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The Effect of Paraformaldehyde Fixation on the Delayed Gadolinium-Enhanced MRI of Cartilage (dGEMRIC) Measurement

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Abstract

The delayed Gadolinium-Enhanced Magnetic Resonance Imaging of Cartilage (dGEMRIC) method allows for both qualitative and quantitative measurement of the spatial distribution of glycosaminoglycan [GAG] in excised cartilage. The objective of this study was to determine the effect of paraformaldehyde fixation on dGEMRIC measurements. Five bovine and seven human cartilage pieces were punched into 5-mm plugs, fixed for 18 h in 4% paraformaldehyde solution, and washed. The magnetic resonance imaging (MRI) parameter T1 was measured prior and post fixation in cartilage without ($T1_0$) and with ($T1_{Gd}$), the ionically charged MRI contrast agent $Gd(DTPA)^{2-}$. Images of tissue before and after fixation were qualitatively very similar. The ratios of $T1_0$, $T1_{Gd}$, and calculated [GAG] after fixation, relative to before fixation, were near or slightly higher than 1 for both bovine cartilage (1.01 ± 0.01, 1.04 ± 0.02, 1.05 ± 0.03, respectively) and for human cartilage (0.96 ± 0.11, 1.03 ± 0.05, 1.09 ± 0.13). Thus, these data suggest that dGEMRIC can be used on previously fixed samples to assess the three dimensional spatial distribution of GAG.

Keywords

cartilage; GAG; fixed charge; fixation; dGEMRIC

The delayed Gadolinium-Enhanced Magnetic Resonance Imaging of Cartilage (dGEMRIC) method allows for both qualitative and quantitative measurement of the spatial distribution of glycosaminoglycan concentration [GAG] in cartilage, and has been used as a nondestructive surrogate for toluidine blue or saffranin O histological staining. Published studies of the validation [1,2] and subsequent use[3–6] of the dGEMRIC method have used

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unfixed tissue. However, there are situations where full three-dimensional analysis of [GAG] may be desirable in tissue that has already been fixed but not sectioned. For experiments in which only one time point is desired, dGEMRIC imaging of fixed samples would allow for much simpler handling of the samples during imaging without concern of degradation. In addition, because many research labs do not have direct access to high-resolution MRI machines at their institution, imaging of fixed samples would permit samples to be sent out for imaging. The availability of a three-dimensional image of an intact fixed sample would also enable evaluation of regions of interest for further staining and histology, depending on the heterogeneity seen in the three dimensional volume.

dGEMRIC imaging exploits the charged nature of the GAG molecule. The cartilage is equilibrated in a bath containing a negatively charged MRI contrast agent, $Gd(DTPA)^{2-}$ (Magnevist, Berlex, NJ). Due to the negative fixed charge of the GAG molecules, the contrast agent distributes inversely proportional to the [GAG]. By measuring the MRI relaxation parameter T1 without contrast ($T1_0$) and with contrast ($T1_{Gd}$), the $[Gd(DTPA)^{2-}]$ in the cartilage can be determined according to the relationship below:

$$[\mathrm{Gd(DTPA)^{2-}}]=\frac{1}{\mathrm{r}}\left(\frac{1}{\mathrm{T1_{Gd}}}-\frac{1}{\mathrm{T1_0}}\right)$$

where $T1_0$, $T1_{Gd}$ = T1 without and with contrast respectively, and r = relaxivity.[7] Fixed charged density, and thus [GAG], can be computed through a modified Donnan Equilibrium theory[1,2] when the equilibrated concentration of gadolinium in the bath is known.

For in vitro studies, this calculation is most commonly done by measuring $T1_{Gd}$ and using previously reported values for r (dependent on temperature and field strength)[7] and $T1_0$.[1,2] However, these previously reported values were measured on unfixed tissue. Fixative agents, such as paraformaldehyde, crosslink tissue proteins. This crosslinking may have some influence on the MRI parameters. Indeed, previous studies of noncartilaginous tissues have found that paraformaldehyde fixation results in a decrease in both T1 and T2 in the brain, liver, and spleen.[8–10] Recent work with bovine cartilage has also shown that fixation leads to a decrease in T2.[11] It remains unknown, however, whether fixation affects T1 of cartilage. Furthermore, because dGEMRIC is essentially a measurement of tissue charge, any direct effect of fixation on the concentration of ionized moieties on the GAG macromolecule would directly affect the dGEMRIC measurement of [GAG].

