A Surgical Model of Middle Cerebral Artery Occlusive Stroke in the Sheep

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Dedicated to the memory of my father, Christopher John Wells.
Gone but never forgotten, your legacy of hard work and discipline will live on forever

And for my incredible wife Vanessa Rose, and my two beautiful daughters, Hannah Rose and Charlotte May

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ABSTRACT

Background: Stroke is an acute neurological injury secondary to vascular pathology, and is the second biggest killer of Australians and the leading cause of adult disability. The rationale of current therapy for occlusive stroke is rapid reperfusion of the ischaemic brain to limit the size of the injury. However, there are no standard neuroprotective therapies that have proven to be beneficial in clinical stroke, despite in excess of 1000 novel drugs showing promise in preclinical rodent studies. The consistent failure of clinical translation in rodent models suggests that they are perhaps not the best choice to simulate the intracranial pathophysiological changes that occur following human cerebral ischaemia, and that a better representative animal model with similar neuroanatomical features is required. Small ruminants such as the sheep have proven to be valuable in traumatic brain injury models, and a surgical model of permanent middle cerebral artery occlusion (MCAO) has recently been developed in the sheep. However, the existing model has a number of shortcomings and is in need of further characterisation before its widespread use in preclinical testing. The aim of this study was therefore to characterise the pathophysiological and radiological response to both temporary and permanent MCAO using a sheep model.

Methods: Several different studies were performed. In the first to determine the feasibility of the project, 18 adult male and female Merino sheep were randomised to sham surgery (n=6), permanent MCAO (n=6) or 2 h temporary MCAO (n=6), and animals had intracranial pressure (ICP) and regional brain tissue oxygen ($PbtO_2$) monitored for 4 h. 6 further animals had magnetic resonance imaging (MRI) after permanent (n=3) or temporary (n=3) MCAO. In the second study, 10 adult Merino sheep were randomised to sham surgery (n=5) or temporary MCAO (n=5), with continuous monitoring of $PbtO_2$ to determine the relationship between duration of temporary MCAO and the development of regional hypoxia. In the third study, 28 adult female Merino sheep were randomised to sham surgery (n=6), permanent

MCAO (n=10) or temporary MCAO (n=12), and monitored for 24 h under light general anaesthesia. MRI was performed in 12 animals (permanent MCAO n=6, temporary MCAO n=6). Stroke volume was calculated after staining fresh brains with 2,3,5-triphenyltetrazolium chloride (TTC).

Results: The first study demonstrated the feasibility of performing surgical MCAO, with significantly larger ischaemic lesion areas on histology and MRI following permanent versus temporary occlusion. The second study demonstrated that $PbtO_2$ fell from a mean baseline of 45.0 +/- 14.1mmHg to a predefined hypoxic threshold of 15mmHg after 42.4 +/- 11.2 minutes of temporary MCAO, at a rate of 1.3mmHg/min. The third study showed a significantly elevated ICP, infarct volumes of 27.4 +/- 6.4%, evidence of space occupying cerebral oedema on MRI and a 30% mortality rate following permanent MCAO monitored for 24 h.

Conclusions: A surgical model of temporary and permanent proximal MCAO stroke has been developed in the sheep. The response of the sheep brain to cerebral ischaemia shares many features with the human brain, particularly following permanent proximal occlusion and the development of space occupying cerebral oedema. The sheep as a representative model of human occlusive stroke appears highly promising for use in preclinical testing, for drugs that demonstrate efficacy in the sheep model may be more likely to successfully translate to clinical stroke.

DECLARATION

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Adam James Wells and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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1. **Wells A**, Vink R, Blumbergs P, Brophy B, Helps S, Knox S, Turner R. A Surgical Model of Permanent and Transient Middle Cerebral Artery Stroke in the Sheep. *PLoS One*, 2012; 7(7):e42157. doi: 10.1371/journal.pone.0042157. Epub 2012 Jul 27

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AUTHOR CONTRIBUTIONS

The following people have contributed to authorship of the manuscripts enclosed in this thesis (in alphabetical order): Peter C Blumbergs, Brian P Brophy, Stephen C Helps, Stephen J Knox, Anna V Leonard, Renée J Turner, Robert Vink.

The individual contributions of each author can be summarised as:

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Realisation of the work: AJW, RJT, SCH, RV, AVL.

Documentation of the work: AJW, RJT, RV, SCH, BPB, PCB, SJK.

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The staff at LARIF at Gilles Plains, headed by Tim Kuchel and Loren Matthews, need high praise in helping to establish the sheep stroke surgery, particularly the overnight experiments and radiology. Diana Pilkington from the Royal Adelaide Hospital MRI department must take credit for establishing the imaging protocol and for performing the studies, and radiologist Steve Knox was instrumental in their interpretation. Establishing an overnight protocol for maintaining a large animal under general anaesthesia was akin to running an intensive care unit and was not an easy task, however was made a lot easier with the help of intensivist Matt Maiden who was concurrently undertaking his own PhD also in a sheep model.

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The amazing artwork from the first stroke model and the arterial blood pressure methodology manuscripts were produced with flair and aplomb by Joshua Burton. Further artwork and figure preparation were contributed to by Tavik Morgenstern. Chris Leigh was incredibly helpful in establishing a protocol for creating a vascular cast of the sheep cerebral arteries; although this never made it into the final thesis, it proved to be a powerful method that would benefit from further exploration.

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Finally I must thank my family for their unconditional love and support, and especially for putting up with my long absences from home whilst performing the overnight sheep experiments.

ABBREVIATIONS

ACA – Anterior cerebral artery

AComA – Anterior communicating artery

ADP – Adenosine diphosphate

AMP – Adenosine monophosphate

ATP – Adenosine triphosphate

ATPases – ATP hydrolases

BBB – Blood-brain barrier

Ca^{2+} – Calcium

CBF – Cerebral blood flow

CCT – Central conduction time

CMR – Cerebral metabolic rate

CMRGlc – Cerebral metabolic rate of glucose

$CMRO_2$ – Cerebral metabolic rate of oxygen

COW – Circle of Willis

CPP – Cerebral perfusion pressure

CSF – Cerebrospinal fluid

CVA – Cerebrovascular accident

CVD – Cerebrovascular disease

CT – Computerised tomography

CTP – Computerised tomography perfusion

DVT – Deep venous thrombosis

DWI – Diffusion weighted imaging

EEG – Electroencephalogram

GLUT – Glucose transporter protein

ICA – Internal carotid artery

ICP – Intracranial pressure

LMWH – Low molecular weight heparin

MAC – Minimum alveolar concentration

MAP – Mean arterial pressure

MCA – Middle cerebral artery

MCAO – Middle cerebral artery occlusion

MRI – Magnetic resonance imaging

Na^+ – Sodium

NIHSS – National Institutes of Health Stroke Scale

NMDA – *N*-methyl-d-aspartate

NO – Nitric oxide

NOS – Nitric oxide synthase

$PbtO_2$ – Partial pressure of brain tissue oxygen

$PbtCO_2$ – Partial pressure of brain tissue carbon dioxide

PCA – Posterior cerebral artery

PComA – Posterior communicating artery

PDM – Perfusion diffusion mismatch

PE – Pulmonary embolism

PET – Positron emission tomography

PWI – Perfusion weighted imaging

rt-PA – Recombinant tissue plasminogen activator

SPECT – Single photon emission computerised tomography

SSEP – Somatosensory evoked potentials

T1WI – T1-weighted imaging

T2WI – T2-weighted imaging

TBI – Traumatic brain injury

TCD – Transcranial Doppler

THAM – tris-hydroxy-methyl-aminomethane

t-PA – Tissue plasminogen activator

TTC – 2,3,5-triphenyltetrazolium chloride

STYLE CONVENTIONS

The abbreviations, punctuations and reference style used in this thesis conform with the guidelines of the *AMA Manual of Style* and the *Style Manual*. The spelling is Australian English and conforms with *The Australian Concise Oxford Dictionary*, except for manuscripts submitted to scientific journals in which spelling is American English.

FINANCIAL SUPPORT

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1. Introduction

1.1 Stroke

A stroke is defined as an acute loss of neurological function secondary to abnormal perfusion of brain tissue (1). This can mean a reduction or termination in the supply of oxygen- and nutrient-providing arterial blood, such as with an obstruction of a cerebral artery, or a bleed into the brain substance, as occurs with rupture of a blood vessel and resultant intracranial haemorrhage; both result in rapid onset of loss of nervous system function but via different mechanisms and with different levels of long term neurological deficit, albeit all fundamentally related to the brain's blood supply (2).

1.1.1 History

The clinical picture of stroke has appeared in writings almost 3000 years old (3). Stroke has had many names over the centuries; originally dubbed apoplexy by Hippocrates around 400 BCE from the Greek *apoplēxia* ("a striking away"), the term suggested that the patient had been "struck down by violence" (4). The term "stroke" was first recorded in the English literature in 1599 with the description of "an excellent Cinnamome water for the stroke of Gods hande (sic)"; it was predominantly a lay term implying a sudden onset and with devastating effects, however physicians at the time continued to prefer the Hippocratic 'apoplexy' (5). In 1628 William Harvey's landmark theories on the circulation of blood within the body were published (6), and later in the 17th century Swiss pathologist Johann Jakob Wepfer distinguished between ischaemic and haemorrhagic stroke, recognising that some patients dying of apoplexy had bleeding on the brain; Wepfer also proposed that

apoplexy could be caused by an obstruction in the path of the brain such that it did not receive enough animal spirits (5). By 1812 the connection between a continuous supply of oxygenated arterial blood to the brain, rather than humors or spirits, and preservation of neurological function was made by the French physician Julien Jean César Legallois: “the maintenance of life in any part of an animal depends essentially on two conditions: one, the integrity of the corresponding portion of the spinal cord and its nerve communications; and the other, the blood circulation in this part” (6, 7). By the beginning of the 19th century with the emergence of modern concepts of medicine as a science, there was a growing trend to classify diseases as separate entities based predominantly on post mortem dissection, with the unification of apoplexy caused by obstruction and those caused by haemorrhage under the term ‘cerebrovascular disease’, and a gradual decline in the use of apoplexy within the scientific and medical community. The re-emergence and professional acceptance of the term ‘stroke’ happened relatively slowly during the 20th century, and mostly due to a shift towards understanding chronic illness and a multidisciplinary approach to healthcare. This team approach to stroke treatment was highlighted in a handbook produced by the British Chest and Heart Association in 1962 titled *‘Modern Views on ‘Stroke’ Illness’*, whereby it was stated “the term ‘stroke illness’ is really a lay term”, but conceded that “’Stroke’ however, is a convenient expression” (8). ‘Stroke’ was thus ultimately adopted as the preferred term for diseases of the brain secondary to acute vascular pathology, and it remains in widespread use by both the professional and lay community today.

1.1.2 Clinical manifestations and basic neurovascular anatomy

Because of the variable underlying mechanisms producing a stroke, the widespread influence the central nervous system has on almost all aspects of whole bodily function and the anatomical architecture in which function exists within the central nervous system (CNS),

there are multiple well characterised patterns of presenting symptoms and signs attributable to different classifications of stroke. The presentation of haemorrhagic stroke, such as occurs with a subarachnoid haemorrhage (classically sudden onset headache with varying degrees of impaired conscious state with or without neurological deficit), is quite different from presentation of ischaemic stroke, such as occurs with proximal middle cerebral artery (MCA) occlusion (MCAO) secondary to focal thrombus (typically painless and associated with contralateral paralysis and paraesthesia, with or without aphasia) (9). As the remainder of the thesis deals with ischaemic stroke involving MCAO, this subtype will be the topic of discussion.

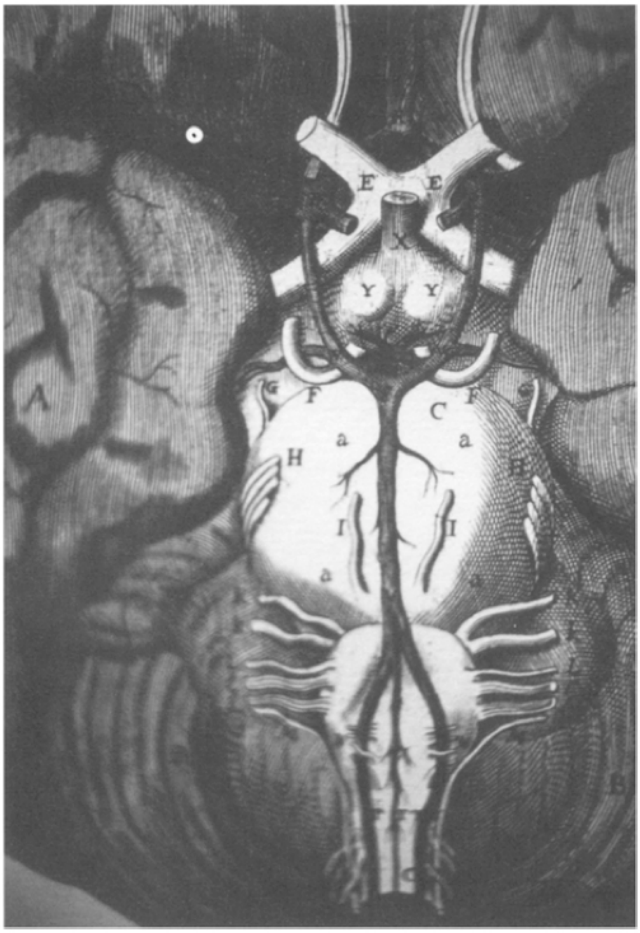


Figure 1.1: The circle of Willis. As depicted by Sir Christopher Wren in Thomas Willis' 1664 work Cerebri Anatome, demonstrating the "multiple conjoynings (sic)" of the two carotid arteries and the two vertebral arteries (6).

The symptoms and signs secondary to a stroke affecting the MCA are dependent upon two criteria: the location of the occlusion along the course of the MCA and the degree of collateral blood supply, and the location of the 'dominant' hemisphere (for speech and language) in that individual. Knowledge of functional neuroanatomy generally allows deduction of probable locations of the occlusion following ischaemic stroke. The brain receives blood from three major paired arteries arising from the circle (polygon) of Willis at the base of the brain (10): the Anterior cerebral artery (ACA), the Middle cerebral artery (MCA) and the Posterior cerebral artery (PCA) (11). The circle of Willis itself receives blood from the Internal carotid artery (ICA) anteriorly and Vertebrobasilar system (VB) posteriorly. Occlusion or reduced flow into the circle from one or more of the feeding arteries may have little effect distally beyond the circle if inflow from the other feeders is sufficient to maintain normal flow beyond into the paired cerebral arteries; likewise, breaking the circle, such as occlusion of the Anterior (AComA) or Posterior (PComA) communicating arteries may be without consequence so long as distal flow is unimpeded (12). Beyond the circle though, the major paired arteries are traditionally classified as end arteries, such that occlusion results in severely restricted flow to the territory supplied by that artery, the end result being necrosis of the brain within that region.

In Rhoton's classification, the MCA arises from the terminal bifurcation of the intradural ICA, coursing laterally in its first part (the M1 segment) before bifurcating (sometimes trifurcating) into inferior and superior trunks that lie on and supply the insula (M2 segment) (11) (Figure 1.2). Coursing now to the circular sulcus of the insula, branches run along the frontoparietal and temporal opercula to reach the superficial part of the sylvian fissure (M3 segment) and then on as superficial terminal cortical branches on the lateral convexity (M4 segment). The cortical MCA branches supply most of the lateral surface of the hemisphere, the temporal pole, the lateral part of the inferior surface of the temporal lobe, and the lateral part of the orbital surface of the frontal lobe (11). There is a strip of cortex that runs from the

frontal pole anteriorly to the occipital pole posteriorly along the medial surface; this is supplied by the ACA anteriorly and PCA posteriorly, and is spared in infarcts involving the cortical MCA territory. From the origin of the MCA in its M1 segment a number of small lenticulostriate perforating branches arise that enter the anterior perforated substance and feed most of the internal capsule, the head and body of the caudate nucleus and the lateral part of the globus pallidus.

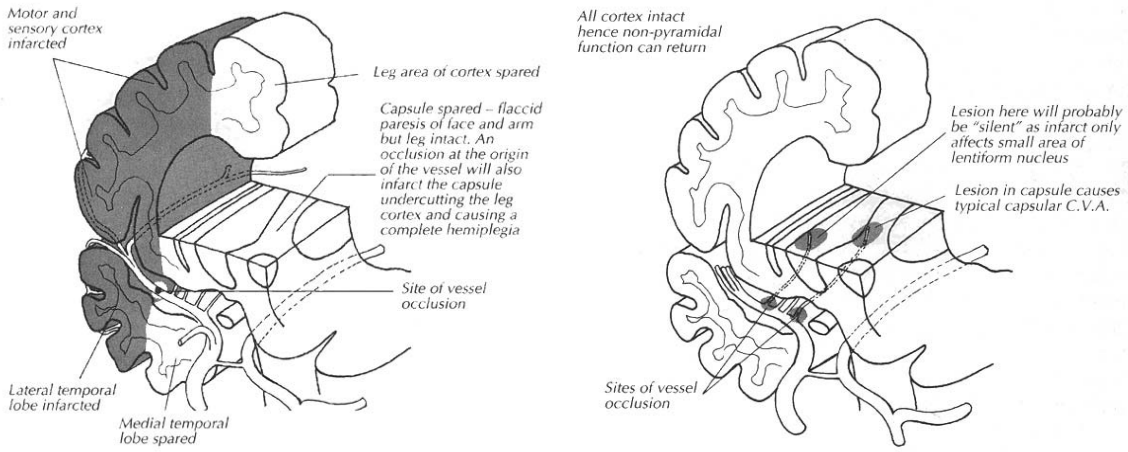


Figure 1.2: Distribution of the MCA. The result of a distal occlusion of the MCA results in a cortical infarct (shaded grey, left). More proximal occlusions affecting the lenticulostriate vessels may spare the cortex (right); complete proximal occlusions will result in ischaemia within the entire MCA territory (9).

In addition to the primary circulation, there is a potential extracranial arterial collateral circulation from anastomoses via the facial, maxillary, middle meningeal and occipital arteries (12). The facial, maxillary and middle meningeal arteries can anastomose with the ophthalmic artery, and the middle meningeal may also form a leptomeningeal collateral circulation, together with the occipital artery posteriorly. The extent of the extracranial

collateral circulation varies greatly between individuals; their ability to contribute significantly to cerebral blood flow (CBF) depends on haemodynamic and metabolic factors, and in the event of acute cerebral ischaemia they may play an important role in maintaining perfusion to the ischaemic tissue, in particular to areas of reduced flow but still potentially salvageable tissue (the *penumbra*, introduced below) (13). Angiogenesis of a significant collateral circulation can be stimulated by angiogenic peptides secreted in relation to ischaemia, unfortunately not quickly enough to maintain normal flow in acute arterial occlusion, but certainly able to make significant contributions in states of chronic hypoperfusion (12).

The dominant lobe is located in the left hemisphere in approximately 95% of right-hand-dominant individuals, and approximately 70% of left-hand-dominant individuals, hence the left hemisphere is the dominant hemisphere in the vast majority of people and is the location for receptive and expressive speech (14). Thus, the location of an occlusion within the MCA will determine how much nervous system function is lost, with complete involvement of all MCA supplied structures from a proximal occlusion resulting in paralysis of the contralateral body, a homonymous hemianopia, and receptive and expressive aphasia if the dominant hemisphere is involved, and perceptual deficits and spatial disorganisation if it is not (15, 16). More distally located MCA strokes have variable neurological deficits depending on location: thalamostriate occlusion classically results in contralateral paralysis secondary to ischaemia affecting the anterior two-thirds of the internal capsule (descending motor pathway), whereas distal occlusion of a more cortical branch may result in contralateral paralysis of the face and upper limb with sparing of the lower limb, the first order motor neurons of which are supplied by the anterior cerebral artery in the medial cortical strip.

1.1.3 Epidemiology and risk factors

In Australia, stroke is the second leading killer behind coronary artery disease and the leading cause of adult disability. There are approximately 60 000 strokes in Australian patients every year, 28% of these being a recurrent stroke, and the incidence is increasing as our population ages, although up to 20% of strokes occur in people aged under 55 years (17). Stroke kills more Australian women than breast cancer and more Australian men than prostate cancer, with 20% of first-stroke sufferers dying within one month, and 33% within one year (17). However, early mortality has shown to be reduced by up to 20% with specialist care within a dedicated stroke unit (18). Approximately 88% of stroke survivors will ultimately be able to live at home, however the vast majority will have some degree of disability. The lifetime cost burden of first-ever stroke on the community is estimated to be $2.14 billion annually (19), although the impact on individuals, their families and the workforce is considerably more than this and difficult if not impossible to estimate in monetary terms (20).

On a global scale, the impact of stroke is even more alarming. The World Health Organization reports that 15 million people suffer a stroke every year and 5.8 million individuals die annually because of stroke, more than all deaths for AIDS-related illness, tuberculosis and malaria combined (21, 22). Two-thirds of people that have had a stroke are living in developing countries with resource constrained health systems, with the overall incidence of stroke in low to middle income countries exceeding that of high income countries by 20% in the last decade (21). As in Australia, stroke is the leading worldwide cause of adult disability, however is second to dementia in low to middle income countries (23). A public health emergency was declared by the World Stroke Organization in October of 2010 to launch their “One in Six” campaign (24), which highlights the fact that 1 in 6 people will suffer a stroke in their lifetime and the urgency for which stroke prevention and novel therapies are required. With around half of all strokes occurring in people aged over 75

years, and with the world's population ageing at an ever increasing rate, particularly in developing countries (25), there has never been a more critical time to develop new therapies to combat stroke.

Risk factors positively influence the pathophysiology of thromboembolic stroke, by encouraging the promotion of vascular disease and focal thrombus formation, or hypercoagulable states and embolism formation, or both. The risk factors associated with the development of ischaemic stroke are now well documented, as are the results of efforts of risk reduction. Simply, risk factors can be divided into those that are modifiable and thus potential targets of health promotion and disease prevention, and those that are not, such as a positive family history.

Although not currently areas that can be addressed, non-modifiable risk factors are still important to consider, for they alert the patient and their primary health care provider that they are at an increased risk for the development of stroke, thus placing even more emphasis on reducing known modifiable risks. They are also areas that may in the future become targets for risk reduction, such as the possibility of gene therapy in patients with a strong family history (26).

Of the modifiable risk factors, hypertension appears to have the strongest association with the development and risk reduction of ischaemic stroke (27-29). There is also evidence for improved morbidity and mortality following modification of other risk factors, including lowering cholesterol levels (30, 31), treating diabetes (32, 33), smoking cessation (34-39), reducing alcohol consumption (40, 41), improved diet and reduced obesity (42-48), increased physical activity (49, 50) and even improved socioeconomic status (20). Atrial fibrillation is a known risk factor for the development of thromboembolic stroke, and anticoagulation treatment with warfarin can reduce the annual incidence of ischaemic stroke by 68%, from around 4.5% untreated to 1.4% (51-53). Long-term antiplatelet therapy initiated in patients

with a previous ischaemic stroke reduces the risk of subsequent serious vascular events such as second stroke, myocardial infarction or death (54). In the North American Symptomatic Carotid Endarterectomy Trial (NASCET), carotid endarterectomy has been shown to reduce the rate of stroke in patients who have already suffered an ischaemic event when ipsilateral carotid stenosis measures 70-99% and when it can be performed with low rates (<6%) of morbidity and mortality (55-57), or in selected patients with symptomatic stenosis of 50-69% or asymptomatic stenosis >60% with very low rates (<3%) of morbidity and mortality (55, 56, 58). Risk reduction through lifestyle modification and adherence to pharmacotherapy is a long-term management solution requiring dedication and good communication with primary caregivers and family members, however it has been shown to be highly effective at steadily reducing mortality when implemented (59).

1.1.4 Ischaemic stroke

Ischaemic stroke, in which brain tissue injury results from blood supply insufficient to prevent cell death by inadequate provision of oxygen and nutrients and removal of waste products, accounts for approximately 80% of all strokes, the remainder being haemorrhagic strokes including intracerebral haemorrhage and subarachnoid haemorrhage (2, 17). Typically ischaemic stroke is the result of focal arterial occlusion from thrombosis or embolism, but may also be secondary to global cerebral hypoperfusion, as in cardiac arrest or hypotension, or secondary to venous thrombosis.

1.1.5 Pathophysiology of cerebral ischaemia

The mechanisms underlying cell death following cerebral ischaemia are now well known.

Before describing the events that occur at a microscopic and macroscopic level after ischaemia, it is worthwhile considering normal brain tissue metabolism and blood supply.

1.1.6 Normal brain metabolism and substrate transport

The brain is a highly aerobic organ, utilising oxygen and glucose almost exclusively for cellular energy production (60). As a percentage of weight, the brain is one of the highest consumers of oxygen in the human body, for although in adults it constitutes less than 2% body weight it receives 25% of the cardiac output and consumes around 20% of all energy produced (61). The brain is highly specialised to function in signalling, and due to its extremely specialised nature, vast appetite for energy and inability to perform any meaningful energy storage, a continuous supply of oxygen and glucose are required for normal functioning.

The brain relies on glucose for energy production predominantly via glycolytic and, to a much lesser extent, tricarboxylic acid cycle metabolism. Energy within the brain is captured within two high-energy phosphate bonds of adenosine triphosphate (ATP) via mitochondrial oxidative phosphorylation. In turn, energy is released by cleavage of the terminal ATP orthophosphate group by ATP hydrolases (ATPases) to produce adenosine diphosphate (ADP), which can then be further hydrolysed to adenosine monophosphate (AMP), all of which are energy producing and generally coupled with energy-dependent reactions within the CNS (62). The vast majority of energy produced within the brain is used in neuronal signalling, most of which is related to the sodium-potassium ATPase pump, and which acts to maintain the transmembrane gradient required for normal signalling. Glucose and oxygen consumption can be measured to give an indication of the cerebral metabolic rate (CMR), which reflects mitochondrial activity and is highly variable between states of activity,

neuroanatomical location and cell type, with highest rates seen as expected within physiologically more active areas of grey matter (63, 64). Neuronal cells have a higher metabolic rate than glial cells, and correspondingly have a higher concentration of mitochondria. There is also likely a degree of heterogeneity within neurons, with higher activity in dendrites and synaptic terminals than in cell bodies and axons. CMR can be expressed as consumption of glucose (CMRGlc) or oxygen ($CMRO_2$), the latter being a reflection of mitochondrial activity and calculated from CBF and the arteriovenous oxygen content difference. At rest, CMRGlc measures around 25-30μmol/100 g/min, and $CMRO_2$ 130-180μmol/100 g/min (65, 66).

Glucose is a hydrophilic molecule that requires facilitated transport via glucose transporter proteins (GLUTs), entering the brain from the peripheral circulation via GLUT1 transporters on endothelial cells (67, 68). Oxygen however is delivered via diffusion down a concentration gradient from inspired pulmonary air, to the peripheral blood stream, then to cells and the extracellular fluid (61). The oxygen carrying capacity of blood is increased dramatically by haemoglobin, which binds oxygen under high oxygen tension (in the lungs) and releases it where it is needed at low tensions (in the tissues). Once in the tissues, oxygen diffusion is relatively inefficient and oxygen molecules can only diffuse for very short distances with the dense capillary network in the CNS ensuring that the distances from blood vessel to each cell is kept to a minimum, although evidence is emerging for pre-capillary arterioles, rather than capillaries themselves, being the principle source of oxygenated blood for brain tissue (69). It is the magnitude of the pressure gradient from the partial pressure of oxygen in capillary blood and the partial pressure of oxygen in the tissues that drives diffusion (70). Oxygen use by mitochondria determines the partial pressure of the tissue, which decreases as consumption increases, thus oxygen delivery can be matched to metabolic needs (61). The concentration of brain tissue oxygen ($PbtO_2$) varies within different regions of the brain being higher in cortical or subcortical grey matter compared with white matter tracts (71), however has been

demonstrated to range from 33.0-47.9mmHg in normal human brain (72), and from 44.0-52.0mmHg in other large gyrencephalic animals such as the sheep (73).

At normal rates of cerebral blood flow (CBF) the fraction of glucose extracted from the blood is about 0.1 whereas the oxygen extraction fraction is about 0.5; glucose supply therefore is usually far in excess of requirements providing a large reserve when demand increases, however the reserve for oxygen is not so great (65). The brain is highly sensitive to hypoxia (or ischaemia) because it has such a low reserve of glycogen. The point at which arterial oxygen pressures become critical as measured by a limitation in ATP generation is debated, however is generally accepted to be around 25-40mmHg (74). As arterial oxygen concentration decreases, thereby reducing the driving force or gradient into the cells to produce cellular hypoxia, pH declines, creatine phosphate hydrolysis increases, ATP concentration decreases together with increases in ADP and AMP, and glucose is rapidly broken down with generation of lactate (62). A dramatic reduction in ATP concentration can occur within minutes of hypoxia, and the effects of continued hypoxia/ischaemia are described in Chapter 1.1.10.

1.1.7 Measuring cerebral blood flow

The need for a method to quantitatively measure CBF in unanaesthetised patients was first identified at a meeting of the Federation of American Societies for Experimental Biology in 1944. At the time, German physician and physiologist Adolf Eugen Fick's principle of cardiac output had been applied by André Frédéric Cournand to calculate output by measuring the rate of pulmonary oxygen uptake and the difference in oxygen concentrations between blood going to and returning from the lungs; blood flow to individual organs could be similarly calculated by determining the difference in concentration between arterial and

venous blood (75). The following year, Americans Seymour Kety and Carl Schmidt published their solution, which was a further adaptation of Fick's principle (76). They proposed that CBF could be determined by measuring the differential rates of change in the arterial and cerebral venous (jugular) concentrations of nitrous oxide, a highly diffusible but inert gas, up to a point at which brain and cerebral blood nitrous oxygen concentrations reached equilibrium (approximately 10-15 minutes). Doing this, they were able to demonstrate a mean CBF of 54 +/- 12mL/100 g/min in 14 healthy young adult males, a value that has been repeatedly validated and oft quoted, and the Kety-Schmidt method of CBF determination gained wide acceptance and use over the next two decades.

As technology has advanced so too have our methods for measuring CBF, with a wide variety of techniques that have unique principles, advantages and disadvantages. CBF can be measured globally or regionally, and as focal arterial occlusion producing ischaemic stroke concerns interrupted local blood flow, it is the regional methods that will form the focus of the following discussion.

Current clinical methods of measuring regional CBF are ideally non-invasive, do not require anaesthesia, permit accurate and reproducible measurements and based on widely available and inexpensive technology (62, 77). All currently used methods are based on neuroimaging with a tracer or contrast agent that has a concentration that can be measured. Nuclear medicine methods use radionucleides as tracers: low energy photon emitting radionucleides in SPECT (single photon emission computerised tomography), and positron emitting radionucleides in PET (positron emission tomography). PET allows for quantification but is mostly limited to research facilities with an in-house cyclotron, whereas SPECT only allows for semiquantification but is much more readily available. Both involve exposure to significant doses of radiation (60).

Perfusion based computerised tomography (CT) imaging (CTP) uses the principle that

iodinated intravascular contrast material can detect CBF. Slow contrast infusion techniques provide a picture of whole brain angiography but not CBF, whereas first-pass techniques allow for quantitative measurement of CBF, but only within limited regions of interest, which is its major disadvantage. It is however readily available and well tolerated (60).

Magnetic resonance imaging (MRI) has been used to measure CBF using exogenous contrast or endogenous (spin labelling of blood water) methods. Contrast methods have a much higher signal to noise ratio than endogenous methods, however endogenous is entirely non-invasive and can be repeated many times (60). MRI methods are not routinely used due to difficulties obtaining quantification, non-standardisation between different centres and the length of the examination compared with CT (62).

Doppler ultrasound methods can measure flow in the carotid and vertebral arteries in the neck, and transcranial Doppler (TCD) can measure velocity in the MCA, but not flow rates (60). Changes in velocity only provide an estimate for changes in flow when the calibre of the MCA does not change; TCD has more clinical use for detecting vasospasm reflected by an increased MCA velocity to indicate a reduction in diameter of the vessel in the setting of subarachnoid haemorrhage (78).

In addition to the non- or less-invasive methods listed above, there are many techniques that have been used in the experimental setting for highly accurate methods of determining CBF, typically with greater degrees of invasiveness (79). Indicator techniques include Kety and Schmidt's nitrous oxide method; if the indicator is radioactive, then the tissue concentration can be measured autoradiographically from brain tissue to allow accurate estimations of regional CBF (80). This method is limited to only one measurement at the end of an experiment. Radioactive microspheres can be injected into the peripheral circulation to lodge within capillaries; these indicators remain intravascular yet still provide measurements of CBF similar to diffusible tracers, and in addition the use of multiple different tracers enables

multiple calculations of CBF within the same animal (81). Clearance methods for measuring CBF were devised as an evolution of the original Kety-Schmidt method, whereby the rate of removal of an indicator from the tissue reflects CBF due to arterial flow washing the indicator from the tissue, with the rate of clearance being exponential and proportional to flow (82-84). Clearance methods require an indicator that is rapidly diffusible across the BBB and is relatively insoluble in water, and can be radioactive or nonradioactive, such as occurs with spontaneous hydrogen oxygenation generating a current flow in an implanted platinum electrode (79, 85). Hydrogen is highly diffusible and lipid soluble, and although the implantation of the platinum electrodes is invasive this technique has been used now for several decades in animal models of regional CBF measurement (86-89).

1.1.8 Cerebral hypoxia and $PbtO_2$

As an alternative to measuring regional CBF and arterial oxygen, measuring $PbtO_2$ theoretically provides a more accurate representation of the regional state of oxygenation where it is needed, within the tissue. Measurements of $PbtO_2$ originally emerged through research into traumatic brain injury (TBI), in an attempt to better understand the effects of low oxygen states on the injured brain and to guide brain tissue oxygenation to prevent secondary hypoxic injury (90, 91).

Clinically, $PbtO_2$ is most frequently measured invasively in neurosurgical and intensive care settings, the majority of cases being in patients with severe TBI, followed by aneurysmal subarachnoid haemorrhage (92). In TBI, use of $PbtO_2$ monitoring to guide therapy has been shown to predict outcome and reduce overall mortality (91, 93). As it is a measurement sourced from a monitoring device its usefulness is maximised by the identification of normal and abnormal oxygen levels, and several investigators have studied the relationship between

$PbtO_2$ and clinical outcome after head injury with thresholds for poor outcome or death ranging from 5-25mmHg (94-96). One study demonstrated that the longer $PbtO_2$ was ≤15mmHg the greater the chance of death, and any episode of PbtO2 ≤6mmHg was associated with an increased chance of death, regardless of its duration (97). Other studies have since validated the prognostic value of 15mmHg for brain injury (98, 99), which has now become a generally accepted treatment threshold (92). $PbtO_2$ has also been used in animal experiments of TBI (73), intracranial haemorrhage (100) and head and neck tumours (101).

$PbtO_2$ can be measured directly via a number of techniques including polarographic oxygen electrodes, fluorescence and phosphorescence optical methods, or indirectly via electron paramagnetic resonance, nuclear magnetic resonance and mass spectrometry (70). The two main types of direct monitoring devices now in use are the Licox (GMS-Integra, Kiel-Mielkendorf, Germany) and the Neurotrend (Codman, Raynham, MA, USA) systems, which differ in their mechanism of oxygen concentration detection. The Licox system has a probe with a polarographic (Clark-type) cell at its tip with a polyethylene wall through which oxygen can diffuse into an inner electrolyte chamber. An electrical current is generated based on the amount of oxygen transformed at the electrode, and the amplitude of the current reflects local $PbtO_2$. The Neurotrend system uses a probe with an optical sensor at its tip connected to a fibre-optic cable. There is a dye connected to the cable, whose properties change depending on gas concentrations and the pH of the regional tissue to determine $PbtO_2$. In addition to $PbtO_2$ and pH, the Neurotrend system can measure the partial pressure of brain tissue carbon dioxide ($PbtCO_2$) and temperature, while the Licox system can only measure $PbtO_2$ and temperature. The tip of the Neurotrend probe samples an area of approximately $2mm^2$, whereas the sampling area for the Licox probe is larger (approximately $14mm^2$), although both are far too small to accurately represent $PbtO_2$ any more than in the very focal area of brain at the probe tip (91, 92). Despite major differences in their mechanism of action

both systems are remarkably accurate, with the polarographic Licox system being marginally superior at lower $PbtO_2$ concentrations and Neurotrend tending to overestimate $PbtO_2$ as values approach zero. Fluorescent systems have been shown to equilibrate faster *in vitro*, with controlled pO_2 decrease 90% response times of 98 +/- 38 seconds for Neurotrend versus 174 +/- 26 seconds for Licox (102).

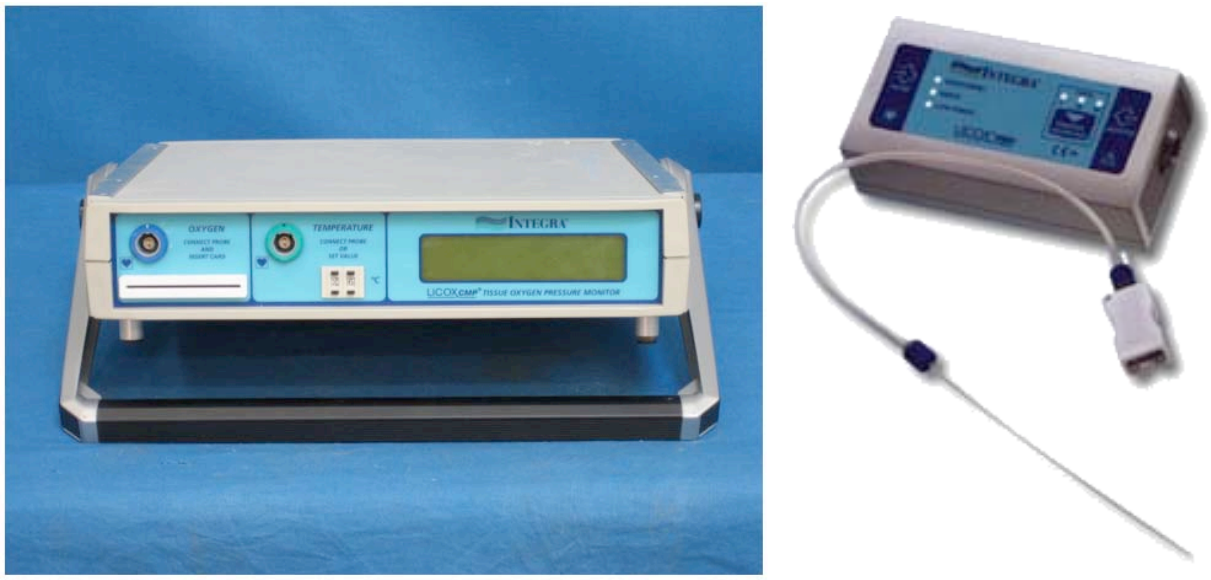


Figure 1.3: The Integra™ Licox® system. The oxygen-sensing probe (model CC1.P1) sits at the tip of the slender microcatheter (right), which is implanted within the brain parenchyma. The distal end of the probe is connected via cable to the CMP monitoring unit (left) for continuous real-time measurement of brain tissue oxygen concentration.

In the context of experimental brain injury, $PbtO_2$ has been demonstrated to be a product of CBF and the arteriovenous difference in oxygen tension, and is not simply an indicator of CBF (103). It must be remembered though that these observations are based on patients with probes within normal looking brain after TBI and with preserved autoregulation, and it seems unlikely that these findings can be correlated to pericontusional brain after TBI (104). After

acute arterial occlusion we know that CBF falls immediately within the supplied territory (105), and it has also been shown that regional $PbtO_2$ is inversely proportional to duration of temporary clip MCAO, suggesting a temporal relationship between cerebral artery occlusion and the development of regional hypoxia (106). Some authors have demonstrated a relationship between CBF and $PbtO_2$, but generally these studies tend to be in cases of TBI or subarachnoid haemorrhage (107), rather than focal arterial occlusion. A correlation between reduced CBF and $PbtO_2$ in global brain insults is intuitive, however a steady drop in regional $PbtO_2$ after acute arterial occlusion when CBF declines immediately would suggest a gradual consumption of oxygen stores after an immediate interruption of oxygen delivery, therefore any correlation between CBF and $PbtO_2$ following MCAO seems doubtful. More importantly, the stand-alone measurement of regional $PbtO_2$ may in fact better reflect the state of increasing hypoxia within the tissue that is important during arterial occlusion, particularly during temporary clip occlusion in cerebrovascular surgery, as described in Chapter 1.1.13.

1.1.9 The penumbra concept

The penumbra, or border zone, surrounds an infarct core and consists of a zone of brain with variable reduction in CBF, enough to cause electrical silence but with maintained morphological integrity and potentially salvageable tissue. The concept of a penumbra was first described by Lindsay Symon's group measuring rates of hydrogen clearance in a baboon model of MCAO (108). Symon demonstrated that after MCAO there were three distinct zones of reduced CBF: an inner core of CBF 6-10mL/100 g/min with neurological electrical failure and an increased concentration of extracellular potassium indicating membrane disruption; a peripheral zone with only mildly reduced CBF and no adverse effect on the neuronal tissue; and an intermediate zone with reduction of CBF below 20mL/100 g/min, in which there was electrical failure. They also demonstrated that the intermediate zone would progress to

infarction in the event that CBF stayed at reduced levels, however if CBF was elevated by raising mean arterial blood pressure the intermediate zone could be rescued and the ultimate size of the resultant infarct was smaller and limited to the core region. Symon's original CBF measurements have been further refined using modern techniques of flow measurement, and thresholds of 17mL/100 g/min for penumbra and 10mL/100 g/min for infarct core are widely reported in the literature (109).

