Potential use of Oxygen as a metabolic bio-sensor in combination with T2* weighted MRI to define the ischemic penumbra.

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Running Headline: Oxygen and T2*-weighted MRI to define ischemic penumbra

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Abstract:

We describe a novel MRI technique, detecting metabolism indirectly through changes in oxyhemoglobin: deoxyhemoglobin ratios and T2* signal change during "**oxygen challenge**" (OC, 5 mins 100% O_2). During OC, T2* increase reflects O_2 binding to deoxyhemoglobin, formed when metabolizing tissues take up oxygen. Here OC has been applied to identify tissue metabolism within ischemic brain.

Permanent MCA occlusion was induced in rats. **Series 1 scanning** (n=5)**:** Diffusion-weighted imaging (DWI), followed by echo-planar T2* acquired during OC, and perfusion-weighted imaging (PWI, arterial spin labeling, ASL). OC induced a T2* signal increased of 1.8%, 3.7% and 0.24% in contralateral cortex, ipsilateral cortex within the PWI/DWI mismatch zone, and ischemic core, respectively. T2* and ADC map co-registration revealed the T2* signal increase extended into the ADC lesion (3.4%).

Series 2 (n=5): FLASH T2* and ADC maps co-registered with histology revealed a 4.9% T2* signal increase in histologically-defined border zone (55% normal neuronal morphology, located within ADC lesion boundary) compared with a 0.7% increase in cortical ischemic core (92% neuronal ischemic cell change, core ADC lesion).

OC has potential clinical utility and, by distinguishing metabolically active and inactive tissue within hypoperfused regions, could provide a more precise assessment of penumbra.

Keywords: T2*; Diffusion-weighted imaging; magnetic resonance imaging; MCAO; oxygen challenge; stroke; rat brain, metabolism, penumbra

INTRODUCTION

Treatment options for acute stroke are currently limited to thrombolysis, the incompletely defined interventions of stroke unit care, early surgical decompression, while anti-platelet therapies reduce the risk of early stroke recurrence. One reason proposed for the failure to translate efficacy of neuroprotective drugs from animal studies to clinical trials was the heterogeneity of stroke pathophysiology, leading to the inclusion of many patients who lacked a biological substrate for drug action (Muir, 2002). Physiological brain imaging to define tissue viability, brain perfusion, or metabolic status, promises to improve patient selection for current therapies or for clinical trials. For example, when patients were selected using the results of magnetic resonance imaging (MRI) diffusion-perfusion mismatch analysis, intravenous thrombolysis was shown to remain effective up to 6 hours after symptom onset, while also having fewer adverse clinical outcomes than use of structural imaging with computed tomography (CT) alone (Kohrmann et al., 2006, Schellinger et al 2007). MRI mismatch analysis may optimize patient selection for reperfusion therapies up to 9h after onset while also providing a biomarker for outcome (Hacke et al., 2005).

The viability, duration and the anatomical extent of the ischemic penumbra vary considerably from patient to patient, and are influenced by many factors. While time since symptom onset correlates broadly with volume of penumbral tissue, clinical features and structural imaging alone do not identify the extent of penumbra accurately on an individual basis (Marchal et al., 1993).

Current physiological imaging techniques used to identify penumbral tissue include Positron Emission Tomography (PET) and MRI. Using PET, the penumbra is best identified as a metabolically active region with reduced cerebral blood flow (CBF) characterized by an increased oxygen extraction fraction (Baron 1999; Marchal et al., 1993). However, few stroke units have PET

facilities able to perform ^{15}O experiments. PET imaging is expensive, with low spatial resolution, and radiation exposure limits repeat imaging in individual subjects. MRI is increasingly available in major stroke centers and offers both neuroanatomical and physiological imaging. The mismatch between the perfusion-weighted images (PWI) and injured tissue in which cellular swelling has already occurred identified on diffusion weighted imaging, (DWI), has been suggested to define the penumbra (Schlaug et al., 1999; Donnan et al., 2002). This provides only an approximation of potentially salvageable tissue (Kidwell et al., 2003). Currently no single apparent diffusion coefficient (ADC) threshold defines non-viability of tissue (Fiehler, et al., 2002; Guadagno, et al., 2004). Indeed, DWI lesions may be partially or even fully reversible when reperfusion occurs within 2-3 hours in animal models (Mintorovitch et al., 1991; Minematsu, et al., 1992) with similar findings in humans (Kidwell, et al., 2000; Fiehler, et al., 2002), although there is also the possibility that they may reappear after a few hours or days.

