Stroke Penumbra Defined by an MRI-based Oxygen Challenge Technique: 2. Validation Based on the Consequences of Reperfusion

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Abstract (word count 200)

MRI with oxygen challenge (T_2*OC) uses oxygen as a metabolic biotracer to define penumbral tissue based on $CMRO_2$ and oxygen-extraction fraction. Penumbra displays a greater T_2* signal change during OC than surrounding tissue. Since timely restoration of CBF should salvage penumbra, T_2*OC was tested by examining the consequences of reperfusion on T_2*OC-defined penumbra.

Ischaemia was induced by intraluminal filament in male Sprague-Dawley rats (n=8) for 109±20min. Penumbra was identified on T_2*-weighted MRI during OC. Ischaemia and ischaemic injury were identified on CBF and apparent diffusion coefficient maps, respectively. Reperfusion was induced by filament withdrawal, and scans repeated. T_2 for final infarct and T_2*OC scans were run on day 7.

T_2* signal increase to OC was 3.4% in contralateral cortex and caudate nucleus and was unaffected by reperfusion. In OC-defined penumbra, T_2* signal increased by 8.4±4.1% during ischaemia and returned to 3.25±0.8% following reperfusion. Ischaemic core T_2* signal increase was 0.39±0.47% during ischaemia and 0.84±1.8%

on reperfusion. Penumbral CBF increased from 41.94±13 to 116.5±25mL/100g/min on reperfusion. On day 7, OC-defined penumbra gave a normal OC response and was located outside the infarct. T_2*OC-defined penumbra recovered when CBF was restored, providing further validation of the utility of T_2*OC for acute stroke management.

Headline: Detecting penumbra using T_2* MRI and O_2 challenge

Key words: ADC, CBF, imaging, MCAO, T_2*

Abbreviations: ADC_{av}= Apparent diffusion coefficient; 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

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Introduction

The most effective intervention for acute ischaemic stroke is reperfusion (Molina and Saver, 2005). As ischaemic stroke refers to the sudden loss of blood flow in a cerebral artery due to a blockage, the mechanical or drug-induced restoration of blood flow with its accompanying nutritive delivery by reperfusion enables salvage of previously injured (penumbral) tissue. In 1996, thrombolysis with recombinant tissue plasminogen activator (rt-PA) was approved by the US Food and Drug Administration for acute ischaemic stroke of less than 3 hours duration. However,

patient ineligibility means that fewer than 10% of all stroke patients can be thrombolysed (Cocho et al, 2005; Molina and Saver, 2005).

In May 2009, the American Heart Association (AHA)/American Stroke Association (ASA) recommended an extension of the window for acute ischaemic stroke, approving rt-PA as a treatment up to 4.5 hours post-symptom onset. The decision was primarily based on findings from the third European Cooperative Acute Stroke Study (ECASS III) trial, which confirmed a significant reduction in disability at the 90-day time period after rt-PA treatment between 3-4.5 hours (Hacke et al, 2008). More recently, a time profile of benefit and harm for alteplase in a pooled analysis of eight randomised trials concluded that one in three patients had improved outcomes when treated between 1 and 3 hours from symptom onset, whilst one in six benefitted in the 3-4.5 hour time window. Significantly, risk may outweigh benefit beyond the 4.5h time point (Lees et al, 2010), and this was supported by a previous Cochrane meta-analysis (Wardlaw et al, 2009).

In MRI thrombolysis studies, patients with penumbral tissue identified by DWI-PWI mismatch experienced improved clinical outcomes up to 6 hours after symptom onset, compared to the standard non-contrast computed tomography (CT)–guided therapy (Köhrmann et al, 2006; Schellinger et al, 2007). This MRI technique, however, has not been validated and is imprecise: DWI lesions have shown to disappear spontaneously or following thrombolysis in both animals and man (Kidwell et al, 2000). As yet, ADC values have failed to distinguish between tissue destined to die and the potentially recoverable penumbra (Guadagno et al, 2004). Similarly, the extent of perfusion deficit is dependent upon the methods and thresholds used to

define it, and it may also include benign oligaemic tissue that is fated to survive (Butcher et al, 2005).

