Stroke Penumbra Defined by an MRI-based Oxygen Challenge Technique: 1. Validation Using [^{14}C]2-Deoxyglucose Autoradiography

Craig Robertson (MRes)[a], Christopher McCabe (PhD)[a], Lindsay Gallagher (HND)[a,] M. Rosario Lopez-Gonzalez (PhD)[c], William M. Holmes (PhD)[a], Barrie Condon (PhD)[b], Keith W. Muir (MD)[d], Celestine Santosh (MD)[b], I.Mhairi Macrae (PhD)[a]

[a] *Glasgow Experimental MRI Centre, Institute of Neuroscience and Psychology, College of Medicine, Veterinary & Life Sciences, University of Glasgow, Glasgow, United Kingdom;*

[b] *Institute of Neurological Sciences, Southern General Hospital, Glasgow G51 4TF, , United Kingdom*

[c] *Department of Clinical Physics, SINAPSE Collaboration, College of Medicine, Veterinary & Life Sciences, University of Glasgow, Glasgow, United Kingdom*

[d] *Division of Clinical Neurosciences, University of Glasgow, Institute of Neurological Sciences, Southern General Hospital, Glasgow G51 4TF, ,UK*

Correspondence to: Craig Robertson

Wellcome Surgical Institute,

Institute of Neuroscience and Psychology,

College of Medicine, Veterinary & Life Sciences

University of Glasgow

Garscube Estate,

Glasgow G61 1QH, UK

Email: c.robertson.1@research.gla.ac.uk

Phone: +44(0)1413305829

Fax: +44(0)1419430215

Support

This work was supported by a MRC Project grant (G0700439); the Translational Medicine Research Collaboration (NS-GU-122) - a consortium made up of the Universities of Aberdeen, Dundee, Edinburgh and Glasgow, the four associated NHS Health Boards (Grampian, Tayside, Lothian and Greater Glasgow & Clyde), Scottish Enterprise and Wyeth Pharmaceuticals; a MRC Capacity Building Area PhD studentship 2007 [to C.R.]; and SINAPSE Collaboration funding [to M.d R.L.-G.] www.sinapse.ac.uk, a Pooling Initiative funded by the Scottish Funding Council and the Chief Scientist Office of the Scottish Executive.

Abstract (word count 196)

Accurate identification of ischaemic penumbra will improve stroke patient selection for reperfusion therapies and clinical trials. Current MRI techniques have limitations and lack validation. Oxygen challenge T_2* MRI (T_2*OC) uses oxygen as a biotracer to detect tissue metabolism, with penumbra displaying the greatest T_2* signal change during OC. [^{14}C]2-deoxyglucose autoradiography was combined with T_2* OC to determine metabolic status of T_2*-defined penumbra.

Permanent middle cerebral artery occlusion was induced in anaesthetised male Sprague Dawley rats (n=6). Ischaemic injury and perfusion deficit were determined by diffusion- and perfusion-weighted imaging, respectively. At 147±32 minutes post-stroke, T_2*-signal change was measured during a 5 minute 100% oxygen challenge,

immediately following by 125 μCi/kg [^{14}C]2-deoxyglucose, i.v.. MRI images were co-registered with corresponding autoradiograms.

Regions of interest were located within ischaemic core, T_2*-defined penumbra, equivalent contralateral structures and a region of hyperglycolysis.

A T_2* signal increase of 9.22±3.9% (mean±SD) was recorded in presumed penumbra which displayed LCMRglu values equivalent to contralateral cortex. T_2* signal change was negligible in ischaemic core, 3.2±0.78% in contralateral regions and 1.41±0.62% in hyperglycolytic tissue, located outside OC-defined penumbra and within the diffusion abnormality. The results support the utility of OC-MRI to detect viable penumbral tissue following stroke.

Running Headline: Detecting penumbra using T_2* MRI and O_2 challenge

Key words: ADC, CBF, imaging, LCMRglu, MCAO, rat

Abbreviations: ADC_{av}= Apparent diffusion coefficient; $CMRO_2$ = cerebral metabolic rate of oxygen**;** DWI = diffusion-weighted imaging; DWI/PWI mismatch = diffusion/perfusion mismatch; MCAO = middle cerebral artery occlusion; OC = oxygen challenge; PaO_2 = Partial Pressure of Oxygen in Arterial Blood; $PaCO_2$ = Partial Pressure of Oxygen in Arterial Blood; PWI = perfusion-weighted imaging; ROI = region of interest; [^{14}C]2-DG = [^{14}C]-2-deoxyglucose.

Word count (excluding references, tables and figure legends): 4218

Introduction

Stroke is a leading cause of morbidity and the second most common cause of mortality worldwide (Donnan et al, 2008). Disability and mortality are reduced by

admission to specialised stroke units, but the only widely approved drug treatment for acute cerebral ischaemia is recombinant tissue plasminogen activator (rt-PA), for which many patients are ineligible. Low treatment rates with i.v. rt-PA (for example 1-7% in the USA following FDA approval (Wardlaw et al, 2009)) partly reflect delay in presentation and institutional barriers to rapid medical assessment (Katzan et al, 2004; Kahn et al, 2005), but also that clinical uncertainty prevents treatment in many circumstances. Such clinical uncertainty includes patients awaking with symptoms in whom onset time is unclear, rapidly improving or mild symptoms, and perceived concern about bleeding risks (Barber et al., 2001). Failure to identify efficacy in both thrombolysis and neuroprotection trials (Kidwell et al, 2001) could have arisen from treatment being administered at a time point when no salvageable (penumbral) tissue remained, but potentially also from recruitment of patients lacking target penumbral tissue even within conventional time windows (Muir, 2002). The possible improvement in safety with more advanced penumbral imaging selection for thrombolysis has also been suggested (Schellinger 2003).

