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Stabilization of T₂ Relaxation and Magnetization Transfer in Cartilage Explants by Immersion in Perfluorocarbon Liquid

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Abstract

Purpose: Magnetic resonance imaging of ex-vivo cartilage measures parameters such as T₂ and magnetization transfer ratio (MTR), which reflect structural changes associated with osteoarthritis. Samples are often immersed in aqueous solutions to prevent dehydration and to improve susceptibility matching. This study sought to determine the extent to which T₂ and MTR changes are attributable to immersion alone and to identify immersion conditions to minimize this confounding factor.

Methods: T₂ and MTR were measured before and after immersion for up to 24 hours at 4°C. Bovine nasal and articular cartilage and human articular cartilage were studied. Experimental groups included undisturbed immersion in Fluorinert FC-770, a susceptibility-matched, hydrophobic liquid with minimal tissue penetration, and immersion in Fluorinert, DPBS or saline, with removal from the magnet between scans. ¹⁹F and ¹H MRI were used to detect cartilage penetration by Fluorinert and swelling, respectively.

Results: Saline and DPBS immersion rapidly increased T₂, wet weight and cartilage volume and decreased MTR, suggesting increased water content for all cartilage types. Fluorinert-immersed samples exhibited minimal changes in T₂ or MTR. No ingress of Fluorinert was detected after two weeks of continuous immersion at 4°C.

Conclusion: Ex-vivo quantitative MR studies of cartilage may be confounded by the effects of immersion in aqueous solution, which may be comparable to or larger than effects attributed to pathology. These effects may be mitigated by immersion in perfluorocarbon liquids such as Fluorinert FC-770.

Keywords

cartilage; MRI; T₂; magnetization transfer; perfluorocarbon

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Introduction

Quantitative magnetic resonance has been used to assess degradation of cartilage ex-vivo (1–5) and in the development of MR methods to study osteoarthritic degeneration in-vivo. For example, increased T_2 has been observed with cartilage degradation in osteoarthritis (OA), presumably due to increased hydration and disruption of macromolecular structure (6). Magnetization transfer ratio, defined as $MTR = 1 - (M_{sat} / M_0)$, where M_{sat} and M_0 represent magnetization with and without off-resonance saturation, respectively, decreases with OA. This reflects decreased exchange between water associated with macromolecules such as collagen and bulk water (7,8).

A standard ex-vivo paradigm is to measure T_2 and MTR following pathomimetic degradation, in which cartilage is exposed to an aqueous enzyme solution (2–5,7,9–13). The extent of T_2 increase and MTR decrease is taken as a measure of degradation. Other MR experiments require immersion of cartilage in aqueous solutions, including estimation of glycosaminoglycan concentration by dGEMRIC (14).

The details of cartilage sample preparation have been observed to directly affect ex-vivo T_2 and MTR measurements (4,5,9,15,16), with one study evaluating the effects of immersion in phosphate buffered versus normal saline (16). However, the effect of prolonged immersion in aqueous solution, either during post-harvest storage (15,17) or scanning, has not been investigated. Of particular concern, water ingress may occur with immersion, leading to substantial increase in T_2 and decrease in MTR. This would clearly represent a major confounding factor in ex-vivo cartilage studies.

Bovine nasal cartilage (BNC), bovine articular cartilage (BAC), and human articular cartilage (HAC) are all used in ex-vivo MR studies. BNC serves as an excellent model for hyaline cartilage while circumventing the confounding effects of the laminar structure and magic angle effect in articular cartilage (18). BAC is a readily-available model of HAC, while the latter is clearly the most relevant for translational research.

In this work, we test the hypothesis that soaking BNC, BAC, and HAC in aqueous solutions leads to increased T_2 and decreased MTR. We tested the additional hypothesis that this confounding effect could be ameliorated by immersion in Fluorinert, a proton-free, hydrophobic, susceptibility-matched perfluorocarbon (19).

Methods

Sample Harvest

BNC—A total of 12 plugs (5 mm diameter) were excised from nasal septa of 2-week-old calves (Research 87, MA) and washed every 30 s during dissection with Fluorinert™ FC-770 (Sigma-Aldrich, St. Louis, MO), hereafter referred to as “Fluorinert”.

BAC—Bovine patellae were excised from the stifle joint of fifteen 2–3 year old cows (Old Line Custom Meat Company, Baltimore, MD). During dissection, patellae were washed every 30 s with Fluorinert. A total of 12 plugs were excised from the weight-bearing region

of the patellae using a 5 mm biopsy punch. Plugs contained full thickness articular cartilage and subchondral bone to maintain structural integrity (20).

