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Improving Neurological outcomes following Neonatal Cardiopulmonary Bypass

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Abstract

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES
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IMPROVING NEUROLOGICAL OUTCOMES FOLLOWING NEONATAL CARDIOPULMONARY BYPASS

Edward J Hickey

Subtle neurological complications are alarmingly frequent following surgical repair of congenital heart disease. Periods of ischaemia during deep hypothermic circulatory arrest (DHCA) have been held responsible, but brain injury is increasingly recognised as multi-factorial. Of particular importance are the systemic inflammatory response and period of reperfusion, both of which contribute exigently to the progression of injury. The aims of this project were to identify techniques whereby the inflammatory and post-ischaemic reperfusion components can be harnessed to modulate the response to DHCA.

In a series of neonatal piglet experiments, strategies for reducing the systemic inflammatory load, methods for protecting against ischaemia through induction of tolerance and identifying facets of the reperfusion period that exacerbate injury have been examined. Furthermore, using a novel technique of contrast magnetic imaging, preliminary studies have been performed aimed at identifying and quantifying the ultrastructural injury resulting from neonatal perfusion techniques.

The results have indicated that: 1). Avoiding allogeneic blood use in neonatal CPB leads to significantly reduced systemic and cerebral inflammation, and that cerebral no-reflow can be avoided; 2). Hypoxaemic reperfusion in cyanotic congenital repair exacerbates the neurological injury sustained during DHCA; 3). Delayed preconditioning through lipopolysaccharide administration can confer robust neurological protection against ischaemia during DHCA, and; 4). Gadolinium MRI may be a useful tool for the quantification of blood-brain barrier injury following CPB and allow further characterisation of the cerebral impact of CPB strategies.

These studies have highlighted areas of current clinical management that can be targeted to improve the neurological recovery following DHCA. In particular this project described the first successful application of delayed cerebral preconditioning to cardiac surgery. This therefore represents a long-awaited step from rodent work closer in the direction of the clinical arena.

All the world is a laboratory to the
inquiring mind.

MARTIN H FISCHER
PHYSICIAN AND WRITER 1879-1962

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INDIVIDUAL CONTRIBUTION

Experimental concept and design was undertaken soley and completely by myself with the following exceptions. Chapter 5 was a continuation of the minicircuit program initiated by Ross Ungerleider and Tara Karamlou, and therefore the experimental and technical concept was in place. The final protocol was detailed by myself with considerable help and support from Tara Karamlou. The circuit was, however, further modified to reduce prime volume further during pilot studies undertaken by myself. Chapter 7 continues from previous studies performed by Steve Tsui and Ross Ungerleider and therefore preliminary findings from Steve Tsui's work were applied in this model. The concept of chapter 8 stemmed from my own reading and research, but Mary Stenzel-Poore was invaluable in planning the application from her experience with rodent models into the large mammal CPB model. Chapter 9 resulted from extensive discussions with Michael Jerosch-Herold about the potential uses of MRI spectroscopy in neonatal assessment of brain injury and exhausting pilot experiments using *ex vivo* brains.

Anaesthesia was undertaken by myself with the help of Loni Socha.

Cardiopulmonary bypass was undertaken by myself in 80% of the experiments described in this project. For the remainder of the experiments, I had the assistance of Jamie You CCP, a clinical paediatric perfusionist.

Surgical execution of experimental protocols was solely undertaken by myself. Loni Socha contributed where assistance was occasionally necessary, but the surgical studies were largely performed without assistance.

Post-operative care was solely undertaken by myself. The histological studies lasted 28hrs from start to finish, and required continuous observation and support. This

laborious existence necessitated the acquisition of monitoring equipment with alarms, continuous bedside presence and the understanding of the veterinary and security staff at OHSU, for which I am grateful.

Tissue processing was performed by myself as a guest in others' laboratories. This included the sectioning and loading of paraffin cassettes, staining for routine light microscopy, staining with histochemical agents and immunohistochemistry. Carolyn Gendron and Jutta Deininger performed the paraffin-embedding and mounted the slides for me, and Nicole Janeba taught me the techniques necessary for immunohistochemistry. I performed the RNA extraction, but the RT-PCR was undertaken by a team experienced in the technique. ELISA for cytokine quantification was performed as a service by an immunology laboratory.

The MRI sequence design, scanning and data analysis was undertaken by Michael Jerosch-Herold, a specialist in MRI spectroscopy.

ACKNOWLEDGMENTS

The work contained within this project has only been made possible due to the enormous hard work and generosity of many people. Many of these individuals have been unrelated to the immediate research team and therefore have only provided assistance through their own goodwill, for which I am enormously grateful.

I approached Ross Ungerleider in the spring of 2004 regarding the possibility of joining his research team. He has an impressive history of undertaking clinically pertinent work in the field of neonatal cardiopulmonary bypass. The opportunities he has provided for me both in the laboratory, and now beyond, will probably be immeasurable for my future career. After following in the footsteps of Steve Tsui and Steve Langley – two highly motivated and successful UK surgeons, I have felt daunted by - but have also thoroughly enjoyed - the challenge of matching their success, however far I may have fallen short! Ross has not only been a focused and helpful mentor, but has provided exceptional support and encouragement throughout the year. His approach to his team – as a boss and a friend - is something to aspire to. I very much hope that I continue to cross paths with both he and Jamie regularly in the future.

Numerous other investigators around the OHSU campus received regular visits from a strange Englishman trying to perform unusual experiments in piglets. Marjorie Grafe MD PhD, was completely invaluable in teaching me the morphological criteria of ischaemic neuronal injury. For six months she told me that despite the horrific ordeals I might be placing piglets through, their brains looked normal! Finally in spring 2005 her

eyes lit up and my reperfusion experiments could start in earnest. I approached Chris Corless MD PhD for guidance on how and where I could have my histology processed. Only a few days into my stay in the United States, I had let my naivety show, as he pointed out that "money talks!" He then suggested I do the staining myself...because "staining is like cooking"...a point for reflection when after many months, the recipe was still not working. I thank him for all his advice and encouragement through this time of need, and especially for allowing me to be a guest in his (entirely female) laboratory.

Kent Thornburg PhD and Richard Traystman MD PhD, both Prinicpal Investigators with their own department to worry about went out of their way to provide me with advice and technical encouragement. In particular, Kent granted me full access to his laboratory and his team, and even helped with the PCR assays.

Jamie You CCP is probably the most over-looked member of Ross's research department. Having to cope with the frustration of changing his career for political reasons when leaving China, he is invaluable as a perfusionist with a surgical background who has considerable experience placing rats and pigs on cardiopulmonary bypass. His advice was always correct. I hope that the research program continues with him in a central role.

Loni Socha had to put up with me for a year. She uncomplainingly watched me through the learning curve, and then kept up with our profuse schedule without recourse. We have done some extraordinary experiments, for which she should feel proud. When she receives a copy of this thesis, I hope she realises that she has been an integral part of it. Chris Komanapalli and Tom Person also witnessed first-hand the highs and lows of my work. Both have interesting projects in hand, and I am therefore certain that they will

continue the research program and generate some exciting studies. I hope they realise they can approach me at any time in the future to discuss ideas.

Tara Karamlou has been on the receiving end of numerous telephone calls. Both our sanities have been questionable at various times during the past year. It has been both useful and fun bouncing ideas, and of course crossing paths at all the meetings. I now have the dubious task of following in her footsteps as the next John Kirklin Fellow, at the Congenital Heart Surgeons' Society Data Centre, Toronto. I look forward to the many meetings we have ahead of us.

Carolyn Dendron, Jutta Deininger and Nicole Janeba have graciously put up with me hijacking space in their lab, and have had to watch the painful process of my immunohistochemistry not working again and again.

Sam, Sonnet, Bob and Krista – all from Kent Thornburg's laboratory – have also guided me through my unconventional agarose recipes and mysterious paraformaldehyde mixes. Thankyou so much for all the help and especially Sam's pies!

This year of research has reaffirmed my previously held intentions to continue with an academic career. It's amazing how people with similar interests find each other. Meeting and working with Mary Stenzel-Poore has been a huge pleasure. Countless ideas have been spawned and untold opportunities await. I hope that we continue working down the same path for some time to come.

Finally, Steve sent me here. He was completely right about how tough things would be, and only he and I understand that. I hope I've done him justice.

PUBLICATIONS AND PRESENTATIONS

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Prizes:

Hawley Seiler Award 2006

52nd Southern Thoracic Surgeons Association Meeting, Nov 2005.

John Parker Medal 2006

Annual Scientific Meeting, Society of Cardiothoracic Surgeon's of Great Britain

The period of research that generated this thesis has largely been responsible for my appointment as the next John Kirklin Fellow at the Congenital Heart Surgeons' Society Data Centre, Toronto, Canada 2006.

LIST OF ABBREVIATIONS AND SYMBOLS

AB	avidin-biotin
ABC	avidin-biotin complex
ACE	angiotensin converting enzyme
ADP	adenosine diphosphate
AIDS	acquired immuno-deficiency syndrome
AMP	adenosine monophosphate
ARDS	adult respiratory distress syndrome
ASD	atrioseptal defect
Asp	aspartate
Ao	aorta
ATP	adenosine triphosphate
BBB	blood-brain barrier
BG	basal ganglia
CA	cornus ammonis
CA	circulatory arrest
Ca^{2+}	calcium
CBF	cerebral blood flow
CD	cluster differentiation (CD11b)
cDNA	complementary DNA
CHD	congenital heart disease
CK-MB	creatine kinase-myocardial bound
cm	centimetres
CNS	central nervous system
CO	cardiac output
COR	cortex
CO_2	carbon dioxide
CpG	phosphorylated cytosine-guanine nucleotide dimer sequence
CPB	cardiopulmonary bypass
CSF	cerebrospinal fluid
DHCA	deep hypothermic circulatory arrest
DHCLF	deep hypothermic continuous low flow
DHFF	deep hypothermic full flow
DNA	deoxyribonucleic acid
DNase	DNA endonuclease
DWI	diffusion weighted imaging
EC	endothelial cell
ECMO	extracorporeal membrane oxygenation
E Coli	Escherichia coli
ELISA	enzyme-linked immunosorbent assay
ELAM	endothelial-leukocyte adhesion molecule
ET-1	endothelin-1
Fas	fas receptor (apoptotic initiator)
FasL	fas-ligand
FF	full flow
FFP	fresh frozen plasma
FOV	field of view
ft	feet

fwd	forward
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
g/dL	grams per decilitre
GM	gray matter
H^+	hydrogen ion
HC	hippocampus
HCO_3^{2-}	sodium bicarbonate
Hct	haematocrit
H&E	haematoxylin and eosin
H-I	hypoxia-ischaemia
HLHS	hypoplastic left heart syndrome
HMWK	high molecular weight kininogen
HR	heart rate
I	flow
ICAM	intercellular adhesion molecule
IE	immediate early
IFN	interferon (subtypes α, β, γ)
IHC	immunohistochemistry
IL	interleukin
i.m.	intra-muscular
iNOS	inducible nitric oxide synthase
i.p.	intra-peritoneal
IRF	interferon regulatory factor
ITU	intensive therapy unit
IU	international units
i.v.	intravenous
IVC	inferior vena cava
K-ATP	potassium-ATP
KCl	potassium chloride
kb	kilo bases
kg	kilograms
KAVD	kinetic assisted venous drainage
KKS	kallikrein-kinin system
kPa	kilopascals
LA	left atrium
LAD	left anterior descending artery
LPS	bacterial lipopolysaccharide (endotoxin)
LV	left ventricular
MAP	mean arterial pressure
µg	micrograms
Mg^{2+}	magnesium
ml	millilitres
µm	micrometres
mM	millimolar
mm	millimetres
mmHg	millimetres of mercury
mmol/L	millimoles/litre
MRI	magnetic resonance imaging
mRNA	messenger RNA
ms	millisecond
MUF	modified ultrafiltration
NEP	neutral endopeptidase
NFκB	nuclear factor-κB
NO	nitric oxide
NOS	nitric oxide synthase
n.s.	not significant
O_2	oxygen
OH	hydroxyl
OL	oligodendrocyte
PA	pulmonary artery
PAF	platelet activating factor
$PaCO_2$	arterial partial pressure of CO_2
PAGE	polyacrylamide gel electrophoresis

PaO_2	arterial partial pressure of O_2
PARP	poly-(ADP-ribose) polymerase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH	potential of hydrogen
PMEA	poly-2-methoxy-ethy-acrylate
PRC	packed red cells
p value	probability value of event occurring by chance
PVL	periventricular leukomalacia
PVR	pulmonary vascular resistance
Q	flow
R	resistance
ΔR1	change in relaxation time pre- and post- contrast
RA	right atrium
rev	reverse
RNA	ribonucleic acid
RNase	RNA endonuclease
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
RV	right ventricle
Sa_{O_2}	arterial oxygen saturation
s.c.	subcutaneous
SD	standard deviation
SEM	standard error of the mean
SOD	superoxide dismutase
SVC	superior vena cava
SVR	systemic vascular resistance
T	tesla
T1	T1-weighted imaging
T2	T2-weighted imaging
TdT	terminal deoxynucleotidyl transferase
TE	echo time
TI	inversion time
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
TNFR	TNFα receptor
TUNEL	terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling
TR	repetition time
TxA_2	thromboxane A_2
US	United States
VAD	ventricular assist device
VAVD	vacuum assisted venous drainage
VSD	ventriculoseptal defect
VR	venous return
WM	white matter
°C	degrees Celsius
±	plus or minus
<	less than
≤	less than or equal to
>	greater than
≥	greater than or equal to

Chapter 1

The History of Cardiopulmonary Bypass

It is often said of the medical profession
that new discoveries are met by captious
criticism and ungenerous mistrust

SIR DOUGLAS POWELL
RESPIRATORY PHYSCIAN, LONDON
1860-UNKOWN

1.1 Artificial Extracorporeal Support

Reports exist in the literature pertaining to the hypothetical concept of extracorporeal cardiopulmonary support as far back as the late 19th Century[4], but the development of clinical systems is attributed largely to the pioneering work of John Heysham Gibbon (figure 1.1) of Massachusetts General Hospital, and subsequently the Jefferson Medical Centre, Philadelphia. Having had his imagination stirred whilst helplessly witnessing the death of a woman in 1931 from a massive pulmonary embolus, he was excited by the possibility of bypassing the cardiopulmonary circulation using an artificial device[5].

"During that long night, helplessly watching the patient struggle for life as her blood became darker and her veins more distended, the idea naturally occurred to me that if it were possible to remove continuously some of the blue blood from the patient's swollen veins, put some oxygen into that blood and allow carbon dioxide to escape from it, and then to inject continuously the now-red blood back into the patient's arteries, we might have saved her life. We would have bypassed the obstructing embolus and performed part of the work of the patient's heart and lungs outside the body."

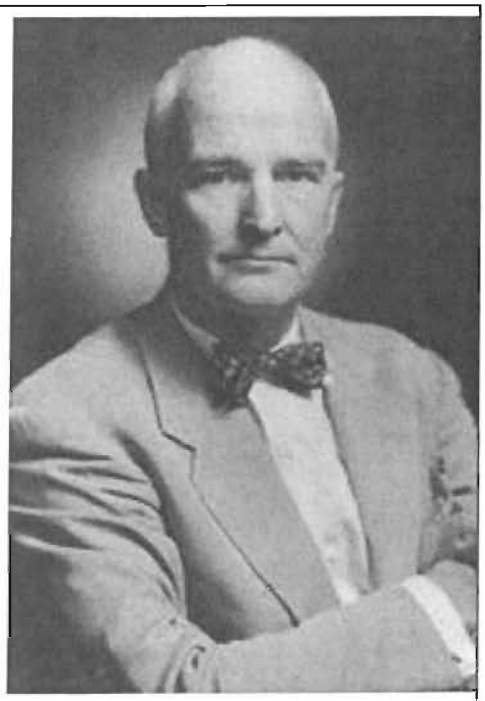

Figure 1.1 John ("Jack") Heysham Gibbon 1903-1973)

Despite meeting with repeated opposition from all whom he approached, he continued his experimentation and investigation independently[6]. In 1935, using a contraption involving two roller pumps, he supported a cat for 26 minutes, which is generally regarded as the first successful application of a heart-lung bypass machine. The

oxygenator in this experiment was a revolving cylinder, into which blood was introduced tangentially at the top. A film of blood therefore descended on the inside of the cylinder and this was the gas-exchange surface. The blood collected at the bottom on a thin edge and was transferred to a glass cup which was warmed with a jacket.

Gibbon's World War II service in the China-Burma-India theatre interrupted his research, but on his return he joined forces with an IBM engineer, Thomas Watson. Watson and his team of engineers invented an improved machine that "minimised haemolysis and prevented air bubbles from entering the circulation" that was tested on dogs and exhibited a mortality rate of 10%. Further improvements culminated in the first human application of his device in 1953 when he successfully repaired an atrial septal defect on a young patient named Celia Bavole[7]. Unfortunately this initial success was not repeated, and Gibbon lost the next five patients, causing him to shy away from the idea of open heart surgery. In fact, Gibbon was not alone in his lack of

Surgeon	Patients	Oxygenator	Year	Lived	Died
Dennis	2	Film	1951	0	2
Helmsworth	1	Bubble	1952	0	1
Gibbon	**6**	**Film**	**1953**	**1 (ASD)**	**5**
Dodrill	1	Autogenous	1953	0	1
Mustard	5	Monkey lungs	1951-3	0	5
Clowes	3	Bubble	1953	0	3
Total	**18**			**1**	**17 (11 in OR)**

Table 1.1 Early experience with the use of CPB. All reported cases 1951-54.

initial success – early attempts with CPB were uniformly dismal (table 1.1): between 1951 and 1953, of all 17 patients placed on CPB, only the one patient of Gibbon's survived (5.5% success) [8]. Gibbon's work did not pass unrecognised, however. Amongst numerous awards in a distinguished career, he received the prestigious Albert Lasker award in 1968.

Meanwhile, at the University of Minnesota, C Walton Lillehei was exploring the concept of *controlled cross-circulation*. This concept involved the use of the child's mother or father as the pump-oxygenator: the parent's femoral vessels were cannulated and arterial blood diverted to the child (figure 1.2). Although this highly innovative approach ("placental circulation") has become synonymous with Lillehei, the technique had in fact previously been in use as an end-stage treatment for uraemia and toxaemia[9]. The infancy of CPB in the clinical arena led to Lillehei embarking on an extraordinary series of operations beginning in 1954. He used this system in 45 open-heart congenital repairs, with success in 27 (60%)[10]. All 45 of the donors survived, and perhaps more extraordinary is the fact that at 30 year follow-up in 1986, 22 (49%) of the original cohort were still alive and leading productive lives[11].

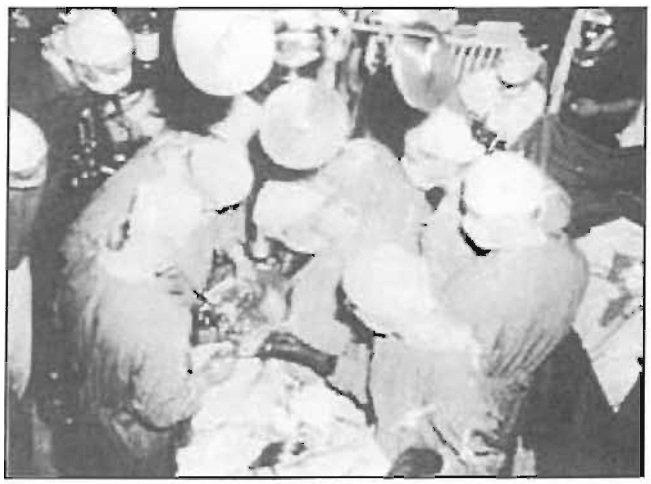

Figure 1.2 A rare image showing the early application of cross-circulation in Lillehei's operating room. To the far right the edges of the surgical drapes can be seen of the donor.

Despite the success using this inspired technique, Lillehei withdrew from the use of controlled cross-circulation only because of paralleled success achieved by the team at

the Mayo clinic, led by John W Kirklin. Kirklin was enthused by experiments conducted in the early 1950s (largely by Gibbon) and put together an impressive team of clinicians, physiologists and engineers, and with the aid of the Gibbon-IBM blueprints, spent two years producing a pump-oxygenator that was deemed fit for formal clinical trial. This Mayo-Gibbon pump-oxygenator was a piece of complex machinery. It included sensors on the venous reservoir controlling occluders that would control the source of pump inflow, up to fourteen wire-mesh screens, each 12 by 18 inches in surface area, and the pump flow rate was automatically controlled by the level in the venous reservoir. Carbon dioxide flow was tailored to the pH (7.43-7.45) and the oxygen flow was constant. Six units of blood were required to prime the pump and after its use, a whole day was spent cleaning it.

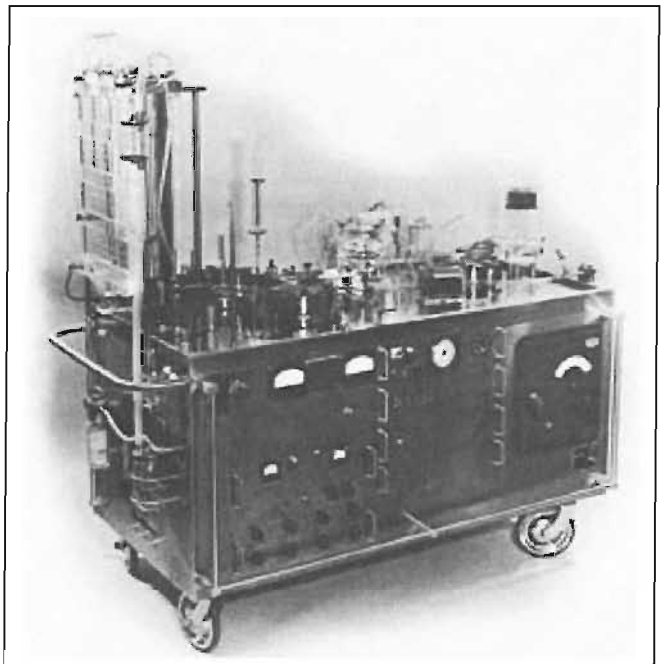

Figure 1.3 Mayo-Gibbon pump oxygenator used by John Kirklin.

Kirklin's team embarked on a prospective study of five (later expanded to 8) patients, irrespective of outcome[12]. The only previous successful repair using such a device was Gibbon's single case of the 45 previously attempted, and families were fully informed of these statistics. Nevertheless, the first patient, a five year-old with a large VSD underwent surgery on March 22nd 1955, and despite the arterial cannula being dislodged and the entire system requiring de-airing, the patient recovered without incident, a tribute to the meticulous energy the team had spent to cover every eventuality. This series was completed with 4 successful outcomes (50%) and was of

huge importance for cementing the place of open heart surgery in the clinical arena and initiating the continued improvements for the development of safe cardiopulmonary bypass that would become routine in hospitals worldwide from the 1960s.

1.2 Hypothermia and Circulatory Arrest

Although frequently considered a subsequent development, (deep) hypothermic circulatory arrest was developed in parallel to CPB, and rapidly became intertwined. Separate from the developments in CPB, Bigelow and colleagues in 1950 initially introduced the concept of cooling in cardiac surgery by demonstrating that moderate hypothermia to 30°C would permit circulatory arrest for 10 minutes. Experiments in dogs were used to study progressively deeper hypothermia in an attempt to permit longer arrest durations, and indeed surface cooling to 20°C did allow survival following 15 minutes of total circulatory arrest[13]. It was quickly apparent, however, that progressive cooling was a precarious business, as bradycardia and inadequate inotropy resulted in hypoxaemia in warmer organs or rendered the circulation at risk of complete collapse through fibrillation. Experimental observations indicated that quinidine or supplemental carbon dioxide could be used to avert fibrillation, but this did not correct the circulatory inadequacy at deep hypothermia, which in addition made rewarming extremely difficult. Nevertheless, this notion was soon applied clinically, in conjunction with inflow-occlusion, for the successful repair of an atrial septal defect in a five year-old girl by Lewis and Taufic[14], and success was also independently reported by others[15].

The marriage of hypothermia and CPB came about in the 1950s when the possibility was raised that damage believed to be attributed to the early oxygenators might be averted by the use of cooling. Much of the early work was undertaken by an

overlooked physiologist in Nashville named Frank Gollan. Using a gas-bubble oxygenator, he reported blood-stream cooling to 4°C and even 0°C[16]. In eerie resemblance to practice 40 years later, he primed with Ringer's solution to produce haemodilution, and corrected the haematocrit by transfusing donor blood on separation from CPB. This remarkable work culminated in a presentation to the American Association of Thoracic Surgeons in 1955 describing a successful series of experimental left atriotomies in animals cooled to 13°C using his oxygenating device[17]. Despite detailed descriptions of the clear and unencumbered operating field - in contrast to the beating-heart and bloody conditions otherwise experienced by surgeons of the day – his efforts (as a physiologist) were largely ignored.

In Europe at the same time, Juvenelle[18], and later Ake Senning, were investigating the impact of cooling on physiological parameters, and in particular showed that hypothermic ventricular fibrillation could be tolerated for prolonged periods, even using an aortic cross-clamp to successfully induce myocardial isolation for almost an hour – almost certainly the first application of this manoeuvre[19].

By the late 1950s, with the concept of cooling becoming more widespread, the team at Duke University collaborated with the radiator division of General Motors to develop a circuit heat-exchanger, and through this described clinical use of circuits close in overall design to those in use today. Their standard protocol proposed maintaining oesophageal temperature at 28-30°C, though they also described successful surgery on a child with severe tetralogy of Fallot who was cooled to 10°C. Technical problems encountered during this tetralogy repair necessitated the complete cessation of the circulation for several periods totaling 22 minutes – perhaps the earliest description of deep hypothermic circulatory arrest[20].

Not all investigators pursued hypothermia in conjunction with full CPB. Charles Drew was a surgeon from Westminster, London, who, despite having visited both Minneapolis and Minnesota to witness the pump-oxygenators these pioneering groups were using, was convinced that the morbidity and mortality they experienced was associated with the use of an oxygenator. He was, however, convinced by the merits of profound hypothermia, and therefore concentrated on developing dual-chamber bypass pumps that made use of the patient's lungs as the oxygenator. Blood from each atrium was drained and pumped, after cooling, into the corresponding outflow artery. The considerable energy spent by his team perfecting this technique on animals in a disused billiards room on the sixth floor of Westminster Hospital made way for a 22-year career using this technique for intra-cardiac repair. He was invited to present his system at a Hunterian Lecture in 1961, and despite artificial oxygenators proving safe, never relinquished his novel biventricular bypass technique[21].