MRI-based diffusion/perfusion (DWI/PWI) mismatch provides an indirect index of the ischaemic penumbra. When used to select patients for thrombolysis, it identified patterns that may define treatment responsiveness (DEFUSE study, Albers, Thijs & Wechsler et al., 2006) and has been used to select patients for an extended time window (Hacke, 2005). However, the technique has not been validated clinically and has a number of limitations. No DWI or apparent diffusion coefficient (ADC) threshold has been determined which can differentiate between irreversibly damaged and potentially recoverable tissue (Guadagno et al, 2004) and when thresholds are applied, the lesions identified can be fully or partially reversed by reperfusion in

animal models and man (Kidwell et al, 2000). Defining a threshold for the perfusion deficit is equally difficult and may include benign oligaemic tissue which is not at risk (Butcher et al, 2005).

We have developed an alternative technique that identifies the penumbra based on its metabolic status which could represent significant improvements on current penumbral imaging. Oxygen challenge (OC) MRI uses a transient hyperoxic challenge to identify changes in the deoxyhaemoglobin:oxyhaemoglobin ratio, detected by T_2*-weighted MRI (Santosh et al, 2008, Dani et al., 2010). Paramagnetic deoxyhaemoglobin and free oxygen in the plasma reduce T_2* signal, whilst diamagnetic oxyhaemoglobin has a minimal influence on T_2* (Uematsu et al, 2007). Following stroke, penumbral oxidative metabolism ($CMRO_2$) is maintained in the face of reduced cerebral perfusion pressure by increasing oxygen extraction fraction (OEF) (Powers, 1991). This increases the deoxy:oxyhaemoglobin ratio in the vasculature resulting in a decreased T_2* signal within penumbra. Increased oxygen delivery during oxygen challenge will convert deoxyhaemoglobin back to oxyhaemoglobin with a resultant increase in T_2* signal, the magnitude of which should be greatest in regions with greatest OEF. T_2* maps can be generated that locate and quantify the percentage change in T_2* signal throughout the territory of the occluded artery. In addition, the maintenance of this increased signal during the oxygen challenge and its return back to baseline following OC is consistent with T_2* signal change indicating oxygen consumption. This technique may therefore yield information on oxygen metabolism that more closely correlates with positron emission tomography (PET) definitions of the penumbra.

In our previous study (Santosh et al., 2008), the OC technique applied to a rodent middle cerebral artery occlusion model (MCAO) defined an inner boundary between metabolically inactive ischaemic core (no T_2* signal increase to OC) and metabolically active penumbra (T_2* signal increase to OC) which overlapped approximately with DWI/PWI mismatch, and correlated with histological appearances of neuronal integrity. The greater magnitude of T_2* signal increase in penumbra during OC compared to normal cortex may differentiate between hypoperfused penumbra and benign oligaemic tissue on the basis of differences in $CMRO_2$, thereby providing a means of defining the outer boundary of the penumbra.

The aim of this study was to provide further validation for the T_2* OC MRI technique: firstly to confirm viability of tissue within regions defined as penumbra using OC. This was achieved by co-registering T_2* OC maps with corresponding [^{14}C]2-deoxyglucose (2-DG) autoradiograms, which provide information on local cerebral glucose utilisation (LCMRglu) as a quantitative representation of metabolic activity. Secondly, regions of interest (ROIs) identified on 2-DG autoradiograms and MRI scans were investigated to determine the LCMRglu and MRI signatures of different tissue compartments in the ischaemic hemisphere.

Materials and Methods

Rodent MCAO surgery

Experiments were performed under license from the UK Home Office and were subject to the Animals (Scientific Procedures) Act, 1986. Male Sprague Dawley rats (289±13g, n=6, Harlan, Bicester, UK), fasted overnight, were initially anaesthetised with 5% inhaled isofluorane in an induction chamber at room temperature. Following

a surgical tracheotomy, animals were artificially ventilated with 2% isofluorane delivered in air, slightly enriched with oxygen (30%) to maintain physiological stability throughout the experiment. Blood gases were sampled at baseline in the 5 min before the start of OC, and half way through 100% O_2 inhalation, and maintained within the normal physiological range apart from increased arterial partial pressure of oxygen (PaO_2) during the oxygen challenge. $PaCO_2$ was maintained between 35 - 45 mm Hg to minimise cerebrovascular reactivity (Table 1). A rectal thermocouple provided continual monitoring of core body temperature which was maintained at 37±0.5^{o}C.

Polyethylene catheters (Portex: external diameter 0.96 mm; internal diameter 0.58 mm; 70 cm long) were placed in both femoral arteries, to continuously monitor blood pressure and conduct blood gas analysis, and a femoral vein for [^{14}C]2-deoxyglucose administration. Middle cerebral artery occlusion (MCAO) was achieved by the intraluminal filament technique (Longa et al., 1989), where a 3-0 nylon monofilament with a bulbed tip was introduced through the internal carotid artery and advanced to block the origin of the middle cerebral artery.

MRI Scanning

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 72 mm birdcage resonator. After stroke surgery, animals were placed prone in a rat cradle, with the head restrained using ear and tooth bars to limit movement, and a linear surface receiver coil (2 cm diameter) placed above the head of the animal.

T_2 scanning

A RARE T_2 sequence (effective TE: 46.8 ms, TR: 5000s; in plane resolution of 97μm; 30 slices of 0.5mm thickness) provided the neuroanatomical template for co-registration of T_2* scans with DWI and PWI scans and 2-DG autoradiograms (Figure 1A & B).