HAC—Tissue from patients undergoing elective knee joint replacement was obtained with informed consent according to a protocol approved by the Institutional Review Board of Medstar Harbor Hospital. After surgery, cartilage was refrigerated in sterile sample cups with no solutions added. A total of 12 osteochondral plugs with 5 mm diameter and 5 mm height were harvested from non-load-bearing regions of the tibial plateau in each sample with minimal visual signs of degradation; these samples were assumed to represent normal cartilage. Experimentation began within two hours of surgery.

Sample Preparation

Directly after harvest, each sample was weighed and placed into a 5 mm × 30 mm glass NMR tube (New Era Enterprises, Vineland, NJ) filled with Fluorinert. Initial MR data were acquired immediately. Samples were maintained at 4°C throughout storage and scanning to minimize degradation. Cold air from a vortex tube (Exair, Cincinnati, OH) was used to refrigerate samples during MR scanning.

Soaking—For each type of cartilage, plugs were randomly assigned to one of four groups of three samples each. Samples from group one (F1) were immersed in Fluorinert within a NMR tube and maintained in the MR instrument undisturbed. Measurements were acquired after 0.5, 1, 3, 4 and 24 hours. Results from F1 define the effect of Fluorinert immersion *per se*. Group two (F2), three (D1) and four (S1) samples were maintained respectively in Fluorinert, DPBS (Gibco, Waltham, MA), and saline (Quality Biological, Gaithersburg, MD). After 0.5, 1, 2, 3 and 24 total hours of immersion, samples in these groups were removed from their baths, washed with Fluorinert, gently blotted dry, weighed and imaged after replacement in Fluorinert. After each imaging session, samples were blotted and returned to their bath. Groups S1 and D1 defined the effect of exposure to saline and DPBS, which may differ (16), while group F2 defined the effect of sample handling without immersion in aqueous solutions.

Data Acquisition

¹H Magnetic Resonance Imaging and Spectroscopy—Data were acquired with a 9.4T Bruker Avance III NMR spectrometer equipped with microimaging gradients and a 5 mm solenoidal coil (Bruker Biospin, Rheinstetten, Germany). Samples were oriented with B_0 at 54.7° to the normal to the articular surface, maximizing the signal-to-noise ratio and minimizing laminar variations in image intensity. In the non-localized experiments, signal from the subchondral region was eliminated using saturation slabs.

T_2 measurements were conducted using a non-localized Carr-Purcell-Meiboom-Gill (CPMG) sequence with 4096 echoes, echo time spacing = 100 μ s, 10 μ s rectangular 90° and 180° pulses, TR = 10 s and eight signal averages. These parameters resulted in linearly-spaced echo times from 0.1 to 409.6 ms and a total acquisition time of 1 min 20 s per sample at each soaking time point. Magnetization transfer-weighted data were acquired using the same CPMG sequence with and without a 3 s pre-saturation pulse with B_1 = 12 μ T applied 6

kHz off-resonance from water. This pulse was applied before slab saturation to avoid regrowth of slab-saturated magnetization. Scan time for the MTR experiment was 3 min 4 s per sample per time point. Cartilage volume measurements were obtained using a 3D FLASH sequence with TE = 1.7 ms, TR = 15 ms, FA = 15°, two averages, 52 $\mu\text{m} \times 52 \mu\text{m} \times 94 \mu\text{m}$ spatial resolution and 3 min scan time.

^{19}F MRI— ^{19}F imaging was performed to detect any penetration of Fluorinert into the cartilage. Experiments were conducted on one sample of HAC using a 5 mm solenoidal coil tuned to ^{19}F . Due to the abundance of protons in cartilage and relatively low coil Q, it was possible to use the same coil to acquire ^1H gradient echo images without retuning. To avoid ghosting due to the multiple ^{19}F chemical shifts in Fluorinert, the largest peak was selectively excited with a 2 ms hermite pulse without a slice gradient. A 3D FLASH sequence was used to acquire images with TE = 4.1 ms, TR = 50 ms, one signal average and 156 μm^3 spatial resolution. Acquisition time was 3.4 min per scan. Matching ^1H and ^{19}F images were acquired using identical parameters, except for flip angle, which was 30° for ^{19}F and approximately 5–10° for ^1H . Baseline scans and scans after two weeks of Fluorinert immersion at 4°C were performed. Regions of interest (ROIs) delineating the cartilage margins were manually drawn on the baseline ^1H image then transferred to the final ^1H image and both ^{19}F images. Mean ^{19}F intensities in these ROIs and matching ROIs in the background of each image were normalized to the mean intensity in the Fluorinert bath at baseline. Normalized intensities before and after soaking were compared to detect possible permeation of Fluorinert into the plug.