There is no current MRI technique that accurately detects tissue viability and which could be used in routine clinical practice to identify patients likely to benefit from therapy such as thrombolysis, flow enhancement or neuroprotection. We hypothesised that alternative techniques that determine tissue metabolic status would represent an advance on current clinical imaging of the penumbra, establishing patient selection criteria for therapeutic strategies outwith current rigid time windows. Oxygen challenge (OC) MRI technique uses a transient hyperoxic challenge to identify changes in deoxyhaemoglobin: oxyhaemoglobin ratios, detected by T_2*-weighted MRI (Santosh et al, 2008). Paramagnetic deoxyhaemoglobin and free oxygen in the plasma reduce T_2* signal, whilst diamagnetic oxyhaemoglobin has a minimal influence on T_2*. 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 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 (Santosh et al, 2008; Robertson, 2011). This technique may therefore yield information on oxygen metabolism that more

closely correlates with positron emission tomography (PET) definitions of the penumbra.

The aim of the current study was to validate the T_2^*OC MRI technique based on the consequences of reperfusion. Our stated hypotheses were that firstly T_2^*OC –defined penumbra should show signs of recovery following early restoration of flow and that its T_2^* response to OC following reperfusion should resemble the signal change in normal, non-ischaemic tissue. This was tested acutely after reperfusion and again at day 7. Evidence of tissue recovery in T_2*OC-defined penumbra was determined from changes in cerebral blood flow (CBF) and apparent diffusion coefficient (ADC) acutely following reperfusion. T_2^*OC maps during ischaemia were also co-registered with T_2 –defined final infarct to confirm that tissue identified as penumbra had not become incorporated into final infarct.

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 (306±12g, n=8, Harlan, Bicester, UK) were initially anaesthetised with 5% inhaled isofluorane in an induction chamber at room temperature. Following intubation, 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 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 mmHg 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 a femoral artery, to continuously monitor blood pressure and conduct blood gas analysis. Middle cerebral artery occlusion (MCAO) was achieved by the intraluminal filament technique, using a modified version of Longa and colleagues' (1989) technique, where a 5-0 silicon rubber-coated monofilament (diameter 0.12mm, length 30 mm; diameter with coating 0.31-0.35mm, and coating length >= 5 mm; www.doccol.com) was introduced through the internal carotid artery to the origin of the middle cerebral artery to preclude flow and induce stroke. The common carotid artery was ligated and the occipital artery branches of the external carotid artery (ECA) were isolated, ligated and dissected. The pterygopalatine branch from the internal carotid artery was also ligated. To ensure complete reperfusion, the ties around the common carotid and pterygopalatine branch were loosened to restore blood flow to MCA territory. Ischaemia was induced for 109±20 minutes, which reflected the time taken to transfer the animal to the scanner and run the ischaemia scan series.

Reperfusion is associated with considerable brain swelling at the 24-48 hr time point. For this reason, 4 animals survived for 7 days. This is a drawback of the intraluminal filament, where the intact skull means there is no control of intracranial pressure.

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.

Scanning protocol

At approximately 1 hour post-stroke, animals underwent MRI scanning which comprised diffusion-weighted imaging (DWI) to detect ischaemic injury, T_2*OC to detect penumbra and arterial spin labelling (ASL) to provide CBF maps of ischaemia. Animals were removed from the magnet, and reperfusion was induced by withdrawal of the intraluminal filament. The scanning sequence was then repeated to confirm reperfusion, and to study the consequences of reperfusion on the tissue defined as penumbra from the earlier DWI, T_2*OC and ASL scans.

Ischaemia-induced damage will continue to evolve following reperfusion in this model and consequently histology or MRI at 24 hrs will not predict final infarct size. Therefore, rats were recovered and rescanned at day 7 to define the final infarct. Choosing this late time point also avoids the confounding effects of brain swelling which are present during the first days after stroke and improves co-registration of final T_2 scans with acute scans. The animals also underwent T_2*OC at this time point.

DWI and PWI scanning

DWI was performed during ischaemia and immediately following reperfusion to assess ischaemically injured tissue (Spin Echo planar (EPI) TE: 43 ms, TR: 4000.3

ms, in plane resolution of 260 µm, 3 directions: x, y, z, B values: 0, 1000 s/mm^2, 8 slices of 1.5mm thickness).

Non-invasive quantitative CBF was carried out on 2 coronal slices within the MCA territory during ischaemia and immediately following reperfusion 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, slice thickness 1.5mm, 16 averages, 4 shots) preceded by 50 hyperbolic secant inversion pulses in a 3s train.

