Regional $T_1$ Relaxation Time Constants in Ex Vivo Human Brain: Longitudinal Effects of Formalin Exposure

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Purpose: Relaxation time constants are useful as markers of tissue properties. Imaging ex vivo tissue is done for research purposes; however, $T_1$ relaxation time constants are altered by tissue fixation in a time-dependent manner. This study investigates regional changes in $T_1$ relaxation time constants in ex vivo brain tissue over 6 months of fixation.

Methods: Five ex vivo human brain hemispheres in 10% formalin were scanned over 6 months. Mean $T_1$ relaxation time constants were measured in regions of interest (ROIs) representing gray matter (GM) and white matter (WM) regions and analyzed as a function of fixation time.

Results: Cortical GM ROIs had longer $T_1$ relaxation time constants than WM ROIs; the thalamus had $T_1$ relaxation time constants similar to those of WM ROIs. $T_1$ relaxation time constants showed rapid shortening within the first 6 weeks after fixation following by a slower rate of decline.

Conclusion: Both GM and WM $T_1$ relaxation time constants of fixed brain tissue show rapid decline within the first 6 weeks after autopsy and slow by 6 months. This information is useful for optimizing MR imaging acquisition parameters according to fixation time for ex vivo brain imaging studies.

Key words: $T_1$-mapping; ex vivo; neuroimaging; brain; MRI; fixation

INTRODUCTION

MRI-pathology correlation studies are very valuable for studying the histological changes underlying abnormal MRI findings (1). MRI of fixed ex vivo human brain tissue is a particularly useful tool for these types of studies because it eliminates the variable of pathologies that arise between time of in vivo MRI and time of death (2). It has also been suggested as a useful tool for forensic neuroimaging (3) and enables very high resolution imaging that cannot occur in a clinically feasible time span. Relaxation time constants are useful as markers of tissue properties in MR imaging studies (4). For example, knowledge about tissue relaxation is necessary for a double inversion recovery sequence, which can suppress the signal from formalin in addition to either gray matter (GM) or white matter (WM). This is useful for studying diseases that present with hyperintense lesions, particularly multiple sclerosis, and when done postmortem, can guide histopathologic sampling for microscopic analysis of pathology (5).

Proper suppression of either GM or WM relies on information about the $T_1$ relaxation time constant of the tissue. Established in vivo $T_1$ relaxation time constants are not appropriate for tissue that has been excised and stored in fixative for varying amounts of time (6). During fixation, the fixative diffuses through the ex vivo brain, which changes the MR signal intensity of the tissue and the contrast between different tissues; this process results in a hyperintense band of formalin on $T_1$-weighted images of fixed tissue that begins in the immediately exposed cortex and radiates inward over time (see in Figure 1) (7,8). This inward diffusion of formalin diminishes the distinction between GM and WM contrast, which is critical for tissue segmentation and volumetric measurements for studies of brain morphology.

It has been previously reported that decline of $T_1$ relaxation time constants occurs after fixation over 3 weeks (7). To our knowledge, there has not yet been a report of $T_1$ relaxation time constants in neuroanatomy-based regions of interest (ROIs). This information is important for studies investigating pathologies that affect specific brain regions. We hypothesized that $T_1$ relaxation time constants decline over time at different rates in different brain regions. In this study, we measured mean GM and WM $T_1$ relaxation time constants in 6 ROIs in five ex vivo human brains fixed in 10% formalin over 6 months. This allowed us to investigate the impact of fixation on ex vivo brain tissue over 6 months and measure the hypothesized decline of the mean $T_1$ relaxation time constants.
METHODS

Tissue Collection and Preparation

Samples were collected from five patients who came to autopsy and did not have neurodegenerative diseases, neurological disorders, infarcts, or hemorrhage during life or at the time of death. Each subject’s brain was hemisected during the brain cutting procedure, which occurred 10–12 days after the date of death. Between the time of death and the brain cutting procedure 10–12 days later, the tissue was kept in 10% formalin at 21°C to begin the fixation process. The intact left hemisphere for each subject was provided for this study. All samples were stored completely immersed in 6 quarts of 10% formalin for each subject was provided for this study. All samples were stored in a closed container. This study was approved by the Mayo Clinic Institutional Review Board and all subjects or appropriate surrogates provided informed consent for the use of tissue for research purposes.

Image Acquisition

Using an inversion recovery spin echo (IR SE) sequence at 3 Tesla (T) (GE Signa, v16, Milwaukee, WI) in a birdcage head coil, single-slice images of brain tissue were acquired at inversion times of 50 ms, 400 ms, 1100 ms, and 2500 ms. Field of view was 18 cm, matrix: $512 \times 128$, slice thickness 3mm, repetition time = 2550 ms, echo time = 14 ms, band width of $\pm 31.25$ kHz. Approximate acquisition time for the full series was 20 min. The single-slice images were acquired approximately 3 mm lateral to the medial surface so that internal structures were not exposed to formalin as a result of hemisection. Baseline scans were acquired in five ex vivo brains that were immersed in 10% formalin for 10–12 days after death. Scans were repeated for each brain every day for 6 days, then at 2 weeks, 1 month, 3 months, and 6 months after the initial scan. The brains were held in a custom-built, water-tight plastic container that was made to fit the shape of the head coil with crosshairs engraved over the center of the lid for reproducible landmarking. The temperature of the scanning room is 20°C. The IR SE sequence used produces low RF deposition, so local heating is not expected.