Similarly, the PWI lesion may include tissue with benign oligemia in addition to penumbra and infarct core, even when optimal thresholds (themselves as yet incompletely defined) are applied (Butcher et al., 2005). Therefore, DWI-PWI mismatch represents no more than a rough approximation of the penumbra, albeit one with some clinical utility. The penumbra *per se* is defined not only as a region of perfusion deficit but also of some remaining metabolic activity. The absence of a marker of tissue metabolism for penumbra definition in current MRI paradigms renders comparability with standard PET findings difficult and leaves interpretation susceptible to the varied approaches used to define the perfusion deficit.

We therefore sought to develop a new MRI technique in which oxygen is employed as a metabolic biosensor to detect tissue metabolism. The technique is based on the different magnetic properties of deoxyhemoglobin and oxyhemoglobin in blood (paramagnetic and diamagnetic, respectively).

Oxygen is carried in the blood in two forms: when air is breathed (~21% oxygen), most combines with hemoglobin to generate oxyhemoglobin but a very small amount dissolves in the plasma (paramagnetic free oxygen). When 100% oxygen is breathed (oxygen challenge, OC), additional oxygen dissolves in the plasma at a rate of 0.003 ml O_2/100ml of blood /mmHg PO_2 (Law and Bukwirwa, 1999).

Hemoglobin enters the brain almost entirely in the form of oxyhemoglobin, and gives up oxygen to metabolically active tissue to become deoxyhemoglobin. Therefore, detecting deoxyhemoglobin *in vivo* can indicate oxygen utilization, and therefore metabolism. Increasing amounts of deoxyhemoglobin (paramagnetic) reduces the T2* signal on a susceptibility-weighted sequence (Turner et al., 1991). During an OC, the additional free oxygen circulates in the plasma where it maintains the level of oxyhemoglobin (diamagnetic), either by directly supplying tissue with oxygen or binding to deoxyhemoglobin. In metabolically active tissue, OC should therefore cause an increase in T2* signal on susceptibility-weighted imaging from the pre-OC baseline (see online supplementary figure 1 for an illustration of the basis of the technique). This increase in T2* signal should reflect the amount of oxygen being taken up by the tissue from the blood. At the end of the OC, free unbound O_2 is no longer present to maintain oxyhemoglobin levels and so deoxyhemoglobin levels will increase as blood flows through metabolizing tissue, and the T2* signal will return to the pre-OC baseline. Therefore, the increase in T2*-weighted signal, its maintenance during the OC and its return back to baseline when the OC is complete, should indicate metabolism within the tissue. This paper presents the results of the OC technique in a rodent stroke model.

MATERIALS & METHODS

Induction of middle cerebral artery occlusion (MCAO)

All experiments were carried out under license from the UK Home Office and were subject to the Animals (Scientific Procedures) Act, 1986. Two series of experiments were carried out and each series consisted of n=5. This was done because the echo-planar T2* sequence provides T2* and has negligible T1 effect. However, image distortion prevents accurate correlation of T2* maps with histopathology. This was achieved in series two using a FLASH T2* sequence. Anesthesia was induced (4-5% isoflurane, echo-planar T2*, series 1, or halothane, FLASH T2*, series 2), in adult male Sprague-Dawley rats (311 ± 23 g, mean ± SD, n=10, Harlan, UK). The animals were tracheotomized, artificially ventilated and anesthesia maintained (1.5-2% of the respective anesthetic) in a mixture of nitrous oxide and oxygen (70:30) during surgery. After surgery, animals were ventilated with air slightly enriched in oxygen (30%) to maintain physiological stability under anesthesia. Body temperature was continuously monitored through a rectal thermocouple and maintained at 37^0C using a water blanket. Polyethylene catheters were placed in both femoral arteries in order to continuously monitor blood pressure and conduct blood gas analysis. MCAO was achieved by the intraluminal filament technique, using a modified version of Longa and colleagues (Longa et al.,1989). Physiological parameters were maintained within the normal physiological range under anesthesia apart from increased arterial partial pressure of oxygen (PaO_2) during the oxygen challenge. $PaCO_2$ was kept as constant as possible (35 - 45 mm Hg) in order to minimize cerebrovascular reactivity.

MRI data acquisition and oxygen challenge

Magnetic Resonance Imaging data were acquired on a Bruker Biospec 7T/30 cm system equipped with an inserted gradient coil (121 mm ID, 400mT/m) and a 72mm birdcage resonator. After surgery, animals were placed prone in a rat cradle, with the head restrained using ear and tooth bars in order to

limit movement, and a linear surface receiver coil (2cm diameter) was placed above the head of the animal.