Data analysis

T_2 was calculated by fitting CPMG data using the “lsqnonlin” nonlinear least squares algorithm in MATLAB (Mathworks, Natick, MA), assuming a two-parameter monoexponential function that included T_2 and proton density (21). The first echo was omitted from this fit, following the analyses of McPhee and Wilman (22) and Milford et al. (21). For consistency, MTR was calculated from the magnitude of the second echo in the CPMG train with and without the presaturation pulse. Cartilage volumes were calculated by 3D intensity-based cluster analysis using Bruker ParaVision 5.1 software.

Statistical Analysis

Repeated measures ANOVA was used to compare time series data for the four groups. The “Two Way RM ANOVA” function in SigmaStat 3.5 (Systat, San Jose, CA) was used to perform this analysis. Time series data are presented as mean percent change from baseline. Error bars indicate the standard deviation over three cartilage samples. To prevent visual confusion resulting from overlap of error bars for different soaking groups, error bars for a given group are shown in only a positive or negative direction; an equal standard deviation of opposite sign is implied for each data point. Statistical significance was defined as $P < 0.05$. Significant differences between time points were identified by paired T-tests. All statistical tests were performed on baseline-normalized data to compensate for variations in sample composition and to test the null hypothesis that T_2 , MTR, wet weight and cartilage volume did not change during immersion.

Results

BNC

Figure 1a shows the relative change in T_2 with immersion. After 30 minutes, T_2 for groups F1_{BNC} and F2_{BNC} decreased by 5.5% ($P=0.15$) and 8.2% ($P=0.09$) from baseline, respectively. Conversely, T_2 for groups D1_{BNC} and S1_{BNC} increased by 22.7% ($P=0.08$) and 33.8% ($P=0.03$) respectively. After 24 hours, T_2 increased by 7.6% ($P=0.177$) and decreased by 11.9% ($P=0.003$) for F1_{BNC} and F2_{BNC}, respectively. In D1_{BNC} and S1_{BNC}, T_2 increased by 35.2% ($P=0.108$) and 39.7% ($P=0.001$), respectively.

Figure 1b shows the relative change in MTR for each group. After 24 hours, MTR values decreased by 6.8% ($P=0.308$) for F1_{BNC}, decreased by 4.8% ($P=0.4$) for F2_{BNC}, decreased by 25.3% ($P=0.12$) for D1_{BNC}, and decreased by 11.3% ($P=0.01$) for S1_{BNC}.

Figure 1c shows the relative change in wet weight for groups F2_{BNC}, D1_{BNC} and S1_{BNC}. After 24 hours, F2_{BNC} sample weight decreased by 9.8% ($P=0.002$). Samples in D1_{BNC} and S1_{BNC} increased in weight by 12.5% ($P=0.001$) and 11.8% ($P=0.07$), respectively.

Figure 1d displays the relative change in cartilage volume for each group. After 24 hours, F1_{BNC} sample volume increased by 5.1% ($P=0.437$) while volume in group F2_{BNC} decreased by 13.2% ($P=0.003$). Groups D1_{BNC} and S1_{BNC} increased in volume by 6.4% ($P=0.129$) and 11.7% ($P=0.104$), respectively.

The T_2 , MTR, weight and volume time courses for each group were compared using repeated measures ANOVA (see Table 1). T_2 time courses differed significantly between all pairs of groups except F1 vs. F2 and D1 vs. S1.

BAC

Figure 2a shows relative changes in T_2 with immersion. After 30 minutes, T_2 for groups F1_{BAC} and F2_{BAC} increased by 1.6% ($P=0.30$) and decreased by 16.4% ($P=0.007$) from baseline, respectively. Conversely, T_2 for groups D1_{BAC} and S1_{BAC} increased by 74.3% ($P=0.002$) and 91.2% ($P=0.002$) respectively. After 24 hours, T_2 increased by 21% ($P=0.128$) and decreased by 28.1% ($P=0.03$) for F1_{BAC} and F2_{BAC}, respectively. In D1_{BAC} and S1_{BAC}, T_2 increased by a total of 88.2% ($P=0.007$) and 136.9% ($P=0.022$) from baseline, respectively.