DWI and PWI were also used to define penumbra from DWI/PWI mismatch and data analysed on two selected coronal slices within the MCA territory (Figure 1 A (vi)). For analysis, the data for the rostral and caudal slices were combined. ASL scans were generated 44±11 min and 59±15 min post-stroke for caudal and rostral slices, respectively. The scanning time for a single ASL slice was approx 6 min and 2 slices were scanned throughout the MCA territory. In addition to this a T_1- weighted image (scan time 10min) was carried out in order to quantify CBF in mL/100g/min.

T_2*- weighted imaging

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, 8 contiguous slices of 1.5mm thickness, 2 averages, temporal resolution 20s, 30 repetitions). Two coronal MRI slices (identified as rostral and caudal 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 4

minutes breathing air, followed by 6 minutes breathing 100% oxygen. This sequence was repeated at day 7 post-stroke.

T_2- weighted imaging

During acute scanning and at 7 days following reperfusion, a sagittal RARE T_2 scan (effective TE: 46.8 ms, TR: 5000s; in plane resolution of 97µm; 18 slices of 0.5mm thickness) was performed, in which the rhinal fissure was used as a neuroanatomical landmark in order to match the geometry as closely as possible with the acute scans. A coronal RARE T_2 sequence (effective TE: 46.8 ms, TR: 5000s; in plane resolution of 97um; 30 slices of 0.5mm thickness) enabled T_2-derived final infarct measurements.

MRI Data Analysis

Defining the ischaemic penumbra with DWI/PWI mismatch

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 caudal and rostral 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**.** Diffusion-perfusion mismatch was calculated as the difference between the perfusion deficit and the ADC lesion area on the corresponding slice. Volumes of DWI/PWI mismatch and thresholded

T_2^*OC -defined penumbra were generated from the data from two coronal slices and the neuroanatomical location compared between the two techniques (Figure 5B).

T_2*OC time course data and defining the ischaemic penumbra

The time course of the T_2* signal change was analysed from ROIs (Figure 1 A(iv)). T_2* percentage signal change was calculated from time course graphs (Figure 2A&B), 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* percentage signal change maps were generated using Image J software (http://rsb.info.nih.gov/ij/). The boundaries of penumbral tissue were 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 1 A(iii)).

Volumetric Analysis of Penumbra

Volumetric analysis of DWI/PWI mismatch and T_2*OC-defined penumbra was carried out over the rostro-caudal extent of the ASL scans.

Regions of Interest (ROIs)

The researcher responsible for ROI placement in the ischaemia scans was blinded to the reperfusion data. ROIs were selected according to specific features on the images (Figure 1): 1. Ischaemic core in caudate nucleus from the thresholded ADC lesion (Figure 1 A (iv), red) and, 2. The contralateral caudate (excluding veins and ventricles) (cerise), manually designated by researcher; 3. Penumbra as defined by

thresholded T_2* percentage signal change (green); 4. Equivalent contralateral cortex (sky blue) and, 5. DWI/PWI mismatch (dark blue).

For the 7 day data, MRI-defined regions of interest were defined and placed within; the final infarct according to the RARE T_2 scan; the thresholded T_2*OC-defined penumbra identified from the acute ischaemic scan series; and two equivalent contralateral ROIs.

Co-registration

To co-register the acute and 7 day scans, linear co-registration was carried out using Analyze. To align the data, ischaemia ASL and T_2^* images were warped to their corresponding DWI slices, and the reperfusion scan series and 7 day scans were warped to the same DWI slice as the ischaemic scan series. The processed data from the T_2*OC and thresholded ADC and CBF maps were co-registered to: a) define ROIs, and b) identify the T_2*OC-defined penumbral tissue. To correlate the T_2-defined infarct with the acute data, the data set at day 7 was also co-registered to the DWI scans generated from the ischaemic scan series.

Statistical analysis

All data are presented as mean ± SD. Data were normally distributed, and as such, mean arterial blood pressure before and during OC was analysed by Student's paired t-test. T_2* signal, ADC and CBF 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 performed to compare changes in T_2* signal, ADC and CBF at the ischaemia and post-reperfusion time points. All data

were tested to confirm normal distribution using the D'Agostino and Pearson normality test.

Results

Acute Data and physiological variables

Oxygen challenge was performed twice, and the mean time to commence OC was 78±15 minutes after MCAO for the scans performed during ischaemia and 180±31 minutes for the set of scans performed following reperfusion. Physiological variables were monitored throughout the experiment. Blood pressure and blood gases recorded immediately before OC for both the ischaemia and reperfusion scan series were within normal physiological levels (Table 1).

