Relaxation Along a Fictitious Field (RAFF) and Z-spectroscopy using Alternating-Phase Irradiation (ZAPI) in Permanent Focal Cerebral Ischemia in Rat

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Abstract

Cerebral ischemia alters the molecular dynamics and content of water in brain tissue, which is reflected in NMR relaxation, diffusion and magnetization transfer (MT) parameters. In this study, the behavior of two new MRI contrasts, Relaxation Along a Fictitious Field (RAFF) and Z-spectroscopy using Alternating-Phase Irradiation (ZAPI), were quantified together with conventional relaxation parameters (T1, T2 and T1p) and MT ratios in acute cerebral ischemia in rat. The right middle cerebral artery was permanently occluded and quantitative MRI data was acquired sequentially for up to 6 hours. The following conclusions were drawn: 1) Time-dependent changes in RAFF and T1p relaxation are not coupled to those in MT. 2) RAFF relaxation evolves more like transverse, rather than longitudinal relaxation. 3) MT measured with ZAPI is less sensitive to ischemia than conventional MT. 4) ZAPI data suggest alterations in the T2 distribution of macromolecules in acute cerebral ischemia. It was shown that both RAFF and ZAPI provide complementary MRI information from acute ischemic brain tissue. The presented multiparametric MRI data may aid in the assessment of brain tissue status early in ischemic stroke.

Introduction

Transverse relaxation time (T2), diffusion and perfusion MRI are established imaging techniques used in the acute phase of ischemic stroke for both diagnosis and disease prognosis [1]. The challenge in accomplishing both these tasks from a single time point MR session has been addressed by the introduction of multiparametric models for tissue outcome assessment, such as ISODATA [2]. For the determination of tissue outcome from a single MRI session, recent studies using quantitative T2 either alone [3] or in combination with T1p [4], [5] MRI have shown great promise. Using preclinical models for ischemic stroke, it has been observed that T1p contrast shows the lesion within seconds from the onset of ischemia, i.e. the drop in blood flow below the level where the energy state of tissue collapses [6] and the degree of absolute T1p change in the early phase of ischemia are in excellent agreement with the extent of neuronal damage which develop in the infarcting brain [7]. A preclinical study addressing the predictive value of multiple MR variables showed that T1p and cerebral blood flow (CBF) were the most valuable single parameters in the early hours of ischemia for predicting the long-term tissue outcome [5]. More recently, the increase in T1p in the ischemic striatum and cortex of rat has been shown to be linear over the 6 first hour of stroke, revealing the potential to provide information about the duration of ischemia from a single time point MR scan [4]. These observations merit further exploration of rotating frame relaxations for evaluation of acute stroke, despite the fact that the exact mechanisms behind the T1p contrast in acute stroke are still much debated [5], [6], [7].

Typically, high specific absorption rate of energy (SAR) is associated with T1p MRI measurements, which may limit its clinical use. To reduce SAR and to create such spin-lock contrast with T1p and T2p contributions, a method entitled Relaxation Along a Fictitious Field (RAFF) was introduced [8]. In an effort to understand biophysical basis of RAFF signal in vivo, it has been concluded that the main processes influencing the magnetization in a two-pool system (free water and macromolecule pool) during a RAFF pulse are dipolar and exchange interactions, and the time course of magnetization can be modeled using the Bloch–McConnell equations for longitudinal and transverse magnetization for both of these pools [9]. The exchange processes that may influence the MR signal are the ones occurring at the average RAFF RF amplitude, typically a few hundred Hz and during the refocusing interval, typically a few thousand Hz.

It has been shown that the MT ratio [MTR = (1 – Msat/M0)], where Msat and M0 are magnetization in the presence and absence of a saturation pulse, respectively decreases in irreversible ischemia [10], [11] and it shows a different time course from the T1p relaxation changes in stroke [12]. It has also been observed that MTR is elevated after a few hours of cerebral ischemia. More
detailed Z-spectral data have revealed that the correlation time of the macromolecular motion and the exchange time between the solid and liquid pools of the two-site exchange model have changed approximately an hour after the onset of stroke [12].