Series 1 (n=5): Diffusion weighted imaging (DWI) was carried out prior to the susceptibility weighted sequence used during the oxygen challenge. The sequence for the DWI was a single shot Spin Echo echo planar imaging (EPI) Diffusion weighted scan (TE: 43 ms, TR: 4000.3 ms, 4 averages, matrix: 96 x 96, FOV: 25 x 25 mm, 3 directions: x, y, z, B values: 0, 1000 s/mm^2, 8 contiguous slices of 1.5 mm thickness). The sequence used to measure T2* changes during the oxygen challenge was a single shot, gradient echo EPI sequence (TE: 20ms, TR: 10s,matrix 96 x 96, FOV 25 x25 mm, 8 contiguous slices of 1.5mm thickness, 2 averages, temporal resolution 20s, 75 repetitions). A separate pulsed arterial spin-labeling sequence (FAIR, Kim et al., 1995) was performed on a single **slice to produce maps of relative cerebral blood flow** (TE: 20ms, TR 8s, matrix 80 x 64, FOV 32 x 25.6mm, slice thickness 1.5mm, 2 averages): 40 pairs of selective and non-selective images were acquired.

Series 2 (n=5): The first MRI sequences consisted of a pilot sequence for positioning and a RARE-T2 (effective TE: 46.8ms, TR:5000 ms, 4 averages, matrix: 256x256, FOV: 25x25 mm, 30 contiguous slices of 0.5 mm thickness**)** to allow selection of histology sections at the same coronal level as the T2* and DWI scans. In order to co-register MRI T2* statistical maps with histological sections (revealing the morphological status of neurons within the ischemic cortex), a second series of experiments was performed using a Flash-2D T2* gradient echo sequence (TE: 21.4 ms, TR: 317.7 ms, matrix: 128 x 128, FOV: 25 x 25 mm, 8 contiguous slices of 1 mm thickness, temporal resolution 37.5 seconds for each volume, 40 repetitions) to acquire the susceptibility-weighted images. The advantage of this sequence over the EPI sequence used in the first series, is that images are less distorted, and can be obtained at higher spatial resolution, making co-registration with histological

sections more accurate. Diffusion weighted imaging was carried out prior to the susceptibility-weighted sequence used during the oxygen challenge. The sequence for the DWI was a Spin Echo EPI Diffusion weighted scan (4 shots EPI, TE: 21.8 ms, TR: 4000.3 ms, 3 averages, matrix: 96 x 96, FOV: 25 x 25 mm, 3 directions: x, y, z, B values: 0, 200, 600, 1200, 2000 s/mm^2, 8 contiguous slices of 1 mm thickness), which was conducted in the same plane as the Flash-2D T2* scan.

In both series of experiments, OC was induced during the T2* scan using the paradigm 5 minutes 20 s with 30% oxygen, 5 minutes 20 s 100% oxygen and a final rest period of 16 minutes with 30% oxygen. Animals were then removed from the magnet and those undergoing FLASH T2* imaging (Series 2), were perfusion fixed (see below) under deep anesthesia for quantitative histopathology of the brain.

Perfusion fixation and histology

Transcardiac perfusion with heparinized saline was followed by 4% paraformaldehyde in phosphate buffer. The brains were left in the skull in fixative to prevent dark cell change artifacts (Brierley et al., 1972), removed 24 hours later, and immersion fixed before being processed and embedded in paraffin wax. Coronal sections of 6 micron thickness were cut on a microtome and mounted on poly-L-lysine coated slides. The cutting plane of these sections was as close as possible to the coronal imaging plane acquired with MRI. Coronal sections were stained with hematoxylin and eosin to identify the histological boundaries between ischemic core, border zone and normal cortex and to determine the level of neuronal ischemic cell change in regions of interest (ROIs) defined on the basis of neuronal morphology**.**

MRI data analysis

ADC and CBF infarction thresholds for permanent MCAO: Apparent coefficient diffusion (ADC) maps were generated from the diffusion **scans over 8 coronal levels throughout the MCA territo**ry and thresholded to 83.5% of the ADC average of the contralateral hemisphere, which has been shown to match most closely the final infarct size in permanent MCAO (Lo et al., 1997). **T2* weighted maps were also generated over the same 8 coronal slices. Single slice ASL was performed to generate a relative CBF map which matched the 5th coronal slice in core MCA territory.** The perfusion deficit was determined using a threshold of 57% of the contralateral hemisphere (Meng et al., 2004). ADC and CBF maps were co-registered to identify the PWI/DWI mismatch area (Figure 1a). **Time course T2* data were also generated from the 5th coronal slice (Figure 3) using a ROI size of 0.61mm^2 containing 9 pixels.**