The successful introduction of hypothermic CPB, and in particular DHCA, changed the course of surgical repair of congenital lesions. From the mid-1950s through the next three decades DHCA enabled progressively more and more complex defects to be challenged such that lesions otherwise incompatible with life were routinely offered the change of survival into adolescence and beyond. Not only did the early mortality continue to improve, to levels of 5% overall in the early 1980s, but this had the knock-on effect of introducing novel haemodynamic physiology into the world of cardiovascular medicine. During this era – the birth of congenital cardiac surgery –the emphasis was understandably almost entirely placed on achieving early operative survival. It was only once operative mortality approached 5% (and currently just under 2% in selected units[22] that attention began to shift towards the long-term quality of life issues for these patients. The realisation that neurological compromise is a frequent occurrence following complex congenital repair has now thrust the study of the cerebral consequences of neonatal CPB strategies firmly centre-stage.

Chapter 2

Cardiopulmonary Bypass as an Instigator of Tissue Injury

2.1 Introduction

In less than 50 years, cardiopulmonary bypass has evolved from a futuristic vision, to a routine practice imposed on patients in the operating theatre daily. However, although early surgical mortality has reduced dramatically, the insult CPB imposes morbidity and quality of life is huge. Cardiopulmonary bypass is a truly systemic insult: a wholly unphysiological state comprising four main insults: abnormal flow dynamics and sheer stresses, systemic inflammation, severe and abrupt hypothermia and periods of relative or total ischaemia.

2.1.1 Flow Dynamics

Shear stresses are generated by the blood pumps, suction devices, abrupt and non-physiological acceleration and deceleration and by the atypical directional flow patterns induced by the cannulae within vessels. Leukocytes are particularly susceptible to shear stress, and in addition to being traumatically disrupted, they can be triggered to degranulate, adhere and initiate phagocytosis[23]. Leukocyte degranulation occurs rapidly on institution of CPB, causing a leukopaenia, exacerbated by transmigration[24]. Levels typically return to baseline within several hours, before the onset of a more persistent leukocytosis lasting several days[25]. This leukocytosis is over and above that caused by the surgical trauma.

Erythrocytes are also susceptible to shear stresses. Haemolysis can yield free haemoglobin, which is reduced in circuits bypassing the oxygenator, or by replacing the membrane for a bubble oxygenator[26]. Free haemoglobin is noted rapidly (within 10 minutes) following the initiation of CPB, and remaining erythrocytes may have a delayed life-span, though it is difficult to separate the progressive post-operative decline in haematocrit from haemorrhage. It has been suggested that negative pressures

associated with suction (and vacuum-assisted drainage) are more damaging than the positive shear forces imposed by the pump though this has not been shown in practice[27].

Considerable energy has been spent studying the potential for pulsatile flow in paediatric CPB, in order to more closely mimic the *in vivo* flow dynamics[28]. In fact it has been the inability to reproduce the normal physiological pulsatility that has led instead to the adoption of laminar flow in paediatric practice. Although experimental data exists to demonstrate reduced post-bypass vasoconstriction and systemic vascular resistance[29] – perhaps related to higher levels of nitric oxide liberation[30] – the overall role of pulsatility in infant CPB has yet to be asserted. This is at least due in part to the increased complexity in the technical aspects of delivering pulsatile perfusion in tiny patients.

2.1.2 Bypass-induced inflammation

The onset of cardiopulmonary bypass (CPB) is associated with generalised activation of the innate immune system[31], and this phenomenon appears to be exaggerated at the extremes of age[32]. No facet of the inflammatory response is spared: neutrophils become primed, complement and kallikrein-kinin systems are activated, inflammatory (and anti-inflammatory) cytokines are generated and activated platelets litter the surface of the extra-corporeal circuit resulting in coagulation disturbances and thrombocytopaenia[31, 33-35]. These processes have a direct role in commonly encountered post-operative complications including capillary leak syndrome, acute lung injury, systemic inflammatory response syndrome, coagulopathies, and multi-organ failure[31, 36]. In addition, recent studies have demonstrated that inflammation contributes exigently to post-CPB cerebral dysfunction. In particular, the apical cytokines (TNFα, IL-1β) and products of neutrophil degranulation (activated oxygen

species) are recognised to be central to the progression of neuronal loss following periods of cerebral ischaemia in virtually every model studied[37-39]. Avenues for harnessing the CPB inflammatory response are therefore of considerable interest.

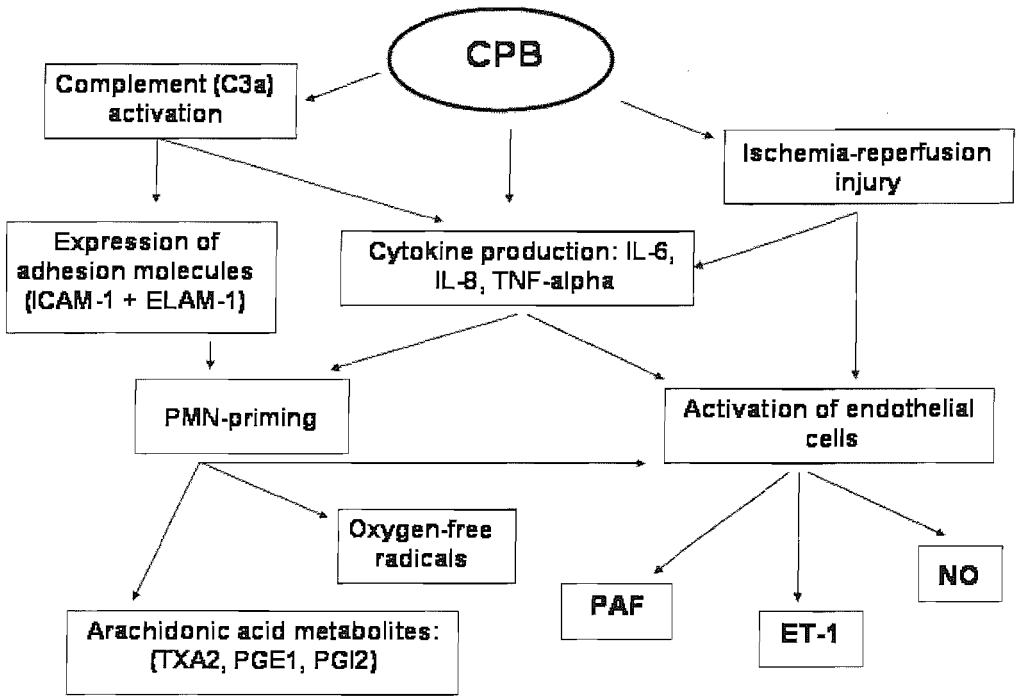

Figure 2.1 Schematic representation of the systemic inflammatory consequences of exposure to the extracorporeal circuit.

2.1.2a Leukocytes

Neutrophil activation during CPB occurs through a number of mediators (in addition to shear stress), including complement products C3a and C5a, and cytokines PAF and leukotriene B4. Neutrophil rolling and adherence is an important step in the onset of tissue injury and is induced via the sequential expression of cell adhesion molecules (CD11b/CD18, E-selectin, ICAM-1, and P-selectin). Pre-treatment with antibodies targeting these cell adhesion molecules (CD18) has been demonstrated to lessen pulmonary ooedema and myocardial reperfusion injury[40]. Flow cytometry studies

have confirmed CD11b/CD18 PMN expression to be elevated immediately following the onset of CPB[41] and on aortic cross-clamp removal[42].

Neutrophil activation results in the release of large amounts of oxygen free-radicals. These include superoxide anion, hydrogen peroxide, hydroxyl radical and singlet oxygen. They all act to disrupt the integrity of membrane lipid bilayers, through a self-perpetuating cascade of lipid peroxidation. Although direct measurement of free-radicals is difficult, quantification of lipid peroxidation end products has demonstrated increased activity following CPB[43, 44]. Correlations between this and complement activity may re-affirm the putative role of the complement cascade in recruiting and activating neutrophils during CPB[45]. The type of oxygenator has also been linked to peroxidation activity, with bubble oxygenators appearing more detrimental[46]. The stages most implicated in free radical generation are the onset of CPB, removal of the aortic cross-clamp and periods of ischaemia-reperfusion (DHCA). Interestingly, free radicals may impair the NO vasodilatation and lead to vasospasm and reperfusion abnormalities (no-reflow) following periods of ischaemia[47], supporting the reports made by Langley et al[48].

2.1.2b Platelet Activation and Sequestration

Platelets are responsible for the primary plug formation during haemostasis. Platelet activation is closely regulated by the endothelial cell. Although the inanimate surface of the CPB circuit activates platelets, endothelium is as important in the process. Endothelial liberation of activated thrombin is a potent stimulator for platelet activation and causes the release of contents from Weibel-Palade bodies, which contain, in addition to thrombospondin and P-selectin, von Willebrand factor[49]. Von Willebrand's factor is the primary "glue" involved in platelet aggregation and adherence, and is one of the principal causes for platelet consumption during CPB.

Activated platelets express glycoprotein binding sites which allow them to be incorporated into fibrin clot. Fibrin has up to six specific binding sites for platelets and therefore links series of platelets together. Overall, during CPB, large numbers of platelets are lost due to their sequestration in the CPB circuit, and especially due to their interaction with endothelial cells in capillary beds. Another important component of endothelial-platelet interaction is the release of P-selectin from Wiebel-Palade bodies. P-selectin is an adhesion molecule that promotes the rolling and adherence of leukocytes, which occurs shortly after the initiation of CPB, is exacerbated by hypoxia-ischaemia (H-I), and may play a central role in the pathogenesis of reperfusion abnormalities (no-reflow)[50].

2.1.2c Inflammatory Mediators

Despite the preponderance of the successful use of CPB in both infants and adults in allowing the repair of complex and otherwise fatal cardiac defects, the main frequently encountered drawback is a systemic, and sometimes severe, inflammatory response. In attempting to breakdown the individual cellular and non-cellular components of the process it becomes apparent that an obligate step in the process is the generation of an extremely complicated and chaotic milieu of harmful cytokines. Attempts at understanding roles of individual mediators has been likened to studying a single fish to understand marine bio-ecosystems[51].

Complement system. One of the first cascades to be activated is the complement system, by virtue of its simplicity. It encompasses a group of 20 or so plasma proteins and is activated in a cascade sequence by both the classic and alternative pathways during CPB. Exposure to the inanimate surface triggers the alternative pathway, liberating C3a and C5a and gastrointestinal endotoxin may be responsible for activation of both pathways.

Numerous intermediate products have partial activity, but the anaphylatoxins C3a and C5a are central in causing mast cell histamine release, increasing vascular permeability, acting as chemotaxins for leukocytes and causing their activation and degranulation. C3a is a potent stimulator of platelet aggregation, while C5a likewise affects leukocytes. Complement is believed to be responsible for the leukopaenia that accompanies prolonged CPB[52].

C3a is elevated during CPB, in proportion to the duration of CPB and remains so for a period after CPB[53]. Complex cardiac operations not utilising CPB are not accompanied by such a rise in C3a[53]. Although C5a is difficult to quantify due to its rapid clearance, elevated C5b-9 ("membrane attack complex") have been documented[35]. The importance of complement lies in the fact that it is one of the earliest arms of the innate immune response to be triggered, and acts in an apical fashion, initiating a huge array of downstream cellular and non-cellular inflammatory responses.

Arachadonic Acid metabolites. Arachadonic acid – principally from the leukocyte cell membrane – leads to the generation of prostanoids and leukotrienes. Thromoboxane A_2 ($TX\text{-}A_2$) is a powerful vasoconstrictor and also aggregates platelets, and although seemingly a brief response, it is elevated with CPB and has been implicated in post-CPB pulmonary hypertension[54]. Blockade of the generation[55] or action[56] of $TX\text{-}A_2$ significantly reduces CPB-related pulmonary injury. The fact that it either directly or indirectly plays an important role in capillary function is implied by the fact that $TX\text{-}A_2$ blockade reduces cerebral no-reflow in piglet models of neonatal DHCA[57]. The prostaglandins (PG) are largely vasodilators and some (PGE_2 and PGI_2) inhibit platelet aggregation. As a result, they may be natural antagonists to the effects of TX-A2. Prostaglandins are also seen to rise with CPB[58].

Leukotrienes (LT) are potent chemotaxins and also increase vascular permeability. Some (LT-B_4) are also long-acting smooth muscle constrictors. Parallel to their known role in the pathogenesis of asthma, it has been suggested that leukotrienes may play a role in ARDS and multi-organ failure following CPB[59].

Cytokines. Inflammatory cytokines are liberated by numerous stimuli including complement activation, activated neutrophils, activated endothelium, platelets and ischaemia. Interleukin (IL)-1β and tumour necrosis factor-alpha (TNFα) are both apical cytokines released by areas of tissue injury (especially ischaemia) and can affect virtually every arm of the immune response. TNFα in particular is now being implicated as one of the major determinants of progression of injury following cerebral ischaemia (see chapter 3), and huge efforts are being made to understand and manipulate this role, especially in the field of stroke research. TNFα is elevated with CPB[60-62], and in proportion to CPB duration. Other pro-inflammatory cytokines including IL-6 and the chemokine IL-8 have also been studied. IL-8 is consistently raised with CPB and is primarily a trigger of neutrophil activation and chemotaxis[60]. Platelet-activating factor (PAF), so named because of its effects on platelets actually has numerous (probably more important) roles as a chemoattractant of neutrophils and the monocyte-macrophage lineage, and has been implicated in both myocardial and pulmonary ischaemia-reperfusion injury during CPB[63]. PAF represents a significant potential therapeutic target in this respect. Certainly in the context of cerebral no reflow, PAF-antagonism improves the cerebral recovery following DHCA[64].

The cytokine network is hugely complicated, and the release anti-inflammatory mediators (for example IL-10) illustrate this. IL-10 suppresses the production of IL-1β, TNFα, IL-6 and IL-8, and its release has been demonstrated during CPB[60, 65]. Overall, in the sea of interacting mediators, the overall sway in one direction or another is likely to be more important than attempting to isolate individual culprits. Certainly in

the case of TNFα, as a true apical cytokine, evidence is mounting that it plays a central orchestrating role in the response to injury. Attempting to decipher the individual contributions of the cytokine network is made more complicated by the realisation that these mediators often play dual roles. For example, TNFα does not simply play a deleterious role in ischaemic injury: TNFα knock-out mice exhibit *more severe injury* following experimental stroke, and protective properties of TNFα are now being identified (see chapter 3).

2.1.2d Kallikrein-Kinin System

A generalised disturbance of microvascular permeability with marked oedema formation and effusions is a significant complication of cardiopulmonary bypass in children and neonates. The kinin peptides – including bradykinin and kallidin - represent a family of low molecular weight inflammatory mediators that are implicated in many physiological and pathological processes, including the regulation of blood pressure, sodium homeostasis, inflammation and the cardioprotective effects of preconditioning. In particular, bradykinin is known to rapidly and reversibly increase vascular permeability by receptor-mediated endothelial cell contraction and intercellular gap formation. Plasma and tissue kallikrein are the enzymes responsible for liberating the active products bradykinin and kallidin respectively, from high molecular weight kininogen precursors[66]. Various alternative pathways also exist, especially in specific disease states. Kinins act via either type 1 (B1) or type 2 (B2) receptors. The B2 is the predominant receptor, though the B1 is induced by tissue injury. They are metabolized by angiotensin-converting enzyme (ACE) and neutral endopeptidase 24.11 (NEP) to inactive septapeptide products. Endogenous kallikrein inhibitors play a role in controlling *in vivo* kinin production, the main ones being α2-macroglobulin, C1 inhibitor, antithrombin III and kallistatin.

Remarkably, kininogen deficiency in healthy humans is relatively asymptomatic, despite clear abnormalities in laboratory coagulation assays. However, kininogen-deficient BNK rats, or B2 receptor knock-out mice display evidence of hypertension, exaggerated responses to fluid and salt administration and impaired natriuesis. Knockout models also exhibit impaired hypotensive responses to bacterial lipopolysaccharide, and in models of carrageenan-induced inflammation, impaired leukocyte accumulation, reduced paw ooedema and reduced nociception.

Studies monitoring levels of bradykinin, kallidin, kallikrein and kallistatin levels have shown that cardiopulmonary bypass is associated with the activation of both bradykinin and kallidin[67]. Experiments using B1 and B2 receptor agonists demonstrated parallel increases, emphasizing the potential for B1 receptors to be upregulated – a process that has been implicated in promoting the inflammatory response to surgery and the systemic inflammatory response to CPB. KKS precursor proteins HMWK, plasma prekallikrein and factor XII (Hageman factor) have been collectively termed the "contact system" due to their requirement for contact with negatively charged artificial surfaces for zymogen activation *in vitro*. The large inanimate surface area of the membrane-oxygenator and tubing provides exactly these conditions known to be associated with KKS activation, and clinical studies in children have confirmed high bradykinin levels during and following CPB[68, 69] and furthermore, body weight gain is directly related to KKS activity[70]. Interestingly, in one of the earliest studies confirming kinin generation in association with neonatal CPB, increasing depth of hypothermia itself was directly linked to increasing liberation of bradykinin[68], challenging the popular notion behind the use of cooling as being a blanket suppressor of pathophysiological processes during CPB.

2.2 Strategies to Reduce CPB-Inflammation

2.2.1 Steroids

Both clinical and laboratory studies have suggested that corticosteroid usage improves the postoperative course after CPB[71-73]. Glucocorticoids blunt neutrophil upregulation, inhibit the expression of adhesion molecules produced by the activated endothelial cells, including endothelial-leukocyte adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1), and therefore reduce diapedesis of leukocytes into injured areas[74]. Ungerleider's group[71, 73] has extensively studied the timing of steroid administration and found that premedication with methylprednisolone (Solumedrol™) 30 mg/kg 12 hours preoperatively is beneficial relative to isolated administration in the pump prime. Bronicki and colleagues demonstrated that preoperative administration of dexamethasone (1 mg/kg) intravenously 1 hour before the pump-run in children produced an eightfold decrease in interleukin-6 and a threefold decrease in TNFα, which improved convalescence[72].

2.2.2 Aprotinin

Aprotinin is a serine protease inhibitor that has known efficacy in reducing the bleeding diathesis that frequently accompanies the use of CPB. Aprotinin was initially shown to reduce inflammatory markers following CPB, but other studies have been equivocal[60]. Others have been unable to demonstrate a significant reduction in inflammatory cytokines with aprotonin, but have shown that it normalises the coagulation index using thromboelastography[75]. These data contradict the notion that aprotinin use, especially during certain neonatal operations such as the Norwood procedure or arterial switch, is associated with an increased risk of thrombosis. Further studies are clearly indicated.

2.2.3 Ultrafiltration and Leukocyte-reduction

Leukocyte filtration during CPB was initially tested in animals in the early 1990's and subsequently used in humans undergoing cardiac surgery[74]. When leukocyte filters are incorporated into the extracorporeal circuit, total leukocyte count and CD18 mediated neutrophil adhesion are significantly attenuated compared to control patient groups, but this has not translated into clinical benefit[74]. In particular, parameters of myocardial and lung function, including respiratory index, blood PaO_2, spirometry and pulmonary vascular resistance, and CK-MB levels were not affected by the use of a leukocyte filter[76]. Equivocal findings, however, are difficult to interpret because ischaemia-reperfusion injury can be mediated initially by other effector cells, and thus occur independent of neutrohpil participation[77].

Intraoperative ultrafiltration, using a conventional or modified approach, has been reported to reduce the rise in total body water following CPB in paediatric patients, as well as the duration of mechanical ventilation[78]. Modified ultrafiltration (MUF) has the advantage of filtering only the patient's extracellular blood volume, resulting in greater haemoconcentration efficacy and more rapid recovery of ventricular function[79]. Similar to the data with leukocyte depletion, however, reduction of inflammatory markers with ultrafiltration have been inconsistent and have not provided durable clinical benefit, likely because many factors including choice of filter and pore size, the haemofiltration rate, and the timing of the procedure importantly influence outcome[60]. In addition, the removal of certain anti-inflammatory cytokines, such as interleukin-10, may actually produce deleterious effects[80].

2.2.4 Biocompatible Coated Circuitry

Heparin coated circuits, and more recently poly-2-methoxy-ethy-acrylate (PMEA) coating, may attenuate inflammation following infant CPB. Jensen and colleagues

showed that the use of a heparin coated perfusion system reduced fibrinolytic activity following bypass in a prospective randomised trial of forty children[81]. A similar reduction in C-reactive protein and complement levels with PMEA-coated circuit was demonstrated by Ueyama et al. in a prospective randomised study comparing heparin-coated, PMEA coated, and conventional circuits[82].

2.3 Hypothermia

The importance of the preservation of tissue viability through the use of hypothermia cannot be overstated in modern surgical practice. It is an integral part of tissue protection and organ support in widely contrasting areas of medicine, and its role in functional recovery following prolonged hypothermic cardiac arrest are well known. Considerable energy is being spent trying to establish protocols for the controlled use of hypothermia following stroke, cardiac arrest, and even in combination with post-surgical extra-corporeal membrane oxygenation (ECMO). However no uniform strategy has yet emerged for the management of therapeutic hypothermic therapy.

The mechanisms whereby hypothermia exerts its neuroprotective effects have not fully been elucidated. The effects of hypothermia on reducing total oxygen consumption and preserving high-energy tissue phosphates are now well known following the pioneering work in the 1950s by Wilfred Bigelow. It should be pointed out that these initial studies opposed the prevailing opinion of the day, which noted that tissue *oxygen extraction increased* during hypothermia. It was only through careful anaesthetic regulation and elimination of shivering that the true effects of temperature on tissue metabolic requirements were realised. Hypothermia causes a reduction in the reaction kinetics of mitochondrial respiration and energy utilisation. This is a central pre-requisite to whatever other protective properties hypothermia conveys, because unless intracellular

ATP concentrations remain within physiologic constraints, neuronal viability will not be preserved. However additional mechanisms may accompany the properties of hypothermia in reducing the metabolic requirements of oxygen consumption in the brain[83].

The onset of hypothermia is initially accompanied by a rise in mean arterial blood pressure due a sympathetic response in association with shivering. In the context of CPB, this may be masked by the anaesthetic state, but thereafter a fall in mean pressure is observed[84]. Although some animals, for example the rat, can maintain unaltered perfusion pressures at temperatures as low as 27°C, temperatures lower than 33-34°C in humans is associated with progressively worsening haemodynamic compromise, and eventually fibrillation. Arterial oxygen tensions during brief hypothermia remain unchanged, but carbon dioxide tensions and pH vary in accordance with experimental methodology of their calculation. $PaCO_2$ and pH are inter-related and their values depend on the type of strategy employed for maintenance of acid-base balance. In the α-stat strategy, the $PaCO_2$ is adjusted at a given lowered core temperature to a value which yields an arterial pH within normal physiological limits. *In vivo* this blood is hypocapneic and alkalotic at the hypothermic temperature. An alternative approach – pH-stat – is to adjust the $PaCO_2$ such that the arterial pH is normal at the hypothermic temperature. This technique utilises the addition of CO_2 and therefore the *in vivo* blood is hypercapneic, and therefore believed to aid in vasodilatation,

Hypothermia itself, in the absence of any other insult has little impact on glucose and lactate concentrations, suggesting that the effects of short term lowering of core temperature have little impact on the catabolic and anabolic processes involving glucose and lactate, such that their constancy is maintained[84].

Within the region of 28-30°C cerebral autoregulation remains intact and cerebral blood flow (CBF) therefore in parallel with the rate of metabolic oxygen consumption[84]. However, in the context of CPB, this linear relationship is sometimes lost, and several reports describe an elevation in CBF with progressive cooling[85]. The reason for this is not known, although some have attributed it to localised hyperaemia resulting from regional ischaemia from micro-emboli, though this is mere supposition. Below 28°C, decreases in cerebral metabolic rate are larger than those in blood flow, and therefore the oxygen delivery capacity of the cerebral circulation is in a metaphorical state of excess where supply increasingly exceeds demands[86]. This is the reason for the descriptions of extremely low flow rates (30ml/kg/min) in meeting cerebral energy demands during deep hypothermic continuous low flow CPB[87].

During periods of ischaemia, hypothermia slows the breakdown of high energy phosphate compounds (ATP, ADP, AMP), as well as the rate of formation of lactate (and inorganic phosphates), and this effect is related to the depth of hypothermia – as expected. Whether this relationship is exponential or linear is not precisely known because few studies have examined numerous temperature points. Overall, however, the breakdown of high energy phosphates (and overall metabolic oxygen consumption) appears to be slowed by 2- to 3-fold for a 10°C drop in core temperature (similar to the rule-of-thumb 7% drop in basal metabolic rate for each 1°C drop from normothermia) [88]. The preservation of energy stores has equally been demonstrated following deep hypothermic CPB strategies[89].

In fact, it is difficult to dissociate the hypothemic impact on biomolecular kinetics from other potentially pathological consequences of hypothermia. For example, although mild hypothermia has been shown in some non-CPB models be protective following head trauma, in others it has been shown to prolong the activation of NFkB and augment the generation of cytokines[90]. Similarly in one model of DHCA, mild post-

CPB hypothermia delayed the recovery from lactic acidosis and energy deficit[91]. These observations may imply that although hypothermia may confer a theoretical protection from severe ischaemia, the inflammatory and metabolic consequences may just reflect slower kinetics. The inflammatory response may be of no lesser amplitude, but simply more drawn out: lactic acidosis may take longer to develop, but may also take longer to recover.

The impact of hypothermia in clinical situations is of huge interest, and unfortunately subject to considerable controversy. For example in the context of CPB, normothermic strategies have gained considerable support and ignited vociferous debate. Although some advocate normothermia as a more physiological state[92], other reports suggest that hypothermia generates a lesser inflammatory response[93-95] although not all[68]. The protective role of hypothermia on brain injury resulting from cardiac arrest is well known[96]. Several clinical areas are being pursued in this light, including neonatal encephalopathy[97], post-cardiac arrest therapy[98, 99], hypoxic encephalopathy[100], and ECMO[101]. Although progression of cerebral injury has been slowed in some animal models of global ischaemia[99], interestingly mild post-operative hypothermia, was not protective following DHCA in studies performed by Jonas' group[102] and in fact worsened outcome in others[91]. However, post-ischaemic *hyperthermia* was shown to have a deleterious effect on histological outcome[102]. It seems, therefore, that the consequences of mild hypothermia are not fully elucidated. Even less is known about the direct cellular ultrastructural consequences of deep hypothermia, and its detailed study would be useful. In the early application of deep hypothermia, extreme temperatures (i.e. below 10°C) were often adopted, and were associated with high incidences of neurologic sequelae[8], although whether this was cause or effect is not known. The use of uninterrupted hypothermic CPB has been shown to trigger cerebral perfusion abnormalities, which may be related to the reduced red cell deformability (and haemolysis) that occurs with deep hypothermia[103].

In the context of hypothermic CPB, the technical application of hypothermia is now realised to be important. For example, "crash-cooling" provides inferior hypothermic protection when compared with more gentle and sustained cooling gradients, and current wisdom is that cooling should be performed for at least 20 minutes, with differences between perfusate and core body temperatures no greater than 10°C to reduce the risk of formation of gas emboli[8, 104, 105]. In fact, maintenance of tissue hypothermia has been proposed by some as the principle protective mechanism of continuous hypothermic perfusion strategies[106].