Image Processing and Analysis

Using fitting code from Barral et al (9) $T_1$ relaxation time constants were calculated and mapped and mean $T_1$ relaxation time constants from GM and WM ROIs were measured using an in-house image analysis program mrview (Fig. 1). ROIs were chosen to represent both GM and WM regions that are either on the brain surface, therefore, directly exposed to formalin (GM in the frontal cortex, GM in the anteromedial temporal lobe, WM in the splenium of the corpus callosum), or exposed through diffusion of formalin into the tissue (GM in the thalamus, subcortical WM in the frontal lobe, WM in the cerebellum). ROIs were drawn manually in mrview. The ROI drawn on the first time point image was copied to the second time point image, and so on. The ROI was adjusted for minor tissue positioning differences at each time point.

Statistical Modeling

Plots of $\ln(T_1$ relaxation time) against $\ln$(time) showed linear patterns, verified both visually and through regressions using spline smoothers taking into account the repeated measures. Given this, lines of the form $\ln(y)=\alpha + \beta \cdot \ln(x)$, where $y$ is $T_1$ relaxation time and $x$ is time, were used to model the progression of $T_1$ relaxation times. Parameters $\alpha$ and $\beta$ for the models were estimated by fitting linear regressions to the natural logs of both the $x$ and $y$ variables. Estimates of the predicted values from each model were then transformed back to the original scale through exponentiation. On the original scale, these models are power functions of the form $y=e^{\alpha x^\beta}$ showing steep initial declines which gradually get shallower, similar to the patterns observed in the data. Because these models only approach a slope of 0, times for the rate of change in $T_1$ relaxation times to reach a small value of 3 ms/week were estimated from the models by taking the first derivative of the equation, then solving for time using the fitted regression coefficients. Confidence intervals for these estimates were obtained by means of the same method using 10,000 simulations per model, and extracting the appropriate (2.5 and 97.5) percentiles of the resulting distributions of times.

We also tested for a difference in the $T_1$ relaxation time constants between the GM ROIs and WM ROIs by adding a GM/WM indicator variable to linear mixed models with $\ln(T_1$ relaxation time constant, ms) as the
outcome and ln(time, days) as the predictor, accounting for the repeated measures in the data. A significant regression coefficient would indicate a shift in the outcome values, with a positive coefficient corresponding to greater $T_1$ relaxation time constants in the GM ROIs.

**RESULTS**

Subjects consisted of three males and two females and were varied on age at death and time between death and initial fixation (Table 1). Consistent with in vivo studies, mean $T_1$ relaxation time constants were longer in the cortical GM ROIs than in WM ROIs in the fixed tissue (coefficient 0.22, SE 0.02, $P < 0.0001$). However, mean $T_1$ relaxation time constants in the thalamus, a dense subcortical GM region which typically does not accumulate iron (10), were similar to those of the WM ROIs; mean $T_1$ relaxation time constants and rate of decline in all WM ROIs are similar. Mean $T_1$ relaxation time constants shortened progressively over 6 months. Figure 2 shows the mean $T_1$ relaxation time constants of the five brains at each time point in each ROI. The model for rate of decline in each ROI is also included in the plots. Shortening of mean $T_1$ relaxation time constants was more rapid within the first month of scanning, which was within 6 weeks after the start of fixation. However, by 6 months of fixation the decline of mean $T_1$ relaxation time constants is slower. Projected timespans to reach a small rate of decay in $T_1$ relaxation time constant is reported for each ROI in Table 2.

**DISCUSSION AND CONCLUSIONS**

In our samples, both GM and WM mean $T_1$ relaxation time constants of fixed brain tissue decline over time. This decline is first rapid and then slows by 6 months with a “knee” around 1 week after the start of scanning in GM ROIs and after 2 weeks of scanning in WM ROIs. Estimates of time it takes to achieve minimal ongoing fixation (3 ms/week) is between 143 and 206 days (Table 2). At this time, there is minimal formalin artifact, but remaining contrast between GM and WM. Longer term storage leads to convergence of GM and WM $T_1$ relaxation time constants and diminished GM/WM contrast (11). The knowledge that the decline in $T_1$ relaxation time constants is less dynamic after 143–206 days, or 4.7–6.8 months, provides a window of time for acquiring scans with minimal formalin artifact while maintaining GM/WM contrast. The shape of the curves suggests a

![Figure 2](image_url)