We now know that the intermediate 'penumbra' zone may not necessarily be electrically silent, and also that it is dynamic, being largest in volume at the onset of ischaemia and progressively shrinking as it is incorporated into the infarct core (110). Therefore, ultimate infarct size is directly proportional to the time delay for the penumbra to be saved from progression, and this is the basis of the concept of early reperfusion therapies described in Chapter 1.1.14.

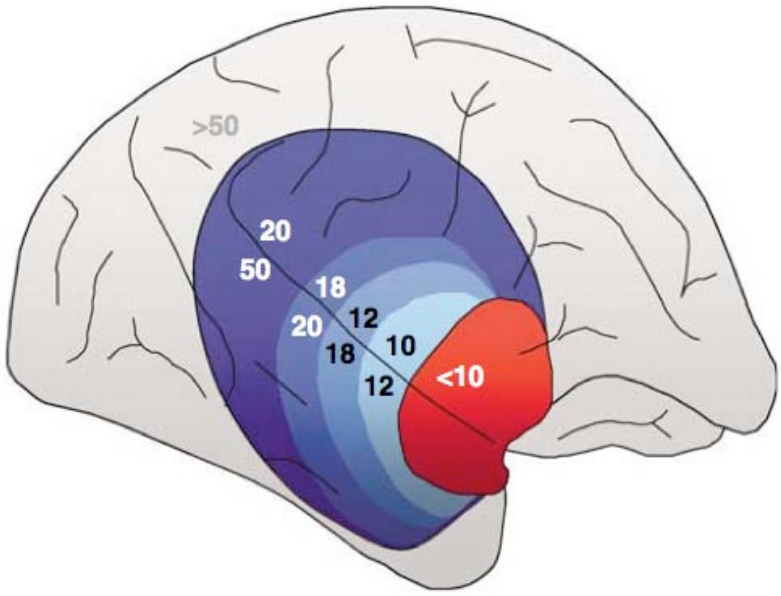


Figure 1.4: The ischaemic penumbra. There is a gradient in reduction of CBF from the stroke core (red) through the penumbra (blue) to normal cortex (grey). Values represent CBF measured in mL/100 g/min (111).

1.1.10 Cellular response to interrupted arterial blood flow

There is a well recognised cascade of events following cerebral artery occlusion that culminate in neuronal cell death, which may not in fact be linearly independent events but a network of individual molecular pathways with variable susceptibility to neuroprotective interventions (112). Oxygen and glucose delivery are severely impaired, resulting in a gross mismatch between ATP use and production, and thereby a rapid decline in cellular ATP concentration (113). Small amounts of ATP are generated via anaerobic glycolysis through the tricarboxylic acid cycle, however this is inefficient and results in lactic acid production, which in turn leads to acidosis (114), although more recently the harmful effect of lactate in cerebral ischaemia has been questioned (115). ATP levels are approximately one-third of normal after around 15 minutes of arterial occlusion (61), and energy failure in the highly energy-dependent brain commences the ischaemic cascade. Initially there is a failure of the sodium-potassium ATPase pumps, resulting in loss of normal transmembrane ion gradients, and therefore calcium influx via voltage-sensitive calcium channels. Cellular uptake of calcium is further driven by extracellular glutamate release and excitotoxicity, also a product of energy failure. As intracellular calcium accumulates lipases are activated, resulting in membrane degradation, exacerbating the loss of the transmembrane gradients. Nitric oxide synthase (NOS) is activated by calcium with calmodulin and generates nitric oxide (NO), a powerful vasodilator and also highly reactive with free radicals and involved in the generation of peroxynitrite, which mediates lipid peroxidation and has cytotoxic effects at a mitochondrial level (61). Mitochondria and cells swell, cell membranes ultimately rupture and intracellular contents are released, invoking an inflammatory response. This is cell death by necrosis, and is the predominant form of cell death within an ischaemic infarct, although there is also a variable degree of programmed cell death (apoptosis), particularly in the penumbra or following reperfusion (114).

The histopathological changes after cerebral ischaemia are well characterised, and like ischaemia affecting tissues elsewhere in the body show a temporal evolution. The first indication of mitochondrial energy failure is the presence of red, eosinophilic neurons ("red" neurons, or "red cell change"). Increasing cytoplasmic eosinophilia with a shrunken and darkly basophilic nucleus follows. Later the cytoplasm becomes uniformly structureless and the nucleus homogenous. Neurons then disintegrate into an eosinophilic debris which is subsequently phagocytised by so-called "foamy" macrophages. Neuronal injury may be remarkably heterogeneous within the infarct; other observed changes include cytoplasmic vacuolation, "ghost neurons" (loss of affinity for Haematoxylin) and neuronal shrinkage. Astrocytes become swollen; within the core they are often necrotic, however they remain viable and become reactive around it (reactive astrocytosis). Endothelial cells become swollen and disintegrate and there is perivascular vasogenic oedema, particularly in the white matter. There is an inflammatory reaction to acute ischaemia, initially with margination of leukocytes around capillaries in the core from around 4 hours after ischaemia. A minority of strokes will have a polymorphonuclear leukocyte infiltrate, but more exhibit a later onset mononuclear inflammatory infiltrate (116).

This cascade encompasses a secondary injury pathway, which occurs in a delayed and stepwise fashion following the onset of ischaemia. Each step in the secondary injury cascade therefore becomes a potential target for therapeutic intervention to minimise tissue injury, maximise neurological function and improve patient outcome following ischaemic stroke.

1.1.11 The big picture: neuroimaging of acute ischaemia

Besides the imaging techniques discussed above in detecting changes in CBF, it is worthwhile briefly considering the neuroimaging characteristics seen on the two commonest forms of

clinical imaging, CT and MRI. CT imaging of the brain has the advantages of being fast, cost-effective and widely available. Unfortunately however, acute stroke often appears like normal brain on non-contrast CT imaging. CTP can demonstrate areas of underperfusion as already discussed, and visualisation of the infarct core can be enhanced with the inhalation of Xenon gas, which penetrates underperfused brain to a lesser degree than normally perfused brain (117). Within around 24 hours however acute ischaemic stroke will become apparent on non-contrast CT scans as an area of hypodensity corresponding with infarcted and metabolically inactive tissue.

Energy failure shifts water from the extracellular space into the intracellular space. High intensity areas on diffusion weighted imaging (DWI) on MRI reflect attenuated diffusion of water, correlating with acute cytotoxic oedema (117). Decreased water diffusion on DWI has been shown to be present as early as 30 minutes after MCAO in animal models, and it has proven to be an early and sensitive diagnostic tool for the detection of ischaemia (118). MRI protocols for perfusion weighted imaging (PWI) can also be used to identify the region of brain being perfused in ischaemia, and since 1996 mismatches between the hyperintense stroke core on DWI and underperfused brain on PWI have been used to identify and calculate the volume of the potentially salvageable penumbra (117). Identifying the so-called perfusion diffusion mismatch (PDM) on MRI has greatly enhanced our ability to rationalise reperfusion therapy in acute cerebral ischaemia, as well as increasing our understanding of the early changes after acute arterial occlusion, particularly in the penumbral zone (119).

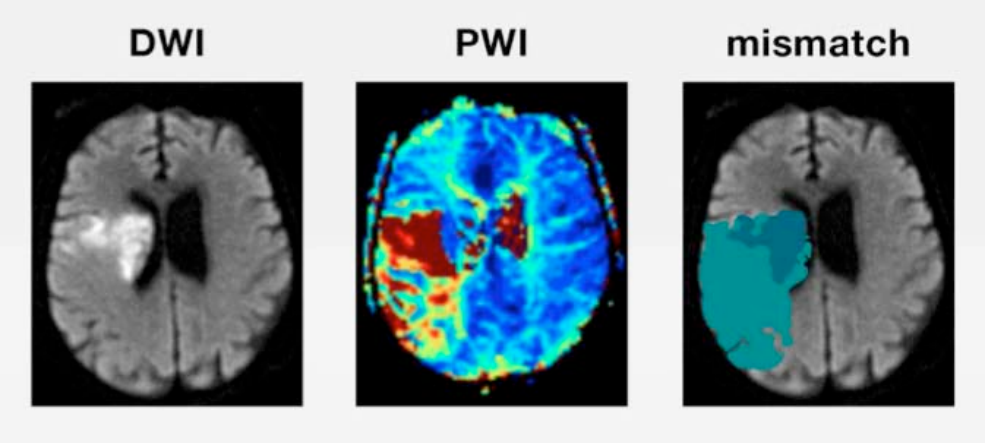


Figure 1.5: MRI of acute ischaemic stroke. The ischaemic core is represented by high signal on diffusion weighted imaging (left), reduced perfusion is represented by the red and yellow areas on perfusion weighted imaging (centre), and the diffusion/perfusion mismatch deficit indicating potentially salvageable brain is indicated by the light blue area (right) (119).

1.1.12 Brain tolerances to ischaemia

After arterial occlusion in focal cerebral ischaemia, CBF reduces immediately within the ischaemic territory, with variable levels of reduced blood flow within the core and also between the core and the surrounding penumbra (114). Two factors are important in the development of cell death, both the degree of flow reduction and the duration (105). A reduction of CBF below 16-18mL/100 g/min results in loss of neuronal electrical activity, however restoration of normal flow re-establishes normal function without neuronal injury. Further reduction of CBF below approximately 8-10mL/100 g/min results in ATP energy failure, ultimately leading to cell death, and these thresholds have been shown to be relatively consistent across different species (114). Within the core where CBF is at its lowest, irreversible injury attributable to energy failure doesn't develop until after approximately the first 5 minutes of arterial occlusion, and reperfusion after 30-60 minutes in small animal models greatly limits the size of the final infarct (113). CBF of 8mL/100 g/min can be

tolerated up to an hour after focal ischaemia in a primate model (120), flow of 10-12mL/100 g/min for around 2-3 hours (121), and 18-23mL/100 g/min has been shown to be tolerated for up to two weeks (110). The heterogeneity in tolerances to ischaemia between cell types is well known, with neurons being much more susceptible than glial cells. However, even amongst the neuronal elements there is a great variability in vulnerability to reduced CBF, with spontaneous activity discontinued at CBF rates anywhere between 6 and 22mL/100 g/min (122). Elisabetta Bandera reviewed human CBF thresholds for penumbra and infarct core in stroke patients using PET or perfusion/diffusion mismatch on MRI and revealed highly variable rates, from 14.1-35.0mL/100 g/min for penumbra, and 4.8-8.4mL/100 g/min for core (109). Although the variability between commercial software prohibited the generation of any clinical recommendations for critical flow thresholds, the mean rates from all studies combined for penumbra (23.0 +/- 8.0mL/100 g/min, n=6) and core (6.6 +/- 1.5mL/100 g/min, n=4) are reasonably similar to flow rates quoted in the literature based on animal studies.

1.1.13 Temporary clip occlusion in neurovascular surgery

Temporary clip occlusion of a parent vessel during neurovascular surgery is a selectively employed technique to help achieve the goals of the operation, usually definitive clipping of the aneurysm or to control intraoperative rupture (123). Understanding that periods of interrupted flow can be tolerated for short durations without permanent neuronal injury is the basis of the concept of safe temporary clip occlusion. British neurosurgeon Sir Geoffrey Jefferson first described the use of cerebral temporary aneurysm clips “for reduction of circulation” in 1928 (124, 125), however the technique wasn’t popularized until 1961 when the pioneering American neurosurgeon J. Lawrence Pool published his experience with temporary clipping for anterior communicating artery aneurysms (124, 126). It was

appreciated early on that the benefits of improved dissection and definitive clipping of the aneurysm associated with temporary stasis of arterial blood flow were offset by the risk of regional ischemia, and in a time dependent manner. This led neurovascular surgeons of the 1960s and 70s to begin investigating time-based thresholds for temporary clip occlusion that avoided permanent ischaemic neurologic injury. Safe time limits for temporary occlusion of the anterior circulation first appeared in 1979 with a landmark study by Japanese cerebrovascular surgeon Jiro Suzuki (127). Suzuki demonstrated that in 215 operated cases of cerebral aneurysm in which the technique of temporary clip occlusion was employed intraoperatively, 38 patients died or demonstrated post-operative neurological deficit, and the maximum safe time limit for temporary occlusion for the M1 portion of the MCA varied depending on core body temperature, from 19 minutes at normothermia to a maximum of 40 minutes at 30 degrees Celsius. Furthermore, Suzuki claimed that intermittent reperfusion could prolong the safe temporary occlusion time. Since then numerous authors have described recommended safe time limits for temporary vessel occlusion (128-138), ranging from as high as 40 minutes (130) to as short as 10 minutes (138), although generally there has been a steady decline in accepted maximum risk temporary occlusion times since Suzuki's work. To date however, all clinical studies reporting safe time limits for temporary clip occlusion are individual case-controlled studies at best with variable end points of neurological injury, from new post-operative clinical deficits to radiological evidence of ischaemia (128-130, 133, 137-141). The 'safe' duration of occlusion is probably highly variable between and even within individuals, with many factors other than the length of temporary occlusion being critical in determining the risk of ischaemic complications including patient age, presence or absence of subarachnoid haemorrhage, grade of subarachnoid haemorrhage, aneurysm configuration and particularly collateral blood supply (138, 139, 142-147). At present, there is insufficient evidence to establish treatment guidelines for safe time limits of temporary cerebral artery occlusion based on time alone.

With increasing sophistication of intra-operative physiological monitoring devices, there have been attempts to individualise the ischaemic complications of temporary occlusion. There are two well-recognised methods of physiological monitoring currently in use to improve safety and minimise ischaemic injury: intra-operative measurements of regional blood flow, and electrophysiological activity (148). Doppler ultrasound can detect changes in flow but correlate poorly with absolute CBF (149). Thermal probes are more sensitive to changes in flow but are still unreliable in predicting ischaemic injury (150). Near-infrared light measurements of haemoglobin oxygen saturation can provide information about hypoxia by detecting changes in venous saturation, but outcomes are still to be validated (151). CBF is coupled with brain electrical activity, with electroencephalogram (EEG) and evoked potential alterations detected with CBF <16mL/100 g/min (152), and although neurons are electrically silent they are still viable for periods of occlusion up to 30 minutes (153, 154). As electrical failure precedes energy failure, monitoring electrical activity during temporary occlusion could help predict and prevent ischaemic complications (148). Changes in amplitude or latency in somatosensory evoked potentials (SSEP) and central conduction time (CCT) are the commonest parameters for monitoring electrical change associated with temporary occlusion in the anterior circulation, although they appear to reflect occlusion of the ICA more than MCA (130, 144, 145). The major disadvantage of electrophysiological monitoring is the inability to continuously record all regions at risk; small infarcts related to lenticulostriate artery occlusion for instance result in hemiparesis despite no abnormality in somatosensory potentials (155). There may also be a considerable time delay between onset of ischaemia and detection of electrophysiological change of up to 3 minutes (156), however SSEP have been shown to reduce the incidence of temporary clip-related injury from 19% to 7.5% in one study (157).

Continuous monitoring of $PbtO_2$ may reflect changes in regional blood flow during temporary occlusion, and several human studies have now investigated the relationship between $PbtO_2$

and stroke risk during aneurysm surgery. Kett-White and colleagues, using a Neurotrend device, reported that $PbtO_2$ <8mmHg for >30 minutes was associated with increased stroke risk (158), whereas Jodicke and colleagues reported the same risk using a Licox system with $PbtO_2$ <15mmHg associated with temporary clip times of >6 minutes (156). Jodicke furthermore showed that monitoring $PbtO_2$ was more beneficial in certain situations, demonstrating that only $PbtO_2$ and not SSEP was a predictor of temporary clip related ischaemia in the ACA (156). Cerejo, reporting on temporary clipping during MCA aneurysm surgery using a Licox probe in unruptured (159) and ruptured (160) aneurysms, did not identify safe time or $PbtO_2$ values predictive of ischemia, but noted CT evidence of infarction in two subarachnoid hemorrhage patients with $PbtO_2$ levels <2mmHg for >2 minutes during temporary clipping. Gelabert-González reported that short periods of temporary clipping producing Licox-detected hypoxia in subarachnoid hemorrhage patients were well tolerated, but without suggesting safe levels of $PbtO_2$ or occlusion times (161). Results from Jodicke's study, in which 15mmHg was identified as a dichotomizing threshold for the prediction of ischemia during aneurysm surgery, together with results from both human (72, 91) and animal (73) TBI studies, support the use of a Licox-measured $PbtO_2$ hypoxic threshold of 15mmHg as a predictor of ischaemic injury.

1.1.14 Stroke treatment concepts

The ultimate stroke treatment goals with current medical technology are to prevent death, limit the size of the infarct and maximise long-term functional outcome in the recovery period after stroke. Therapeutic options for ischaemic stroke to help achieve these goals include expectant management, non-surgical therapies and surgical interventions (162).

Ischaemic stroke was long thought to be a disease unamenable to therapeutic intervention,

thus treatment was directed towards minimising complications associated with neurological deficit, minimising secondary brain injury, identifying and addressing risk factors for recurrent stroke and rehabilitating the patient. In the 1950s new technologies including interventional angiography and anticoagulation were emerging, giving hope that these developing techniques could restore blood supply to the ischaemic brain. Davidson's "Principles and Practice of Medicine" in 1956 suggested careful use of anticoagulation in ischaemic stroke (163), and at about the same time carotid endarterectomy was emerging as a surgical technique for the treatment of stroke. Although anticoagulation eventually progressed to the currently used thrombolytic therapies, it became apparent that endarterectomy procedures that restored flow rarely restored function, and is today used as a preventative therapy rather than an acute interventional one (164).

Chemical thrombolysis emerged as a technique to reperfuse the ischaemic brain based on the concepts described above, particularly the theory of a potentially salvageable penumbra. Generally it is performed via intravenous injection of a thrombolytic agent to dissolve a focal blood clot, however more recently intra-arterial administration guided to the region of occlusion has become a popular alternative. The first generation of thrombolytic agents, streptokinase and urokinase, emerged in the 1950s; initial trials were associated with increased mortality secondary to the development of intracerebral haemorrhage, however this was probably due to a delay in patient enrolment amongst other factors (165), and they were subsequently mostly abandoned. Second generation thrombolytics included tissue plasminogen activator (t-PA), and were more fibrin-selective than first generation agents. Justin Zivin's group was the first to demonstrate a beneficial effect of t-PA in a rabbit model of embolic stroke in 1985 (166). By 1996 recombinant t-PA (rt-PA) was approved for use in ischaemic stroke up to 3 hours after the stroke ictus based on the results of several large multicentre randomised controlled trials, particularly NINDS, ECASS I and II, and ATLANTIS (167, 168). In fact, of over 1000 experimental treatments in ischaemic stroke,

only intravenous thrombolysis with rt-PA has successfully translated from animal research to the clinical setting (169). Although a later combined analysis of these three trials demonstrated that clinical outcome decreased with time to treatment and that the chance of a good outcome was increased by 2.8 (CI 1.8-4.5) if therapy was initiated within 90 minutes (170), the therapeutic window for intravenous rt-PA was extended to 4.5 hours when a systematic review of further randomised controlled trials demonstrated an improved outcome without a significant difference in mortality (171-173), and this window remains the recommendation today. Even though it is an effective treatment with a well documented yet acceptable adverse event profile including haemorrhagic transformation within the ischaemic tissue, the current biggest problem with thrombolysis use is the small proportion of patients who actually receive this therapy estimated to be as little as 2-5%, secondary to the strict eligibility criteria and narrow window of opportunity (174).

More recently, mechanical clot retrieval devices have emerged to reperfuse the ischaemic territory, in which the device is placed at the site of arterial occlusion endovascularly under direct radiological vision, either alone or in conjunction with intravenous or intra-arterial thrombolysis. Focal clots can be aspirated proximal to the occlusion as in the Penumbra® system, or snared within a coil or basket advanced through the clot distal to the occlusion, as in the MERCI® retrieval system (175). These devices have a superior ability to restore flow to large calibre cerebral vessels compared with chemical thrombolysis, and are approved for use up to 8 hours after the stroke ictus providing treatment options for patients in whom thrombolytic therapy is contraindicated or fails or those who present beyond the narrow rt-PA window. However, long term results with regards to functional outcome have been disappointing proportional to the excellent rates of recanalization, and more studies are needed before they can become a recommended therapy (176).

Another non-surgical therapy that has been extensively investigated but remains to yield a

successful clinical therapy is neuroprotection, which is the restriction of injury secondary to ischaemia to prevent the death of potentially salvageable neurons (177). A complex cascade of biochemical events as outlined in Chapter 1.1.10 occurs after cerebral ischaemia, with one or more components of this cascade, such as glutamate release and receptor activation, intracellular calcium influx, free radical production, inflammation and apoptosis, all being potential targets for direct neuroprotection (174). Disappointingly however, no neuroprotective therapies have successfully translated from seemingly promising animal experiments (174, 177).

Surgical therapies for ischaemic stroke can be divided into therapies that restore blood supply and therapies that treat the consequences of stroke. As mentioned above, there were initial high hopes for surgical restoration of blood flow after cerebral artery occlusion, however the significant delays in achieving restoration surgically in such a time critical event as ischaemic stroke resulted in abandonment of surgery for non-surgical interventions in the acute setting. Flow restoring surgery is still employed for cerebral ischaemia, however as a preventative measure in cases of chronic ischaemia (178, 179). Much more commonly, surgical intervention is utilised following ischaemic stroke in the event of the development of space occupying cerebral oedema, in which case surgery is a life saving measure, as described below.

1.1.15 The blood-brain barrier, cerebral oedema, the Monro-Kellie doctrine and raised intracranial pressure

A barrier between the CNS and the peripheral circulation was first proposed by Paul Ehrlich and his student Edwin Goldmann in 1885, with German physician Max Heinrich Lewandowsky coining the term ‘blut-hirn schranke’ (blood-brain barrier) in 1900 (180). One

of the brain's responses to ischaemic injury is the breakdown of the blood-brain barrier (BBB), what we now recognise as a physical barrier between the endothelium and the extracellular space, composed of endothelial cells, tight junctions, neurons, astrocytic foot processes, pericytes and the extracellular matrix, and the primary function of which is to maintain fluid homeostasis within the brain distinct from the peripheral circulation (181)**.** Interruption of the BBB can result in a net flux of fluid from the vasculature into the brain parenchyma and thus a net increase in volume of the brain (182). After ischaemic stroke and the cascade of events that result from energy failure, there is depletion of ATP, failure of the sodium-potassium ATPase pump, increased intracellular potassium, lactic acidosis and release of extracellular glutamate, all of which have been implicated in disruption of the BBB (181, 183). Endothelial cells swell within minutes of ischaemia onset, protease induction causes degradation of the extracellular matrix, and all of these changes are exacerbated by the inflammatory response, release of nitric oxide and generation of oxygen free radical species. The situation is further complicated with reperfusion, however is highly dependent on the duration and severity of ischaemia and the animal species. When the volume of cerebral ischaemia is large, the disruption of the BBB can be so great that the amount of extra water transferred into the brain from the peripheral circulation as oedema fluid can increase the overall volume of the brain considerably, and with space occupying effect (182).

A net increase in brain water volume after BBB breakdown results in a larger sized brain attempting to fit inside a skull that has not changed in size. The pathophysiology of increased intracranial volume was initially described by Scottish anatomist and surgeon Alexander Monro (secundus) in his 1783 monograph *'Observations on the structure and functions of the nervous system'*. Monro recognised that the cranium was a rigid box ("for being enclosed in a case of bone"), and that "the substance of the brain, like that of other solids of our body, is nearly incompressible" (184). He also proposed that "the blood must be continually flowing out of the veins, that room may be given to the blood which is entering by the arteries", and

that “the quantity of blood within the head must be the same, or very nearly the same, at all times… a quantity of blood, equal in bulk to the effused matter, will be pressed out of the cranium”. Although apparently not aware of CSF being a normal intracranial component (despite having the interventricular foramen named for him), he correctly surmised that any increase in the volume of the cranial contents occurs at the expense of volume of another.

In 1824 George Kellie, an anatomist and student of Monro (secundus), and still unaware of the existence of CSF, published a manuscript *‘An account of the appearance observed in the dissection of two of three individuals presumed to have perished in the storm of the 3rd, and whose bodies were discovered in the vicinity of Leith on the morning of the 4th, November 1821 with some reflection on the Pathology of the Brain’*. Kellie felt his observations regarding estimations of the amounts of blood in the cerebral veins of humans and animals after death confirmed Monro’s hypothesis. He stated “the brain itself, little compressible, is contained within a firm and unyielding case of bone, which it exactly fills, and by which it is defended from the weight and pressure of the atmosphere – a force constantly acting on every part of the system – a force therefore which must be constantly operating to maintain the plenitude of the vascular system within the head. If these premises be true, it does not then appear very conceivable how any portion of the circulating fluid can ever be withdrawn from within the cranium, without its place being simultaneously occupied by some equivalent; or how anything new or exuberant can be intruded without an equivalent displacement”. These two Scottish physicians thus gave their names to the theory describing intracranial volume shift, the Monro-Kellie doctrine.

The CSF was first described by the pioneering French experimental physiologist François Magendie in 1825, and the incorporation of CSF as one of the intracranial components in the Monro-Kellie doctrine came 21 years later by English physician George Burrows. Burrows recognised that as intracranial blood volume increased CSF exited the cranium into the spinal

space, only to return as intracranial blood volume decreased again in a cyclical fashion. Thus, the complete doctrine states that the central nervous system and its accompanying fluids are enclosed in a rigid container whose total volume tends to remain constant, and an increase in volume of one component (e.g. brain, blood or CSF) must be at the expense of a decrease in volume of one or both of the other elements, otherwise intracranial pressure (ICP) will increase.

The ICP is defined as the pressure within the cranial cavity, with normal adult human values approximately 5-15mmHg. Three different pressures contribute to ICP: atmospheric pressure, hydrostatic pressure and filling pressure. Atmospheric pressure is transmitted through the vasculature and is variable secondary to altitude, however ICP is usually reported relative to atmospheric pressure, therefore this contribution is generally ignored (185). Hydrostatic pressure depends on the weight of the fluid of the contents of the skull and spinal canal above the point of measurement, relative to the cross-sectional area at that level (186, 187). Filling pressure is a product of the volume of the intracranial contents and their elastance (pressure change per unit of volume change) and the relationship is non-linear in both normal and pathological conditions, as demonstrated with increased volume in the Monro-Kellie doctrine.

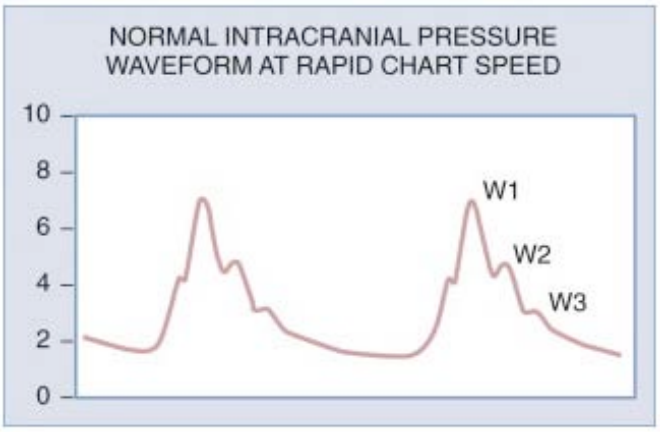


Figure 1.6: Intracranial pressure waveform. The waveform is cyclical and three distinct smaller components can be seen, the percussion wave (W1), the tidal wave (W2) and the dicrotic wave (W3) (188).

Furthermore, what we frequently quote as the ICP is an average pressure of a waveform with again three components: the baseline level, and two superimposed rhythmic components secondary to cardiac and respiratory activity, which cyclically alter the blood volume within the cranial cavity. Further analysis of the ICP waveform reveals at least three constant peaks (Figure 1.6): the percussion wave (also known as W1, the largest peak) from pulsations in large intracranial arteries, the tidal wave (W2) attributable to brain elastance, and the dicrotic wave (W3) separated from the tidal wave by the dicrotic notch, corresponding to the dicrotic notch of the arterial waveform. Information about the compliance of the intracranial contents can be garnered from the pulsatile components (pulse amplitude and frequency), and can be an early sign of a decompensating system (189, 190).

As intracranial volume increases so does pressure, but as Henry Ryder first demonstrated in a study on rhesus monkeys in 1951 it does so in a hyperbolic pattern (Figure 1.7) (191). Initially in the horizontal or flat portion of the curve, increased volume results in only a small increase in ICP, because compensatory mechanisms are able to maintain ICP within a normal range (the period of spatial compensation). As compensatory mechanisms are exhausted and compliance decreases however, small increases in volume result in progressively larger increases in ICP (the period of spatial decompensation), up to the point where ICP approaches mean arterial pressure (MAP), when the curve once again flattens out (188). The effects of increased ICP are twofold. First, there is a reduction of cerebral perfusion pressure (CPP), which is calculated as MAP minus ICP. The brain is excellent at autoregulating CBF for a wide range of CPP but with a lower limit of around 50mmHg, therefore increases in ICP can reduce CPP below its lower threshold if MAP does not increase accordingly (192, 193). Once the autoregulatory reserve has been exhausted CBF falls resulting in ischaemia, then development of a vicious cycle of cytotoxic oedema and raised ICP. The second effect of raised ICP is the formation of pressure gradients within the neuraxis (194, 195). Pressure is generally conducted uniformly from one part of the CNS to another via the CSF, however the

human brain in particular is relatively compartmentalised secondary to the strong fibrous dura mater and its intracranial folding, the falx cerebri and the tentorium cerebelli, hence pressure gradients may occur between compartments (196). Depending on the site of focal space occupying pathology, there are several well recognised anatomical herniation syndromes including transtentorial herniation (a central downwards shift of the hemispheres and basal ganglia through the tentorial incisura, compressing the diencephalon), uncal herniation (a medial shift of the uncus and hippocampal gyrus into the tentorial notch, distorting the brainstem) and subfalcine herniation (a medial shift of the cingulate gyrus below the falx cerebri, compressing vascular structures) (197-199). Herniation syndromes that compress the brainstem or diencephalon are the most serious complication of raised ICP and can result in decreased consciousness, respiratory abnormalities, altered cardiovascular function and ultimately death (188).

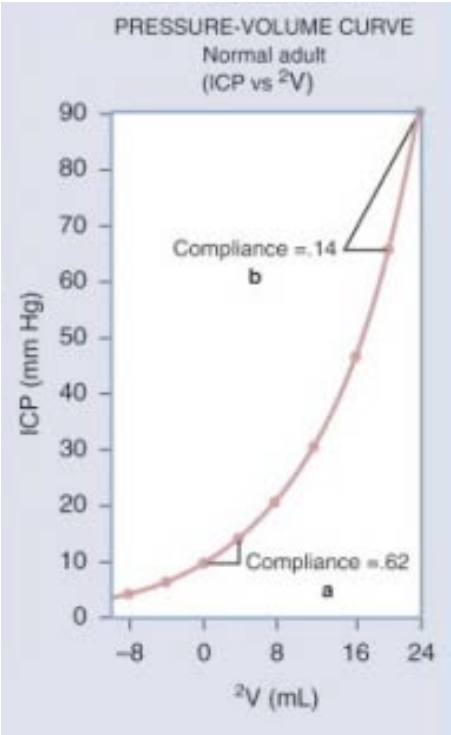


Figure 1.7: Intracranial pressure/volume curve. In physiological conditions, increasing the volume of the intracranial contents results in a small rise in intracranial pressure, however as compensatory mechanisms are exhausted compliance decreases and small increases in intracranial volume result in large increases in intracranial pressure (188).

1.1.16 Malignant MCA stroke

A small percentage of strokes affecting the MCA territory are so large and result in so much cerebral oedema they cause a significant rise in brain volume and ICP and have an untreated mortality rate of approximately 80% due to transtentorial herniation and brainstem compression (200, 201). These were dubbed “Malignant MCA stroke” by German neurologist Werner Hacke in 1996, due to their rapid progression and high mortality rate (202). Malignant MCA strokes make up only 10-15% of all ischaemic strokes involving the supratentorial brain, however they account for a disproportionately high early in-hospital mortality rate of 28.9% of all patients admitted to a stroke unit (203). Survivors have a significantly greater incidence of severe disability than smaller volume MCA stroke sufferers (204), and in those developing malignant oedema there is a short window after stroke onset, lasting between less than 24 hours up to around 5 days, in which the space occupying effect of the oedema is so large that it is life threatening.

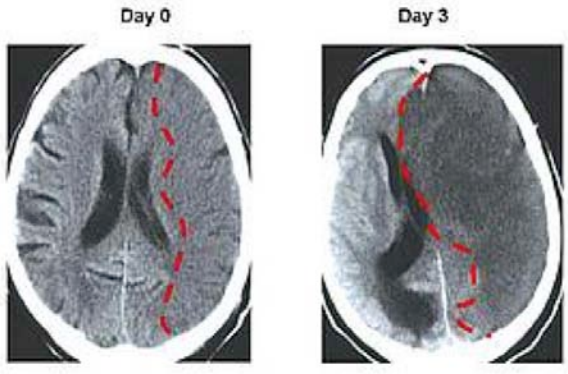


Figure 1.8: Malignant MCA stroke. CT scans taken from the same patient demonstrating large volume left-sided MCA stroke on the day of admission to hospital indicated by the dashed red line (left), and massive increase in size of the infarct volume secondary to vasogenic oedema by day 3, resulting in a shift of intracranial contents to the contralateral hemisphere (right). Note also the change in density of the stroke lesion, from almost normal on day 0 to hypodense by day 3 (205).

In Hacke's original description, 55 patients out of a total of 1025 admitted to a single stroke unit over a three-and-a-half year period had CT evidence of complete MCA territory infarction within 48 hours of the ictus and required neurocritical care for clinical evidence of raised ICP or respiratory failure. 26 patients had an ICP monitor inserted for therapeutic monitoring of anti-oedema therapy and/or barbiturate coma. The mortality rate from all included patients was 78%, with death between days 2 and 7 (median day 4) and resulting from transtentorial herniation in all patients. Mean ICP over the entire monitoring period was 19.4mmHg; in patients who died, mean highest ICP was 43mmHg (range 30-80mmHg), and in those that survived it was 28mmHg (range 23-38mmHg) (202).

Although beneficial in the setting of head injury, monitoring ICP after large volume MCA stroke remains controversial (206, 207). Often these patients have only a small decline in conscious state; placing ICP monitors in patients deemed at risk of developing a malignant course would usually require a general anesthetic with its associated risks in the setting of acute cerebral ischaemia. What is more, it has been shown that severe brainstem compression can occur despite relatively normal ICP; midline shift and clinical signs of herniation can occur even with ICP <20mmHg (208), clinical signs of herniation precede raised ICP in most if not all patients, and in the event of an elevation in ICP medical therapies to reduce pressure are relatively ineffective (209). Routine monitoring of ICP in large volume MCA stroke is therefore not recommended, however randomised clinical trials comparing surgical decompression versus aggressive medical control of ICP are lacking (210).

Therapies for malignant ischaemic oedema can be divided into those that reduce the volume of the oedema (anti-oedema therapy), and those that increase the volume of the intracranial compartment (surgical decompression). There are a number of non-operative treatments investigated for managing space occupying cerebral oedema, including head elevation, hypothermia, hyperventilation, mannitol, glycerol, tris-hydroxy-methyl-aminomethane

(THAM), barbiturates and corticosteroids (211). 30 degree head elevation helps reduce ICP in head injured patients, but in acute stroke it does so to only a small degree and at the expense of a significant reduction in CPP and therefore is not recommended as a routine treatment (212, 213). Hyperventilation lowers ICP by inducing hypocarbia-induced vasoconstriction, and also therefore negatively affects CBF and is not recommended (214). Hypothermia is a widely studied neuroprotective therapy, and although there are concerns with adverse effects including cardiac arrhythmia, infection, coagulopathy and rebound intracranial hypertension, external cooling has been shown to reduce mortality and improve functional outcome in small clinical trials and larger randomised trials are ongoing (215-220). Mannitol is frequently used as an osmotic agent in head injury but may also have some neuroprotective qualities (221-223). It may be detrimental in cerebral oedema associated with BBB disruption and there is insufficient evidence to support its use following ischaemic injury, although data is generally lacking (224, 225). Glycerol crosses the BBB more readily than mannitol and may be associated with less rebound intracranial hypertension, but again evidence to support its routine use is lacking (226). THAM neutralises acidosis related vasodilatation to reduce ICP and has been demonstrated to reduce ICP in animal models and clinical trials of head injury, but is yet to be investigated in cerebral ischaemia (227-229). Barbiturates reduce cerebral metabolic activity and therefore substrate requirements and CBF; they may be helpful for treating ICP refractory to other medical managements, but as with most other novel therapies data is lacking (200). Corticosteroids help stabilise the BBB and are beneficial in oedema related to brain tumours which like ischaemia is also associated with BBB disruption, but have not shown to improve mortality or functional outcome in ischaemia (230). Other experimental anti-oedema therapies such as the oral hypoglycaemic agent glibenclamide which acts on the SUR1 receptor have shown promise in small animal studies (210, 231), however have not yet translated successfully to the clinical setting.

Surgical decompression is one of the oldest medical procedures known; trephined skulls can

be dated back 7000 years to the Neolithic period (232), and trepanation was described in ancient Greek and Roman literature (233), but was significantly absent in the definitive ancient Egyptian surgical treatise, the Edwin Smith Papyrus (234). More recently, Kocher proclaimed "if there is no CSF pressure, but brain pressure exists, then pressure relief must be achieved by opening the skull" (235). Decompressive craniectomy alters the "rigid box" component of the Monro-Kellie doctrine by increasing the size of the intracranial cavity, and has proven effective when non-surgical anti-oedema therapies have failed, such as in malignant stroke. A large craniectomy coupled with duraplasty allows the oedematous brain to swell away from the midline, whereas undecompressed brains with focal space occupying oedema push against the midline and compromise the vital centres within the brainstem secondary to uncal and transtentorial herniation. Decompressive craniectomy is utilised for pathological conditions associated with raised ICP secondary to an increase in brain volume in which a focal pathology (such as tumour or extradural haematoma) cannot itself be removed. Removing the overlying bone increases the volume of the intracranial cavity exponential to the size of the bone removed; suboptimal craniectomies of diameter 6cm increase intracranial volume by $9cm^3$ and are associated with secondary injury attributable to herniation through the craniectomy defect (236), whereas doubling the diameter of the craniectomy to 12cm results in an $86cm^3$ increase in intracranial volume and avoids iatrogenic injury (237). Besides inadequate decompression, other complications associated with craniectomy include infection, haemorrhage, subdural haematoma, and longer term, hydrocephalus or low pressure headaches associated with the syndrome of the trephined (207). The technique as applied to ischaemic stroke first garnered interest in the 1990s with experimental rodent MCAO demonstrating no mortality and smaller stroke volumes with early decompression, compared with 35% mortality within 48 hours of animals managed non-operatively (238, 239). In early non-randomised clinical studies investigating the protective role of decompression in MCA stroke, there appeared to be a reduction in mortality, which was not associated with increasing the number of severely disabled survivors. Two small

randomised clinical trials were initiated in the early 2000s (HeADDFIRST in North America and HeMMI in the Philippines) (162), however definitive data was lacking until the pooled analysis of the results of three separate European randomised studies, each investigating the effect of decompression on functional outcome in patients with malignant MCA stroke (240-242). This ultimately demonstrated that in young patients (age <60 years) with malignant MCA stroke, decompressive craniectomy within 48 hours of stroke onset was associated with reduced mortality and increased number of survivors with a favourable functional outcome (243). The inclusion criteria for each trial were slightly different, particularly the neuroimaging criteria (which ranged from CT evidence of infarction involving at least two-thirds of the MCA territory in DESTINY and HAMLET to infarct volume on diffusion-weighted MRI of more than 145 cm^3 in DECIMAL), age (18-55 years in DECIMAL, 18-60 years in DESTINY) and time to inclusion and randomisation from onset of symptoms (<24 hours for DECIMAL, <36 hours for DESTINY and <45 hours for HAMLET). Nevertheless, all three trials shared a similar design and had the same primary outcome measure (functional outcome). The authors of the pooled analysis correctly pointed out that the decision to perform decompression should be made on an individual basis, for although craniectomy saves lives it appears to be at the cost of producing a far greater number of survivors with moderately severe disability (modified Rankin Scale score of 4; unable to attend to own bodily needs without assistance, and unable to walk unassisted). A large criticism of the pooled analysis was that it used mRS ≤4 as a 'favourable' outcome when mRS ≤3 (moderate disability, requires some help but able to walk unassisted) is generally accepted as favourable in the literature (244). Further criticisms were directed towards the inability to extrapolate the findings to patients aged greater than 60 years and the optimal timing to perform decompression (206, 207). Nevertheless, surgical decompression by craniectomy and duraplasty in select patients with large volume MCA stroke is a very powerful intervention with a 50% absolute risk reduction in mortality (95% CI 33-67) (243).