2.4 Hypoxia-Ischaemia

Hypoxia-ischaemia (H-I) during neonatal CPB is either intentional or unintentional. Intentional ischaemia is an integral part of the CPB strategy and implemented either by reducing the flow rates (or distribution) or terminating flow completely (circulatory arrest). These are by convention both performed at moderate or deep hypothermia due to the protective effects discussed previously.

Unintentional severe hypoxia-ischaemia in the infant is usually a result of technical mishap. Typically the cause would either by gaseous embolisation (microscopic from the circuit or macroscopic from a dislodged cannula, or running the venous reservoir dry, for example) or a major flow distribution problem arising from misplacement of the cannulae.

Little has been determined regarding the intracellular responses to ischaemia during hypothermic CPB. This is in contrast to the enormous energy spent on elucidating the cellular impact of ischaemia in the fields of stroke or peri-natal brain injury. The mechanisms being elucidated in these pathologies does however provide considerable

insight the processes that may be involved in ischaemia during CPB, and is reviewed in detail in the following chapter.

Chapter 3

Neurological Injury following Neonatal CPB

3.1 Introduction

Historically, neurological injury following neonatal CPB has almost exclusively been attributed to periods of global hypoxia-ischaemia (H-I) sustained during deep hypothermic circulatory arrest (DHCA) [107]. While it was in the context of DHCA that brain injury has been brought to the forefront of our attention, it is now becoming increasingly clear that brain injury is actually multifactorial, with many causes, and many confounding factors[108]. For example, DHCA is frequently utilised to repair some of the most complex congenital defects in infants with many co-existing problems. The fact that these conditions are fatal without surgical intervention means that we have no "control" population with which to compare. Only in the advent of novel imaging techniques are we now beginning to realise that the incidence of pre-operative brain injury is in itself significant, and attention is now focusing on identifying children pre-operatively at high-risk of subsequent injury[109]. Pre- and perioperative factors are likely influence the susceptibility of cerebral tissue to injury even before a period of H-I is imposed. Finally, the reperfusion period is now placed centre-stage in the propagation of tissue injury, and its manipulation is likely to represent one of the key potential areas for the future. In addition, superimposed upon the entire operative window is the added component of the inflammatory response – both local and systemic – that is now recognised to pose almost as great a threat to cerebral homeostasis as H-I[70, 110-112].

3.2 Incidence

A survey of six North American hospitals in 1988-89 reported an incidence of brain injury post-congenital repair of between 2 and 25%[113]. This variability in reporting is largely due to discrepancies in the techniques or thresholds for detecting clinical deficit. Initial reports of brain injury following neonatal CPB related to the observation

of dramatic defects such as choreoathetosis and cerebral palsy[107]. A general consensus is now instead emerging that although gross motor defects are uncommon, subtle behavioural, neurocognitive and developmental outcomes occur with alarming frequencies[114, 115]. In the much publicised Boston Circulatory Arrest Trial, for example, evidence of subtle neurological insults was present in as many as a third of infants exposed to deep hypothermic CPB strategies. Although clearly this study represented a subset of infants with complex defects who were exposed to extreme CPB strategies, perhaps the most important outcome to emerge from this trial was that eliminating cerebral ischaemia through the use of continuous low flow did not significantly alter the incidence of cerebral injury. Perhaps this serves to emphasise the multi-factorial nature of brain injury following infant cardiac surgery.

3.3 Experimental Investigation

The very nature of its ease of use and clear, bloodless operating field ensured that DHCA became a routine part of the cardiac surgeon's practice during the two decades following its inception. Continued success dictated that progressively more complex lesions would be challenged, requiring more lengthy periods of whole-body anoxia. Certainly, lengthy ischaemic periods are detrimental, as has been confirmed in recent detailed series[116], but experimental models generally require inordinately long durations of circulatory arrest in order to generate gross injury, which makes it very difficult to elucidate the threshold for a "safe duration of DHCA".

Experimental studies have been undertaken in wide-ranging species and with numerous end-points as indicators of cerebral well-being. Initial models depended largely on metabolic and cerebral blood flow responses to DHCA. Predictably, lengthy (clinically irrelevant) DHCA durations result in exhaustion of metabolic supplies, tissue acidosis

and lactate production[86], confirmed more recently using elegant MRI spectroscopic techniques[117]. Numerous studies have confirmed the cerebral no-reflow response as being a consistent feature of DHCA[64], and this response has been linked to outcome[118, 119]. Experimental techniques that reduce cerebral no-reflow, including the use of steroids[71], leukocyte filtration[118], PAF antagonism[64], TX-A_2 inhibition[57], free-radical scavengers[48] or intermittent perfusion[120] have all been interpreted as beneficial. Subsequently, attempts were made to characterise the histological injury sustained during DHCA. This has been problematic, because of the extremely long durations of DHCA required to generate detectable injury in many models. Typically these range from 60-150 minutes, whereas clinical DHCA is fortunately infrequently longer than 60 minutes. These models have nevertheless provided significant insight into the mechanisms of neuronal loss, including regional susceptibility[121], confirming the dominant role of free-radical-mediated reperfusion injury[119] and have been used to compare management techniques for neurological impact[122]. **They have not, however, been useful for providing information on the "safe" duration of DHCA.** Although the lack of overt histological damage has been used to imply safety[123], this is **not** an appropriate conclusion. Subtle ultrastructural changes (on electron microscopy) occur in the absence light microscopic changes[120], and moreover, rat models of global cerebral ischaemia reveal an elevated apoptotic index as late as six weeks following the insult[124, 125]. Instead, it is likely that much of the clinical injury sustained (regardless of CPB strategy) reflects a very subtle and low rate of apoptosis over a significant time (certainly spanning the entire clinical duration) that is occurring at a time of cerebral plasticity. These changes are then reflected in the subtle neurodevelopmental abnormalities that so frequently occur following congenital repair[114].

3.4 Mechanism of Injury

3.4.1 Cerebral Microvascular Response to Ischaemia

Microvessel ultrastructure in the central nervous system (CNS) is remarkably preserved across species. Penetrating arteries consist of an endothelial cell layer, a basal lamina derived from extracellular matrix, and smooth muscle, which becomes progressively less significant with arborisation. An extension of the sub-arachnoid space forms the Virchow-Robins space, surrounding cortical penetrating arterioles until it becomes the *glia limitans* – the boundary between astrocyte end-feet and the vessel – which fuses with the basal lamina at capillary level.

The permeability barrier – the blood brain barrier (BBB) – is formed by the intact inter-endothelial cell tight junctions, a basal lamina and surrounding astrocytes, with their end-feet processes. The proximity of the cellular components (endothelial cells and astrocytes) implies an intricate process of interaction to enable rapid fluctuations in transport requirements as necessary. In addition, individual endothelial cells exhibit differential functional characteristics, such that amino acid, glucose and other transmembrane transport proteins vary according to microvessel diameter[126]. Expression of cell adhesion molecules for regulation of leukocyte adhesion also varies, predominating in the post-capillary venule.

The overall architecture of cortical pial vessels appears complex. In the grey matter, penetrating vessels divide into hexagonal arrays of vessels to supply columns of cortical neurons, which in turn interconnect with horizontal arrays of vessels. At the grey-white matter interface, the density of vessels significantly decreases, in accordance with the greatly reduced metabolic requirements of the white matter tissue. The architecture of the deep nuclei and basal ganglia is far less understood.

During normal blood flow, not all capillaries are perfused. During hypercapnia and anaesthesia, substantial increases in local blood flow can occur, suggesting that a cerebrovascular reserve exists which can be recruited in the short term (i.e. separate from neovascularisation). In the case of abrupt cerebral ischaemia, however, it is unclear how significant any such reserve is, or what role it plays in the period of reperfusion[126].

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During a period of ischaemia-reperfusion, the cerebral microvasculature becomes the staging platform for a series of complex intercellular interactions. The interface between the vascular compartment and the plasma column is significantly altered, the central theme being the promotion of microvascular obstruction by leukocyte and platelet interactions.

3.4.2 The Endothelial Unit and No-Reflow

The vascular endothelium plays a pivotal role in the systemic host response to injury, and is activated by both systemic inflammation and ischaemia. From a relatively dormant and benign resting state, exposure to cytokines, lipopolysaccharide, C3a and C5a, products of neutrophil activation and ischaemia, endothelium undergoes profound alterations in its gene expression profile and function. Cell adhesion molecules are expressed to facilitate neutrophil rolling, adhesion and transmigration. Cytokines and cell surface signaling molecules are expressed to participate in the orchestration of the inflammatory response.

The concept of "no-reflow" was introduced following early perfusion studies involving dye tracers[127] (figure 3.1). They noted that after periods of ischaemia, despite restoring the blood flow to pre-ischaemic levels, some areas of the myocardium remain unperfused. This was termed "no-reflow" and was defined...

"...the inability to perfuse previously ischaemic myocardium, even when blood flow has been restored to the large arteries supplying the tissue."

This phenomenon is now recognised to be a common feature of ischaemia during CPB, particularly in relation to cerebral ischaemia sustained during DHCA (cerebral no-reflow) [64, 120]. The pathogenesis of cerebral no-reflow appears to involve several aspects, and an activated endothelial unit is central[128]. There are two main components to no-reflow: 1) a "physiological" component characterised by a sustained vasoconstriction (or impaired vasodilatation. This is physiological in the sense that in theory it might pharmacologically or otherwise be reversed; 2) a "non-physiological" component characterised by obstructed capillary beds. This is non-physiological in the sense that it represents a mechanical obstruction that therefore cannot be overcome by vasoactive mediators alone[129].

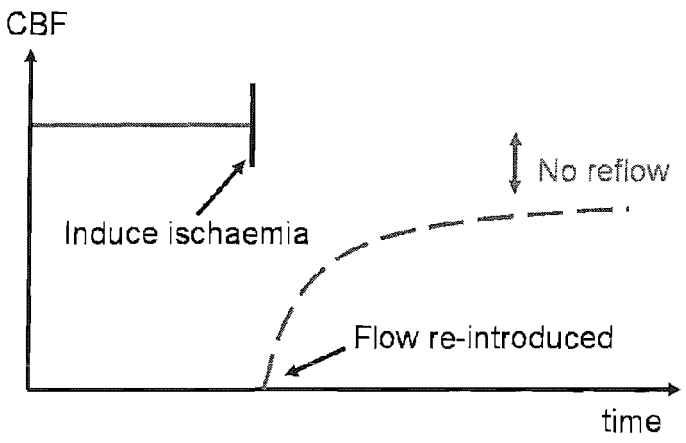


Figure 3.1 Schematic representation of cerebral no-reflow phenomenon. Following a period of relative or total tissue ischaemia, when flow is re-introduced perfusion does not return the baseline pre-ischaemic level. This discrepancy in perfusion is termed "No-Reflow" and has been attributed to both mechanical and physiological (vasoactive) components.

Physiological no-reflow. During periods of H-I, products of anaerobic metabolism build up (lactate, adenosine, H+...), all of which are potent vasodilators involved in local capillary bed autoregulation. The predicted outcome following a period of H-I would therefore be a reflex reactive hyperaemia, in response to these metabolites to recover the oxygen debt and restore aerobic metabolism. In fact, even in normothermic

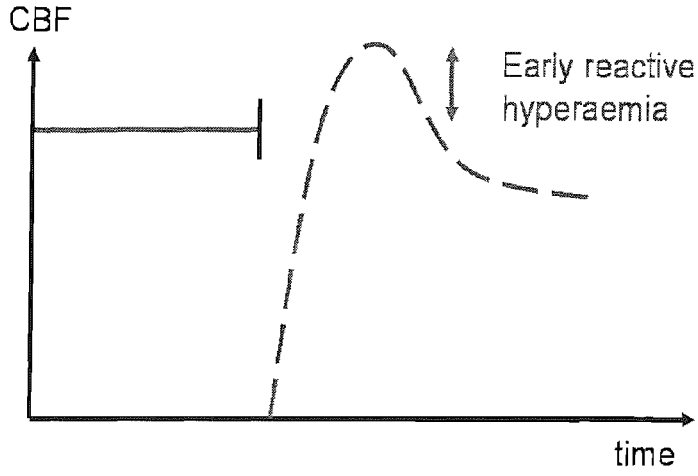


Figure 3.2 Schematic representation of the reactive hyperaemic response to global ischaemia.

models of severe global neonatal H-I, a brief reactive hyperaemia *is* seen in the early reperfusion period, before a more sustained depression of tissue perfusion (no-reflow) [130, 131] (figure 3.2). The difference between the observations in these models and the events occurring following ischaemia during CPB is the lack of pre-existing systemic inflammation associated with the use of CPB.

Endothelin-1 is a potent long-acting vasoconstrictor that is elevated by both CPB and ischaemia[132] and may be partly responsible for the sustained depression in post-ischaemic depression in myocardial blood flow following periods of ischaemia[133]. Because endothelin concentrations are elevated following ischaemia, investigators have examined its role following cerebral ischaemia. Endothelin-1 is indeed elevated following DHCA[134], and in fact techniques that reduce the overall circulating levels of endothelin-1 following DHCA (such as ultrafiltration of the perfusate during the circulatory arrest period) are associated with improved cerebral outcome. The arachadonic acid metabolite thromboxane A_2 (vasoconstrictor) has also been implicated, as its direct inhibition has been shown to reduce cerebral no-reflow in neonatal piglet models of DHCA[57]. Furthermore, thromboxane A_2 release may be mediated in part by endothelin-1, exacerbating the overall vasoconstrictor response following periods of ischaemia[135].

Down-regulation of counteracting vasodilator mechanisms may also contribute. Endothelium releases PGI_2, adenosine and NO all of which act on underlying pre-capillary sphincters to augment blood flow. Following ischaemia, endothelial generation of prostanoid vasodilators may be impaired, allowing unopposed vasoconstriction to occur. The common pathway for mediation of vasodilatation is through the production of NO. Certainly following periods of ischaemia or hypoxia, the nitric oxide axis appears to by dysregulated[136] and nitric oxide synthase, which mediates the production of NO through the conversion of the amino acid L-arginine to L-citrulline is deficient following ischaemia-reperfusion[137]. Free-radicals and activated oxygen species are potent inhibitors of NO[47], it may be that arteriolar constriction may be fundamentally due to this inability for NO vasodilatation to be triggered.

Non-physiological no-reflow. A central feature of the no-reflow phenomenon appears to be capillary plugging by adherent neutrophils. Neutrophil elastase has been suggested as a mechanism of proteolytic endothelial basement membrane degradation leading to detachment, swelling and destruction of endothelial cells, contributing to the cellular debris occluding capillary beds[138]. In addition, in non-CPB stroke models, endothelial cells have been shown to undergo apoptosis, a process leading to impaired capillary function[139], and perhaps obstruction through endothelial casts or ooedema. Platelets, which become activated upon exposure to the inanimate surface of the extracorporeal circuit, are also believed to adhere to capillary endothelium, disrupting the microvascular perfusion[140, 141]. Monoclonal blocking antibodies to tissue factor preserves myocardial blood flow following ischaemia[141]. Direct inhibition of platelet activation through antagonism of glycoprotein IIb/IIIa has very recently been shown to significantly reduce cerebral no reflow following DHCA[142]. Overall, the contribution of adherent leukocytes and platelets, endothelial ooedema and casts and inflammatory debris (fibrin) hare collectively believed to result in mechanically

occluded capillary beds that contribute to reperfusion abnormalities following periods of either total or relative ischaemia during CPB.

The basal lamina represents the second architectural component beyond the endothelium to undergo structural changes. Ischaemia initiates a gradual decrease in expression of basal lamina components laminin-1 and –5, collagen IV and fibronectin, which is paralleled by accumulation of haemoglobin. Loss of basal lamina architecture is mirrored by loss reactivity and differentiation of endothelial cells as evidenced by their profile of cell surface integrins and selectins[143]. Some of these changes in basal lamina structure may be associated with local generation of proteases, initiated by ischaemia. In primate models, rapid up-regulation of pro-matrix metalloproteinases and plasminogen activators parallels basal lamina alterations, and mice knock-out for MMP-9 show decreased BBB permeability following cerebral H-I[143].

3.4.3 Endothelial unit and the Blood Brain Barrier

A feature common to both reperfusion injury following H-I and more severe haemorrhagic transformation following experimental stroke is the abnormally permeable capillary bed resulting from disruption of the blood-brain barrier (BBB). The endothelial unit ***is*** the BBB - a uniquely structured arrangement of the endothelial cells, perivascular basement membrane and astroglial cells that collectively impart its special qualities as the gate-keeper between the blood and the environment within which the delicate neurons reside. Apart from areas associated with control of homeostasis (choroids plexus, respiratory areas of the brain stem), the BBB is otherwise only selectively permeable - characterised by a low level of vesicular transport and tightly juxtaposed endothelial cells - such that specific membrane transport proteins are necessary to allow for the controlled passage of molecules including glucose, amino acids, lipoproteins, hormones and drugs. The BBB therefore serves to provide a buffer against harmful fluctuations in the systemic circulation.

In animal models of acute cerebral ischaemia, scanning electron microscopy has revealed a rapid change in morphology of the endothelial unit, including membrane invaginations, endothelial cell contraction, microvillar formation and loss of astrocyte foot-processes[144]. One of the most profound observations was the dramatic rise in the permeability of the BBB to intravenously injected protein chasers, including horseradish peroxidase[145]. This early (within 12 hrs) BBB disruption has now been confirmed in human patients suffering regional stroke using magnetic resonance imaging markers[146], which is interestingly *potentiated* by early thrombolysis (reperfusion injury) [147].

The significance of the loss of this barrier is only beginning to be appreciated. This is partly due to the uncertainty as to whether BBB injury is a primary or secondary event, however the importance of this distinction is becoming less with the realisation that the extent of BBB injury *per se* appears to influence both pathological and functional outcome. For example, BBB injury has been directly implicated in the pathogenesis of haemorrhagic transformation, a harmful inflammatory process following stroke[110]. and, the elevated BBB permeability seen in stroke patients following early thrombolysis correlates with worse functional outcome[147].

Ischaemia is not the only insult to affect the BBB, and in fact the propensity for central nervous system tumours to grossly damage the BBB is a well recognised phenomenon that accounts for the use of intravascular contrast imaging in their assessment. Local and systemic sepsis, including cytokines, bradykinin and toxins all result in BBB injury[148].

3.4.4 Pre-Operative Brain Injury

Although the neurological assessment of neonates is difficult, several studies have confirmed that infants with congenital cardiac disease have a significant incidence of brain injury pre-operatively. In a study of 131 infants, neurobehavioural problems have been present in as many as 50%[149]. The advent of magnetic resonance imaging has introduced a safe and sensitive method by which subtle neurological lesions can be identified. Mild ischaemic lesions (in the form of PVL – see below) was present in 16% of a series of infants undergoing surgical repair (which increased to over 50% post-operatively) [150], an observation subsequently confirmed in repeated studies[151]. An MRI study of ten infants undergoing arterial switch operation revealed pre-operative lesions in 4[152].

Numerous pre-operative factors contribute to cerebral injury (below), not least the direct association of genetic and chromosomal abnormalities with congenital cardiac lesions. Particularly prominent is the high incidence of Down's syndrome in congenital cardiac disease (15%), which is associated pathologically with microscopic calcification and (more rarely) cerebrovascular defects, and clinically with developmental problems[153]. The CATCH-22 spectrum (microdeletions at 22q11 region of chromosome 22, which includes di George) is present in 5-10% children with congenital heart disease, and display developmental delay and hypotonic motor problems[154].

3.4.5 Prematurity and Periventricular Leukomalacia

Prematurity as a cause of peri-natal brain injury is of enormous importance, becoming increasingly apparent as survival of pre-term infants has become more routine. Thus, of the 57,000 infants born in the US each year with a birthweight below 1500g[155], 90% will now survive the neonatal period, though 10% of these will later display spastic

motor deficits (cerebral palsy), and as many as 25-50%[156] will manifest cognitive or behavioural problems that interfere with educational progression. This enormous morbidity is primarily due to the extreme fragility of certain areas of the immature brain, and specific "temporal windows" of this susceptibility to injury are now being recognised. Peri-natal hypoxia-ischaemia (H-I) is the single most common cause of brain injury in the premature infant, and white matter lesions are a characteristic feature, known as ***peri-ventricular leukomalacia*** **(PVL). The relevance of PVL to neonatal cardiac surgery, is that many of the risk factors, compounding factors and triggers overlap considerably between the two clinical scenarios,**

The neuropathology of PVL consists of localised necrosis of all cellular elements deep in the cerebral white matter, with subsequent cyst formation (figure 3.3). A more diffuse component is associated with less severe injury. Both are related to a specific developmental window of extreme fragility of immature oligodendrocye precursors (OL) [157]. These OL cells are or course destined to become mature OLs, which subsequently form myelin of cerebral white matter. This OL-related injury is therefore characterised by a deficiency in cortical myelination, loss of white matter volume, and the clinical manifestation of spastic motor disorders. Cortical myelination in humans is largely a post-term event in humans, hence the manifestation of these lesions later in the post-natal period.

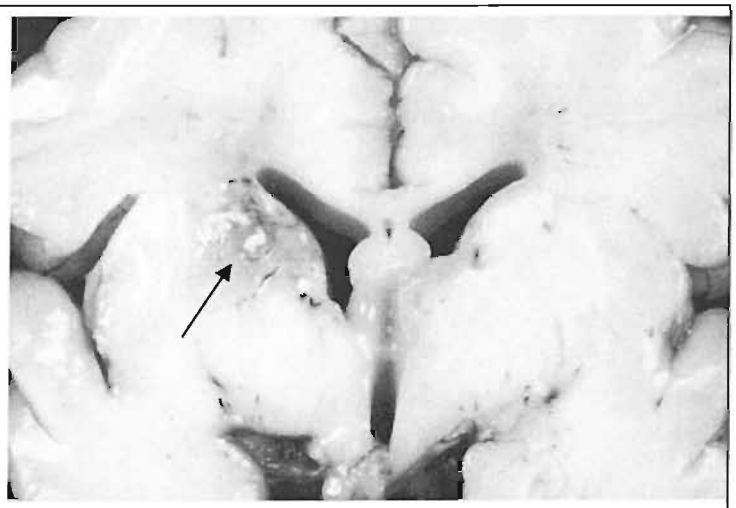


Figure 3.3 Periventricular leukomalacia (PVL). Necrotic white matter adjacent to the lateral ventricles (arrow).

The pathogenesis of PVL comprised three major factors that each relate either to the propensity for cerebral ischaemia, or of specific vulnerability of immature OLs.

1). **Vascular anatomy**. The blood supply to the cerebral white matter is through short-penetrating and sparser long-penetrating arteries. Many of these arteries do not fully develop until the post-term period. Thus these deep cortical regions are associated with the most precarious blood supply. The vulnerability of deep cortical white matter has been confirmed by positron emission tomography (PET) data revealing that even in normal or near-normal pre-term white matter, CBF values are frequently measured as <5ml/100g/min (the threshold for viability in the adult brain is approximately 10ml/100g/min) and some studies have reported ranges as low as 1.6-3.0ml/100g/min[158]. The low deep cortical white matter blood flow in the preterm infant therefore implies a minimal margin for safety for tissue oxygenation.

2). **Impaired auto-regulation of CBF**. At least in a subset of premature ventilated infants, studies using xenon clearance identified impaired cerebrovascular auto-regulation. Thus their circulation is instead pressure-passive, such that an overall drop in cardiac output is directly mirrored by a drop in flow through the cortical penetrating arteries to an area under precarious supply.

Support for a causal involvement of CBF is provided by the association between clinical events causing (even brief) periods of hypotension and the occurrence of PVL. Included in these are examples of congenital cardiac defects including hypoplastic left heart syndrome, patent ductus arteriosus with retrograde cerebral diastolic flow and severe illness, regardless of cause, requiring extra-corporeal membrane oxygenation[156].

3). **The specific maturation-dependent susceptibility** of developing OL precursors in the pathogenesis of PVL has emerged through studies using models of mid-to-late gestational cerebral H-I in sheep and rats[159, 160]. It should be emphasised that **injury in a pre-gestational window of susceptibility is a key feature of PVL** and therefore post-gestational studies either lose relevance or are studying different entities. From these early observations, elegant cell culture studies defined the immunocytochemical profile of the OL precursors dominant in the developmental window of maximal vulnerability[157]. Using this model, several important questions have been addressed. First, OL precursors are exquisitely sensitive to free radical attack which can be completely prevented by scavengers. Second the mode of cell death (by histological criteria, TUNEL and caspase-3 staining) appears to be apoptosis, rather than necrosis[161]. Interestingly, apoptosis seems to occur in a biphasic pattern, with an initial spell occurring in the first 24 hrs, and a second wave occurring up to a week later[125]. Third, the maturation dependence was confirmed by the demonstration that mature OLs are resistant to free-radical injury[161]. Lastly, and most importantly, the same group confirmed that it is the inadequate *processing* of free radicals in immature OLs that confers their susceptibility. Free radicals are usually processed by defense mechanisms including especially glutathione peroxidase and catalase. If these fail, in the presence of Fe2+, the Fenton reaction generates the hydroxyl radical, with catastrophic consequences. The application of iron chelators, thereby starving immature OLs of Fe2+, rendered them *resistant* to H-I and free radical injury[161].

Inflammation and PVL. In discussing the role of prematurity in brain injury in infants destined to undergo surgical repair of congenital heart defects, of particular importance is the impact of inflammation and the associated cytokines. A number of clinical and epidemiological series suggested a link between maternal/foetal infection and PVL[112]. Similarly the induction of maternal intra-uterine infection in foetal rabbits has resulted in WM lesions[162]. Furthermore in cell culture models, either TNFα or

IFNγ alone (in the absence of H-I) can trigger apoptotic cell death, and TNFα potentiates the toxicity of IFNγ[163, 164].

The high incidence of prematurity and low birth weight in infants with CHD, and especially the high incidence of associated co-morbidity, including the innate risks of intensive care therapy (sepsis, artificial nutrition, numerous parenteral medications, artificial ventilation, etc), dictates that infants born with severe congenital heart defects represent a group highly at risk of suffering from PVL lesions. Immaturity, neuronal vulnerability, hypoxia-ischaemia-reperfusion injury, free radical liberation and systemic inflammation are the hallmark feature in the pathogenesis of PVL – a major cause of cerebral palsy in infants. These are all integral components of neonates undergoing complex congenital repair of cardiac lesions. Recent MRI studies have detected a high incidence of PVL lesions in infants both pre- and post-congenital repair [151].