An alternative way for detecting MT is Z-spectroscopy using Alternating-Phase Irradiation (with Sine Modulation), ZAPI(SM) [13]. In ZAPI, the irradiation pulse is applied at the frequency of the water resonance and the saturation is targeted to macromolecular spins by using amplitude-modulated RF (alternating phase: AP). With sinusoidal modulation, the frequency profile of a ZAPI pulse displays two off-resonance peaks and the frequency of these depends on the period of the RF modulation. With suitable selection of irradiation power and modulation frequency, direct saturation of water can be tailored to be negligible. Only macromolecular spins will experience saturation and by selection of a range of modulation frequencies, the line shape and $T_2$ distribution of the macromolecule pool can be probed. When a ZAPI sequence is run without modulation (i.e. constant wave: CW) at a frequency offset from water, the experiment simplifies into a conventional MT experiment. To illustrate the difference, Figure 1 shows ZAPI and CW Z-spectra measured in boiled egg white with three different RF amplitudes. The details and practical considerations of ZAPI have been discussed in [13].

For this acute cerebral ischemia study we had two aims: first, to investigate the time courses and magnitude of changes in the RAFF and ZAPI parameters, and to compare these to $T_1$, $T_2$ and MT. Second, to infer the effects of known changes in tissue water content and dynamics to these novel MRI contrasts during acute cerebral ischemia [14].

**Methods**

**Ethics Statement**

All animal procedures were approved by the Animal Care and Use Committee of the University of Eastern Finland and conducted in accordance with the guidelines set by the European Community Council Directives 86/609/EEC.

### Animal Models

Adult male Wistar rats (280–330 g, $n=9$), were subjected to permanent middle cerebral artery occlusion (MCAO) using the procedures described by Longa et al [15]. The occluding thread was left in place for the duration of the MRI scanning and the animal was sacrificed thereafter. Sham-operated animals ($n=2$) underwent similar procedure, but without the occlusion. The purpose of the sham animals was only to ensure that the setup is reliable and stable and that the operation produces the expected lesion; the effects of ischemia in the MRI parameters were evaluated by comparing the ipsi- and contralateral values in the experimental animals. All operations and scanning were performed under isoflurane anesthesia with a constant flow of 70/30 N2O:O2 through a nose cone. The core temperature was monitored online and was maintained close to 37°C by circulating warm water in a heating pad placed under the torso. Breathing rate was also monitored online throughout the MRI study (SA Instruments Inc., NY, USA). Arterial blood gases and pH were analyzed immediately before MR scanning (i-Stat Co., East Windsor, NJ, USA).

### MRI and Data Analysis

The MRI experiments were performed in a horizontal 4.7 T MagneX Scientific Inc. (Yarnton, UK) magnet interfaced to a Varian Unity Inova console (Varian Inc., Palo Alto, CA, USA). MRI was acquired at several time points for of up to 7 hours, with 60 minute intervals during MCAO. A volume coil was used as a transmitter and a quadrature half-volume coil as a receiver (Rapid Biomedical GmbH, Rimpar, Germany). Except for diffusion, a fast spin-echo (FSE) readout ($128\times64$, echo spacing 10 ms, FOV $25.6\times25.6$ mm²) was used for all imaging with different preparation blocks. In all cases the data was collected from a single axial slice positioned in the middle of the striatum, 5 mm caudally from the olfactory bulb.

The trace of the diffusion tensor ($D_{xx}=(1/3)\text{ Trace}[D]$) image was used to localize the acutely ischemic tissue. $D_{xx}$ was quantified as an average of diffusion values measured along the three
orthogonal directions (b-value 356 s/mm²) using a spin-echo sequence (time to repetition (TR) 1 s, time to echo (TE) 55 ms). Cerebral blood flow maps were acquired with a continuous arterial spin labeling technique with a 3 s labeling pulse (γB1 = 200 Hz) applied 2 cm from the imaging slice, and a 500 ms post-labeling delay [5]. Eight label-control pairs were used to estimate CBF using a water partition coefficient, A, of 0.9.