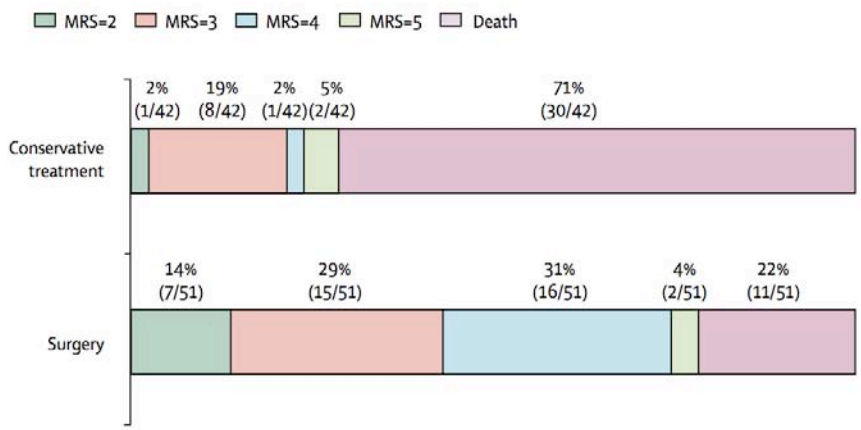


Figure 1.9: Outcome after decompressive craniectomy for malignant MCA stroke. Distribution of mRS scores or death following surgery or non-surgical management for malignant MCA stroke, from the pooled analysis of three randomised controlled trials. mRS, modified Rankin score (243).

The optimum time to perform surgical decompression remains controversial: performed too late secondary ischaemia and herniation from space occupying oedema is not prevented, and too early risks performing a highly invasive intervention on a subgroup of patients who may not progress to a malignant course (206, 207). The original pooled analysis suggested no added benefit when surgery was performed early (within 24 hours) versus late (up to 48 hours), however other authors have suggested a reduction in mortality by up to 50% when patients are operated within 24 hours (245), or even ultra-early within 6 hours (246) of MCA stroke, suggesting that early prediction of a malignant course my be beneficial by offering decompression early within the course of the disease. With that in mind, several researchers have attempted to identify factors that predict a malignant course after MCA stroke. Stroke volume measured radiologically seems the most logical and is also the most investigated parameter; the larger the infarct, the more BBB disruption and vasogenic oedema, and the more likely that patient's pressure/volume relationship will occupy the decompensatory

component of the curve. An MRI DWI volume >145cm^3 in the MCA territory within 14 hours of the ictus had 100% sensitivity and 94% specificity for predicting a malignant progression in one study (247), and CT hypodensity involving >50% of the MCA territory was a positive predictor of fatal brain oedema (OR 6.3, 95% CI 3.5-11.6, p<0.001) (248). Involvement of the ACA or PCA in addition to the MCA was also associated with an increased risk for malignancy (OR 3.3, 95% CI 1.2-9.4, p=0.02). As a percentage of hemispheric volume, malignant strokes occupy between 39 and 47.6% of the ipsilateral hemisphere, compared with only 4.6-13% of the ipsilateral hemisphere in non-malignant stroke (249). Factors other than radiological stroke volume have also been investigated, including raised white cell count, systolic blood pressure >180mmHg, past medical history or hypertension or heart failure, and patients presenting with a National Institutes of Health Stroke Scale (NIHSS) of ≥20 on admission who also present with nausea or vomiting (207). There has also been emerging interest in blood markers, including serum levels of the astroglial protein S100B which has shown to be predictive of a malignant course with 94% sensitivity and 83% specificity when measured 24 hours after stroke onset (250). Further identification and characterisation of serum markers appear promising, not only for the future identification of patients who develop a malignant course, but also for novel non-surgical therapeutic agents.

1.1.17 Reperfusion injury and haemorrhagic transformation

Reperfusion is the major focus of interventional therapy for acute cerebral ischaemia, and when it occurs it can greatly limit the size of the resultant infarct in a time dependent manner, providing a rationale for early intervention. The therapeutic reperfusion window however is relatively short; once it has closed, reperfusion can have deleterious effects and is known as reperfusion injury. Reperfusion injury results from a number of different processes including endothelial activation, oxygen free radical production, an inflammatory response, increased

cytokine production, vasogenic oedema and haemorrhagic transformation, with BBB disruption and postischaemic hypoperfusion being common mechanisms (181, 251).

Haemorrhagic transformation occurred in 6.4% of patients treated with rt-PA within 3 hours of stroke in the original NINDS clinical trial and only 0.6% of patients given placebo (167), but mortality and functional outcome was improved in the treatment group in spite of the increased complication rate. ECASS III demonstrated that rt-PA was effective up to 4.5 hours after stroke with an increased incidence of intracerebral haemorrhage (2.7% versus 0.3%) but a non-significant reduction in mortality (6.7% versus 8.2%) (171). Further systematic reviews have demonstrated improved functional outcome when rt-PA was given between 3-4.5 hours after stroke onset, despite an increased incidence of haemorrhagic transformation (172). Robust data regarding haemorrhagic complications with thrombolysis beyond 4.5 hours are lacking, however the beneficial effects of reperfusion will eventually be overtaken by the deleterious effects of intracerebral haemorrhage with increasing time to thrombolysis, and at present the available evidence only supports use of intravenous thrombolysis to a maximum of 4.5 hours after stroke onset (20). The added challenge of reperfusion injury after thrombolysis requires a unique approach to the development of therapeutic strategies to maximise outcome after ischaemic stroke, and animal models that allow for reperfusion will be vital in their development (174, 252).

1.1.18 Current therapeutic guidelines

Several countries and organisations have developed best-evidence treatment guidelines for managing stroke, including Australia's National Stroke Foundation (20). The most recent revised edition, published in 2010 by the Australian Government, National Health and Medical Research Council, is a critical appraisal of current primary evidence in the literature

for both acute and post-acute stroke care in a contextual summary for Australian patients and health care workers, with an emphasis on a multidisciplinary team approach, and with special consideration for Aboriginal and Torres Strait Islander people. The following is a short summary of current approved therapies and treatment recommendations for acute cerebral ischaemia.

1.1.18.1 Standard medical therapy

Treatment of stroke patients in specialist stroke units significantly reduces death and disability compared with conventional care within general hospital wards; all patients with stroke should be admitted to hospital and treated in a stroke unit with a multidisciplinary team (18). Blood pressure of more than 220/120 mmHg should be cautiously reduced (by no more than 10-20%), and supplemental oxygen given only to patients who are hypoxic (253, 254). Blood glucose should be checked and measures to ensure euglycaemia should be initiated, particularly if the patient is diabetic. Fever is associated with poorer outcomes after stroke, and antipyretic therapy should be initiated in addition to finding and treating potential causes of fever, typically chest or urinary tract infection (255, 256).

Early seizures (<7 days) occur in 2-33% of patients after stroke, however long term anticonvulsant therapy should be reserved for patients with recurrent seizures, and the preferred medication and duration of therapy are as yet unclear. Hydration needs to be assessed and dehydration prevented with appropriate supplementation (257). Swallowing should be formally assessed to ensure a safe, functional swallow to prevent aspiration and associated complications, and nasogastric tube feeding commenced in patients without a functional swallow (258). Urinary catheterisation should be avoided with the exception of acute urinary retention.

Early mobilisation, adequate hydration and antiplatelet therapy are recommended to help prevent deep venous thrombosis (DVT) and pulmonary embolism (PE) (259), however low molecular weight heparin (LMWH), or unfractionated heparin if LMWH is unavailable, may be cautiously used in acute ischaemic stroke patients at high risk of DVT/PE (260, 261). Thigh-length anti-embolic stockings are not recommended for DVT/PE prevention post-stroke (262). All stroke patients with decreased mobility and other risks for developing problems related to pressure ulcers should be evaluated regularly and provided with appropriate pressure relieving aids and strategies (263). Treatment for airways obstruction should be initiated in patients found to have obstructive sleep apnoea (264, 265).

1.1.18.2 Reperfusion therapies

Thrombolysis with intravenous rt-PA in acute ischaemic stroke should be undertaken within 4.5 hours of stroke onset only in patients satisfying strict inclusion and exclusion criteria (172, 173). Intravenous rt-PA should be administered as early as possible to limit the size of the infarct, and should only be used in specialist hospitals with adequate expertise and equipped for rapid stroke assessment. Intra-arterial thrombolysis with urokinase or recombinant pro-urokinase can be used up to 6 hours from stroke onset in carefully selected patients (173), however the expertise to perform intra-arterial drug delivery is currently limited to only a handful of institutions within Australia. There is currently little clinical evidence to support the use of mechanical clot retrieval devices, however early data are encouraging and more patients are needed for enrolment into continuing clinical trials.

1.1.18.3 Neuroprotection therapies

There have been many purported neuroprotective drugs and therapies for acute ischaemic stroke studied in clinical trials, however none have demonstrated any clear benefit and therefore are not recommended for routine clinical use. Some methods that initially appeared promising, such as active cooling, have shown to have no reduction in the combined risk of death or dependency in a Cochrane review, and a non-significant increase in infection (255). Statin therapy appears to have a slight neuroprotective effect; patients who are on a statin therapy before stroke appear to do better and those on statins who have the drug withdrawn after stroke appear to do worse, hence patients already on statin therapy who suffer an ischaemic stroke should continue with this drug (266). All other agents that have shown early promise in animal or small clinical trials should only continue to be used as part of a randomised controlled trial.

1.1.18.4 Surgery and management of ischaemic cerebral oedema

Patients aged 18-60 with large MCA infarction should be urgently referred to a neurosurgeon if surgery can take place within 48 hours of the stroke onset for consideration of decompressive hemicraniectomy (243). Corticosteroid use to reduce oedema volume has no benefit and in fact causes harm, and is therefore contraindicated (230). Osmotherapy (with glycerol or mannitol) and hyperventilation may be used in patients awaiting neurosurgical consultation or those who are deteriorating on the way to the operating room, however as stand-alone therapies themselves there is insufficient evidence that they increase long-term outcome (226, 267).

1.1.19 Summary

Stroke is a devastating condition that disables or kills millions of people every year. Our understanding of cerebral blood flow and the pathophysiological mechanisms that cause and result from cerebral ischaemia has improved considerably in the last century, but despite advances in reperfusion therapies and ongoing randomised controlled trials a cure for stroke for the vast majority remains no closer. The establishment of clinical guidelines has nevertheless improved mortality and functional outcome after stroke, highlighting the importance of evidence based medicine in cerebral ischaemia. Mortality after malignant MCA stroke is significantly improved with decompressive craniectomy and duraplasty, however it continues to have a disproportionately high mortality compared with all supratentorial strokes; alternative treatments for space occupying cerebral oedema associated with ischaemia need to be explored and compared with decompression in animal and clinical trials.

1.2 Stroke research

Stroke research can be divided into work investigating pathophysiology and mechanisms of disease, and clinical trials translating applied knowledge into therapies that improve survival and functional outcome or disease prevention. Clinical trials are the ultimate test for general acceptance or otherwise of therapies in the medical community, however knowledge gained from basic science research, particularly animal models that aim to replicate human disease, are the cornerstone for advancing our understanding of this highly complex condition. The following section deals with stroke basic science research, particularly animal models of stroke.

1.2.1 History of stroke research

The present day understanding of the complex events that occur following cerebral ischaemia is the result of an evolution of centuries of investigation and knowledge, commencing with the scientific studies on the circulation that London physician Thomas Willis published his seminal work *Cerebri Anatome* (6). Working with Richard Lower and Sir Christopher Wren, the cerebral circulations of man and a range of different species were examined. Lower wrote of observations in carotid artery occlusion in dogs, neurological deficit attributable to reduced cerebral blood flow, and the significance of patent collateral vessels in maintaining function (6).

The next great advances came in the 18th century from of the understanding of the chemistry of respiration, particularly the role of oxygen, by the French chemist Antoine Laurent Lavoisier. A connection between cerebral blood flow or air deprivation and neurological function was forming, culminating in Legallois' observations on oxygenated arterial blood supplying the brain. Others began experiments on cerebral artery occlusion, such as London surgeon Sir Astley Cooper in 1836: "(after ligating both carotid arteries and vertebral arteries in the rabbit) respiration almost directly stopped; convulsive struggles succeeded; the animal lost consciousness and appeared dead. The (vertebral clamps were) removed – It laid upon its side, making violent convulsive efforts, breathed laboriously and its heart beat rapidly. In two hours it had recovered" (268). Later in the 19th century the site of respiration was confirmed as being at the tissue level, and haemoglobin was isolated from the blood and chemically characterised.

Contemporaries of Willis in the 17th century had recognised the brain received a greater proportion of blood per organ weight than almost anything else in the body, yet technological advances delayed any real attempts at determining CBF until Hill and Nabarro's measurement of oxygen and carbon dioxide contents of arterial and venous blood of dogs at rest and during

seizures in 1895 (269). Canine CBF was reported to be 138mL/100 g/min by Paul Jensen in 1904, and only 12mL/100 g/min in muscle, however Jensen was actually reporting flow in the whole head rather than just brain flow, thereby overestimating CBF (270). Further calculations by Harold Himwich made from the observations of Jensen and others revealed a CBF of 60mL/100 g/min, comparable to values quoted today. Later advances came in the discovery of a relationship between rates of CBF and states of consciousness or arousal. Oxidative metabolism of carbohydrates had by now been established as the major energy source of the nervous system, and the observation of membrane potentials and neuronal signalling lead to the proposal that the majority of oxidative metabolism is devoted to neural conduction (271). Finally it was deduced that the brain stores no oxygen, has no significant stores of glycogen and requires a steady flow of arterial blood to maintain its appetite for metabolic substrates; and that a failure of CBF, or hypoxia, or hypoglycaemia will rapidly be followed by irreversible neurological damage if homeostasis is not restored (272).

The modern era of stroke research began in the 1950s with the explosion of research technologies and support for research by major government and private funding agencies. Modern concepts of CBF were established by Kety and Schmidt, and Louis Sokoloff collaborated to investigate regional differences in flow in both normal and pathological states in studies using autoradiographic analysis of CBF in cats (273). Sokoloff was also instrumental in establishing regulation of CBF by factors such as blood pH, pCO_2 and pO_2, and the effect each had on cerebral function and metabolism (274). Finally, the introduction of computerised axial tomography and its derivatives, MRI and PET, have heralded a new era in neuroimaging and measurements of CBF *in vivo*. As can be seen, animals have played a vital role in the establishment of our current knowledge of CBF, brain metabolism and the effects of energy failure, and the contributions of animal studies, past present and future, cannot be underestimated.

1.2.2 Animal models of cerebral ischaemia

Animal models of ischaemic stroke have been categorised into global or focal ischaemia, and further still by the species of animal used (275). Global models investigate the response to a reduction or cessation of total cerebral blood flow, such as occurs in cardiac arrest. Focal models of ischaemia can be further considered by the location of the occlusion (MCA, ICA, basilar, multifocal etc), the duration of occlusion (permanent versus temporary) and the method of occlusion (intracranial vascular occlusion, microcirculatory occlusion or extracranial vascular occlusion) (276). Temporary arterial occlusion with reperfusion tends to produce a variable and less predictable amount of ischaemic injury but better mimics human stroke, particularly when associated with thrombolysis reperfusion or spontaneous recanalisation (277, 278). Survival is also greater in temporary occlusion models, which may be more suitable to assess long-term outcomes. The site where the artery is occluded will also affect the size of the stroke, with more proximal MCAO for instance producing infarcts involving both subcortical and cortical structures, while more distal occlusions tend to spare subcortical areas, although proximal occlusion may sometimes spare the cortex in the event of good collateral flow from adjacent territories (so-called “striatocapsular” infarct) (15).

Focal models typically involve occlusion of a major cerebral blood vessel, most commonly and most clinically relevant being the MCA. There have been multiple techniques of producing permanent and transient MCAO in various animal species, including: a transorbital approach in cats (279, 280), dogs (281, 282), monkeys (283) and rats (284); a retro-orbital approach in cats (285); a transcranial approach in rats (286-290), cats (291), dogs (292), gerbils (293) and monkeys (294); an intraluminal occlusion model with embolic devices in rats (295), rabbits (296), dogs (297) and monkeys (298); an intraluminal occlusion model via a retractable thread in rats (299, 300), mice (301, 302), rabbits (303) and monkeys (304); a clot embolism model in rats (305-308) and dogs (309); and a vasospastic occlusion model

with endothelin-1 in rats (310) and cats (311). Frequently CBF is monitored with transcranial Doppler or PET during arterial occlusion to demonstrate a reduction from pre-occlusion baseline, or evidence of neuroelectrical activity suppression via EEG is accepted as evidence of reduced regional CBF (312-314). Traditionally, the MCA was approached surgically transcranially, retro-orbitally or transorbitally (with enucleation) but with substantial dissection required, dural opening and variable retraction injury to the brain. In 1986 though, a new rodent method of intraluminal thread occlusion allowing for either permanent or transient occlusion was published by Koizumi, in which a silicone coated 4/0 suture was introduced into the ICA and advanced past the origin of the MCA (299). The two greatest advantages of this approach are the relative ease with which the occlusion can be achieved compared with direct surgical approaches, and the simplicity with which the circulation can be reperfused (315). The intraluminal approach is associated with a 12% incidence of subarachnoid haemorrhage however, and transecting the external carotid artery results in ischaemia of the muscles of mastication, making eating and swallowing difficult and producing a reversible weight loss (249). Unsurprisingly the intraluminal thread model of rodent MCAO was rapidly adopted as the preferred animal model for stroke research, and the more complicated, invasive and costly larger animal models were used sparingly although not forgotten entirely, and may in fact now be experiencing a resurgence in the literature.

For the purposes of stroke research, animal species are generally divided by size into small (rats, mice etc.) and large (cats, dogs, pigs, nonhuman primates etc.). Small animals have the advantages that they are cheaper to supply and house, allow for investigation of genetic knockout mutations, and produce consistent and reproducible infarcts (249, 299). Sophisticated physiological monitoring is however somewhat limited in small animals, and there are growing concerns that their lissencephalic brains and other neuroanatomical differences limit their translation to human neurological disease. Most large animals on the other hand have gyrencephalic brains and otherwise similar neuroanatomy to humans,

sophisticated neurological and physiological monitoring is able to be performed (often with the same hardware and techniques used clinically), and regional imaging techniques such as MRI and PET are also easier in the physically larger brains. Large animals are generally more expensive to supply and house than rodents, anaesthetic regimes are more complicated, the presence of a rete mirabile (316) in many large animals precludes an endovascular approach to vessel occlusion necessitating complex and invasive intracranial surgery, and the resultant infarct is often more variable than equivalent rodent models. In addition, there are considerable animal welfare concerns with large animals, particularly nonhuman primates, and rodent models are frequently seen to be more ethically acceptable.

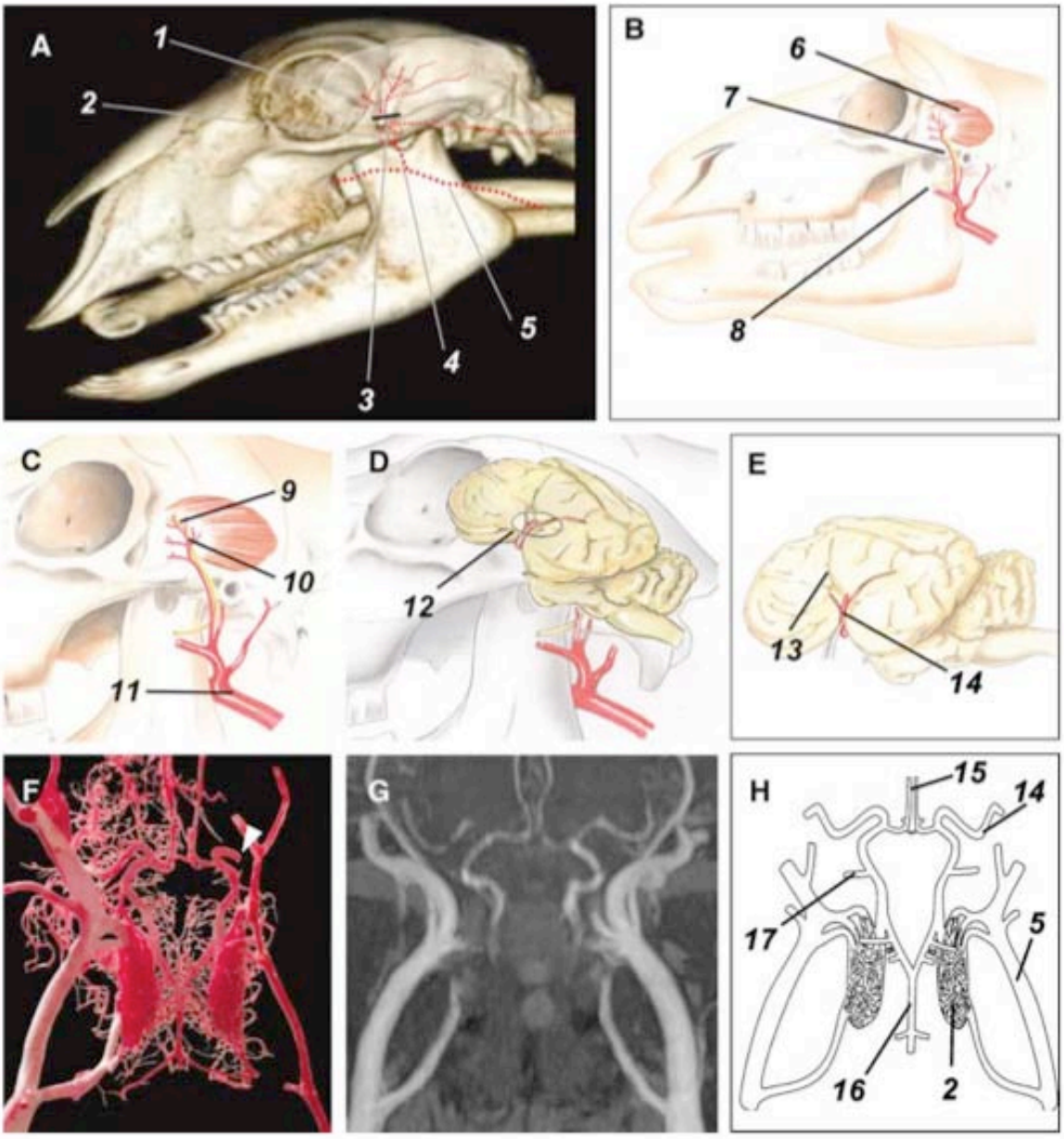
The distinction between small and large animals is almost certainly an oversimplification, and neuroanatomical evolution from a smooth, lissencephalic brain (as in the rodent) to a complexly infolded gyrencephalic brain (as in humans and many nonhuman primates) would suggest greater similarities in normal and abnormal states in species sharing neuroanatomical features (317). One general rule of experimental stroke models is that infarcts tend to become more uniform the lower the animal species (276). Although they are quadrupeds like the rodent, ruminants including sheep have gyrencephalic brains like the human and also dense white matter tracts and a strong fibrous tentorium cerebelli (318). There are clear continuing ethical problems with the use of certain species, particularly nonhuman primates but also cats and dogs, therefore ruminants may offer a suitable compromise, particularly sheep which are abundant, of moderate cost and easier to handle and house than larger subspecies, such as cows.

The effects of animal MCAO vary considerably depending on the species, strain, method of occlusion and duration of occlusion, with or without reperfusion. Rodent MCAO typically results in large volume hemispheric strokes akin to human malignant MCA stroke, yet the mortality rate in rodents nowhere near approximates that in humans (249), further alluding to

problems in translating lissencephalic rodent stroke to the gyrencephalic human brain. Permanent MCAO results in different lesion size depending on the strain of rat, with larger volume strokes in Fischer-344 and Sprague-Dawley rats than Wistar rats, and larger volumes again in spontaneously hypertensive rats (319). Reperfusion via the intraluminal thread model improves survivability, and medium- to long-term functional outcome assessments are now well established with this method (320). Cat models of MCAO result in large hemispheric stroke, but unlike rodents they are associated with progressive cerebral oedema and mortality rates approaching human malignant stroke (321). In Gerbils, which lack a PComA, bilateral occlusion of the common carotid artery results in global cerebral ischaemia, and unilateral occlusion causes severe neurological deficit in 30-40% and death within several days (319). Regarding gyrencephalic nonhuman primates, MCAO was extensively studied in baboons in the 1970s with sophisticated neurobehavioural monitoring able to be performed, which demonstrated neurological deficits including facial and upper limb weakness with some recovery over months to years, similar to human MCAO. Space occupying ischaemic cerebral oedema has been investigated in rodent models of MCAO, and although oedema and midline shift have been observed radiologically (322), ICP is usually recorded from the posterior fossa which is problematic in a species that lacks a significant tentorium cerebelli and therefore any meaningful compartmentalisation between brain and cerebellum (320, 323-325). Because of these intrinsic anatomical properties, the rodent is therefore probably a poor model of malignant MCA stroke. There have been attempts to measure ICP after large volume MCA stroke in large animal models over the years, however most of these studies have been limited to very short monitoring periods of only 6-12 hours after stroke or plagued by technical difficulties and outdated ICP monitoring technologies (280, 326-328).

A sheep model of focal MCA occlusion ischaemic stroke was described in 2008 by a group in Leipzig, Germany, headed by Johannes Boltze (329). Via an invasive retro-orbital craniotomy, Boltze's group characterised the effects of selectively and permanently occluding

the prebifurcation MCA trunk, one post-bifurcation branch, or both branches in sheep maintained for up to 6 weeks after surgery. MRI and PET examinations were undertaken at varying intervals during the survival period, and behavioural phenotyping of neurofunctional disability was performed. Radiological findings were correlated with gross pathological and microscopic histopathological changes. With 100% survival 6 weeks post-stroke and uncomplicated species-specific animal housing, Boltze's group surmised that their surgical approach to permanent MCAO could be used as an alternative to existing large animal models, particularly for investigating the efficacy of novel therapies. Although a seemingly robust model, their method of MCAO was limited to permanent occlusion only without reperfusion via a mechanism unnamed in their methodology, and their description of the highly complicated approach to the MCA was generally lacking: "after exposure of the skull bone surface, a hole was drilled into the parietal plate using a set of neurosurgical burrs and was then extended using Kerrison rongeurs. On local incision of the dura mater, MCA or its branches…were permanently occluded" (329). They noted that infarct volumes were significantly larger with greater number of MCA branches occluded, with infarct volumes calculated on DWI MRI figures were quoted in mL (rather than as a percentage of hemisphere or supratentorial brain involved). Mortality secondary to space occupying cerebral oedema was conspicuously absent, despite the authors claiming complete proximal MCAO in 10 out of 30 animals and an extended monitoring period well beyond the known window for the development of malignant MCA stroke. Furthermore, representative MRI figures of total MCAO demonstrated cortical infarction but sparing of the deep subcortical grey matter supplied by perforators coming very proximally from the M1 segment, and evidence of increased diffusion adjacent to the "drill hole", which was actually located very distally on the lateral surface of the brain, not inferiorly at the skull base where the MCA takes origin. This evidence suggests that in this study "total" MCAO refers only to the MCA at the level of the



Intraoperational situs, topographical anatomy and cerebral blood supply. Principle of the surgical procedure and the most important anatomic structures in the operational field. (**A**) gives 3D CT reconstruction of a sheep skull (obtained in planning experiments before the study). (**B**) to (**E**) show illustrations drawn from series of photographs focusing on important structures. (**1**) MCA supply area (schematic drawing of branches and capillaries); (**2**) rete mirabile epidurale rostrale; (**3**) approximate location of total MCAO; (**4**) ramus caudalis ad rete mirabile epidurale; (**5**) maxillary artery (note: all structures are projected on lateral bone surfaces but can be found behind these surfaces); (**6**) temporal muscle; (**7**) A. auricularis caudalis; (**8**) N. facialis (N. VII); (**9**) N. auriculopalpebralis; (**10**) A. temporalis superficialis; (**11**) A. carotis externa; (**12**) drill hole; (**13**) Sulcus centralis; (**14**) MCA. Blood supply of the sheep brain is illustrated in (**F**) to (**H**). Pictures show a basal view of the circulus arteriosus and are adapted from Förschler *et al*, 2007. (**F**) A corrosion cast made by intra-arterial delivery of Mallocryl M (Laborchemie Apolda, Jena, Germany) at day 43 after MCAO. Because of the MCAO location (white arrow head) the distal supply area of the left MCA is not filled with Mallocryl M. (**G**) Time of flight MRA of a control animal not subjected to MCAO. (**H**) The corresponding scheme drawing. (**15**) A. cerebri anterior (rostralis); (**14**) MCA; (**5**) A. maxillaris, which supplies the (**2**) rete mirabile epidurale rostrale; (**16**) A. basilaris; (**17**) A. choroidea rostralis.

Figure 1.10: Approach to the MCA in and neurovascular anatomy of the sheep. In the description by Boltze et al., a small craniotomy was performed to occlude the prebifurcation MCA or one or both of its branches (A-E). Sheep neurovascular anatomy (F-H); the rete mirabile is clearly demonstrated, and the site of MCA occlusion can be seen at the white arrowhead in *F* (329).

bifurcation, not at its origin. Boltze correctly identified that the presence of a *rete mirabile* (Latin: ‘wonderful net’) in the sheep precluded an intraluminal approach to MCAO and necessitating a direct surgical approach to the MCA. Absent in rodents, it was also refuted as a structure of human vascular anatomy by the famed Flemish anatomist Andreas Vesalius in his 1543 volume *De Humani Corporis Fabrica* (330), however is generally present in a large number of vertebrates and particularly ungulates, including pigs, oxen and sheep, but not horses (331, 332). Absence of a *rete mirabile* in the sheep would significantly reduce the technical difficulty of MCAO by exchanging the complex transcranial surgical approach for a simpler intraluminal approach as per the rodent model, and also allow for reperfusion. Despite its disadvantages however the sheep model appears exciting as an alternative to current rodent models of MCAO.

1.2.3 Other models

We assume that animal models are an accurate representation of human health and disease for a wide variety of biological systems, including the CNS. When assessed with formal scientific validation however, animal models are frequently not helpful in contributing to the development of clinical interventions, or are substantially different from clinical outcomes (333). The quality of the methodology may play a part and is a variable factor, however interspecies differences are undeniable and generally unmodifiable. Non-animal models require formal validation studies before their acceptance, which should be extended to animal models if we are to continually ethically justify their ongoing use.

The CNS is highly complicated with multiple integrated physiological systems. Although an exciting prospect, replicating the effects of MCAO for instance in a computational model is a very long way from becoming a reality with current technology. Any non-animal models

would require a highly sophisticated interaction between oxidative metabolism, cellular chemistry and genetics, CBF, the BBB, hydrostatic and osmotic forces, the immune response, the cerebrospinal fluid and ICP. It is difficult to assess more than one of these factors at a time *in vitro*, let alone all of them. Techniques to better investigate the study of basic metabolism, physiology and anatomy in living humans have been proposed (334), but will not permit trialling novel therapies. *In vivo* animal models are currently the only method of investigating the complex responses of the CNS to ischaemic injury. Considering the current need for animal models of stroke, the general lack of validity for acceptance and the overwhelmingly poor clinical translation especially in stroke research (169, 335), hypotheses should be carefully chosen and animals should be relevant to them, particularly when there are no non-animal alternatives (336).

1.2.4 Failings and research priorities

Brazilian researcher Juliana Casals has noted that "the success of stroke studies in animals depends on the choice of the experimental model species", further arguing that "it is the most important aspect of experimental design", and that "an inadequate model may lead to limitations that compromise results and analyses" (15). Although highly important, the choice of animal model is but one component of preclinical testing that needs careful consideration to improve clinical translation. There is nevertheless a large discrepancy between neuroprotective and restorative drugs that are successful in preclinical testing and those that translate to the clinical setting, and much has been written regarding the role that the choice of animal model plays in this translational failure.

The Stroke Therapy Academic Industry Roundtable (STAIR) committee was formed in order to help address these failures, and published their initial recommendations for standards

regarding preclinical neuroprotective and restorative drug development in 1999 (252). The group, chaired by Marc Fisher, intended to propose mechanisms to improve the translation of neuroprotective and restorative drugs from animal models to clinical therapies, based on a consistent and clear failure of these drugs to be effective in humans despite preclinical success. STAIR recommendations included dose of drug used, optimal window of opportunity, physiological monitoring, outcome measures, target populations, sex differences, genetic manipulation, combined pharmacological agents and appropriate animal models. Regarding animal models, the recommendations were to study permanent occlusion models prior to reperfusion models; preclinical testing should begin with rodents, and then proceed to cats or nonhuman primates before entering large human clinical trials. The need for standardised neurobehavioural testing was also recognised. The vast dissimilarities between rodent and human brain were highlighted, together with the role that gyrencephalic primate models can offer, particularly with regard to neurological recovery, and stroke recovery drugs that have shown promise in rodents could be explored in primates (252).

Initial STAIR Preclinical Recommendations

1. Adequate dose-response curve
2. Define the time window in a well-characterized model
3. Blinded, physiologically controlled reproducible studies
4. Histological and functional outcomes assessed acutely and long-term
5. Initial rodent studies, then consider gyrencephalic species
6. Permanent occlusion then transient in most cases

Figure 1.11: The initial STAIR recommendations. From Fisher, M. et al. Update of the stroke therapy academic industry roundtable preclinical recommendations (252).

The STAIR group has since released multiple publications regarding the designing of clinical trials, advancing development of acute therapies, improved technology and outcome measures, thrombolytic and neuroprotectant therapy, and extended window therapy trials since their first recommendations (337-341). In addition, in 2009 they also released an update on their original 1999 recommendations for preclinical testing, based on the observations that the original recommendations were not closely adhered to nor rigorously validated. Further to their original recommendations, they suggested that initial evaluations in young, healthy male animals should be followed by studies in females, aged animals and animals with comorbid conditions (including hypertension, diabetes and hypercholesterolaemia) to improve clinical translation (342). Other updated recommendations included defining dose response and therapeutic windows, the use of multiple outcome measures including histopathological and behavioural endpoints, the use of basic physiological monitoring, reproducibility in multiple laboratories, and the use of multiple species, particularly animals with gyrencephalic brains (such as cats or primates) following early success in rodents or rabbits (342). In addition to histology and neurobehaviour, South Australian stroke researcher Renée Turner has suggested that death as an outcome measure, which is important in human clinical trials and a component of the original rabbit thrombolysis study (166), should also be included in preclinical rodent trials to avoid skewing results biased towards surviving animals (343), and ideally this should be extended to large animal models too.

Turner and others have identified the failings of using young healthy male animals to replicate human stroke, which is generally associated with multiple medical comorbidities including advanced age, hypertension, hypercholesterolaemia, diabetes and smoking (344-347). A better model of stroke may not be simply in the choice of animal used, but the ability to incorporate the effects of human comorbidities within the model (334). The use of anaesthesia in stroke models is a further confounding factor, as many anaesthetic agents have neuroprotective qualities and can limit the resultant infarct size after MCAO (348). Models in

which animals are prepared under anaesthesia, and the stroke subsequently performed after recovery from anaesthesia, are more likely to replicate human stroke in which ischaemia results from acute thrombus occlusion of a cerebral artery in the awake patient, rarely the anaesthetised one (343).

A working party was also established in Europe to attempt to identify research priorities that could potentially result in major advances in the field. The Stroke Research Workshop, headed by Stephen Meairs, met in Brussels in October 2005 with the objective to "identify research activities that could potentially result in major advances in the areas of stroke prevention, treatment and recovery" (349). Areas of interest included reperfusion therapies, optimising delivery of care, neuroprotection, recovery after stroke, cerebrovascular biology, stroke imaging and stroke prevention. Animal models were specifically acknowledged in identification of stroke injury mechanisms, and were also identified for ongoing use in evaluating stem cell therapies and the development of small animal models of lacunar stroke.

Unfortunately in many developed countries, stroke is regularly given a low funding priority in relation to its large impact on society. Considering the clinical and financial burden that it has on the world it is relatively surprising that stroke is so poorly funded, especially when compared with the other two most common causes of death in developed nations, heart disease and cancer. Over the last three decades in the United States of America, the United Kingdom and Europe for instance, stroke research has consistently been underfunded when compared to either cancer or heart disease by a ratio as much as 50:1 (350). Such disparities between research and the burden of disease will only amplify as the world's population ages (351); it has been predicted that stroke will account for 6.2% of the total burden of illness by 2020 (349). Without the development for more effective treatment and prevention of stroke, this burden can only be expected to increase exponentially.

1.2.5 Potential targets for future therapies

Neuroprotection is the holy grail in stroke research, and just as elusive; despite thousands of experimental drugs being investigated and hundreds showing promise in preclinical studies, all but rt-PA have failed to translate to successful clinical therapies (169, 352). As always however, there are a few agents currently being investigated that are showing early promise, and will likely still need to be combined with reperfusion therapies.

Although associated with improved functional outcome, rt-PA is frequently associated with a considerably variable treatment response and neurotoxicity, and nonneurotoxic thrombolytics are now under investigation (349). Desmoteplase (from vampire bat saliva) is more fibrin-specific than rt-PA and is nonneurotoxic, tenecteplase is a genetically modified product of alteplase, and Ancrod and Batroxobin are snake venom derivatives (353); all but Batroxobin are undergoing various Phase II or III clinical trials (354).

Neuroprotection in cerebral ischaemia has been the focus of preclinical animal testing for decades. Iron chelators can upregulate hypoxia-inducible-factor-1α and increase transcription of survival genes; several iron chelators have reduced stroke volume in animal models (355-357). Animal models of granulocyte colony-stimulating factor have also showed early success in reduction of stroke volume and improved neurological outcome via anti-apoptosis mechanisms (358). Free radical scavengers such as ebselen (359), or trappers such as NXY-059 (355), have been developed based on experimental stroke models and clinical trials are ongoing. *N*-methyl-d-aspartate (NMDA) receptor antagonists modulate excess glutamate release (360, 361), and combination therapy of prostaglandin E1 and lithium have shown to reduce infarct volume and neurological deficit in rodent models of focal stroke (362). Oral hypoglycaemic drugs used in diabetic patients have possible neuroprotection and/or anti-oedema qualities; pioglitazone reduces plasma TNF-α levels and improves antioxidant levels (363), and glibenclamide blocks the sulfonylurea channel SUR1 to mediate cerebral oedema

after ischaemia and has been demonstrated to be superior to decompressive craniectomy in a rodent model of malignant MCA stroke (210, 231), as well as being neuroprotective in Wistar rats (364). Transplanted stem cells are showing promise in minimising injury and enhancing plasticity after cerebral ischaemia, and have been shown to migrate into areas of injury in animal models (365). Some, all or none of these agents, in combination with reperfusion (366), may lead to new treatment strategies for ischaemic stroke that may treat the stroke core, rather than targeting the ischaemic penumbra.

1.2.6 Summary

Animal models of cerebral ischaemia have existed for hundreds of years, and are becoming increasingly sophisticated, particularly since the latter half of the 20th century. The substantial knowledge of the pathophysiological events relating to cerebral arterial occlusion is directly attributable to what we have learned from models of focal and global ischaemia. Unfortunately, the intricacies of human stroke are far more complicated than developing different ways to occlude cerebral arteries in different species of laboratory animal, which may explain why preclinical testing of novel therapeutic agents has failed to translate to successful clinical therapies. A worldwide underfunding of stroke research demands that we be smarter and more efficient in developing animal models that better replicate the human condition, so that preclinical testing may result in improved clinical translation.

2. Aims and hypotheses

Based on what we currently know regarding acute ischaemic stroke, the urgent need for novel therapeutic agents, the shortcomings in translation with current rodent models of middle cerebral artery occlusion and the promise of the sheep as an exciting, intermediary large animal alternative to the rodent, the body of the work of this study was aimed at the characterisation of the acute changes following proximal MCAO in a sheep model. The experiments that follow each constitute a manuscript either published in the peer reviewed scientific literature or submitted for consideration of publication addressing the aims and hypotheses below. A fourth manuscript is included describing a technical note for experimental invasive arterial blood pressure monitoring which shows promise for highly accurate and reliable systemic blood pressure readings, and which has been adopted amongst others in the laboratory as the preferred method for blood pressure recording.