Acute T_2 percentage signal change to OC*

During MCAO (ischaemia scans) the T_2* signal change during OC varied in magnitude across the hemisphere ipsilateral to the stroke (Figure 2A & C) with the smallest change in ischaemic core and the largest in the dorsolateral cortex (OC-defined penumbra). T_2* signal increase in the penumbra ROI was significantly greater than in the contralateral cortical ROI ($p<0.01$, Figure 2C). In ischaemic core caudate nucleus, mean T_2* signal during OC was significantly reduced compared to the contralateral caudate nucleus ROI ($p<0.05$).

Following reperfusion (mean OC scan time = 180±31 min following stroke onset and 71 min from initiation of reperfusion), T_2* signal increase in the penumbral ROI reduced significantly from 8.4±4.1% to 3.25±0.81% ($p<0.001$). In DWI/PWI mismatch, T_2* % signal changed from 5.48±2.8% during ischaemia to 2.49±1.04%

following reperfusion. There were no significant T_2* percentage signal changes from ischaemia to reperfusion in any of the other ROIs; T_2* % signal change in ischaemic core caudate nucleus changed from 0.39±0.47% during ischaemia to 0.83±1.7% following reperfusion, contralateral cortex changed from 2.97±1.8% to 2.61±1.1%, and contralateral caudate nucleus changed from 3.35±2% to 2.593±1.3%.

Severity of ischaemia and tissue viability

During ischaemia, blood flow in the OC-defined penumbra, DWI/PWI mismatch and ischaemic core caudate nucleus were significantly reduced compared to the equivalent contralateral ROIs (Figure 3A, $p<0.001$). CBF values in the OC-defined penumbra were significantly higher than the DWI/PWI mismatch defined penumbra ($p<0.05$). On reperfusion, mean CBF in ischaemic core caudate nucleus increased from 4.3±5.3 to 31±74 mL/100g/min, but remained significantly reduced compared to the equivalent contralateral ROI (Figure 3B, $p<0.001$). Mean CBF in the OC-defined penumbra increased significantly from 41.94±13 to 116.5±25 mL/100g/min ($p<0.001$, Figure 3B). Mean CBF in DWI/PWI mismatch also increased significantly from 16.8±14 to 104.4±50 mL/100g/min ($p<0.001$). Blood flow in contralateral caudate nucleus and cortex did not change significantly following reperfusion; 143±20 to 121±14 and 161±24 to 127±36 mL/100g/min, respectively.

During ischaemia, ADC values (mean scan time=72±8 min post-stroke) in the OC-defined penumbra, ischaemic core caudate nucleus and DWI/PWI mismatch were significantly reduced compared to the equivalent contralateral ROIs (Figure 1 A (i) & 3C, $p<0.001$, $p<0.001$ and $p<0.01$, respectively) and remained significantly reduced in the DWI/PWI mismatch and ischaemic core caudate nucleus compared to equivalent

contralateral ROIs following reperfusion (mean scan time=172±35 min post-stroke, $p<0.05$ and $p<0.001$, respectively), whilst the OC-defined penumbra was no longer significantly reduced. Mean ADC values were not significantly different between ischaemia and reperfusion scans in any of the six ROIs, Figure 3C & D)

Day 7 T_2-defined infarct volume and OC T_2* % signal change

Final infarct volume was 107±69 mm^3. In the surviving animals, infarcts were located subcortically with minimal cortical damage whilst the surviving animals tended to have smaller infarcts than the animals that died prematurely. Both ipsilateral and contralateral hemispheric volumes were calculated to show that there was no residual oedema at day 7 (622±27 mm^3 and 624±30 mm^3 for the ipsilateral and contralateral hemispheric volumes, respectively).

T_2* % signal change on day 7 in the OC-defined penumbra ROI (1.69±0.6%) was not significantly different from data for the equivalent contralateral cortex (1.72±0.6%, Figure 4B). The T_2* signal change in the T_2-defined final infarct ROI was significantly reduced – displaying a negative signal (-0.88±0.6%) compared to the equivalent contralateral caudate nucleus (1.72±0.6%) ($p<0.01$, Figure 4B).

Evidence of penumbral salvage

Co-registration of the T_2^* OC defined penumbra ROI (derived from the T_2^* percentage map during ischaemia) onto the RARE T_2 scan at day 7 (Figure 5), revealed that this region was not incorporated into the final infarct in any animal, providing evidence that penumbra had been salvaged by reperfusion.

