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EFFECTS OF FORMALIN FIXATION ON MYELIN WATER FRACTION MRI MEASUREMENTS IN HUMAN WHITE MATTER: A NOVEL *EX VIVO* STUDY

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INTRODUCTION

Myelin is an essential component within the human brain, and is responsible for efficiently transmitting neural signals between regions. However, there are certain neurodegenerative diseases (e.g., multiple sclerosis, etc.) where myelin is lost or damaged. As a result, sensitive and specific myelin measurements are important for better understanding of brain damage and plasticity.

Various magnetic resonance imaging (MRI) techniques are widely used to non-invasively study brain anatomy and tissue microstructure. Among these, multi-component T_2-relaxation myelin water imaging (MWI) has proven to be specific and sensitive to white matter (WM) myelination [1], and has been effectively used to understand neurological diseases such as multiple sclerosis [1]–[3], Alzheimer's Disease [4], schizophrenia [5], and phenylketonuria [6] in earlier *in vivo* studies. The ratio of myelin water content to the total water content, called the myelin water fraction (MWF), can be calculated from the short T_2-relaxation component ascribed to water trapped within the myelin sheath (T_2 ~10-40 ms) and longer T_2-relaxation components arising from intra- and extra-cellular water (T_2 ~100-200 ms) [2]. A few histopathology–based *ex vivo* studies have successfully validated MWF as a marker of myelin concentration [7]–[9]. However, these studies used formalin as a fixative, and formalin is known to alter certain MR properties, such as T_1- and T_2-relaxation times [10]–[12]. However, to the best of our knowledge, no previous study has formally investigated and/or characterized formalin-induced MWF signal changes, which could have important implications for *ex vivo* MWI studies.

In this paper, we have therefore characterized the effects of formalin fixation on MWF signals and discussed possible implications for future studies and clinical applications.

METHODOLOGY

Subject Preparation

Two neurologically-healthy human brain specimens were obtained from deceased females (71 and 74 years old) with prior approval from the University of Manitoba Biomedical Research Ethics Board (BREB). Neither brain had any history of neurological disorders nor any incidental findings or pathologies (e.g., previously undiagnosed neurodegenerative diseases, tumors, lesions, hemorrhages, infarcts, etc.). After carefully removing the brain from the cranium at 33 hours and 55 hours after death each specimen was immersed in 10% phosphate buffered formalin (PBF) solution inside an air- and liquid-tight MRI-compatible container [13] that was changed every few days to allow sufficient fixation. All MRI scans were performed at room temperature (22°C ± 1°C) in a whole-body 3T Siemens Magnetom Verio scanner (Siemens Healthcare, Erlangen, Germany), using a 12-channel head coil and 4-channel flex coil, at the same 13 time points (i.e., 0, 12, 24, 46, 120, 168, 211, 288, 336, 500, 672, 840 and 1032 hours) after immersion in the PBF solution.

Image Acquisition

A multi-echo 3D combined Gradient and Spin Echo (GRASE) sequence was used to acquire images with 32 different echo times [14], and was used for multi-component myelin water imaging measurements [2]. Scan parameters were: Repetition time (TR) = 1030 ms, First

Echo Time (TE1) = 10 ms, Echo Train Length (ETL) = 32, Echo Spacing (ESP) = 10 ms, Flip Angles = 90° (excitation) and 180° (refocusing), Matrix Size = 160 x 120 x 22, Field of View (FOV) = 180 x 240 x 110 mm^3, Resolution = 1.25 x 1.25 x 5 mm^3, Acquisition Time = 15 min. Conventional T_1-weighted images were obtained using a 3D magnetization prepared rapid acquisition gradient echo (MPRAGE) sequence, that was used for image segmentation to generate binary masks and spatial normalization. The parameters for this sequence were: TR = 1900 ms, TE = 2.49 ms, Inversion time = 900 ms, Flip Angle = 9°, Matrix Size = 512 x 512 x 176, Resolution = 0.49 x .49 x 0.98 mm^3, FOV = 250 x 250 mm^2, and Scan Time = 5 min.