2.1 Aims

1. To develop and characterise a transcranial surgical approach to occlusion of the proximal middle cerebral artery in the anaesthetised sheep
2. To trial and evaluate different methods of permanent and temporary proximal middle cerebral artery occlusion
3. To characterise the intracranial pressure response, regional brain tissue oxygen response, magnetic resonance features and histopathological features following hyperacute (4 hours) middle cerebral artery stroke in the sheep with both permanent and temporary (2 hours) arterial occlusion
4. To investigate the relationship between duration of temporary arterial occlusion and the development of regional brain tissue hypoxia

5. To characterise the intracranial pressure response, regional brain tissue oxygen response, magnetic resonance features and histopathological features following acute (24 hours) middle cerebral artery stroke in the sheep with both permanent and temporary (2 hours) arterial occlusion

2.2 Hypotheses

1. A transcranial surgical approach to the proximal middle cerebral artery can be developed in the sheep
2. The sheep proximal middle cerebral artery can be occluded either permanently or temporarily to allow for reperfusion
3. After permanent arterial occlusion, regional brain tissue oxygen will decline permanently, and after temporary occlusion, regional brain tissue oxygen will decline for the period of occlusion and rise again towards baseline levels following reperfusion
4. In the first 24 hours after arterial occlusion with large volume middle cerebral artery stroke in the sheep there will be radiological features of space occupying cerebral oedema, raised intracranial pressure and early mortality attributable to cerebral herniation, similar to human malignant middle cerebral artery stroke

3. Preliminary experimental design

The development and characterisation from scratch of a surgical model to access and occlude the sheep proximal MCA required considerable thought, planning and preliminary experiments before proceeding to the final experiments. In the very beginning due diligence took place in the form of familiarising with the current literature regarding surgical access to the sheep MCA, neuroanatomical texts relating to sheep brain, head and neck anatomy particularly, as well as examination and dissection of various dry sheep skulls that were available in the laboratory.

Examination of dry sheep skulls achieved two specific things. First, the position of the anterior and middle cranial fossae and their relationship to external skull landmarks were made clear, in particular the location that a craniotomy would need to be placed in order to access the basal anterior cerebral circulation and overlying structures, such as the zygomatic arch and the coronoid process of the mandible. Secondly, we were able to trial various methods of head positioning; human neurosurgical patients frequently have their heads stabilised in a 3-pin rigid fixation device (such as the Mayfield skull clamp, Integra LifeSciences, New Jersey, USA), and with the delicate nature of the planned sheep neurovascular surgery it was felt that a similar fixation device may be beneficial. As the human calvarium is relatively large it is possible to place 3 separate pins around the equator of the skull; the sheep skull however consists of a very long snout, prominent orbits and only a relatively small calvarium, hence a pinned mechanism of head fixation is not possible. Various methods of fixation directly to bone were trialled, eventually leading to the development of a 4-pin screw device that fixed directly to the bony snout, which itself was ultimately abandoned in favour of a much simpler sand bag and sports tape support and retraction system.

The next stage involved dissection of culled sheep heads obtained from carcasses of animals

from other researchers' experiments, with the approval of the University of Adelaide and SA Pathology Animal Ethics Committees. Six culled male Merino sheep heads were obtained, frozen, and thawed as required prior to dissection. Specifically, the goals in dissecting these culled heads were to clearly establish the relationship of sheep external surface anatomy landmarks to the contents of the intracranial cavity. Or otherwise put, that a craniotomy would provide sufficient access to the proximal MCA in order to occlude it and produce a stroke. Dissection of these culled heads proved invaluable; the extent of removal of the mandibular coronoid process became very clear, and the small access to the cerebral circulation, the narrow working channel and the need for not only magnification but also good illumination became apparent. A simple operative microscope was trialled, however ultimately simple loupe magnification with a head mounted LED light source (Surgical Acuity, Wisconsin, USA) proved simpler and superior.

The initial anaesthetic regime was important. The goal was to provide adequate analgesia and anaesthesia, but to avoid as much as possible neuroprotection and thus any impact that anaesthesia could have on the size of the stroke produced. An effective sheep general anaesthesia regime had been used for years by other researchers within our department, consisting of inhalational isoflurane and intravenous ketamine. Isoflurane can be neuroprotective about certain concentrations, however when monitored appropriately and when used in conjunction with other agents (such as ketamine) its dosage can be kept below neuroprotective levels; this is further discussed in chapter 5.6. Our first live animal experiments were conducted with the isoflurane and ketamine regime, and this was not altered significantly for the duration of the project.

In the first series of 4-hour experiments, a total of 32 animals were randomised to sham surgery (n=6, 3 male), permanent MCAO (n=10, 9 male) or temporary MCAO with reperfusion (n=16, 12 male). As one of the original aims was to determine which techniques

could be established for different methods of permanent and temporary occlusion the groups within the original 32 animals were not uniform, therefore only 18 representative animals were included in the experiments in chapter 4.1 (n=6 per group). For permanent occlusion, electrocautery with bipolar diathermy was always the first choice, with other options considered including silk ligation and a Weck ligature clip. The ease with which the proximal MCA could be reached with the bipolar forceps tips and the consistency of permanent occlusion with this method resulted in its exclusive use for permanent MCAO. Post-mortem examination of each brain subjected to permanent occlusion revealed a small area of electrical damage at the proximal MCA, which was helpful in confirming the site of occlusion however demonstrated that this method was not without collateral, albeit relatively small, injury to the brain tissue adjacent the vessel. A plexus of sympathetic nerves runs on cerebral arteries for neurogenic control of cerebral autoregulation and damage to them following electrocautery is inevitable, however the effects of this in the setting of permanent MCAO are unknown.

Temporary MCAO with reperfusion was more problematic; the aim was to apply a focal external pressure to the MCA sufficient to occlude the arterial lumen and cease blood flow through it but without damaging the vessel or particularly the endothelium, such that releasing the pressure would restore blood flow to pre-occlusion levels, and via a mechanism that could reach the depths of the proximal MCA in the skull base with technical efficiency. Temporary aneurysm clips have become increasingly sophisticated to provide a closing force sufficient to reduce or eliminate blood flow in cerebral vessels, but without producing any long-term endothelial damage (367). The closing force of an aneurysm clip is an extremely important factor particularly in small arteries, where excessive forces are not tolerated and which may result in endothelial injury and clot formation (368). Temporary aneurysm clips are designed to have a much lower closing force than their permanent counterparts and therefore less propensity to cause injury, however have still been demonstrated to cause mild to moderate focal endothelial injury in an experimental piglet model with temporary occlusion of the

common carotid and subclavian arteries for times of up to 30 minutes (369). Closing force increases exponentially with increasing distance from the fulcrum of the clip (the “lever law”) (368), such that the closing force at the base of the blades in a temporary clip could in fact be higher than at the tip of the blades in a permanent clip (370-372). For this reason, all attempts were made to occlude the vessel in this study with the blades nearest the tip rather than the base to minimise the possibility of injury and maximise the chance of reperfusion in the relatively small (approximately 1-1.5mm diameter) sheep MCA. Although we did not specifically look for histological evidence of focal endothelial injury at the site of placement of the temporary aneurysm clip, we did observe for macroscopic evidence of focal thrombus in every post-mortem, particularly in animals that had a delayed or absent rise in $PbtO_2$ following clip release, and found none.

Because of the (relative) ease of atraumatically placing and removing the temporary aneurysm clip at the proximal MCA with the clip applicator it was adopted as the preferred method of temporarily occluding the vessel. Several attempts were made early on to occlude the vessel with a silk ligature snare. With this method, a 4/0 silk suture was fed around the proximal MCA after it had been freed from its overlying arachnoid, and a small diameter plastic catheter sheath was passed over both free ends of the suture. The snare was tightened simply by advancing the sheath along the suture loop, progressively shrinking the snare; once tight enough to occlude the vessel, the sheath was then held in place with an artery forceps, which could then be released to loosen the snare and allow reperfusion. This method was attempted on 2 occasions, and was found to be technically very demanding and also resulted in focal trauma to the MCA with associated vasospasm.

Furthermore, the duration of temporary MCAO required several attempts before a standardised time was decided upon. For temporary MCAO in the development studies, occlusion times that were trialed ranged from 30 to 120 minutes. As has been demonstrated

repeatedly, the duration of cerebral arterial occlusion in experimental models of ischaemia generally determines the size of the resultant infarct (276, 277, 319, 373). Rodent models of MCAO with occlusion times of 30 minutes are always associated with some degree of infarction (277); in addition, temporary intraluminal rodent MCAO produces substantial penumbra after 60-90 minutes of occlusion (374). As expected, temporary occlusion in this study for only 30 minutes (n=1) or 60 minutes (n=2) resulted in a smaller sized infarcts than in animals exposed to 2 hours of occlusion. Given that the initial study employed an observation time of 4 hours, it was elected to standardise temporary occlusion to 2 hours to allow the same amount of time for reperfusion, and ultimately in the first cohort of 32 animals the combination of 2 hours MCAO with a temporary aneurysm clip followed by 2 hours of reperfusion was used 13 times (6 being included in the original 4-hour methodology manuscript (chapter 4.1), 5 included in the cortical hypoxia threshold manuscript (chapter 4.2), and the remainder being excluded due to technical reasons).

In summary, the initial methodology development was a complicated procedure involving more than 3 months of work before being attempted in a live, anaesthetised animal, and which still required further development following the first live animal experiments. There were many technical considerations and obstacles before the final successful and reproducible methodology was characterised; the results of the characterisation form the following chapters.

4. Experiments: manuscripts published or being considered for publication

4.1 A Surgical Model of Permanent and Transient Middle Cerebral Artery Stroke in the Sheep

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4.1.1 Abstract

Background: Animal models are essential to study the pathophysiological changes associated with focal occlusive stroke and to investigate novel therapies. Currently used rodent models have yielded little clinical success, however large animal models may provide a more suitable alternative to improve clinical translation. We sought to develop a model of acute proximal middle cerebral artery (MCA) ischemic stroke in sheep, including both permanent occlusion and transient occlusion with reperfusion.

Materials and methods: 18 adult male and female Merino sheep were randomly allocated to one of three groups (n=6/gp): 1) sham surgery; 2) permanent proximal MCA occlusion (MCAO); or 3) temporary MCAO with aneurysm clip. All animals had invasive arterial blood pressure, intracranial pressure and brain tissue oxygen monitoring. At 4 h following vessel occlusion or sham surgery animals were killed by perfusion fixation. Brains were processed for histopathological examination and infarct area determination. 6 further animals were randomized to either permanent (n=3) or temporary MCAO (n=3) and then had magnetic resonance imaging (MRI) at 4 h after MCAO.

Results: Evidence of ischemic injury in an MCA distribution was seen in all stroke animals. The ischemic lesion area was significantly larger after permanent (28.8%) compared with temporary MCAO (14.6%). Sham animals demonstrated no evidence of ischemic injury. There was a significant reduction in brain tissue oxygen partial pressure after permanent vessel occlusion between 30 and 210 mins after MCAO. MRI at 4 h demonstrated complete proximal MCA occlusion in the permanent MCAO animals with a diffusion deficit involving the whole right MCA territory, whereas temporary MCAO animals demonstrated MRA evidence of flow within the right MCA and smaller predominantly cortical diffusion deficits.

Conclusions: Proximal MCAO can be achieved in an ovine model of stroke via a surgical

approach. Permanent occlusion creates larger infarct volumes, however aneurysm clip application allows for reperfusion.

4.1.2 Introduction

The current clinical treatment of acute occlusive middle cerebral artery (MCA) stroke is directed towards early reperfusion of the ischemic brain in a timely fashion by thrombolysis or thrombectomy (171, 375). However, the majority of MCA occlusion (MCAO) stroke patients either do not meet the strict criteria or fail to receive significant reperfusion, and are managed with best medical therapy and decompressive craniectomy for the malignant MCAO variant (242, 243, 376). Over 1000 agents have shown to be efficacious in preclinical evaluation, with only tissue plasminogen activator translating into a successful clinical therapy (166, 169, 377). This poor translation has led many researchers to believe that rodent models have limited predictive value and that alternate large animal models are likely to become important in future translational research (319, 378). Given that the number of individuals that will suffer a stroke in the next decade will rapidly increase (350), novel therapies that can limit or reverse ischemic injury at a cellular level are urgently required.

An ovine model of permanent focal cerebral ischemia has recently been developed to capitalize on the advantages of large animal translation (329), however this model offers only permanent vessel occlusion and the technique of MCA exposure and occlusion was only briefly described. Accordingly, the aims of our study were to develop a sheep model of surgical proximal MCA exposure and occlusion, to establish the viability of different methods of vessel occlusion including those facilitating reperfusion, and to determine the extent and pattern of ischemic injury with different methods of occlusion with or without reperfusion. We describe in detail a technique we have developed of focal MCA occlusion in the sheep that allows investigation of either permanent or temporary proximal vessel occlusion.

4.1.3 Materials and methods

4.1.3.1 Experimental Procedure

All experimental protocols were approved by the Animal Ethics Committees of the University of Adelaide and SA Pathology, and conducted according to guidelines established for the use of animals in experimental research as outlined by the Australian National Health and Medical Research Council code of practice for the care and use of animals for scientific purposes (7th edition, 2004).

4.1.3.2 Animals and Experimental Design

18 adult male and female Merino sheep 18-24 months old (mean weight 50.1 +/-5.8kg) were allocated to the study. Animals were intra-operatively randomized to permanent occlusion (n=6), 2h temporary vessel occlusion followed by reperfusion (n=6) or sham surgery (n=6). Anesthesia was induced with intravenous thiopentone (1000mg in 20mL, Jurox Pty Ltd, Australia) and maintained with 1.5% inhalational isoflurane (Veterinary Companies of Australia Pty Ltd, Australia) in a mixture of oxygen and room air, plus intravenous ketamine (Parnell Australia Pty Ltd, Australia) infusion 4.0mg/kg/hr via a femoral venous line. These two agents were used to avoid the intrinsic neuroprotective properties of either above certain doses, but to maintain a twilight general anesthesia. With the animal supine, an arterial catheter was placed in the right femoral artery for continuous blood pressure monitoring and periodic arterial blood gas sampling. The animal was then placed prone in the sphinx position, burr holes were placed symmetrically in left and right parietal bones posterior to the coronal suture and approximately 20mm from the sagittal suture, dura was perforated and skull bolts secured. In the left bolt, a Codman microsensor intracranial pressure (ICP) probe (Codman &

Shurtleff Inc., MA) was calibrated and inserted intraparenchymally to a depth of approximately 15mm, and in the right a LICOX brain tissue oxygen ($PbtO_2$) probe (Integra LifeSciences, NJ) was inserted and secured to a depth of approximately 10mm, such that its oxygen sensing tip was positioned in the parietal lobe cortex supplied by the right MCA.

4.1.3.3 Surgical Approach to the MCA

With the animal still in the sphinx position, the head was tilted to the left ninety degrees and secured on a support to facilitate a right MCA approach, utilizing gravity to lift the cerebrum away from the bony skull base during intracranial surgery. Wool between the eye and ear was shorn and a 50mm vertical incision made terminating at the zygomatic arch. Temporalis and other muscles of mastication were divided and stripped from the coronoid process of the mandible. Removing the coronoid process is necessary to perform a craniotomy adequate to access the proximal MCA, and it was thus fractured at its base level with the zygomatic arch after muscle stripping (Figure 4.1.1A-B). The remaining masticators were then divided and stripped from the outer table of the cranium with monopolar diathermy (Covidien, Ireland) as far forward as the fibrous ring attaching the posterior orbit to the concave border of the parietal bone. A small craniotomy was performed over the junction of the parietal and squamous temporal bones with a high-speed pneumatic drill using a 5mm cutting burr (Midas Rex, Medtronic, MN), taking care not to breach the dura beneath. Bone was carefully removed anteroinferiorly with a Kerrison rongeur from the greater wing of sphenoid to aid in later exposure of the proximal MCA at the circle of Willis. Our goal was for the inferior margin of the craniotomy to run approximately level with floor of the middle cranial fossa, with the concave dural surface marking the junction of frontal lobe with temporal lobe in the center of the craniotomy (Figure 4.1.1C). The anterior component of the craniotomy was frequently the most difficult part of the exposure but also the most critical in order to achieve

satisfactory proximal MCA access. An adequate amount of bone removal over the squamous temporal was necessary to aid safe brain retraction and manipulation to access the terminal ICA.

A horseshoe shaped durotomy was performed with an inferiorly based flap. With a well-placed craniotomy, the cortical branches of MCA and its bifurcation point were seen running in the subarachnoid space on the surface of the brain. All intradural work was carried out with loupe magnification and a head mounted light source (Surgical Acuity, WI). Careful suction of cerebrospinal fluid (CSF) from the arachnoid cisterns of the anterior circulation promotes brain relaxation, and increases visibility in addition to minimizing retraction injury. The cortical branches were followed proximally, and with gentle upwards retraction of the anterior temporal lobe and careful aspiration of CSF from the prechiasmatic cistern, the terminal internal carotid artery (ICA) was identified looping around the optic tract medial to where the free tentorial edge meets the roof of the cavernous sinus (Figure 4.1.1D). Refer to online supplementary material (Video S1) for a multimedia file demonstrating the surgical approach to the proximal MCA, including placement of a temporary aneurysm clip.

4.1.3.3.1 Neurovascular Anatomy

The sheep neurovascular anatomy has been reported previously (331, 332), however for the purposes of this model a brief description of the relevant surgical anatomy is described and illustrated. As with most ruminants, the sheep has an extradural rete mirabile at the skull base from which the intradural ICA arises to provide blood supply to the majority of the supratentorial structures. Similar to the human, the terminal intradural ICA bifurcates to the anterior cerebral artery (ACA) and MCA, however in the human the intradural ICA course is short whereas in the sheep the it runs anteriorly for a short distance, sweeping forwards from its dural origin lateral to the optic tract adjacent to the optic chiasm and inferior to the large

olfactory lobe, where the ACA and MCA originate (Figure 4.1.2). The MCA runs forwards to approach the optic nerve but then turns back on itself posterolaterally in its proximal segment on the undersurface of the posterior frontal lobe. It turns again to run up laterally on the cortical surface and bifurcates early, just anterior to the inferior pole of the temporal lobe, into anterior and posterior trunks. These trunks are often visible for quite a distance on the lateral cortical surface at the junction of frontal and temporal lobes, before separating and dividing further into terminal cortical branches. Unlike the human, the sylvian fissure in the sheep is shallow and does not require dissecting to access the MCA along its course from origin to its terminal branches.

4.1.3.3.2 MCA Occlusion

After exposure of the proximal MCA, animals were randomized into one of three groups as follows: 1) sham, in which the proximal MCA was dissected but not occluded; 2) permanent occlusion, in which the proximal MCA was occluded via Malis bipolar diathermy forceps (Valleylab Inc., CO); and 3) temporary occlusion with reperfusion, in which the proximal MCA was occluded with application of a Sugita temporary mini straight aneurysm clip (Mizuho Medical Inc, Japan) which was removed 2 h later. The exposed brain was irrigated with saline during surgery to prevent drying out of the cerebral cortex, particularly for temporary occlusion animals in which there was a 2 h delay between aneurysm clip application and wound closure. The site of vessel occlusion was standardized to the proximal MCA within 3mm of its origin, as depicted by the asterisk in Figure 4.1.1D.

After sham surgery, permanent MCA occlusion or aneurysm clip release, the dura was approximated and closed watertight with ethyl cyanoacrylate (Bostik, Australia) and reinforced with dental acrylic cement (Lang Dental, IL) which was manipulated into the edges of the craniotomy, maintaining the shape of the cranial cavity and homeostasis of intracranial

pressure dynamics. The wound was closed in layers and the head was then returned to a neutral position for monitoring under anesthesia.

4.1.3.4 Histological Examination

At 4h following the onset of vessel occlusion or sham surgery, animals were administered intravenous heparin (5000I.U./5ml; Pfizer, NY) and killed via common carotid perfusion fixation with 10% neutral-buffered formalin (379). The head was stored overnight at 4°C to minimize artifact from premature tissue manipulation (380). Brains were removed and stored in formalin for a minimum of 7 d prior to being processed, embedded in paraffin wax and sectioned coronally at 5 mm intervals for histological examination by H&E, albumin immunohistochemistry (dilution 1:20000, ICN Pharmaceuticals Australasia Pty Ltd, Australia), and Weil's stain. Infarct area was determined by calculating the percentage of infarct volume on H&E in a whole coronal section at a level through the optic chiasm, encompassing head of caudate, anterior thalamus, putamen, internal capsule and the area of greatest cortical MCA supply. Edema was corrected for by a modified Swanson calculation (381).

4.1.3.5 Magnetic Resonance Imaging

A further 6 animals (mean weight 55.8 +/-5.2kg) were randomized to permanent MCAO (n=3) or 2h temporary MCAO followed by reperfusion (n=3) via an identical protocol outlined above but without insertion of ICP or $PbtO_2$ monitors. At 4 h after vessel occlusion animals were placed in a 1.5T Siemens Sonata (Siemens AG, Munich, Germany) Magnetic Resonance Image (MRI) machine for a sequence protocol that included magnetic resonance angiography (MRA), diffusion weighted imaging (DWI), fluid attenuated inversion recovery

(FLAIR), and T1 and T2 weighted images.

4.1.3.3.6 Statistical Analysis

All data is expressed as mean +/- standard deviation. Physiological data (arterial blood pressure, ICP, $PbtO_2$, PO_2, PCO_2) was analyzed using two-way analysis of variance (ANOVA) followed by individual Bonferroni tests (Prism Version 5.0d, Graphpad, CA). Physiological parameters were analyzed pre-MCAO or sham surgery, and at 30 minute intervals until the completion of the experiment. Lesion volume data was analyzed by individual student t-test. A p-value of $p<0.05$ was considered significant.

4.1.4 Results

4.1.4.1 Surgery

All experimental procedures were carried out without complication. There were no on-table mortalities or unexpected events. The mean time from the beginning of the operation to vessel occlusion or sham surgery was 76.1 +/-18.7 mins (range 53 to 105 mins).

4.1.4.2 Physiological Parameters

Basic physiological parameters are expressed in table 4.1.1. For all groups, there was no statistically significant difference for mean arterial PO_2, PCO_2, or blood pressure at any of the recorded time intervals. Mean ICP was 7.1 +/- 1.4mmHg prior to craniotomy, falling to 5.2

+/- 0.3mmHg upon opening the dura and rising to 10.0 +/- 2.0mmHg by the end of the 4 hour monitoring period, with no significant difference between groups (Figure 4.1.3). Mean $PbtO_2$ was 40.6 +/- 7.2mmHg prior to vessel occlusion or sham. $PbtO_2$ remained stable throughout the monitoring period in sham animals, however decreased in both MCAO groups for minimum values of 5.6 +/- 1.0mmHg in permanent occlusion animals at 240 mins and 30.5 +/- 17.0mmHg in transient occlusion animals at 90 mins (Figure 4.1.4). For almost the entire duration of the monitoring period, $PbtO_2$ after permanent MCAO remained significantly reduced (30-210 mins), however after temporary MCAO $PbtO_2$ returned to and remained at baseline levels upon release of the aneurysm clip after a period of non-significant reduction during clip application (41.2 +/- 11.9mmHg at 150 mins vs 44.8 +/- 11.4mmHg for sham).

4.1.4.3 Post mortem and gross pathological changes

Macroscopic changes consistent with acute cerebral ischemia were demonstrated in all permanent and temporary vessel occlusion animals. Sham animals demonstrated evidence of surgery in the region of the right proximal MCA, however the vessel was patent, the brain well perfused in all territories and macroscopically showed no evidence of injury. Permanent occlusion animals showed softening of the MCA supplied cortex adjacent to the firm formaldehyde perfuse-fixed brain of the anterior and posterior cerebral artery territories, whereas temporary occlusion and sham animals had uniformly perfuse-fixed brains. On coronal sections, permanent occlusion brains were swollen in the MCA territory with ipsilateral compression of the lateral ventricle and midline shift towards the contralateral side. Temporary occlusion animals demonstrated subpial petechial hemorrhages but a lesser degree of swelling. The proximal pre-bifurcation MCA was confirmed as the vessel occluded in both MCAO groups at post mortem.

4.1.4.4 Histopathology and infarct area

No microscopic evidence of ischemic injury was observed in sham animals. Conversely, MCAO animals demonstrated signs of ischemic injury within the MCA territory (cortical and subcortical structures), as evidenced by areas of pallor on H&E staining correlating with albumin extravasation and pallor on Weil staining (Figure 4.1.5). The H&E changes seen were due to microvacuolation of the neuropil which varied in different areas from fine to coarse microvacuolation. The cortical neurons in the coarsely vacuolated areas showed a spectrum of change varying from marked cytoplasmic shrinkage and pyknotic nuclei to early eosinophilia of the shrunken cytoplasm ('red neurons' corresponding with acute ischemic change, Figure 4.1.6A). Vesicular swelling of astrocytic nuclei was present before neuronal changes were evident at 4 h survival (Figure 4.1.6B).

The ischemic area was significantly larger in permanent occlusion animals than temporary occlusion (Figure 4.1.7, 28.8% vs 14.6%, $p<0.01$); in permanent MCAO there was evidence of ischemia involving the majority of the MCA territory, as well as subcortical structures (caudate nucleus, putamen), whereas ischemic areas in temporary MCAO animals were typically patchy in the MCA supplied cortex and with variable involvement of sub-cortical structures (Figure 4.1.5). A small amount of albumin extravasation was visible at the site of the craniotomy in sham animals suggesting not-insignificant injury to the underlying cortex secondary to the surgical approach and/or closure.

4.1.4.5 Magnetic Resonance Imaging

Permanent MCAO animals were characterized by complete proximal MCA occlusion on MRA with poor collateralization (Figure 4.1.8A), and restricted diffusion through the entire right MCA territory including the majority of the basal ganglia (high DWI signal in Figure

4.1.8B). There was increased signal at the site of craniotomy on T2 weighted imaging consistent with surgical manipulation but no FLAIR signal abnormalities identified, no blood products visualized and no evidence of mass effect (Figure 4.1.8C).

Temporary MCAO animals demonstrated a focal deficit on MRA at the proximal MCA consistent with clip occlusion but with reconstituted flow to the distal MCA branches (normal distal MCA filling, Figure 4.1.8D). DWI showed restricted diffusion at the right caudate head and genu of the internal capsule in addition to areas of restricted cortical diffusion (Figure 4.1.8E). As per permanent MCAO, there was T2 evidence of surgical manipulation but no FLAIR signal abnormalities and no blood products identified (Figure 4.1.8F).

4.1.5 Discussion

Animal models remain the most widely used approach to study the pathogenesis of ischemic stroke and determine the efficacy of various drug therapies. Large animal models of experimental stroke were common until the mid 1980s when the intraluminal thread model of MCAO was introduced in the rat (299), and although the rodent model offers many advantages, such as low cost, known physiological database and the ability to investigate knock out genes (249), the biological interspecies differences and poor clinical translation suggests that an animal model that is more clinically relevant is necessary (277, 378, 382). Despite the inherent disadvantages of working with large animals (277), they are potentially better suited to investigate stroke pathology and study treatment efficacy (319). There are a number of non-primate large animals models of acute ischemic stroke in the current literature, each with their own inherent advantages and disadvantages. Rabbits have an arterial anatomy that permits endovascular autologous clot occlusion of various intracranial arteries including the MCA (383) and the basilar artery (384), however their brains are lissencephalic like the

rat and not generally representative of the human gyrencephalic brain. Cats are of a similar size to rabbits and have a gyrencephalic brain, however their vascular anatomy precludes an endovascular approach to occlusion and the surgical approach is transorbital (313). Both rabbits and cats are considerably smaller than sheep or pigs, and although weight itself does not predict phylogenetic order, larger brains allow for easier surgical manipulation and limit the requirement of higher resolution imaging. Boltze et al. have demonstrated permanent extrinsic MCAO in a sheep model, however their site of surgical occlusion targets distal cortical branches and spares the proximal trunk and deep perforators (329); it also does not allow for reperfusion. Watanabe et al. similarly produced permanent MCAO in a pig model in which the vessel was occluded proximally, however this required a transorbital approach and subsequent persistent craniotomy defect (385). Non-human primates (NHP) remain the closest to humans phylogenetically, however endovascular autologous thromboembolic NHP models produce unreliable anterior circulation stroke patterns and their use worldwide is increasingly limited by restricted ethics approval (319, 386).

One reason why NHP are so attractive for translational stroke research is their remarkable anatomical similarity to the human brain, such that acute proximal MCAO results in basal ganglia and white matter infarction in addition to cortical stroke (386). Considering the importance of anatomical similarity, the sheep brain appears highly promising as a substitute for human stroke research. Key features are its gyrencephalic pattern, dense white matter tracts, strong fibrous dura mater and tentorium cerebelli, and large size compared with the rodent, rabbit or cat brain. The neurovascular anatomy is very similar to the human (319) with the exception of an extradural rete mirabile (330), which precludes an endovascular or embolic approach to MCAO. In addition to a large area of cerebral cortex, the human MCA may give rise to perforating lenticulostriate arteries which take origin from the first two MCA segments, the majority arising from the prebifurcation M1 segment with a smaller number from the postbifurcation M1 and less commonly M2 segments, but may also arise from the

ACA or ICA (11). Human lenticulostriates supply the head and body of the caudate nucleus, the superior part of the internal capsule and the lateral part of the globus pallidus after traveling through the anterior perforated substance. The surface of the well developed rhinencephalon in the sheep corresponds to the anterior perforated substance (387); perforating arteries in this region are derived from the ACA and MCA, either as single trunks from parent vessels or as trunks divided into cortical branches of the olfactory lobe. These perforators have many anastomoses creating conditions for a rich collateral supply (387), with the exception of arterioles distributed to the amygdaloid nucleus (388, 389). To include the deep perforating branches when performing an occlusive MCA stroke it is important to expose the artery proximally at its origin at the terminal ICA bifurcation.

Transient ischemia in addition to permanent vessel occlusion is a major advantage of this study and has the ability to significantly improve upon the existing ovine model (329). Furthermore, we have demonstrated significantly different lesion areas between transient and permanent proximal MCAO. Temporary vessel occlusion with reperfusion strengthens the translational potential, and recent STAIR recommendations have suggested that preclinical testing of neuroprotective agents should be conducted in multiple species and with both temporary and permanent occlusion models (342). MCAO by means of an aneurysm clip provides further advantages. The clip applicators are ergonomically easy to handle, are adept at placing clips within small surgical exposures under the guidance of illuminated magnification, and temporary clips allow for repositioning with little risk of significant endothelial damage (369) if initial placement is deemed inadequate. In addition, aneurysm clips can be placed essentially atraumatically after freeing the MCA from its overlying arachnoid. The clip may be left on the vessel for any length of time before reperfusion, or alternatively could be left on permanently. For permanent vessel occlusion we used bipolar electrocautery, which we found easier to consistently occlude the MCA at its most proximal part with less bony dissection and brain retraction and little or no arachnoid dissection. It

does, however, create a discrete area of focal electrocautery trauma to the adjacent brain tissue. Parenchymal injury may be limited by using a low current setting and restricting cauterization to only a short segment of the proximal MCA under direct visualization. An aneurysm clip left on permanently would avoid electrical trauma but at the cost of increased surgical difficulty.

$PbtO_2$ and Cerebral Blood Flow

The use of a LICOX system to measure $PbtO_2$ has advantages and disadvantages, and although it is not a true surrogate for cerebral blood flow (CBF), it may correlate well with regional CBF, particularly after brain injury (390). In the context of this model, in which we measured the partial pressure of oxygen within a focal area of brain tissue at the tip of the probe, $PbtO_2$ may be seen as a reasonable substitute for measuring CBF. Cerebral arteries are classically thought of as end-arteries, therefore detection of brain tissue hypoxia within one vascular zone should be representative of all cerebral structures supplied by that artery. However considerable inter-individual differences in collateral supply means this is not always the case (12). Furthermore, problems with consistent placement of the probe tip within the same vascular zone could influence the $PbtO_2$ results, such that if the LICOX probe tip lay within a watershed zone partially supplied by posterior or anterior cerebral arteries, or even placed too deep to be representative of a cortical MCA zone. In such circumstances, $PbtO_2$ levels may have remained falsely elevated when in fact the ischemic core was grossly hypoxic. Indeed, this was observed in 2 temporary MCAO animals, in which there was no reduction of $PbtO_2$ despite demonstrable ischemic change on H&E, and contributed to the wide standard deviations and lack of significance in this group. $PbtO_2$ levels in permanent MCAO animals were significantly reduced, reaching a minimum level of 5.6mmHg by 240 mins after MCAO. Normal values for $PbtO_2$ in the human are 33.0-47.9mmHg (72), and in

the sheep 44.0-52.0mmHg (73), which compares favorably with our mean baseline measurement of 40.6mmHg. In animal models and clinical data of traumatic brain injury there is evidence that neurological outcomes are poorer the longer $PbtO_2$ is <15mmHg, and in permanent MCAO animals this occurred within 120 mins and persisted for the remainder of the experimental period, however did not fall below 30mmHg in the temporary MCAO group. There was a noticeable drop in $PbtO_2$ levels in sham animals at 240 mins, which can be explained by missing data at this time point in 4 of the 6 animals in this group; this also contributed to the lack of statistical significance between sham and permanent MCAO at 240 mins. Despite its disadvantages, the LICOX system is easy to use and minimally invasive, provides rapid, continuous and accurate measurements (72), is freely commercially available and is widely reported in the literature with regard to both human and animal outcomes, particularly in relation to traumatic brain injury (73, 91).

Surgical approach and ICP

Previous large animal models have frequently utilized a transorbital approach to the MCA in order to minimize brain retraction (276). We ultimately elected to approach the MCA transcranially for several reasons. Firstly to avoid enucleation; in anticipation of evolving this method into a survival MCAO stroke model, we felt that clinical observation of both eyes and visual fields would be important. Secondly, a well-placed transcranial approach gives direct access to the proximal MCA, from its origin to beyond its cortical bifurcation, providing the option of performing vessel occlusion at the main trunk or at one or more distal branches. Finally our approach allows for watertight dural closure with replacement of the removed bone with a suitable substitute, which we found to be important for ICP dynamics. A watertight dural closure can be achieved with cyanoacrylate alone, which we observed to not produce any significant heat during polymerization, however the ethyl monomer is recognized

to produce focal cerebral injury (391) which may explain the small amount of albumin uptake on immunostaining of sham animals. This could potentially be eliminated by using the less traumatic isobutyl cyanoacrylate monomer, or a commercial albeit more expensive dural closure product such as DuraSeal (Confluent Surgical Inc., MA). Secondary brain injury such as raised ICP can contribute significantly to morbidity and mortality associated with large strokes (200), so maintaining the integrity of the closed cranial cavity after vessel occlusion is an important component of this model. ICP decreases on opening the dura and aspirating CSF, however rises again to pre-craniotomy levels towards the end of the 4-hour monitoring period after dural and bone reconstruction and as CSF reaccumulates. Our results demonstrated a relatively low early mean ICP after temporary clip occlusion, which may in part be explained by the craniotomy remaining open for 2 h longer in this group than the other two groups. This delayed closure is necessary to remove the aneurysm clip, however postpones reconstitution of ICP homeostasis after craniotomy and needs to be taken into consideration. The supratentorial space can generally be considered a uniform compartment with regards to ICP, however ICP gradient development after unilateral stroke remains controversial. In one model of NHP stroke, significant gradients were observed in animals with >20% infarct volume (326). Another report in malignant human stroke demonstrated equal bilateral ICP changes for the majority of the monitoring period, only developing a gradient after the development of transtentorial herniation and brainstem compression (392). Clearly having the $PbtO_2$ sensor placed within the ischemic tissue was important, hence the LICOX monitor was placed ipsilaterally, and although the sheep brain is considerably larger than the rodent and permits insertion of multiple probes, having the current setup versus two ipsilateral probes was favorable to limit overcrowding in the surgical field. Considering the area of ischemic tissue at 4 h in the permanent MCAO group, we predict to observe a significant ICP rise in longer periods of monitoring as occurs in human malignant MCA stroke (393), and this has been identified as an area of future study to help confirm the translational importance of this model.

MRI

MRI adds significantly to the translational potential of our model. Firstly, we have demonstrated with MRA that permanent MCAO results in complete and sustained occlusion of the proximal MCA, and that 2 h of temporary MCAO with an aneurysm clip results in restoration of blood flow distal to the occlusion. This is consistent with the $PbtO_2$ data, which suggests that regional CBF is restored after temporary occlusion but completely interrupted after permanent MCAO. This also suggests that a temporary aneurysm clip does not injure the proximal MCA or its endothelium sufficient to prevent reperfusion. Secondly, the patterns of diffusion deficits in both MCAO groups at 4 h were similar to the size of infarct on H&E at the same time point (Figures 4.1.5 and 4.1.8). Diffusion weighted imaging remains highly sensitive and specific in early clinical detection of acute ischemia (394), and our DWI results improve the translational strength of this model. Finally, lack of early T2 signal change or evidence of mass effect is also consistent with human stroke, in which cerebral edema and raised ICP can take several days to develop (200).

Study limitations and future directions

Animals were only monitored for 4 h after vessel occlusion or sham to demonstrate histological evidence of cerebral ischemia and prove the feasibility of the surgical approach and different methods of vessel occlusion. However, 4 h is insufficient to comment on final infarct volume or early mortality rates, nor is neurological outcome assessable in a non-survival study. The area of ischemia at 4 h after permanent MCAO may be predictive of final stroke volume, however larger infarcts could potentially develop with longer survival, especially with temporary MCAO and reperfusion in which cell death can be delayed by days, or with the development of secondary injury phenomena such as raised ICP after large

strokes. Therefore, the model in its current setup provides no measureable outcomes for which to test novel therapeutic agents besides early histological changes, MRI diffusion or brain tissue oxygen partial pressures.

In regards to approaching the sheep MCA, the surgery itself is technically demanding when compared with intraluminal rodent models. We found that a thorough knowledge of the relevant anatomy is paramount in safely and efficiently performing the surgery. Another disadvantage of the approach is the amount of extracranial bone and muscle dissection required, which may be a source of not insignificant morbidity for the sheep and may not in its present form be necessarily suitable for a survival study. A more rostral craniotomy that avoids removal of the coronoid process limits the amount of soft tissue and bone dissection and has already been used in a survival study (329), however this approach only provides access to the distal cortical MCA branches, not the important pre-bifurcation proximal segment and associated deep perforating branches.

It would be difficult to achieve the 28% stroke area we observed after permanent MCAO when targeting only the distal vasculature; performing a proximal occlusion and producing complete MCA territory stroke is an important model to simulate the human condition. Malignant MCA stroke is typically associated with larger infarct volumes (395, 396), so for an animal model to replicate malignant MCA stroke and its associated comorbidities, including cerebral edema, raised ICP and high early mortality, occluding the MCA proximally and probably permanently is critically important. Alternatively, smaller cortical-based infarcts sparing the subcortical structures could be performed by transient proximal MCAO followed by reperfusion or by the distal MCAO method as described by Boltze et al. (329), with each approach having its advantages and disadvantages.

4.1.6 Conclusions

We have produced a large animal model of proximal MCA vessel occlusion in the sheep whereby temporary or permanent vessel occlusion is possible. This model may represent a supplement to the currently used small animal models to improve the translation from experimental studies to clinical therapies.

4.1.7 Acknowledgements

We would like to thank Mr Joshua Burton for contributing the superb sheep anatomy illustrations and Ms Diana Pilkington for the MRI acquisition.

4.1.8 Figures

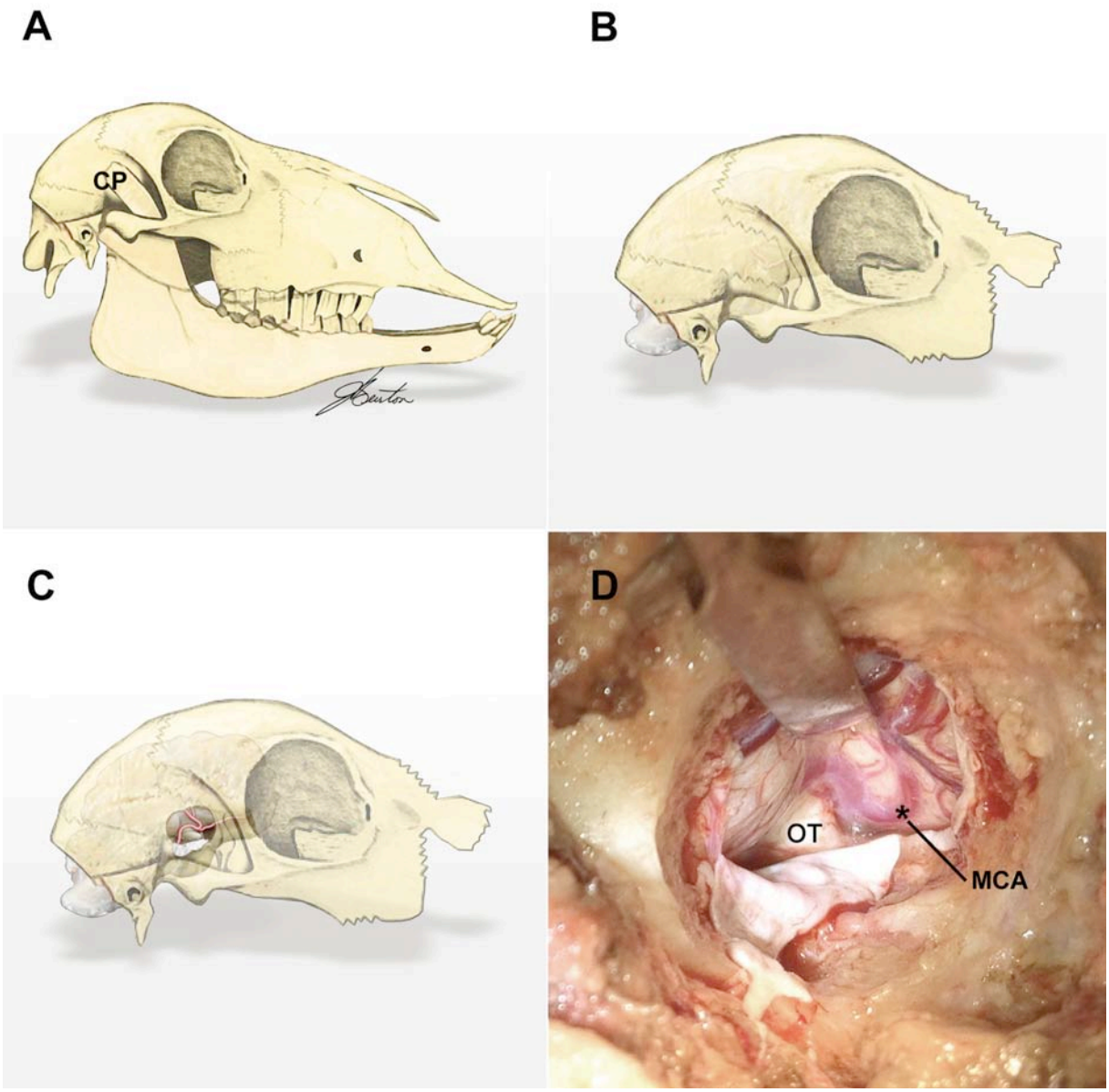

Figure 4.1.1: Surgical approach to the proximal MCA. The coronoid process of the mandible (A) is fractured near its base and removed to expose the skull overlying the MCA (B). A small craniotomy and durotomy reveals the proximal part of the vessel (C). Intraoperative photography demonstrating the proximal MCA looping at the skull base (D); asterisk marks the point of occlusion. CP, coronoid process; MCA, middle cerebral artery; OT, optic tract.