Volumetric Analysis of Perfusion Deficit, ADC lesion and Penumbra

The volume of perfusion deficit, determined at 52.8±14.2 min post-stroke, was 110±3 mm^3 and the ADC lesion volume determined at 72±8 min post-stroke was 91.6±33 mm^3 generating a DWI/PWI mismatch volume of 20.2±15 mm^3. The thresholded T_2*OC-defined penumbral volume, determined at 78±15 min was 17.3±9 mm^3. The mismatch- and T_2*OC-defined penumbral volumes were approximately 22% and 19% of the volume of the ADC-defined ischaemic core, respectively. Although the volume of penumbral tissue was similar for the two methods, there were spatial differences with regards to the physical location (Figure 5(iv)).

The perfusion deficit volume in the 4 animals that survived to day 7, was 110.3±6 mm^3 determined at 53.5±12 min post-stroke, and the ADC lesion volume was 88.6±21 mm^3 determined at 68.3±8.4 min post-stroke. DWI/PWI mismatch volume was 25.7±14 mm^3 and the thresholded T_2*OC-defined penumbra, determined at 82.5±15 min was 18.9±8 mm^3. The mismatch- and T_2*OC-defined penumbral volumes were approximately 29% and 22% of the volume of the ADC-defined ischaemic core, respectively. The T_2-defined final infarct volume was 71.7±22 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). Evidence for ongoing metabolism in the OC T_2*-defined penumbra was provided by co-

registration of [^{14}C] 2-deoxyglucose autoradiography with MRI, displaying detailed information on the adjacent tissue compartments within the ischaemic hemisphere which demonstrate markedly different levels of glucose metabolism (Robertson et al, 2011). Depending on the duration and severity of the ischaemic insult, at least some of the tissue regarded as penumbra may recover if blood supply is promptly restored. If blood supply is restored and the tissue recovers, it should no longer demonstrate an increased OEF and its response to OC should be similar to non-ischaemic tissue. We tested the validity of OC T_2* by timely restoration of CBF and final infarct measurement to determine tissue salvage.

The aim of this study was to validate the OC T_2* MRI technique by identifying penumbral tissue during ischaemia and comparing the T_2* response to OC in this tissue on reperfusion and 7 days later, when the fate of this tissue was confirmed by a T_2 scan. T_2* MRI sequences should reflect changes in the penumbra associated with restoration of flow and confirm its amenability to salvage. Evidence of recovery in the OC-defined penumbra was verified acutely by assessing changes in CBF and ADC following reperfusion and T_2-derived final infarct volume after 7 days. 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 T2* 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.

There are varying definitions of penumbra which have been devised using different techniques. The penumbra can be described as a region of decreased protein synthesis

and preserved adenosine triphosphate (ATP) (Hossmann, 1993), unlike the ischaemic core, which experiences reductions in both protein synthesis and ATP. Following the progressive reduction in blood flow, decreased protein synthesis is one of the first biochemical or molecular changes that can be identified (Dienel, Pulsinelli and Duffy, 1980; Bergstedt, Hu and Wieloch, 1993), declining when flow reduces below 50% (Mies et al, 1990). Whilst this method may more accurately delineate penumbra, it requires a terminal autoradiographical technique which cannot be used in recovery models of stroke. As such, the current definition of penumbra used for the study was tissue with reduced CBF but preserved CMRO2 and raised OEF. Additionally, the pathophysiological mechanisms of ischaemic damage differ between permanent and transient stroke. In permanent MCA occlusion, peri-infarct depolarisations in oligaemic tissue are responsible for the evolution of penumbra into the infarct core, whereas delayed secondary energy failure is implicated in the evolution of damage (Folbergrova et al, 1992).

Acute Data

As expected, during ischaemia, the tissue demonstrating the greatest T_2^* increase was localised to a cortical boundary zone between the MCA and anterior cerebral artery territories which overlapped the DWI/PWI mismatch area. A smaller T_2^* response was recorded in non-ischaemic tissue, with negligible response within ischaemic core. These MR findings were consistent with our previous studies (Santosh et al, 2008; Robertson et al, 2011).