T_2* scanning

The sequence used to measure T_2* changes during OC was a single shot, gradient echo (EPI) sequence. (TE: 20ms, TR: 10s,matrix 96 x 96, FOV 25 x25 mm^2, 8 contiguous slices of 1.5mm thickness, 2 averages, temporal resolution 20s, 75 repetitions). Two coronal MRI slices which corresponded to territory supplied by the middle cerebral artery were selected for analysis. The paradigm for the T_2* weighted oxygen challenge sequence was 5 minutes breathing air, followed by 5 minutes breathing 100% oxygen, and then 15 minutes breathing air.

DWI scanning

DWI was performed to identify ischaemically injured tissue (Spin Echo planar (EPI) TE: 43 ms, TR: 4000.3 ms, in plane resolution of 260 um, 3 directions: x, y, z, B values: 0, 1000 s/mm^2, 8 slices of 1.5mm thickness).

Arterial Spin Labelling (ASL)

Non-invasive quantitative CBF was carried out on 2 coronal slices within the MCA territory using a form of pseudo-continuous ASL based on a train of adiabatic inversion pulses (Moffat et al, 2005). The sequence employs a spin-echo echo-planar-imaging (EPI) imaging module (TE 20ms, TR 7000ms, matrix 96 x 96, FOV 25 x 25

mm^2, slice thickness 1.5mm, 16 averages, 4 shots) preceded by 50 hyperbolic secant inversion pulses in a 3s train.

[^{14}C] 2-Deoxyglucose autoradiography

At the end of the MRI scanning session, animals were quickly removed from the magnet, and a bolus of [^{14}C]2-deoxyglucose injected intravenously at a steady rate over 30 seconds (125 μCi/kg in 0.6 ml heparinised saline, Perkin-Elmer). Plasma glucose and ^{14}C were analysed from fourteen timed arterial blood samples over 45 minutes by glucose oxidase assay and liquid scintillation analysis, respectively. At 45 minutes, animals were rapidly killed by intravenous injection of sodium pentobarbitone and the brains quickly dissected out, frozen (isopentane, -40^{o}C) and processed for quantitative autoradiography. Coronal cryostat sections (20μm) were exposed to X-ray film (Kodak Biomax MR film) for 3 days with a set of ^{14}C standards (Amersham Biosciences, GE Healthcare). Autoradiograms were analysed using a computer digitised image analysis system (MCID v4, Interfocus).

Quantitative optical density measurements were taken from 5 regions of interest (ROIs, defined in detail below and shown in Figure 2): 1. ischaemic core; 2. penumbra; 3. an intermediate region of increased glucose utilisation at the boundary of the ischaemic core (see Figure 2C); 4. & 5. contralateral regions homotopic to the penumbra and ischaemic core, respectively. Optical density values were converted into ^{14}C tissue concentrations using the calibration curve derived from the set of ^{14}C standards. The ^{14}C tissue concentrations along with the plasma glucose and ^{14}C plasma concentrations were used to calculate LCMRglu (μmol/100g/min) in ROIs using the operational equation of Sokoloff et al (1977).

Glucose utilisation for each ROI was generated from ten autoradiographic images covering the rostro-caudal extent of each MRI slice (1.5mm) to accurately equate T_2* signal change maps with glucose utilisation.

Defining the ischaemic penumbra with T_2* OC

The time course and size of the T_2* signal change was analysed within ROIs (see Figure 3). T_2* percentage signal change was calculated from time course graphs, where the average baseline signal was subtracted from the peak signal during oxygen challenge. This value was then divided by the average baseline signal and multiplied by 100. T_2*OC percentage signal change maps were generated using Image J software (http://rsb.info.nih.gov/ij/)

Penumbral tissue was defined using a threshold based on the empirical rule: the mean plus 2 Standard Deviations (SD) of the T_2* value of the contralateral hemisphere, excluding the ventricles (see Figure 2B).

Defining the ischaemic penumbra with DWI/PWI mismatch

DWI and PWI (using ASL) were used to define mismatch prior to the T_2*OC protocol and data analysed on two selected coronal slices within the MCA territory (Figure 1). Quantitative ADC_{av} maps, in units of square millimetres per second, were calculated using the Stejskal-Tanner equation (Stejskal and Tanner, 1965). ADC maps and CBF maps were generated using Image J software. A 16.5% reduction of mean contralateral ADC was used to determine ischaemic lesion volume, which has been shown to match closely the final infarct size following permanent MCAO in Sprague Dawley rats (Lo et al, 1997). PWI was carried out on the 5^{th} and 6^{th} coronal slices within core MCA territory and the perfusion deficit area was calculated based on a

57% reduction of mean contralateral CBF (Meng et al., 2004). ADC and CBF maps were overlaid to identify the DWI/PWI mismatch area defined as the difference between the perfusion deficit and the ADC lesion area on the corresponding slice. For analysis, the data for the rostral and caudal slices were combined.

Volumetric analysis of penumbra, hypo- and hyperglycaemia

Volumetric analysis of penumbra (in terms of mismatch and the T_2*OC), ADC defined lesion, perfusion deficit and hypo- and hyperglycaemic tissue on 2-deoxyglucose autoradiograms were performed and data expressed as the mean volume within the MCA territory included in the ASL scans. For 2-deoxyglucose autoradiograms, images were analysed to cover the same rostro-caudal extent as the MR images, and were thresholded to determine the region of severely reduced glucose use (glucose values below 10 μmol/100g/min) and the hyperglycolytic band (tissue with glucose values above 60 μmol/100g/min).

Regions of Interest

Circular ROIs were selected according to specific features on the images (Figure 2), and to ensure placement solely within the areas of interest and uniformity in ROI size, ROIs were chosen manually (spanning 80 pixels). The researcher responsible for ROI placement was blinded to the autoradiograph data during MRI ROI placement and *vice versa*. MRI-based ROIs were defined and placed within 1. Ischaemic core in caudate nucleus within the thresholded ADC lesion (Figure 2E, red) and, 2. Its mirror contralateral region (sky blue), manually designated by researcher; 3. Penumbra as defined by thresholded T_2* percentage signal change (Figure 2B, green); 4.