Both echo-planar and FLASH T2* maps were analyzed using SPM. Before the data were analyzed statistically, motion correction and a spatial smoothing filter of 0.4mm and high pass filtering (1700s) were applied. The expected time evolution of the T2* signal during the OC was and then applied to the data on a voxel-by-voxel basis by applying a general linear model using an uncorrected statistical threshold of $p<0.005$ (Friston et al., 1995). **To align the data, DWI, ASL and Flash 2D data were scaled to their corresponding Rare T2 slices. Rare T2 slices were warped to the corresponding histology image (which was manually segmented and greyscaled), using second order non-linear warping (AIR). The warp parameters were then applied to the DWI and Flash 2D results, allowing the imaging results to be compared to the histology.** The processed data from the T2* OC, thresholded ADC and CBF maps, were co-registered: a) to locate the boundary on the thresholded ADC maps and the location of the PWI/DWI mismatch (Figure 1); and b) to define regions of interest (ROIs): IC, (ischemic core, in the center of the cortical zone of ADC abnormality), MM (PWI/DWI mismatch, ipsilateral cortex with ADC < 83.5% and CBF <57% of contralateral

hemisphere,), BZ, (border zone, within the area displaying ADC < 83.5% contralateral hemisphere and a positive T2* signal change), and NC, (normal cortex, homotopic region of the contralateral cortex) (Figure1a and 1c). All data are presented as mean ± standard deviation unless otherwise indicated.

Histological Analysis

An experienced neuropathologist (DG) who was "blinded" to the FLASH-T2* and ADC maps, selected the histology sections that best matched the anatomical MRI scan at the level of the anterior commissure. Identification of the boundaries between ischemic core, border zone and normal cortex was achieved by studying the neuronal morphology combined with changes in the neuropil in the form of pallor of staining and microvacuolation (see Figure 2). ROIs representing **ischemic core** (**IC**, where the majority of cells and neuropil showed the irreversible features of ischemic cell change, Figure 2a), four adjacent fields within the watershed, border zone (**BZ,** mixed population of cells with abnormal and normal morphology, Figure 2b) and adjacent **normal cortex** (**N**, normal cell morphology and neuropil, Figure 2c) were identified on each section within cortical layers 3-5. These regions were photographed at x 200 magnification with further magnification (x 4) during printing, for an assessment of the percentage of neurons displaying either normal morphology or ischemic cell change (Tamura et al, 1981). The ROIs were then superimposed onto the processed FLASH-T2* OC and ADC maps using Automated Image Registration software (Woods et al., 1998). OC T2* signal time course data from these regions were then extracted.

Statistical Analysis

Physiological variables before and during OC were analyzed by Student's paired t-test. T2* signal changes in different regions of interest were analyzed by one-way analysis of variance followed by Student's t-test with a Bonferroni correction. The influence of OC on relative CBF

was analyzed by averaging data within each group prior to OC, and during OC, subtracting one from the other and applying a 1 sample t-test.

RESULTS

Influence of oxygen challenge on physiological variables

In Series 1, OC was performed 154±74 minutes (mean±SD, n=5) after the onset of the MCA occlusion. Physiological values recorded before and after OC are detailed in Table 1. All physiological variables remained within the normal physiological range during OC except for the expected increase in arterial partial pressure of oxygen during the inhalation of 100% oxygen. PaO_2 increased 3.5 fold during OC **($p<0.05$).** This resulted in an increase of approximately 5% in the oxygen carrying capacity of plasma, since every mmHg rise dissolves an extra 0.003ml O_2/100ml blood, once hemoglobin is fully saturated (Law and Bukwirwa, 1999). **OC also induced a small, statistically significant increase in mean arterial pressure ($p<0.005$).**

ADC, CBF and echo-planar T2 data from MCA territory following stroke (Series 1)*

Thresholded ADC maps identified tissue injured by focal ischemia, thresholded ASL maps identified the perfusion deficit, and co-registration revealed the PWI/DWI mismatch area (**MM,** Figure 1a) **which was 16.05±5.44 mm^2 (n=5)**

Echo-planar T2* statistical maps revealed the distribution of significant T2* signal change in response to OC (Figure 1b). In all 5 animals, the hemisphere contralateral to MCAO, showed a significant increase in T2* signal intensity during the OC **(Figure 1a&b, NC 1.8±0.68%)** while regions in the ischemic core displayed no significant change in T2* signal **(Figure 1a&b, IC, 0.24±0.42%).** Co-registration of ADC, CBF and T2* statistical maps revealed a positive T2* signal

change within the PWI/DWI mismatch zone (MM, 3.7±1.4%, Figure 1a) **which was significantly greater than the T2* signal change recorded in contralateral cortex (p<0.05). Similarly, the T2* signal change in ischemic core was significantly smaller than in contralateral cortex (p<0.05).** Some tissue within the zone of ADC abnormality (outlined in blue, Figure 1c) also displayed a significant T2* signal increase on OC (BZ 3.5±2.4%). The time courses of the T2* signal change in NC, BZ, and IC are presented in Figure 3 a-c. **OC did not significantly influence relative CBF in the border zone (change in normalised signal -0.008, 95% CI -0.066 to 0.081, p=0.78) but did induce a small decrease in CBF in the contralateral cortex (-0.10, 95%CI -0.199 to -0.001, p=0.048, Figure 3d).**