Figure 2b shows relative changes in MTR for each group. After 24 hours, MTR values decreased by 4.0% ($P=0.038$) for F1_{BAC}, increased by 3.6% ($P=0.029$) for F2_{BAC}, decreased by 13.1% ($P=0.0067$) for D1_{BAC}, and decreased by 14.8% ($P=0.0066$) for S1_{BAC}.

Figure 2c presents relative changes in wet weight for groups F2_{BAC}, D1_{BAC} and S1_{BAC}. After 24 hours, F2_{BAC} sample weight decreased by 1.2% ($P=0.029$). Samples in group D1_{BAC} and S1_{BAC} increased in weight by 8.4% ($P=0.04$) and 6.6% ($P=0.05$), respectively.

Figure 2d displays relative changes in cartilage volume. After 24 hours, the sample volume in group F1_{BAC} increased by 1.8% ($P=0.033$) while F2_{BAC} sample volume decreased by

3.8% ($P=0.63$). $D1_{BAC}$ and $S1_{BAC}$ increased in volume by 25.2% ($P=0.007$) and 27.0% ($P=0.028$), respectively.

T_2 , MTR, weight and volume time courses were compared among groups using repeated measures ANOVA (Table 1). T_2 time courses differed significantly in all pairs of groups, while MTR time courses were significantly different except for D1 vs. S1. Cartilage volume time courses differed significantly except for F1 vs. F2 and D1 vs. S1.

HAC

Figure 3a shows percent changes in T_2 with immersion. After 30 minutes, T_2 for groups $F1_{HAC}$ and $F2_{HAC}$ decreased by 3.8% ($P=0.19$) and 6.2% ($P=0.29$) from baseline, respectively. Conversely, T_2 values for groups $D1_{HAC}$ and $S1_{HAC}$ increased by 85.2% ($P=0.009$) and 18.3% ($P=0.076$), respectively. After 24 hours, T_2 decreased from baseline by 1.5% ($P=0.53$) and 18.5% ($P=0.19$) for groups $F1_{HAC}$ and $F2_{HAC}$, respectively. In groups $D1_{HAC}$ and $S1_{HAC}$, T_2 increased by 117.5% ($P=0.01$) and 46.8% ($P=0.005$), respectively.

Figure 3b shows percent changes in MTR for each group. After 24 hours, MTR increased by 0.3% ($P=0.447$) for $F1_{HAC}$, increased by 3.3% ($P=0.06$) for $F2_{HAC}$, decreased by 14.1% ($P=0.01$) for $D1_{HAC}$, and decreased by 8.0% ($P=0.058$) for $S1_{HAC}$.

Figure 3c presents percent changes in wet weight for groups $F2_{HAC}$, $D1_{HAC}$ and $S1_{HAC}$. After 24 hours, $F2_{HAC}$ decreased in weight by 9.3% ($P=0.09$). Sample weight in $D1_{HAC}$ and $S1_{HAC}$ increased by 13.2% ($P=0.12$) and 3.6% ($P=0.26$) respectively.

Figure 3d displays percent changes in cartilage volume with immersion. After 24 hours, $F1_{HAC}$ sample volume decreased by 0.6% ($P=0.347$). During this time, $F2_{HAC}$ volume decreased by 11.5% ($P=0.20$). Groups $D1_{HAC}$ and $S1_{HAC}$ increased in volume by 21.9% ($P=0.05$) and 12.0% ($P=0.01$), respectively.

The T_2 , MTR, weight and volume time courses were compared between sample groups using repeated measures ANOVA (Table 1). This analysis showed that T_2 and MTR time courses differed significantly between pairs of groups except for F1 vs. F2. Similarly, cartilage volume time courses differed significantly between pairs of groups except when comparing D1 and S1 or F1 and F2.

^{19}F MRI

Figure 4 shows matching 2D slices from 3D gradient echo images of a HAC sample immersed in Fluorinert for two weeks with ROIs signifying cartilage margins in the baseline ^1H image. Normalized mean ^{19}F intensity in the Fluorinert bath was 1.000 ± 0.069 at baseline and 0.995 ± 0.068 after two weeks while background ROI intensities were 0.037 ± 0.020 and 0.039 ± 0.020 , respectively. In the cartilage ROI, normalized mean ^{19}F intensity was 0.076 ± 0.100 at baseline and 0.082 ± 0.125 at two weeks. These data indicate that a negligible amount of Fluorinert penetrated the cartilage matrix during two weeks of immersion at 4°C .