Accordingly, in this study, we compared $T1_0$, $T1_{Gd}$, and dGEMRIC calculations of GAG before and after paraformaldehyde fixation to evaluate whether the standard dGEMRIC protocol can be applied to fixed tissue.

MATERIALS AND METHODS

Sample Preparation

Two sources of tissue were used: bovine nasal cartilage, and visually intact human articular cartilage. A full bovine nasal septum was obtained from a tissue vendor (Covance, Princeton, NJ) wrapped in saline soaked gauze and frozen to −20°C for later use. Human tibial plateau tissue, including bone and overlying cartilage, was obtained from tissue removed during total joint replacement surgery (New England Baptist Hospital), in accordance with approved human studies protocols. The human tissue was wrapped in saline, soaked in gauze, and frozen to −20°C upon receipt, a procedure validated in our previous studies on analysis of cartilage by ex vivo dGEMRIC.[4]

Tissue was later thawed by leaving the samples at room temperature for 2–5 h, after which the saline soaked gauze was removed, and specimens were cut in preparation for study. Bovine cartilage was punched into plugs measuring 5 mm in diameter and cut to a 2 mm thickness. Full-thickness human cartilage was removed from the bone using a scalpel and punched into plugs measuring 5 mm in diameter. A flat was cut from the circumference to provide a fiducial marker. Human plugs had varying thickness.

Overall Protocol

The samples were equilibrated overnight at 4°C in Hank's Balanced Salt Solution (HBSS, Invitrogen, Carlsbad, CA), weighed, imaged to measure $T1_0$, and then equilibrated over night in HBSS containing 1 mM $Gd(DTPA)^{2-}$ (Magnevist, Berlex Imaging, Wayne, NJ) and imaged again to measure $T1_{Gd}$. Then, samples were fixed for 18 h in a paraformaldehyde solution. Postfixation, the equilibration, weighing and imaging sequence used for unfixed tissue was repeated.

MR Imaging

A total of five bovine nasal cartilage and seven human articular cartilage plugs were imaged individually in an 8.5T magnetic resonance microimaging system (Bruker Instruments, Billerica, MA). Each imaging session provided a series of 10 or 11 T1-weighted images, using a saturation recovery sequence with TRs ranging from 100–5000 or 7500 ms for $T1_0$ and 100–2700 or 3600 ms for $T1_{Gd}$. For the bovine samples, multislice imaging was used to cover the entire sample. For the human samples, the imaging section was positioned to obtain an image across the cartilage/bone in the center of the sample.

$T1_0$ and $T1_{Gd}$ were computed for each pixel by curve fitting the T1-weighted images using custom software written in Matlab® (The Math Works, Natick, MA). The average $T1_0$ and $T1_{Gd}$ of the cartilage were computed for each imaging slice. Using a previously validated modified Donnan theory[1,2] assuming a relaxivity of 4.6 $(mM–s)^{-1}$,[7] average [GAG] was calculated from these $T1_0$ and $T1_{Gd}$ values for the given slice.

Fixation

The samples were fixed for 18 h in 4% paraformaldehyde solution. The solution was made by dissolving 2 g of paraformaldehyde powder (Sigma Chemical, St. Louis, MO) in 50 mL of HBSS and 5 μL of 10 M sodium hydroxide (NaOH, Acros, Geel, Belgium). The solution was placed in a 60°C water bath for 2 h in order to allow the paraformaldehyde to properly dissolve.

Statistics

A paired Student *t*-test was performed on each data set using SigmaStat (Systat, San Jose, CA) to assess whether there were significant differences in wet weight, $T1_0$, $T1_{Gd}$, and [GAG] before and after fixation ($p<0.05$).

RESULTS

For the bovine samples, the ratios of weight after fixation to that before fixation were 1.00 ± 0.01 ($p>0.7$), indicating that hydration was not affected by this fixation protocol.