T1ρ MRI maps were collected with an on-resonance continuous wave (CW) spin lock (SL) preparation block AHP-SL-AHP (AHP = adiabatic half passage). The duration of the SL pulse ranged from 8 to 64 ms with SL amplitude (B1,SL) of 1700 Hz (40 μT), repetition time (TR) of 2.5 s, T2 was measured with an adiabatic double refocusing block, with an echo time (TE) of 2.5 s. T1 was measured using an inversion recovery sequence with five inversion times (TI 5–1500 ms, TR 3 s). All relaxation maps were calculated using three-parameter nonlinear fits.

For measuring TRAFF, the sine/cosine pulses with the previously described pulse design [8] were used, with a peak amplitude (γB1) of the RAFF pulse train of 625 Hz and five RAFF pulse train durations, linearly spaced between 0 and 144 ms. Two measurement, with and without initial inversion (H1-pulse, Tp = 4 ms, γB1 = 2.5 kHz) were performed and simultaneous fitting to the non-zero steady state model [8] was applied to the data. Fitting of TRAFF was performed on pixel-by-pixel basis to obtain TRAFF maps. The Bloch–McConnell equations for signal decay during the RAFF pulse train were used to simulate TRAFF change at two time points during stroke [8]. In these simulations, the values for relaxation times, pool sizes and exchange dynamics were incorporated from earlier work [16], in which a two-site exchange model was used. Dav and CBF time courses are presented in Fig. 2 B–C. In stroke animals, the average Dav decreased by 42 ± 9% (from the average value of 0.94 ± 0.11 × 10⁻⁹ m²/s in the contralateral hemisphere) by 100 min of MCAO, indicating severe ischemia in all brain regions studied. The apparent diffusion in normal brain was slightly higher than the generally reported which could have been due to the relatively low b-value range used. However, the diffusion drop caused by ischemia was typical for severe ischemia.

**Results**

In the sham-operated animals, no systematic ipsi-contra differences in any MRI parameters quantified were seen. The values for MT parameters and relaxation in sham animals were similar to the values in the contralateral side in the ischemia group. In stroke animals, the average Dav decreased by 42 ± 9% (from the average value of 0.94 ± 0.11 × 10⁻⁹ m²/s in the contralateral hemisphere) by 100 min of MCAO, indicating severe ischemia in all brain regions studied. The apparent diffusion in normal brain was slightly higher than the generally reported which could have been due to the relatively low b-value range used. However, the diffusion drop caused by ischemia was typical for severe ischemia.
increase occurred approximately an hour after induction of ischemia (Fig. 3C). The delay, or even an initial shortening of $T_2$, has been associated with increased capillary and venous deoxygenation and blood volume, resulting from compromised but non-zero blood flow in and near the ischemic region [17]. $T_{RAFF}$ displayed a time course more resembling that of $T_2$ than of $T_1$ (Fig. 3D). The sensitivity of different relaxation times to ischemia was quantified by analyzing the respective correlations.
When the brain areas were analyzed separately, for all parameter pairs the $R^2$ values were high (0.87 or higher), as all parameter changes were close to linear. When the samples were pooled (data from the five brain areas combined, Fig. 2A), the correlation between $T_1$ and RAFF was steeper than that of RAFF and $T_{1p}$ and RAFF and $T_2$, due to the smaller range in $T_1$. Furthermore, the regression lines (Fig. 4) show that the correspondence between RAFF and $T_2$ is very close to the line of unity, whilst other relaxation parameters either correlate with offsets or with different slopes.

RAFF simulations were performed to predict the change in $T_{RAFF}$ in two time ranges: (a) from pre-ischemic state at 0 min (estimated by contralateral values) to 135 min, and (b) from 135 min to 245 min. Due to limitations of the model in taking into account variety of relaxation mechanisms in tissue, the baseline of $T_{RAFF}$ is inexact, predicting a value of 102 ms for pre-ischemic condition, while the measured value is 120 ms. However, the computed changes in $T_{RAFF}$ in the ischemic tissue (6.5% for (a) and 6.6% for (b)) are in excellent agreement with the values observed in the S1 region (Fig. 3D). When the simulations were run with oscillating water frequency, mimicking diffusion in field gradients around capillaries, the computed $T_{RAFF}$ was further decreased (94, 98, 100 and 100 ms, for oscillations frequencies of 1, 10, 100 and 1000 Hz, respectively). The simulation for the system of finite line width also decreased the value of computed $T_{RAFF}$ giving a value of 100 ms.