Reintroduction of flow into the penumbral region restores arterial oxygen levels and therefore the oxygen extraction fraction is expected to reduce. The reduction in T_2^*

signal change in OC T_2^*-defined penumbra following reperfusion is consistent with our stated hypothesis and provides evidence that the tissue is metabolising aerobically (Figure 2 B). The extent of reperfusion was apparent on CBF maps which demonstrated that blood flow in penumbral tissue attained levels similar to the contralateral cortex (Figure 3B). However, withdrawal of the intraluminal filament did not universally lead to complete reperfusion, as flow was still compromised in the ADC-defined ischaemic core (31.74±38 mL/100g/min, Figure 3D). Evidence of tissue recovery following reperfusion was evident in the OC-defined penumbra, in which the ADC value stayed within the normal range (0.63±0.06 $x10^{-3}mm^2/sec$). Also, reperfusion increased the ADC value of some tissue within the ischaemic core to above the pre-defined viability threshold (shown by change in lesion size following reperfusion (Figure 1A to B (i)).

Day 7 data

Our hypothesis stated that reperfused penumbral tissue should exhibit a normal T_2^* response to OC and this was confirmed on the day 7 response scan (Figure 4B). Following reperfusion, irreversibly injured (ischaemic core) tissue should no longer metabolise oxygen or extract oxygen from the blood. Oxyhaemoglobin:deoxyhaemoglobin ratios would therefore be expected to remain static during OC. Within the T_2- defined infarct on day 7 post-stroke a negligible negative T_2^* signal change was observed during OC. This is most likely to be due to the presence of paramagnetic free oxygen dissolved within the plasma flowing through non-metabolising irreversibly damaged tissue which will result in a reduction in T_2^* signal. Therefore, after 7 days, T_2^*OC enabled reperfused, non-metabolic tissue to be differentiated from metabolic tissue.

DWI/PWI mismatch technique & its limitations

Individually, PWI and DWI/ADC provide valuable information on the location and severity of ischaemia, and tissue injury, respectively. Combined, they provide an approximate and indirect assessment of the location and size of penumbra. PWI and DWI scans were included in the scanning routine of the current study to provide a reference for comparison with T_2* identification of penumbra. However, DWI/PWI mismatch has a number of limitations in detecting 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 shown in the current study and by others, DWI-defined 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 (Butcher et al, 2005) and accurate MRI thresholds for defining the perfusion deficit have yet to be determined. As such, the inner and outer margins of the penumbra may not be adequately delineated using the mismatch technique and the region of decreased CBF using PWI frequently overestimates the final lesion size (Kucinski et al, 2005)

Translation of T_2*OC to the clinic

Recently, Dani et al (2010) demonstrated the first clinical application of OC during T_2*-weighted MRI, detecting differences in vascular deoxyhaemoglobin levels between tissue compartments following stroke. The percentage signal change maps generated from the OC data could discriminate between grey and white matter on the

contralateral hemisphere, consistent with the higher metabolic demand in grey matter. ROIs selected within the DWI lesion displayed a reduced T_2* percentage signal change compared to the non-ischaemic hemisphere. With patients scanned in the hyperacute phase, penumbral tissue (defined by DWI/PWI mismatch) had a significantly higher T_2* percentage signal change in 3 out of 4 patients compared to normal tissue. This increase in metabolic status was less evident in patients scanned at later time points, in line with the likelihood that penumbral tissue may have been recruited into the irreversible ischaemic core. These preliminary clinical data support the potential for this novel MRI technique to delineate penumbral tissue in acute stroke by its metabolic status. Crucially, administration of oxygen with T_2* scanning can be performed quickly and easily with widely available hardware. This technique may be used acutely or more importantly to detect existing penumbral tissue in patients unable to present within the 3-4.5 hour time window for thrombolysis.

Limitations of the study

Time to reperfusion varied slightly between rats due to the time taken to carry out the MRI scanning protocol during ischaemia. This may have contributed to mortality and explain why in some rats little or no tissue salvage was seen following reperfusion. Reperfusion is associated with considerable brain swelling at the 24-48 hr time point. For this reason, only 4 animals survived for 7 days. This is a drawback of the intraluminal filament, where the intact skull means there is no control of intracranial pressure.

Summary

Salvage of the OC-defined cortical penumbra may therefore be evident by attenuation of the T_2* percentage signal change following reperfusion, which is shown by restoration of CBF in the OC-defined penumbra. Therefore, cortex identified as penumbra using the Oxygen Challenge is capable of recovery when blood flow is restored, which provides further validation of the utility of OC MRI for acute stroke management.

