Paraformaldehyde and Karnovskys Fixatives Alter Microstructure in an Erythrocyte Ghosts Tissue Model

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INTRODUCTION

MRI studies of chemically-fixed biological samples have become increasingly common. The fixatives typically used for MRI investigations, paraformaldehyde and gluteraldehyde, achieve fixation by cross-linking protein amino groups with methylene bridges to render tissues metabolically inactive and structurally stable [1]. These sample properties permit long scan times and thus, fixed samples are well-suited to high-resolution, multidimensional acquisition schemes with MR images that are devoid of motion or flow artifacts. Further, 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; for example, high-resolution diffusion tensor studies of rat spinal cords [2] have been facilitated by the use of chemical fixation. Unfortunately, there is no consensus on proper fixation methods, nor is much known about how chemical fixation alters the MR properties of tissue. In this study, we compared the effects of chemical fixation with 4% paraformaldehyde and a modified Karnovsky's solution (2% paraformaldehyde / 2% glutaraldehyde) on tissue microstructure by measuring the water diffusion properties of a previously described erythrocyte ghost tissue model [3]. Results of this study indicate that chemical fixatives commonly employed for MRI investigations uniquely alter tissue microstructure.

METHODS

Erythrocyte ghosts were prepared as previously described [3] and suspended in buffer containing 1% agarose at 37ºC. The suspension was placed in a glass tube and cooled to gel the agarose, thus forming a tissue model (referred to as "GhoGel") that could be immersed in fixative solutions. The GhoGel was cut into 1-cm lengths and briefly washed with phosphate buffered saline (PBS, pH 7.4, 300 mOsm/kg). GhoGel samples (N = 3 per fixative type) were then immersion-fixed in PBS solutions containing 4% paraformaldehyde or modified Karnovsky's solution (2% paraformaldehyde / 2% glutaraldehyde). After 7 days of fixation, samples were washed with PBS for 12 hrs prior to NMR measurements. This eliminates the detrimental effects to SNR caused by free fixative, which substantially shortens T_2 in unwashed tissue samples [4]. GhoGels then were placed in 3-mm NMR tubes. All MR data were acquired at 20ºC using a 14 T magnet interfaced to a Bruker spectrometer, equipped with triple axis 300 G/cm shielded gradients. Water diffusion was measured with a pulsed gradient spin echo sequence at 6 diffusion times (5 - 50 ms). The diffusion gradients were linearly incremented in 32 steps to produce *b*-values between 0 and 16000 s/mm^2 for all diffusion times. MR data were analyzed using a two-compartment model incorporating exchange between compartments, intracellular restriction and extracellular tortuosity [5]. The analysis provided an index of cell size, extracellular apparent diffusion coefficient (ADC), intracellular fraction and the rate of exchange between intra- and extracellular water (intracellular residence time). The "initial slope" water ADC was also calculated from the initial region of the diffusion signal attenuation curves (b = 0 - 1000 s/mm^2).

RESULTS

The diffusion-weighted signal attenuation curves for GhoGel samples fixed in 4% paraformaldehyde and modified Karnovsky's solution are shown in Fig. 1. The initial-slope ADC of water in GhoGel samples were statistically different ($P < 0.001$); 0.41 ± 0.01 μm^2/ms for samples fixed with 4% paraformaldehyde and 0.53 ± 0.01 μm^2/ms for samples fixed with modified Karnovsky's solution. The signal attenuation plots (Fig. 1) also indicate that increasing the diffusion time causes greater signal attenuation at high *b*-values for samples fixed in Karnovskys; this suggests that a faster rate of water exchange exists between the intra- and extracellular compartments in samples fixed with Karnovskys solution. The results of fitting the two-compartment exchange model to the data demonstrate statistically significant differences for extracellular ADC, restriction diameter and intracellular residence time between the two fixative types (Table 1). The data suggests that GhoGels fixed by 4% paraformaldehyde may have a larger intracellular fraction and a longer mean intracellular residence time (slower water exchange rate) than samples fixed by the modified Karnovsky's solution.

DISCUSSION

We observed significant differences in the water diffusion properties of erythrocyte ghosts tissue model after treatment with two solutions commonly used for the fixation of MRI and histological samples. Analysis with a two-compartment exchange model suggested that the intracellular fraction was larger and the water exchange rate between intra- and extracellular compartments was slower in erythrocyte ghosts fixed with 4% paraformaldehyde in PBS. These findings suggest that ghosts fixed with 4% paraformaldehyde are larger than those fixed with Karnovsky's solution. The two compartment model parameters reported in Table 1 also differ from the published values for unfixed erythrocyte ghosts [3], but further studies will be required to directly compare GhoGel samples before and after fixation. The difference reported may be attributed to fixative-specific changes of membrane permeability to water and/or cell volume changes. Membrane water permeability may be altered by fixative-induced membrane protein crosslinking [1] or structural changes to membrane protein channels such as Aquaporin [6]. Apparent changes in cell volume during chemical fixation were not predicted since paraformaldehyde and gluteraldehyde are believed to exert no significant effects on osmolality [1]. Our data demonstrate that fixative solutions can significantly alter tissue microstructure, and thus may alter the results obtained in diffusion MRI studies of *ex vivo* samples. We next plan to study how chemical fixation affects the MR properties of cortical brain slices, which unlike GhoGels, also model the cellular heterogeneity and extracellular matrix of *in vivo* nervous tissue. Future studies also will investigate fixative-induced changes in tissue microstructure using correlative techniques such as electron and confocal microscopy.

REFERENCES

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Fixative solution for GhoGel samples	Extracellular ADC (μm^2/ms)	Restriction diameter (μm)	Intracellular residence time (ms)	Intracellular fraction (no units)
4% Paraformaldehyde	1.23 ± 0.05	2.78 ± 0.01	20.0 ± 2.5	0.52 ± 0.01
Karnovskys	1.10 ± 0.03	2.33 ± 0.04	14.7 ± 2.2	0.42 ± 0.02
t-test (P-value)	0.022	<0.001	0.069	<0.001

Table 1 – Results of two compartment exchange analysis for the two sample groups

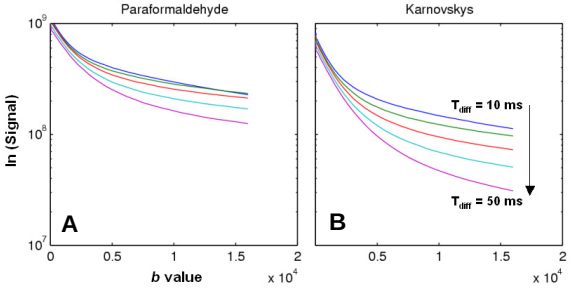

Figure 1 – log(signal) plotted against *b*-value for samples fixed in (A) 4% paraformaldehyde and (B) Karnovskys solution, acquired at diffusion times of 10, 17, 25, 35 and 50 ms.