Equivalent contralateral cortex (white), and, 5. A cortical hyperglycolytic region selected from the 2-DG autoradiogram (Figure 2C, yellow).

Co-registration and Regions of Interest Analysis

The processed data from the T_2*OC, 2-DG, thresholded ADC and CBF maps, were co-registered to: a) generate ROI data, and b) identify the location of the DWI/PWI mismatch (Figure 1C and D). Linear co-registration was first carried out using Analyze and then DWI, ASL, T_2^*- and 2-DG images were warped to their corresponding RARE T_2 slices using AIR 5.2.6 (Automated Image Registration).

Statistical analysis

All data are presented as mean±SD. Data were normally distributed, and as such, physiological variables at baseline (pre-OC) and during OC were analysed by Student's paired t-test. T_2* signal, ADC, CBF and LCMRglu values in different ROIs were analysed by one-way analysis of variance followed by Student's paired t-test with a Bonferroni correction for multiple comparisons. A paired t-test was used to analyse the temporal evolution of the ADC-derived lesion volume. All data were tested to confirm normal distribution using the D'Agostino and Pearson normality test.

Results

Physiological variables

Mean time to commence OC was 147±32min after MCAO. Physiological variables were recorded immediately before and during OC (Table 1). PaO_2 increased

significantly (262%, $p<0.0001$) as expected during OC, $PaCO_2$ did not change and MABP increased significantly (11%, $p<0.05$). The mean plasma glucose prior to scanning was 9.8±2.7 mmol/l.

T_2* percentage signal change to OC

T_2* percentage signal change during OC was measured over two coronal slices. Thresholded T_2* maps revealed a region of increased signal which corresponded approximately with the DWI/PWI mismatch (Figure 2B&F). Examining the individual T_2* time course data, (Figure 3) T_2* signal increase in this penumbral ROI was 9.2±3.9%, significantly greater than in the contralateral ROI (2.76±0.3%, $p<0.001$, Figure 4). In ischaemic core, mean T_2* signal during OC was reduced (-0.49±1.6%) compared to the contralateral caudate nucleus ROI (3.56±0.94%, $p<0.01$). In the hyperglycolytic ROI, there was a small T_2* signal increase of 1.4±0.6% which had a significantly smaller signal response than the contralateral cortex ($p<0.05$) and penumbral ROI ($p<0.001$).

Glucose use values in ROIs

[^{14}C]2-deoxyglucose autoradiography confirmed glucose use within the T_2*OC -defined penumbra, which was not significantly different from the contralateral ROI (22.67±2.1μmol/100g/min compared to 20.1±2.3 μmol/100g/min, Figure 5). Glucose use was below the limit of detection in ischaemic core (Figure 2C& 5) and was markedly increased in the hyperglycolytic ROI (79.7±12 μmol/100g/min, compared to contralateral cortex 20.1±2.3 μmol/100g/min, $p<0.001$). The hyperglycolytic ROI (279% increase in LCMRglu) occurred within the boundary of the ADC lesion. It

displayed a small increase in T_2*signal to OC which was significantly smaller ($p<0.05$) than the T_2* increase in contralateral cortex (Figure 4).

Absolute glucose utilisation values were low compared to most values from the literature, which are generated from conscious animals. In order to confirm the validity of our 2-DG technique, in an additional animal, anaesthesia was withdrawn following MRI scanning and [^{14}C]2-deoxyglucose autoradiography carried out 2 hours later when the animal was fully conscious. Glucose use values were similar to reported values, and proportionally higher in each ROI, when compared to anaesthetised animals: contralateral cortex 41.67 μmol/100g/min; contralateral caudate nucleus 46.7 μmol/100g/min; penumbra, 69 μmol/100g/min (62% increase); hyperglycolytic ROI, 91 μmol/100g/min (122% increase); ischaemic core, 1.43 μmol/100g/min (97% decrease relative to contralateral caudate nucleus). Therefore, the low glucose utilisation values in the MRI study were most likely due to the level and duration of anaesthesia (See Supplementary Material).

To dismiss the possibility that the presence of the hyperglycolytic band may be a confound of O_2 administration, an additional rat underwent MCAO, and when fully conscious [^{14}C]2-deoxyglucose autoradiography was carried out without OC. The hyperglycolytic band was present, suggesting the OC *per se* was not responsible for generating hyperglycolytic tissue.

Severity of ischaemia and tissue viability

On quantitative CBF maps (Figure 2D), contralateral cortex and caudate nucleus ROIs had mean CBF values of 185±45 and 165±31 mL/100g/min, respectively. CBF within ischaemic core, hyperglycolytic and penumbra ROIs was significantly reduced

(7.9±4.7, 21±0.2, and 37±66 mL/100g/min, respectively, all $p<0.001$) relative to the equivalent contralateral ROI (Figure 6A).

On ADC maps (Figure 2E) ROIs in contralateral cortex and caudate nucleus had a mean ADC of 0.73±0.06 and 0.74±0.02 $x10^{-3}$ mm^2/sec, respectively (Figure 6B). ADC values were significantly reduced in ischaemic core and hyperglycolytic ROIs (0.47±0.04 and 0.51±0.08 $x10^{-3}$ mm^2/sec, respectively $p<0.001$) while in the T_2* OC - defined penumbral ROI, mean ADC value (0.71±0.04$x10^{-3}$ mm^2/sec) was not significantly reduced compared to contralateral cortex ($p>0.05$, Figure 6B).