Conflict of interest

The authors declare no conflict of interest.

References

Aronowski J, Strong R, Grotta JC (1997) Reperfusion injury: demonstration of brain damage produced by reperfusion after transient focal ischemia in rats. J Cereb Blood Flow Metab 17:1048–1056

Baron JC, Bousser MG, Comar D, Soussaline F, Castaigne R (1981) Noninvasive tomographic study of cerebral blood flow and oxygen metabolism in vivo: potentials, limitations and clinical applications in cerebral ischemic disorders. Eur Neurol 20:273-284

Baron JC. Ischemic stroke studied by 150-labeled compounds: misery perfusion and luxury perfusion. In: Heiss W-D, Pawlik G, Herholz K, Wienhard K, eds. Clinical efficacy of positron emission tomography. Dordrecht: Martinus Nijhoff; 15-23

Bergstedt K, Hu BR and Wieloch T (1993) Postischemic changes in protein synthesis in the rat brain: effects of hypothermia. Exp Brain Res 95:91-99

Cocho D, Belvis R, Marti-Fabregas J, Molina-Porcel L, Diaz-Manera J, Aleu A, Pagonabarraga J, Garcia-Bargo D, Mauri A, Marti-Vilalta JL (2005) Reasons for exclusion from thrombolytic therapy following acute ischemic stroke. Neurology 64:719–20

Folbergrova, J., Memezawa, H., Smith, M., and Siesjo, B. (1992). Focal and perifocal changes in tissue energy state during middle cerebral artery occlusion in normo- and hyperglycemic rats. *J. Cereb. Blood Flow Metab.* **12:**25–33

Hossmann KA (1993) Disturbances of cerebral protein synthesis and ischemic cell death. Prog Brain Res 96:161-177

Khatri P, Wechsler LR, Broderick JP (2007) Intracranial hemorrhage associated with revascularization therapies. Stroke 38(2):431-40

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

Köhrmann M, Jüttler E, Fiebach J, Huttner H, Siebert S, Schwark C, Ringleb P, Schellinger P, Hacke W (2006) MRI versus CT-based thrombolysis treatment within

and beyond the 3 h time window after stroke onset: a cohort study. Lancet Neurol 5:661–667

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

Lees KR, Bluhmki E, von Kummer R, Brott TG, Toni D, Gotta JC, Albers GW, Kaste M, Marler JR, Hamilton SA, Tilley BC, Davis, SM, Donnan, GA, Hacke W (2010) for the ECASS, ATLANTIS, NINDS, and EPITHET rt-PA Study Group Investigators. Time to treatment with intravenous alteplase and outcome in stroke: an updated pooled analysis of ECASS, ATLANTIS, NINDS and EPITHET trials. Lancet 375:1695–703

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

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

Mies G, Paschen W, Edhardt G and Hossmann KA (1990) Relationship between blood flow, glucose metabolism, protein synthesis, glucose and ATO content in experimentally-induced glioma (RG1 2.2) of rat brain. J Neurooncol 9:17-28

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

Molina CA, Saver JL (2005) Extending Reperfusion Therapy for Acute Ischemic Stroke: Emerging Pharmacological, Mechanical, and Imaging Strategies
Stroke. 36:2311-2320

Molina, C.A., and Alvarez-Sabin, J (2009) Recanalization and Reperfusion Therapies for Acute Ischemic Stroke. *Cerebrovasc Dis* 2009;27 (Suppl. 1):162-167

The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group (1995) Tissue plasminogen activator for acute ischemic stroke. N Engl J Med 333:1581-1588

The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group (1997) Intracerebral hemorrhage after intravenous t-PA therapy for ischemic stroke. Stroke 28:2109– 2118

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

Saver JL (2004) Number needed to treat estimates incorporating effects over the entire range of clinical outcomes. Arch Neurol. 61(7):1066-70

Schellinger PD, Thomalla G, Fiehler J, Köhrmann M, Molina CA, Neumann-Haefelin T, Ribo M, Singer OC, Zaro-Weber O, Sobesky J (2007) MRI-based and CT-based thrombolytic therapy in acute stroke within and beyond established time windows: an analysis of 1210 patients. Stroke 38:2640–2645

The ATLANTIS, ECASS, NINDS rt-PA Study Group Investigators (2004) Better outcome with early stroke treatment: a pooled analysis of ATLANTIS, ECASS, and NINDS rt-PA Stroke Trials. Lancet 363(9411):768-74

