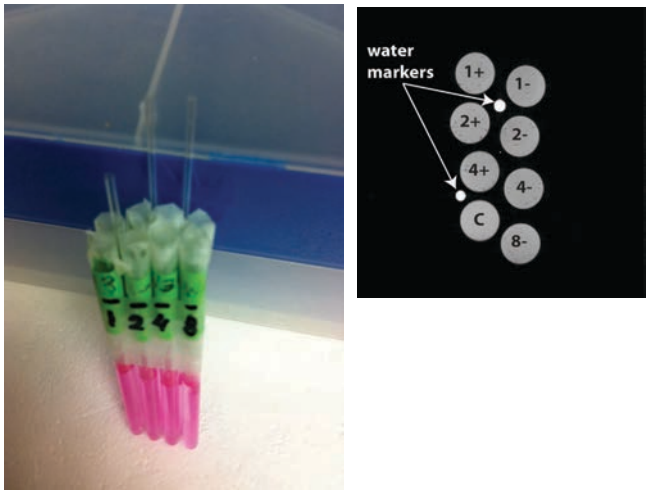

Figure 2: Schematic of MRI measurements and a representative spin echo MRI image of 'week 3' chondrogenic alginate bead samples. The samples marked with 1-, 2-, 4- and 8- are without the aid of ascorbic acid and are of 1, 2, 4 and 8 million cells/ml cell densities, respectively. The samples 1+, 2+ and 4+ are with 1, 2 and 4 million/cells with the aid of ascorbic acid, respectively. The beads with 8 million cells/ml were exploded during the course of the study and are not included in the analysis. Water-filled capillaries were used as markers for sample positioning. The beads were 2 - 4 mm diameter, and fitted to the size of the inner diameter of a 5 mm NMR tube. Although less accurate than measuring the each sample group separately, this arrangement allowed the group measurement in a fast manner.

consists of a vertical wide bore magnet (89mm) and a 600MHz (^{1}H) Varian Unity/Inova NMR spectrometer equipped with gradient coils with a maximum strength of 95 Gauss/cm providing in-plain imaging resolution up to 10 microns. The inner diameter of the radiofrequency coil used (30mm) allowed us to measure multiple samples at the same time (shown in Figure 2) thus reducing inter-scan errors.

Diffusion weighted spin echo MRI sequence was used for the measurement of ADC. The experimental parameters are: TE/TR = 50 ms/ 9000 ms, FOV = 30 mm x 30 mm, slice thickness = 2 mm, Matrix size = 64 x 64, number of slices = 10, 7 b-values: 92, 367, 825, 1466, 2291, 3299 and 4491 s/mm^2.

C. MRI data processing

All diffusion weighted image intensities were fitted to the single exponential decay curve using a custom written Matlab program. The diffusion coefficient was calculated using a circular region of interest (ROIs) for each slice covering the whole bead/tube volume because the bead contrast was not clearly visible in diffusion maps. Since most beads were at the bottom of the tube, only 5 bottom slices were taken into account while calculating ADC. The mean nADC was determined by taking the ratio nADC = $ADC_{TEbeads}/ADC_{control\_beads}$ for each slice and then taking the average of the nADC.

IV. Results and Discussion

Figure 3 shows the nADC for tissue-engineering construct groups as a function of growth time. The ADC is not specific to the particular ECM component and represents the tissue microenvironment that includes restricted and hindered water motion within the tissue. In the context of monitoring tissue-engineering growth, we can say that the lowering of the nADC value is generally correlated with increased macromolecules.

A few points can be noted from the figure:

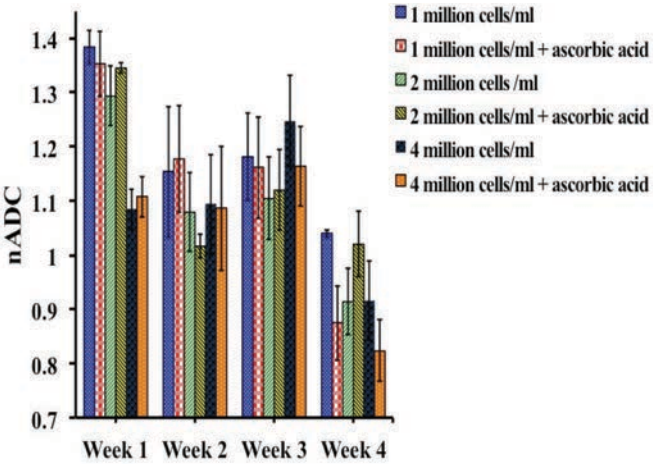

Figure 3. Normalized ADC (nADC) for chondrocytes seeded in alginate beads as a function of culture time. The error bars are for SEM (standard error of the mean). The differences in normalized Apparent Diffusion Coefficient (nADC) values between different groups are astonishing and provide insight into how MRI can be used to give understanding of tissue growth.

- With increased culture time from Week 1 to Week 4, a reduction in the nADC was observed for all sample groups. This signifies a higher macromolecule synthesis with time. The null hypothesis that there is no difference between Week 4 and Week 1 sample groups using two-tailed paired t-statistics was tested The calculated p-values were found to be lower than 0.005 for all sample groups thus rejecting this hypothesis. The difference between Week 4 and Week 1 sample groups is found to be statistically significant for all sample groups.
- When we compare the effect of cell density, the average nADC values are lower for high cell density when compared with low cell density. However, this difference was not found to be statistically significant for our study.
- The addition of a vitamin cofactor (ascorbic acid) was designed for additional collagen synthesis. When we look at the groups with and without ascorbic acid, for 1 and 4 million cells/ml, the ascorbic acid group shows lower nADC when compared with the group without such an addition. This represents a higher macromolecule synthesis for these sample groups.

Figure 4 shows the percent nADC change for all groups from week 1 to week 4 as a function of ascorbic acid and the cell density. It can be seen that the percent change with ascorbic acid is higher as compared to without this additional cofactor for both 1 and 4 million cells/ml groups. For the sample groups with 2 millions cells, this effect was unexpectedly reverse. Surprisingly, however, the low cell density shows a higher percent change in the nADC value going from week 1 to week 4 as shown in Figure 4. This suggests a higher percent of macromolecular synthesis for lower cell density.

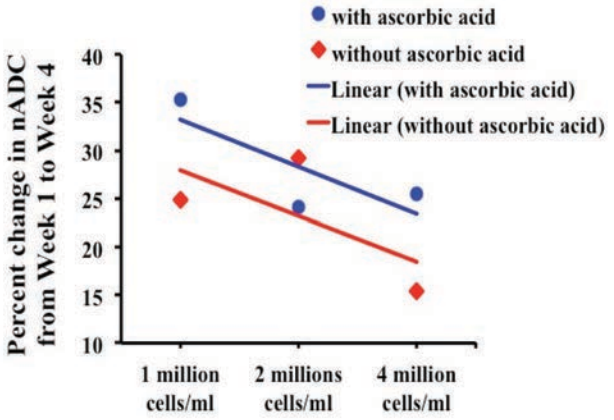

Figure 4: Change in % values of nADC from Week 1 to Week 4 as a function of vitamin cofactor and cell density. The trend-line is plotted as a guide to eye. A few points to be noted: (a) a lower cell density corresponds with a higher percent change in nADC showing the increased ECM synthesis during the four-week of growth period and diminishing return in ECM growth with increased cell density. (b) the change in nADC is higher for the groups with ascorbic acid when compared to the groups without ascorbic acid.

V. CONCLUSIONS

This study shows the sensitivity of normalized ADC (nADC) in identifying growth for chondrocytes in alginate beads with a varying ECM amount. We show that nADC is a sensitive tool for identifying tissue growth and differentiating between different cell densities and different macromolecule synthesis. However, this study has its own limitations. One of the limitations is that using the nADC alone it was not possible to assign growth with statistical significance. Further use of the multivariate principal component-based analysis in conjunction of other popular methods for cartilage assessment, such as T_2 maps, will improve the sensitivity of this method without the need to increase the sample size. We plan to undertake such a study in the future.

ACKNOWLEDGMENT

M. Kotecha thanks Dr. Boris Epel for providing the Matlab program Kazan Viewer and Arbuz GUI for data analysis.

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