Image Preprocessing

All images were reoriented to the anterior commissure - posterior commissure (AC-PC) plane using Statistical Parametric Mapping (SPM12) [Wellcome Trust Centre for Neuroimaging, London, UK] to facilitate subsequent image processing steps across the time points. The T_1-weighted images were segmented to generate binary masks that were later used for skull-stripping. Using MATLAB [The MathWorks Inc., Natick, MA, USA], the MWF within each voxel was obtained from the geometric mean of the multi-exponential T_2-distribution from myelin water, divided by the sum of geometric means of the T_2-distribution of intra- and extra-cellular water and the myelin water. The T_1-weighted image of 4th time point of each subject was used as a reference to perform co-registration of the images, enabling comparable assessment across the subjects and time points. Spatial normalization of the T_1-weighted images was performed in MRIStudio [Johns Hopkins University, Baltimore, MD, USA] by using the John Hopkin's University (JHU) T_1–weighted brain template in Montreal Neurological Institute (MNI) space for the first four time points. This included a two-stage warping procedure, consisting of a 12-parameter affine (linear) transformation, followed by high-dimensional, non-linear normalization with the large deformation diffeomorphic metric mapping (LDDMM) algorithm [15]. For time points 5 to 13, the same normalization parameters from time point 4 were applied, since the shape of the brain was assumed to remain unchanged and the image contrast was reduced during the later time points. Region of interest (ROI) analyses were performed using ROIEditor (a part of MRIStudio) and 3D ROIs were chosen from the JHU_MNI_SS ("Eve") atlas in four deep WM structures, including the genu of the corpus callosum (GCC), splenium of the corpus callosum (SCC), optic radiation (OR), and internal capsule (IC) (Fig. 1). For bilateral ROIs, corresponding values were averaged across both hemispheres.

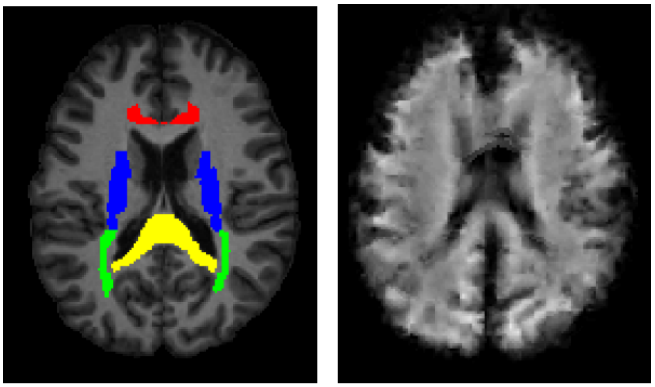

Figure 1: *(a) Four white matter ROIs [Red = genu of the corpus callosum (GCC), Yellow = splenium of the corpus callosum (SCC), Green = optic radiation (OR), Blue = internal capsule (IC)] overlaid on the JHU_MNI_T1 template. (b) Representative MWF image from Subject 2 at 336 hours.*

Statistical Analyses

All data were analyzed using SPSS 23.0 (IBM, NY, USA). Spearman rank-correlation coefficient (r_s) was used to assess correlations between MRI measures and fixation time. Two-tailed tests were performed, and only ROIs showing Bonferroni-corrected p-values <0.05 (i.e., <0.0125, corrected for multiple comparisons across brain regions) were considered to be statistically significant.

RESULTS

For each subject, MWF was plotted as a function of fixation time (hours) in four deep WM structures, and Spearman correlation coefficients and p-values were calculated (Fig. 2). The apparent MWF exhibited statistically significant increases in all four regions, and all of the MWF estimates beyond the third time-point (i.e., 24 hours) were at least twice as large as the initial baseline .