Penumbra defined by DWI/PWI mismatch

For comparison, penumbra, as defined by DWI/PWI mismatch was mapped over two coronal slices from the co-registered ADC and ASL scans (Figure 1C & D, 2F)**.**

Evolution of ADC-derived lesion volume

DWI scans generated at the start and conclusion of the MRI scanning session provided information on the evolution of ischaemic injury over time. A significant increase in the ADC-derived lesion volume (106±66mm^3 at 52±11 minutes compared to 185±130mm^3 at 106±8 minutes post-stroke, $p<0.05$) highlighted the progression of damage in the acute stroke period and the concomitant loss of penumbral tissue.

Volumetric analysis of penumbra, hypo- and hyperglycaemia

The volume of perfusion deficit determined at 76.4±13.2 min post-stroke, was 103.1±21 mm^3, the ADC lesion volume, determined at 106±8 min post-stroke was 80.7±30 mm^3, generating a DWI/PWI mismatch volume of 22.3±19 mm^3. The thresholded T_2*OC-defined penumbra, determined at 147±32min, was 15±9 mm^3.

Mismatch- and T_2*OC-defined penumbral volumes were approximately 28% and 19% of the volume of the ADC-defined ischaemic core, respectively. The volume of severely reduced glucose use in ischaemic core was 52.8±18 mm^3 and the volume of the hyperglycolytic band was 21.1±16 mm^3 (total volume incorporating the region of severely reduced glucose use and the hyperglycolytic band was 73.9±26 mm^3).

Discussion

We previously described the oxygen challenge MRI technique in a focal cerebral ischaemia model and compared it with DWI/PWI mismatch and histologically-defined neuronal morphology (Santosh et al., 2008). We have also demonstrated feasibility of the technique in clinical use in acute stroke (Dani et al, 2010). The current study provides evidence of ongoing metabolism in T_2*OC -defined penumbra and provides more direct validation for the technique. In addition, co-registration of [^{14}C]2-deoxyglucose autoradiography with MRI has provided detailed information on the adjacent tissue compartments within the ischaemic hemisphere which demonstrate markedly different levels of glucose metabolism. We propose that the T_2*OC technique indirectly identifies penumbra from its higher oxygen extraction fraction and how this influences deoxy/oxyhaemoglobin ratios and T_2* signal. However, we acknowledge that other factors may give rise to an increase in T_2* signal in the penumbra. For example, an increased cerebral blood volume in penumbral tissue may increase deoxyhaemoglobin in this region, thus magnifying the T_2* response.

MRI-defined DWI-PWI mismatch identifies penumbra in a similar, but not exact neuroanatomical location (Figure 2). However, the underlying assumptions are

undermined by reversibility of the DWI lesion with reperfusion, uncertainty over relevant perfusion thresholds, and the variable metabolic state of DWI lesion voxels. Information on metabolic state should improve penumbral definition. BOLD MRI offers information on oxygen consumption and delivery (Baird & Warach, 1999; Kavec et al, 2001), but static T_2*-weighted MRI under normoxic conditions has not adequately delineated penumbra in ischaemic stroke patients (Tamura et al, 2002; Grohn & Kauppinen, 2001), possibly because deoxyhaemoglobin is not rapidly cleared in ischaemic conditions (Giesler, et al, 2006). We hypothesised that mapping dynamic changes in T_2* in response to oxygen challenge should improve discrimination between penumbra and surrounding tissue compartments (Santosh et al, 2008).

Our results demonstrate a region of significant T_2* increase during OC localised to a cortical boundary zone between the MCA and anterior cerebral artery territories which overlapped the DWI/PWI mismatch area. This region displayed metabolism not significantly different from contralateral normal tissue, and significantly greater than infarct core which is consistent with previous [^{14}C]2-deoxyglucose studies in acute focal ischaemia (Belayev et al, 1997; Tohyama et al, 1998).

A smaller T_2* signal increase to OC, compared to penumbral tissue, would also be expected in normal metabolising tissue (as shown in Santosh et al., 2008) and in all six animals, the hemisphere contralateral to MCAO showed measureable increases in T_2^* signal intensity during OC (Figure 3&4). By co-registering thresholded T_2* maps with glucose use autoradiograms, a metabolic signature of normal tissue was generated to allow comparison with the ischaemic hemisphere. Our MR findings were

consistent with the previous study (Santosh et al, 2008), in which the ischaemic core, defined by DWI, was notable as a region with negligible T_2* signal change. It registered undetectable glucose use values, thus confirming metabolic inactivity in this tissue compartment where $CMRO_2$ and OEF would be expected to be markedly reduced or absent.

An intermediate tissue compartment, identified as hyperglycolytic on autoradiograms, displayed a small (sub-threshold) T_2* signal change and lay inside the boundary of the ADC abnormality (Figures 2, 3 & 5). This may point to residual oxygen utilisation on the border of the ADC abnormality (Figure 4), in tissue with ADC and CBF values intermediate between the ischaemic core and T_2*-defined penumbra (6A & B). The presence of hyperglycolysis within the ADC lesion suggests the tissue is still metabolically active, and may not be irreversibly injured at this time point. This region may therefore be critically injured and employing anaerobic glycolysis (Nedergaard and Astrup, 1986) which cannot sustain viability. Since some oxygen is being extracted from the blood, this suggests some oxidative metabolism persists but may indicate tissue destined for rapid, irreversible damage. This supports recent BOLD MR studies that propose the ADC lesion may not only incorporate tissue that is destined to die, but also severely injured, still viable tissue (Kidwell et al, 2002). A number of studies have reported reversibility of the ADC lesion following reperfusion (Mintorovitch et al, 1991; Minematsu et al, 1992: Kidwell et al, 2000). Hypermetabolic tissue displaying a small T_2* % signal change may therefore indicate an intermediate compartment of the ischaemic penumbra situated within both the ADC abnormality and the perfusion deficit, whose fate may be being determined by

secondary insults post-stroke, such as pre-terminal spreading depolarisations which may exhaust ATP levels in vulnerable cells.