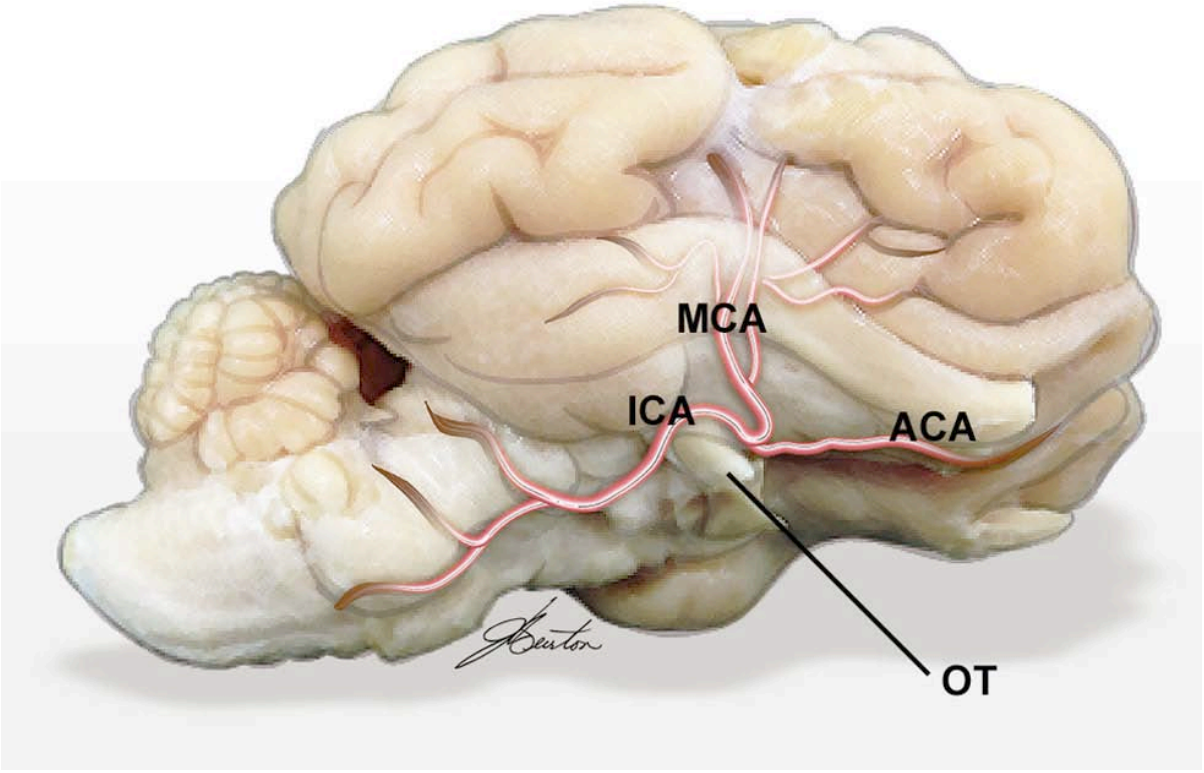

Figure 4.1.2: Neurovascular anatomy of the sheep, anterior circulation, inferolateral view, right side. ICA sweeps around the optic tract before bifurcating terminally into MCA and ACA. MCA loops backwards before running laterally on the surface of the brain and dividing into terminal branches. ACA, anterior cerebral artery; ICA, internal carotid artery; MCA, middle cerebral artery; OT, optic tract.

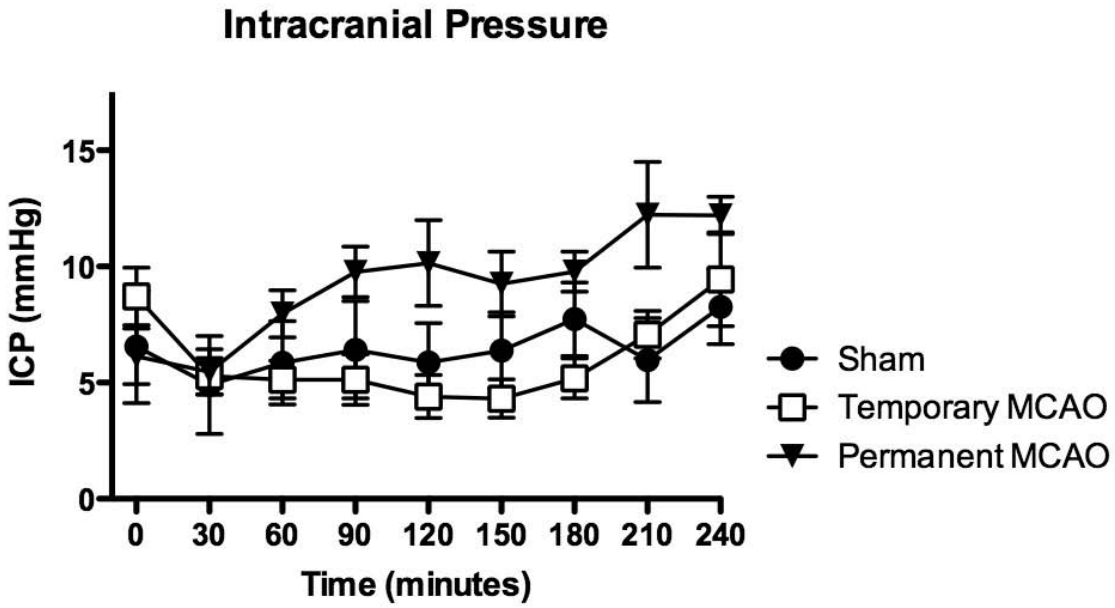

Figure 4.1.3: ICP after surgical MCAO, first 4 hours. ICP falls initially in all three groups due to dural opening and CSF aspiration. After skull reconstruction, ICP slowly rises above pre-craniotomy levels after permanent MCAO, but without any significant difference between groups.

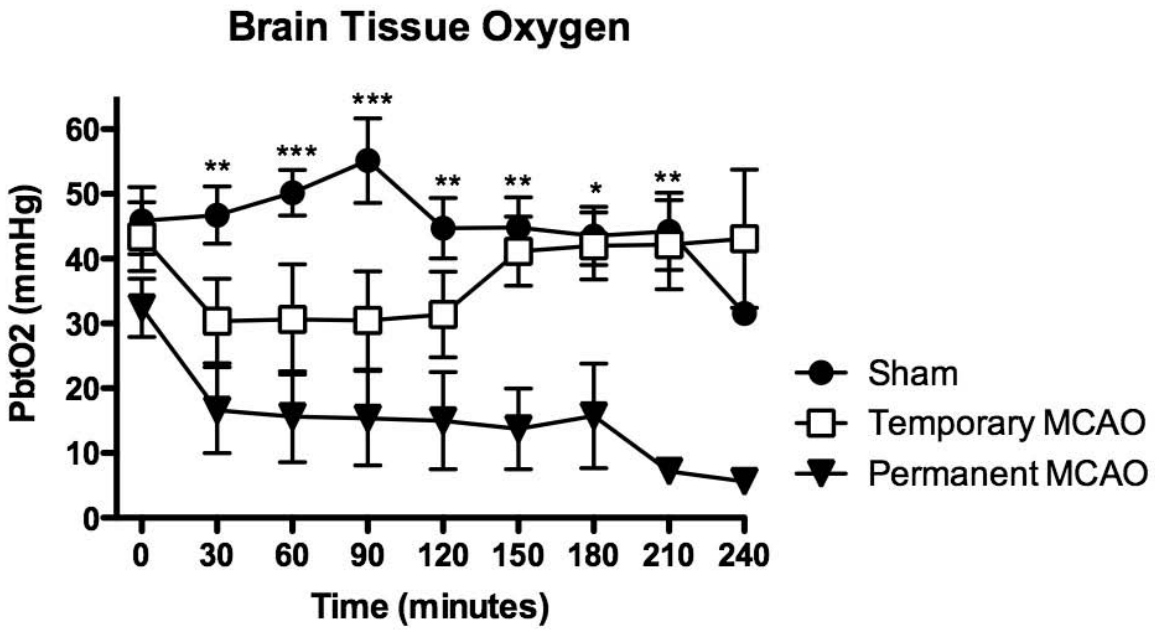

Figure 4.1.4: PbtO2 after surgical MCAO, first 4 hours. After permanent MCAO, $PbtO_2$ falls significantly compared to sham. $PbtO_2$ begins to rise again upon release of the temporary aneurysm clip in the transient occlusion group, however remains low in the permanent occlusion group. Sham animals demonstrate no significant change for the duration of the monitoring period. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (permanent MCAO compared to sham).

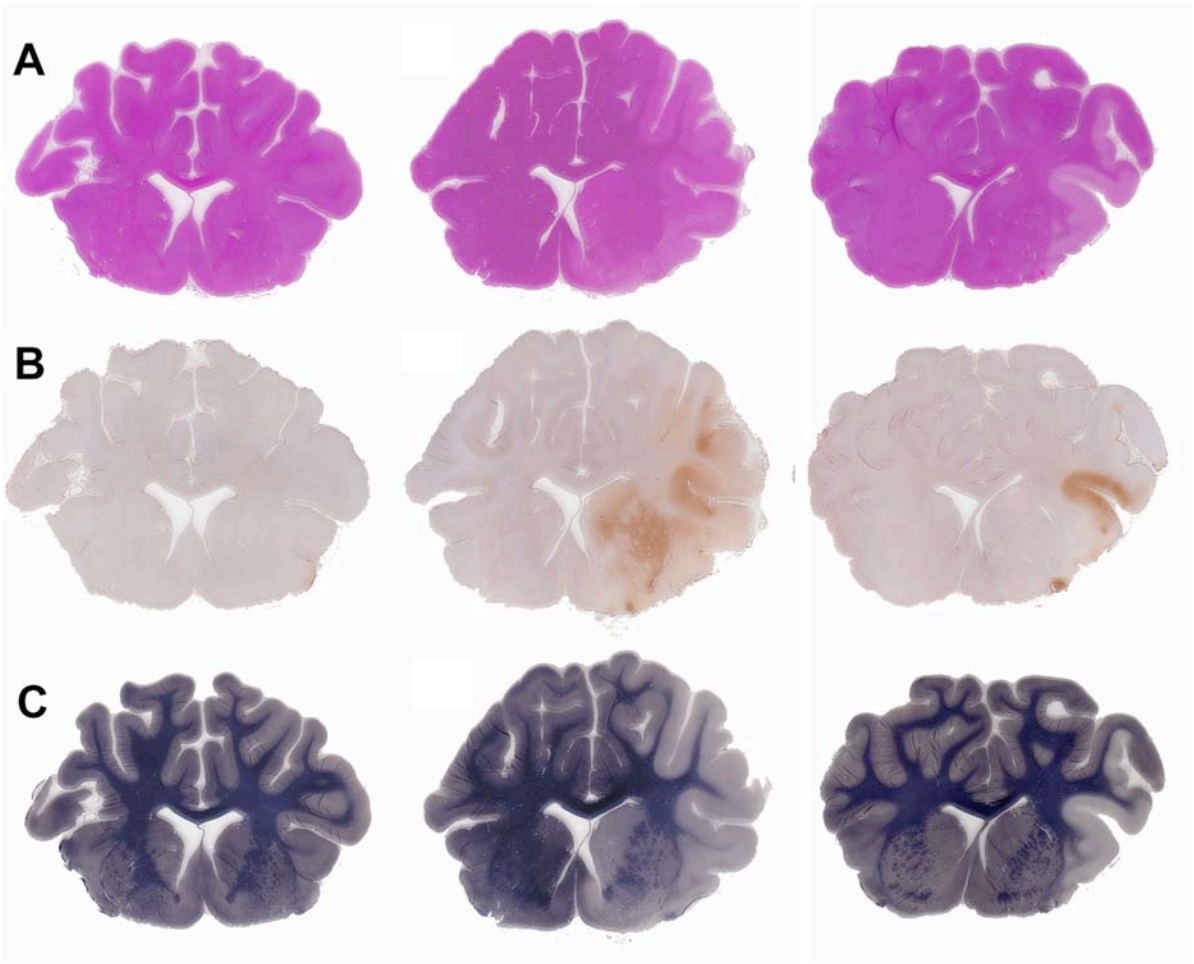

Figure 4.1.5: Histopathology. (A) H&E stain for sham (left), permanent MCAO (center), temporary MCAO (right). (B) Albumin immunostain for sham (left), permanent MCAO (centre), temporary MCAO (right). Weil stain for sham (left), permanent MCAO (centre), temporary MCAO (right). MCAO, middle cerebral artery occlusion.

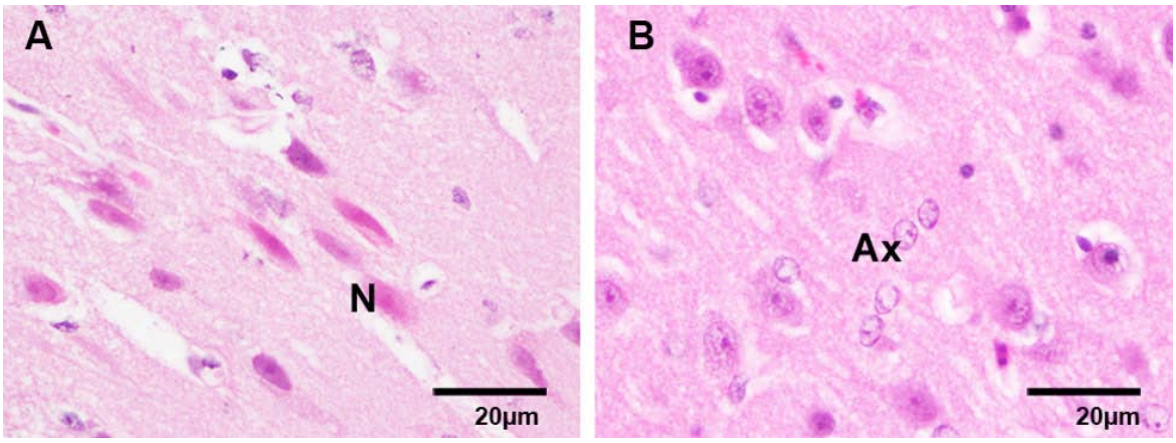

Figure 4.1.6: Histopathology. 'Red neurons' (A) and astrocytic nuclear swelling (B). H&E x400. N, neuron; Ax, axon.

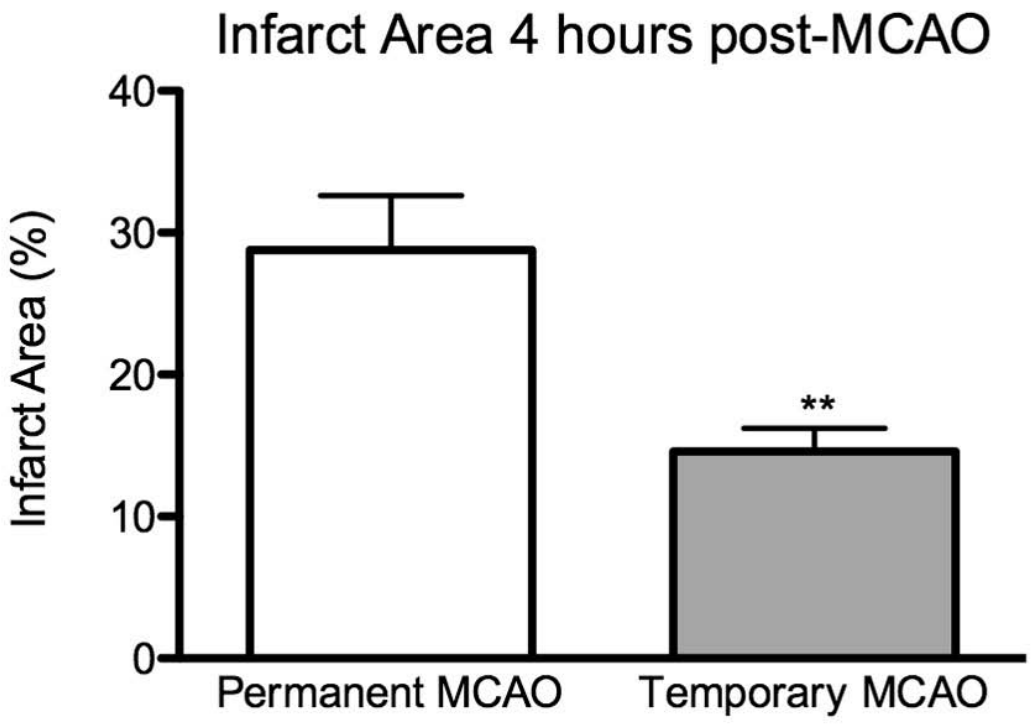

Figure 4.1.7: Infarct area at 4 hours. Infarct areas expressed as percentage of ischemic brain tissue identified on H&E staining on coronal section level with the optic chiasm; areas have been corrected for edema. **, $p<0.01$

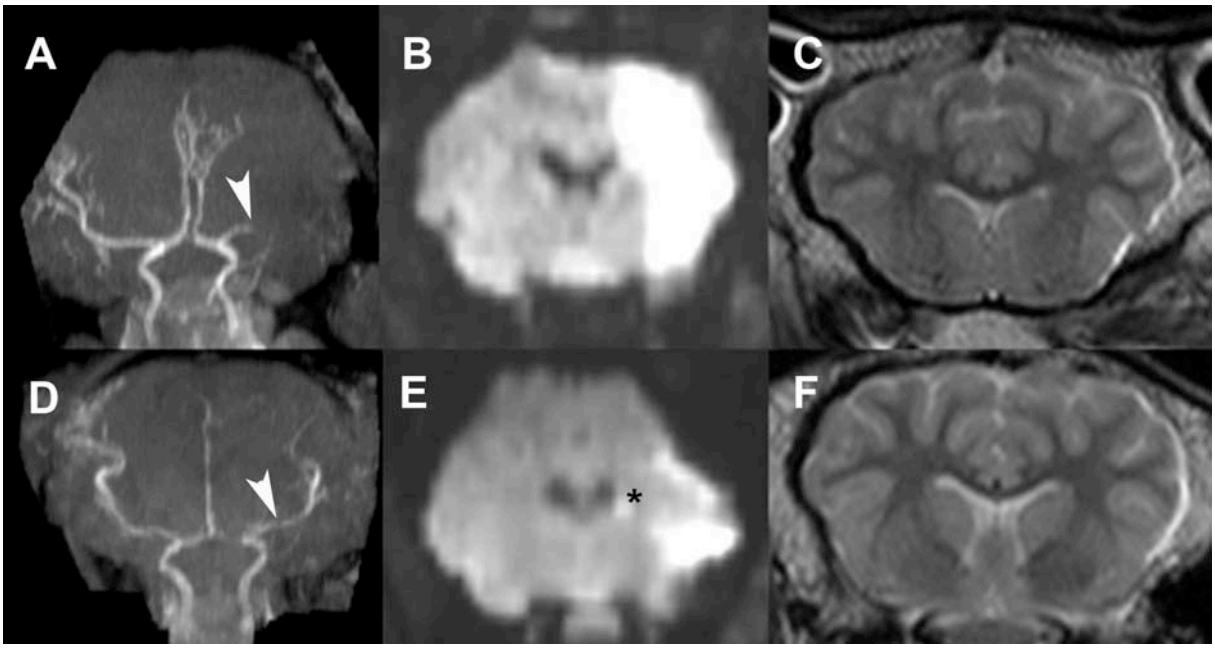

Figure 4.1.8: MRI at 4 hours after MCAO, coronal orientation. Permanent MCAO MRA (A), DWI (B) and T2 weighted imaging (C). Temporary MCAO with reperfusion MRA (D), DWI (E) and T2 weighted imaging (F). Arrowhead on MRA indicates site of arterial occlusion. Asterisk indicates restricted diffusion at caudate head after temporary MCAO. DWI, diffusion weighted imaging; MCAO, middle cerebral artery occlusion; MRA, magnetic resonance angiogram; MRI, magnetic resonance imaging.

Group	PO_2 (mmHg) +/- SD	PCO_2 (mmHg) +/- SD	ABP (mmHg) +/- SD
Sham	112.1 +/- 15.7	37.0 +/- 5.0	104.5 +/- 13.5
Permanent MCAO	101.4 +/- 25.8	41.2 +/- 8.6	94.7 +/- 7.6
Temporary MCAO	115.2 +/- 20.5	39.4 +/- 8.6	101.3 +/- 2.9

Table 4.1.1: Physiological data. Mean blood gas and arterial blood pressure measurements for all time intervals by group. MCAO, middle cerebral artery occlusion; PO_2, arterial partial pressure of oxygen; PCO_2, arterial partial pressure of carbon dioxide; ABP, mean arterial blood pressure.

4.1.9 Supporting Information

Video S1. Surgical approach to the proximal Middle Cerebral Artery, demonstrating placement of a straight mini aneurysm clip to produce temporary arterial occlusion. http://www.plosone.org/article/fetchSingleRepresentation.action?uri=info:doi/10.1371/journal.pone.0042157.s001

4.2 Regional brain tissue oxygen response to temporary aneurysm clip occlusion of the proximal middle cerebral artery

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Running head: Regional brain oxygen with temporary MCAO

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4.2.1 Abstract

Background: Temporary arterial occlusion risks cerebral ischemia in a time dependent manner. Current recommended safe time limits for temporary occlusion during cerebrovascular surgery are around 10-15 minutes. Regional brain tissue oxygen tension ($PbtO_2$) has been used to predict ischemic adverse events during temporary arterial occlusion, however the relationship between $PbtO_2$ and duration of arterial occlusion has not been previously investigated.

Methods: 10 adult Merino sheep had a right frontotemporal craniotomy and randomization to either 2-hours temporary proximal middle cerebral artery occlusion (MCAO) followed by reperfusion (n=5), or sham surgery (n=5). $PbtO_2$ was monitored via a right parietal LICOX probe. Animals were perfuse fixed and brains examined with Haematoxylin and Eosin.

Results: Following MCAO, $PbtO_2$ fell to a defined ischemic threshold of 15mmHg from a mean baseline of 45.0 +/- 14.1mmHg after a mean of 42.4 +/- 11.2 minutes, at a rate of 1.3mmHg/minute. The rate then slowed to 0.2mmHg/min until clip release. Temporary MCAO resulted in variable ischemic injury limited to the MCA territory.

Conclusions: Regional $PbtO_2$ decline after MCAO is rapid until the onset of hypoxia, then continues to fall but at a slower rate. Higher baseline $PbtO_2$ levels predict a longer time to hypoxia. Ischemic complications during cerebrovascular surgery may be better avoided by individualizing the risk with regional $PbtO_2$ monitoring, rather than by measuring duration of temporary clipping in minutes. The linear decline of $PbtO_2$ following MCAO combined with a relatively long mean time to develop hypoxia provides a scientific rationale for ultra-early intervention for ischemic stroke.

Key words: animal models; brain ischemia; intraoperative monitoring; neurosurgery; vascular surgery

4.2.2 Introduction

By weight, the brain is one of the highest consumers of oxygen in the body, and relies upon a continuous supply to maintain aerobic metabolism (61). There is a complex relationship between the interruption in supply of oxygenated arterial blood, brain tissue oxygenation and the development of cerebral ischemia, however it is generally accepted that the relationship is time dependent and that neurons within the ischaemic core begin to die within minutes after arterial occlusion (397). This understanding of a short duration of aerobic reserve and rapid development of neuronal necrosis justifies the brain's ability to tolerate seemingly only very short periods of interrupted arterial blood flow. Reperfusion is employed frequently in ischemic stroke, and therapies such as recombinant tissue plasminogen activator (rt-PA) are approved for use up to 4.5 hours following stroke onset, however this is based on the assumption that the ischemic core is lost and that the penumbra is the target for salvage (375, 398). Despite this, there is emerging evidence for improved outcomes in thromboembolic stroke with earlier reperfusion (399, 400), particularly in the 28.3% of ischaemic stroke patients who arrive within the "Golden Hour" (401), suggesting that the brain may in fact be more tolerant of temporary arterial occlusion than previously thought.

Temporary clipping of parent vessels is selectively employed during cerebrovascular surgery for short intervals in order to safely dissect and definitively clip an aneurysm, particularly with complex vascular anatomy, or in the event of intraoperative rupture and uncontrolled hemorrhage (123). From its first description by Jefferson almost 100 years ago and its popularisation by Pool over 50 years ago (124, 126), temporary clipping has become increasingly accepted as a useful surgical adjunct but at the risk of cerebral ischemia, which is

thought to be in a time dependent manner (106). In 1979 Suzuki described the first safe time limits measured in minutes for temporary occlusion of proximal vessels of the anterior circulation (127), and since then numerous authors have reported their own experiences (128-138). Over time and with increasingly sophisticated perioperative monitoring and neuroimaging, the recommended temporary occlusion time has decreased from almost 20 minutes in Suzuki's paper to as little as 10 minutes in Ha's 2009 recommendations (138), however the risk of iatrogenic stroke remains up to 20% (128, 135, 156). Surprisingly, most recommendations in the literature are not generally made on any scientific evidence but on review of historical data.

As an alternative to relating duration of temporary clipping to risk of ischemia, Jodicke and colleagues used a commercially available partial pressure of brain tissue oxygen concentration ($PbtO_2$) monitor to identify thresholds of cerebral hypoxia and stroke risk (156). Regional $PbtO_2$ declines following temporary aneurysm clip occlusion of a parent vessel in a time dependent manner (106, 402), and Jodicke identified 15mmHg as a dichotomizing threshold for the development of procedure related ischemia. Others have used various monitored endpoints during intraoperative temporary vessel occlusion to predict the risk of iatrogenic stroke, including $PbtO_2$ and somatosensory evoked potentials, however the predictive value of these endpoints remains highly variable (156, 159-161). Any relationship between duration of temporary occlusion and monitored indications of ischemia such as regional $PbtO_2$ has never before been explored. We therefore set out to investigate the relationship between duration of temporary proximal middle cerebral artery occlusion (MCAO) and regional $PbtO_2$ in a newly characterised large animal model of temporary MCAO with reperfusion. We aimed to determine the mean time for regional $PbtO_2$ to fall below a defined ischaemic threshold of 15mmHg after prolonged MCAO, the rate of decline from baseline to hypoxia, the mean lowest $PbtO_2$ recorded during prolonged temporary MCAO, and the mean time for the regional hypoxia to be restored above the ischaemic

threshold upon release of the temporary aneurysm clip.

4.2.3 Methods

4.2.3.1 Experimental procedure

All studies were approved by the Animal Ethics Committees of the University of Adelaide and SA Pathology, and conducted according to guidelines established for the use of animals in experimental research as outlined by the Australian National Health and Medical Research Council code of practice for the care and use of animals for scientific purposes (7th edition, 2004).

4.2.3.2 Animals and experimental design

10 adult male and female Merino sheep (54.0 +/- 6.4kg) were randomized to temporary MCAO (n=5, 2 male) or sham surgery without occlusion (n=5, 1 male). Anesthesia was induced with intravenous thiopentone (Jurox Pty Ltd, Australia) and maintained with 1.5% inhalational isoflurane (Veterinary Companies of Australia Pty Ltd, Australia) mixed with oxygen and room air, plus 4.0mg/kg/h intravenous ketamine (Parnell Australia Pty Ltd, Australia). Blood pressure was monitored invasively via the right femoral artery and arterial blood gas analysis was performed hourly. A LICOX microcatheter system (Integra LifeSciences, NJ) was used for continuous $PbtO_2$ measurement. A right parietal burr hole was placed 1cm posterior and inferior to the horn bud, and the probe was inserted and secured to a depth of 6mm, such that its tip was consistently within the superficial cortex of the middle cerebral artery (MCA) supplied antero-inferior parietal lobe. The probe was allowed to

equilibrate for a minimum of 1 hour prior to MCAO or sham surgery. A right-sided craniotomy was then performed using a technique previously described in order to access the proximal MCA (402). Animals randomized to temporary MCAO had placement of a straight mini Sugita temporary aneurysm clip (Mizuho Medical Inc, Japan) at the proximal M1 segment of the MCA, just beyond the terminal carotid bifurcation. Baseline $PbtO_2$ values were recorded for 20 minutes prior to temporary clipping. The clip was removed after 2 hours of temporary occlusion, the dural and craniotomy defects were closed watertight with methylcyanoacrylate and acrylic respectively, and the animal monitored for a further 2 hours of reperfusion under general anesthetic. Animals randomized to sham surgery had dissection of the proximal MCA sufficient to place an aneurysm clip but without performing MCAO; baseline $PbtO_2$ values were recorded over a 20-minute period prior to vessel dissection. At 4 hours following MCAO onset or sham surgery animals underwent bilateral common carotid perfusion fixation with 10% formalin. Brains were sectioned coronally for 5 sections at 5mm intervals, with the middle section level with the origin of the MCA. They were then examined histologically with Haematoxylin and Eosin to assess the volume of ischemic injury as a percentage of whole brain using a modified Swanson calculation (381).

4.2.3.3 Statistical analysis

All data are expressed as mean +/- standard deviation, except for $PbtO_2$ descent and ascent times which are also expressed as median with interquartile range. Physiological data including $PbtO_2$, mean arterial blood pressure (MABP), arterial oxygen tension (PaO_2) and arterial carbon dioxide tension ($PaCO_2$) were analyzed using two-way analysis of variance (ANOVA) followed by individual Bonferroni tests (Prism, Graphpad, CA). Mean $PbtO_2$ descent and ascent times were analyzed using a two-tailed unpaired t test, whereas median $PbtO_2$ descent and ascent times were analyzed using a two-tailed Mann Whitney test. $PbtO_2$

descent times were compared with $PbtO_2$ decline via non-linear regression analysis. Likewise, stroke volumes were compared with the duration that the brain remained hypoxic also via non-linear regression analysis. A p value of <0.05 was considered significant.

4.2.4 Results

4.2.4.1 Surgery and basic physiological parameters

There were no adverse outcomes or complications from the surgical approach or LICOX microcatheter placement. There was no significant difference in mean arterial oxygen tension (107.1 +/- 14.4mmHg for sham vs. 112.1 +/- 15.9mmHg for MCAO, p>0.05) or mean arterial carbon dioxide tension (42.5 +/- 9.1mmHg for sham vs. 39.3 +/- 7.7mmHg for MCAO, p>0.05). MABP was significantly higher in MCAO animals compared with sham (103.9 +/- 24.1mmHg vs. 90.6 +/- 19.3mmHg, p=0.04), however despite this MABP remained within a physiological range at all time points in each animal within each group.

4.2.4.2 $PbtO_2$

$PbtO_2$ results for individual animals in the MCAO group are presented in Table 4.2.1. Mean $PbtO_2$ values plotted at 5-minute intervals are represented in Figure 4.2.1. Baseline $PbtO_2$ in MCAO animals was 45.0 +/- 14.1mmHg, compared with 49.3 +/- 15.2mmHg in sham animals (p>0.05). The mean time for $PbtO_2$ to fall below 15mmHg after MCAO was 42.4 +/- 11.2 minutes (range 28.8-59.6 minutes; T1, Figure 4.2.2a, b), and the lowest mean $PbtO_2$ recorded was 6.7 +/- 2.8mmHg (range 3.5-10.1mmHg) at a mean time of 110.8 +/- 11.9 minutes after clip application.

The mean time for $PbtO_2$ to recover above 15mmHg was 25.3 +/- 47.4 minutes after clip release (range 1.8-109.9 minutes; T2, Figure 4.2.2a), which was 19.1 minutes faster than the mean descent to hypoxia time (p=0.455). As the $PbtO_2$ times to hypoxia were not normally distributed they were also expressed as median values, with a median descent time of 41.3 minutes (interquartile range 18.1) versus a median ascent time of only 4.4 minutes (interquartile range 57.1; p=0.151). The mean highest $PbtO_2$ recorded after clip release was 31.5 +/- 13.1mmHg, compared with a mean highest $PbtO_2$ of 50.5 +/- 11.8mmHg in the corresponding time period in sham animals ($p<0.05$, Figure 4.2.2b). As a percentage of baseline, the mean highest post-MCAO $PbtO_2$ level was 70.4 +/- 20.0% (Table 4.2.1). $PbtO_2$ levels in MCAO and sham animals were also compared at the two time points representing mean duration for $PbtO_2$ to fall below 15mmHg (42.4 minutes) and lowest $PbtO_2$ reading (110.8 minutes; Figure 4.2.2b). At 42.4 minutes, mean $PbtO_2$ in MCAO animals was 14.9 +/- 2.7mmHg, compared with 52.2 +/- 9.1mmHg in sham animals ($p<0.0001$). At 110.8 minutes, mean $PbtO_2$ in MCAO animals was 7.6 +/- 2.5mmHg, compared with 48.1 +/- 12.3mmHg in sham animals ($p<0.0001$).

The relationship between baseline $PbtO_2$ and time for $PbtO_2$ to fall below 15mmHg showed a positive linear correlation (p=0.01, Pearson r correlation coefficient 0.95), corresponding to a $PbtO_2$ descent of 1.3mmHg/minute after temporary aneurysm clip MCAO (Figure 4.2.3). With prolonged MCAO following the development of hypoxia, $PbtO_2$ continued to descend but at a slower rate (0.2mmHg/minute, $p<0.05$, Pearson r correlation coefficient 0.95).

4.2.3.3 Histopathology

Sham animals displayed no evidence of ischemic injury on H&E examination. MCAO resulted in variable ischemic injury within the MCA territory despite identical vessel occlusion times and LICOX-measured hypoxia in all animals. Two animals demonstrated no

injury at all (animals 2 and 5, Table 4.2.1), one animal had a small stroke volume (2.3%, animal 1), and two animals demonstrated stroke volumes greater than 10% (animal 3, 13.3%; animal 4, 22.0%). In animals with evidence of ischemic injury, the head of the caudate nucleus was always involved, with variable involvement of the MCA supplied cortex and subcortical grey matter (Figure 4.2.4). The mean stroke volume was 7.5 +/- 9.8% (range 0-22.0%); of animals that showed evidence of ischemic injury, the mean stroke volume was 12.5 +/- 9.9% (range 2.3-22.0%). The duration that $PbtO_2$ stayed below the ischemic threshold of 15mmHg was not predictive of the volume of ischemic injury at 4 hours after temporary MCAO (p=0.512).

4.2.4 Discussion

Temporary clip occlusion was employed in 35.4% of all aneurysms treated surgically in one large single surgeon series (123), with the MCA being the second most likely vessel to be temporarily clipped after the internal carotid artery. The risk of temporary occlusion related ischemia is estimated to range between 16.3-29.3%, and the risk is thought to increase exponentially with length of interrupted blood flow (138, 397). Of all the variables associated with stroke during temporary occlusion, duration of clip application is frequently cited as the most important parameter, with some authors suggesting that it is the only factor that needs to be considered when assessing risk for ischemic complication (138). Cerebral hypoxia has also been identified as a risk for ischemic complications, and regional $PbtO_2$ has been shown to be inversely proportional to duration of temporary occlusion (106), but surprisingly the relationship between temporary MCAO and time for the brain to become hypoxic has never before been investigated. Considering how vital oxygen supply and metabolism is in the context of ischemic stroke, the general paucity in the literature of experimental studies investigating a relationship between regional brain tissue oxygenation and cerebral artery

occlusion is remarkable.

Temporal thresholds of cortical hypoxia

Recommendations for safe temporary occlusion during cerebrovascular surgery are frequently said to be in the order of 10-15 minutes, however the mean (42.4 minutes) and median (41.3 minutes) times for the MCA supplied cortex to develop hypoxia defined at $PbtO_2$ <15mmHg in our experience were considerably longer. The exception is Jabre and Symon's series, which reported that 40 minutes was the longest that temporary occlusion of the MCA could be performed and still achieve an excellent outcome (130). Mean and median data do not translate to clinical recommendations due to unacceptable sensitivity and specificity values; likewise, historical data suggesting acceptable temporary occlusion times based on retrospective analysis of the development of radiological or clinical stroke are have limited generalizability. The identification of guidelines for safe temporary artery occlusion times measured purely in minutes therefore seems highly unlikely owing to significant interindividual differences. We have however identified a relationship between baseline $PbtO_2$, duration of MCA occlusion and the development of cortical hypoxia, suggesting that there may yet be a way of individualizing the risk of ischemic complications during temporary occlusion.

This relationship reinforces the temporal relationship between duration of ischemia and the development of neurological deficit, and provides a clinical rationale for ultra-early intervention (403). If the mean time for the ischaemic core to become hypoxic is approaching an hour as our results suggest and is a representation of the human state, then there is a great impetus on emergency and stroke physicians to develop techniques to provide rapid intervention to the 72.9% of patients who arrive within the Golden Hour after ictus and do not receive thrombolysis. Furthermore, it is essential for the community to recognize the urgency

of stroke and that emergency services provide urgent transfer to hospital for the 71.7% of patients who take longer than 1 hour to be seen in a tertiary institution (401).

Regional brain tissue oxygenation to identify risk of ischemic complications

$PbtO_2$ monitors provide real-time feedback of the regional state of brain oxygenation during temporary occlusion, although the definition of hypoxia related to $PbtO_2$ remains problematic. Clinical thresholds for temporary clipping using $PbtO_2$ feedback have now been produced in several human studies using both LICOX and Neurotrend (Diametrics Medical, High Wycombe, UK) devices (156, 158-161). However, these thresholds are still by necessity based on retrospective analyses of the development of neurological deficit or radiological evidence of ischemia. Jodicke's suggestion that regional $PbtO_2$ of 15mmHg could be used as a dichotomizing threshold for ischemic injury was associated with 90% sensitivity but a positive predictive value of only 56% (156); it nevertheless correlates well with other animal and human experimental data in traumatic brain injury in which 15mmHg is a well recognised threshold for worse outcome (72, 73, 91). However a distinct problem exists with using a set $PbtO_2$ threshold when baseline pre-clip measurements are already below this value, even though normal values for both sheep (73) and human (72) should be well above it. $PbtO_2$ can be highly variable between and even within individuals, and can be affected by not only cerebral blood flow (CBF) and arterial partial pressure of oxygen but also depth of placement in the brain, particularly between grey and white matter (71, 404). Although our mean baseline of 45.0 +/- 14.1mmHg for MCAO animals was comparable to the literature, our range was 32.7-67.1mmHg (Table 4.2.1), highlighting the interindividual variability. In several reported human series $PbtO_2$ values have shown considerable variability and baseline levels have been much lower. In Cerejo's series of unruptured MCA aneurysms mean baseline $PbtO_2$ was only 9.6mmHg (range 2.3-27.3mmHg), with baseline values <10mmHg

found in 5 out of 8 cases (159), while Jodicke reported a mean of 23.9mmHg, with a huge range from 2.0 to 67.2mmHg (156). Perhaps in an attempt to circumvent the problem of low baseline levels, an a priori relative reduction in baseline $PbtO_2$ of 20% as a predictor of ischemic complications was employed by Jodicke but was associated with only 50% sensitivity and positive predictive value of only 42% (156), suggesting that reduction in $PbtO_2$ as a percentage of baseline correlates poorly with stroke risk. Fixed numeric thresholds on the other hand have been demonstrated to be associated with both regional CBF and development of stroke (72, 156, 405). However, the inter- and intraindividual variability in $PbtO_2$ need to be taken into account and great care must be taken to standardize probe tip placement.