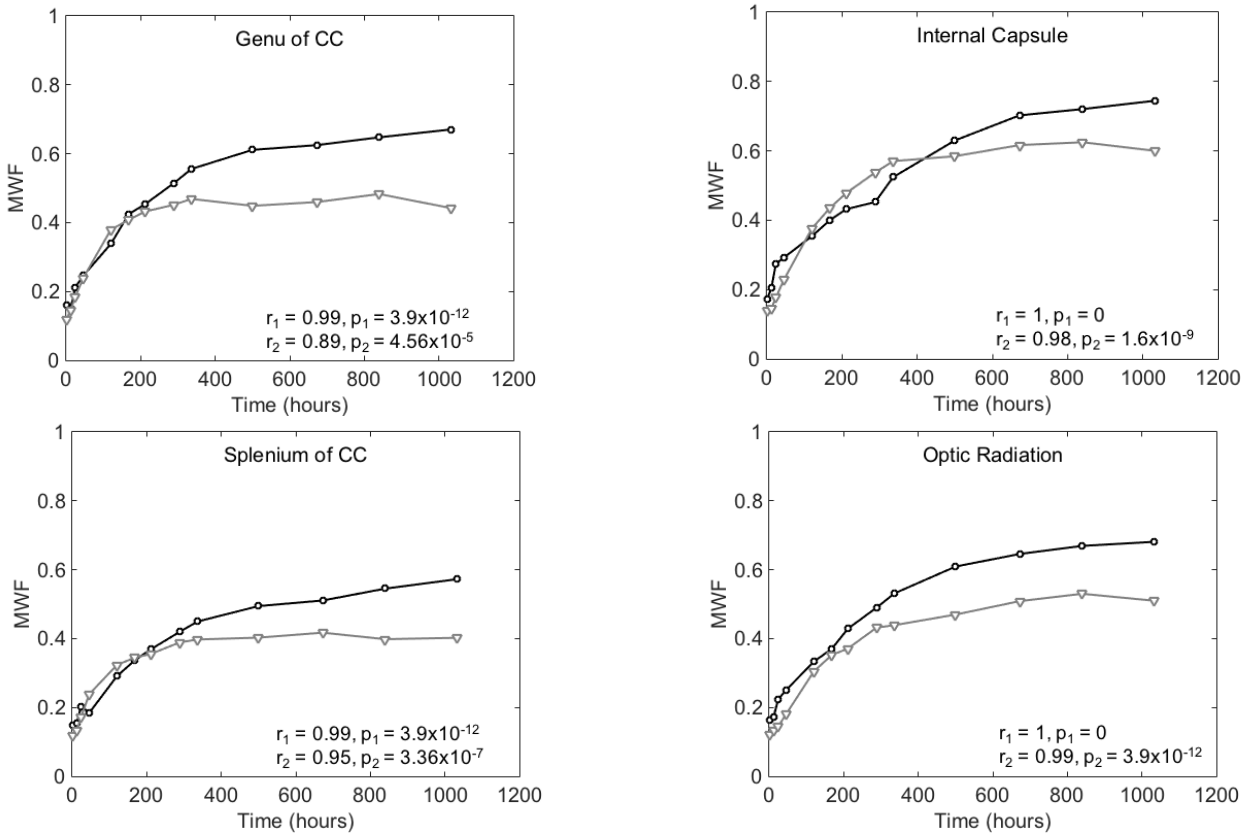

Figure 2: *MWF vs. formalin fixation time are shown for all four WM structures. The x and y axes show the time in hours and MWF respectively. The black line represents Subject 1 and the grey line indicates Subject 2. The plots demonstrate MWF changes throughout the fixation process.*

DISCUSSION

Our findings demonstrate that MWF measurements were significantly and progressively altered by formalin fixation. Although, the exact mechanism underlying this change is not yet understood, it may reflect a greater reduction of intra- and extra-cellular water [8] – i.e., due to the chemical reaction between water and formalin [12] – compared to myelin-bound water. Although further investigation is required to understand the exact cause(s) for these changes, our findings indicate that researchers must exercise caution when comparing *in-vivo* MWF values with *ex-vivo* values.

Although the study design was optimized as much as possible, there are a few limitations that warrant a brief discussion. Due to both ethical and logistical constraints, our sample size was necessarily low (n=2); and the postmortem intervals (PMI) (i.e. time between death and fixation) of 33 hours and 55 hours in this study may have had a contributing effect, since PMI is also known to affect MRI properties. Partial volume effects (due to tissue segmentation, subcortical structures adjacent to formalin, limited spatial resolution, and imperfect spatial normalizations to the MNI template) may have had an effect on the precision of the MWF measurements. However, the fact that the MWF increases were so statistically significant and highly replicable (across both subjects and regions) suggests that the main findings are independent of these limitations (i.e., sample size, PMIs, and/or partial volume effects).

Unfortunately, we could not evaluate the MWF changes beyond 1032 hours in this study, even though other studies have suggested that fixation continues to change MRI properties up to 1142 hours [16] or even a year [17]. We hope that future experiments with a larger sample size, a longer study period, and minimum PMI will replicate and extend the current findings.

In conclusion, this study characterized the time-dependent effects of formalin fixation on MWF MRI measurements in *ex vivo* human brain specimens, with a particular focus on white matter structures. The changes of MWF indicate that formalin gradually diffuses inward from the cortical surface, and tissue fixation continues to affect MWF estimates until, and perhaps beyond our maximum fixation time of 1032 hours. Our results lead to the conclusion that *ex vivo* myelin water imaging should be performed immediately after formalin fixation to avoid erroneous interpretations.

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