Wardlaw JM, Murray V, Berge E, del Zoppo GJ (2009) Thrombolysis for acute ischaemic stroke. Cochrane Database of Systematic Reviews 4. Art. No.: CD000213

Yang GY and Betz AL (1994) Reperfusion-induced injury to the blood-brain barrier after middle cerebral artery occlusion in rats. Stroke 25:1658–1665

Table 1

PHYSIOLOGICAL DATA (n=8)				
	Values during ischaemia scans		**Values for reperfusion scans**	
MABP	BASELINE 89.4±9	DURING OC 98.3±6*	BASELINE 86.9±9	DURING OC 93.5±10**
$PaCO_2$ (mmHg)	41.8±8		42.8±7	
PaO_2 (mmHg)	90±13		89.5±9	
BLOOD pH	7.324±0.05		7.302±0.03	

Titles and Legends to Figures

Figure 1 A. Ischaemia Scan Series and B. Post-Reperfusion Scan Series, with ROIs superimposed on their ADC maps (images (i) and iv); (ii) OC T_2* % signal change map, (iii) Thresholded OC T_2* map, (v) CBF map (mL/100g/min), and (vi) DWI/PWI overlay (mismatch tissue shown in red). ROIs were defined as follows;

Green ROI - The penumbra was defined by applying a threshold to display the greatest T_2* percentage signal change excluding veins and ventricles (iii).

Red ROI - Ischaemic core within the caudate nucleus, derived from the ADC lesion (i).

Sky blue ROI - The contralateral cortex, equivalent to OC-defined penumbra

Cerise ROI - The contralateral caudate nucleus, equivalent to the ADC-derived lesion

Dark Blue ROI - The ROI defined by the DWI/PWI mismatch (vi) derived from the thresholded ADC (i) and CBF Maps (v)

Table 1. Baseline Physiological Variables for the Ischaemia Scan Series and the Reperfusion Scan Series

Data expressed as mean±SD. * $p<0.05$ and ** $p<0.001$, Student's paired t-test.

Figure 2. EPI T_2* Signal time course during ischaemia (A), and following reperfusion (B), **and mean T_2* percentage signal change from baseline for ROIs during ischaemia (C) and following reperfusion (D).** A. and B.; Positive T_2* signal changes were recorded during OC in contralateral caudate nucleus and cortex, the DWI/PWI mismatch, and the OC T_2*-defined penumbra. All data were normalised to the average signal over the 4 minutes prior to OC from 8 animals. The blue box represents the period of 100% oxygen inhalation (OC).C and D; Horizontal lines

represent means. **, p<0.01, relative to contralateral cortex ROI. # , p<0.05, relative to contralateral caudate nucleus.

Figure 3. Mean CBF and ADC in selected ROIs during ischaemia and following reperfusion. A and B; Mean CBF during ischaemia and following reperfusion, and, C and D; Mean ADC during ischaemia and following reperfusion, respectively. Horizontal lines represent means. ***, p<0.001, **, p<0.01 relative to contralateral cortex ROI. ###, p<0.001, ##, p<0.01, relative to contralateral caudate nucleus ROI. $, p<0.05

Figure 4. T_2* percentage signal change at day 7 in selected ROIs. A. MRI ROIs Derived from (i) Acute T_2* % Signal Change Maps During Ischaemia (ipsilateral cortex penumbra - green); (ii) RARE T_2 Scans at day 7 post-stroke (infarct ROI-yellow) and (iii) T_2* % Signal Change Maps at day 7 (contralateral cortex and caudate – sky blue and red, respectively). ROIs were then superimposed onto T_2* % signal change maps generated 7 days after stroke to generate EPI T_2* signal time course graphs (B). Normalised signal from 4 animals. All data were normalised to the average signal over the 4 minutes prior to OC. Blue box represents period of 100% oxygen inhalation.

Figure 5. Comparison of neuroanatomical location of thresholded OC-defined penumbra in relation to T_2-defined final infarct and DWI/PWI mismatch. Acute T_2*% signal change map (i) was thresholded to identify penumbra (ii) and superimposed upon the RARE T_2 (iv) – penumbra drawn in green. The final infarct (drawn in yellow) was derived from the RARE T_2. The T_2*OC penumbra ROI was

superimposed upon the DWI/PWI mismatch (iii) in order to compare the difference in the spatial locations of the mismatch and the T_2*OC penumbra.