FLASH T2 data and histology (Series 2)*

FLASH T2* signal change during OC was similar to that seen with the echo-planar sequence. Experiments were repeated using FLASH T2* to provide improved image resolution for co-registration with histology sections. **MR scanning started 145 ± 24 mins (mean ± SD, n=5) after MCAO and** perfusion fixation of the brains was carried out under deep anesthesia at 224 ± 4 mins. Histologically-derived ROIs (IC, BZ & N, Figure 4a), chosen following definition of the boundaries between ischemic core, border zone and normal cortex, were superimposed onto the processed T2* and ADC maps. Ninety-two percent of neurons displayed ischemic cell change (Table 2) in ischemic core which co-registered within the zone of ADC abnormality and displayed a small 0.7±2 % positive T2* signal change during OC (IC, Figure 4d, 5d). Histopathological analysis of the border zone demonstrated a gradual decrease in the number of neurons showing ischemic cell change moving from BZ0- BZ3 (Figure 4a, Table 2) which co-registered with abnormal ADC values and a 4.9±2.5% positive T2* signal change (BZ, Figure 4d, 5c).

ROIs identifying normal cortical histology (N, Figure 4a) and a homotopic region in contralateral cortex, co-registered with normal ADC values and a positive T2* signal change on OC (3.5±1.4 % and 3±1.1%, respectively, Figure 5a and b).

DISCUSSION

In this paper, we describe a new "oxygen challenge" technique, **using oxygen as a biosensor to detect tissue oxygen utilization and define the ischemic penumbra. Increased T2* signal, indicative of continuing oxygen utilization, mapped onto the zone of PWI/DWI mismatch and beyond this, into the ADC lesion, in a recognized rodent stroke model. Histology confirmed the existence of neurons displaying normal morphology within this defined penumbra.**

With **PWI/DWI mismatch, the current standard technique for identifying penumbra, accuracy in defining the perfusion and diffusion thresholds to predict infarction is crucial but as yet, standard thresholds have not been set for either clinical or pre-clinical imaging. Further, these thresholds vary, depending on time elapsed since blood vessel occlusion, and for PWI, extracranial stenosis can further complicate accurate determination of the perfusion deficit. In the current series, we used published thresholds calculated for three hours post-stroke in permanent MCAO in rodents (Meng et al., 2004, Lo et al., 1997) to define the PWI/DWI mismatch but acknowledge that determining in house thresholds could further improve penumbra definition. The oxygen challenge technique, offers the prospect of significant improvements over PWI/DWI mismatch in defining penumbral tissue by providing information on remaining metabolism (oxygen uptake).**

Basis for the OC technique: The magnetic properties of oxyhemoglobin and deoxyhemoglobin were demonstrated as long ago as 1936 (Pauling and Coryell, 1936). Deoxyhemoglobin is paramagnetic,

and water molecules around red blood cells are affected by the local magnetic field distortions due to its presence. This results in a reduction of the T2* value which can be detected with susceptibility-weighted imaging. Oxyhemoglobin is diamagnetic and has no such effect. Ogawa et al were able to demonstrate an MR signal change *in vivo* when the relative amounts of oxy- and deoxyhemoglobin were altered in the brain (Ogawa et al., 1990) and this Blood Oxygen Level Dependent (BOLD) contrast effect has since been used to demonstrate brain activation in response to external stimuli (functional MRI or fMRI, see **Kwong et al PNAS 1992**).

BOLD imaging was applied to animal and human stroke for identification of penumbral tissue, but produced limited results. Reductions in BOLD T2 and T2* have been observed in animal models of global and focal ischemia (Kavec et al., 2001; Roussel, et al., 1995), in response to reductions in CBF. Similarly Geisler and colleagues using BOLD imaging in acute stroke patients (Geisler et al, 2006) reported significant reductions in T2′ (quantitative T2* corrected with spin-spin effects) in the ischemic hemisphere which they hypothesized was due to increased deoxyhemoglobin and oxygen extraction fraction in penumbral tissue. T2' signal decreases were evident within the ADC lesion, in regions outside the ADC lesion later progressing to infarction, and in surviving tissue, compared to the unaffected hemisphere, but variability was such that there was no significant difference in T2' between these 3 ipsilateral ROIs, preventing estimation of a threshold or the clear-cut delineation of the penumbral tissue. Detecting changes in deoxyhemoglobin with BOLD imaging therefore does not provide an adequate surrogate marker for active metabolism. However, providing dynamic information on changes in oxy: deoxyhemoglobin ratios during OC and mapping statistically significant intensity changes in T2*-weighted images, allows identification of tissues responding and not responding to oxygen challenge (Figure 1b, 4b) and makes it possible to discriminate metabolizing tissue within penumbra from non-metabolizing tissues in ischemic core.