3.4.6 Cyanosis

Cyanosis is intrinsic to almost 40% of infants born with CHD. It occurs on delivery, with the transition from the foetal circulation, or on duct closure, or more gradually with age, due to progressive right ventricular hypertrophy. Although many compensatory mechanisms occur in chronic hypoxia, including polycythaemia, neovascularisation, and augmentation of cardiac output, these adaptive responses may take time to develop. It is unclear; however, whether in lesions involving the abrupt transition to a cyanotic state (such as left ventricular hypoplasia) tissue injury occurs during this transition. *In vitro* models using cultured rat neurons exposed to prolonged oxygen deprivation demonstrated an elevated rate of apoptosis[165], but more recently, an *in vivo* rat model in which rats were placed in a 10% O_2 atmosphere immediately at birth to mimic cyanotic heart disease failed to demonstrate an increased rate of

apoptosis or ceramide concentration at 1 or 4 weeks over controls. This implies that even abrupt institution of severely cyanotic conditions can be tolerated well in the long-term. However, other factors may influence the cerebral response to chronic hypoxia, including acidosis[166], periods of haemodynamic compromise, inflammation/sepsis and prematurity, so the picture is not altogether clear. In fact, Limperopoulos *et al.* reported the interesting finding of a *lower* incidence of neurological compromise in cyanotic infants than acyanotic ones[149].

3.4.7 Intra-Operative Factors

The perfusion management has advanced enormously since initial data emerged to implicate periods of DHCA in the pathogenesis of brain injury following neonatal CPB. Among the developments include the characterisation of optimal pH management strategies[122, 167], improving cooling and rewarming techniques, and the elimination of routine haemodilution[168].

3.4.8 Reperfusion Injury

The period of reperfusion following ischaemia is now recognised to be of paramount importance in every area of cardiovascular medicine. Re-instigating capillary flow results in a flood of oxygenated blood containing inflammatory mediators, activated and primed leukocytes and oxygen[128]. These provide the fuel for the generation of activated oxygen species – in a metabolically precarious tissue bed – that result in the breakdown of lipid bilayers membranes. Once activated, this process is self perpetuating, as activated oxygen species further fuel their own production in a series of cascade reactions. Interrupting or dampening this process results in tissue preservation in virtually every clinical scenario, and techniques such as free radical scavengers have also been proved effective in improving the response following DHCA. Of particular importance is the fact that reperfusion injury is centrally related to the systemic

inflammatory response, as products of neutrophil degranulation themselves are initiators of the process.

3.5 The use of DHCA in modern practice

The historical link between brain injury and DHCA has resulted in numerous novel – and occasionally complex – continuous perfusion strategies, aimed at reducing or eliminating any period of cerebral ischaemia. These include continuous low-flow CPB[169-171], antegrade selective cerebral perfusion[172], retrograde cerebral perfusion, combinations of circulatory arrest and selective cerebral perfusion[173] [174] and more recently, normothermic full-flow techniques[92, 175, 176]. Perhaps, the propensity for the numerous strategies suggests that they all have limitations. Certainly, evidence is beginning to accumulate to suggest continuous CPB as being more inflammatory[62, 177], and regional perfusion techniques themselves are associated with perfusion deficits[178-180]. The recent preponderance of normothermic continuous perfusion strategies carries narrower margins for safety, and possibly a more pronounced systemic inflammatory response.

Despite the neurological concerns surrounding the use of DHCA, and the significant body of experimental evidence demonstrating irreversible neuronal injury following its use, DHCA is still frequently used in modern neonatal congenital repair. Experimental models have provided considerable insight into the consequences of ischaemia during CPB, but by necessity require amplification of the ischaemic insult, and therefore lengthy periods of DHCA are used, not necessarily relevant to clinical practice. More importantly, DHCA presents several attractive benefits. Most notably, the operating field during DHCA is optimal, and cannot be paralleled by any continuous perfusion technique. Secondly, during periods of circulatory arrest, the blood volume is not dynamically exposed to the extracorporeal circuit, and therefore the total duration of

exposure to CPB is usually less (even more so because the surgical repair may be faster due to the enhanced access and clarity). This is reflected in an overall lesser systemic inflammatory response, recently confirmed experimentally[62], and a trend observed in studies reported here (chapter 5). Systemic inflammation is now realised to contribute significantly to the amplification of neuronal loss, and therefore reducing ischaemia through continuous perfusion is at the expense this exaggerated inflammatory response. Thirdly, the paranoia surrounding periods of hypothermic ischaemia – largely resulting from studies in the 1980s, when ischaemic periods were longer and CPB management rudimentary by today's standards – has generated numerous complex strategies involving continuous cerebral perfusion. These include continuous low flow, retrograde cerebral perfusion, selective cerebral perfusion, and more recently, continuous normothermic full-flow. These either are associated with perfusion abnormalities of their own, involve complex additional surgical intervention or in the case of normothermic strategies, are at the expense of the safety margins that hypothermia presents. In addition, normothermia may be associated with even more deleterious inflammatory consequences than hypothermic strategies (see chapter 10). Perhaps most importantly, the introduction of continuous perfusion strategies has *not* had the effect of reducing injury following neonatal CPB. In the most detailed and lengthy follow up series to date, the incidence of neurological deficit was not significantly different between infants exposed to either DHCA or deep hypothermic continuous low flow CPB[114]. Both groups were, however, significantly cerebrally deficient compared to normal infants. Perhaps this exemplifies the contribution of associated pre-, intra- and post-operative factors or otherwise the fact that continuous perfusion introduces additional insults that counteract the elimination of ischaemia. Considerable vociferous debate exists[181] surrounding the adoption of respective strategies, and clear answers are not presently available – largely because of the lack of effective experimental techniques to investigate *clinically relevant* CPB strategies. What is clear, however, is that DHCA remains a heavily utilised technique in modern practice, perhaps contrary to popular opinion. No better evidence exists to support this than the response of

participants at sessions of the American Association of Thoracic Surgeon's meeting (2003) paediatric session. This data does not otherwise exist in the public domain, and provides an invaluable insight into the current use of DHCA.

Figure 3.4-6 Audience response sessions from annual scientific meetings of the Congenital Heart Surgeons' Society, American Association of Thoracic Surgeons and Society of Thoracic Surgeons (2003-2004). Attendees provided anonymised responses to questions put forward by the chairing panel.

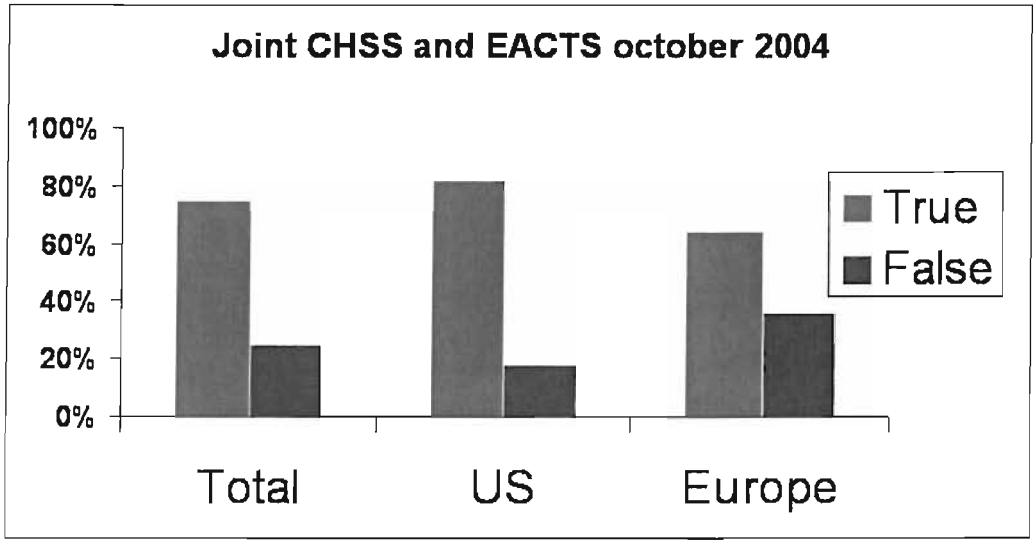
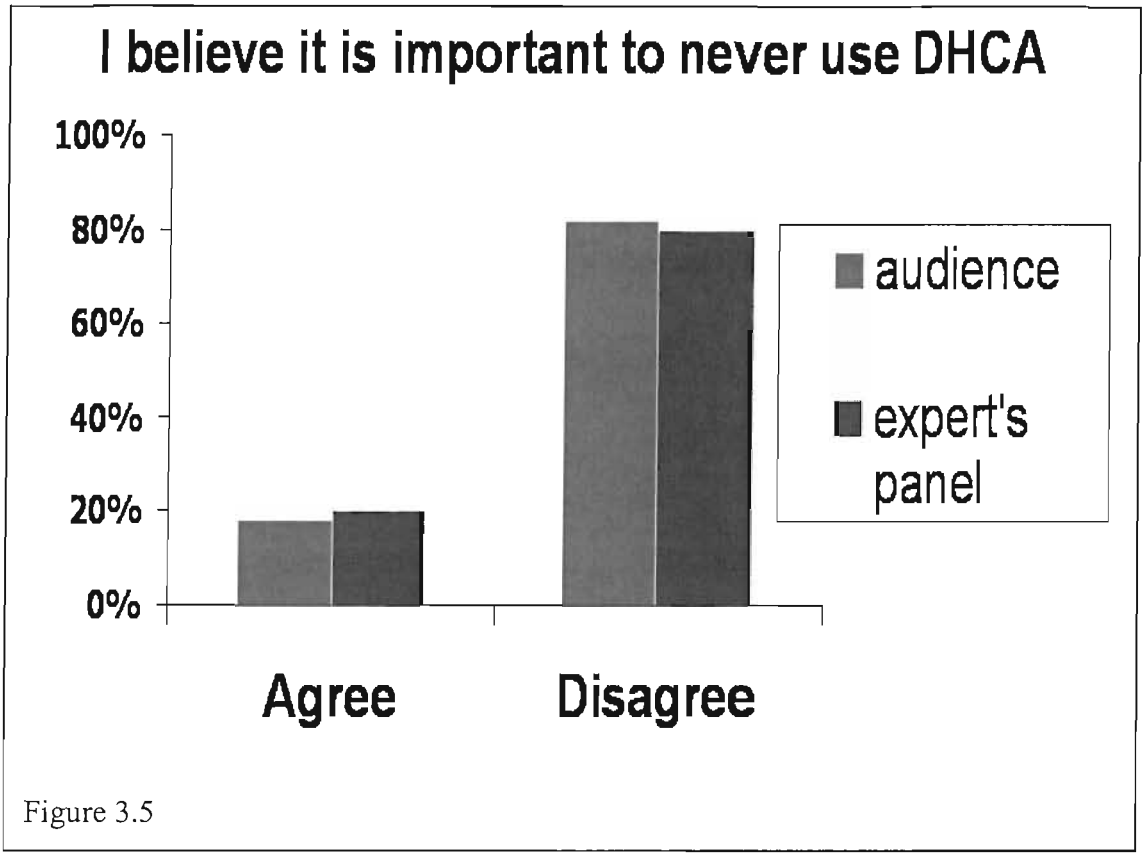


Figure 3.4



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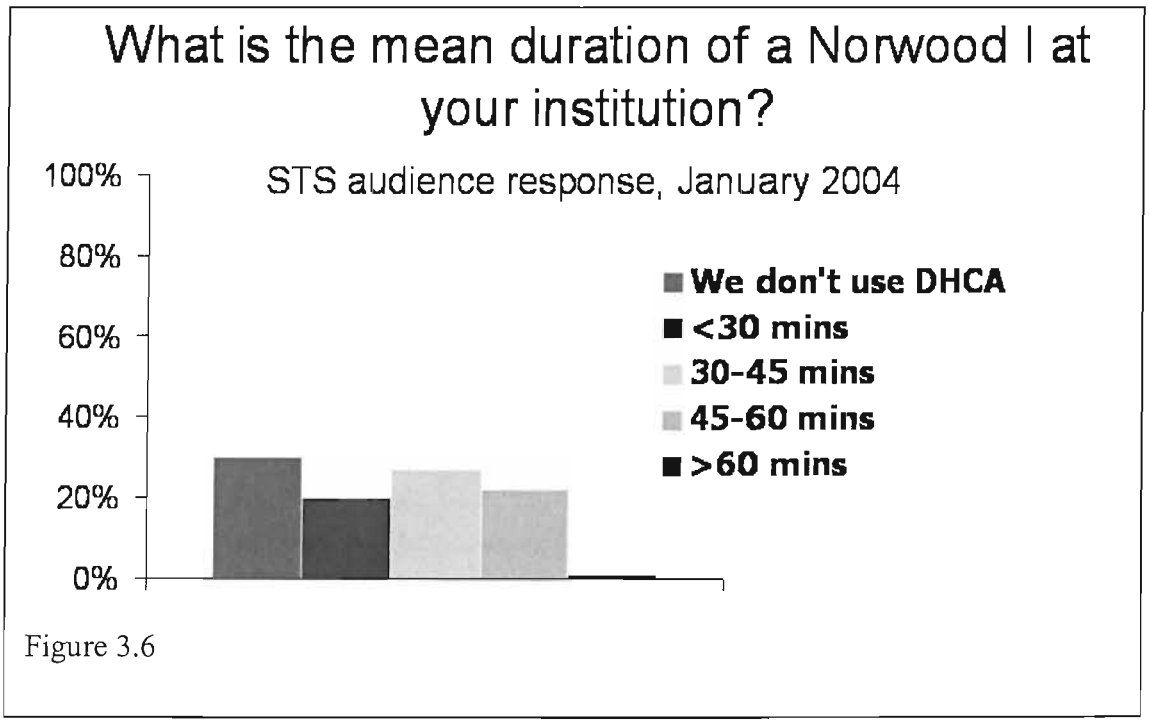


Figure 3.6

Despite the historical concern surrounding the use of DHCA, and enormous effort being spent to devise alternative techniques, this series of questions implies several important points:

- **Although the majority of surgeons try to avoid its use...**
- **...very few surgeons feel it never has a place in their practice**
- **...and 70% use it for complex defects (stage 1 Norwood for example)**
- **...and even in modern practice, almost 25% typically use uninterrupted DHCA for durations greater then 45 minutes during a Norwood I.**

Despite the concern surrounding the impact of DHCA on neurological recovery, it is therefore still a frequently utilised technique. It follows, therefore, that the experimental study of DHCA remains highly pertinent to clinical practice and that although neurological injury is now increasingly recognised to be a multifactorial process, methods of protecting against neuronal loss during DHCA are of huge significance.

Chapter 4

Experimental Techniques

4.1 Animals

All animal experiments were conducted with the approval of the institution's Animal Care and Use Committee. The animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH publication 85-23, revised 1995) and were housed in the institution's NIH-approved animal facility before the experiments. The experimental subjects in all studies were male uncastrated piglets aged 1-3 weeks. Despite the wide variety of animal species used for the study of cardiopulmonary bypass, including rat, rabbit, guinea pig, dog, sheep and swine, size precludes the use of small mammals in neonatal research. Neonatal swine are universally accepted to be an excellent correlate for the study of clinical cardiovascular physiology[182], and have therefore been adopted as the preferred model for the investigation of neonatal cardiopulmonary bypass. The anatomy is relatively similar, especially the cerebral circulation. The neonatal piglet heart and great vessels are comparable in size to human infants, though considerably rotated, such that the inter-ventricular septum lies anteriorly in the pig, with both ventricles immediately visible on pericardotomy (see figure 4.4). This results in corresponding rotation of the outflow tracts, such that the aorta is *posterior,* lying behind the right atrial appendage and pulmonary artery (figure 4.5), which can make instrumentation awkward.

The use of neonatal swine is not without problems. Though developmentally slightly more mature than human neonates (and fully myelinated) such that they are independently mobile, like all mammals they are initially nourished through breast-milk. In the commercial sector, enormous pressure is placed on early weaning to maximize the breeding capacity of the sow. Despite this, piglets maternally separated within one month of birth invariably perish due to inadequate nutrition. In well over one hundred piglets involved in this project, all separated within 1-2 week's age, only

one died. This is a reflection of the considerable efforts by numerous staff members to ensure the adequate hydration and weaning of animals onto semisolids, including bottle feeding and the use of electrolyte replacement drinks (Gatorade™). Diarrhoea and weight loss due to bacterial overgrowth (or parasites) nevertheless is usual 7-10 days after weaning, and therefore animals should be experimented upon shortly after settling into the animal housing, and not if there is evidence of diarrhoea or weight loss.

The preference for male sex is based on the recent identification of ooestrogen in conferring powerful neuroprotection to cerebral ischaemia[183]. This has generated huge interest in the field of stroke research. Oestrogen protects against, and testosterone exacerbates[184] ischaemic and chemical neuronal injury, and exogenous ooestrogen is protective even in neonatal males[185]. The mechanisms are beginning to be elucidated[183, 186], and clearly this represents an area of huge interest for future CPB study. In the light of these discoveries, it is inappropriate to use mixed sexes for neurological study, and all the piglets were therefore male uncastrated animals.

4.2 Preparation and Monitoring

Free access to food and water was allowed up until the time of surgery. The piglets were induced with 0.3ml IM telazole into the buttock, and once drowsy, were weighed had a diathermy pad placed over their shaved back. After placing ties to gently restrain their limbs, anaesthesia was induced by placing a mask over their snout and allowing them to inspire pure oxygen and 1-2% isofluorane. A saturation probe was placed in the side of the mouth. Intravenous cannulae were inserted into both veins in both ears and a saline drip commenced. The entire neck, chest, abdomen and both groins were scrubbed with betadine solution and surgical drapes placed around the animal.

Surgical tracheostomy was performed (figure 4.1) and an endo-tracheal tube (3-4.5Fr) inserted to allow controlled ventilation at 12-16/min, tidal volume 10ml/kg to maintain arterial saturations and end-tidal CO2 within the normal range. Temperature probes were then inserted into the oropharynx and rectum.

The right groin was then dissected to expose the femoral artery and veins (figure 4.2), which were skeletonised and cannulated with V8 polyethylene. An arterial blood sample would be analysed for baseline data, and then the lines connect to a pressure recorder for continuous invasive blood pressure and central venous pressure recording. The groin wound was then closed with an appropriate suture.

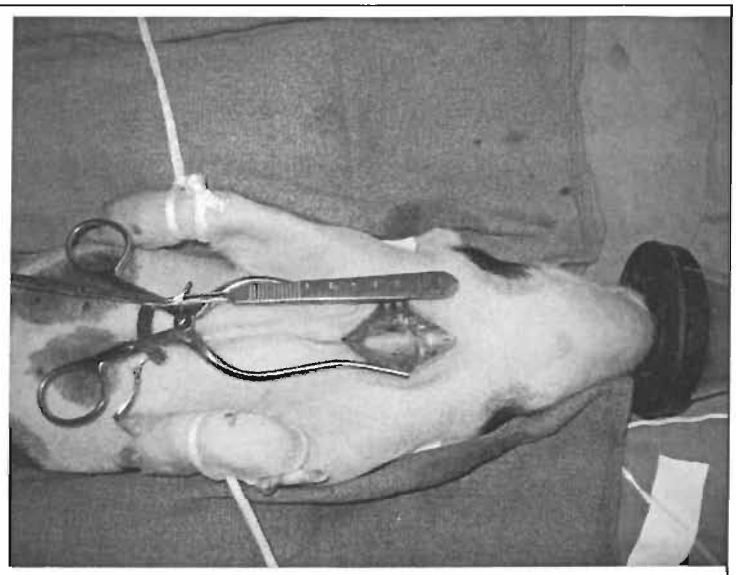


Figure 4.1 Anaesthetic induction followed by surgical tracheostomy. Gentle limb restraints were applied and the trachea exposed through a midline neck incision.

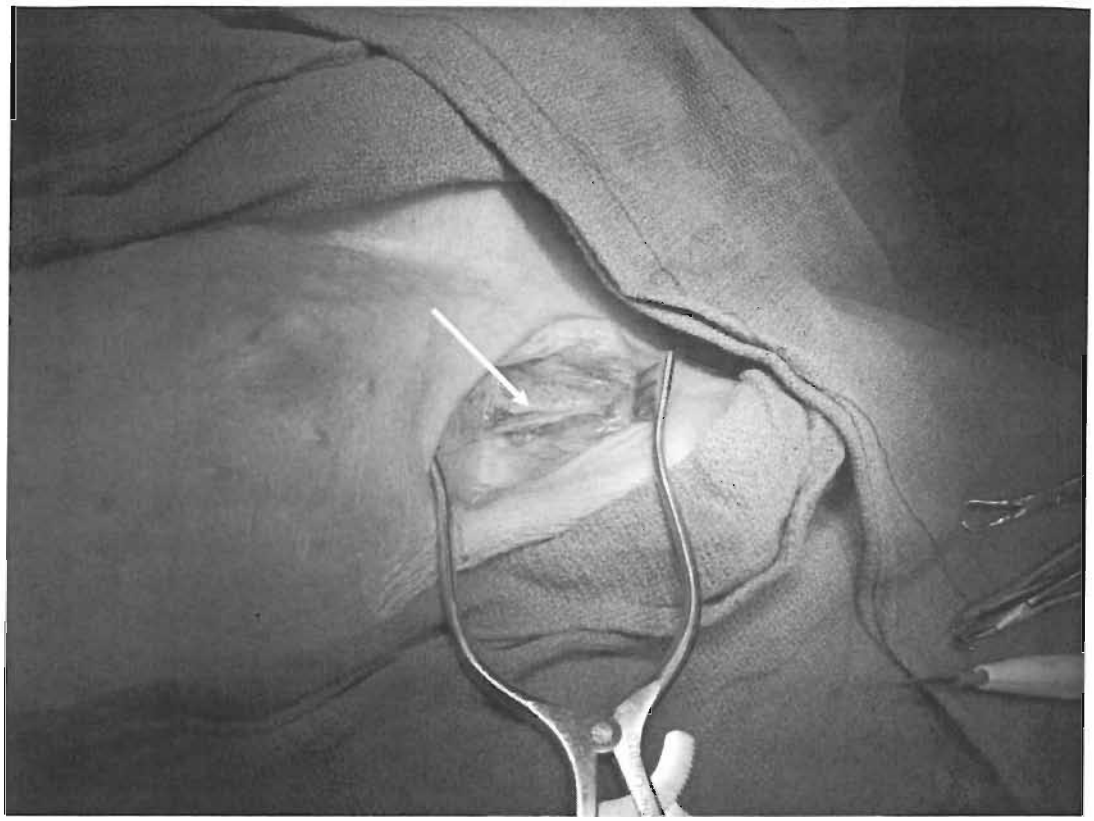


Figure 4.2 Surgical exposure of the femoral vessels (arrow).

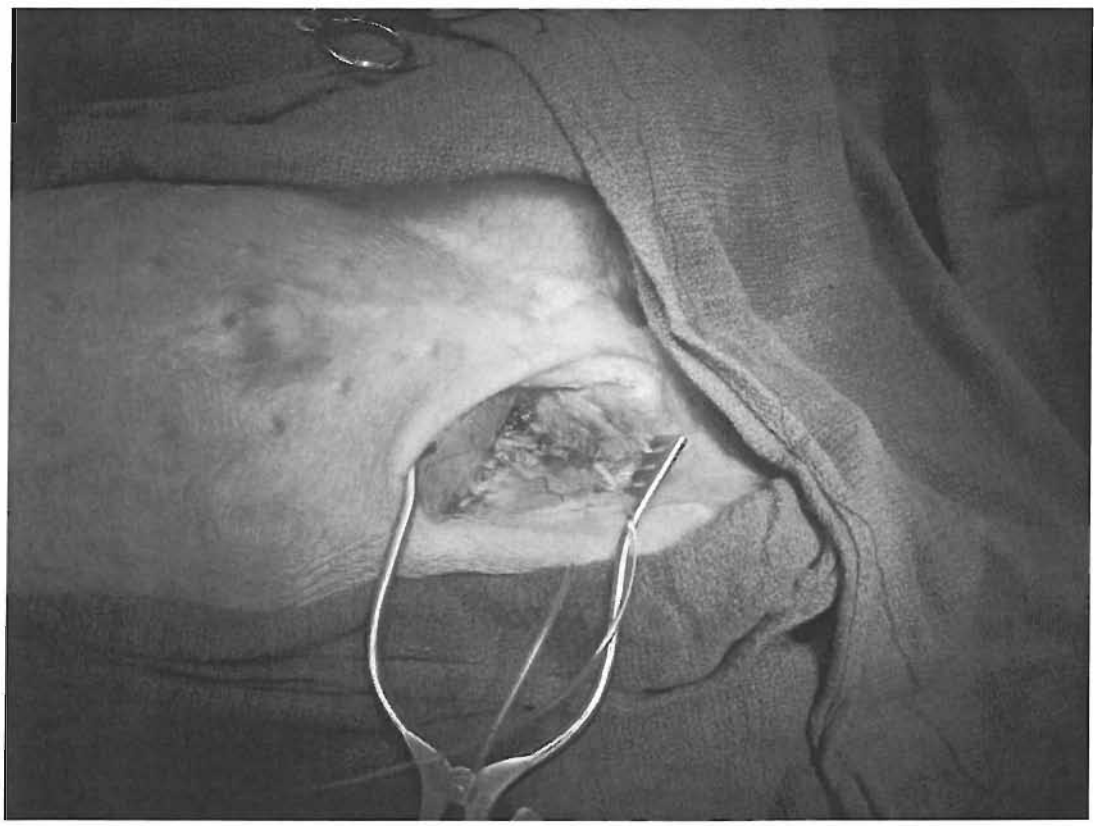


Figure 4.3 Cannulation of the femoral vessles with V-8 catheters.

Midline sternotomy was then performed (figure 4.4) and bone wax used to minimise bleeding. After inserting the chest spreader, the pericardium was opened in the midline down to the ventricular apex, and, after sweeping the thymus to the left, up to the superior vena cava, before hitching it with stay sutures. A 2/0 silk tie was then place around the right atrial appendage to allow its retraction to the right (figure 4.5). By retracting the pulmonary artery left, the aorta was exposed deep inside the wound into which a partial-thickness 5/0 prolene purse-string was placed. A corresponding purse-string was then inserted in the right atrium.

For those studies requiring cardiac output or pulmonary artery pressure measurement, the aorto-pulmonary window was gently opened and an ultrasonic flow-probe (Transonic systems, NY) placed around the pulmonary artery, and a V5 polyethylene cannula inserted through a purse-string in the right ventricular outflow tract and then advanced until the pressure trace confirmed its position in the pulmonary artery.