During ischemia, MT signals, as detected by ZAPI and CW-MT, were increased at the first time point. The time courses for MT parameters are presented in Figure 5. CW-MT (Fig. 5A and B) shows larger differences between the hemispheres than ZAPI MT parameter (Fig. 5C), most likely due to early negative BOLD effect (resulting in line broadening) and changes in $T_1$ relaxation contributions to MT via direct saturation of water. However, there was no further increase in ZAPI MT parameter during the evolution of the stroke. This is strikingly different to the time course of all the relaxation parameters, especially $T_{1p}$. This suggests that neither RAFF nor $T_{1p}$ are MT-driven relaxation processes. The uncoupling was confirmed by correlation analysis in which changes in MT parameters and relaxation times were compared. All $R^2$ values, ranging from 0.10 to 0.39, for linear correlation were positive but statistically insignificant.

Removing the longer macromolecular $T_2$ components from the MT process with the $T_2$ filter in ZAPI (Fig. 5D-F), showed a similar course to the conventional MT: The ZAPI MT parameter decreases at the first time point and remains low throughout all time points studied. The amplitude of change is naturally smaller due to the fact that the overall MT is smaller with stricter filter (macromolecules with shorter $T_2$), because fewer macromolecular protons contribute to the signal. A simple measure of macromolecular $T_2$ distribution is $S(n \mu s)/S(100 \mu s)$, in which $n = 80, 60$ or $40 \mu s$. In ischemic tissue (Fig. 2A: S1, S2, C1) this parameter for $\tau$ of 60 and 40 $\mu s$ was significantly increased at the 60 minute time point (Fig. 6 B and C), which may suggest early changes in macromolecular dynamics in ischemic brain parenchyma. The direction of change indicates that while the ZAPI MTR is increased, i.e. $S(100 \mu s)$ is decreased, there is no corresponding decrease in signal measured with the selection of macromolecules with shorter $T_2$. These differences were reversed as the stroke...
evolved, and the few significant data points in later stages of ischemia actually represent cases where $S(n_{\text{ms}})/S(100\text{ ms})$ decreases. This trend is clearly visible in the data for $t=40\text{ ms}$ (Fig. 6 C), even if all the points are not statistically significant.

Figure 7 shows sample images of one animal imaged at one and five hours post MCAO. Signal-to-noise ratios for the MRI parameter maps were as follows: $T_1$ 56, $T_2$ 56, $T_{1p}$ 43, $T_{\text{RAFF}}$ 44, $D_\text{av}$ 10 and CBF 15. For GW-MT and ZAPI the corresponding ratios for MT-maps varied between 86 and 133.

Discussion

We have characterized the time courses for RAFF and $T_{1p}$ relaxation parameters, as well as ZAPI and conventional MT, in the acute phase of permanent focal cerebral ischemia in rat. It is evident that the temporal evolutions of the relaxation parameters studied are unrelated to MT-driven processes as detected by CW-MT MRI. We show that, $T_{\text{RAFF}}$ in brain parenchyma can be described by Bloch–McConnell equations and the changes caused by ischemia in $T_{\text{RAFF}}$ are predicted accurately by these equations using a two pool model [8]. The current data demonstrate that the ZAPI MT parameter is elevated in early minutes of ischemic stroke, and furthermore, the data measured with the ZAPI $T_2$ filter suggest changes in macromolecular $T_2$ distribution during the evolution of stroke. Overall, the MRI data obtained may provide complementary information for tissue status assessment for established MRI techniques used to image acute ischemic stroke.