Within normal metabolizing tissues, the increased oxygen delivery during OC will convert deoxyhemoglobin back to oxyhemoglobin, resulting in an increase in the T2*-weighted signal (Supplementary online Figure 1). This signal increase is maintained during OC and the signal returns to baseline after OC (Figure 3a & 5a). In ischemic core, in our model, there is no restoration of flow and consequently no significant change in T2* signal is evident during OC (Figure 1b, 3c & 5d). **Border zone between core and normal cortex, as defined by the PWI/DWI mismatch (Figure 3b) or histology (Figure 5c) displayed an increased T2* signal compared to normal cortex. Previous 2-deoxyglucose studies suggest penumbra metabolism within the normal range at 2 hours post-stroke, with loci of hypermetabolism evident on autoradiograms (Belayev L et al., 1997, Tohyama et al., 1998). Although PET studies detect reduced cerebral metabolic rate of oxygen ($CMRO_2$) values within penumbral tissue relative to non-ischemic tissue, values are higher than in ischemic core and preserved relative to CBF, leading to high oxygen extraction fraction in humans (Furlan et al., 1996) and baboons (Giffard et al., 2004). Therefore, since penumbral tissue is metabolic but with restricted blood flow and a high oxygen extraction fraction, the higher level of deoxyhemoglobin in the blood should give rise to a more positive T2* signal change during OC. In our study, the penumbral tissue ROI, as defined by PWI/DWI mismatch, did display the greatest change in T2* during OC (3.7%) which supports the possibility that T2* change during OC reflects oxygen extraction fraction.**

The box car design used in this study provided information on the rise and fall time of the T2* weighted signal which will support further optimisation of analysis paradigms in future studies. There is a possibility for low frequency signal drifts to be classed as statistically significant using this design (false positive), because of the low frequency of this boxcar design, Although it is expected that the contribution from drifts will be minimal, future studies will attempt to account for any such drifts.

For an overview of the basis of the OC technique and T2* responses in ischemic core, penumbra and normal cortex see the supplementary online illustration.

A region of positive T2* signal change was also found within the zone of ADC abnormality (3.4%), in the watershed area of the ipsilateral dorso-lateral cortex (BZ0-BZ3 containing increasing percentages of morphologically normal neurons on histology, Figure 4a & d, Table 2). This suggests some tissue within the ADC lesion is still viable and metabolizing oxygen and is supported by reports that injured tissue, identified by DWI or ADC maps, has the potential to recover with early reperfusion and may not evolve into an infarct (Mintorovitch et al. 1991; Minematsu et al. 1992: Kidwell et al., 2000; Fiehler et al., 2002). Additional studies, **incorporating early reperfusion of ischemic tissue, are planned to validate the accuracy of a positive T2* signal in identifying metabolizing tissue capable of recovery.** We are also developing the technique further to define the boundaries between penumbra and benign oligemic tissue (destined to recover). This may be possible by combining T2* signal change during OC with a better defined assessment of the perfusion deficit. Alternatively, since both echo-planar and FLASH sequences revealed T2* signal changes in MM and BZ of a greater magnitude than contralateral and histologically normal ipsilateral cortex, the OC technique may also provide a means of defining the outer boundary of the penumbra, the focus of subsequent OC studies.

Changes in CBF, CBV, tissue oxygenation and oxidative metabolism can all **influence** the T2* signal (Ramsay et al., 1993 and Corfield et al., 2001), so it was important to discount the possibility that factors other than tissue metabolism were influencing the signal change during OC. $PaCO_2$ was regularly monitored and maintained within the physiological range (Table 1) to avoid any influence of CO_2 on CBF. OC induced a small increase in MAP and could therefore have increased CBF, if

autoregulation was affected in the ischemic hemisphere. **However, ASL revealed no increase in CBF in border zone or contralateral cortex during OC (Figure 3d). The physiological significance of the reduction in CBF in contralateral cortex requires further investigation given the borderline statistical significance and the fact that CBF was determined on a single slice within core MCA territory. Future studies on OC and CBF will employ multislice CBF maps throughout the brain and parenchymal laser Doppler flowmetry in ROIs.**

Previously, Kety and colleagues (Kety et al, 1947) reported a decrease in CBF during hyperoxia in conscious, normoventilating volunteers, **later confirmed by Floyd and co-workers (Floyd et al., 2003)**. **Similarly, hyperoxia induced a small decrease in CBF in anaesthetized rat (5.6%, Matsuura et al., 2001), anaesthetized mouse (9%, Shin et al., 2007) and in non-human primates (9%, Zhang et al., 2007).** However, ischemic tissue appears to respond differently to hyperoxia and in two recent animal MCAO studies, **extended periods of hyperoxia have been shown to induce a modest increase in CBF in the ipsilateral hemisphere. For example, after ~10 mins of 100% O_2 in a clip-induced distal MCAO model in mice (Shin et al., 2007) and after ~ 120 mins of 100% O_2 in intraluminal filament-induced permanent MCAO in rat (Henninger et al., 2007)**. **Importantly, in the Henninger study, with the same species, strain and stroke model as the current study, there was no evidence of a significant influence of 100% O_2 on CBF over the first 120 mins of 100% O_2.**