Alternatively, there may yet be an argument for using a percentage reduction of $PbtO_2$ baseline as a threshold for hypoxia and risk of ischemic complications. There is good evidence for a reduction in CBF and stroke risk, with reported thresholds differentiating between benign oligemia and penumbra of 17mL/100 g/min, and between penumbra and core of 10mL/100 g/min. A review by Bandera identified 6 studies that reported a CBF threshold for penumbra in stroke (109), resulting in a mean value of 23mL/100 g/min (range 14.1-35mL/100 g/min). Given that normal average CBF is 50mL/100 g/min (406), it would be reasonable to suggest that the cerebral ischemic threshold is between a third to a half of normal flow. Although not strictly a measure of CBF, $PbtO_2$ is a product of CBF and arteriovenous difference in oxygen tension (103, 104), and in the context of cerebral artery occlusion $PbtO_2$ may be a reasonably accurate representation of CBF. Repeat analysis of our results demonstrated that the mean time for $PbtO_2$ to decline to 50% of baseline after MCAO was 18.4 +/- 10.0 minutes (Table 4.2.1), which is considerably closer to current clinical recommendations for safe temporary clip occlusion times and may more accurately represent the true pathophysiological state and ischemic risk. To investigate the relative importance of fixed thresholds versus relative reduction in regional $PbtO_2$, it would be interesting to determine the effect on various outcomes (including neurobehaviour, radiology and

histopathology) of releasing the temporary clip at the point when regional $PbtO_2$ dropped to 15mmHg versus the at the point when regional $PbtO_2$ dropped to 50% of baseline.

In addition to defining the time for the development of hypoxia following MCAO, we have identified the rate that regional $PbtO_2$ declines as being 1.3mmHg/min until $PbtO_2$ reaches 15mmHg, which then slows to 0.2mmHg/min during prolonged temporary occlusion. This two-speed rate of decline suggests a change in oxygen metabolism either side of the 15mmHg threshold. These rates also have significant implications on thromboembolic ischemic stroke and reperfusion, and provide an excellent pathophysiological rationale supporting the ultra-early treatment of ischemic stroke patients. The highly consistent rates of decline we observed have major implications in cerebrovascular surgery, for the brain could be kept above the hypoxic threshold for longer periods of temporary occlusion with higher pre-clip baseline $PbtO_2$ levels, which may be achieved via increasing arterial oxygen tension in the patient prior to temporary clip application.

Histological evidence of ischemic injury

Due to our methodology it is not possible to comment on $PbtO_2$ values and the risk of histological evidence of ischemia. Interesting to note though there was considerable variation in the effect of temporary occlusion despite identical vessel occlusion times. Furthermore, we found no correlation between the duration that regional $PbtO_2$ was below the purported hypoxic threshold, which itself was a product of baseline $PbtO_2$, and the size of the resultant infarct (Table 4.2.1; Figure 4.2.4). In our series of prolonged MCAO, the mean duration that the cortex was hypoxic was 102.9 +/-49.0 minutes, ranging from 64.8-188.6 minutes (Table 4.2.1). $PbtO_2$ ascent times were generally very short with the exception of animal 5, which took 109.9 minutes to rise above the threshold upon clip release (Table 4.2.1). Post mortem examination in this animal revealed no focal thrombus of the proximal MCA associated with

the temporary clip, and the prolonged recovery time was attributed to vasospasm associated with vessel manipulation or microthromboemboli. Incredibly, this animal demonstrated no histological evidence of ischemia despite recording both the lowest absolute $PbtO_2$ (3.5mmHg) and highest percentage reduction from baseline (90.2%). Many of these seemingly contradictory results are probably attributable to the nature of ischemic injury related to reperfusion. The pathophysiological effects of cerebral ischemia with reperfusion are complex and remain poorly understood, however considerable interindividual variations (particularly in mammals with gyrencephalic brains and including but not limited to collateral blood supply, the inflammatory response and endothelial physiology) make variable pathologic response to identical periods of MCAO a recurrent theme (398). The short monitoring period of only 4 hours after MCAO probably contributed to the variable histology results, as final infarct size may take much longer to become apparent, particularly after reperfusion (181). $PbtO_2$ ascent times generally contributed very little to total hypoxia times in this model of prolonged temporary occlusion, however could potentially contribute significantly during shorter, more clinically relevant periods of temporary MCAO, and the extra time that the hypoxic brain requires to rise to normal should always be taken into account by the surgical team. The key observation here is that reversal of cerebral ischemia after temporary clip release in our experience is typically quite rapid but not instantaneous.

4.2.5 Conclusions

We have demonstrated in an ovine model of proximal temporary MCAO that the mean time to develop regional hypoxia as measured with a LICOX probe is 42.4 minutes. $PbtO_2$ levels decline linearly, initially fast until the onset of hypoxia, then slowly as hypoxia proceeds, and a higher baseline $PbtO_2$ is predictive of longer time to cortical ischemia following MCAO. Despite prolonged MCAO and the development of regional hypoxia, histological evidence of

ischemia is highly variable or even absent 4 hours after MCAO.

4.2.6 Acknowledgements

None.

4.2.7 Disclosure/Conflict of Interest

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

4.2.8 Titles and legends to figures

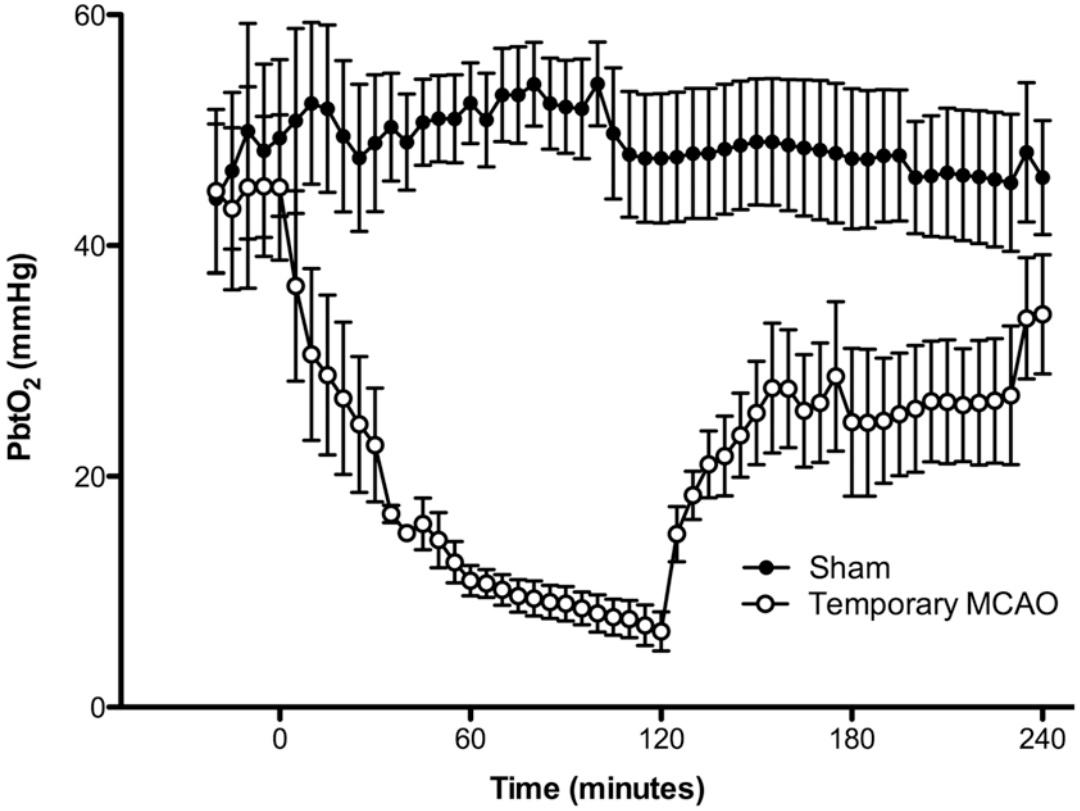

Figure 4.2.1: Mean PbtO2, 5-minute intervals, temporary MCAO versus sham. A temporary aneurysm clip is applied to the proximal middle cerebral artery in the MCAO group at time = 0 minutes, and released to allow reperfusion at time = 120 minutes. MCAO, middle cerebral artery occlusion; $PbtO_2$, brain tissue oxygen concentration.

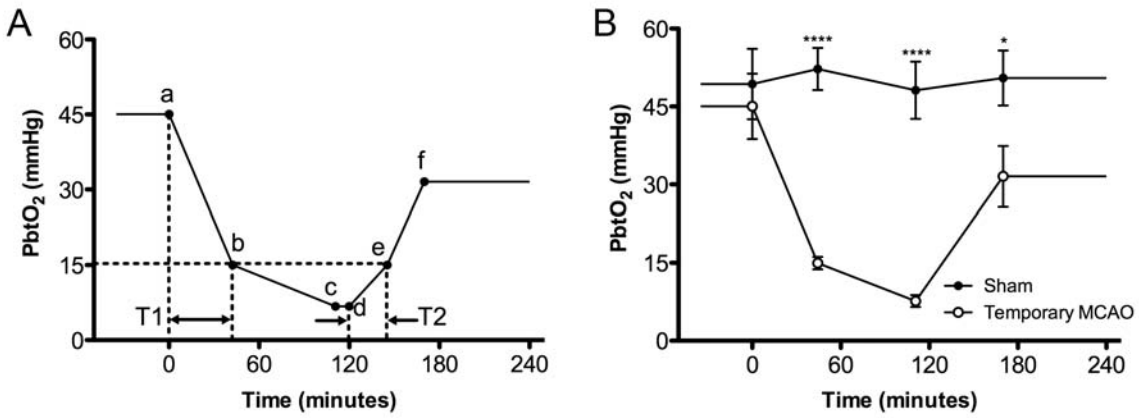

Figure 4.2.2: $PbtO_2$ response to temporary occlusion. a) Schematic of the $PbtO_2$ response to temporary MCAO. The aneurysm clip is applied to the proximal middle cerebral artery at point *a*, $PbtO_2$ falls to 15mmHg at point *b*, $PbtO_2$ reaches its lowest value at point *c*, the clip is removed at point *d*, $PbtO_2$ rises above 15mmHg at point *e*, and point *f* is the mean maximum reperfusion $PbtO_2$ value. b) Comparison of $PbtO_2$ at time points *b*, *c* and *f* during temporary MCAO and sham surgery. *, $p<0.05$; ****, $p<0.0001$; MCAO, middle cerebral artery occlusion; $PbtO_2$, brain tissue oxygen concentration.

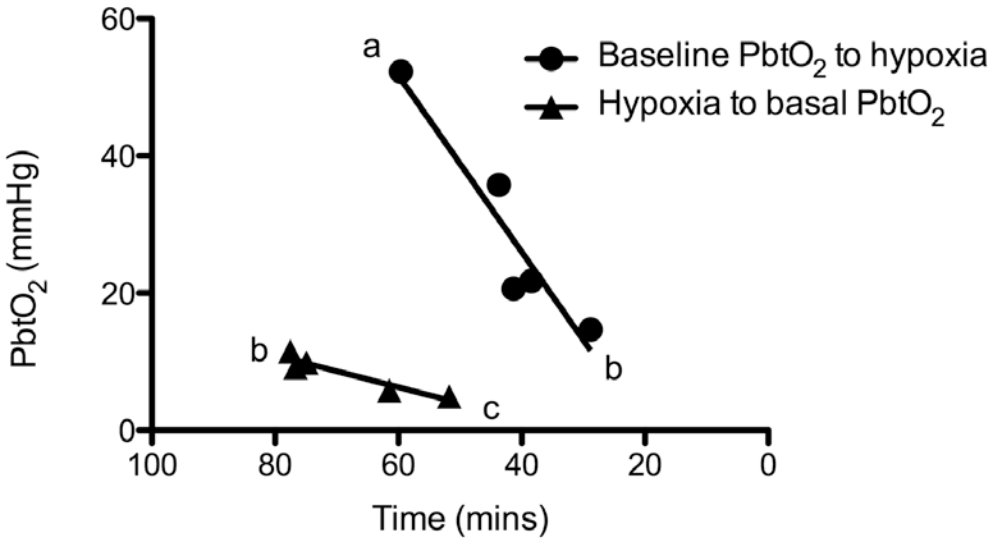

Figure 4.2.3: $PbtO_2$ decline following clip occlusion. Non-linear regression analysis, straight line fit, comparing baseline $PbtO_2$ with time for $PbtO_2$ to fall below 15mmHg after MCAO (point *a* to *b*), and time for $PbtO_2$ to fall from 15mmHg to the lowest recorded value (point *b* to *c*). MCAO, middle cerebral artery occlusion; $PbtO_2$, brain tissue oxygen concentration.

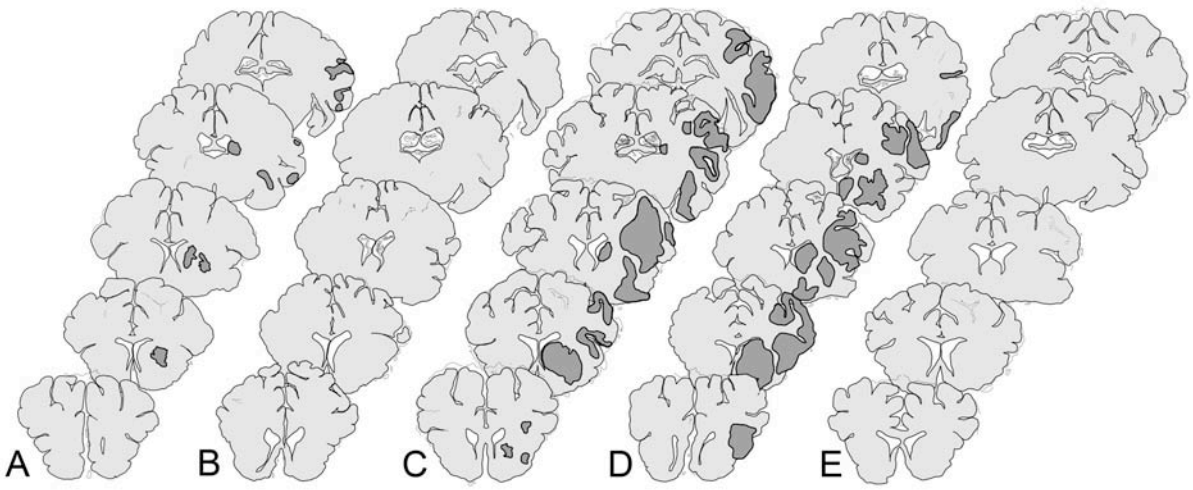

Figure 4.2.4: Histopathology, coronal stack. Schematic representation of calculated stroke volumes from H&E staining, corresponding with animals in Table 4.2.1: MCAO animal 1 (a), 2 (b), 3 (c), 4 (d) and 5 (e). Normal brain is shaded light grey, ischemic brain is shaded dark grey.

MCAO animal	Baseline $PbtO_2$ (mmHg)	Time for $PbtO_2$ to descend <15mmHg (mins)	Time for $PbtO_2$ <50% baseline (mins)	Lowest $PbtO_2$ (mmHg)	Lowest $PbtO_2$ (% of baseline)	Time for $PbtO_2$ to ascend >15mmHg (mins)	Duration $PbtO_2$ <15mmHg (mins)	Highest reperfusion $PbtO_2$ (mmHg)	Highest reperfusion $PbtO_2$ (% of baseline)	Stroke Volume (% of whole brain)
1	67.1	59.6	31.6	10.1	15.1	4.4	64.8	43.3	64.5	2.3
2	32.7	28.8	13.2	9.2	28.1	1.8	93.0	29.2	89.3	0
3	50.9	43.7	11.5	5.1	10.0	8.2	84.5	46.1	90.6	13.3
4	36.8	38.4	26.5	5.8	15.8	2.1	83.7	23.8	64.7	22.0
5	35.6	41.3	9.0	3.5	9.8	109.9	188.6	15.2	42.7	0

Table 4.2.1: Individual $PbtO_2$ and stroke volume data, MCAO group.

4.3 Malignant middle cerebral artery stroke in an ovine model: Raised intracranial pressure following permanent but not transient proximal middle cerebral artery occlusion

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Running headline: Ovine malignant middle cerebral artery stroke.

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4.3.1 Abstract

Background: Malignant middle cerebral artery (MCA) stroke has a disproportionately high mortality due to the rapid development of refractory space occupying cerebral edema. Animal models are crucial to the development of successful anti-edema therapies, however poor clinical translation is frequently associated with the predominately used rodent models, such that large animal gyrencephalic models of malignant MCA stroke are urgently needed.

Methods: 28 adult female Merino sheep were randomized to sham surgery (n=6), temporary proximal MCA occlusion (MCAO, n=12) or permanent MCAO (n=10). Intracranial pressure (ICP) and brain tissue oxygen were monitored for 24 hours under light general anesthesia. MRI was performed in 12 animals (temporary MCAO n=6, permanent MCAO n=6). Brains were removed and stained with 2,3,5-triphenyltetrazolium chloride (TTC) to calculate stroke volume.

Results: Temporary MCAO demonstrated no increase in ICP, radiological evidence of ischemia within the MCA territory but without space occupying edema, and TTC infarct volumes of 7.9 +/- 5.1%. Permanent MCAO resulted in significantly elevated ICP, radiological evidence of space occupying cerebral edema and TTC infarct volumes of 27.4 +/- 6.4%. There was a 30% early mortality rate following permanent MCAO, which was associated with a significant increase in ICP and a trend towards larger TTC stroke volumes compared with animals that survived.

Conclusions: Permanent proximal MCAO in the sheep results in space occupying cerebral edema, raised ICP and early mortality similar to human malignant MCA stroke, and this animal model may prove useful for preclinical testing of anti-edema therapies that have shown promise in rodent studies.

4.3.2 Introduction

Malignant middle cerebral artery (MCA) stroke consists of a rapid neurological deterioration secondary to space occupying cerebral edema following large volume MCA territory infarction (200). It accounts for 10-15% of all supratentorial strokes but a disproportionately high 30-day mortality rate approaching 80% (202, 407), compared with around 20% for all ischemic strokes (17). Death results from rapid edema formation, raised intracranial pressure (ICP) and transtentorial herniation, typically within 2-5 days of stroke onset but often as early as 24 hours (200, 202, 393). Decompressive craniectomy has proven to be an effective therapy to significantly reduce the number of dead or severely disabled patients following malignant MCA stroke (243), however it is highly invasive and not without its own morbidity and mortality (207). Nevertheless, it remains a powerful treatment for a deadly condition for which we currently have no non-surgical therapeutic options, despite several novel therapies having proven beneficial in preclinical rodent studies (231, 408).

There are a number of reasons why experimental therapies have failed to translate clinically, one of which may be attributable to the animal model in which they are tested. The rodent intraluminal thread model of MCA occlusion (MCAO) has become the cornerstone of animal stroke research and a first line for trialing novel therapies (299), but potentially at the costs of rendering large animal models obsolete and of continued failed translation. Although our understanding of the pathophysiological mechanisms of cerebral ischemia at a cellular and molecular level has increased considerably due to knowledge gained from rodent models of

stroke (15), the gross neuroanatomical differences between small animal lissencephalic brains and large animal gyrencephalic brains make it inappropriate for rodent intracranial pathophysiology to simulate the human state after large cerebral insults (323). This is particularly relevant in the context of space occupying lesions and raised ICP, in which the rodent brain frequently is associated with an inconsistent ICP response and low or variable mortality despite very large insults (320, 323, 324).

An ovine model of gyrencephalic MCAO has previously been characterized, including descriptions of magnetic resonance imaging (MRI), positron emission tomography, behavioral phenotyping and histopathology after permanent occlusion of one branch, two branches or the main MCA trunk (329). More recently, we have described the surgical approach to the sheep proximal MCA at its origin from the terminal internal carotid artery, demonstrating almost complete MCA territory ischemia following permanent proximal MCAO and smaller lesions with transient occlusion and reperfusion (402). Despite histological evidence of large areas of ischemia, blood-brain barrier (BBB) dysfunction and early edema development, we were unable to demonstrate significantly elevated ICP or radiological evidence of space occupying edema or cerebral herniation, which we attributed to our short monitoring period of only 4 hours. We hypothesized that a longer period of monitoring would reproduce the intracranial pathophysiological changes in human MCA territory stroke. We therefore set out to characterize the ICP, radiological and histopathological response to permanent and transient ovine proximal MCAO in which the monitoring period was extended to 24 hours after stroke onset.

4.3.3 Materials and Methods

4.3.3.1 Experimental procedure

All studies were approved by the Animal Ethics Committees of the University of Adelaide and SA Pathology, and conducted according to guidelines established for the use of animals in experimental research as outlined by the Australian National Health and Medical Research Council code of practice for the care and use of animals for scientific purposes (7^{th} edition, 2004).

4.3.3.2 Animals and experimental design

28 adult female Merino sheep 18-36 months old (mean weight 59.5 +/- 7.3kg) were allocated to the study. Animals were intra-operatively randomized to permanent right proximal MCAO (n=10), 2 hours of temporary aneurysm clip right proximal MCAO followed by reperfusion (n=12) or sham surgery (n=6). The general anesthesia and surgical protocol, including insertion of a left hemisphere Codman ICP monitor (Codman & Shurtleff Inc., MA) and right hemisphere brain tissue oxygen ($PbtO_2$) LICOX probe (Integra LifeSciences, NJ), has been previously described (402). In addition to the established protocol, intramuscular antibiotics (Cephalexin 15mg/kg, Virbac Pty Ltd, Australia) were administered at induction and 12 hourly until the end of the experiment, an indwelling urinary catheter was inserted for urine collection and output measurement, and intravenous fluids were administered as an infusion via the femoral venous catheter (Hartmann's solution 4mL/kg/h, Baxter Pty Ltd, Australia). To maintain a neutral acid-base status, the strongly alkaline saliva drool was collected and periodically returned via an orogastric feeding tube, and sodium bicarbonate 8.4% (Pfizer Australia Pty Ltd, Australia) was added to the intravenous infusion as required. Core body

temperature was monitored and normothermia was maintained with a heating pad and blankets. Animals were monitored under light general anesthesia as previously described for 24 hours after MCAO or sham.

4.3.3.3 Magnetic Resonance Imaging

12 animals (permanent MCAO n=6, temporary MCAO n=6) had imaging in a 1.5T Siemens Sonata MRI scanner (Siemens AG, Munich, Germany) at the end of the monitoring period using the protocol previously described (402). Radiological infarct volume was calculated as a percentage of whole brain on coronal section using the diffusion weighted images (DWI), corrected for edema using a modified Swanson calculation (381). Cerebral edema was calculated on coronal T2 weighted imaging sequences (T2WI) as a percentage of whole brain uncorrected. Midline shift was assessed on axial T1 weighted imaging sequences (T1WI), and measured in millimeters at the level of the interventricular foramen. Cerebral herniation and brainstem compression was identified on axial and sagittal T1WI.

4.3.3.4 Histological Examination

At the end of the 24 h monitoring period, or after MRI, animals were administered intravenous heparin (5000I.U./5ml; Pfizer, NY) and killed via common carotid perfusion fixation with cold TRIS-buffered saline. The brains were subsequently rapidly removed, sliced into 10mm coronal slices using a custom made matrix, and a 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich Pty Ltd, Australia) stain was performed to determine infarct volume as previously described in rodent stroke (409); non-infarcted tissue stains red/pink in color and infarcted tissue remains a pale cream/white color. Brain slices were incubated in 3% TTC at 37℃ under dark room conditions for 20min, turning once. Anterior and posterior

sides of all brain slices were photographed on a flatbed scanner (Canon CanoScan LiDE700F, Canon Inc., Japan). The degree of infarction was determined by an observer blinded to the surgery groups and experienced in the evaluation of infarct determination, with infarct volume calculated as a percentage of whole brain corrected for edema via the modified Swanson calculation (381). Sections were then immersion fixed in 10% neutral-buffered formalin for a minimum of 7 days prior to being processed for histological examination by H&E, albumin (dilution 1:2000, Dako, Glostrup, Denmark) and Caspase-3 (dilution 1:1000, Abcam, Cambridge, UK) immunohistochemistry, and Weil's stain**.**

4.3.3.5 Statistical Analysis

Data is expressed as mean +/- standard deviation. For ICP and $PbtO_2$ results, which were not normally distributed and did not have uniform variance, raw data underwent a logarithmic exponential transformation and were then expressed as geometric mean with standard deviation (410). Physiological data (arterial blood pressure, ICP, $PbtO_2$, PaO_2, $PaCO_2$, core body temperature) was analyzed using two-way analysis of variance (ANOVA) followed by individual Bonferroni tests (Prism Version 5.0d, Graphpad, CA). Physiological parameters were analyzed pre-MCAO or sham surgery, and at hourly intervals until the completion of the experiment, with the exception of arterial blood gas data which was collected 4 hourly. Lesion volume data and MRI characteristics were analyzed by individual student t-tests. A p-value of $p<0.05$ was considered significant.

4.3.4 Results

4.3.4.1 Surgery and basic physiology

There were no surgical complications. Mean arterial blood pressure, PaO_2, $PaCO_2$ and core body temperature were within physiological parameters with no difference between groups throughout the monitoring period. All sham and temporary MCAO animals survived the 24-hour monitoring period. Of the permanent MCAO animals, 3 out of 10 (30%) died prematurely secondary to raised ICP and brainstem compression, characterized initially by an elevation in mean ICP and widening of the ICP pulse amplitude, followed by a rise and then fall in end tidal CO_2, and finally arterial hypotension and cardiac arrest. Mean time to death in animals dying prematurely was 17h3m +/- 2h46m (range 14h30m – 20h0m). One of the non-survivors had MRI examination at the point of arterial hypotension but prior to cardiac arrest.

4.3.4.2 Intracranial Pressure

Intracranial pressure after MCAO is expressed in Figure 4.3.1. The mean pre-operative ICP was 6.4 +/- 1.9mmHg, with no difference between groups. ICP rose above pre-operative levels in the sham group and was maintained at approximately 10mmHg for the duration of the monitoring period (Figure 4.3.1). In temporary MCAO animals, ICP rose slightly higher to 13.0 +/- 2.0mmHg by 24 hours, however with no overall significant difference compared with sham animals.

After permanent MCAO, ICP rose significantly compared with sham ($p<0.0001$), and remained elevated for the duration of the monitoring period with a continuous upwards trend (Figure 4.3.1a). Permanent MCAO animals that died prematurely also had a significantly

elevated mean ICP compared with permanent MCAO animals that survived (27.0 +/- 1.3mmHg vs. 18.5 +/- 1.3mmHg, $p<0.0001$), which was sustained for the duration of the monitoring period (Figure 4.3.1b).

4.3.4.3 Brain Tissue Oxygen

$PbtO_2$ following MCAO is expressed in Figure 4.3.2. The mean baseline $PbtO_2$ for all animals was 45.4 +/- 1.3mmHg, with no difference between groups. In sham animals, $PbtO_2$ showed a gradual decline over the first 8 hours, to eventually plateau at approximately 30-35mmHg for the remainder of the experiment. In temporary occlusion animals baseline $PbtO_2$ was 44.8 +/- 1.4mmHg prior to aneurysm clip application, falling to 10.1 +/- 2.3mmHg 2 hours after MCAO, immediately prior to clip removal and reperfusion ($p<0.0001$). It then rapidly rose to 22.5 +/- 2.2mmHg 1 hour after reperfusion, then gradually climbed to 29.1 +/- 2.5mmHg 9 hours following reperfusion, at which point any difference to sham lost significance. Temporary MCAO $PbtO_2$ plateaued at approximately 28-30mmHg for the remainder of the experiment.

Permanent occlusion animals demonstrated a baseline of 45.6 +/- 1.4mmHg, which declined significantly following MCAO to a low of 7.0 +/- 2.3mmHg at 8 hours ($p<0.0001$), and continued to stay low to eventually plateau at approximately 7mmHg by 24 hours ($p<0.0001$). Permanent MCAO $PbtO_2$ remained the lowest of all three groups at the conclusion of the experiment, with temporary MCAO approaching sham levels (Figure 4.3.2).

4.3.4.4 MRI

MRI characteristics are expressed in Table 4.3.1, and Figures 4.3.3 and 4.3.4. MRA

confirmed no flow beyond the proximal MCA in all permanent MCAO animals, and reperfusion of the right MCA territory in all temporary MCAO animals (Figure 4.3.3a, c). The mean DWI deficit for permanent MCAO animals was 25.4 +/- 6.8%, compared with 10.7 +/- 3.9% for temporary MCAO animals (p=0.001). Cerebral edema measured on coronal T2WI revealed a mean volume of 25.0 +/- 4.9% for permanent MCAO animals, and 5.4 +/- 4.1% for temporary MCAO animals (p<0.0001, Figure 4.3.4). Mean midline shift was 3.3 +/- 0.6mm for permanent MCAO animals, compared with 1.0 +/- 0.8mm for temporary MCAO animals (p=0.0002). None of the temporary MCAO animals demonstrated transtentorial herniation and brainstem compression, whereas herniation was present in 3 out of 6 (50%) permanent MCAO animals, seen on MRI as crowding around the foramen magnum, effacement of the cisterna magna and tonsillar herniation (Figure 4.3.4f). These animals also demonstrated significant local mass effect with sulcal effacement. Permanent MCAO animals that herniated (n=3, including 1 animal that died prematurely) demonstrated a mean DWI lesion volume of 27.7 +/- 5.5%, a mean T2WI edema volume of 27.4 +/- 3.6%, and mean midline shift of 3.6 +/- 0.5mm; permanent MCAO animals that did not herniate (n=3, all survived) demonstrated a mean DWI lesion volume of 23.2 +/- 8.4% (p=0.479), a mean T2WI edema volume of 22.6 +/- 5.4% (p=0.269), and mean midline shift of 3.0 +/- 0.7mm (p=0.298).

4.3.4.5 TTC and Histology

TTC staining was performed in 5 of 6 sham animals, 11 of 12 temporary MCAO animals, and 8 of 10 permanent MCAO animals. There was no TTC evidence of ischemia in any of the sham animals. All temporary MCAO animals demonstrated TTC pallor within the right MCA cortex (Figure 4.3.5); subcortical structures were involved in 6 of 11 animals, most frequently affecting the head of caudate nucleus. The mean TTC infarct volume for temporary MCAO

was 7.9 +/- 5.1%. Permanent MCAO animals demonstrated significantly larger TTC infarct volumes, generally affecting the whole MCA supplied cortex and typically most of the subcortical structures (caudate, putamen, thalamus), with mean TTC infarct volumes of 27.4 +/- 6.4% ($p<0.0001$). One permanent MCAO animal demonstrated infarction in the contralateral posterior cerebral artery territory (PCA), attributed to raised ICP, uncal herniation and occlusion of the PCA on its passage through the tentorial notch; this animal died prematurely at 20 hours following MCAO and had a highest mean ICP of 63.1 +/- 7.8mmHg with a pulse amplitude of 24.7mmHg in the hour prior to death. Animals that herniated on MRI tended to have larger TTC infarct volumes than permanent MCAO animals that did not herniate (27.9 +/- 3.0% vs 23.6 +/- 9.4%, $p=0.485$), and TTC infarct volumes of permanent MCAO animals that died prematurely tended to be larger than those that survived (31.8 +/- 1.3% vs 26.0 +/- 6.9%, $p=0.301$), however neither of these comparisons were statistically significant.

Histopathological examination revealed different patterns of injury between the two MCAO groups (Figure 4.3.6). Temporary MCAO sections stained for H&E demonstrated acute ischemic cell change universally within the stroke core. In the penumbra, there was evidence of an inflammatory response characterized by astrocytic swelling and migration of polymorphonuclear cells and granulocytes into the parenchyma (Figure 4.3.7a). There was no inflammation within the ischemic core. Permanent MCAO demonstrated larger areas of acute ischemic cell change corresponding to TTC examination, frequently confined to the grey matter and sparing the white matter (Figure 4.3.7b). There was little evidence of an inflammatory reaction at the periphery of permanent MCAO sections.

The Weil stain as a marker of cerebral edema and albumin immunostain as a marker of BBB disruption corresponded well with MRI T2WI evidence of edema (Figure 4.3.6). The Caspase-3 stain confirmed necrosis, and not apoptosis, as the underlying mechanism of cell death within the ischemic core following permanent MCAO; there was however considerable

apoptosis within reperfused ischemic territories, as well in the contralateral hemisphere of permanent MCAO animals (Figure 4.3.8).

4.3.5 Discussion

Decompressive craniectomy for malignant MCA stroke improves early survival and functional outcome by preventing brainstem compression secondary to transtentorial herniation (207). Rather than managing the consequences of space occupying edema, novel therapies directed towards limiting BBB permeability (411) and vasogenic edema (210, 408) after ischemic stroke have frequently proven to be as effective as or superior to decompression in rodent models. However, the significant differences between rodent and human intracranial anatomy, particularly the delicate rodent tentorium cerebelli (323, 412, 413), make translation of pathology involving raised ICP and transtentorial herniation questionable. The initial STAIR guidelines included a recommendation for the testing of neuroprotective and restorative drugs in gyrencephalic species after initial rodent studies, secondary to the consistent failure of seemingly promising treatments to translate from small animal models of ischemia (169, 252). The neuroanatomical properties of the sheep (402), together with the physiological and histological outcomes we have demonstrated, suggest that an ovine MCAO model may be a suitable and ethically less debatable gyrencephalic alternative to non-human primates for preclinical testing after preliminary rodent work.

The sheep fulfills other STAIR criteria by allowing for extensive physiological monitoring including arterial blood pressure, arterial blood gas analysis, multiple blood draws from the same animal at multiple time points, electrolyte and renal function, hemoglobin and hematocrit, fluid balance and core body temperature. Furthermore, we have demonstrated multiple outcome measures for proximal MCAO in the sheep, including physiological (ICP, $PbtO_2$), radiological (DWI stroke volume, T2WI edema, midline shift and herniation

syndromes) and pathological (TTC and immunohistopathology). Early mortality occurred in 30% of our sheep within 24 hours of permanent MCAO secondary to cerebral herniation and brainstem compression. Like previous non-human primate models, and of course large volume human stroke, early mortality after permanent MCAO is a consistent finding, hence large animal non-primate models that replicate this provide a powerful tool for investigating therapies targeting malignant ischemic cerebral edema and mortality. Because of this and the extent of extracranial dissection required to reach the proximal MCA, this model of permanent proximal MCAO is not suitable for long-term survival studies, however a survival ovine model of smaller volume permanent MCA stroke has previously been characterized and is in use by Boltze and colleagues (329). Additional advantages of the present model are that it allows for reperfusion and has investigated the effects of MCAO in female animals, both of which are recommendations made by the original STAIR committee (252).

ICP

Raised ICP as an outcome measure is used frequently in human and animal studies of traumatic brain injury, but less commonly in the setting of acute cerebral ischemia. This is relatively surprising given our extensive knowledge of the pathophysiological changes associated with ICP dynamics and space occupying lesions and the availability and ease of use of ICP monitors, but perhaps less surprising considering that the majority of ischemic strokes are not associated with clinically relevant intracranial hypertension. Nevertheless, intracranial hypertension has been identified as a cause of early in-hospital mortality in up to 28.9% of all patients admitted to a stroke unit (203), with raised ICP generally being identified on radiological or clinical findings rather than by direct measurement of pressure.

Hacke's original description of malignant MCA infarction included 26 patients in whom an epidural ICP probe was inserted to monitor the effects of anti-edema therapy and barbiturate

coma placed after radiological identification of space occupying edema (202). Baseline ICP was 19.4mmHg, and over 4-10 days the mean highest ICP recorded in patients that died was 43mmHg compared with a mean highest ICP of 28mmHg in those that survived. Ropper and Shafran reported their ICP findings in 6 patients in an earlier series (414), and a larger series by Schwab suggested ICP>35mmHg was a poor prognostic sign (209), however the clinical usefulness of ICP monitoring for ischemic stroke is questionable at best and is not as useful an indicator to perform surgical decompression as clinical and neuroimaging findings (209, 415). Experimentally however it is simple to perform and provides a quantifiable numerical value from which the physiological effectiveness of anti-space occupying edema therapies can be assessed.

Several authors have described ICP after rodent stroke, however measurements tend to be taken from within the posterior fossa (324, 325). Due to the underdeveloped tentorium in the rat an infratentorial ICP monitor is probably an accurate reflection of supratentorial pressure following space occupying cerebral edema, however lack of significant compartmentalization between cerebrum and cerebellum makes transtentorial herniation following rodent MCAO unlikely. Nevertheless, rodent MCAO is associated with space occupying cerebral edema and midline shift (322), and decompression has shown to improve mortality rates and neurological function (239), suggesting that ICP following large volume rodent MCA stroke is still a contributing factor to outcome. There have been several studies investigating ICP after ischemic stroke in gyrencephalic species including cats, dogs and baboons, however almost all have reported relatively short monitoring periods of only 6 to 12 hours post stroke (326-328). O'Brien and Waltz performed transorbital MCAO in cats and measured ICP for up to 15 days via a number of different methods, however were plagued by technical difficulties (321). The present study therefore is the only report of ICP after permanent and temporary MCAO in a gyrencephalic species for a clinically relevant minimum 24 hours.

ICP was measured in the contralateral hemisphere in order to avoid overcrowding in the

ipsilateral hemisphere. There is some experimental evidence to suggest that a significant pressure gradient exists between hemispheres in gyrencephalic brains with unilateral space occupying pathology including large volume stroke (326), whereas others believe pressure is transmitted uniformly within the supratentorial compartment (416). A human case report suggested that gradients may only be occurring at the time of decompensation and intracranial shift (392). Recording ICP in the right hemisphere in our study may have yielded higher mean pressures particularly after permanent MCAO, or bilateral recordings may have added more information to the question of interhemispheric pressure gradients following MCA stroke. Even so, our contralateral recordings still demonstrated a significant elevation in ICP following ischemic space occupying cerebral edema.

$PbtO_2$

We have previously characterized the regional $PbtO_2$ response to permanent and temporary MCAO in the sheep, however only for the first 4 hours (402). We demonstrated reduced $PbtO_2$ in temporary occlusion animals that returned to sham levels 30 minutes following reperfusion, and a sustained reduction in $PbtO_2$ in animals with permanent MCAO. The present study demonstrates similar results, however with some important differences. Firstly, it took 7 hours for $PbtO_2$ in temporary MCAO animals to approach and lose any significance with sham levels, more than 6 hours longer than our initial study. Second is that temporary MCAO $PbtO_2$ levels fell much lower in the present study, almost to permanent MCAO levels, and in fact declined faster than the permanent MCAO group. Possible explanations for these differences include surgical experience and improved technique, and the logarithmic exponential transformation with geometric mean data analysis used in the present study, both of which should result in more accurate data collection and analysis compared with the previous study. Similar to our previous study and important to note, we observed no rebound

hyperoxia up to 22 hours after reperfusion.

MRI

The development of a malignant course following MCA stroke is most frequently associated with large stroke volume, however factors such as inflammation and BBB breakdown may also be important mechanisms (417). Nevertheless, large territory human MCA stroke volumes, such as DWI stroke volume >145cm^3 (242) or Computerized Tomography ischemic changes affecting at least two-thirds of the MCA territory with basal ganglia involvement (241) or space occupying edema (418), have shown to be predictive of a malignant course and an improved outcome following surgical decompression (243). Midline shift is a frequently associated, albeit relatively late, finding, and the amount of midline shift has been shown to correlate well with edema volume in experimental rodent studies (322). When compared with the smaller infarcts of temporary MCAO animals, we found permanent MCAO was associated with significantly larger diffusion deficit and edema volume (approximately 25% of the supratentorial brain) as well as radiological evidence of raised ICP secondary to focal space occupying pathology. Furthermore, permanent MCAO animals that had radiological evidence of raised ICP tended to have larger DWI, T2WI and TTC volumes, however the study was underpowered to detect a significant difference.

Histopathology

Ischemic injury on H&E for both permanent and temporary MCAO mirror TTC and DWI evidence of ischemia, and similarly the Weil stain correlated well with T2WI cerebral edema. Albumin extravasation confirms that disruption of the BBB is the source of cerebral edema in

this model, and is significantly more prevalent following permanent MCAO. The general paucity of apoptosis within permanent MCAO core also helps confirm that necrosis is the predominant mechanism of cell death, however energy-dependent cell death is a prominent feature following reperfusion. Apoptosis was also a feature of non-ischemic brain following permanent MCAO indicating secondary injury mechanisms associated with raised ICP. The Caspase-3 immunostains suggest there are different mechanisms and therefore potentially different targets for reducing post-ischemia cell death, depending on the presence or absence of early reperfusion or space occupying edema.

4.3.6 Conclusions

We have demonstrated a malignant course following permanent proximal MCAO in an ovine model, characterized by intracranial hypertension, space occupying cerebral edema, large diffusion deficit and a 30% mortality within 24 hours of stroke. Temporary MCAO followed by reperfusion results in smaller stroke volumes, localized cerebral edema without space occupying effect, no rebound brain tissue hyperoxia, and ICP similar to sham. This model provides a powerful tool for the preclinical investigation of novel therapies targeting malignant cerebral edema that have shown efficacy in rodents.