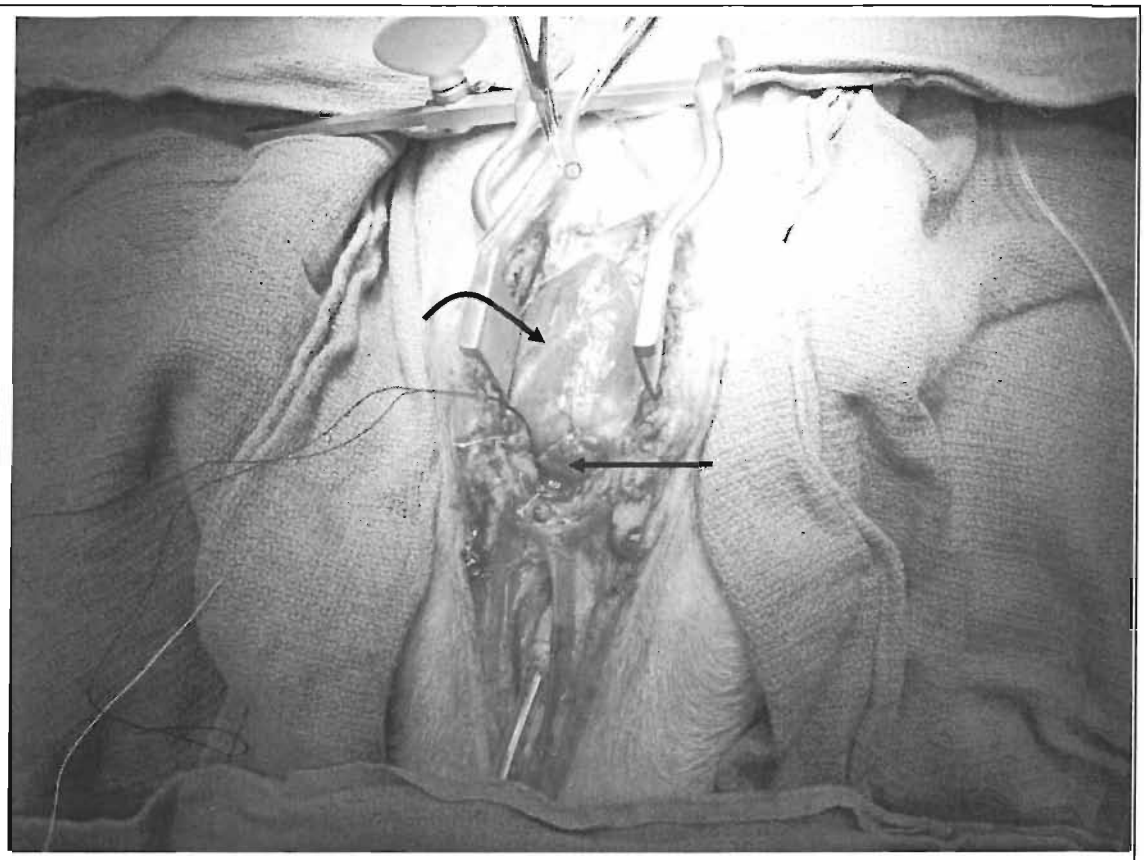


Figure 4.4 Median sternotomy and pericardium opened to expose the mediastinal structures. The right atrium (straight arrow) and pulmonary artery lie directly anterior to the posteriorly placed aorta. The left anterior descending artery is visible in the midline (curved arrow).

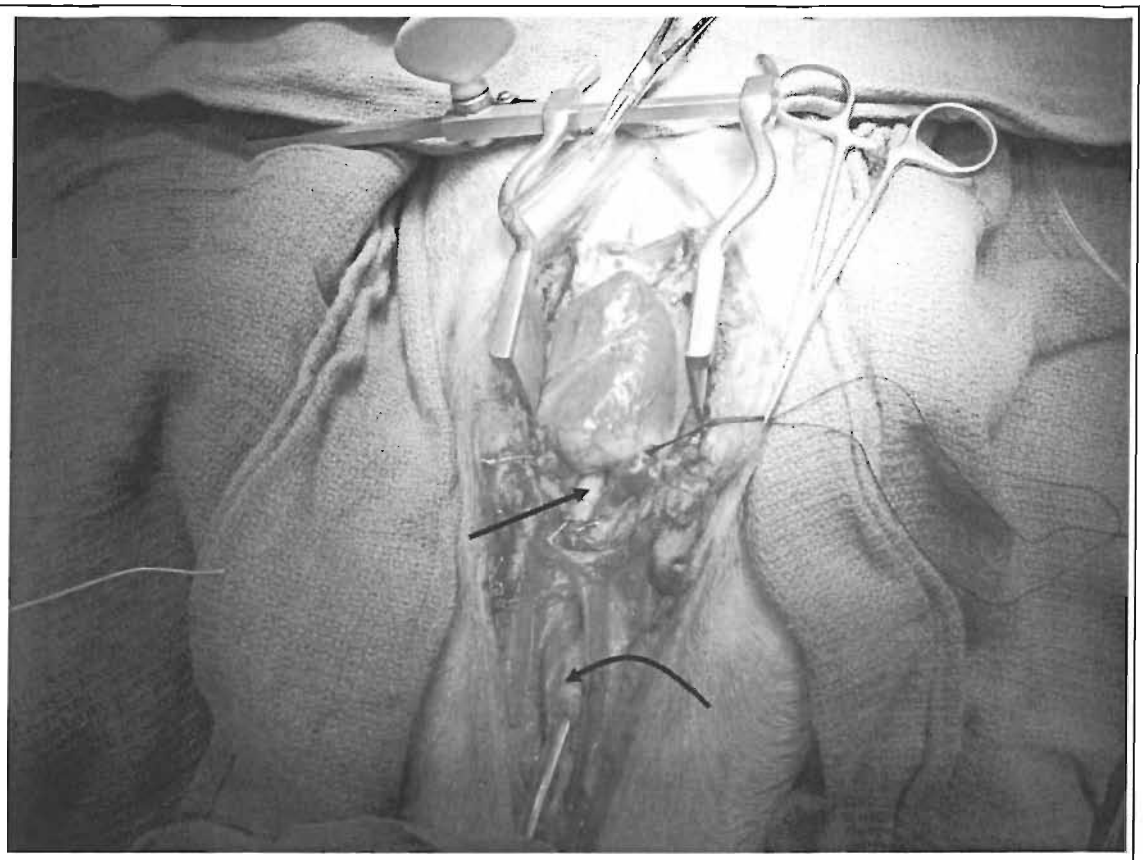


Figure 4.5 The right atrial appendage has been retracted to reveal the short and posteriorly placed aorta (arrow). The endotracheal tube is visible at the bottom of the picture entering the trachea (curved arrow).

4.3 Cardiopulmonary Bypass

The cardiopulmonary bypass circuit consisted of a Capiox Baby RX™ (Terumo Cardiovascular, Japan) hollow fibre oxygenator-reservoir and heat-exchanger unit, a COBE™ roller head cardiopulmonary bypass pump, and polyethylene tubing (3/8" pump and arterial lines and 1/4" venous lines). Temperature probes were connected to the venous return and arterial outflow lines. A pressure manometer line was connected to the arterial outflow line. The circuit was primed with 2ml heparin and fresh whole blood. This blood was obtained by exsanguinating an adult pig using sterile technique

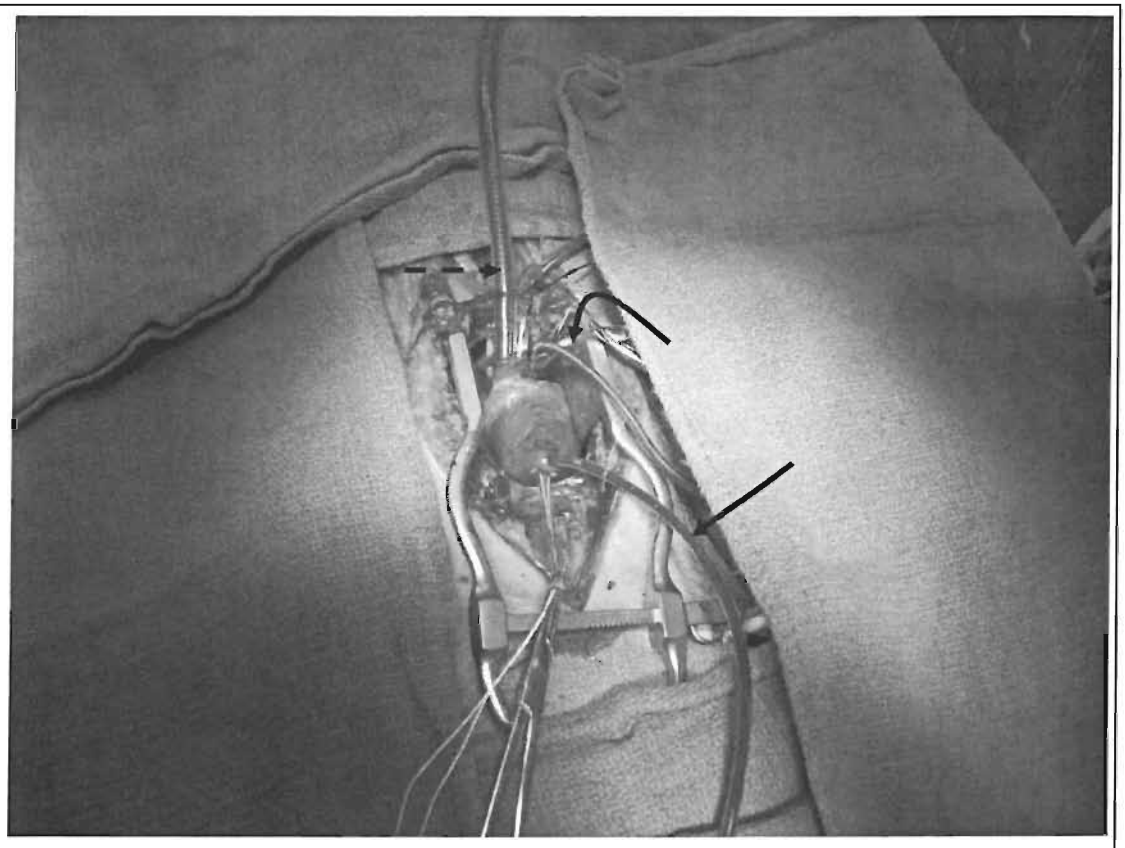


Figure 4.6. Full instrumentation of the heart for CPB. An apical left ventricular vent was routinely inserted for decompression of the ventricle (arrow). A size 8Fr DLP™ arterial cannula (curved arrow) and 20Fr venous cannula (dotted arrow) are visible.

and intravenous broad-spectrum antibiotics (cefazolin 25mg/kg i.v.), under general anaesthesia and ventilation, via the carotid artery. The blood was stored in sterile

citrate-phosphate-DDD bags and refrigerated before use. After priming and de-airing the circuit and warming to 37°C, a blood gas sample would be analysed to ensure the acid-base balance was within normal limits.

After systemic heparinisation (500iu/kg), cardiopulmonary bypass was instituted via a 6-8Fr arterial DLP™ (DLP Inc, MI) and 18-22Fr venous DLP™ cannulae inserted via the purse-strings (figure 4.6). Flow rates would gradually be increased until full-flow was reached (100-150ml/kg/min), and the rate adjusted to achieve a mean arterial pressure of 50mmHg. The venous cannula would be adjusted to ensure maximal venous drainage and an empty right ventricle before the ventilators were switched off. Vacuum-assisted drainage was used to enhance venous return, by connecting the closed venous reservoir to a further pump head through a regulator set at -20mmHg.

After 10 minutes of CPB at 37°C with a mean arterial pressure of 50mmHg, a baseline set of arterial and sagittal blood gas samples were analysed for baseline oxygen extraction data. Thereafter, the piglet was exposed to the CPB strategy according to the experimental protocol.

Cooling was performed by circulating ice water through the heat-exchanger at a temperature no greater than 10°C different from the venous blood temperature, for a period of at least 20 minutes until the target temperature was reached in both probes. The oro-pharyngeal temperature typically cools significantly more rapidly than the rectal, and therefore the heater-cooler periodically would be adjusted to allow as equal cooling as possible. Blood gases were managed using a pH-stat strategy during cooling and α-stat strategy during re-warming; this is the technique employed by protocol in the clinical pediatric cardiac surgical department and theoretically aids in cerebral vasodilatation during cooling. Re-warming was undertaken by passing water heated to no greater than 10°C more than the venous inflow temperature through the heat-

exchanger. At 25°C, and before any bicarbonate addition, a blood gas would routinely be performed to allow comparison between the animals. Acidosis would be corrected by sodium bicarbonate addition to the pump. At 30°C, after lignocaine 0.1ml/kg and calcium gluconate 1ml/kg, the heart would be defibrillated as necessary with 2J applied via internal paddles. Once the core temperature had reached 35°C, after 3 sustained breaths to 30mmHg, the ventilator would be reconnected, the rate adjusted according to the blood gas data.

Once the core temperature had achieved 36°C, the animal was separated from CPB in conjunction with a continuous infusion of intravenous noradrenaline (.01-.1μg/kg/min). The animals invariably have the clinical features of a dynamic circulation with a low systemic vascular resistance, and the introduction of noradrenaline infusions (as opposed to periodic adrenaline boluses) resulted in significantly more successful and smooth weaning compared with earlier attempts while introducing the model.

4.4 Fluorescent Microspheres

Radioactive microspheres have been the gold standard for measuring regional organ blood flow, though the use of radioactivity is becoming increasingly problematic due to higher costs of storage and disposal, and the desire to reduce employee radiation exposure. Recently, fluorescently labeled microspheres have been demonstrated to be a reliable alternative for the determination of blood flow[187].

The microspheres are polystyrene 15.5μm diameter ±2% spheres labeled with up to ten fluorescent colours, five of which were used in this study: ^{198}gold, ^{175}ytterbium, ^{176}lutetium, ^{140}lanthanum and ^{153}samarium (Biopal™). The microspheres exceed the

diameter of capillaries (8μm), and therefore after injection into the systemic circulation, they become lodged in end-organ capillary beds during the first-pass. Quantification of regional organ blood flow is by comparison of the proportion of microspheres within a given tissue sample to a reference blood sample withdrawn at a controlled rate from the femoral artery. It is therefore crucial that during the injection, the microspheres are dispersed evenly throughout the circulation and the site of injection is therefore important. The ideal injection site is via a left atrial catheter, which ensures excellent atrial and ventricular mixing before ejection throughout the circulation. Alternatively, for experimentation with CPB, an injection through a side-port of the arterial cannula allows though mixing before the microspheres reach the aortic root.

In this series of experiments, following ultrasonic agitation to ensure complete suspension, 5 million (2ml) microspheres were injected either via a side-port of the arterial cannula or via a V5 polyethylene cannula in the left atrial appendage, depending on the experimental protocol. The micropsheres were injected over a period of 20 seconds and chased with 10 ml of arterial blood. A reference sample was withdrawn at a controlled rate (2-4ml/min) via the femoral artery, commencing 10 seconds prior to microsphere injection and continuing for 3 minutes, to ensure complete first-passage of the micropheres. At the end of the experiment, end-organ tissue sample were taken and weighed, before being dried, together with the reference sample, overnight at 70°C. The dried samples were then shipped externally for quantification of fluorescence (Biopal™ Inc).

$$Q_i = (Q_{ref} * Int_i/Int_{ref})$$

Where Q_i and Q_{ref} are the flow in the sample and reference withdrawal speed respectively, and Int_i and Int_{ref} are the fluorescent intensity in the sample *i* and reference

blood sample respectively. The calculated flow would then be normalised for sample tissue weight to be expressed as ml/min/100g.

4.5 Tissue processing

Preservation of tissue dates back to the early Egyptians, and the use of aldehyde fixatives does not seem to follow far behind in antiquity[188]. Since that time, DNA has been discovered and the entire human genome has been mapped. This new era of recombinant DNA technology, which requires the extraction of intact nucleic acid from tissues, has altered the profile of the ideal fixative. Prior to the 1970s, ideal attributes included preservation of enzymatic reactivities, maintenance of linkage between proteins, lipids and other cellular constituents, and of course, minimisation of process-related changes in morphology. Since the 1970s, however, such techniques need to include the preservation of nucleic acid, with minimal damage.

Fixatives can generally be classified into aldehyde and non-aldehyde fixatives. The standard aldehyde fixative is formaldehyde, as a 37% solution (formalin). Paraformaldehyde is a close relative with stable high molecular weight polymers. Aldehyde fixatives act by forming methylene cross-links between groups on proteins, including amino-, imino-, amido-, guanidyl-, hydroxyl-, carboxyl-, SH-, and aromatic groups. Many of these cross-links are apparently reversible on washing, although some formaldehyde will remain in irreversible linkage[189]. Glutaraldehyde is the strongest cross-linker followed by formaldehyde and paraformaldehyde. Although cross-linking is desirable for routine histology in providing excellent retention of morphology, cross-linking can destroy or mask antigens and obscure DNA targets by cross-linking histones. Therefore, for immunohistochemistry and either *in situ* nucleic acid labelling or extraction, minimal tissue cross-linking is ideal.

Non-aldehyde fixatives include alcohols such as ethanol and methanol, ketones (acetone), organic acids (citric acid) and composite fixatives (such as Streck tissue fixative) and tend to work by precipitating proteins. The mechanism behind this is to disrupt hydrogen bonds between amino acids, thereby altering their secondary structure. Antigenicity is usually well maintained. However, the lack of cross-linking can affect cellular morphology these are not, therefore, commonly used agents.

An alternative for the study of nucleic acid is to harvest tissue fresh. Nucleic acid, despite its chemical stability, becomes rapidly unstable after death due to the action of exo- and endonucleases. Therefore for the recovery of intact RNA, tissue must be extracted as rapidly as possible after death and immediately flash-frozen in liquid nitrogen (-196°C). In addition, to eliminate RNA contamination from the cellular constituents of blood, organs should ideally be flushed with saline prior to its sampling.

As brain tissue undergoes morphological changes extremely rapidly which precludes fresh harvest of cerebral tissue prior to both sampling for nucleic acid study and subsequent fixation for histology and IHC, two parallel strategies were adopted for processing tissue from each cerebral hemisphere. This involved the fresh harvest of tissue for nucleic acid analysis on one side followed immediately by perfusion fixation of the contra-lateral side. This is in contrast to small mammal (i.e mice, rats) experimentation, where cost allows one to have separate groups for each limb of tissue analysis protocol.

Prior to euthanasia, the chest was re-opened and clot removed to expose the aorta and right atrium. 5/0 purse-strings were then inserted into the aortic root immediately distal to the previous cannulation site in the aorta and circumferentially around the previous

right atrial purse-string. A modified circuit was used for the perfusion-fixation of the animal. An adult venous reservoir was connected in-line with the polyethylene venous lines and a pump line passing around a COBE™ pump-head to the arterial cannula. The perfusate was loaded into the top of the reservoir.

After systemic heparinisation (1000iu), the aorta was cannulated with an 8Fr DLP™ cannula and connected to the circuit. The right atrium was then cannulated and the 20Fr DLP™ connected to an exsanguinations line. The animal was then exsanguinated and perfused with 3 litres of normal saline at a mean arterial pressure of 40mmHg. Euthasol™ (Baxter Healthcare, Deerfield, IL) was simultaneously administered. A left craniotomy was immediately performed (figure 4.7) and the entire left cerebral hemisphere excised intact (figure 4.8). The hippocampus was dissected (figure 4.9) and snap-frozen in liquid nitrogen.

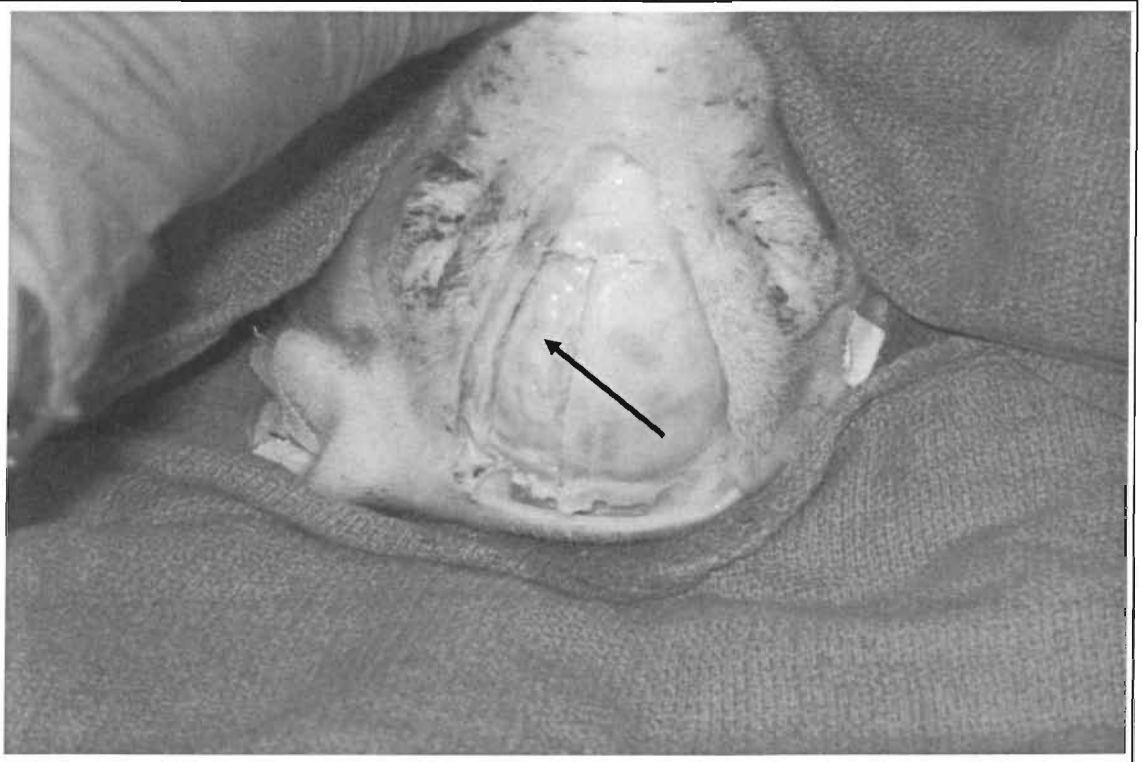


Figure 4.7 A left craniotomy is performed during the exsangunation process. A triangle of scalp has been raised to expose the cranium. Using an oscillating saw, a strip of cranium has been carefully resected leaving the meninges intact (arrow).

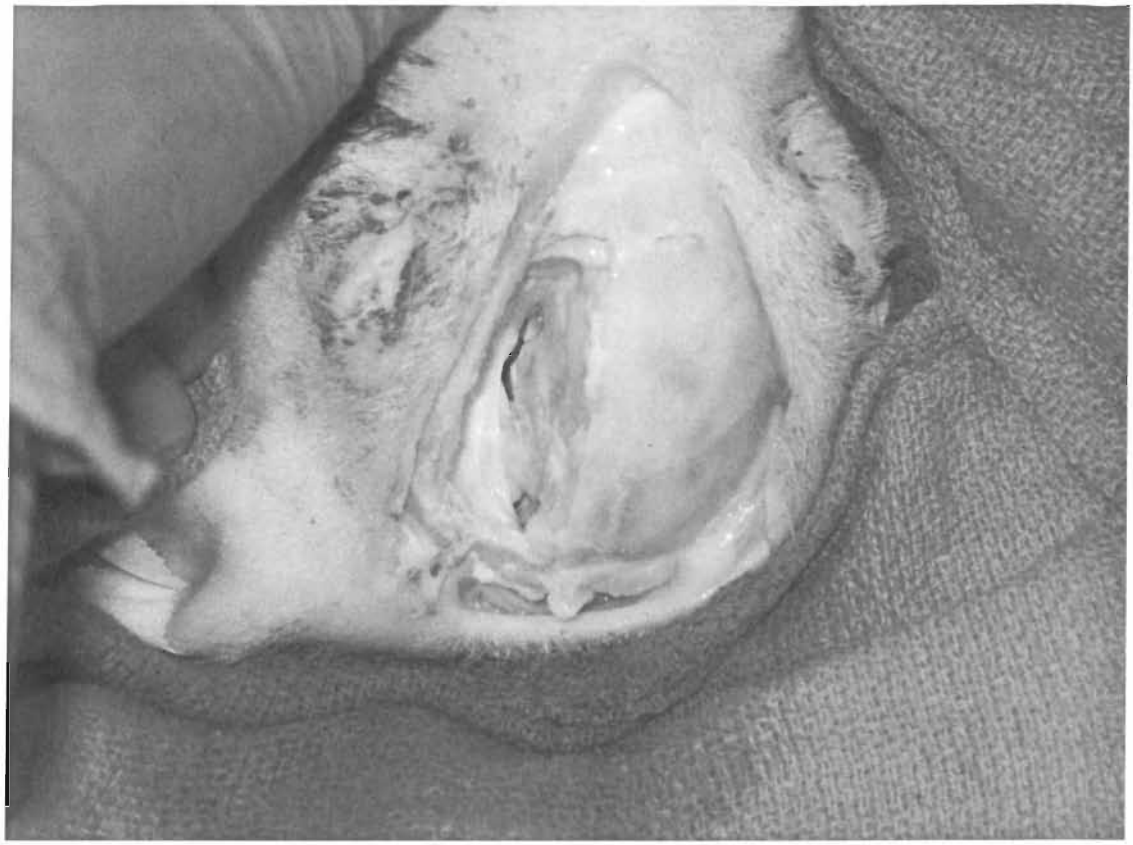


Figure 4.8 The meninges have been removed to expose the cortex. Through the craniotomy, a scalpel has been used to resect the entire left cerebral hemisphere intact. This was then snap-frozen before perfusion-fixation of the right hemisphere.

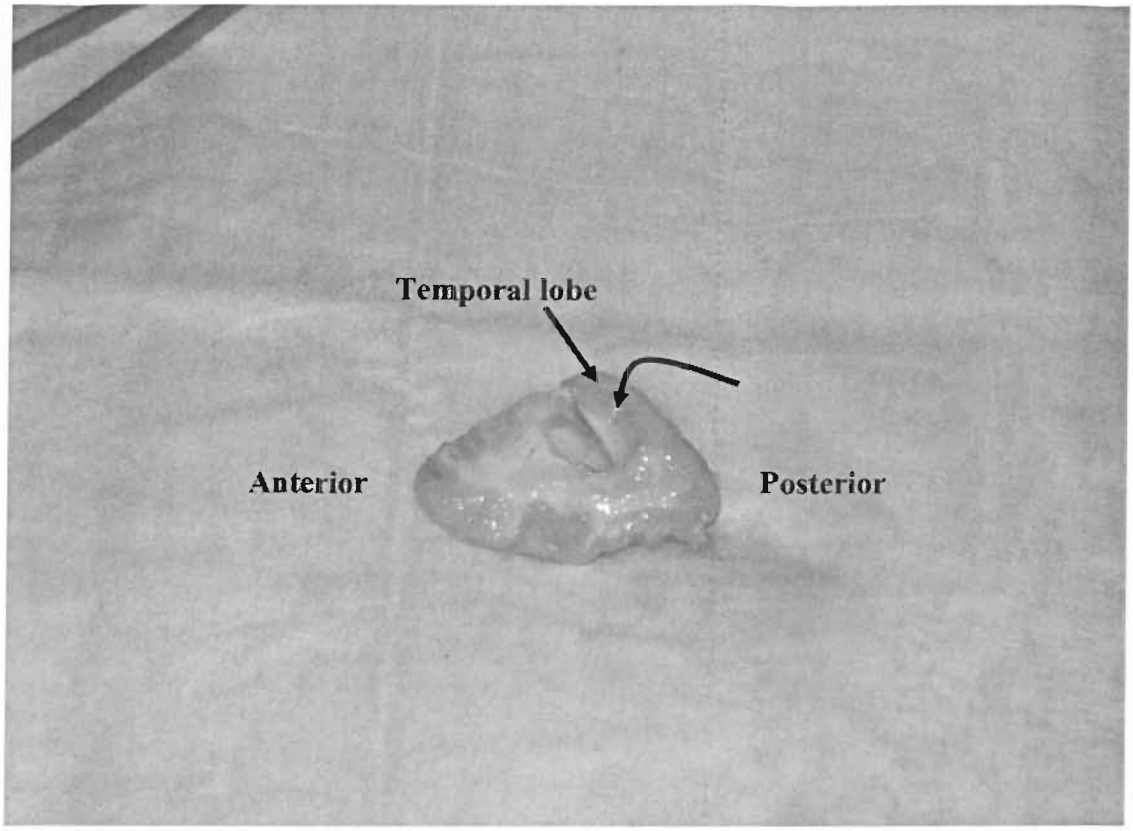


Figure 4.9 The left hemisphere resected. This figure shows the medial surface of the left cerebral hemisphere, from which the thalamus has been removed. The hippocampus is visible behind where the thalamus has been removed from (curved arrow).