Ventilation with 100% oxygen can cause a decrease in tissue T1 (Tadamura et al., 2005). Therefore, for the FLASH T2* sequence (TR 317ms), which has a T1 component, a reduction in tissue T1 during OC would result in a positive signal change (Haacke et al., 1999) in addition to the increase seen from T2*. This would lead to a component of the response which was unrelated to oxy: deoxyhemoglobin ratios and tissue metabolism. In the EPI sequence (TR 10s) however, there is no

such effect, as the magnetization is fully relaxed before the next repetition. Hence the EPI sequence was primarily T2* weighted and any signal increase during OC should be due to changes in the oxy: deoxyhemoglobin ratio or perfusion**. To produce a positive T2* signal change, CBF would have to increase in response to OC, which was not shown in our study.**

In a number of animals, positive T2* signal changes were identified in loci within the ipsilateral striatum (Figure 4b), a region normally identified as ischemic core in permanent MCAO models. The distribution of the positive signal change corresponded to the position of the penetrating arterioles arising from the lenticulo-striate branches of the MCA (Scremin, 1995). This finding may be explained by the fact that MCAO was induced by the intraluminal filament method. We speculate that depending on how well the filament occludes the origin of the MCA, it is possible that a small amount of residual flow through these arterioles may occur and be sufficient to maintain metabolism in tissue immediately adjacent to these blood vessels causing a positive T2* signal on OC.

In these first studies we specifically chose a permanent MCAO model to simplify interpretation of the OC data. However, as mentioned above, further validation of the technique will involve applying the OC technique in models of transient MCAO. In tissues which are reperfused, but where there is no remaining tissue metabolism, there should be little or no conversion of oxyhemoglobin to deoxyhemoglobin. During OC, the oxygen not bound to hemoglobin should remain in a free state within the plasma and as this oxygen is paramagnetic, the T2* signal change should be negative, depending on the concentration of the free oxygen. Therefore following reperfusion, we predict the ischemic core should show a negative T2* signal change during OC, enabling reperfused, non-metabolic tissue to be differentiated from metabolic tissue**. The technique as described in the current paper has a number of limitations which should, however, be straightforward to resolve. For example, it should be possible to shorten the interval between MCAO and OC to**

~60 mins, the size and number of ROIs can be increased and, upgrading to continuous ASL would permit a mismatch volume, rather than an area to be determined for comparison with T2* changes. Partial volume effects and cross modality comparison with different resolution remains one of the challenges of this method. The AIR based method used for co-registration of MRI scans with histology sections depends on the use of data sets that contain similar contrast and signal information. Some error will result from differences between the histology and MR scanning data but is expected to be small when compared with plane slice errors. The most significant errors will result from errors in the histology slice plane with respect to the MR scanning plane. Imaging planes were selected by staff with knowledge of the histology procedure, to minimize these errors. We used a RARE sequence with an in plane resolution of 97 x 97 microns in order to achieve a precise co-registration. The mismatch of resolution between the histology and the BOLD (Flash) sequence was resolved by sampling the same field of view (FOV) size on the histology sections. The results from this last assessment thus reflect an average over a FOV similar to the voxel size acquired in MRI. The ROI has also been chosen to be in the middle of the mismatch region in order to limit the partial volume effect that could lead to inhomogeneous tissue in that voxel. To sample the z dimension, the histological assessment has been performed over 3 sections chosen to cover the 1 mm MRI slice.

Finally, a significant literature now exists on the beneficial influence of hyperoxia on ischemic tissue and its potential to salvage ischemic brain, extend the time window for acute stroke treatment, reduce the frequency of peri-infarct depolarizations, induce ischemic tolerance and enhance post-stroke recovery (Singhal, 2007, Shin et al., 2007). MRI studies in rodent stroke have revealed hyperoxia-induced recovery of ADC values with prolongation of PWI/DWI mismatch and the time window for beneficial reperfusion, and significant reduction in infarct volumes when hyperoxia was commenced within 30 mins of stroke and lasted for at least 105

minutes (Singhal et al, 2005, Henninger et al., 2007). OC used as a diagnostic tool involves only 5 minutes of 100% O_2 so it is debatable whether this would influence penumbra fate. However, temporal changes in T2* during and following OC may provide additional important information on tissue viability.