Limitations of the DWI/PWI mismatch technique

Despite its limitations, the DWI/PWI mismatch model provides information on location and severity of ischaemia (derived from perfusion-weighted imaging, PWI), tissue viability status (apparent diffusion coefficient, ADC values derived from diffusion-weighted imaging, DWI), and therefore approximate location and size of penumbra. PWI and DWI scans were therefore included in the scanning routine for comparison with T_2*OC identification of penumbra. A number of studies have shown that differentiation between viable and non-viable tissue is difficult, using the diffusion abnormality (Kidwell et al, 2000; Fiehler et al, 2002) which correlates poorly with final infarct (Li et al, 1999). As mentioned above, DWI lesions may be recoverable following prompt reperfusion in animal models and man, and may not be destined for infarction (Schlaug et al, 1997; Mintorovitch et al, 1991; Kidwell et al, 2000). Additionally, the perfusion deficit may incorporate tissue with benign oligaemia (destined to survive) even when optimal MRI thresholds (themselves as yet incompletely defined) are applied (Butcher et al, 2005). As such, the inner and outer margins of the penumbra may not be adequately delineated using the mismatch technique which has been shown to frequently overestimate the final lesion size (Kucinski et al, 2005)

Limitations

The [^{14}C] 2-DG method may not accurately define LCMRglu because the increase in radioactivity registered may either be a result of real glucose consumption or a

methodological artifact from an increase in the lumped constant. In normal conditions, the lumped constant in the 2DG technique is stable over a large spectrum of plasma glucose concentrations (Sokoloff et al, 1977). With reduced glucose supply – such as during ischaemia - the lumped constant may increase considerably (Suda et al, 1990; Vannucci et al, 1989; Gilland and Hagberg, 1996). As there is reduced glucose availability, the glucose distribution volume decreases and the lumped constant increases because 2-DG is the preferred sugar for phosphorylation by hexokinase. As such, local values of the lumped constant should be estimated, which may be derived by measuring the brain uptake of methlyglucose (Dienel et al, 1991, Gjedde et al, 1985). Another limitation is the lack of a gold standard or independent identification method for penumbra to compare T_2*OC with.

Summary

The T_2*OC technique represents a clinically-translatable method of yielding metabolic information from the ischaemic brain that may improve delineation of penumbral tissue after acute ischaemic stroke. By exploiting the different magnetic properties of oxy- and deoxyhaemoglobin, the dynamic response to oxygen administration may detect tissue capacity for oxygen utilisation and define the ischaemic penumbra on the basis of oxygen extraction and metabolism. This study therefore supports the utility of the oxygen challenge technique to provide information on oxygen metabolism and tissue viability following stroke.

Supplementary information is available at the *Journal of Cerebral Blood Flow & Metabolism* website - www.nature.com/jcbfm

Conflict of interest

The authors declare no conflict of interest.

References

Albers GW, Thijs VN, Wechsler L, Kemp S, Schlaug G, Skalabrin E, Bammer R, Kakuda W, Lansberg MG, Shuaib A, Coplin W, Hamilton S, Moseley M, Marks MP (2006) Diffusion and perfusion imaging evaluation for understanding stroke evolution: The DEFUSE study, Ann Neurol 60:508–517

Baird AE, Warach S (1999) Imaging developing brain infarction. Curr Opin Neurol 12:65–71

Barber PA, Zhang J, Demchuk M, Hill MD, Buchan AM (2001) Why are stroke patients excluded from TPA therapy?: An analysis of patient ineligibility. Neurology 56:1015-1020

Baron JC (1999) Mapping the ischaemic penumbra with PET: implications for acute stroke treatment. Cerebrovasc Dis 9:193–201

Belayev L, Zhao W, Busto R, Ginsberg MD (1997) Transient middle cerebral artery occlusion by intraluminal suture: I. Three-dimensional autoradiographic image-

analysis of local cerebral glucose metabolism-blood flow interrelationships during ischemia and early recirculation. J Cereb Blood Flow Metab 17:1266−1280

Butcher KS, Parsons M, MacGregor L, Barber PA, Chalk J, Bladin C, Levi C, Kimber T, Schultz D, Fink J, Tress B, Donnan G, Davis S (2005) Refining the perfusion–diffusion mismatch hypothesis. Stroke 36:1153–1159

Dani, KA, Santosh C, Brennan D, McCabe C, Holmes WM, Condon B, Hadley DM, Macrae IM, Shaw M, Muir KW (2010) T2* Weighted Magnetic Resonance Imaging with Hyperoxia in Acute Ischemic Stroke. Annals Neurol 68(1):37-47

Dienel GA, Cruz NF, Mori K, Holden JE, Sokoloff L (1991) Direct measurement of the lambda of the lumped constant of the deoxyglucose method in rat brain: determination of lambda and lumped constant from tissue glucose concentration or equilibrium brain/plasma distribution ratio for methylglucose. J Cereb Blood Flow Metab 11(1):25-34

Donnan GA, Fisher M, Macleod M, Davis SM (2008) Stroke. Lancet 371 (9624): 1612–23

Fiehler J, Foth M, Kucinski T, Knab R, von Bezold M, Weiller C, Zeumer H, Röther J (2002) Severe ADC decreases do not predict irreversible tissue damage in humans. Stroke 33:79–86

Gilland E, and Hagberg, H (1996) NMDA Receptor-Dependent Increase of Cerebral Glucose Utilization After Hypoxia-Ischemia in the Immature Rat. J Cereb Blood Flow Metab 16: 1005-1013

Ginsberg MD (1990) Local metabolic responses to cerebral ischemia Cerebrovasc Brain Metab Rev 2(1):58-93.