Discussion

It has been shown that OA begins with proteoglycan loss and collagen disruption (23). Enzymatic degradation is often applied to healthy ex-vivo animal cartilage to mimic these in-vivo effects. During both enzymatic degradation and imaging, cartilage samples are routinely immersed in aqueous solutions to ensure enzyme activity and for susceptibility matching and prevention of dehydration, respectively (16). Thus, changes in cartilage MR parameters solely due to prolonged immersion in aqueous solutions are an important experimental concern. One approach to minimizing these effects is to maintain the samples in Fluorinert during scanning, but the consequences of this practice have not been previously explored.

The decrease in T_2 and increase in MTR observed in group F2 may be attributed to loss of hydration due to air exposure while samples were repeatedly unpacked, blotted, weighed and repacked for scanning. This is supported by the concomitant loss in wet weight and cartilage volume observed for these samples. In contrast, this did not occur in samples that remained undisturbed in Fluorinert (Group F1). It is clear from the relative stability of T_2 , MTR and cartilage volume over 24 hours that undisturbed immersion in Fluorinert greatly retards changes in sample hydration. In contrast, in groups D1 and S1, T_2 , wet weight and cartilage volume increased and MTR values decreased substantially after soaking periods as short as thirty minutes. This change can be explained by increased hydration upon direct exposure of all cartilage surfaces to aqueous solution, including the transitional and deep zones. In contrast, articular cartilage in-vivo is exposed to synovial fluid only at its articular surface. Thus, the excision of in vitro samples for scanning may facilitate rapid influx of water and swelling during immersion in aqueous solutions. Notably, in BNC and BAC, T_2 was longer at each time point in samples immersed in saline than in those immersed in DPBS. This is consistent with measurements by Wang and Xia (16). Furthermore, the T_2 increase upon soaking cartilage in aqueous solutions is comparable to that observed in enzymatic degradation experiments (3,4,9,16). Thus, changes in T_2 observed during these experiments may have been misinterpreted as mirroring natural cartilage degradation.

For each parameter, some differences were observed among the three types of cartilage studied. For the Fluorinert groups (F1 and F2), results were similar for all types of cartilage. Specifically, T_2 , MTR and cartilage volume all exhibited very small changes in group F1 compared to F2, D1, and S1. Group F2 displayed similar trends of decreased T_2 , weight and volume and increased MTR regardless of cartilage type. Similarly, in all tissues, T_2 increased markedly with exposure to DPBS or saline. In D1_{BNC}, this change was smaller than in D1_{BAC} or D1_{HAC}. In general, each parameter showed qualitatively similar trends for the three cartilage types, although the magnitude of these changes varied with tissue type, especially for soaking in saline or DPBS.

Given the promising performance of Fluorinert immersion in stabilizing cartilage T_2 , MTR and volume over 24 hours at 4°C, we conducted further experiments with ^{19}F and ^1H imaging to determine whether Fluorinert penetrates HAC during prolonged refrigerated storage. These experiments strongly suggest that such penetration is negligible over two weeks. This is consistent with the fact that Fluorinert contains a mixture of hydrophobic

perfluorocarbons such as perfluorotributylamine. Immersed in Fluorinert, hydrated tissue samples are thus expected to absorb little perfluorocarbon liquid and to give up little water to the bath. Although the lack of Fluorinert penetration was demonstrated in a single sample of HAC, this sample may represent a worst case in that it was harvested from the tibia of an older patient undergoing knee replacement; while the sample was macroscopically intact, microscopic degradation may have rendered it more permeable to fluids of any kind than cartilage excised from younger humans or animals with fully-intact knee joints. Moreover, partial depletion of glycosaminoglycans in HAC, resulting in a loss of (negative) matrix fixed charge density, may have allowed a greater concentration of phosphate ions to equilibrate in HAC samples immersed in DPBS than can occur with BAC or BNC samples. As described by Zheng and Xia (24) and Wang and Xia (16), the presence of phosphate ions increases exchange rates between water pools in cartilage, leading to longer T_2 ; this phenomenon may explain why HAC exhibited a greater increase in T_2 with soaking in DPBS versus saline while an opposite and smaller trend was observed for BNC and BAC.

While immersion in Fluorinert cannot inhibit tissue degradation due to endogenous proteases, such immersion can effectively impede sample dehydration during long experiments. Our results indicate that for sample stability during scanning, immersion in Fluorinert is much more effective than immersion in saline or DPBS and may be simpler than other proposed methods (25). It should be noted that, in addition to FC-770, other perfluorocarbon mixtures in the Fluorinert family as well as those of the Fomblin (19) and Galden families should be suitable for this purpose. However, Fluorinert is not a suitable solvent for enzymatic degradation, leaving open the question of the most appropriate means of performing ex-vivo pathomimetic degradation of cartilage.