In conclusion, our novel hypothesis, that positive signal changes on T2* weighted imaging during OC provide an index of tissue metabolism in ischemic tissue, is supported in these first series of experiments by CBF, histological findings and comparison with the PWI/DWI mismatch technique. Further studies are required to identify the boundary between penumbra and benign oligemic tissue and to confirm viability of tissue within regions defined as penumbra using OC. If confirmed, this new MRI technique should be able to discriminate between metabolically active and inactive tissues in the ischemic brain, enable serial imaging and thereby provide an alternative and improved means of defining the ischemic penumbra.

Disclosure/Conflict of Interest: The authors have no conflict of interest to declare concerning this research

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Figure Legends

Figure 1

Series 1 a) Co-registration of thresholded ADC and CBF maps illustrating PWI/DWI mismatch. ROIs used to extract T2* signal changes: IC signifies ischemic core and lies within the dark red region defined by an ADC value <83.5% of the average contralateral value (Lo et al., 1997) and blood flow <57% of the average contralateral value (Meng et al., 2004); MM signifies mismatch and lies within the adjacent pink region which defines the PWI/DWI mismatch (ADC>83.5% and CBF<57%), NC signifies contralateral cortex. BZ, representing the ROI within the border zone, defined in c) as the overlap of positive T2* signal within the region of ADC abnormality; b) corresponding echo-planar T2* oxygen challenge statistical map. **Colored voxels correspond to a significant T2* signal increase during the inhalation of 100% oxygen, p<0.005**; c) co-registration of region of ADC abnormality (ADC<83.5%) and T2* map. The region within the ADC lesion boundary which demonstrated a positive T2* change during OC is outlined in blue. ROIs used to extract T2* signal changes: BZ border zone (positive T2* within ADC lesion boundary). All ROIs have been marked on Figure 1a & c for clarity.

Figure 2: Histological photomicrographs of ROI in dorso-lateral cortex ipsilateral to permanent MCAO from a representative animal. Pictures from hematoxylin & eosin stained sections have been captured at x 200 magnification.

a) Ischemic Core. Note darkly stained triangular neurons that have the features of the ischemic cell process within microvacuolated, pale stained neuropil.

b) Border Zone. Scattered throughout the ischemic cortex are neurons (circled) which display a normal morphology.

c) Normal Cortex. Neurons display normal morphology.

Figure 3: **Series 1** a-c Time course of the echo-planar T2* signal change during OC; a) contralateral cortex, b) border zone and c) ischemic core (from ROIs NC, BZ & IC, respectively, defined in Figure 1c). Graphs display signal intensity changes (%) against scan number for each animal. All data were normalized to the average signal over the 5 minutes prior to OC; d) Relative CBF (rCBF) assessed using arterial spin labeling in contralateral cortex and border zone. Data (n=5) were normalized to the average signal prior to OC in the contralateral cortex and presented as mean ± SD. **Change in rCBF during OC was analyzed by a 1 sample t-test. OC did not significantly influence CBF in the ipsilateral border zone (-0.008, 95% CI -0.066 to 0.081, p=0.78) but induced a small decrease in CBF in the contralateral cortex (-0.10, 95%CI -0.199 to -0.001, p=0.048).**

The gray bar represents the period of 100% oxygen inhalation.

Figure 4: **Series 2** Co-registration of histology, FLASH T2* oxygen challenge map and ADC map from a representative animal: a) the histological section displaying adjacent fields where neurons were counted and assessed for features of ischemic cell change by an investigator with no prior knowledge of the MRI maps: BZ border zones, IC, ischemic core, N, normal cortex. Numerical data are presented in Table 2;

b) T2* statistical map overlaid onto histology section. Colored voxels correspond to a significant T2* signal increase during the inhalation of 100% oxygen, $p<0.005$; c) localization of region of ADC abnormality, shaded white and defined as tissue displaying an ADC value of < 83.5% of the average contralateral value (Lo et al., 1997); d) the area of overlap (outlined in blue) between the ADC abnormality (c) and the T2* map (b). When mapped onto the T2*/ADC maps for each of the 5 animals, IC always fell within a region showing ADC abnormality and no T2* signal change, BZ fell within the ADC/T2* overlap region or on the boundary on the T2* map between no signal change and a positive signal change, and N fell within the region displaying a normal ADC value and a positive signal change on the T2* map.

Figure 5: **Series 2** Time course of the FLASH T2* signal change during OC; a) contralateral cortex, b) normal ipsilateral cortex, c) border zone (BZ0) and d) ischemic core (ROIs defined in Figure 4a). Graphs display signal intensity changes (%) against scan number for each animal. All data were normalized to the average signal over the 5 minutes prior to the oxygen. The gray bar represents the period of 100% oxygen inhalation.