In ischemic stroke, the supply of oxygen is severely compromised or completely interrupted, leading primarily to a collapse of the energy state followed by a cascade of damaging events in the brain tissue. Several simultaneous processes occur with different timescales, such as membrane depolarization, ionic and water shifts between extracellular and intracellular space, and activation of destructive proteases and lipases. A steady increase in longitudinal relaxation throughout the observation time window is evident, chiefly due to increased overall water content. Unlike $T_1$ and $T_2$, $T_1$ is already elevated at the first time point and the slope of subsequent increase is smaller than that of $T_{1p}$ and $T_2$. A similar linear increase is seen in transverse relaxation, but in some brain regions this is preceded by an early reduction (Fig. 3C). The diffusion recovery in the peripheral C3 region in the last time point (Fig. 2B) is reflected in longitudinal relaxation parameters (Fig. 3A and B).

The temporal evolution of $T_{\text{RAFF}}$ in stroke resembles that of transverse relaxation (Fig. 3D). Indeed, $T_{\text{RAFF}}$ relaxation is accelerated during the first hour of ischemia, especially in S2 and C1 regions, (Fig. 3D). The initial decrease and/or plateau in $T_2$ [17] is associated with high concentration of deoxygenated hemoglobin and dilated microvessels in the poorly perfused, but still viable, tissue in the early minutes of the insult. In such a matrix, the apparent $T_2$ measured from the parenchyma is a net result of shortened blood $T_2$, increased cerebral blood volume (CBV) and accelerated dephasing by extravascular field gradients. In this application of RAFF, half of the magnetization relaxes via $T_2$ type relaxation and it seems that the RAFF contrast in stroke is dominated by this relaxation pathway. However, when the $T_{2p}$ is measured using a train of adiabatic pulses, the plateau evident in $T_2$ disappears [16]. The difference between RAFF and T2 MRI may be due to selection of $B_1$. In our study the value of $\gamma B_1$ was only 20% of the value used previously in [16].

The measured values of $T_{\text{RAFF}}$ are longer than the simulated relaxation times. Our RAFF simulations employ a classical two-site exchange model with six ($2\times3$) differential equations. The model assumes that the chemical shift between water and macromolecules remains constant and that both pools have fixed Larmor frequencies. However, when the simulations are run for a more realistic model system, the number of relaxation channels is
Table 1. The measured relaxation rates in the sham-operated animals.

| Brain region | T1 [ms] | T2 [ms] | Tp [ms] | TRAFF [ms] | MTR CW 10 kHz [%] | MTR CW 5 kHz [%] | MTR ZAPI 100 μs [%] |
|--------------|--------|--------|--------|------------|-------------------|------------------|-------------------|
| S1           | 1070±18| 57.0±1.0| 73.6±1.4| 114.9±2.3  | 15.6±1.1          | 28.6±1.3         | 16.9±1.7          |
| S2           | 1086±18| 56.8±1.5| 73.8±1.8| 118.9±4.5  | 15.4±1.4          | 27.9±1.3         | 16.5±1.9          |
| C1           | 1139±29| 64.4±2.1| 83.8±2.2| 133.7±6.5  | 14.7±1.7          | 26.0±1.8         | 15.2±2.4          |
| C2           | 1120±15| 55.7±0.9| 72.7±2.2| 112.1±3.7  | 15.9±1.9          | 27.9±1.6         | 16.3±2.5          |
| C3           | 1092±19| 54.8±0.8| 71.6±1.5| 107.8±2.8  | 15.8±1.9          | 28.3±1.4         | 16.9±1.8          |

The figures presented are mean values of areas in both hemispheres and the six time points. No systematic variation across the hemispheres, between the time points or between the animals was observed. These values agree closely with the relaxation data measured in the contralateral hemispheres of the stroke animals as well.

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Figure 5. Progression of the MT changes for conventional MT and ZAPISM. Conventional MT (marked CW) is shown at offsets of 10 and 5 kHz, and ZAPISM (marked AP) on resonance, with full MT (τ = 100 μs) and with T2 filtering (τ = 80, 60 and 40 μs). The values are (ipsi-contra)/contra changes at brain regions S1-C3, at time points one (TP1) to six (TP6). The results from Student’s t-tests are given by: p<0.05 = † and p<0.01 = ‡. doi:10.1371/journal.pone.0069157.g005
Figure 6. Time courses for relative ipsi-contra differences in $T_2$-filtered ZAPISM MT. The values of parameter $S(n\ \mu s)/S(100\ \mu s)$ are shown at time points one (TP1) to six (TP6) in the different brain regions S1-C3. The results from Student's t-test are given by: $p<0.05 = \dagger$ and $p<0.01 = \ddagger$. 