**FIG. 2.** $T_1$ relaxation time constants over 6 months of fixation. Plots show mean $T_1$ relaxation time constants (ms) in each ROI over 6 months after the start of fixation. The x-axis shows the time in days, where $x=0$ is the day of death and the curves begin 10–12 days after that when the first scan was acquired. The baseline mean $T_1$ relaxation time constants are in tissue that has been in fixative for 10–12 days, so the mean $T_1$ relaxation time constants are shortened compared with in vivo measurements. The y-axis shows mean $T_1$ relaxation time constant in each ROI. Colored lines represent the five subjects’ brain hemispheres and black lines show the best fit curves.

| Subject | Age at death (y) | Sex | Death to fixation interval (hours) |
|---------|-----------------|-----|-----------------------------------|
| Subject 1 | 58 | F | 8 |
| Subject 2 | 58 | F | 21 |
| Subject 3 | 86 | M | 22 |
| Subject 4 | 57 | M | 17 |
| Subject 5 | 60 | M | 17 |

Table 1

Subject Characteristics

Table shows demographics information that could contribute to baseline intersubject mean $T_1$ relaxation time constant variability. Colored font indicates the colored curve in Figure 2 corresponding to the subject.
change in the dynamics of the tissue fixation process; earlier, the tissue is in the process of protein cross-linking during the fixation process and as more cross-linking occurs, the mean $T_1$ relaxation time constant shortens. As more of the tissue in the region has reached its fixed stage, the decline is less evident. It has been shown that fully fixed tissue acts as a barrier to further diffusion of fixative into cells (12). Intersubject variability might be due to differences in characteristics listed in Table 1.

A previous study reported stabilization of decline of mean $T_1$ relaxation time constants at 3 weeks (7), however, our results show that decline of mean $T_1$ relaxation time constants only slows after 2 weeks and does not consistently reach stabilization by 6 months. Their measurements were acquired using tissue immersed in 20% formalin, while ours were acquired using tissue immersed in 10% formalin, which suggests an impact of fixative concentration and type of fixative, size of the specimen, magnetic field strength of the scanner (13-15), $T_1$ estimating method, and $T_1$ curve fitting code. This should be considered when using published mean $T_1$ relaxation time constants for assigning acquisition sequence parameters. Our study design did not include refreshing the formalin during the 6-month period, as is done in some, but not all pathology laboratories. When stored for long periods, for example, during the 6-month period, as is done in some, but not all pathology laboratories. When stored for long periods, for example, the tissue is in the process of protein cross-linking, which occurs over approximately 30 days (16).

At the cellular level, speed of fixation is dependent on the rate of chemical reactions with the cellular components (18). Every cell experiences a period when they are exposed to a gradient of formalin as it diffuses in Srinivasan et al (12). Many factors influence penetration of formalin as well as the subsequent fixation (16). Factors influencing penetration rate are pH, fixative concentration (19), uniform exposure of tissue at all surfaces, length of time in fixative, a prior history of formalin exposure, pressure (20), and presence of blood vessels which are thought to accelerate fixative uptake in submerged tissue through capillary action (16,19). Factors influencing fixation rate are time, pH, temperature, existing cross-linking from prior formalin exposure, and viscosity (12,19).

Our data at each time point consists of four IR SE scans acquired with four different inversion times. In each subject, the mean $T_1$ relaxation time constant of each ROI was calculated at every time point. The mean $T_1$ relaxation time constant was calculated using an exponential fit of 4 inversion times to calculate the $T_1$ recovery rate assuming a monoexponential fit. It is possible that a biexponential fit would better represent the mixture of fixed and unfixed tissue that is being captured in our anatomical ROIs at each time point. To have a reasonable estimation for a biexponential fit, a higher number of inversion time measurements would be needed (21). Therefore, a future study with a higher number of inversion times may clarify whether a biexponential fit would better estimate the $T_1$ recovery rates of fixed and unfixed tissue in each ROI at each time point.

In conclusion, ex vivo brain MRI can provide valuable information, but the fixation process can cause artifacts that impact GM and WM segmentation. In addition, length of time in fixative should be noted when assigning acquisition sequence parameters. Tissue properties may need to be characterized separately for cortical GM,

### Table 2

| Region               | Tissue type | Direct formalin exposure | Days (CI)          |
|----------------------|-------------|--------------------------|--------------------|
| Frontal cortex       | GM          | Yes                      | 165 (143-183)      |
| Thalamus             | GM          | Yes                      | 170 (150-192)      |
| Anteromedial temporal lobe | GM    | Yes                      | 206 (186-223)      |
| Subcortical WM       | WM          | No                       | 165 (154-174)      |
| Splenium of corpus callosum | WM | Yes                      | 143 (119-163)      |
| Cerebellar WM        | WM          | No                       | 154 (124-189)      |

*aThe table shows the estimated time in days that it would take for each ROI to show very little change in $T_1$ relaxation time constant (slope of 3 ms/week), indicating that fixation is complete. GM = gray matter; WM = white matter.*
subcortical GM, and WM. When possible, scans should be acquired after an appropriate length of time for the fixation process of ex vivo tissue to be minimal and lead to fewer artifacts.

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