4.3.7 Disclosure/Conflict of Interest: None.

4.3.8 Figures and Legends

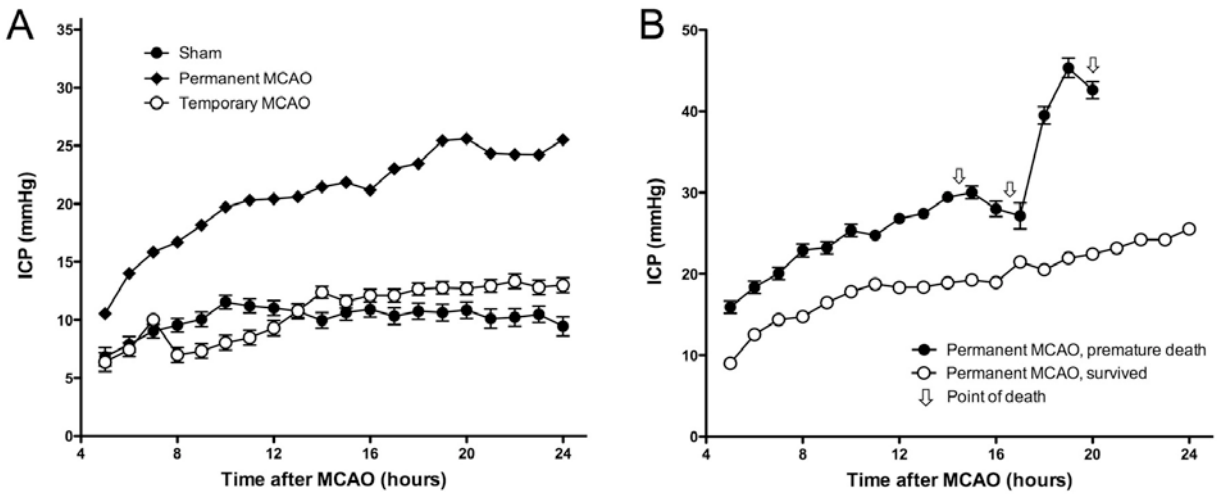

Figure 4.3.1: Mean ICP, 24 hours. Mean ICP following sham surgery, temporary MCAO or permanent MCAO (a). Mean ICP following permanent MCAO, animals that died within the 24-hour monitoring period versus animals that survived (b). ICP, intracranial pressure; MCAO, middle cerebral artery occlusion.

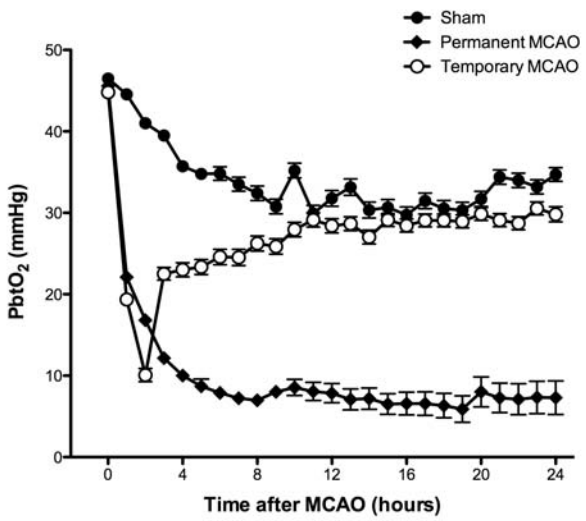

Figure 4.3.2: Mean $PbtO_2$ following MCAO or sham, 24 hours. $PbtO_2$, partial pressure of brain tissue oxygen; MCAO, middle cerebral artery occlusion.

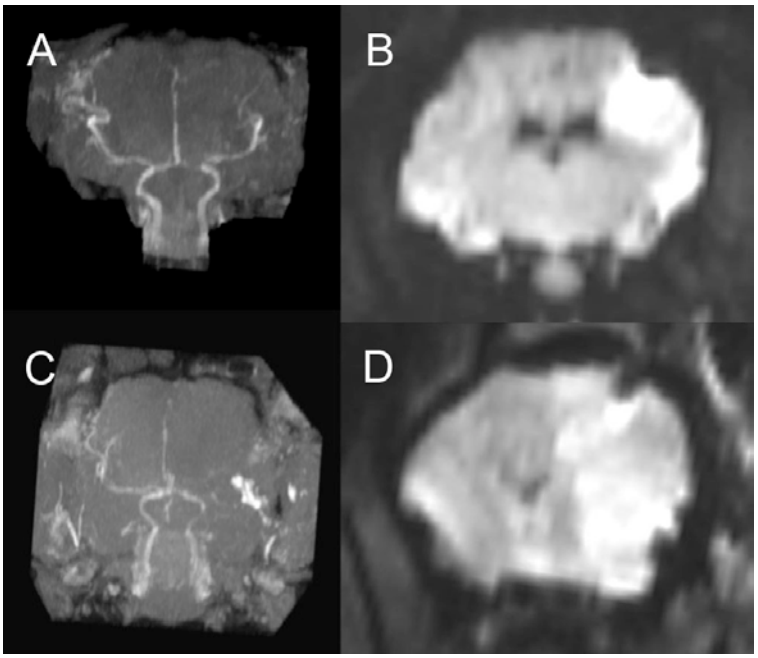

Figure 4.3.3: MRI findings at 24 hours, MRA and DWI. Temporary MCAO MRA demonstrates reperfusion in the right MCA territory (a), and diffusion deficit on DWI in the right MCA cortex (b). Permanent MCAO MRA shows no flow beyond the right proximal MCA (c), and a larger diffusion deficit involving the whole MCA territory including subcortical structures (d). DWI, diffusion weighted imaging; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; MRA, magnetic resonance angiography.

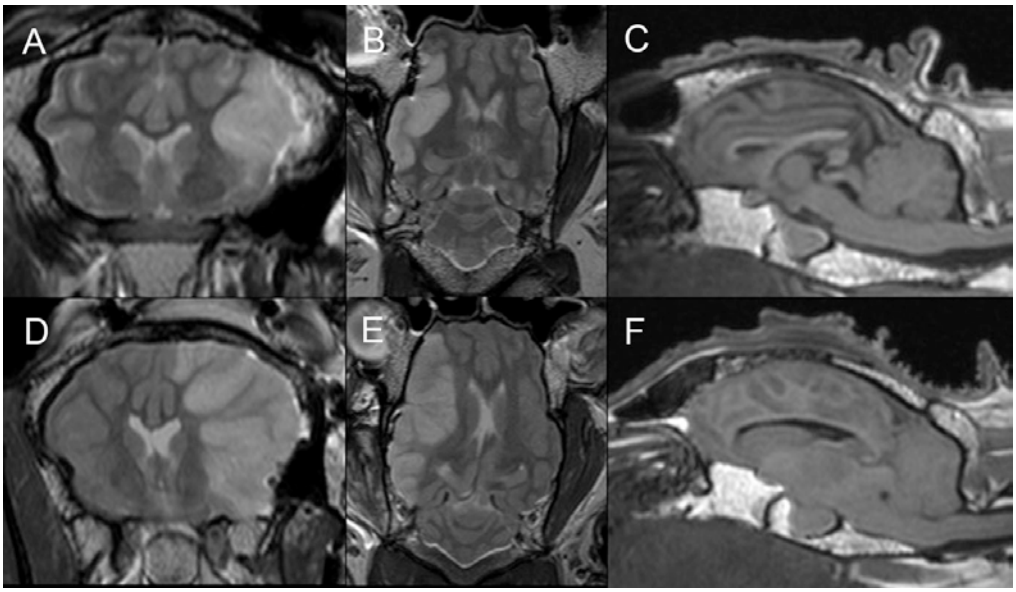

Figure 4.3.4: MRI findings at 24 hours, T1 and T2 weighted imaging. Temporary MCAO demonstrates cerebral edema in a right MCA distribution similar to the diffusion deficit in *Figure 4.3.3b* on T2 coronal imaging (a), and no mass effect or midline shift on T2 axial imaging (b). Sagittal T1 sequences show preserved basal cisterns and posterior fossa CSF spaces (c). T2 coronal (d) and axial (e) imaging after permanent MCAO show cerebral edema distributed as for the diffusion deficit in *Figure 4.3.3d*, with associated mass effect and midline shift. Sagittal T1 imaging demonstrates effacement of the basal cisterns and cisterna magna, tonsillar herniation and brainstem compression (f). MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion.

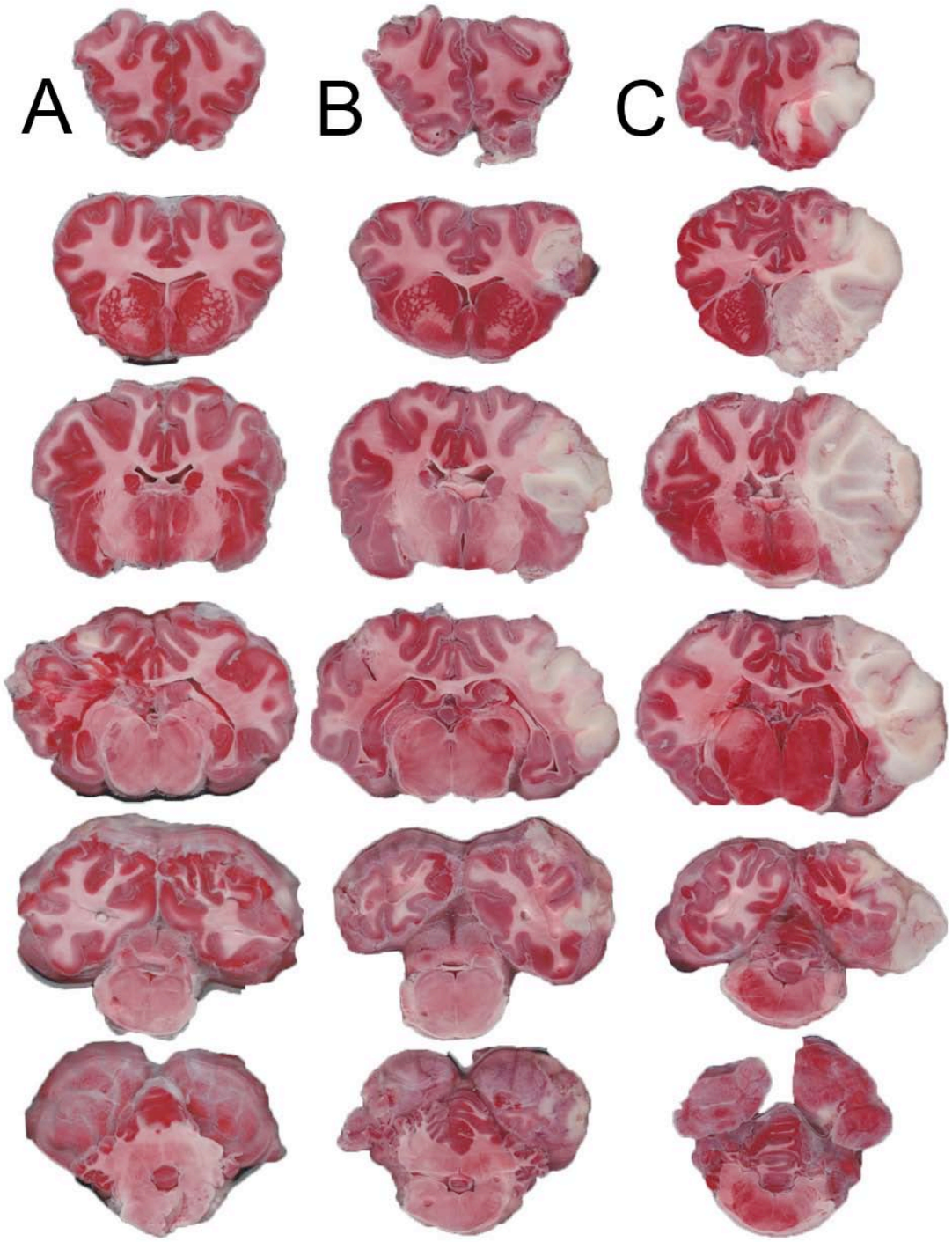

Figure 4.3.5: TTC at 24 hours, coronal stack. Unstained brain tissue represents cerebral ischemia. There is no evidence of ischemia in sham animals in the left column (a), small cortical ischemia in temporary MCAO animals in the center column (b), and large MCA territory ischemia in permanent MCAO animals in the right column (c). MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; TTC, 2,3,5-triphenyltetrazolium chloride.

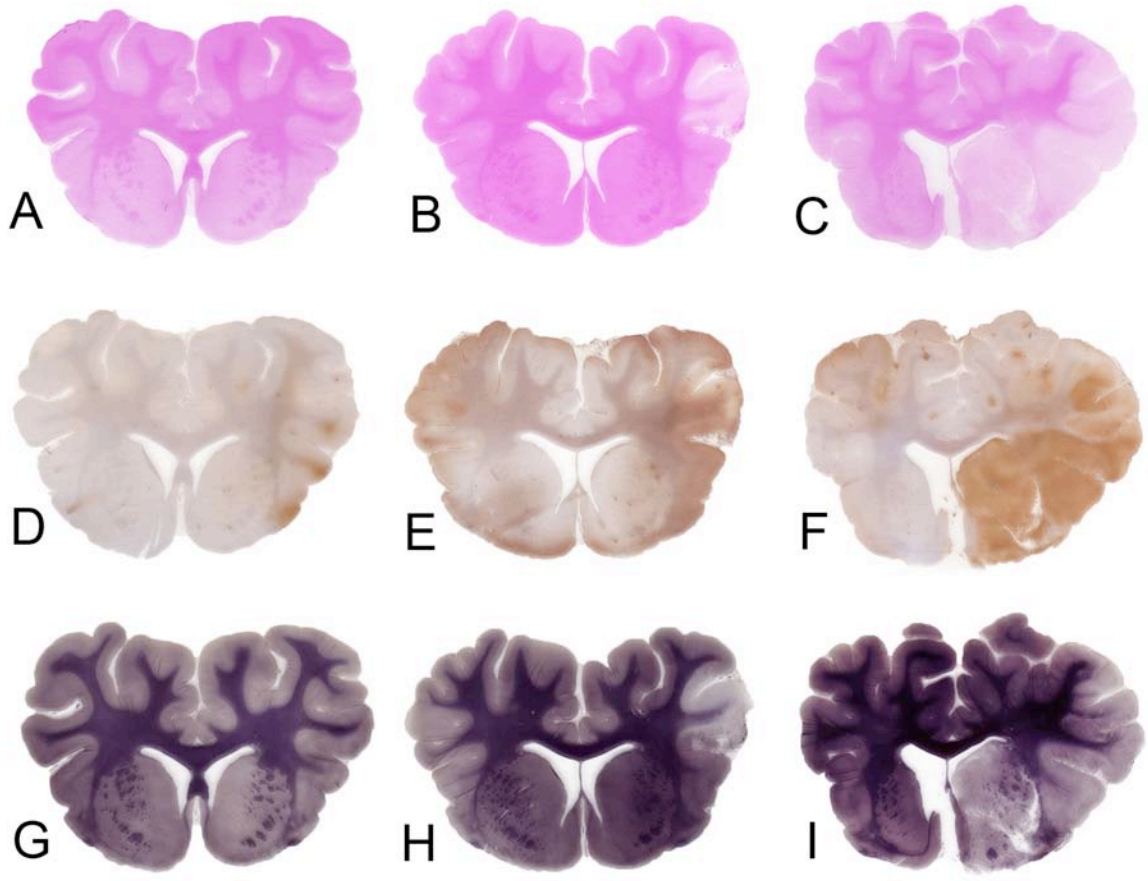

Figure 4.3.6: Histopathology, coronal section. Section level with the origin of the MCA. H&E for sham surgery (a), temporary MCAO (b) and permanent MCAO (c). Albumin immunostain for sham (d), temporary MCAO (e) and permanent MCAO (f). Weil stain for sham (g), temporary MCAO (h) and permanent MCAO (i). MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion.

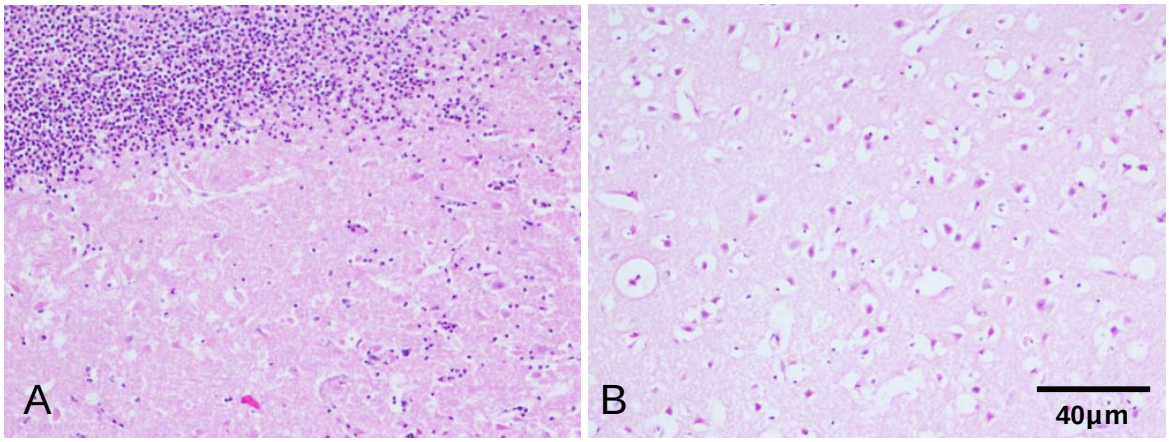

Figure 4.3.7: Histopathology, H&E, x200. Inflammatory reaction within the penumbra of reperfused animals characterized by migrating polymorphonuclear cells and axonal swelling (a). Dense area of necrosis within the ischemic core of permanent MCAO animals (b).

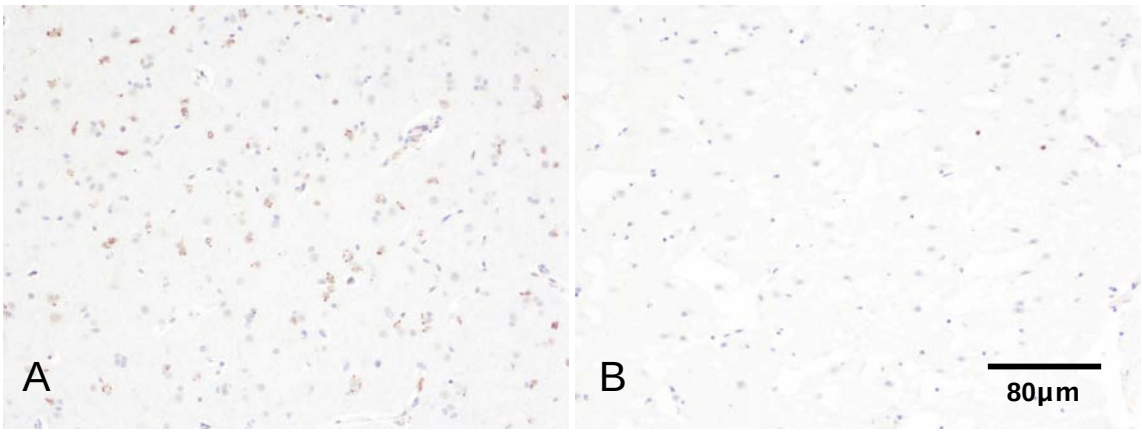

Figure 4.3.8: Caspase-3 immunostain for apoptosis, x100. Prominent apoptosis within the reperfused ischemic core (a), with relative sparsity within the core of permanent MCAO animals (b).

Animal	MCAO (Permanent/ Temporary)	TTC stroke volume (%)	MRI characteristics at 24 hours			
			DWI volume (%)	T2WI volume (%)	Midline shift (mm)	Herniation (Yes/No)
1 *	P	30.9	33.8	31.5	3.8	Y
2	T	2.6	14.7	2.3	0	N
3	P	32.8	32.4	26.1	3.1	N
4	T	14.1	7.5	3.7	0.9	N
5	P	24.9	23.3	24.7	4.1	Y
6	P	23.8	21.0	25.3	3.4	N
7	T	5.8	13.9	5.1	1.6	N
8	T	4.8	7.1	1.9	1.6	N

9	T	0.9	7.0	5.9	0	N
10	P	14.1	16.1	16.3	2.5	N
11	P	28.1	25.9	26.0	3.1	Y
12	T	12.4	14.1	13.2	1.9	N

Table 4.3.1: MRI characteristics and TTC stroke volume at 24 hours after MCAO. Animal 1 (*) died prematurely at 14.5h after permanent MCAO. DWI, diffusion weighted imaging; MCAO, middle cerebral artery occlusion; MLS, midline shift; MRI, magnetic resonance imaging; T2WI, T2 weighted imaging; TTC, tetrazolium chloride.

4.4 Invasive arterial and intracranial pressure monitoring using a Millar type strain gauge tip pressure transducer

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4.4.1 Abstract

Blood pressure monitoring is an important physiological variable in experimental animal research. Recording accurate physiological pressure is most accurately done with a Millar-type tip transducer, which can be placed into the lumen of a blood vessel, or directly into a normally turgid organ such as the brain. We describe an adaptation to a commercially available strain gauge tip transducer designed for intracranial pressure monitoring. Our adaptation provides for intra-luminal blood pressure recording in addition to sampling blood for gas or other analysis. We implant an identical probe into the brain for simultaneous recording of intracranial pressure. The advantages of this system include being able to place the transducer at a site independent of the catheter tip and recording highly accurate blood pressure at high frequency. This method exhibits zero drift and allows for arterial blood sampling without catheter obstruction for time periods exceeding 24 hours.

4.4.2 Introduction

Invasive arterial blood pressure monitoring remains an important component of animal research, and is typically performed with a measuring device coupled to an indwelling catheter. The device may consist of a pressure transducer at the tip of the catheter, or a transducer external to the animal coupled to a fluid column through which pressure waves are conducted. Tip transducers have an excellent frequency response suitable for investigating high frequency components (419), however they are generally more expensive than fluid

coupled transducers.

There are several commercially available tip transducers, typically comprising of a Millar type peizoresistive transducer attached to an indwelling vascular catheter, with or without a lumen for blood sampling (Millar Instruments, Inc., TX). The Millar catheter tip transducers with lumen come in size 4-7Fr, are reusable but non-sterile, and those without a lumen commence from size 1Fr, and again are reusable but non-sterile.

As an alternative to current ready-made tip transducers, we describe a method of adapting a commercial intracranial pressure (ICP) transducer for intravascular use. The Codman Microsensor (Codman & Shurtleff Inc., MA) consists of a pressure sensing microchip transducer mounted in a titanium case in its 1.2mm diameter tip attached to a 0.7mm diameter nylon lead measuring 100cm long. For intracranial use, the pressure sensing tip is placed in the brain parenchyma and secured to the skull via a locking rubber bung connected to a Luer-Lok plastic bolt screwed into a burr hole. The distal end of the lead is attached to a Codman ICP Express Monitoring System (Codman & Shurtleff Inc., MA), providing a mean, systolic and diastolic ICP on a digital display. Waveform analysis is not integral to the system however and requires post-processing with an analogue-digital converter. A single Codman Microsensor can be used for days or weeks in the same patient with a high level of accuracy and no appreciable drift (420).

An alternative for experimental studies is to use an adaptor cable to connect the Codman pressure transducer to an ADInstruments PowerLab system (ADInstruments, Australia). The PowerLab system will record the analogue signal from the Codman microsensor at typical frequencies of 1 kHz or greater. Having access to an accurate pressure waveform from a large central artery and the brain simultaneously makes post-processing of the waveform a useful adjunct to studies of cerebral pathology associated with deranged ICP dynamics, such as traumatic brain injury and space occupying stroke.

Using the Codman Microsensor, the interface cable, an ADInstruments bridge amplifier and recording on LabChart v7.2.4 (ADInstruments, Australia), we have successfully monitored arterial blood pressure with a Codman Microsensor via a catheter in the right femoral artery. We have also been able to reliably sample arterial blood periodically via the same catheter for extended periods without problems associated with catheter tip obstruction secondary to clot formation.

4.4.3 Method

The methodology was established in a series of studies characterizing an ovine model of middle cerebral artery stroke (402). All experiments were approved by the Animal Ethics Committees of the University of Adelaide and SA Pathology, and conducted according to guidelines established for the use of animals in experimental research as outlined by the Australian National Health and Medical Research Council code of practice for the care and use of animals for scientific purposes (7^{th} edition, 2004). The anaesthesia protocol consisted of inhalational isoflurane 1.5% and intravenous ketamine 4mg/kg/h. Animals were ultimately monitored in the sphinx position, and were administered intramuscular antibiotics at the time of induction and 12 hourly until the completion of the experiment.

The sheep femoral artery runs relatively superficially and parallel with both the femoral vein and nerve in the groin, and can easily be dissected and cannulated with the animal supine and hind legs extended. Using a sterile technique, we isolated a 3cm segment of the femoral artery, tying off the distal end and applying a vascular clamp proximally. For the catheter, we used a 12Fr gauge 42cm long sterile spinal manometer tube (Baldwin Medical Australia, Australia), which has an external diameter of 4mm but an internal diameter large enough to allow free passage of the 1.2mm Codman Microsensor tip. The male-taper Luer-Slip end was

cut off whereas the female-taper Luer-Lok end was left intact, then the cut end of the manometer was inserted in the femoral artery to a depth of approximately 15-20cm and secured around the artery with silk ties. Two stopcocks were attached to the female-taper end, one running parallel in continuity with the catheter for insertion of the pressure transducer, the second attached perpendicular to the first for fluid inflow and blood sampling (Figure 4.4.1). The Codman Microsensor was passed through a male-taper Luer-Lok locking rubber bung and connected to the first stopcock. With this setup the Codman Microsensor can then be advanced within and independent of the lumen of the arterial catheter, thus providing catheter tip blood pressure readings from distal aorta or beyond to more proximal anatomical locations, in theory limited only by the length of the Codman Microsensor lead and the ability to guide it. Fluid exchange occurred at the second stopcock, and the tap of this stopcock could be turned to any position without interfering with the passage of the nylon Codman Microsensor lead. At the end of the experiment the Codman Microsensor was removed and its 2-point accuracy confirmed in a simple water column of known height. All other arterial catheter components were discarded, however the Codman Microsensor was sterilized in 70% alcohol for reuse and therefore experimental cost reduction.

We have successfully used this method for measuring arterial blood pressure in over 40 sheep for monitoring periods exceeding 24 hours. With the catheter advanced at least 15 cm into the femoral artery, combined with a fluid inflow of up to 30mL/h of 0.9% saline, there have been no problems associated with blood clotting and catheter obstruction. Prior to the catheter tip method we used a fluid coupled external transducer, however this setup would frequently clot and obstruct within 4-6 hours, prohibiting blood sampling and damping the arterial waveform and mean arterial blood pressure. By comparison, the waveform recorded via the Codman Microsensor is physiologically accurate, undamped, directly coupled with other physiological parameters recorded simultaneously by the same means such as ICP, and highly consistent in monitoring periods exceeding 24 hours with no appreciable drift (Figure 4.4.2), however

admittedly this was not confirmed via an alternative blood pressure monitoring technique.

Others in our group have used the Codman Microsensor to monitor blood pressure in rabbits via the femoral artery without a vascular catheter, however this method precludes blood sampling from the same artery. A catheter-less method of Codman Microsensor blood pressure monitoring could therefore be applied to any species of animal with an artery large enough to accommodate the 1.2mm diameter tip. Other disadvantages include the acquisition cost of the hardware, however we have sterilized the Codman Microsensors for repeated use and have found them to be very robust; breakages associated with insertion are rare, and the same Microsensor may be used for many animals.

The Codman Microsensor ICP monitor used for measuring blood pressure via a tip transducer is thus a viable alternative to current commercial products, however adds the ability to advance the pressure sensing tip independent of the vascular catheter, a waveform pattern uncontaminated by fluid inflow artifact, coupling with other physiological parameters such as ICP and reliable blood sampling.

Acknowledgements:

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Conflict of interests:

None.

4.4.4 Figures

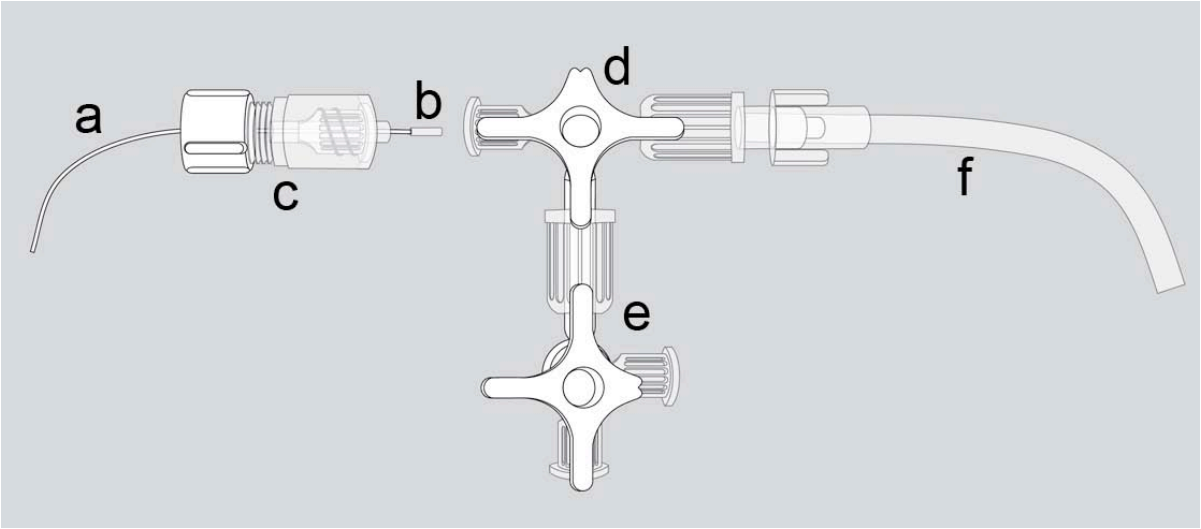

Figure 4.4.1: Configuration of femoral artery catheter and connectors. *a*, Codman microsensor lead; *b*, Codman microsensor tip; *c*, locking rubber bung; *d*, stopcock 1; *e*, stopcock 2; f, femoral catheter.

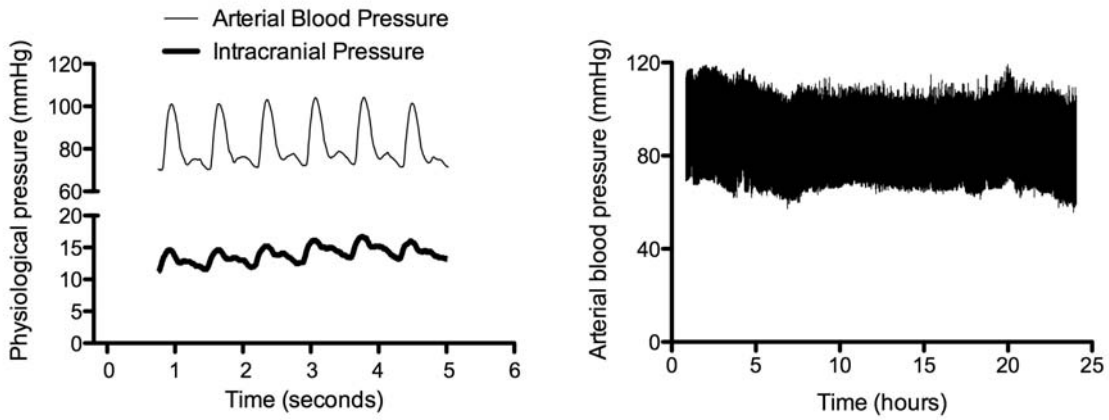

Figure 4.4.2: Waveform measurements. *Left*: typical waveform for arterial blood pressure and intracranial pressure as measured simultaneously with Codman Microsensors in the sheep. *Right*: Arterial blood pressure measured with a Codman Microsensor in the distal aorta over a 24 hour period.

5. General Discussion

5.1 The sheep as a model of MCAO stroke

The sheep model presented in the current thesis has the potential to improve the chances of successful translation of stroke therapeutics from experimental studies to clinical trials by filling the substantial void between preclinical rodent models and clinical trials of novel stroke therapies created by the gradual decline of experimentation of nonhuman primates. It is worthwhile comparing this model to the original 1999 STAIR recommendations (below in *italics*) (252), which have provided an excellent and universally accepted framework for improving the design and rigorous testing of preclinical stroke studies (347, 421-423).

Permanent MCAO models should be studied first, followed by transient (reperfusion) models. The model characterised in this thesis allows for the study of both permanent and transient stroke. This is in an advance on the only other existing sheep MCAO model, within which only permanent occlusion is achievable. We have found significant differences in pathophysiology between permanent and temporary MCAO, which further emphasizes the importance of testing novel therapies in both models. Neuroprotective agents used in conjunction with thrombolytics could be trialed in the temporary MCAO model, with clip release simulating thrombolysis at the time of thrombolytic drug administration.

Permanent and transient model studies should be initially completed in rats, then possibly in cats or primates before beginning large clinical trials with humans. The present sheep model, in either permanent or temporary MCAO guise, or both, is a good model within which to validate agents of outstanding promise identified in rodent studies before proceeding to clinical trials. It may also make a suitable and more ethically tolerable alternative to nonhuman primates in pre-clinical testing.

Blood pressure, blood gases, haemoglobin, glucose and CBF should be monitored for as long as possible; temperature should be monitored and maintained as constant as possible. It has been demonstrated that invasive arterial blood pressure by the newly described methodology (chapter 4.4), frequent arterial blood gas sampling, haemoglobin/haematocrit and glucose as well as simple electrolyte measures, and core body temperature (as well as brain temperature with the Licox probe) can all be monitored successfully for periods exceeding 24 hours. Direct measurements of CBF were not investigated in this study, however indirect reference to CBF via regional brain tissue oxygen was continuously measured, again successfully, and may in fact be just as or even more important than CBF measurements following MCAO (discussed below).

At least 2 outcome measures should be considered, functional response and infarct volume, over the acute phase (1 to 3 days) and the long term (7 to 30 days). The present study utilised multiple outcome measures, including mortality, ICP, radiological evidence of space occupying oedema, and infarct volume. It did not however evaluate neurobehaviour and function for reasons discussed below, and this may be an area of improvement in the future. Nevertheless, a more thorough evaluation of outcome was included in the present study than simply infarct volume as described in many rodent studies (424-426).

Only in acute treatment studies (typically up to 6 hours or intensive monitoring) can all the physiological variables that affect outcome be comprehensively assessed. This may be true in small animals such as rodents but in large animals, the current sheep model included, intensive monitoring of physiological variables such as ICP, BP and blood gases (as described above) can be carefully and successfully monitored for periods in excess of 24hrs. This provides yet another advantage of the sheep as a large animal model of stroke.

It is necessary to follow animals for longer than 24 hours as effects of compounds shown to be initially effective may be lost over time. One of the limitations of the present study is that

the monitoring period was limited to 24 hours; the main reason for stopping at this time point was based on the practicalities of performing overnight experiments and ethics committee approval. A 24-hour monitoring period quickly escalates to a 32 hour experiment performed over two consecutive days; with only 2 researchers involved in the vast bulk of the theatre setup, stroke surgery, monitoring period and post-mortem studies in alternate but overlapping shifts, longer studies would be a significant strain, as well as limit the number of studies possible per working week. Longer monitoring periods, such as 36 hours, were originally considered, however would have pushed the total experiment time into a third day, testing the boundaries of endurance of such a small surgical team. Although all animals developed a degree of systemic acidosis with variable treatment success via methods described below, there was nothing else observed or measured to suggest that the animals would not tolerate prolonged periods of general anaesthesia beyond 24 hours, however the practical implications of doing so would probably require more staff to share the monitoring duties. Alternatively, modification to include survival studies would prolong the post-stroke survival time, and also allow for the inclusion of post-stroke behavioural assessment.

It is desirable to demonstrate that drugs improve functional outcome after experimental ischaemia. In its present form, it is generally felt that the surgical approach to generate proximal MCAO is not conducive to a survival study, however future studies are planned to investigate modification of the surgical approach to facilitate post-stroke survival. The amount of bone and soft tissue dissection to masticatory structures could be devastating in a ruminant, although there is no evidence in the literature to support or refute this. In addition, it would be reasonable to expect that mortality rates following proximal MCAO would be even higher in the first 24 hours in unanaesthetised sheep; survivors would almost certainly be highly disabled. Furthermore, a survival model of sheep permanent MCAO already exists (329), consisting of significantly smaller infarct volumes, a craniotomy placed above and away from the important masticatory structures and a 0% mortality rate, as further discussed

below. The present model may be suitable for survival studies investigating malignant MCA stroke, however would need to be rigorously tested and monitored to ensure animal welfare.

Replication of improved function in a species additional to rodents may optimize the chance of success in clinical trials; evaluation in larger species (such as cats or primates) is desirable. Despite its many advantages, the current model in its present form may not be the large species for which to evaluate improved function, however a modification to the approach may be appropriate. As discussed, functional outcome studies may be better served by the Boltze approach (329), or the present approach modified to involve less soft tissue dissection but at the cost of a more distal occlusion, whereby temporary occlusion techniques could still be performed. The cost of performing distal MCAO is smaller infarct volume and less neurological disability, therefore to maximise lesion volume and functional disability in a survival model would require a modification to the surgical approach to the proximal MCA to minimise disability related to the approach itself. Importantly, it must be remembered that the sheep as a species is appropriate to assess outcome for a variety of neurological conditions including stroke, and future studies are planned to include such assessment with this model.

The age and species of the animal may influence its relevance to human stroke. Neuroprotective agents should be examined in male and female animals to separate possible sex differences. We have used both male and female sheep in the current studies. Female sheep were primarily chosen for the 24 hour study because of the perceived need for urinary catheterisation to prevent urinary retention and monitor strict fluid balance, and the convoluted urethra in the male sheep that prevents catheter insertion (T. Kuchel, personal communication, May 2011). Because of the impending switch from rams to ewes between the initial (4 hour) methodology study and the 24 hour study, a number of 4 hour stroke experiments were performed in females to ensure no differences in physiology or stroke volumes, and there were none. That said, all of the 24-hour stroke experiments were

undertaken in females, hence no comparisons could be made between gender in malignant MCA stroke. Care needed to be taken not to use ewes that were or had recently been lactating; in the instance that they were, they were found to be highly resistant to intravenous induction of anaesthesia, and also required a much higher concentration of inhalational isoflurane to maintain general anaesthesia, far beyond the neuroprotective dose (below), and therefore potentially destabilising the experiment. These findings of a general resistance to anaesthesia were also observed by co-researchers who had experience in lactating sheep. Generally we used animals aged 18-24 months; on one occasion an animal was found to be severely hypoxic on first blood gas analysis, which was not correctible despite multiple attempts at restoring normal respiratory physiology. This animal was euthanased before commencement of the stroke surgery component of the experiment, and records indicated that the animal was up to 7 years old and therefore quite elderly, and was probably suffering from a subclinical pneumonia pre-operatively. Although it has been noted that the vast majority of experimental stroke studies are performed on young animals with no predisposition to the common medical comorbidities associated with human stroke (334), there are likely significant difficulties maintaining anaesthesia in elderly animals and a significant mortality unrelated to stroke may need to be accepted if elderly animals are to be used.

5.2 Comparisons with existing models

As summarised in Chapter 1.2.2, the Leipzig model of ovine MCAO was developed to address the problem of poor clinical translation with rodent models and in reference to the initial STAIR recommendations (329). Similarities and differences can now be highlighted between the two current sheep MCAO stroke models.

Location and method of arterial occlusion. Although they claimed “total proximal MCAO”,

Boltze's group apparently only exposed the MCA as proximal as the prebifurcation segment. Their craniotomy was sited far too superiorly above the skull base to reach the terminal ICA without significant and deleterious brain retraction, but in doing so they avoided removing the coronoid process, and although not alluded to in their manuscript it was later confirmed that their method of permanent MCAO was also with bipolar diathermy coagulation (R. Turner, personal communication, June 2011). They did not report methods of temporary occlusion with reperfusion. The current model has built upon this and also used bipolar diathermy for permanent MCAO, however vessel occlusion was performed at the very proximal MCA, and reperfusion techniques of occlusion were also explored and characterised.

Closing the cranial cavity. There is no comment in the Leipzig model regarding dural closure or reconstructive cranioplasty. Considering that their animals survived for 6 weeks with no mortality it would seem intuitive that some attempt at preventing postoperative CSF leak was made and achieved, albeit this may have been simply via soft tissue and skin closure. Duraplasty and cranioplasty were vital to the current model to prevent both CSF leak and herniation of cranial contents through the surgical site, therefore maintaining ICP dynamics as close as possible to the clinical condition. This was essential given that ICP was one of our main outcomes.

Neurophysiological monitoring. No invasive intracranial monitoring devices were utilised in the Leipzig model. The current model used both Licox $PbtO_2$ and Codman microsensor ICP monitoring intraoperatively and in the postoperative monitoring period. $PbtO_2$ was not measured in the Leipzig model, however CBF was via PET in 5 animals with total proximal MCAO. CBF was not directly measured in the current model, which utilised $PbtO_2$ as an indicator of perfusion instead. Changes in ICP dynamics could be suggested based on MRI findings in the Leipzig model, however no evidence of space occupying oedema was demonstrated.