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The ZAPI experiment can be considered as a dual off-resonance irradiation [13]. Side lobes fall at ±1/(2τ), which is ±5 kHz for τ of 100 μs, and with decreased τ, the off-resonance frequency increases. Side lobes experience lower B1 than the nominal irradiation B1, and thus the direct saturation lines are narrower and contamination from one side lobe is less than what is observed in conventional MT at the corresponding off-resonance frequency. Even if there are two side lobes in ZAPI, the ZAPI experiment results in higher signal than a conventional off-resonance MT experiment with similar rms power, Fig. 1 and [13], suggest that the direct saturation component is smaller in ZAPI. The dual direct saturation contribution in ZAPI is an advantage in the case of B0 shifts across the sample: in conventional MT the amount of direct saturation will change as a function of water resonance, while in ZAPI the changes in the direct saturation from the two side lobes will act to balance each other, as long as the B0 inhomogeneity remains small compared to the offsets of the irradiation. These practical aspects of ZAPI experiments have been discussed in more detail in [13].

The time courses for ZAPI and conventional MT differ in acute ischemia. This may be due to the influence of relaxation changes which influence the standard off-resonance CW-MT signal more than the ZAPI signal. According to ZAPI T2 filter experiments, there is a shift in the T2 distribution of the macromolecular pool at the early time points of acute stroke: the ratio between signal with ‘T2-filtered’ MT and full MT increases (Fig. 6). This may be a result of an increase in ZAPI MTR, as S/(100 μs) decreases whilst S/(<100 μs) remains unchanged. However, previous MT work [12] revealed apparent T2 changes in the liquid pool only, but no change in the macromolecule pool. This suggests that the changes in ZAPI originate from modification in the distribution of shorter T2 components of the macromolecules. This change is not observed throughout the evolution of the stroke, in fact there is a shift to the opposite direction in the last two ZAPI MTR time points. The changes detected by the ZAPI T2 filter are small, much smaller than the inherent differences in T2 seen between brain and muscle tissue [13]. However, the ZAPI technique is sensitive to these small changes and may prove a useful detection tool in other conditions in which the changes in macromolecular composition and consequently in T2 distribution and line shape are larger.

An important technical note is that both MT and RAFF MRI are more demanding for B1 homogeneity than the other relaxation parameters used in this study. Potentially, B1 inhomogeneity induces additional noise when averages from different experiments are computed. However, there was no systematic difference between the hemispheres in the sham-operated animals and therefore, B1 inhomogeneity must have been high. Another issue, which is related to demanding RF pulse sequences, is tissue heating. In both RAFF and ZAPI the root mean square RF amplitudes are very small, however, which keeps the SAR low.

The entire MRI protocol took about 30 minutes to acquire. The protocol in this study was not optimized for temporal resolution, but rather we wanted to run the MT experiments at steady state. The images were acquired with a segmented FSE readout. For all these methods used, there are faster acquisition paradigms and
pulsed MT techniques that can be applied if time is paramount. The slow acquisition also means that the time points for different parameters do not match exactly; the relaxation times were measured first, followed by CW-MT and ZAPI-MT. However, as the order was kept the same and six time points were measured, we believe this is not a serious problem for interpretation and comparison of the time courses.

In conclusion, we have shown that both RAFF and ZAPI provide complementary MRI information from the acutely ischemic rat brain tissue. From a mechanistic point of view, MT and relaxation changes during evolving stroke are not correlated. Early ZAPI signal changes reflect altered macromolecular T₂ distribution rather than net water accumulation. The multi-parametric MRI results may be useful for multiparametric assessment of brain tissue status early in acute ischemic stroke.

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**Author Contributions**

Conceived and designed the experiments: KTJ TL RAK OHJG JN. Performed the experiments: KTJ. Analyzed the data: KTJ TL JN. Wrote the paper: KTJ TL RAK OHJG JN.

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