The Effects of Formalin Fixation and Crosslinking on MRI Contrast in Bovine Nasal Cartilage Explants

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Introduction: Fixation of tissue samples is useful in microimaging experiments not only because the resulting stability of the sample permits time-consuming acquisition of high-resolution, high quality 3D images but also because samples may be immediately processed for histological analysis after imaging. However, other than in neural tissues, there have been few reports in the literature on the effects of fixation on T_1 , T_2 or spin density and resulting MRI contrast and none of these have involved cartilage. Moreover, no systematic study of fixation effects on magnetization transfer (MT) parameters in tissue has appeared, despite the fact that MT-weighted images often give superior delineation of morphology and pathology in a variety of tissues, such as articular cartilage. The goal of the present work is to examine the effects of formalin fixation on MT and T_2 contrast in cartilage in detail. We have chosen to focus on these two parameters since T_2 and MT-weighted images have been shown to give excellent contrast in articular cartilage imaging in vivo. Moreover, quantitative studies have shown that T_2 is sensitive to cartilage matrix content, while MT parameters have been used extensively as indicators of collagen content in cartilage.

Methods: Bovine nasal cartilage (BNC) was chosen as a model system due to its relatively homogeneous, reproducible composition. 8 mm diameter disks were excised from the nasal septa of 4-5 month old calves. From each disk, a central 3 mm core was then removed. The resulting "donut" samples were randomly separated into four groups of 7 samples each. All samples from a given group were threaded onto a 3 mm Pyrex tube and inserted into a 10 mm glass NMR tube. Each tube was filled with 5 ml of DPBS with added protease inhibitors (PI, Sigma P2714), pH 7.5 ± 0.1. Four NMR tubes, containing samples from each group, were fastened together and loaded into a 30 mm ^{1}H resonator (Bruker) and maintained at +4°C by a stream of cold air generated by a vortex tube (Exair, Cincinnati). MRI was performed on a Bruker DMX400 NMR spectrometer equipped with 3-axis shielded gradients. A 1 mm sagittal or coronal slice was defined through the center of each tube, permitting samples in all four tubes to be imaged simultaneously. ^{1}H T_2 was measured using a 64-echo CPMG sequence with TE=10ms and TR=15s. MT-weighted images were obtained using a spin echo sequence with TE=10ms and a presaturation pulse with +6 kHz offset and B_1=12μT. The presaturation pulse length t_p was varied from 0.1 to 4.6 s in 7 steps while TR was fixed at 5 s. Other MR parameters included MTX=256×128, FOV=4.0×1.5 cm (V×H) and NEX=2. T_2 was calculated by a 3-parameter fit of the mean pixel intensity in each cartilage sample at each echo time. MT ratio, MTR = $1 - M_{ss}/M_0$, and apparent MT exchange rate, $k_m = MTR/T_{1sat}$ were calculated from a fit of mean pixel intensity to the function $M(t_p) = M_{ss} + (M_0\text{-}M_{ss})\exp(-t_p/T_{1sat})$. After initial T_2 and MT measurements, fresh solutions of 10% neutral formalin (Surgipath) in DPBS were prepared to give 0%, 0.1%, 1% and 10% formalin with pH 7.5. The fluid in each tube was replaced with one of these four fixative solutions. The cluster of tubes was returned to the magnet and T_2 and MT scans were repeated as described above. Following MRI, the cluster was stored at +4 °C. MRI was repeated in this manner over 13 weeks. Immediately after the last "post-formalin" MRI session, all fluids were replaced with 0.66M $NaBH_3CN$ (Aldrich) in normal saline (pH 8.8). Tubes were then stored at +4 °C overnight to permit reduction and acid-stabilization of immature crosslinks and formalin-methylated amino acids, thus allowing their detection by later amino acid analysis (AAA). Fluids were then replaced with fresh DPBS and the tubes were again stored at +4 °C. Washing was repeated a total of four times over two days to remove residual $NaBH_3CN$ and/or formalin. After the final washing, fluid in each tube was replaced with fresh PI solution. MRI scans were then acquired to give final T_2 and MT values. After scanning, samples from the 0% and 10% dose groups were removed, blotted, weighed and stored at –20 °C. A portion of each thawed sample was later excised, digested and analyzed for glycosaminoglycan (GAG) content by the standard DMMB assay. Another portion was hydrolyzed in 6M HCl for 18 hours at 108°C and analyzed for hydroxyproline (hyp) by the DMB assay and for mature natural crosslinks (PYR and DPD) by HPLC with fluorescence detection. A separate portion was dried, hydrolyzed and subjected to AAA using ninhydrin absorbance detection. Biochemical data was analyzed using a two-tailed unpaired T-test. The dependence of MR data on dose group was evaluated using a one-way repeated measures ANOVA analysis. Appropriate post-hoc tests were performed for pairwise comparisons between post- and pre-formalin values within each group, and for comparisons between 0% and 10% groups at each of the three time points (pre-formalin, post-formalin, and post-washout). All MRI data for cartilage samples are presented as mean ± standard error. T_2 data for bath fluids are shown as mean ± std. dev., where the standard deviation reflects both the noise level of the images and the quality of the fit.