MRI and stroke lesion volume. MRI was used in both studies to assess stroke lesion volume. Although expressed in mL, the Leipzig results can be translated to percentage of whole brain affected knowing that the normal sheep brain weighs approximately 130g (427, 428), and therefore has a volume of approximately 130mL. For total proximal MCAO, Boltze found stroke volumes on postoperative day 1 of 16.3 +/- 5.2mL (12.5% of whole brain), compared with 27.4 +/- 6.4% of whole brain after permanent MCAO in the current 24-hour study. Boltze's volumes fall accordingly with 2-vessel (8.7 +/- 3.9mL, 6.7% stroke) or 1-vessel occlusion (5.6 +/- 3.6mL, 4.3% stroke). The significantly smaller stroke volumes compared with the current findings further suggest distal rather than proximal MCAO was achieved in this model.

Early mortality. Mortality within 6 weeks of stroke was not a feature of the Leipzig model. In contrast, and even while fully anaesthetised, the current model demonstrated a 30% mortality rate within 24 hours of permanent proximal MCAO secondary to raised ICP, cerebral herniation and brain stem compression, and attributable to the size of the infarct and the resultant space occupying cerebral oedema, which again was not present in the Leipzig model. Clearly, such differences in mortality reflect the lesion size in each model. Early mortality secondary to large stroke volume and space occupying cerebral oedema closely resembles large volume human MCA stroke, therefore is a good representation of the clinical condition.

Neurobehavioural phenotyping. Behavioural assessment following MCAO is a big advantage of the Leipzig model and is an essential component of pre-clinical evaluation of potential stroke therapeutics. A common veterinary neurological examination scale was used assessing items including consciousness, oral intake, ataxia and motor function (429). This scale could potentially be adapted to the current model in a survival study, however more emphasis may be needed for assessing consciousness and other manifestations of raised ICP. Therefore,

although superficially similar, the Leipzig model and the current model of ovine MCAO are fundamentally quite different. The Leipzig model can be summarised as a survival model of small permanent MCA cortical stroke, whereas the current model is a non-survival investigation into complete MCA territory permanent or temporary stroke with significant mortality secondary to space occupying cerebral oedema and raised ICP. Although aspects of each could be adapted for the other, for instance performing a distal Leipzig occlusion using current model reperfusion techniques, each model should be considered unique with their own uses for investigating the pathophysiology of and novel treatments for MCA stroke both in the acute and chronic phase.

5.3 Limitations of the current model

As eloquently stated by Susan Reinwald, “the overriding caveat to using large lower-order species is to take the time in advance to understand and appreciate the limitations and strengths of each animal model” (430). There are a number of possible limitations of the current model, and this is despite careful consideration on how to eliminate or reduce the impact of each. The surgical technique has a steep learning curve but has now been refined to the point of subjective reproducibility between two different operators. Despite this, the amount of collateral extracranial tissue damage required to access the very proximal MCA has been difficult to reduce. Attempts were made to mobilise the coronoid process out of the temporal fossa by opening the mouth preoperatively and therefore reduce its projection into the surgical field, however this maneuver did not help accessing the skull base and the coronoid process still required dissection and removal. Other methods at surgically mobilising the coronoid process with the aim of reconstructing it to restore functional mastication post-operatively were tried with variable success in anticipation of later developing a survival model of this approach, but would still expect to have significant post-operative pain and

therefore difficulty with oral intake. Of note, the rodent intraluminal thread model of MCAO transects the ipsilateral external carotid artery which supplies muscles of both mastication and swallow, therefore difficulties with eating and maintaining normal weight post-operatively in the rodent are common and are noted to have no influence on the final infarct volume, but do result in poorer performance in behavioural measures (249). Therefore assumptions regarding mastication after removal of the coronoid process in the sheep need to be methodologically investigated before ruling the technique out as prohibitory for survival studies.

A commercial cyanoacrylate glue was applied to the dura to prevent CSF leak in the absence of a suitable duraplasty product. Although cyanoacrylates have previously been shown to produce chemical and thermal injury to adjacent brain, no such damage attributable to the glue was observed in any of animals in this project. Indeed, specialised commercial, albeit far more expensive, dural sealant substances are available and may be considered in future studies in order to minimise any effects this may have on CNS injury.

CBF was not directly measured in the current study. The Leipzig model demonstrated with PET a relative reduction in CBF of 42.6 +/- 1.7% 24 hours after total proximal MCAO in a region of interest of size 14.3 +/- 1.3mL, with CBF decreasing to 27.8 +/- 5.6% at 14 days and then increasing to 37.2 +/- 6.4% at 42 days (329). Considering the relatively small stroke volume compared with the current model, there may have been a degree of collateral supply contributing to an incomplete CBF reduction; with the larger volumes achieved in the current model it would be interesting to determine CBF via PET, particularly in the core where collateral supply is minimal or absent.

In general, large animal laboratory experiments are costly, particularly when compared with rodent studies (431). Despite their translational difficulties, rodent models are still vital in initial drug development prior to testing in large animal models (252). Although the sheep experiments performed in the current model were relatively expensive and require a well

established and funded laboratory, they are still very cost effective alternative when compared with nonhuman primate models (430), which themselves are yet to be proven to be superior particularly with regard to neuroprotection in stroke, and are associated with significant ethical issues (432).

Young, generally healthy adults were used almost exclusively in this project. This helped reduce cost and complications secondary to the physiological effects of age, however as a result this remains a model of MCA stroke in health, not illness. An improved model may further characterise the effects of permanent and temporary MCAO in aged or transgenic sheep (433, 434), but bearing in mind the anaesthetic issues discussed above (252).

5.4 Methods and duration of arterial occlusion

Electrocautery was clearly identified as the best method for producing permanent MCAO, however many methods for temporary MCAO were considered, before ultimately settling on aneurysm clip application as the method of choice for this study. One method considered but not tried was the Rose Bengal photothrombosis technique. Rose Bengal (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein) is a stain commonly used in ophthalmology to identify conjunctival injury, but when injected intravenously focal platelet aggregation can be stimulated following exposure to green light of wavelength 560nm to the vessel (435). Vessel occlusion occurs over approximately 5 minutes (436). Reperfusion occurs either via slow spontaneous recanalisation as can occur over time in human ischaemic stroke (437), or with administration of various thrombolytic agents including rt-PA, which has allowed investigation of both thrombolytic and neuroprotective agents in rodent models of cerebral ischaemia (438). Furthermore, in rodent models the laser can be stereotactically illuminated through the skull eliminating the need for craniotomy (439); the skull base anatomy of the

sheep is complicated and the sphenoid bone of varying thickness which probably prevents illumination through the intact skull, however it could conceivably be at the very least thinned with the pneumatic drill, still avoiding perforating the inner table of bone and durotomy. Finally, although the mechanism of arterial occlusion is slow compared to instant occlusion seen clinically with focal thrombus formation or thromboembolism, the rate of reperfusion is clinically much more accurate with slow vessel recanalisation compared with instant reperfusion seen with mechanical occlusion models (Figure 5.1). Given the many advantages of the photothrombosis approach to MCAO it will be considered for future modifications to the present model.

Some have argued that strokes produced via photothrombosis produce atypical lesions unlike human stroke, and that endothelin models produce lesions more like surgical MCAO (440). Endothelin-1 is a naturally occurring peptide that produces dose dependent and severe vasoconstriction, and can be injected stereotactically onto the MCA to produce intraluminal occlusion and reduction in CBF sufficient to cause cerebral ischaemia (277). As the drug effect wears off the MCA lumen slowly opens again to gradually reperfuse the ischaemic territory, akin to thrombolysis. However, the rate of reperfusion is difficult to control and standardise (441). The ability to inject the drug stereotactically is a major advantage, and could possibly be performed in the sheep transorbitally without enucleation, however periods of MCAO remain relatively short at approximately 20-60 minutes (276, 277, 442).

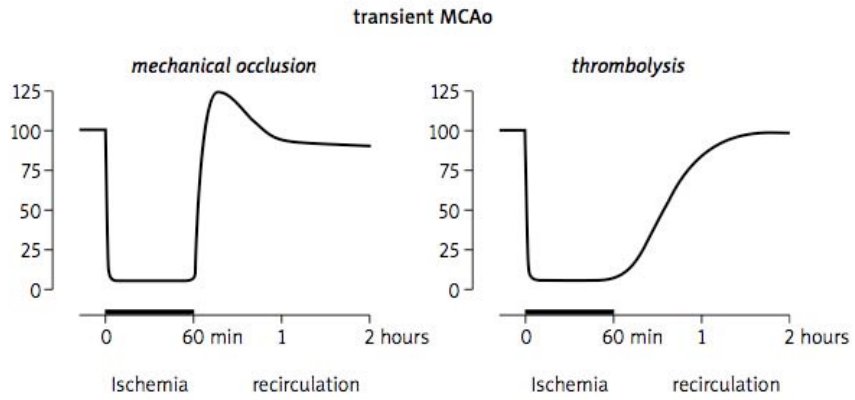

Figure 5.1: Flow profiles after temporary MCAO. Arterial occlusion from an external (mechanical, left) or intraluminal source (thromboembolism, right) both result in an immediate reduction of flow distal to the occlusion. Release of mechanical occlusion results in almost instantaneous reperfusion (with rebound hyperperfusion), however clot thrombolysis results in slow restoration of flow dependent upon the speed of revascularisation (378).

A recent systematic review of outcome after ischaemic stroke reported 14 clinical trials consisting of 2450 patients treated with intravenous t-PA in the literature, with the mean time to treatment being 143 minutes (95% CI 131-155) (443). It must also be remembered that after treatment with t-PA the time to recanalisation will be less than 1 hour in the majority but can be as much as 3 hours after drug administration (444), such that 2 hours of experimental temporary occlusion may be too short to accurately represent the duration of arterial occlusion in clinical stroke. The choice of 2 hours for temporary occlusion was better justified by the results of chapter 4.2, in which the mean lowest $PbtO_2$ recorded during prolonged temporary occlusion was 110.8 minutes. Note however the large variations in both 4-hour and 24-hour model stroke volume following 2 hours of temporary MCAO followed by reperfusion in this model, particularly compared with the fairly uniform size stroke that permanent MCAO generated. Variability in final stroke volume can make screening of neuroprotective agents problematic, however it is actually an accurate reflection of the variability encountered in

human ischaemic MCA stroke (445). The great advantage of course with temporary occlusion using aneurysm clips is that there is theoretically no limit to the duration of occlusion, such that a temporary clip can be placed for 5 minutes or 5 hours.

5.5 Developing a 24-hour model of MCAO

The change from a single day, 4-hour experiment, to an overnight, 24-hour experiment was far from a simple extension of the existing protocol. Many aspects of the surgical protocol required careful consideration to ensure success, and further refinements were necessary as the project progressed. First to consider was the switch from a non-sterile protocol to a fully sterile surgical setup. As the initial study monitored for only 4 hours sterility was not felt to be an issue, hence instruments were not sterilised, the surgical field not prepared aseptically and the surgeon was not gowned and gloved in a sterile manner. All metal instruments were able to be autoclaved, however certain hard plastic ones were tested in the autoclave to check their suitability for sterilisation; those that failed the autoclave test were either subsequently replaced with sterile single use items for each experiment or were sterilised in 70% alcohol if no sterile consumable replacement could be found. A pneumatic sterilisable Midas Rex drill was obtained (courtesy of Professor Nigel Jones, Adelaide, South Australia) to replace the electric drill used initially in the 4-hour study. The skin was prepared with betadine and draped in a sterile manner, and the surgeon wore a cap, mask and sterile gloves and gown. Intramuscular antibiotics were administered at induction and periodically throughout the experiment. In an attempt to demonstrate that antibiotics did not affect stroke lesions, they were also administered to the last subgroup of 4-hour stroke animals.

It was deemed desirable to monitor urine output via an indwelling bladder catheter to observe a normal fluid balance and ensure normal renal and electrolyte physiology. However, the

highly convoluted male sheep urethra mean that they are not good subjects for urinary catheterisation (446). Therefore, the decision was made to switch gender to female animals, and like the initiation of antibiotics, the last subgroup of 4-hour stroke animals also included females, again in an attempt to demonstrate that there were no gender difference in stroke volumes. As well as monitoring urine output, intravenous fluids (compound sodium lactate Hartmann's solution, Baxter Healthcare Pty Ltd, Australia) were administered at a rate of 3mL/kg/hour continuous infusion to maintain euvolaemia.

Due to significant problems with obstruction of the femoral arterial catheter within the 4-hour experiments, a longer lasting arterial catheter was desirable; this led to the development and refinement of the Codman microsensor arterial blood pressure monitor setup (chapter 4.4). Generally, the Codman microsensor catheter method would provide a highly accurate arterial blood pressure as well as reliable arterial blood sampling for the entire (32+ hour) experiment.

Continuous general anaesthesia for extended periods also proved challenging. Ventilation parameters needed to be carefully set and regularly adjusted to maintain normal respiratory physiology, and intermittent airways suction via the endotracheal tube was necessary to prevent secretion accumulation and pulmonary oedema developing (447). Anaesthesia could be maintained for the entire experiment generally with the same protocol, however as the animals were kept only lightly sedated the researcher needed to remain vigilant to the animal showing signs of wakefulness, and anaesthesia briefly deepened as required.

Careful consideration of arterial carbon dioxide and serum sodium concentrations was necessary to maintain normal intracranial physiology, and this was closely related to the animal's acid base status. Initial experiments demonstrated progressive development of an acidotic state as the animal lost its highly alkaline saliva through drool dripping from its mouth. Normally in ruminants, excess saliva production is swallowed, however in the intubated and anaesthetised animal the saliva would drool in volumes up to 1.5 to 2 litres in a

24-hour period, with production exacerbated by ketamine (448). This led to replacement of saliva via an orogastric feeding tube, as well as maintenance of a neutral acid base status by adding sodium bicarbonate to the intravenous fluid as required. In animals that became acidotic there was a compensatory decline in $PaCO_2$ that could not be corrected via slowing the ventilation rate, and hence ICP in hypocapnic animals was not a reflection of true ICP after space occupying oedema (449). Likewise, serum sodium levels were monitored and controlled with periodic orogastric boluses of water to avoid hypernatraemia, which can also lower ICP in acute injury (450).

5.6 Anaesthesia and neuroprotection

The purported neuroprotective properties for ischaemia of various anaesthetic agents are well documented (451-457). Even very light anaesthesia with the powerful intravenous agent propofol has been shown to reduce infarct volume in experimental rodents (348). Most anaesthetic agents suppress neural transmission and therefore energy requirements, and can offer some degree of protection to the ischaemic brain (458). The anaesthetic protocol used in the current study included inhalational isoflurane, a highly volatile agent of halogenated molecules, and intravenous ketamine, a weak noncompetitive (NMDA) antagonist that has sympathomimetic and sedative-hypnotic properties (459).

Halogenated anaesthetics induce amnesia, analgesia and muscle relaxation, but via an unknown mechanism. They are known as decoupling agents because they increase CBF but at the same time decrease CMR and $CMRO_2$ (460). Isoflurane also lowers systemic arterial blood pressure in a dose-dependent manner. Although generally accepted to have a neuroprotective role in experimental stroke, some have shown that cerebral infarction is actually delayed rather than prevented with its use (461). The potency of inhalational

anaesthetics may be measured as the concentration of the agent in the lungs at 1 atmosphere that is needed to prevent motor response in 50% of subjects exposed to a pain stimulus; although it is by definition a median value, it is termed the Minimum Alveolar Concentration (MAC), and a lower MAC value represents a more potent volatile anaesthetic (462). The MAC value for isoflurane in humans has been shown to be age dependent but is around 1.2, and MAC can be calculated based on expired concentrations of isoflurane (463). MAC isoflurane values in sheep for general anaesthesia are in the range 1.0-1.5 (464). With human MAC isoflurane values of 1.1 CBF is not significantly increased, however at 1.6 MAC (approximately 1.9% end tidal isoflurane via the Nickalls calculation) $CMRO_2$ is significantly depressed, and isoflurane becomes neuroprotective (465). 1.2 MAC is appropriate for neurosurgical anaesthesia, corresponding with approximately 1.4% end tidal isoflurane. Therefore, for all experiments in this project end tidal isoflurane was strictly maintained between 1.4-1.9% to provide appropriate anaesthesia yet avoiding neuroprotection against ischaemia.

Ketamine was for years considered to be relatively contraindicated in ischaemic brain injury because it increased CBF, $CMRO_2$ and ICP; more recent discovery of its NMDA antagonist effects suggested that it may have neuroprotective properties through inhibition of apoptosis proteins and modulation of the inflammatory response (466). Furthermore, its effects on the cerebral circulation can be further mediated by coadministered anaesthetics, with no change in CBF or ICP observed in patients given ketamine with isoflurane (467). Rodent stroke studies have demonstrated no neuroprotective effect of ketamine at low doses given before or immediately after temporary cerebral artery occlusion, however have shown protective qualities when given in much higher doses (468). Ketamine has the specific advantage of possessing analgesic properties, and although inhalational anaesthetics are generally preferred for prolonged procedures, combination inhalational/ketamine regimes have been popular for and successful in anaesthetising small ruminants since the 1970s (469). The addition of a low

dose ketamine infusion to sub-neuroprotective MAC isoflurane produced a synergistic relationship between the two anaesthetic agents, permitting a lower MAC isoflurane and therefore enabling good anaesthesia and systemic blood pressure but avoiding inadvertent neuroprotection as a confounding factor when characterising the stroke model (470).

5.7 Malignant MCA stroke

The most powerful clinical treatment for morbidity and mortality associated with space occupying ischaemic cerebral oedema remains surgically increasing the volume of the intracranial cavity to allow room for the brain to swell and limit or prevent secondary injury (235, 241, 267, 415, 471). Compared with non-operative management, decompressive craniectomy has been demonstrated to significantly reduce the mortality rate following malignant MCA stroke but without increasing the number of severely disabled survivors (243), albeit with a few caveats and controversies (206, 207). The fact that no effective therapies for limiting ischaemic cerebral oedema exist is a reflection on the current lack of knowledge in the pathophysiology of space occupying oedema secondary to large volume MCA stroke. The effects of surgical decompression are two-fold: prevention of cerebral herniation, and restoration of physiological ICP and CBF to the compromised but otherwise salvageable brain (472). Decompression is a powerful intervention; a small clinical series of patients with raised ICP secondary to aneurysmal subarachnoid haemorrhage demonstrated an immediate reduction in ICP from a mean of 59mmHg before decompression to a mean of 10mmHg after decompression and duraplasty, coupled with a rapid rise in $PbtO_2$ from a mean of 6mmHg to a new mean of 23mmHg post-operatively (473). Following the development of a focal space occupying infarct, ICP reduction following surgical decompression improves CBF to the non-ischaemic brain and therefore prevents secondary injury, but also optimises flow through collateral vessels supplying the ischaemic penumbra to limit final stroke volume

(415). In the context of TBI, surgical decompression for raised ICP allows a diffusely swollen brain to expand with the possibility of exacerbating axonal stretch injury (474-476). In malignant MCA stroke this seems to be less of an issue; unilateral decompression overlying the oedematous infarct is unlikely to further damage the underlying necrotic tissue, however many controversies regarding decompression following stroke remain and may possibly be addressed by their investigation in a suitable animal model.

Models of malignant MCA stroke have previously been explored in both small (238, 239, 322, 324) and large animals (326, 328). The clinical features of human malignant MCA stroke, characterised by a high mortality rate, radiological features of large volume MCA territory stroke and space occupying oedema with or without raised ICP, have been difficult to replicate in animal models. For species such as the rat, with substantially different neuroanatomy to the human particularly with respect to tentorial compartmentalization, intracranial dynamics associated with raised ICP have questionable translational significance (323). Novel agents in rodent models of malignant MCA stroke have claimed efficacy or superiority to decompressive craniectomy (210, 231, 477), but as per the STAIR recommendations further preclinical testing is required in a gyrencephalic large animal species before proceeding to clinical trials. The anatomical features of the sheep brain are conducive to the formation of space occupying cerebral oedema, tentorial compartmentalization and the development of cerebral herniation, a consistent intracranial hypertension response to large ischaemic injury, a size sufficient to perform sophisticated neuroradiological imaging, and also to perform experimental decompressive craniectomy (discussed below). Therefore the present model, which accurately replicates the important components of the clinical condition, will become extremely valuable for preclinical testing of novel therapeutic agents, particularly when outcomes are as devastating as they are in malignant stroke.

5.8 Regional tissue oxygenation in MCAO

In experimental models of TBI, $PbtO_2$ may be a reasonably accurate reflection of CBF (390). In brain injury $PbtO_2$ is composed of CBF and $PbtO_2$; more recently, it has been demonstrated that $PbtO_2$ is a product of CBF and the arteriovenous difference in oxygen tension, but only in normal-appearing tissue after traumatic injury and only when autoregulation is preserved (103, 104). But the relationship between CBF and $PbtO_2$ during acute arterial occlusion has not been defined. We know that regional CBF within the core declines dramatically immediately after occlusion (114), however results from the current model have demonstrated that regional $PbtO_2$ takes considerable time to fall to hypoxic levels after MCAO. Therefore, in the setting of ischaemic stroke, $PbtO_2$ should be considered a poor reflection of CBF. This is inherently intuitive; after arterial occlusion, substrate delivery ceases however consumption continues until substrate levels have been depleted, or are at concentrations below levels required for normal consumption. Oxygen extraction fraction in normal brain is quite high at around 0.5, and CBF is generally regulated tightly to oxygen requirements, however in cerebral ischaemia mitochondrial function is also regulated to substrate availability such that in hypoxic states normal cell function will progressively decline to the point of loss of cellular integrity and cell death. Therefore, although substrate delivery ceases immediately after MCAO, substrate consumption will continue unabated for as long as possible, followed by a slower consumption rate in hypoxic states. In ischaemic stroke, $PbtO_2$ is therefore a reflection of oxygen availability and consumption at a cellular level, and not a reflection of CBF. This is demonstrated by the two-speed decline in regional $PbtO_2$ in chapter 4.2, where $PbtO_2$ declines rapidly initially with cessation of substrate delivery but ongoing consumption, then the rate of decline falls as consumption reduces in the presence of hypoxia ($PbtO_2$ <15mmHg) and a reduced requirement for oxygen. To state there is no relationship between CBF and $PbtO_2$ in this context assumes that CBF falls to zero upon arterial occlusion, however it must be noted that CBF was not directly measured and to state emphatically that no relationship exists

would require further experiments measuring both parameters after MCAO. Similarly, the rate of $PbtO_2$ ascent after reperfusion should be a reflection of diffusion of oxygen from arterial blood to the regional brain tissue and not CBF which recovers almost immediately as shown in Figure 5.1, whereas $PbtO_2$ takes 4.4 minutes (median) to rise above the ischaemic threshold upon reperfusion. Again, this relationship needs to be explored with further studies measuring both CBF and $PbtO_2$.

Is $PbtO_2$ a better indicator than CBF of the state of regional tissue health after MCAO? It would seem reasonable to suggest so. Of the two vital substrates for normal brain function, oxygen and glucose, it is oxygen that has the highest extraction fraction and therefore the lower reserve, hence $PbtO_2$ measures the brain substrate that declines the fastest following arterial occlusion. Furthermore, cellular integrity could theoretically be maintained so long as regional $PbtO_2$ stayed above the hypoxic threshold, the duration of which following MCAO has been demonstrated to be a reflection of baseline pre-occlusion $PbtO_2$ levels. The importance of $PbtO_2$ is also supported in studies investigating regional oxygenation in temporary aneurysm clip occlusion, such as Jodicke's dichotomising threshold of $PbtO_2$ <15mmHg for likelihood of poorer outcome (156). Again, this is intuitive; so long as $PbtO_2$ stays above the hypoxic threshold then the substrate is in sufficient supply so that neurons can still function through oxidative phosphorylation, and cellular integrity is maintained. $PbtO_2$ declines linearly at a steady rate following MCAO, thus increasing PaO_2 (and therefore increasing the pre-occlusion baseline $PbtO_2$) will prolong the time for $PbtO_2$ to fall below the hypoxic threshold. Another possible mechanism to prolong the time to hypoxia would be to reduce the rate of consumption such as with decreasing metabolic demand, which may slow the rate of hypoxia development. These theories give justification to clinically relevant techniques of oxygen therapy to increase PaO_2 and burst-suppression or barbiturate use to slow cerebral metabolism used in neurosurgical anaesthesia (135). Monitoring regional $PbtO_2$ during temporary arterial occlusion in neurovascular surgery has exciting implications for

real-time detection of patients at risk of ischaemic complications. However, it is fundamentally important to realise that the operating surgeon will work as expeditiously as possible to reperfuse the ischaemic territory in a timely fashion or employ intermittent reperfusion to minimise the incidence of iatrogenic stroke. Even though it may be desirable to reperfuse the brain once $PbtO_2$ levels reach 15mmHg, release of the temporary clip will only occur if it is appropriate for the stage of the operation, which generally means a controlled aneurysm, even if it is still bleeding (478).

The implications for ischaemic stroke are much more complex. Stroke patients do not have the same luxury as neurovascular surgery patients in being anaesthetised and ventilated, nor have their PaO_2 strictly controlled and measureable prior to arterial occlusion, nor have immediate access to neuroprotection. Pre-oxygenating prior to arterial occlusion is not possible in this group of patients, nor is administering barbiturates, nor limiting the duration of arterial occlusion to several minutes. Nevertheless, there are still lessons in regional $PbtO_2$ learnt from temporary occlusion studies that can be applied to permanent MCAO and ischaemic stroke. The most important of these is the prolonged duration for regional $PbtO_2$ to decline to hypoxic levels, at least in the anaesthetised sheep brain. As mentioned in chapter 4.2, if the mean duration for regional $PbtO_2$ to fall below the hypoxic threshold approaches an hour, this could have significant implications in limiting cerebral infarction and consequently morbidity and mortality following cerebral artery occlusion if stroke patients were treated in an ultra-early emergency fashion, from community recognition of stroke symptoms and signs, to hospital transfer, acute imaging and reperfusion institution. There is a growing trend towards recognising stroke as a “brain attack” or “code stroke” and a medical emergency (479-483), and the prolonged manner in which regional $PbtO_2$ declines after MCAO demonstrated in this model gives justification to emergency stroke treatment, particularly with possible salvation of the previously unsalvageable, the stroke core. It is easy to remember that rt-PA has a therapeutic window of up to 4.5 hours after stroke onset (484); we

must also remember that earlier reperfusion results in better outcomes (485). Ischaemic stroke is a medical emergency (486); time is brain (487); the earlier that oxygenated arterial blood flow is restored, it seems, the better.

5.9 Proposed uses for the current model

No animal model is ever likely to exactly mimic human stroke, and the clinical relevance of animal models is regularly questioned (334). Nevertheless, our understanding of the pathophysiological mechanisms of cerebral ischaemia has increased considerably due to knowledge gained from experimental models, albeit with poor translation of novel therapies. Although it has its limitations, the current model has the potential to not only further our understanding of mechanisms but also help further develop therapeutic agents for cerebral ischaemia.

Temporary proximal MCAO in a large gyrencephalic brain has the potential to simulate human pathophysiology of temporary aneurysm clip occlusion in neurovascular surgery and ischaemic stroke managed with thrombolysis or mechanical clot retrieval revascularisation. As a model for prolonged temporary proximal MCAO the current technique is relatively accurate: animals are similar weight to adult humans, brains are gyrencephalic, neurovascular anatomy is grossly similar including a variable collateral leptomeningeal supply, the artery is reached via microsurgical techniques, and the artery is occluded with a Sugita temporary aneurysm clip. Thus, the $PbtO_2$ and variable histology findings presented in chapter 4.2 are probably a good representation of (prolonged) human temporary proximal MCAO. Further clinical relevance could come from performing shorter duration temporary clip occlusion and correlating this with regional $PbtO_2$, MRI, histopathology and especially functional outcome. Other techniques previously explored for neuroprotection during temporary clip occlusion,

such as hypothermia and burst suppression (127, 133-135, 139), could also be explored with the current model and correlated with these same outcomes to determine if neuroprotection increased the duration of ischaemic injury-free temporary occlusion time. Finally, the importance of regional $PbtO_2$ as a predictor of stroke risk during temporary occlusion could be determined by performing a series of experiments whereby a temporary clip was placed until the point of Licox-measured hypoxia and then released, however ideally this would be a survival study with assessment of neurological status in addition to radiology and histopathology being the important outcome measures. A whole host of potentially neuroprotective drugs that have shown promise after temporary MCAO could be trialed in this model to fulfilling one of the original STAIR recommendations (252), and preferably in combination with a thrombolytic agent to better mimic the clinical condition.

The model of permanent proximal MCAO shows great promise in further developing our understanding of novel non-surgical therapies for space occupying cerebral oedema. The great advantage of the current model is that unlike the rodent brain, the sheep brain shares many neuroanatomical features with the human brain, particularly intracranial compartmentalisation secondary to dural infolding, and the ability to develop raised supratentorial ICP and herniation syndromes. Further analysis and processing of the ICP data may reveal patterns that reflect decreasing intracranial compliance and other predictors of a malignant ICP state, and these could further be correlated with various blood markers which may in future help predict those patients with MCA stroke that are at an increased risk of developing life threatening cerebral oedema. Several studies have demonstrated improved functional outcome in rodent models of large volume MCA stroke with novel anti-oedema agents compared with decompressive craniectomy (210, 231, 408), and these should be replicated in a suitable large animal model such as the current one prior to advancing to clinical trials. The exciting results of J Marc Simard’s group, in which the oral hypoglycaemic agent glibenclamide has been shown to be superior to decompressive craniectomy following rodent MCAO (210), could

easily be replicated in the present sheep model of malignant MCA stroke, or any other number of anti-oedema drugs that have shown promise in rodent stroke models. Extending the current model to perform a post-stroke decompressive hemicraniectomy with duraplasty would not be difficult; there are no major anatomical obstacles to accessing the sheep calvarium, the scalp is extremely folded and mobile in this area, and pericranium could easily be harvested for duraplasty. The only real consideration with this approach would be placement of the Licox probe, which would prohibit hemicraniectomy in its current location, but could possible be altered such that its entry point was closer to the midline but its tip still angled in towards the ischaemic core. Future studies investigating properties of novel anti-oedema therapies in this model should therefore randomize intervention to vehicle, drug + vehicle, or decompression, and the effect of decompression on physiological outcomes following permanent proximal sheep MCAO needs to be characterised.

There is also the possibility that some of the considerable controversies surrounding surgical decompression for malignant MCA stroke, particularly timing of intervention, correlation with ICP and/or MRI, and the intracranial pathophysiological changes occurring, may be investigated in the current sheep model. A better understanding of the pathophysiological processes occurring during the development of space occupying cerebral oedema may result in the discovery of new and successful novel therapeutic agents.

5.10 Future directions

There are several modifications that could extend the current model to improve its translational ability. First is the need to develop an approach to proximal MCAO that is conducive to a survival study as already noted. Behavioural function is such an important outcome measure in experimental and clinical trials that it must be a component of large

animal preclinical models. There are two concerns regarding survivability in the current model as previously discussed: animal welfare (pain and mastication) and significant mortality secondary to space occupying cerebral oedema. Pain and discomfort could be limited with analgesia, however restricted temporomandibular function is of concern for oral intake and particularly swallowing saliva, therefore fluid and acid-base status would need to be strictly monitored. Mortality itself is a particularly useful outcome measure (343), and although it has some ethical considerations it is important to observe the effects of novel agents on mortality in a preclinical model that replicates human cerebral ischaemia, including malignant MCA stroke.

Different surgical access to the proximal MCA may help resolve some of the issues with developing a survival study. A transorbital route provides good access with minimal brain retraction but is associated with enucleation, which is less desirable in assessing functional behaviour post-stroke. Craniotomies that keep the coronoid process intact limit access to only the distal portion of the MCA, therefore the important lenticulostriate vessels are excluded from occlusion and a predominantly cortical stroke results (329). The peculiarities of the sheep cerebral circulation are thought to prohibit an intraluminal approach to the MCA, as in Koizumi's rodent model. The ICA arises from the rete mirabile, which itself is formed from three branches of the internal maxillary artery, and although normal blood flow is almost entirely via the carotids there is a small amount of supply from the vertebrobasilar system posteriorly which joins the anterior circulation in the circle of Willis (316). The sheep occipital artery provides a connection between the common carotid and the vertebral, and although it would initially require an experienced interventional radiologist and a fully equipped angiography suite, it may be possible to gain access to the sheep anterior cerebral circulation intraluminally via the posterior cerebral circulation. Clearly an intraluminal model akin to the rodent model would be less invasive than the current technique, and may even be set up similar to the rabbit thromboembolic model (166), in which the stroke initiating device

is prepared and implanted under anaesthesia but not triggered until the animal is fully awake, which better replicates human stroke and avoids the potential neuroprotective effects of anaesthesia (343). If the current surgical technique of accessing the proximal MCA is to still be utilised however, then a photothrombotic method of arterial occlusion and reperfusion should be explored to possibly avoid durotomy as well as better replicating recanalisation and restoration of CBF after thrombolysis.

Finally, this model must be used to further investigate novel therapeutic agents that have shown promise in rodent models, as well as therapies that target ischaemic cerebral oedema. Applied to the current 24-hour model of permanent proximal MCAO, outcomes of ICP <20mmHg, reduction in radiological infarct and oedema volume, midline shift and herniation, and a significantly improved or abolished early mortality would warrant establishing a clinical trial of the same agent in the majority of patients with malignant MCA stroke who do not fit the criteria and are not eligible for decompressive craniectomy, prior to a randomised clinical trial comparing decompression with successful novel agents.

6. Conclusions

A new model of permanent or transient proximal MCA stroke has been characterised in the sheep. The anatomical features of the sheep brain, together with its size and the pathological and radiological changes following MCAO, indicate that the sheep is a relatively accurate representation of human MCA stroke. The different pathological mechanisms following permanent or transient vessel occlusion can be investigated via this model, as can the intricate, and as yet incompletely understood, events associated with space occupying cerebral oedema following malignant MCA stroke. Furthermore, experimental studies investigating the efficacy of novel therapeutic agents for neuroprotection and/or anti-oedema treatment using a sheep preclinical model may address some of the significant problems associated with failed clinical translation in rodent models. The significance of these findings is that the sheep may become a crucially important and widespread intermediary step from initial rodent studies to clinical trials in the development of urgently needed novel therapies for ischaemic stroke.

7. Appendices

7.1 Publications

1. Chapter 4.1

   Wells A, Vink R, Blumbergs P, Brophy B, Helps S, Knox S, Turner R. A Surgical Model of Permanent and Transient Middle Cerebral Artery Stroke in the Sheep. *PloS one*, 2012; 7(7):e42157.

7.2 Papers prepared and/or submitted for publication

1. Chapter 4.2:

   Wells A, Vink R, Brophy B, Helps S, Turner R. Regional brain tissue oxygen response to temporary aneurysm clip occlusion of the proximal middle cerebral artery. *International Journal of Stroke.*

2. Chapter 4.3

   Wells A, Vink R, Helps S, Blumbergs P, Turner R. Malignant middle cerebral artery stroke in an ovine model: Raised intracranial pressure following permanent but not transient proximal middle cerebral artery occlusion. *Journal of Neuroscience.*

3. Chapter 4.4

   Wells A, Helps S, Leonard A, Vink R, Turner R. Short communication: Invasive arterial and intracranial pressure monitoring using a Millar type strain gauge tip pressure transducer. *Research in Veterinary Science*.

7.3 Presentations at scientific meetings

1. **Wells A**. *A surgical model of occlusive middle cerebral artery stroke in the sheep*. Neurosurgical Society of Australasia Annual Scientific Meeting, Coolum 2010.
2. **Sandoz B**, Anderson R, Finnie J, Wells A, Turner R, Vink R. *Reproducible brain injuries produced in a sheep model by direct impact.* 23rd Conference of the International Society of Biomechanics, Brussels, Belgium 2011.
3. **Turner R**, Wells A, Helps S, Vink R. *Characterisation of a novel model of middle cerebral artery occlusion in the sheep*. 25th International Symposium of Cerebral Blood Flow, Metabolism and Function, Barcelona, Spain 2011.
4. **Wells A**, Helps S, Brophy B, Vink R, Turner R. *A surgical model of occlusive middle cerebral artery stroke in the sheep*. Stroke Society of Australasia, 22nd Annual Scientific Meeting, Adelaide 2011.
5. **Wells A**, Helps S, Vink R, Turner R. *Large animal stroke: MCA occlusion in the sheep*. Stroke Society of Australasia, 22nd Annual Scientific Meeting, Adelaide 2011.
6. **Wells A**, Turner R, Helps S, Brophy B, Vink R. Temporal thresholds for cortical ischaemia following temporary aneurysm clip occlusion of the middle cerebral artery in the sheep. Royal Australasian College of Surgeons 80th Annual Scientific Congress, Adelaide 2011.
7. **Turner R**, Wells A, Helps S, Vink R. *Characterisation of a new model of middle cerebral artery occlusion in the sheep*. 31st Annual meeting of the Australian Neuroscience Society, Auckland, New Zealand 2011
8. Wells A, Vink R, Helps S, **Turner R**. MCA Occlusion in the sheep: 24hr Monitoring study. 32nd Annual meeting of the Australian Neuroscience Society, Gold Coast 2012.
9. **Wells A**, Vink R, Blumbergs P, Brophy B, Helps S, Turner R. *Middle cerebral artery stroke in a large animal ovine model: temporary occlusion with reperfusion, and*

permanent occlusion producing "malignant" stroke with raised intracranial pressure and early mortality. Australian Society for Medical Research (SA Division) Annual Scientific Meeting, Adelaide 2012.

10. **Wells A**, Vink R, Blumbergs P, Brophy B, Helps C, Turner R. *Characterisation of a Surgical Ovine Model of Acute Proximal Middle Cerebral Artery Occlusion: Brain Tissue Oxygen, Intracranial Pressure and Magnetic Resonance Imaging*. Neurosurgical Society of Australasia Annual Scientific Meeting, Gold Coast 2012.
11. **Wells A**, Helps S, Brophy B, Vink R, Turner R. *Temporal Thresholds for Cortical Hypoxia Following Temporary Aneurysm Clip Occlusion of the Middle Cerebral Artery*. Royal Australasian College of Surgeons (SA Branch) RP Jepson Medal, Adelaide 2012.
12. **Wells A**, Vink R, Helps S, Turner R. *Characterising a New Large Animal Model of Middle Cerebral Artery Occlusive Stroke: From Conception to Realisation, the ACNR Sheep Experience*. Frontier Technologies in Nervous System Function and Repair Workshop, Mt Lofty 2012.
13. Wells A, Helps SC, Vink R, **Turner RJ**. Magnetic resonance imaging of MCA stroke in the sheep. 33rd Annual Meeting of the Australian Neuroscience Society, Melbourne, Australia 2013.

7.4 Prizes

Nimmo Prize 2011, full-time research category, for the best presentation by a medical researcher working in the University of Adelaide, Royal Adelaide Hospital or Institute of Medical and Veterinary Science

7.5 Techniques

7.5.1 TTC preparation and use

Fresh brain tissue was prepared for TTC staining according to the following method:

1. At the end of each study, sheep were perfused with 4°C TRIS saline via both common carotid arteries using the Santoreneos method (379)
2. Brains were rapidly and carefully removed via a craniotomy made with an electric oscillating saw
3. Brains were cut in 1cm thick sections in a coronal orientation, with the zero reference at the terminal ICA (approximately the optic chiasm)
4. Coronal sections were incubated in a warm solution containing: 3% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich Pty Ltd Australia, 3g/100mL 20mMol TRIS saline buffer, pH 7.4) for 10 minutes each side (total 20 minutes incubation)
5. Sections were then scanned on both sides with a flatbed scanner (Canon CanoScan LiDE700F, Japan), placed individually into cassettes, then into 10% formalin for diffusion fixation for a minimum 7 days; fixed brain sections were then processed, embedded, cut and stained in the usual fashion

7.5.2 Modified Swanson calculation for calculating stroke volume

In 1990 Swanson and colleagues described a semiautomated method for measuring experimental brain infarct volume, particularly to determine the volume of the surviving gray matter rather than the infarct volume, and to minimize error introduced by distortion and overestimation of stroke volume introduced by oedema formation (381). Swanson's calculation was modified for use to calculate the quoted stroke volumes of MRI, TTC or H&E stained brains corrected for oedema represented in this thesis based on the following method:

1. Coronal sections of whole brains were scanned on a Canon CanoScan LiDE700F flatbed scanner and opened in Adobe Photoshop CS5 Extended version 12.0 x64
2. The Window:Histogram function and Lasso Tool were used to determine the area (in pixels) of:
    a. The ischaemic injury (*Vii*)
    b. The ipsilateral hemisphere (*Vih*)
    c. The contralateral hemisphere (*Vch*)
3. Stroke *area* (expressed as percentage coronal section) for individual sections to correct for oedema is calculated:

$$\%Stroke = 100 \times \left(\frac{Vii \times Vch}{2Vih^2} \right)$$

4. Stroke *volume* (expressed as percentage of whole brain) is a summation of individual oedema corrected stroke area in pixels, divided by the total brain area in pixels, multiplied by 100
5. This method is valid for processed brain sections cut, stained and scanned on slides, brains stained with TTC and scanned directly on the flatbed scanner, or digital MRI images (DWI, T1WI or T2WI images)

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