The venous cannula was then connected to the circuit and 2 litres of ice-cold 4% paraformaldehyde re-circulated through the pump circuit at a mean arterial pressure of 50mmHg. Paraformaldehyde is toxic; therefore impermeable bags were sealed around the entire head during this fixing process to collect the perfusate draining via the hemi-craniotomy. After 20 minutes, the circuit was stopped and the brain extracted and immersed in 4% paraformaldehyde for 3 days before sectioning and paraffin embedding.

The piglet brain is illustrated in figure 4.10 and 4.11. After several preliminary sections and making reference to a swine atlas of neuroanatomy[182], it was decided to make four coronal sections through the following anatomical locations (figure 4.12):

1. 2mm anterior to the optic chiasm (frontal lobe (neocortex))
2. 3mm posterior to the optic chiasm (frontal lobe and caudate (basal ganglia))
3. through the mammillary bodies (hippocampus)
4. through the middle of the cerebellum

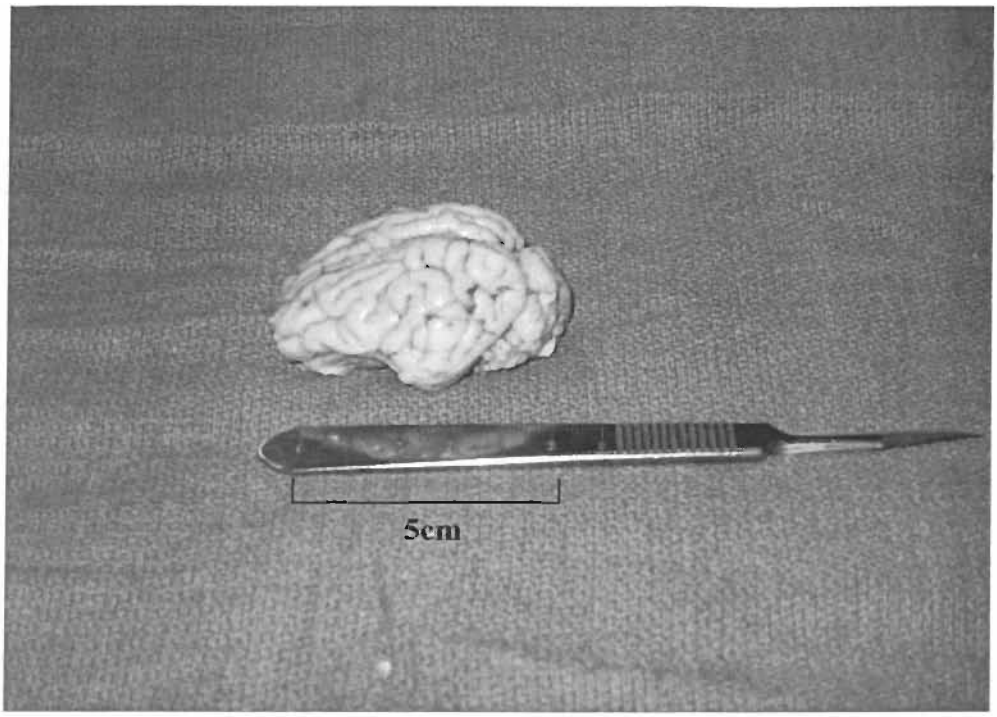


Figure 4.10 An excised piglet brain immediately after perfusion fixation.

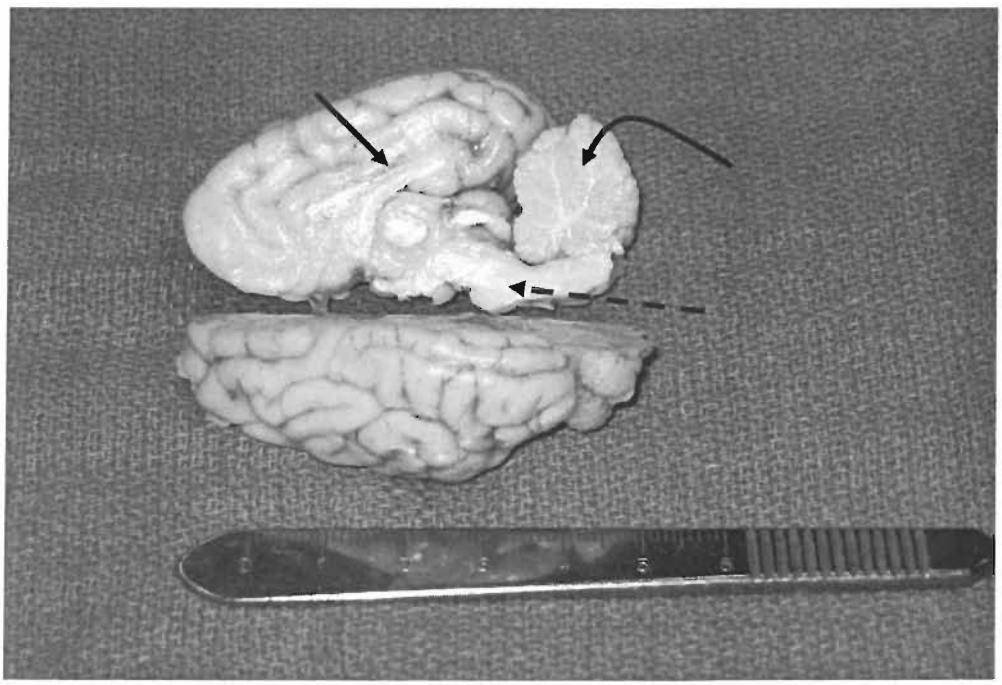
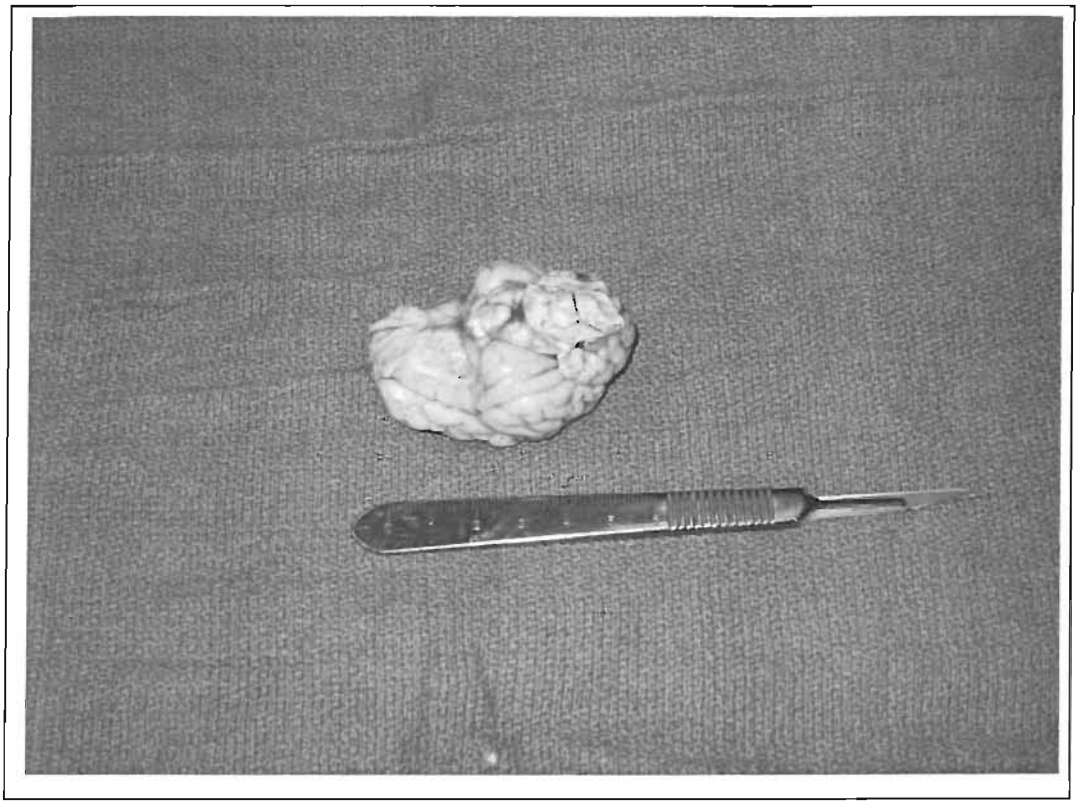


Figure 4.11 The brain sectioned in the mid-sagittal plane to demonstrate the midline structures including corpus callosum (arrow), cerebellum (curved arrow) and brainstem (dotted arrow).



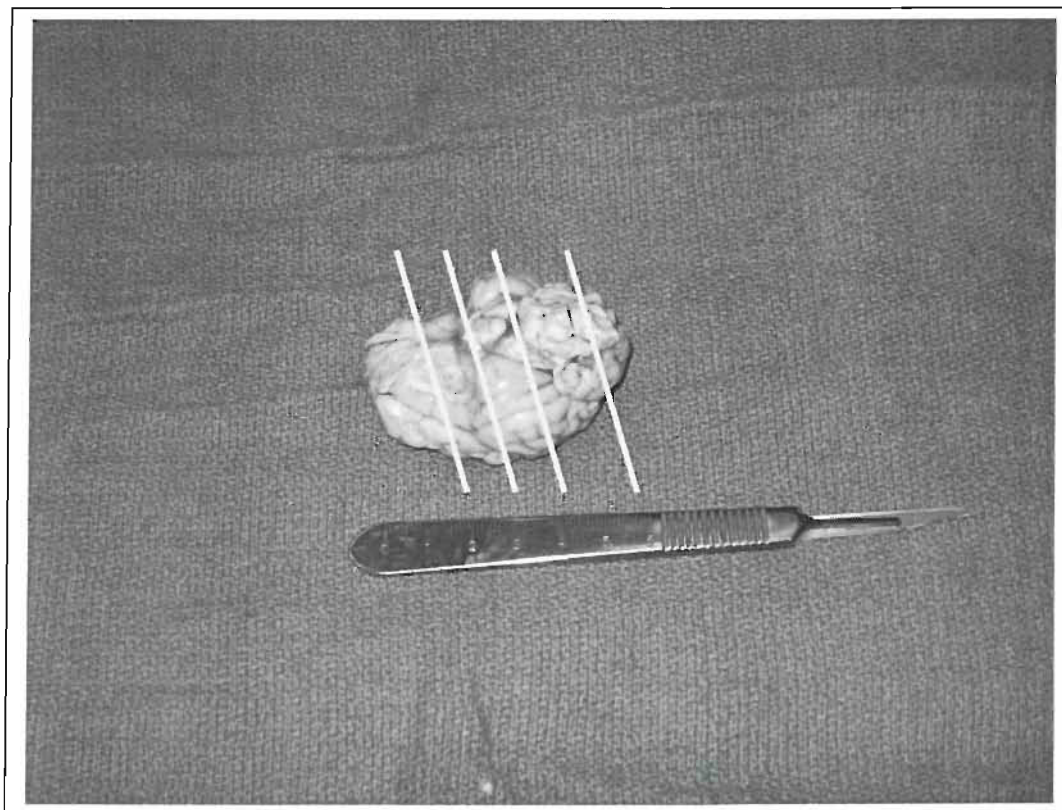


Figure 4.12 The four coronal sections for the histological samples.

The samples were loaded into cassettes and paraffin embedded. Blocks were then cut using a 5μm microtome to yield blank paraffin-embedded slides for subsequent staining. Routine haematoxylin and eosin staining was carried out on an automated stainer. Blocks and slides are illustrated in figures 4.13 and 4.14 to provide an indication of the anatomical regions represented.

Figure 4.13a Slice 1. Frontal lobe cortex

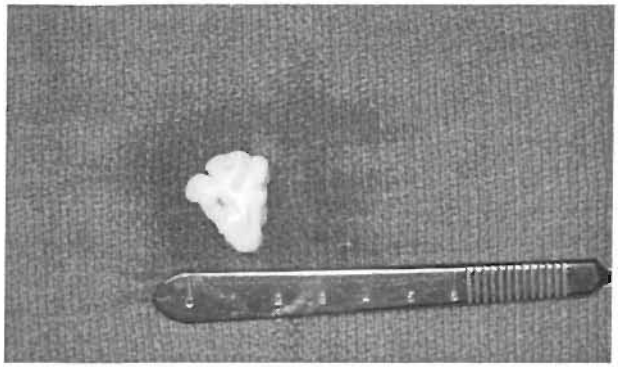


Figure 4.13b Slice 2. Frontal lobe and caudate (arrow).

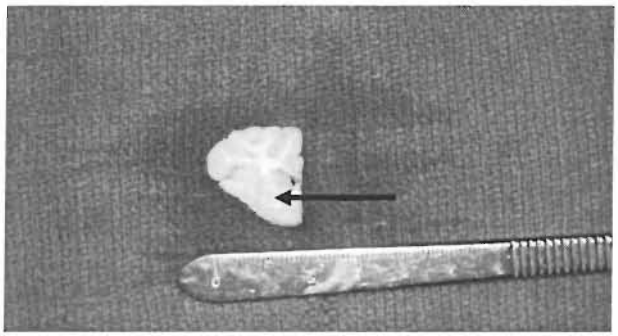
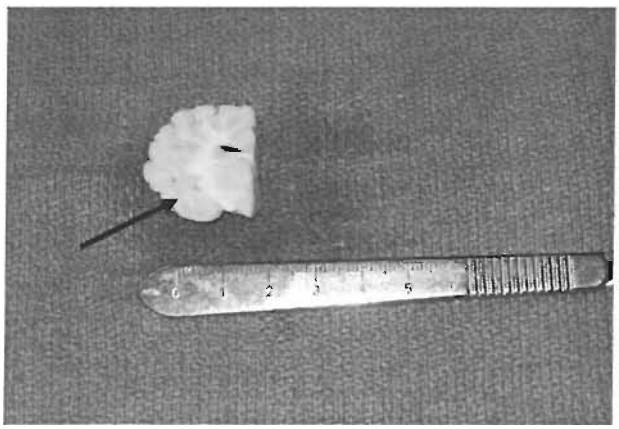


Figure 4.13c Slice 3 through the hippocampus (arrow)



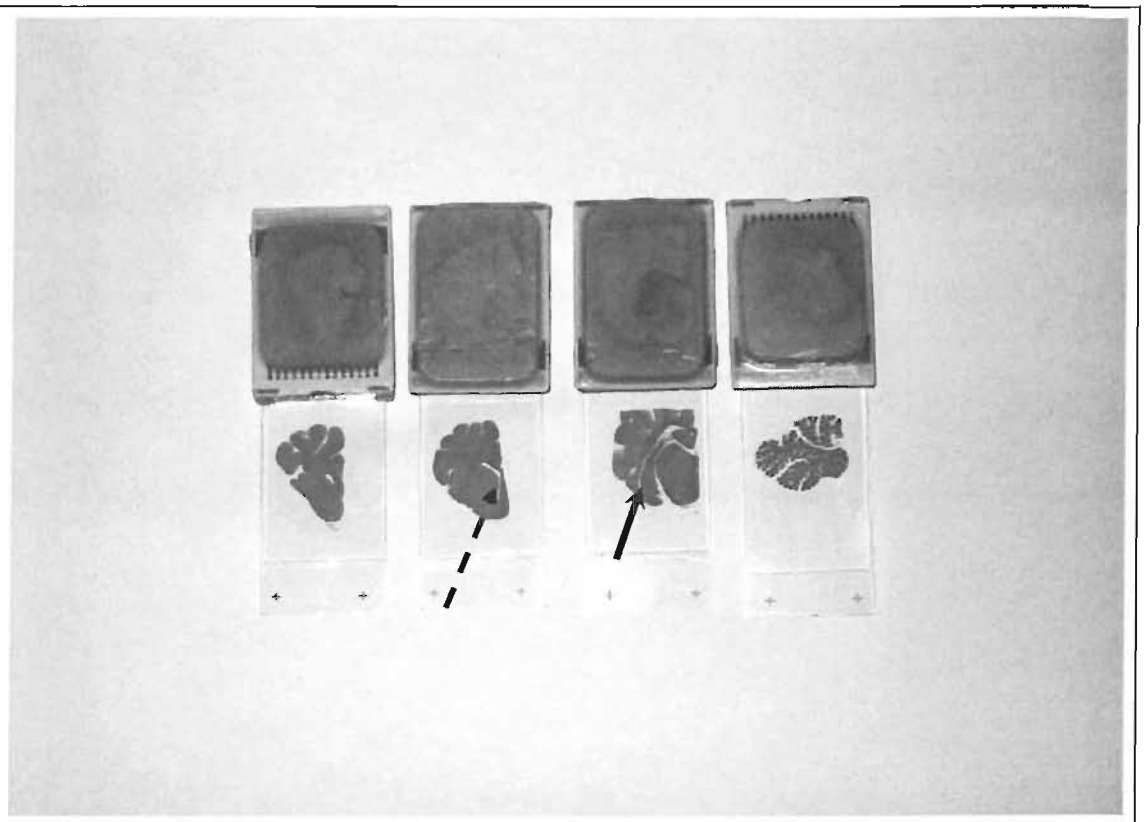


Figure 4.14 Tissue sections embedded in paraffin blocks, and the corresponding haematoxylin and eosin stained slides. The hippocampus is indicated by the arrow and the caudate by the dotted arrow.

4.6 Special Histological Stains

4.6.1 Fluoro-Jade™

Conventional techniques using routine staining and light microscopic examination for the determination of neuronal degeneration is not straight-forward. Morphological criteria such as neuronal shrinkage, vacuolation and hyperchromatism though associated with degeneration can also be artefact, resulting from tissue fixation and processing[1]. Therefore, unless a very high threshold is set for meeting the criteria for degeneration, routine light microscopy is prone to false positives, but also to miss a significant proportion of neurons that are actually injured[1]. More sensitive staining methods include suppressed silver techniques, in which normal neurons remain unstained and degenerating neurons stain black, but these are labour-intensive and capricious procedures.

Schmued and co-workers, in pursuit of a more ideal histochemical tracer, screened a variety of fluorescent anionic dyes under various conditions and identified Fluoro-Jade™ as a compound capable of selectively staining degenerating neurons in brain sections with greater sensitivity and specificity than haematoxylin and eosin or Nissl-type stains[190]. Fluoro-Jade™ is an anionic fluorochrome that has a green iridescence with excitation peak at 480nm and an emission peak at 525nm. The filter used for visualization is a fluorescein/FITC filter. The mechanism by which degenerating neurons are stained is not known. However, it is likely that injured neurons exhibit strongly basic properties, rendering their affinity for the strongly acidic Fluoro-Jade™ (and aversion for the basic nissl dyes sometimes used). It has also been inferred[190] that the cytoplasm need not be disrupted, as membrane permeabilising agents do not affect its staining. Perhaps the principal advantages of its use are that staining is quick and simple, and makes interpretation straight-forward, for those untrained in

neuropathology. Despite its discovery in this context over eight years ago, remarkably, Fluoro-Jade™ has been little adopted: only six original articles describe its use in a medline review of the literature. Interestingly, though use has only been described in staining degenerating neurons, it has recently been noted to also stain ischaemic renal tubular cells in the context of acute tubular necrosis[191].

Following de-paraffinisation, slides were immersed for 20 minutes in 0.06% potassium permanganate before washing several times in deionised water. Fluoro-Jade™ crystals were made up to a 0.0004% solution in 0.01% acetic acid and the slides immersed for 20 minutes before thoroughly air-drying in a warm incubator. After further washes in xylene, slides were cover-slipped and viewed using an epifluorescent microscope with blue (450-490nm) excitation light. A barrier filter allowing passage of all wavelengths longer than 515nm results in a green emission colour from cells stained positively with Fluoro-jade™.

4.6.2 Immunohistochemistry for irreversible cell injury

4.6.2a Background

Apoptosis is a regulated physiological process leading to cell death characterised by cell shrinkage, membrane blebbing and DNA fragmentation, before phagocytic removal of the debris. Although much has been made of the differences between this and the necrotic pathway (an unphysiological response to injury), many intracellular processes are common to both (including DNA fragmentation), and under routine light microscopy, despite earlier assertions to the contrary, most investigators question the reliability in making a distinction.

TUNEL

DNA fragmentation appears to proceed in a regulated fashion. In several well-researched models, it has been demonstrated that large fragments between 50kb and 300kb are initially produced by endonucleolytic degradation of higher-order chromatin organisation, which can be seen on pulsed-field gel electrophoresis[192, 193]. Activation of Ca^{2+}- and Mg^{2+}-dependent endonucleases then shortens the fragments further by cleaving the DNA at linker sites between nucleosomes[194]. The ultimate DNA fragments are multimers of approximately 180kb nucleosomal units, which appear as the familiar "DNA ladder" on standard agarose gel electrophoresis.

The fragmentation of DNA has been hijacked as a useful route by which apoptosis can be measured *in situ*. By using the TUNEL assay[195], double stranded DNA breaks are detected by enzymatically labelling the 3'-OH termini with modified nucleotides. Labelling in this fashion correlates well with other techniques for identifying apoptotic cells, whereas by contrast, normal or proliferative nuclei express relatively insignificant numbers of blind 3'-OH ends, and do not stain in such assays. DNA damage and fragmentation can, of course, result from other stimuli, and demonstration of fragmented DNA is not therefore specific for apoptosis.

The principle behind TUNEL involves the enzymatic (terminal deoxynucleotidyl transferase, TdT) template-independent addition of digoxigenin-labeled and unlabeled nucleotides on the 3'-OH end of DNA at the double-stranded breaks between nucleosomes. The incorporated nucleotides therefore form an oligomer (of undetermined length) of digoxigenin-conjugated nucleotide and unlabeled nucleotides in a random sequence. This proportion of labeled to unlabeled nucleotides has been optimised to provide the maximal number of binding sites for the digoxigenin antibody.

This anti-digoxigenin antibody is conjugated to a peroxidase reporter molecule which generates intense chromagen staining when the reaction substrates are applied.

Caspase-3.

The caspases are a family of cysteine proteases which are centrally involved in the regulation of apoptosis. Figure 4.15 illustrates the apoptotic pathway, and indicates the central involvement of caspase-3. Initiator caspases (including caspase-2, -8, -9, -10, -11 and -12) are coupled to pro-apoptotic signals. Once activated, these caspases cleave and activate downstream effector caspases (including caspase-3, -6 and -7). These in turn execute apoptosis by cleaving proteins following specific aspartate (Asp) residues. Activation of Fas and TNFR by FasL and TNFα, respectively, leads to the activation of caspase-8 and -10, whereas DNA damage leads to the activation of caspase-2. Cytochrome c released from damaged mitochondria is couple to the activation of caspase-9, a caspase critical for intracellular amplification of apoptotic signals. Caspase-11 is induced and activated by pathological proinflammatory and pro-apoptotic stimuli and leads to the activation of caspase-1 to promote inflammatory response and apoptosis by directly processing caspase-3. Caspase-12 is specifically activated under endoplasmic reticulum stress conditions.

Caspase-3 is either partially or totally responsible for the proteolytic cleavage of key proteins such as poly (ADP-ribose) polymerase (PARP), which is a critical step in the final execution of cell death. Caspase-3 is itself activated through proteolytic processing: its inactive zymogen is cleaved into activated P17 and P12 fragments, adjacent to an aspartic acid residue (Asp). Caspase-3 immunohistochemistry detects endogenous levels of large fragment (P17) of activated caspase-3 adjacent to Asp175. The primary antibody does not recognise uncleaved caspase-3 or the other caspases (or fragments thereof). Although the majority of immunohistochemical targets are

cytoplasmic in their staining, caspase-3 staining can be with cytoplasmic or nuclear – which may be related to timing. Although caspase-3 is typically cytoplasmic in nature, it cleaves PARP, which is nuclear in location, and therefore must necessarily be nuclear at some point in its activation.

Although the precise mechanisms involved in cell death was of lesser significance to these studies, the use of these immunohistochemical stains was an attempt to simplify identification of irreversibly damaged cells. DNA fragmentation as identified by TUNEL is a late event in the demise of the cell. Caspase-3 activation precedes DNA fragmentation and the identification of cleaved Caspase-3 might be a more sensitive tool for identifying cells subsequently destined to perish. Importantly, the mechanisms involved in neuronal death were *not* the subject of study, and therefore distinction between apoptosis and necrosis was not important for the purposes of these investigations.

