Effect of Fixative Osmolality on Biological Tissue Microstructure Studied with an Erythrocyte Ghost Model

P. E. Thelwall[1], T. M. Shepherd[1], G. J. Stanisz[2], S. J. Blackband[1]

[1]Department of Neuroscience, McKnight Brain Institute, University of Florida, Gainesville, Florida, United States, [2]Imaging Research, Sunnybrook and Women's CHS, University of Toronto, Toronto, Ontario, Canada

Introduction

MRI studies of chemically fixed biological samples have become increasingly common. Fixed samples permit long scan times and are thus well suited to high resolution, multidimensional acquisition schemes and images that are devoid of motion or flow artifacts. Furthermore, it is often possible to isolate tissues or organs of interest as fixed samples and to employ high field magnets with smaller RF coils to improve the signal-to-noise ratio (SNR) per unit time. For example, MRI-based 3D histological studies of mice and mouse organs are under development for phenotyping (1) and high-resolution diffusion tensor studies of rat spinal cord injury have been facilitated by the use of chemical fixation (2). Unfortunately there is no consensus on optimal fixation methods, nor is much known about how chemical fixation alters the MR properties of tissue. In this study we assessed the effects of altering the osmolality of fixative solutions on tissue microstructure. A simple tissue model composed of erythrocyte ghosts (3) was employed and tissue microstructure was assessed by MR measurement of water diffusion, analysed with a two compartment exchange model (3,4).

Paraformaldehyde is not believed to contribute to osmotic gradients experienced by tissues during fixation because it freely crosses membranes and the osmotic properties of cell membranes are altered by contact with paraformaldehyde (5). However, the buffer solution used for chemical fixation (e.g. saline) can effect tissue microstructure via osmotic gradients because it penetrates into tissue more rapidly than paraformaldehyde. We postulated that differences in tissue microstructure could be induced in tissue samples placed in 4% paraformaldehyde solutions buffered with hypo- and hyperosmotic phosphate buffered saline (PBS). We also predicted that these changes would be preserved despite washing fixed tissue samples repeatedly in isotonic PBS after chemical fixation. If these postulates are valid then consistent fixation methods are essential for comparing MR data acquired from chemically fixed samples. If fixation has the potential to preserve microstructural changes such as cell swelling, it may be possible to employ chemical fixation methods to study highly dynamic tissue changes during ischemia by preserving highly transient microsturctural states for later detailed investigation with time-intensive MR methods.

Methods

Erythrocyte ghosts were prepared as previously described (3) and suspended in buffer containing 1% agarose at 37 ºC. The suspension was placed in a 2.2 mm ID glass tube and cooled to gel the agarose, forming a tissue model that could be immersed in fixative solution (refered to as "GhoGel"). The GhoGel then was cut into 1 cm lengths, briefly washed with 1× PBS (pH 7.4, 300 mOsm/kg), then immersion-fixed in PBS solutions containing 4% paraformaldehyde (n = 3 for each fixative osmolality). Fixative solutions contained 0.66×, 1× and 1.33× PBS to yield solution osmolalities of 200, 300 and 400 mOsm/kg. After 7 days of immersion-fixation, the samples were washed for 12 hrs in 1× PBS for 12 hrs to eliminate free fixative, which substantially shortens T_2 and reduces SNR for unwashed tissue samples (6). GhoGels 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, 10, 17, 25, 35 and 50 ms), 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 analysed using a two compartment model incorporating exchange between compartments, intracellular restriction and extracellular tortuosity (3,4). The analysis provided an index of cell size, extracellular apparent diffusion coefficient, intracellular fraction and the rate of exchange between intra- and extracellular water. "Initial slope" water ADC was also calculated from the initial region of the diffusion signal attenuation curves (b = 0 – 1000 s mm^{-2}).

Results and Discussion

Figure 1 shows plots of log(Signal) vs *b*-value at diffusion times of 17 – 50 ms for samples fixed with 4% paraformaldehyde in 200, 300 and 400 mOsm/kg fixative solutions; clear differences in the signal attenuation curves can be seen. Table 1 shows the ADC calculated from the initial slope of these plots (for *b*-values between 0 and 1000 s mm^{-2}) and results from the two compartment exchange model, indicating changes in cell size, mean intracellular residence time for water, and intracellular fraction. There are large differences in the initial slope ADC of samples fixed at different osmolalities, suggesting cell swelling in hypotonic fixative and cell shrinking in hypertonic fixative. The parameters from the two compartment exchange model fits support this hypothesis. Samples fixed in hypotonic solution (200 mOsm kg^{-1}) demonstrated a higher intracellular fraction than those fixed in isotonic solutions, whilst samples fixed in hypertonic solution (400 mOsm kg^{-1}) showed a faster exchange rate between intra- and extracellular compartments. Further MR studies are underway to increase sample size to clarify the microstructural changes caused by the different fixative buffer osmolalities. In addition, correlative methods such as electron microscopy and optical methods are being investigated to augment the MR measurements.

The results demonstrate that the morphology of tissue microstructure present at the time of fixation can be maintained despite subsequent washing of samples in isotonic PBS prior to MR investigation. This illustrates the importance of consistent fixation methods if diffusion weighted MR datasets are to be compared. Furthermore, this suggests the opportunity to fix subtle microstructural changes in a tissue (such as those caused by ischemia in brain tissue) for later in-depth study with diffusion-MRI, with the aim of investigating the microstructural basis for contrast changes in diffusion weighted MRI *in vivo*. Future studies are planned to investigate the effect of fixative osmolality on tissue microstructure in perfused brain slices (7) with the aim of fixing microstructural alterations caused by experimental perturbation.

Acknowledgements and References

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Figure 1 – Log (Signal) against *b*-value for GhoGel samples fixed in 400, 300 and 200 mOsm kg^{-1} 4% paraformaldehyde solutions. Data acquired at diffusion times of 17 – 50ms are shown.

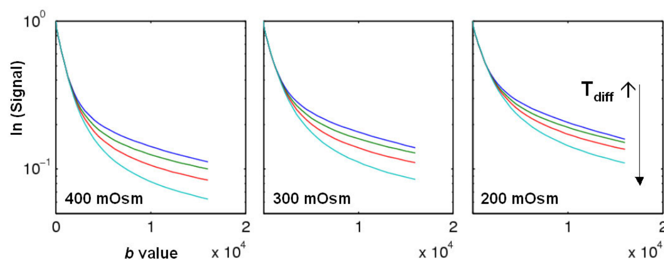

Table 1 – Initial-slope ADC and results of two compartment exchange model fitting. Trends in initial slope ADC, mean intracellular residence time and intracellular fraction can be seen with decreasing fixative osmolality.

Fix Osmolality / mOsm kg^{-1}	Initial slope ADC / µm² ms^{-1}	Two compartment exchange model: results of fitting			
		Intracellular restriction diameter / µm	Apparent extracellular ADC / µm² ms^{-1}	Mean intracellular residence time / ms	Intracellular fraction
400	0.64 ± 0.05	2.8 ± 0.04	1.30 ± 0.04	16.5 ± 0.004	0.37 ± 0.03
300	0.57 ± 0.02	2.8 ± 0.13	1.27 ± 0.07	19.7 ± 2.8	0.38 ± 0.03
200	0.47 ± 0.004	3.0 ± 0.07	1.27 ± 0.02	19.5 ± 0.7	0.48 ± 0.01