Gjedde A, Wienhard K, Heiss WD, Kloster G, Diemer NH, Herholz K, Pawlik GJ (1985) Comparative regional analysis of 2-fluorodeoxyglucose and methylglucose uptake in brain of four stroke patients. With special reference to the regional estimation of the lumped constant Cereb Blood Flow Metab. 5(2):163-78

Grohn OH, Kauppinen RA (2001) Assessment of brain tissue viability in acute ischemic stroke by BOLD MRI. NMR Biomed14:432-440

Guadagno JV, Warburton EA, Aigbirhio FI, Smielewski P, Fryer TD, Harding S, Price CJ, Gillard JH, Carpenter TA, Baron JC (2004) Does the acute diffusion-weighted imaging represent penumbra as well as core? A combined quantitative PET/MRI voxel-based study. J Cereb Blood Flow Metab 24:1249–1254

Hacke W, Kaste M, Fieschi C, von Kummer R, Davalos A, Meier D, Larrue V, Bluhmki E, Davis S, Donnan G, Schneider D, Diez-Tejedor E, Trouillas P (1998) Randomised double-blind placebocontrolled trial of thrombolytic therapy with intravenous alteplase in acute ischaemic stroke (ECASS II). Second European-Australasian Acute Stroke Study Investigators. Lancet 352:1245–1251

Kahn JH, Viereck J, Kase C, Jeerakathil T, Romero R, Mehta SD, Kociol R, Babikian V (2005) The use of intravenous recombinant tissue plasminogen activator in acute ischemic stroke. J Emerg Med 29(3):273-277

Katzan IL, Hammer MD, Hixson ED, Furlan AJ, Abou-Chebl A, Nadzam DM (2004) Utilization of intravenous tissue plasminogen activator for acute ischemic stroke. Cleveland Clinic Health System Stroke Quality Improvement Team. Arch Neurol 61(3):346-50

Kavec M, Gröhn OHJ, Kettunen MI, Silvennoinen MJ, Penttonen M, Kauppinen RA (2001) Use of spin echo T2 BOLD in assessment of misery perfusion at 1.5 T. MAGMA 12: 32–39.

Kidwell, CS, Liebeskind, DS, Starkman, S, and Saver, JL (2001) Trends in acute ischemic stroke trials through the 20th century. Stroke 32: 1349–1359.

Kidwell CS, Saver JL, Mattiello J, Starkman S, Vinuela F, Duckwiler D, Gobin YP, Jahan R, Vespa P, Kalafut M, Alger JR (2000) Thrombolytic reversal of acute human cerebral ischemic injury shown by diffusion/perfusion magnetic resonance imaging. Ann Neurol 47:462–469

Kidwell CS, Saver JL, Starkman S, Duckwiler G, Jahan R, Vespa P, Villablanca JP, Liebeskind DS, Gobin YP, Vinuela F, Alger JR (2002) Late secondary ischemic injury in patients receiving intraarterial thrombolysis. Ann Neurol 52:698 –703

Kucinski T, Naumann D, Knab R, Schoder V, Wegener S, Fiehler J, Majumder A, Röther J, Zeumer H (2005) Tissue at risk is overestimated in perfusion-weighted imaging: MR imaging in acute stroke patients without vessel recanalization. Am J Neuroradiol 26:815– 819

Li F, Han S, Tatlisumak T, Liu KF, Garcia JH, Sotak CH, Fisher M (1999) Reversal of acute apparent diffusion coefficient abnormalities and delayed neuronal death following transient focal cerebral ischemia in rats. Ann Neurol 46:333–342

Lo EH, Pierce AR, Mandeville JB, Rosen BR (1997) Neuroprotection with NBQX in rat focal cerebral ischemia. Effects on ADC probability distribution functions and diffusion–perfusion relationships. Stroke 28:439–447

Longa EZ, Weinstein PR, Carlson S, Cummins R (1989) Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 20:84-91

Meng X, Fisher M, Shen Q, Sotak CH, Duong TQ (2004) Characterizing the diffusion/perfusion mismatch in experimental focal cerebral ischemia. Ann Neurol 55:207–212

Minematsu K, Li L, Sotak CH, Davis MA, Fisher M (1992) Reversible focal ischemic injury demonstrated by diffusion-weighted magnetic resonance imaging in rats. Stroke 23:1304–1310

Mintorovitch J, Moseley ME, Chileuitt L, Shimizu H, Cohen Y, Weinstein PR (1991) Comparison of diffusion- and T_2-weighted MRI for the early detection of cerebral ischemia and reperfusion in rats. Magn Reson Med18:39–50

Moffat BA, Chenevert TL, Hall DE, Rehemtulla A, Ross BD (2005) Continuous arterial spin labeling using a train of adiabatic inversion pulses. J Magn Reson Imaging 21:290-6

Muir KW (2002) Heterogeneity of stroke pathophysiology and neuroprotective clinical trial design. Stroke 33(6):1545-50

Muir KW, Santosh C (2005) Imaging of acute stroke and transient ischaemic attack. J Neurol Neurosurg Psychiatry 76:19-28

Nedergaard M, Astrup J (1986) Infarct rim: effect of hyperglycemia on direct current potential and [14C] 2-deoxyglucose phosphorylation. J Cereb Blood Flow and Metab 6:607-615

Powers WJ (1991) Cerebral hemodynamics in ischemic cerebrovascular disease. Ann Neurol. 29(3):231-40