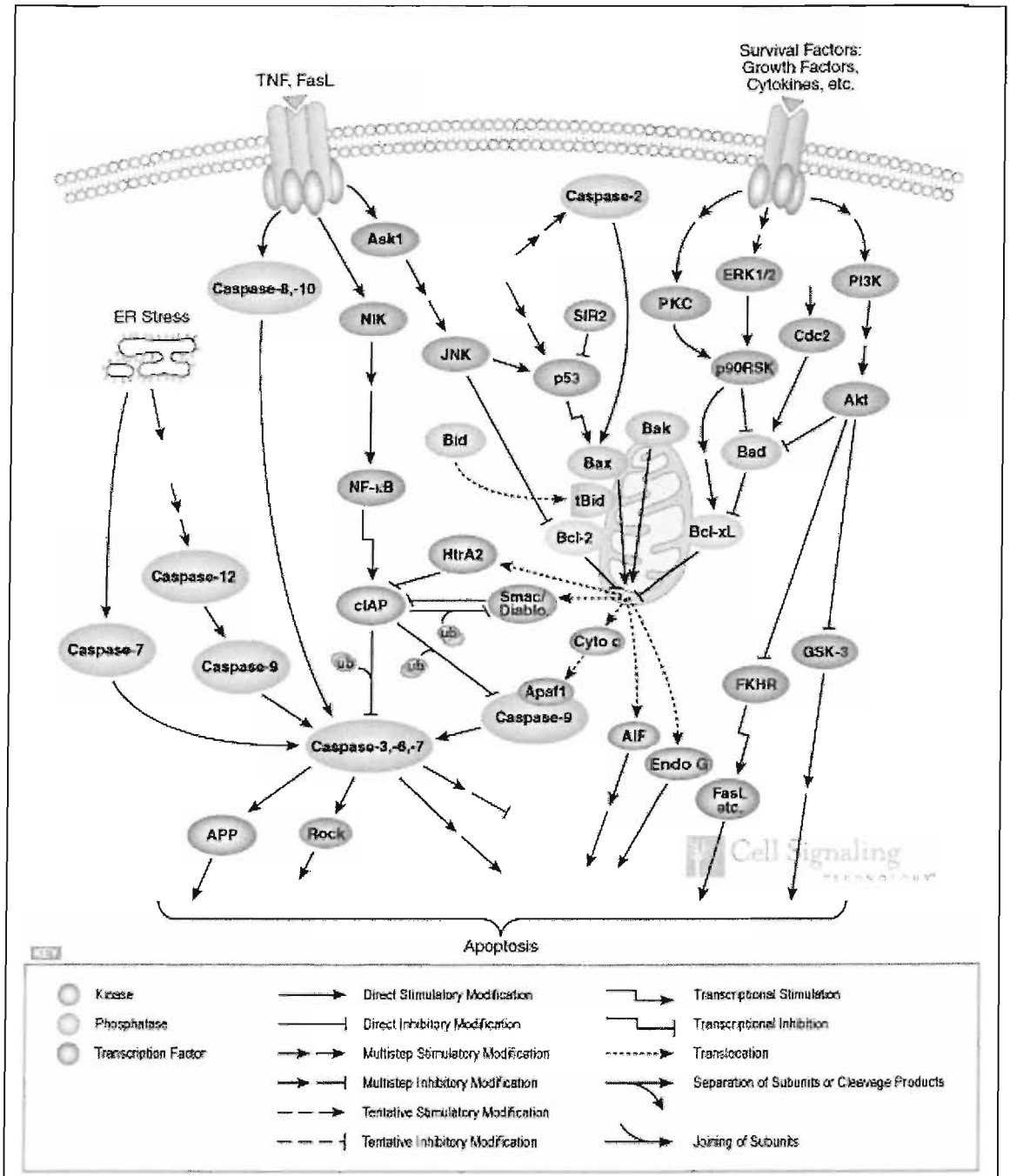


Figure 4.15 Diagram of Caspase family of proteins. Caspase-3 is an executioner caspase, that coordinates the death response. (Taken from Cell Signaling Technology, USA).

4.6.2b TUNEL Methods

In situ labelling by TUNEL was performed using kits from either Chemicon International (USA) or Sigma (MO). Following sample de-paraffinisation, antigen retrieval was undertaken either by the recommended proteinase-K protein digestion enzyme (20μg/ml) for 15 minutes at room temperature or by steaming for 10 minutes. Background peroxidase activity was then quenched in a 3.0% hydrogen peroxide solution before washing and application of an equilibration buffer. The TdT enzyme was then applied for an incubation period of 1 hour at 37°C. This reaction was stopped and the anti-digoxigenin conjugate applied to the slides for an incubation period of 30 minutes at room temperature. The peroxidase substrate was then applied to allow staining for a 3-6 minute period before washing and counterstaining with either methyl green or eosin and coverslipping. For each immunostaining procedure, controls included human tonsil positive control, a negative control lacking primary antibody and an experimental negative control (i.e. tissue not exposed to an ischaemic period).

4.6.2c Caspase-3 Methods

In situ staining for cleaved Caspase-3 (Asp175) was performed using the ABC immunoperoxidase method (Cell Signaling Technology, USA). Briefly, following deparaffinisation and antigen retrieval by steaming in sodium citrate buffer (pH 6.0) for 10 minutes, slides were allowed to cool on the bench. Peroxidase quench was applied to neutralise background peroxidase activity prior to incubation with a blocking solution to reduce background staining. Incubation with primary antibody was carried out overnight at 4°C in a humidified chamber to prevent the sections from drying out. On a negative control slide, the primary antibody was substituted by buffer lacking the antibody. A secondary antibody was subsequently added for 30 minutes at 25°C, and after several washes in PBS, the AB reagents were added. Following addition of the chromagen-substrate, the reaction was allowed to proceed for 10-15 minutes or until a

brick-red colour emerged. At this point the reaction was terminated with deionised water and the slides counter-stained with haematoxylin before cover-slipping. For each immunostaining procedure, controls included human tonsil positive control, a negative control lacking the primary antibody and an experimental negative control (i.e tissue not exposed to an ischaemic period).

4.7 Nucleic Analysis

Whole tissue RNA was extracted from hippocampal tissue samples using a RNeasy® Lipid Tissue Mini Kit (Qiagen Inc, Valencia, CA), which is designed for optimal lysis of tissues rich in fat, such as brain and adipose tissues. Briefly, a 100mg (frozen) sample of tissue was lysed with 1ml QIAzol™ lysis reagent and immediately rotor-stator homogenised at 7000rpm before centrifuging with 200µl chloroform for phase-separation. The lysis reagent is a monophasic solution of phenol and guanidine thiocyanate, designed to facilitate lysis of fatty tissues and inhibit RNases. The clear aqueous phase containing nucleic acid was removed, an equal volume of 70% ethanol added to provide appropriate binding conditions and the sample spun through an RNeasy™ Mini spin column twice. The spin column is a silica-gel-membrane to which the RNA binds, separating it from the effluent. After washing the column with buffers, the RNA was eluted by spinning with RNeasy™ RNase-free water into 30µl aliquots.

Once eluted, viability of whole RNA was verified by PAGE in an agarose gel, to ensure clear bands of 18S and 28S ribosomal RNA. Automated spectrophotometry was then performed to quantify concentration and purification of RNA prior to either light-cycler quantitative RT-PCR or microarray genome survey by external laboratories.

4.8 Cytokine Analysis

Blood samples were centrifuged at 3000rpm for 10 minutes at 4°C and the serum removed and stored at -20°C. Quantification of serum TNFα was undertaken by enzyme-linked immunosorbent assay (ELISA) (Sigma Aldrich, St Louis, MO) by an external laboratory.

4.9 Statistical Analysis

All data were entered into datasets and analysed using SAS® statistical software (Cary, NC). Data are expressed as mean ± standard error of the mean (SEM). Data was assumed to be normally distributed. Comparison between two animal experimental groups was undertaken using paired students *t* tests. Comparison between more than two animal experimental groups was undertaken using analysis of variance (ANOVA). Significance was considered $p<0.05$.

Chapter 5

Use of a Miniaturised Circuit to avoid Cerebral No-Reflow

5.1 Abstract

Objectives: We have successfully developed a miniaturised bloodless prime circuit for neonatal cardiopulmonary bypass (CPB), which has previously been shown to elicit significantly reduced systemic inflammation. We studied the effects of this circuit on cerebral re-perfusion, because the pathophysiology of "no-reflow" is believed to have an inflammatory component

Methods: Neonatal piglets were randomised to CPB with miniaturised circuitry using either blood (group 1) or bloodless (group 2) prime. At 18°C, piglets were subjected to 60 minutes of either: A, circulatory arrest (DHCA) or B, continuous low-flow bypass (DHCLF). Animals were then rewarmed and separated from CPB. Analysis of cerebral blood flow (CBF) was undertaken pre- and post-CPB. In addition, quantification of circulating TNFα and intracerebral TNFα mRNA was performed.

Results: All haemodynamics including cardiac output were similar. The final haematocrit in group 2 was 22% (vs 28%, $p<0.05$). The CBF fell in every animal in group 1A, but increased in every animal in group 2A. The use of DHCLF was not associated with pronounced trends in either prime group. Final serum TNFα concentrations were significantly higher in group 1B (3166±843pg/ml) than group 2B (439±192pg/ml), $p<0.05$. Irrespective of CPB strategy employed, the use of a blood prime generated significantly higher levels of intra-cerebral TNF mRNA.

Conclusions: This is the first report describing a hyperaemic response in the early stages following neonatal DHCA, which we attribute to avoiding allogeneic whole blood. DHCLF has been introduced to avoid cerebral ischaemia, but is associated with more inflammation. The analysis of circulating and intra-cerebral TNFα in this study suggests that DHCLF in conjunction with a bloodless prime might offer advantages through avoiding ischaemia, no-reflow and the detrimental inflammatory response.

5.2 Introduction

The onset of cardiopulmonary bypass (CPB) is associated with generalised activation of the innate immune system[31], and this phenomenon is exaggerated at the extremes of age[32]. No facet of the inflammatory response is spared: neutrophils become primed, complement and kallikrein-kinin systems are activated, inflammatory (and anti-inflammatory) cytokines are generated and activated platelets litter the surface of the extra-corporeal circuit resulting in coagulation disturbances and thrombocytopenia[31, 33-35]. These processes have a direct role in commonly encountered post-operative complications including capillary leak syndrome, acute lung injury, systemic inflammatory response syndrome, coagulopathies, and multi-organ failure[31, 36]. In addition, evidence is accumulating to suggest that inflammation directly exacerbates the progression of injury following periods of cerebral ischaemia. In particular, the apical cytokines (TNFα, IL-1β) and products of neutrophil degranulation (activated oxygen species) are recognised to be central to the progression of neuronal loss following periods of cerebral ischaemia in virtually every model studied[37-39]. Avenues for harnessing the CPB inflammatory response are therefore of considerable interest. One potential target unique to the neonate is the enormously disproportionate extra-corporeal volume with respect to patient size. Attempts to bring this ratio more in-line with that of older children and adults (through circuit miniaturisation) are fraught with technical and practical difficulty, but early experimental models suggest that these efforts will improve patient recovery. Although the benefits of miniaturisation may, at least in part, be due to the smaller circuit surface area, avoiding the use of allogeneic blood appears to be a major mechanism in reducing CPB inflammation.

Conventional neonatal cardiopulmonary bypass (CPB) requires the use of a blood prime in order to prevent unacceptable haemodilution. Severe haemodilution is not only a problem for red cell-dependent gas transport, but also for platelet and humoral factor-

dependent coagulation and protein-dependent intravascular oncotic pressure[196, 197].

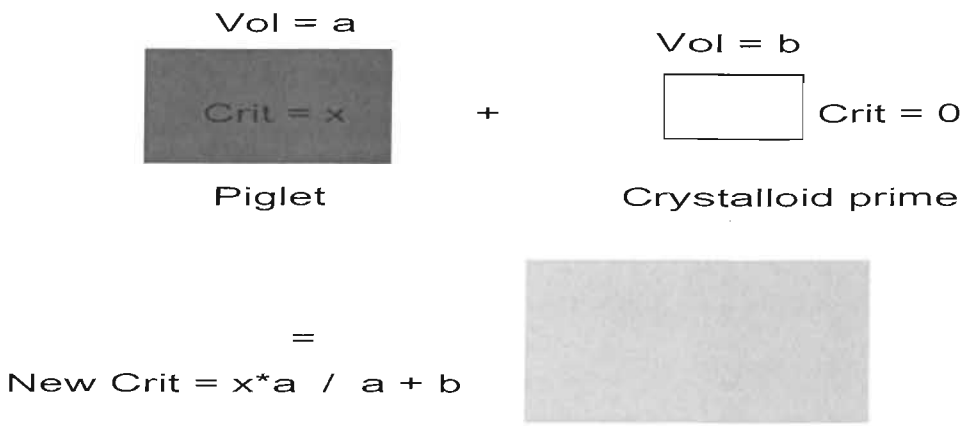


Figure 5.1 The haemodiluting effects of using a bloodless prime. In order to prevent haemodilution below a predetermined level the only variable that can be controlled is the circuit size (vol b) through miniaturisation.

Until only recently, blood transfusions have been used liberally with a seemingly risk-free attitude since their first description in the mid-17th century[198]. Aside from the potential for transmissible infection, allogeneic blood is now recognised to be a trigger for both immunosuppression and systemic inflammation[199, 200]. Among the described immunomodulatory consequences are decreased CD4+ and IL-2R+ve Helper-T cells, increased CD8+ Suppressor-T cells, increased circulating B cells, decreased IL-2 production and elevated complement, eicosanoids and inflammatory cytokines[200, 201]. A more pragmatic approach is therefore advocated in the administration of blood products in many clinical applications, including trauma and surgical resuscitation, due to accumulating evidence indicating deleterious inflammatory consequences and clinical outcome[201]. More recently the use of blood in both adult and infant CPB has been scrutinised, and early indications have confirmed that avoiding its use is associated with improved recovery [202, 203].

5.3 The development of Miniaturised Circuits

Accumulating evidence surrounding the deleterious effects of blood use has prompted the investigation of circuit miniaturisation for infant CPB. A small feasibility study by Lau et al. in neonatal piglets showed that a low-volume, asanguineous prime can be achieved safely, and that total blood requirements were significantly reduced from 314 ± 32 mL with use of a conventional neonatal circuit to 47 ± 6 mL with use of the miniaturised circuit[204]. Previously, Wabeke et al. had demonstrated that a smaller prime (90 vs 330 ml) and the use of vacuum-assisted venous drainage in a rabbit model normalised resistance in the peripheral microcirculation[205]. Based on the initial promising data obtained in animals, serious efforts to reduce circuit size and priming volumes began in the conduct of neonatal CPB. Jansen et al. found that colloid osmotic pressure was maintained in infants primed with a low-volume circuit (245 ml) compared to infants receiving a higher volume prime (364 ml) [206]. This translated into improved postoperative fluid balance and superior cardiopulmonary function in these infants post-operatively. More recently, Fukumura and colleagues used a low-volume prime in conjunction with dilutional ultrafiltration in 19 neonates with transposition of the great arteries undergoing arterial switch operation[207]. The miniaturised circuit improved systolic blood pressure and reduced both postoperative water gain and duration of ventilatory support. Reduction in inflammatory mediators was similarly shown by Fromes et al. who incorporated biocompatible components into a minimal extracorporeal circuit in adult patients[208].

Infant CPB commonly involves the use of deep hypothermic CPB strategies. In particular, deep hypothermic circulatory arrest (DHCA) has been frequently used for the repair of complex congenital defects due to its simplicity and bloodless operating field. However periods of ischaemia during DHCA have been implicated in neurological injury that occurs clinically following its use[107, 121, 209]. A consistent feature of DHCA in experimental models is the abnormal recovery of cerebral blood flow (CBF)

following the ischaemic period, a phenomenon termed "no-reflow" [120], and furthermore methods that alleviate no-reflow are associated with improved neurological recovery[118, 119]. Cerebral no-reflow is believed to have both mechanical and physiological components[143]. Inflammatory debris, including leukocytes, platelets, endothelial casts and fibrin may occlude the capillary bed[210], and an imbalance in vasoactive mediators (including endothelin-1, nitric oxide and eicosanoids) may contribute to dysfunction of cerebral autoregulation[211, 212]. In order to reduce unwanted complications of cerebral ischaemia, including no-reflow, several novel strategies have been introduced, including deep hypothermic continuous low flow (DHCLF) [169], although not necessarily improving outcome[114, 213]. The consequences for cerebral reperfusion following DHCLF are less well defined, but cerebrovascular abnormalities have been described to a lesser degree[214, 215].

I aimed to examine the benefits a bloodless prime circuit may confer on cerebral recovery following deep hypothermic CPB. Because the pathophysiology of cerebral no-reflow may have an inflammatory component, and because experimental models of neonatal CPB using a bloodless prime has been associated with a reduced systemic inflammatory load, I hypothesised that our bloodless prime circuit may have a beneficial impact on the cerebral no-reflow phenomenon. I therefore placed animals on CPB using a further miniaturised circuit primed with either whole blood or crystalloid. After exposing them to a strategy of either DHCA or DHCLF, I examined cerebral perfusion using the fluorescent microsphere technique[187]. In addition I examined both the systemic and local cerebral inflammatory response through the quantification of circulating TNFα or its intra-cerebral mRNA.

5.4 Materials and Methods

5.4.1 Animal Groups and Surgical Procedures

Animal Groups and Surgical Procedures

Twenty neonatal piglets (2-5kg) were randomised to two groups according to the composition of the CPB prime. Group 1 (n=10) were exposed to a miniaturised circuit primed with fresh whole blood harvested from an adult donor pig on the day of surgery; group 2 (n=10) were exposed to a miniaturised circuit with a modified asanguinous prime. During CPB, animals within each group were randomly subdivided further to a strategy of either DHCA (A) or DHCLF (B) (figure 5.2, overleaf).

Adolescent donor swine (40-50kg) were used to supply blood for the extracorporeal circuit in group 1. Following anaesthetic induction with inhaled isofluorane (1.5%) blood was harvested sterilely through cannulation of the carotid artery and stored at -4°C in citrate-phosphate-dextrose solution until use (CPB Blood Pack Unit, Baxter Healthcare, Deerfield, IL) later the same day.

The piglets were premedicated with Telozole™ (8mg/kg) (Baxter Healthcare, Deerfield, IL) and anaesthesia induced with inhaled 1.5% isofluorane. Peripheral venous access was obtained via the marginal vein of the pinna and used to administer a bolus of fentanyl citrate (10μg/kg). After intubation through a surgical tracheostomy, anaesthesia was maintained with isofluorane (0.5%-1.0%) and a fentanyl citrate infusion (25μg/hr). An infant pressure cycled ventilator (Sechrist Industries, Anaheim, CA) was used for ventilation, with peak inspiratory pressures of 25mmHg, and positive end-expiratory pressures of 3mmHg. Arterial PaO_2 of 200 to 250 mmHg was achieved by adjusting the partial pressure of oxygen in either the ventilator or CPB oxygenator. An arterial

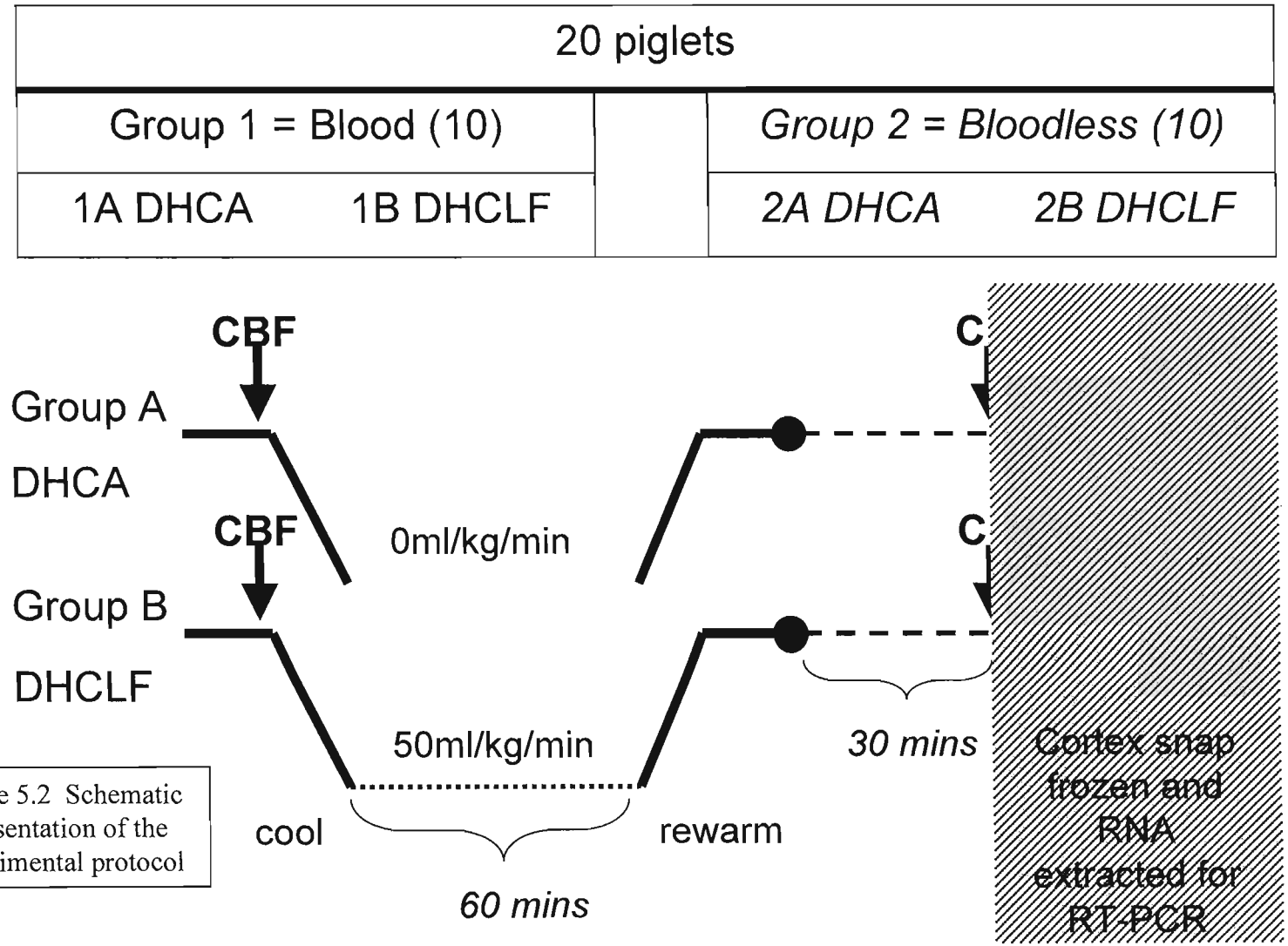


Figure 5.2 Schematic representation of the experimental protocol

$PaCO_2$ of 35 to 45 mmHg was maintained by adjusting the minute ventilation or the rate of sweep gas of the CPB circuit. Rectal and oesophageal temperature probes were placed to ensure accurate measurements of core temperatures during cooling and rewarming intervals. Catheters (V-8; Bolab, Lake Havasu, AZ) were inserted into the right femoral artery and vein for blood gas monitoring and measurements of mean aortic and central venous pressure respectively.

The heart was exposed through a median sternotomy. A pulmonary artery ultrasonic flow probe (Transonic Systems, Ithaca, NY) was placed around the main pulmonary artery for measurement of cardiac output. A V-5 catheter (Bolab, Lake Havasu, AZ) was inserted into the left atrial appendage for post-CPB injection of fluorescent microspheres. Systemic heparinisation was given intravenously to maintain the kaolin activated clotting time greater than 480 seconds. After instrumentation and baseline data acquisition, the aortic root (8Fr) and right atrial appendage (22Fr) were cannulated through purse-string sutures (DLP Inc, Grand Rapids, MI). Normothermic CPB was established at a rate of 100-150ml/kg/min to maintain mean arterial pressures of 40-50 mmHg. The animal was then perfusion-cooled using a pH-stat strategy to 18°C over 20 minutes. During cooling and rewarming a temperature difference of no greater than 10°C was permitted between the core temperature and the perfusate. The heart was then packed in ice. At the end of the cooling period, the animals were randomised to their CPB strategy. In groups 1A and 2A (DHCA), CPB was ceased and the circulatory volume drained into the reservoir and the arterial line clamped for 60 minutes. In groups 1B and 2B (DHCLF), the pump flow was reduced to 50 ml/kg/min for the 60 minute period. At the conclusion of this interval, full-flow CPB was reinstituted and rewarming initiated using an alpha-stat strategy with the use of sodium bicarbonate (8.4%) as necessary. The animal was rewarmed over a minimum duration of 30 minutes, ensuring a mean arterial pressure of 50 mmHg, and weaned from CPB. Thirty

minutes following discontinuation of CPB, the animal was euthanised with Beuthanasia-D (1ml/10kg) (Baxter Healthcare, Deerfield, IL).

5.4.2 Miniaturised Circuit

The miniaturised extracorporeal circuit includes the following: a Cobe™ Century (Cobe Cardiovascular Inc, Arvada, CO) roller pump console; an infant oxygenator-reservoir (Capiox®-Baby RX-05, Terumo, Japan); Cobe™ tubing packs to include a 3/16" internal diameter polyvinyl chloride (PVC) arterial line (60cm) and a 1/4" PVC venous line (30cm). The pump boot was a 3/16" line (raceway 43cm). Venous return was vacuum assisted (-20mmHg). A left ventricular vent was routinely inserted through an apical purse-string for decompression of the ventricular cavity. The prime constituents for the experimental groups are listed in figure 5.3. Cardiopulmonary bypass was instituted as follows: the oxygenator, pump line and arterial line were

Prime Constituents:	Albumin 32ml Bicarbonate 12ml Crystalloid 12ml Heparin 2ml Fentanyl 2ml **Total: 60 ml**
Circuit Priming Volume:	Oxygenator (O) 43ml Arterial Pump (P) 6ml Arterial Line (A) 10ml

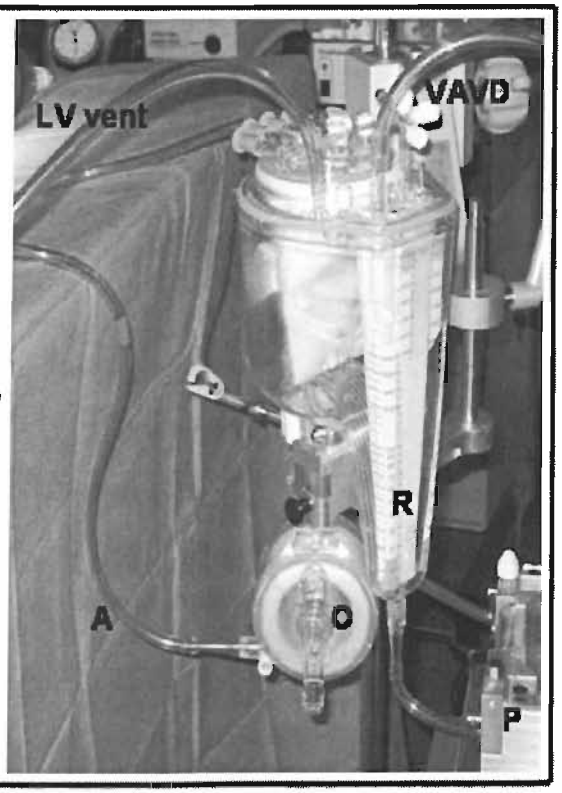


Figure 5.3 Miniaturised circuit and prime constituents. R, reservoir; VAVD, vacuum-assisted venous drainage line; O, oxygenator; A, arterial line.

primed antegrade, leaving the reservoir empty. The aorta was then cannulated and the arterial line de-aired. The right atrium was then cannulated, venous blood drained into the reservoir and CPB gradually instituted immediately thereafter.

5.4.3 Data Acquisition and Sample Preparation

Data for pulmonary and cardiac function were obtained at baseline prior to CPB and again 30 minutes after the discontinuation of CPB using a Gould TA-6000 chart recorder (Gould, Valley View, OH) and Powerlab 4.2 software (AD instruments, Castle Hill, Australia). All animals were allowed to stabilise for 10 minutes prior to each data acquisition point. The measurements included arterial blood gas (Instrumentation Laboratories Synthesis 10, Lexington, MA), arterial blood pressure, and pulmonary artery flow. Pressure and flow data were sampled for 10 seconds and digitised at 500Hz and stored as a computer file for later analysis.