Results: Fig.1 shows a typical sagittal MT-weighted MR image of cartilage samples from one of the four dose groups. In all such images, cartilage samples could easily be distinguished from surrounding fluid and signal intensity was highly homogeneous within each cartilage disk. A typical region of interest for quantitative analysis of one of the samples is indicated by red color in the Figure. Prior to addition of formalin and after washout, bath fluid T_2's for all four tubes were comparable. Addition of formalin resulted in bath fluid T_2's that were markedly shorter for increased formalin concentration: 246.1±7.3ms vs 40.2±1.4ms for 0% vs 10% formalin, respectively. Cartilage T_2 values are shown in Fig. 2, where blue, red and yellow bars represent values measured before formalin, after 13 weeks of storage in formalin at +4°C and after thorough washing. Significant increases in T_2 for the 0% and 0.1% groups and a significant decrease for the 10% group were observed upon equilibration with formalin. Interestingly, after washout, the mean T_2 of samples in the 10% group remained significantly shorter than that measured before fixation while all other groups showed T_2's indistinguishable from pre-formalin values. Figs. 3 and 4 show analogous results for cartilage MTR and k_m, respectively. In all groups and at all time points, no MT effect could be observed in bath fluids. After 13 weeks of fixation, only small changes in cartilage MTR were observed, but after washout of residual formalin, MTR increased markedly with formalin dose. Similar, but much larger effects were observed for k_m, including a full 54% increase upon fixation and washout in the 10% dose group. Overall, after 13 weeks of equilibration and thorough washout, k_m was 121% higher in the 10% group relative to the 0% (unfixed) group while T_2 was 43% shorter and MTR was 42% greater in fixed samples. Comparing biochemical data for samples from the 0% and 10% groups after equilibration and washout, we found no significant differences in the content of sulfated GAG, hyp (and thus total collagen) or mature natural crosslinks. A small but significant difference was noted in water content: 80.6±1.5% vs 77.2±0.9% for 0% vs 10% groups, respectively. AAA showed a significantly lower relative abundance of lys, hyl and tyr in the 10% group while all other assignable amino acids and natural crosslinked species showed no significant differences in concentration between groups. Finally, several new peaks were observed in the AAA chromatograms of each of the formalin-fixed samples that were not observed for any of the 0% controls. These peaks could not be assigned to any known natural amino acids and are assumed to arise from formaldehyde-modified residues.

Discussion: Cartilage samples imaged following immersion in formalin showed much shorter T_2's than unfixed samples. This effect is partially attributable to the presence of hydrated formaldehyde (methylene glycol) and its oligomers. After these were washed away, however, T_2 remained depressed in the fixed samples, despite the fact that total collagen and GAG content were similar in fixed and unfixed samples. Similarly, MTR and, particularly k_m were elevated in samples imaged after fixation, an effect which became only more pronounced after washing. Thus, in the presence of formalin, cartilage-fluid contrast is exaggerated in MT-weighted images, while cartilage T_2 can become much shorter than in fresh tissue. After washing, decreased T_2 and increased MT effects persisted in fixed samples despite constant GAG and total collagen contents and negligible differences in water content. We conclude that T_2, MTR and k_m in cartilage are all dependent on properties other than simple biochemical content. The observed depletion in lys, hyl and tyr with fixation and the appearance of new AAA peaks is consistent with both the replacement of lys and hyl with their N_ε-(di)methyl derivatives and/or formation of lys-CH_2-tyr and hyl-CH_2-tyr crosslinks in addition to the endogenous crosslinks present in the cartilage prior to fixation. The formation of new, formalin-induced crosslinks might increase MT contrast by increasing macromolecular rigidity, thereby increasing ^{1}H T_1's and/or reducing motional averaging of dipolar couplings between free and bound water protons. In cartilage, collagen molecules are quite rigid in the absence of fixation due to the presence of natural crosslinks. However, fixation may create crosslinks within or between molecules of proteoglycan core protein, collagens II, IX and XI which could decrease the mobility of GAG side chains. Thus, the observed increase in MT effects upon fixation and washout might be attributable to magnetization transfer involving the large GAG-associated water pool, which has been assumed to be negligible in cartilage due to GAG mobility. We conclude that T_2 and especially MT effects in cartilage may depend upon unexplored characteristics such as crosslinking status and collagen-proteoglycan interactions.

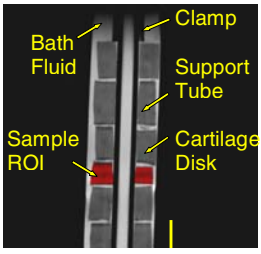

Fig. 2: Cartilage T_2 (ms) vs Formalin Concentration (%)

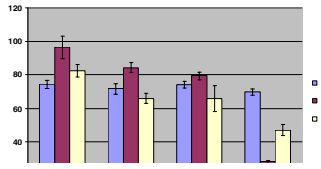

Fig. 3: Cartilage MT Ratio vs Formalin Concentration (%)

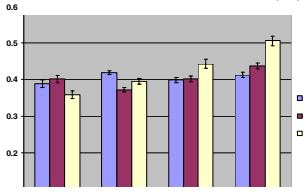

Fig. 4: Cartilage App. MT Rate (s^{-1}) vs Formalin Concentration (%)