Chemical Fixation Alters the Water Microenvironment in Rat Cortical Brain Slices - Implications for MRI Contrast Mechanisms

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INTRODUCTION

MRI studies of chemically-fixed tissues are common, yet little is known about how chemical fixation alters the MR properties of tissue. Parafromaldehyde and gluteraldehyde solutions achieve fixation by cross-linking protein amino groups via methylene bridges to render tissues metabolically inactive and structurally stable [1]. These sample properties permit long scan times so that fixed tissues are well-suited to high-resolution, multidimensional acquisition schemes with MR images that are devoid of motion or flow artifacts. Furthermore with fixed samples it is often possible to isolate tissues or organs of interest and employ high-field magnets with smaller RF coils to improve the signal-to-noise ratio (SNR) per unit time (e.g. 50-μm-resolution diffusion tensor studies of rat hippocampi [2]). Recent studies on a simple erythrocyte ghost tissue model suggest that aldehyde fixatives, particularly 4% paraformaldehyde, significantly alter mean intracellular residence time [3,4]. In this followup study, we compared the effects of chemical fixation with 4% paraformaldehyde and a modified Karnovsky's solution (2% paraformaldehyde / 2% glutaraldehyde) on the T_1, T_2 and water diffusion properties of rat cortical brain slices. Water diffusion data were analyzed with a two-compartment exchange model [5]. This study demonstrates significant perturbation of nervous tissue microstructure by aldehyde fixation that should be accounted in MRI studies of fixed tissue.

METHODS

Vibratome-cut slices of rat isocortex (500-μm thick) were procured from male P30 Long-Evans rats and imaged using a multislice perfusion chamber inside a 600 MHz spectrometer with a 10-mm birdcage coil as described previously [6]. Cortical slices from each rat were divided into fresh and fixed samples. Fresh, or unfixed, slices were imaged promptly in artificial cerebrospinal fluid (300 mOsm/kg, pH 7.4). Chemically fixed slices were immersion-fixed in 4% paraformaldehyde or Karnovsky's solution (2% paraformaldehyde, 2% glutaraldehyde) for 7-10 days, imaged in fixative, then washed 4-5x in PBS (300 mOsm/kg, pH 7.4) over 12 hours and re-imaged. Water diffusion measurements required a pulsed-gradient spin-echo multislice sequence with 12 diffusion-weighted images using diffusion gradients aligned with the read gradient (0-940 mT/m) and diffusion times (T_d) of 10, 20 and 35 ms, echo times of 23.5, 33.5 and 48.5 ms respectively, and a repetition time of 1.5 s. T_1 and T_2 were measured in slices with partial saturation (TR = 150 ms – 10 s) and multi-echo sequences (TE = 10 ms, 30 echos). Data were analyzed with a two-pool diffusion model with exchange [4] that accounts for restricted diffusion in the intracellular space, extracellular water diffusion mediated by tortuosity and water exchange between intra- and extracellular compartments. The model estimates the apparent diffusion coefficient of water in the extracellular space, average cell dimensions, the mean water intracellular residence time and the intracellular volume fraction. Tissue microstructure and MR relaxation properties were thus determined under living, fixed and fixed-washed conditions. These parameters were compared statistically using ANOVA.

RESULTS

Both 4% paraformaldehyde and Karnovsky's solution fixatives significantly reduced cortical brain slice T_2 ($P < 0.05$) [Fig. 1] such that data for the two-compartment exchange model were unobtainable from slices in fixative. Washing aldehyde-fixed samples in PBS appeared to restore T_2 to pre-fixation values by removal of excess fixative [7], but interestingly T_2 was statistically longer than in fresh slices for slices fixed with 4% paraformaldehyde then washed in PBS ($P < 0.05$). Similarly, slice T_1 was reduced from 2.02 ± 0.05 s in unfixed slices to 1.596 ± 0.043 s and to 1.499 ± 0.050 s upon fixation with 4% paraformaldehyde and Karnovsky's solution respectively ($P < 0.05$). Unlike the T_2 effects of free fixative solutions, removal of excess fixative by washing in PBS did not significantly alter the T_1 of fixed slices.

Slices in either aldehyde fixative solution showed significantly increased rates of water exchange between intra- and extracellular compartments and an increase in calculated intracellular diameter after fixation and washing [Table 1]. Slices fixed in Karnovsky's solution and washed showed a significantly decreased intracellular ADC, whereas slices fixed in 4% paraformaldehyde and washed showed an increased intracellular fraction.

DISCUSSION

Free, unreacted aldehyde fixative solution alters the T_2 relaxation properties and reduces SNR of cortical slices as previously described [7]. However, our data demonstrates that the chemical reactions during aldehyde fixation cause permanent alteration to the relaxation and diffusion properties of tissues, as washing fixed nervous tissue samples does not restore their MRI properties to pre-fixative values. These data agree with previous studies on fixation of gel-immobilised erythrocyte ghosts [3,4]. The large increase in membrane permeability to water after fixation suggests disruption of cell membrane integrity. This suggests fixative may significantly impact the fractional anisotropy and mean diffusivity measurements of diffusion tensor imaging, which relies on the restrictive effects of cell membranes imparting anisotropic diffusion properties to water molecules. These results are significant to all *ex vivo* microimaging studies – investigators should be cautious when extrapolating data from fixed tissue samples to *in vivo* MRI studies.

REFERENCES

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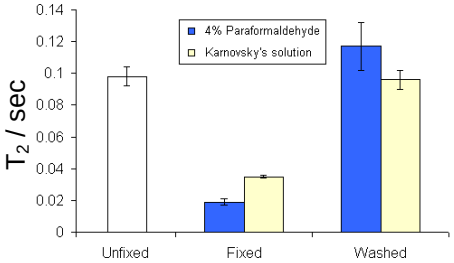

Figure 1 – T_2 of tissue water protons in rat cortical slices after procurement, after fixation in 4% paraformaldehyde or Karnovsky's solution, and after washing in 1 × PBS

Sample	n	D_{intra}	ADC_{extra}	a / μm	k / s^{-1}	V_{intra}
Unfixed	11	1.83 ± 0.07*	0.58 ± 0.08	2.34 ± 0.34*†	14.19 ± 4.53*†	0.68 ± 0.03
Karnovsky's solution	10	1.37 ± 0.50*†	0.85 ± 0.27	3.15 ± 0.26*	56.91 ± 6.66*	0.66 ± 0.12
4% Paraformaldehyde	11	1.72 ± 0.14†	1.28 ± 0.88	3.16 ± 0.26†	53.03 ± 6.57†	0.76 ± 0.12
1-way ANOVA		0.003	0.017	<0.001	<0.001	0.069

Table 1 – Analysis of tissue water diffusion analysis in fresh and fixed-washed brain slices, employing a biophysically appropriate analysis method. D_{intra} = intracellular diffusion rate, ADC_{extra} = extracellular diffusion rate moderated by tortuosity, a = intracellular diffusion diameter (cell size), k = intracellular water exchange rate (proportional to inverse of intracellular residence time), V_{intra} = intracellular volume fraction. * or † Denotes values that were statistically different by posthoc Tukey multiple comparisons test.