Santosh C, Brennan D, McCabe C, Macrae IM, Holmes WM, Graham DI, Gallagher L, Condon B, Hadley DM, Muir KW, Gsell W (2008) Potential use of oxygen as a metabolic biosensor in combination with T2(*)-weighted MRI to define the ischemic penumbra. J Cereb Blood Flow Metab 28:1742–1753

Schellinger PD, Fiebach JB, Hacke W (2003) Imaging-Based Decision Making in Thrombolytic Therapy for Ischemic Stroke. Stroke 34:575 -583

Schlaug G, Siewert B, Benfield A, Edelman RR, Warach S (1997) Time course of the apparent diffusion coefficient (ADC) abnormality in human stroke. Neurology 49:113–119

Sokoloff L, Reivich M, Kennedy C, Des Rosiers M.H, Patlak CS, Pettigrew KD, Sakurada O, Shinohara M (1977) The [^{14}C]deoxyglucose method for the measurement of local cerebral glucose utilisation: theory, procedure and normal values in the conscious and anaesthetised albino rat. Journal of Neurochemistry 28:897-916

Stejskal EO, Tanner JE (1965) Spin diffusion measurements: spin echoes in the presence of a time-dependent field gradient. J Chem Phys 42:288–292.

Tamura H, Hatazawa J, Toyoshima H, Shimosegawa E, Okudera T (2002) Detection of deoxygenation-related signal change in acute ischemic stroke patients by T2*-weighted magnetic resonance imaging. Stroke;33:967

Tanaka K, Greenberg JH, Gonatas NK, Reivich M (1985) Regional flow-metabolism couple following middle cerebral artery occlusion in cats. J Cereb Blood Flow Metab 5(2):241-52.

Tohyama Y, Sako K, Yonemasu Y (1998) Hypothermia attenuates hyperglycolysis in the periphery of ischemic core in rat brain. Exp Brain Res 122:333–338

Uematsu H, Takahashi M, Hatabu H, Chin C, Werli S, Werli F and Asakura T (2007) Changes in T1 and T2 observed in brain magnetic resonance imaging with delivery of high concentrations of oxygen. J.Comput.Assist.Tomogr 31:662-665

Vannucci RC, Christensen MA, Stein DT. Regional cerebral glucose utilization in the immature rat: effect of hypoxia-ischemia. *Pediatr Res.* 1989;26:208–214

Wardlaw JM, Sandercock PAG, Murray M (2009) Should more patients with acute ischaemic stroke receive thrombolytic treatment? BMJ 339:b4584

Table 1

PHYSIOLOGICAL DATA (n = 6)		
	BASELINE	**DURING OC**
MABP	82.4 ± 7	91.4 ± 6.7*
$PaCO_2$ (mmHg)	34.8 ± 7	36.3 ± 8.5
PaO_2 (mmHg)	85.8 ± 7.4	310.6 ± 84.1**
BLOOD pH	7.32 ± 0.04	7.32 ± 0.04

Titles and Legends to Figures

Table 1: Physiological variables at baseline and during oxygen challenge. Data expressed as mean±SD, * $p<0.05$ and ** $p<0.0001$, Student's paired t-test.

Figure 1: RARE T_2 neuroanatomical templates for caudal (A) and rostral (B) middle cerebral artery territory slices of interest. Corresponding coregistered PWI and DWI threshold images displaying DWI lesion (white) superimposed on Perfusion deficit (red) revealing DWI/PWI mismatch areas for caudal (C) and rostral (D) slices.

Figure 2: Representative MRI maps and 2-DG autoradiograms from the rostral slice in a stroke rat. A) T_2* OC % signal change map, B) Thresholded T_2* OC map, C) [^{14}C] 2-deoxyglucose (2-DG) autoradiogram, D) CBF map (mL/100g/min), E) ADC map (x10^{-3} mm^2/sec), and F) DWI/PWI overlay (mismatch tissue shown in red). ROIs were defined as follows

Green ROI: Penumbra – the thresholded region displaying the greatest T_2* percentage signal change excluding veins and ventricles (B).

Yellow ROI: Hyperglycolytic region – identified by the dark band within the ipsilateral cortex on the [^{14}C]2-deoxyglucose autoradiogram (C).

Red ROI: Ischaemic core – within the ADC-derived lesion in the caudate nucleus

White ROI: Contralateral cortex – homotopic to the penumbra ROI

Sky Blue ROI: Contralateral caudate nucleus – homotopic to the ischaemic core ROI

Figure 3: EPI T_2* OC signal time course: Time course T_2* signal change during OC in: ADC-derived ischaemic core (Red); Cortical hyperglycolytic region from 2-deoxyglucose autoradiogram (Yellow); a region corresponding to the greatest T_2*% signal change increase adjacent to hyperglycolytic band (Green); corresponding regions on: the contralateral cortex (Black) and caudate nucleus (Sky Blue). Each line represents data from a single animal. Within each animal, all data were normalized to the average signal over the 5 mins prior to OC. Blue box represents period of 100% oxygen inhalation.

Figure 4: T_2* percentage signal change from baseline in ROIs. Individual animal data with horizontal lines representing means. ***, $p<0.001$ and *, $p<0.05$ relative to contralateral cortex ROI. ##, $p<0.01$ relative to contralateral caudate nucleus

##, p<0.01 compared to contralateral caudate nucleus

Figure 5: LCMRglu in ROIs. Individual animal data with horizontal lines representing means. ***, p<0.001 relative to contralateral cortex. ###, p<0.001 relative to contralateral caudate nucleus.

Figure 6: CBF (A) and ADC (B) in ROIs. Individual animal data with horizontal lines representing means. ***, ### (p<0.001) relative to contralateral cortex and contralateral caudate nucleus, respectively.