As shown in the representative examples of Figure 1, there was a close visual correspondence between the images before and after fixation. When comparing the parameters $T1_0$, $T1_{Gd}$, and [GAG] obtained from dGEMRIC, we observed small but statistically significant differences in measurements before and after paraformaldehyde fixation for the bovine plugs, and no statistically significant differences for the human

cartilage (Table 1). Indeed, the ratios of the average $T1_0$, $T1_{Gd}$, and [GAG] before and after fixation were close to 1 for both bovine cartilage (1.01 ± 0.01, 1.04 ± 0.02, 1.05 ± 0.03) and human cartilage 0.96 ± 0.11, 1.03 ± 0.05, 1.09 ± 0.13) (Table 1).

DISCUSSION

Unlike previous findings in noncartilaginous tissues,[8–10] these data clearly indicate that fixation of either bovine or human cartilage had a very modest, if any, effect on the T_1 measurements—$T1_0$, $T1_{Gd}$, and calculated [GAG] by dGEMRIC. The lack of hydrational changes in the cartilage with fixation shown here and in a prior study[11] may account for why the $T1_0$ did not change. Thus, these data suggest that the standard dGEMRIC protocol can be used on previously fixed samples to assess the spatial distribution of GAG. These data further suggest that [GAG] quantified using dGEMRIC measurements postfixation may be slightly greater than what might have been computed prefixation. This fact may need to be recognized when comparing fixed and unfixed tissue, but is highly unlikely to impact conclusions when comparing similarly handled samples.

The long times during which the tissue was incubated in solution could raise questions about whether there was a loss of GAG from the tissue into the bathing medium. Although we did not measure GAG release into the bathing medium, we noted that the consistency of the measurements before and after fixation suggests there was no appreciable loss of GAG. Furthermore, this consistency indicates that the fixation process itself does not appreciably affect the ability of the fixed charge on the GAG macromolecules to influence the concentration of contrast agent. The finding that $T1_{Gd}$ was slightly, but consistently, higher after fixation could be explained in a number of ways, including a modest decrease in the volume accessible to $Gd(DTPA)^{2-}$ (thereby slightly decreasing the average concentration), blocking of charged groups on proteins such as collagen, and slight fixation-associated differences in relaxivity.

The absolute GAG values calculated by dGEMRIC for bovine cartilage is comparable to previous studies reporting biochemically measured values that ranged from 50 mg/mL to over 100 mg/mL.[11–14] The absolute GAG values found for human cartilage is comparable to previous findings that report a range of GAG values from 20–80 mg/mL.[15–21]

The ability to use the dGEMRIC technique on fixed cartilage allows for much simpler tissue handling during the imaging, and opens experimental avenues previously closed. One of the challenges in evaluating cartilage using histology is the practical inability to routinely evaluate the cartilage everywhere in the joint. This finding offers the opportunity to take advantage of the intrinsic ability of nondestructive imaging to visualize the three-dimensional volume. By analyzing the whole joint, one might more easily note particular spatial patterns or perhaps identify regions of particular interest that could be further evaluated using histological approaches.