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1. Time course of percent change (mean $\pm$ standard deviation) in T_2 (a), magnetization transfer ratio (b), wet weight (c), and cartilage volume (d) for **bovine nasal cartilage (BNC)** immersed under different conditions. A dashed gray line indicates zero change in each parameter. Weight change was not monitored in the F1 group as these samples were left undisturbed in the magnet for 24 hours. Note the increase in T_2 and wet weight and decrease in magnetization transfer ratio (MTR) for groups D1 (DPBS) and S1 (saline) over as little as 30 minutes of soaking, indicating the marked effect of immersion in aqueous solutions on MR parameters. These effects were much smaller in group F1, for which samples were maintained in Fluorinert.

2.

Time course of percent change (mean $\pm$ standard deviation) in T_2 (a), magnetization transfer ratio (b), wet weight (c), and cartilage volume (d) for **bovine articular cartilage (BAC)** immersed under different conditions. A dashed gray line indicates zero change in each parameter. Note the substantial increase in T_2 , wet weight and cartilage volume and decrease in MTR for groups D1 (DPBS) and S1 (saline) over as little as 30 minutes of soaking, indicating the marked effect of immersion in aqueous solutions on MR parameters. This effect was much smaller in group F1, for which storage in Fluorinert was implemented.

3. Time course of percent change (mean $\pm$ standard deviation) in T_2 (a), magnetization transfer ratio (b), wet weight (c), and cartilage volume (d) for **human articular cartilage (HAC)** immersed under various conditions. A dashed gray line indicates zero change in each parameter. Note the substantial increase in T_2 , wet weight and cartilage volume and decrease in MTR for group D1 (DPBS) over just 30 minutes of soaking. Group S1 (saline) showed a smaller, but noticeable increase in T_2 , wet weight and cartilage volume and decrease in MTR. These changes were negligible after 24 hours immersion in Fluorinert (group F1).

4. ^1H and ^{19}F gradient echo images of a human articular cartilage sample acquired at baseline and after two weeks of immersion in Fluorinert FC-770 at 4°C. All images were acquired with identical geometrical parameters. A region of interest (labeled “1H_ROI”) was drawn around the cartilage in the baseline ^1H image and copied to all other images. Note that the subchondral bone, visible as a void at the bottom of each ^{19}F image, is dark in both ^1H and ^{19}F images due to its short T_2 and exclusion of Fluorinert. These images illustrate the lack of penetration of Fluorinert into the cartilage over the time scale of the experiments in the present study.

For each cartilage type, *P*-values are shown for repeated measures ANOVA analyses comparing time courses for T₂, magnetization transfer ratio (MTR), wet weight and cartilage volume measurements in pairs of sample groups. An asterisk indicates statistical significance.

Table 1:

Comparison	Bovine Nasal Cartilage (BNC)				Bovine Articular Cartilage (BAC)				Human Articular Cartilage (HAC)			
	T ₂	MTR	Weight	Volume	T ₂	MTR	Weight	Volume	T ₂	MTR	Weight	Volume
D1 vs. F1	0.0158*	0.0103*	N/A	0.919	4.07E-04*	6.25E-05*	N/A	0.0155*	7.55E-06*	9.96E-05*	N/A	4.98E-04*
D1 vs. F2	3.88E-03*	7.98E-03*	8.28E-04*	0.0391*	4.92E-05*	2.83E-06*	0.0220*	0.0105*	1.78E-05*	4.05E-05*	0.0145*	1.62E-04*
S1 vs. F1	2.40E-03*	0.0672	N/A	0.413	5.07E-05*	6.62E-05*	N/A	0.0106*	0.0124*	0.0256*	N/A	3.66E-03*
S1 vs. F2	8.07E-04*	0.0477*	1.41E-03*	0.0148*	1.07E-05*	2.93E-06*	0.0508	7.33E-03*	1.31E-03*	3.56E-03*	0.0881	8.15E-04*
D1 vs. S1	0.149	0.206	0.305	0.478	0.0207*	0.925	0.43	0.769	1.39E-04*	9.55E-04*	0.133	0.076
F2 vs. F1	0.235	0.772	N/A	0.0419*	0.0135*	3.36E-04*	N/A	0.702	0.0654	0.124	N/A	0.136