Acknowledgments

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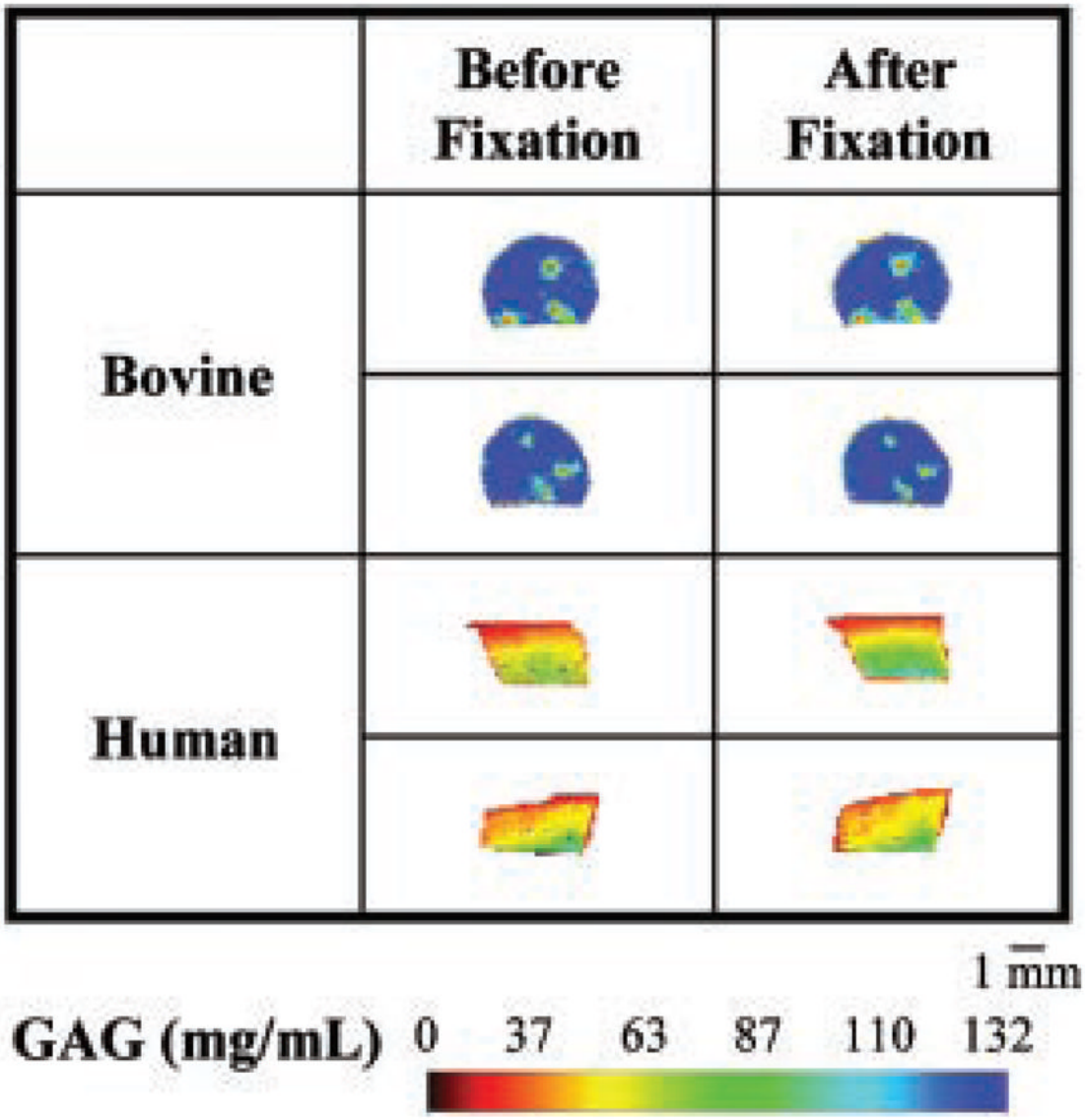

Figure 1.
Representative dGEMRIC images of bovine and human cartilage before and after paraformaldehyde fixation. The similarity between the before and after images supports the observations made from the averaged data (in Table 1), and suggests that dGEMRIC imaging can be used in previously fixed tissue.

Table 1

Average $T1_0$, $T1_{Gd}$, and [GAG] Values before and after Fixation for Bovine (N = 5) and Human (N = 7) Cartilage

		Before	After	Ratio	*p*-value
Bovine N = 5	$T1_0$ (ms)	1820 ± 54	1836 ± 45	1.01 ± 0.01	0.09
	$T1_{Gd}$ (ms)	736 ± 65	768 ± 66	1.04 ± 0.02	0.01
	[GAG] (mg/mL)	116 ± 13	123 ± 13	1.05 ± 0.03	0.01
Human N = 7	$T1_0$ (ms)	1762 ± 18	1683 ± 88	0.96 ± 0.11	0.34
	$T1_{Gd}$ (ms)	364 ± 42	374 ± 38	1.03 ± 0.05	0.16
	[GAG] (mg/mL)	53 ± 13	57 ± 10	1.09 ± 0.13	0.15

The ratio of after/before was calculated for each sample and then averaged. A p value refers to the comparison of before and after fixation using a paired t-test.