5.4.4 Analysis of Cerebral Blood Flow

Cerebral blood flow measurements were determined by the reference-sample, fluorescent-labelled microsphere technique[216]. The technique adopted in the current study was identical to that used in previous reports from our laboratory[217]. Suspensions of microspheres with a diameter of 15.5 ± 0.1 μm (Biopal Inc, MA) containing approximately 5 x 10^6 microspheres were used for each injection. Two different fluorescent-labelled colours were used in each piglet and these were chosen at random from five different microsphere colours used in our laboratory (gold, ytterbium, lanthanum, lutetium or samarium). For each flow measurement, 5 x 10^6 micropsheres were injected in to the systemic circulation via either the side-arm of the aortic cannula (data point 1) or through the left atrial cannula (data point 2), to ensure thorough mixing of the microspheres with blood before arrival at the aortic root. The injection was performed over 30 seconds and flushed with warm saline flush. A reference blood

sample was withdrawn from the distal aorta at a constant rate (3ml/min) using a Harvard syringe pump (Harvard Apparatus, South Natick, MA) via the femoral artery catheter. This withdrawal commenced 10 seconds before microsphere injection and continued for a total of 3 minutes. At the end of the experiment, the brain was removed and the cerebral hemispheres dissected. Three representative samples of cortical grey matter of approximately 2g each were weighed fresh and dried together with reference blood samples overnight in an oven at 70°C. Samples were subsequently analysed to estimate the quantity of each type of fluorescent-labelled microsphere present in each sample (Biopal™, Biophysics Assay Laboratory, Worcester, MA). The withdrawal rate of the reference blood sample and the ratio of counts from a cortical specimen to the reference blood sample allowed calculation of the cerebral blood flow. Cerebral blood flow measurements are expressed in millilitres per 100 grams of brain per minute by normalising for fresh tissue.

5.4.5 TNFα assay

An enzyme-linked immunosorbent assay (ELISA) porcine cytokine kit (R&D Systems Inc., Minneapolis, MN) was used to assay the serum concentration of TNF-α. The sensitivity of the ELISA kit was 2.8 to 5.0 pg/ml, and the range for the standards was from 0 to 1500 pg/ml.

5.4.6 Analysis of Intra-cerebral TNFα mRNA

The Primer Designer 4 program (Scientific & Educational Software PO Box 72045, Durham, NC 27722-2045 USA) was used to design pairs of 20mers to amplify a 146bp region of pig GAPDH (GenBank Accession # AF017079), a 178bp region of pig 18S (GenBank Accession # AY265350.1), and a 111bp region of pig TNFa (GenBank Accession # NM_214022). Primer Sequences were as follows: Pig GAPDH (Fwd: tcg gca tcg tgg aag gac tc, Rev: agc ctt ggc agc acc agt ag); Pig 18S ribosomal RNA (Fwd:

cgg aac tga ggc cat gat ta, Rev: tcg gaa cta cga cgg tat ct, 3' phosphorylated Rev: tcg gaa cta cga cgg tat ct-phosphate); Pig TNFa (Fwd: cca atg gca gag tgg gta tg, Rev: ttg atc tcg gca ctg agt cg (used in RT reaction), Rev2: agg acc tgg gag tag atg ag (used in PCR reaction). Total RNA was isolated from 50-100mg chunks of flash frozen pig forebrain grey matter using the RNeasy Tissue Lipid Mini Kit (Qiagen Inc., Valencia, CA, USA). DNase treatment was performed on the columns during the RNA extraction using the RNase-Free DNase Set (Qiagen), according to the manufacturer's protocol. A series of dilutions was made from a single pig forebrain RNA sample (1:2, 1:10, 1:40, 1:100, 1:400 and 1:1000). These dilutions were used to make a standard curve. A 1:25 dilution was made of each sample RNA. For each standard RNA and sample RNA, a reverse transcription reaction was performed with SuperScript II RNase H- RT (Invitrogen, Carlsbad, CA, USA).

The primers used for reverse transcription were the Pig 18S Rev, 3' phosphorylated 18S Rev, Pig GAPDH Rev, and Pig TNFa Rev primers (2 pmol of each primer, except the pig 18S Rev; 0.2 pmol of pig 18S Rev was used). The 3'phosphorylated 18S rev is phosphorylated at the 3' end and cannot be extended. This limits the amount of 18S that is reverse transcribed, so that it does not overwhelm the RT reaction and prevent the reverse transcription of other genes.

An RT- control reaction was run for each sample, under identical conditions, omitting the RT enzyme and replacing it with sterile water. Four replicates of one of the RT reactions were run, to give an estimate of the reproducibility of the results. Following reverse transcription, the cDNA samples were purified using QIAquick® PCR purification columns (Qiagen Inc., Valencia, CA, USA). Purified samples were eluted in 30µl 10mM TrisCl pH 8.5.

5.4.6a Real-time PCR

All PCR reactions were performed on a LightCycler Instrument (Roche Diagnostics, Indianapolis, IN, USA), using the Quantitect SYBR Green PCR Kit (Qiagen). The experimentally determined optimal cycling conditions are listed in the chart below.

	Temperature	**Temp transition rate**
Denaturation	95°C, 15 min	20°C/s
Amplification (40 cycles)		
Denaturation:	94°C, 15 s	20°C/s
Annealing:	55°C, 20 s	20°C/s
Extension:	72°C, 30s *1 fluorescence reading at 72°C*	2°C/s
Melting Curve	95°C, 0 s	20°C/s
	65°C, 15 s	20°C/s
	95°C, 0s Continuous fluorescence readings	0.1°C/s
Cooling	40°C, 30s	20°C/s

Table 5.1 PCR reaction conditions.

Fluorescence readings were taken at the end of each amplification cycle, as indicated, to monitor the amount of double-stranded DNA present. During these cycles, fluorescence was plotted as a function of cycle number, to show when reactions were in the log-linear phase of amplification.

During the melting curve, fluorescence was monitored continuously while the temperature was raised from 65°C to 95°C at a rate of 0.1°C/s. Fluorescence was plotted as a function of temperature to allow the determination of the specific Tm for each product. During initial PCR reactions, the size of each PCR product was verified by running it on a 1.2% agarose gel with a 1Kb+ ladder (Life Technologies). In subsequent runs the melting curve analysis was used to verify the correct product. Quantification values were generated only from samples showing only a single product that melted at the expected temperature.

Relative Quantification:

For each primer pair (GAPDH fwd + rev, 18S fwd + rev, and TNFa fwd + rev), a separate PCR reaction was run for six standard curve samples, each unknown sample, and the four RT replicate samples. At the completion of the PCR reaction, the C_T (threshold cycle) was determined by the 2nd derivative maximum method, using the LightCycler Version 3.5 Software (Roche). The C_T for each standard curve sample was plotted versus the log of the concentration in that sample. cDNA from unknown samples was diluted 1:25, and the concentration of each amplicon was calculated based on the equation derived from the standard curve. Calculated values indicated expression levels of the gene being amplified relative to its expression level in the standard pig forebrain RNA upon which the standard curve was based. Concentrations of the TNFa were calculated for each sample. These concentrations were normalised by dividing by the concentration of the housekeeping gene, either 18S or GAPDH, in that sample.

5.5 Results

5.5.1 Arterial Blood Gas and Haemodynamic Data

Haemodynamic and arterial blood gas data were similar during the course of the experiment, with no significant differences between groups (table 5.2). Cardiac output corrected for body weight reveals slightly lower values in group 2B pre-CPB and group 2A post-CPB, these differences did not approach statistical significance in either case. Importantly, adequate post-CPB perfusion is indicated in all groups by the similar mean arterial pressures and satisfactory acid-base status (sodium bicarbonate was not administered following wean from CPB). Arterial tensions of carbon dioxide, a powerful stimulus for cerebral vasodilatation were similar between groups at both data acquisition points.

Following page: Table 5.2 general piglet parameters. *$p<0.05$ compared with pre-CPB values. **$p<0.05$ compared with corresponding group using a blood prime.

Variable	Prime	CPB Strategy	Pre-CPB	Post-CPB
MAP mmHg	1: Blood	1A: DHCA	47.0 ± 2.9	45.8 ± 2.9
		1B: DHCLF	47.4 ± 3.0	43.4 ± 3.3
	2: Bloodless	2A: DHCA	53.8 ± 4.2	45.4 ± 4.7
		2B: DHCLF	51.8 ± 4.3	50.4 ± 4.6
CO ml/min/kg	1: Blood	1A: DHCA	94.2 ± 10.7	89.3 ± 5.7
		1B: DHCLF	90.0 ± 4.7	81.7 ± 11.4
	2: Bloodless	2A: DHCA	86.2 ± 6.1	75.1 ± 6.5
		2B: DHCLF	76.5 ± 4.0	88.6 ± 9.1
Hct %	1: Blood	1A: DHCA	27.2 ± 2.1	28.8 ± 1.9
		1B: DHCLF	26.4 ± 1.3	28.4 ± 1.2
	2: Bloodless	2A: DHCA	29.0 ± 1.4	21.6 ± 1.4*, **
		2B: DHCLF	31.6 ± 0.8	22.0 ± 0.6*, **
pH	1: Blood	1A: DHCA	7.40 ± .07	7.36 ± .05
		1B: DHCLF	7.42 ± .08	7.34 ± .05
	2: Bloodless	2A: DHCA	7.40 ± .07	7.30 ± .01
		2B: DHCLF	7.46 ± .05	7.36 ± .05
Base deficit mmol/l	1: Blood	1A: DHCA	-4.2 ± 2.0	-4.0 ± 2.7
		1B: DHCLF	-2.8 ± 1.4	-5.2 ± 1.4
	2: Bloodless	2A: DHCA	+0.2 ± 1.2	-5.6 ± 2.6
		2B: DHCLF	-2.0 ± 1.5	-3.2 ± 0.4
PaO_2 mmHg	1: Blood	1A: DHCA	469 ± 25	331 ± 72
		1B: DHCLF	428 ± 46	324 ± 65
	2: Bloodless	2A: DHCA	464 ± 29	273 ± 79
		2B: DHCLF	419 ± 62	366 ± 58
$PaCO_2$ mmHg	1: Blood	1A: DHCA	37.8 ± 3.7	40.0 ± 3.5
		1B: DHCLF	33.6 ± 5.0	39.8 ± 3.0
	2: Bloodless	2A: DHCA	36.0 ± 4.1	43.6 ± 2.0
		2B: DHCLF	33.8 ± 2.4	37.6 ± 2.2

5.5.2 Cerebral Blood Flow

Cerebral blood flow data for the two data acquisition points is given in figure 5.4. We experienced considerable heterogeneity between animals when calculating cerebral flow using the fluorescent microsphere method. However, the advantage in using microspheres is that each animal serves as its own control. Information is mostly usefully gleaned by examining the change in cerebral blood flow within animals in each group before and after CPB (figure 5.5). In both groups exposed to DHCLF (1B and 2B), the response was widely varied, with both large increases and large decreases observed in post-CPB CBF, though an insignificant trend towards elevated post-CBF CBF was observed following bloodless prime DHCLF (group 2B). The effects following DHCA were more consistent. Every animal in group 1A (blood prime DHCA) experienced a fall in post-bypass CBF (mean -45% ±28 from baseline) and every animal in group 2A (bloodless prime DHCA) experienced an elevation in post-CPB CBF (mean +28% ±14 from baseline), $p<0.01$. Post-bypass, the calculated CBF was only 16.9% ±11.3 of the overall cardiac output in group 1A, compared with 31.8% ±5.9 in group 2A, $p<0.05$ (figure 5.6).

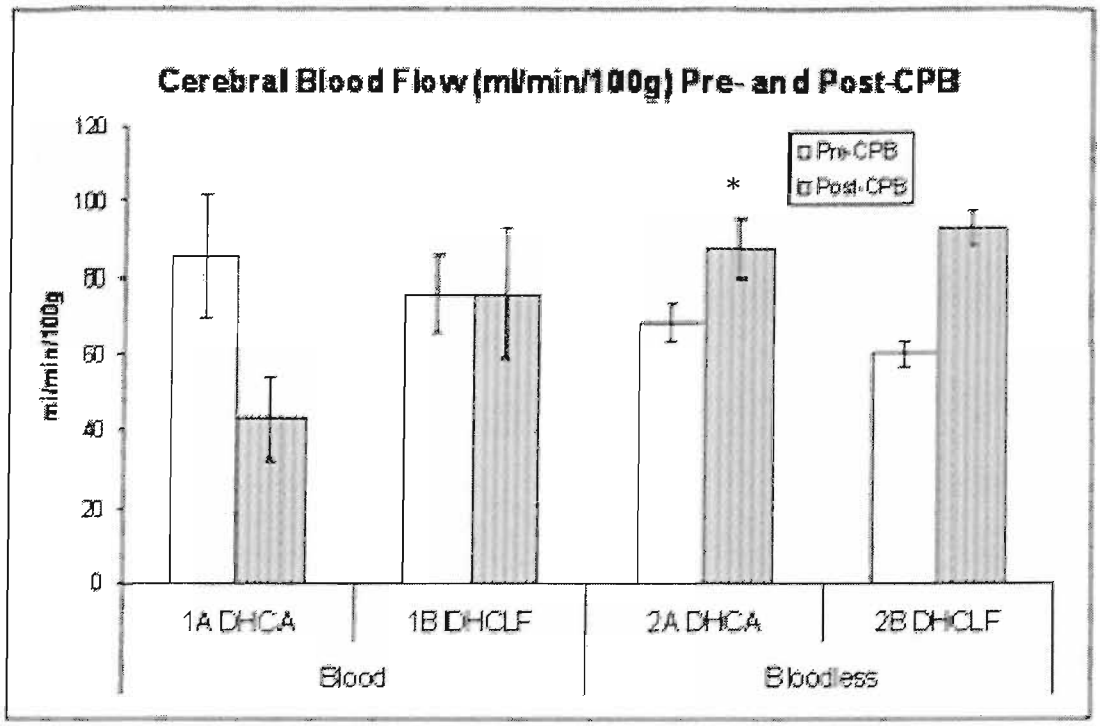


Figure 5.4 Calculated cerebral blood flow pre-and post-CPB in the experimental groups (ml/min/100g). * $p<0.05$ compared with corresponding group using a blood prime.

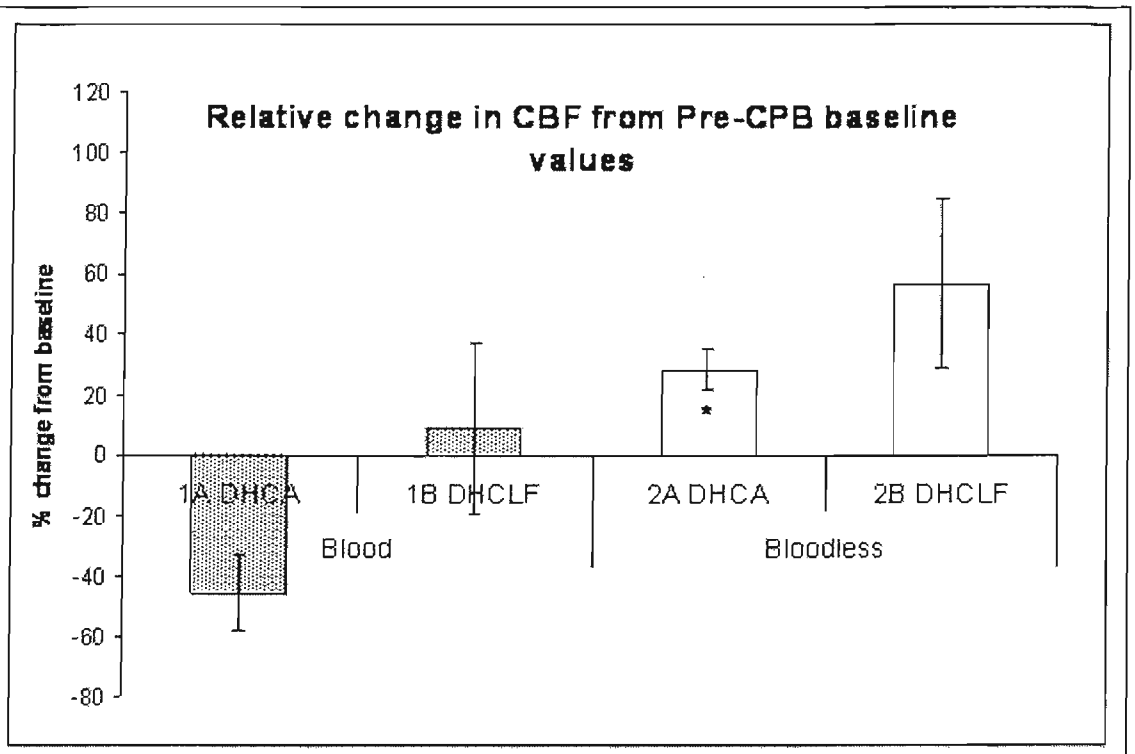


Figure 5.5 Change in CBF from pre-CPB baseline values within each experimental group. * $p<0.01$ compared with corresponding group using a blood prime.

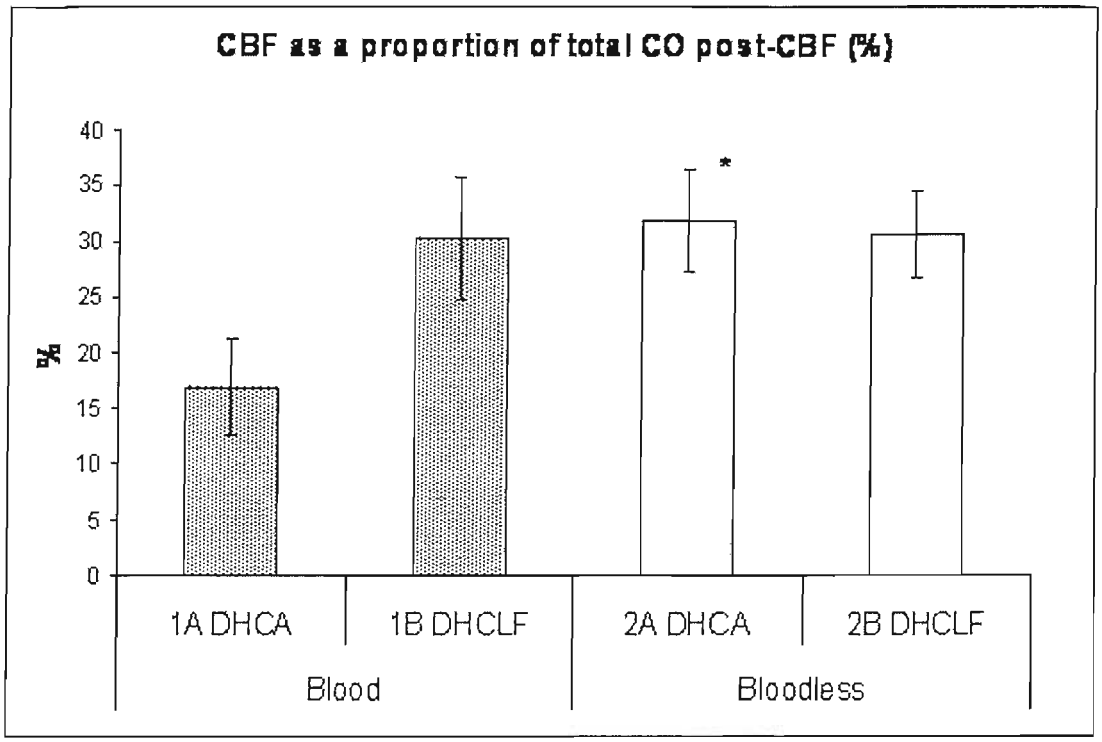


Figure 5.6 Post-bypass calculated CBF as a percentage of total cardiac output.

* $p<0.05$ compared with corresponding group using a blood prime.

5.5.3 Circulating TNFα Load

Circulating TNFα concentrations were significantly higher post-CPB following DHCLF with a blood prime (group 1B; 3166 ±1887 pg/ml) than DHCLF using a bloodless prime (group 2B; 439 ±429 pg/ml), $p<0.05$ (figure 5.7). By contrast, TNFα concentrations were similar between groups exposed to DHCA, regardless whether blood (group 1A; 1179 ±2045 pg/ml) was used or not (group 2A; 1626 ±1861 pg/ml). To examine the potential for the high circulating TNFα levels originating directly from the donor animal during the harvesting process, three samples of donor blood were analysed, revealing a mean level of 213 pg/ml (range 87-386).

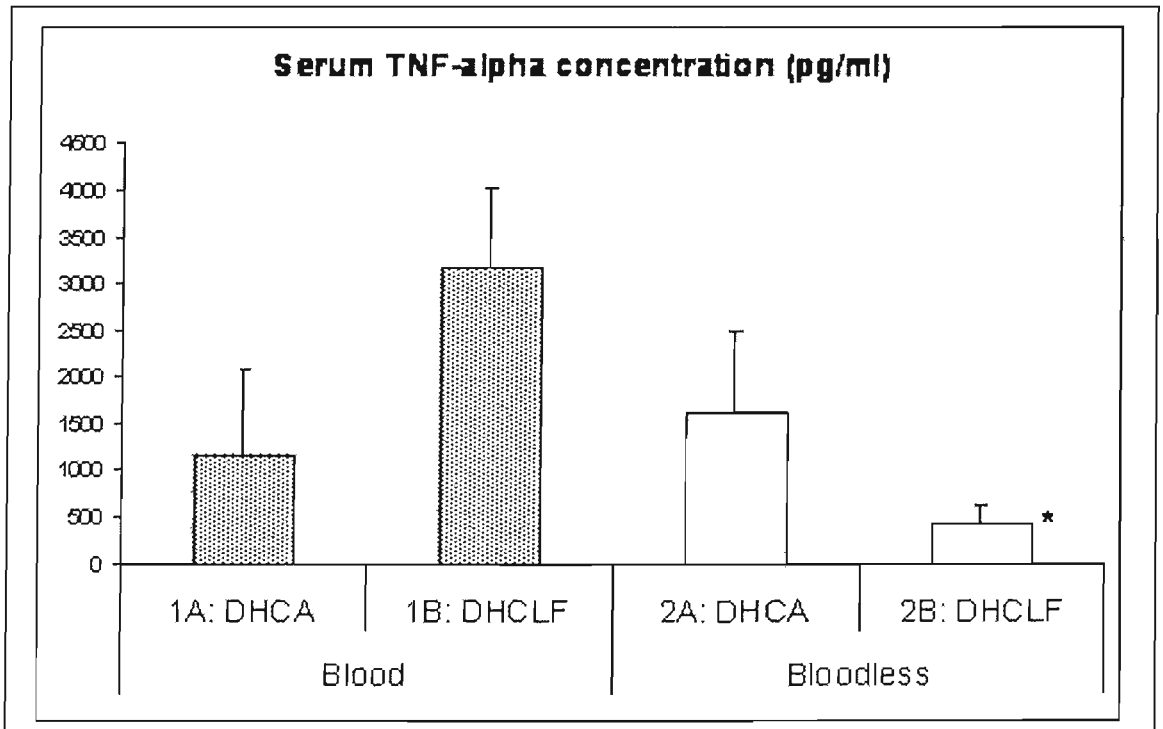


Figure 5.7 Serum TNFα concentration at the conclusion of the experiment. * $p<0.05$ compared with corresponding group using a blood prime.

5.5.4 Intra-cerebral expression of TNFα mRNA

Quantification of TNFα mRNA within sampled tissue was made by comparison to two reference household genes GADPH and ribosomal 18S RNA (figure 5.8). Following DHCLF with a blood prime (group 1B), mean TNFα mRNA levels were elevated 2.2 ±2.2 or 2.2 ±2.6 fold (GADPH and 18S RNA respectively), whereas the same strategy

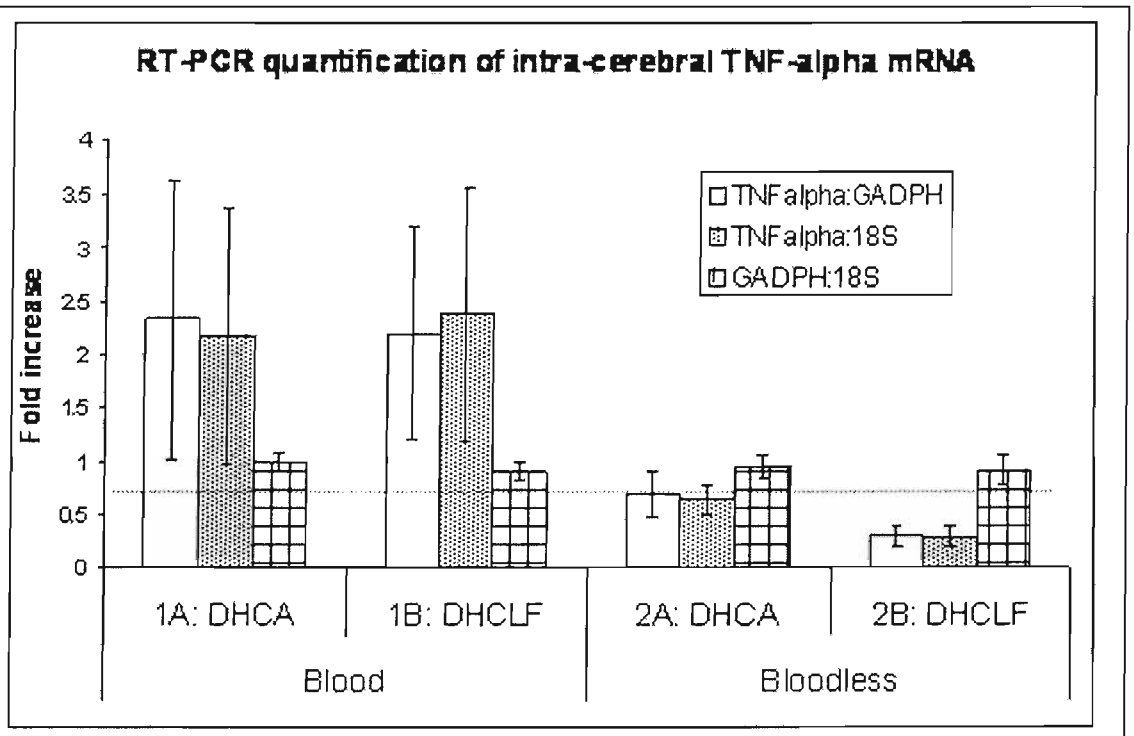


Figure 5.8 Quantification of intra-cerebral TNFα mRNA, by polymerase chain reaction. Levels are expressed as relative fold increases or decreases compared to two house-keeping genes (GADPH and 18S ribosomal RNA).

using a bloodless prime (group 2B) revealed only negligible levels (0.31 ±.23 and 0.30 ±.19 fold respectively), $p<0.05$. A similar pattern was observed with a strategy of DHCA, though this was not significant (2.3 ±2.9 and 2.2 ±2.8 vs. 0.70 ±0.46 and 0.64 ±0.31; $p = 0.13$ and 0.12 respectively). Overall, irrespective of the CPB strategy, the

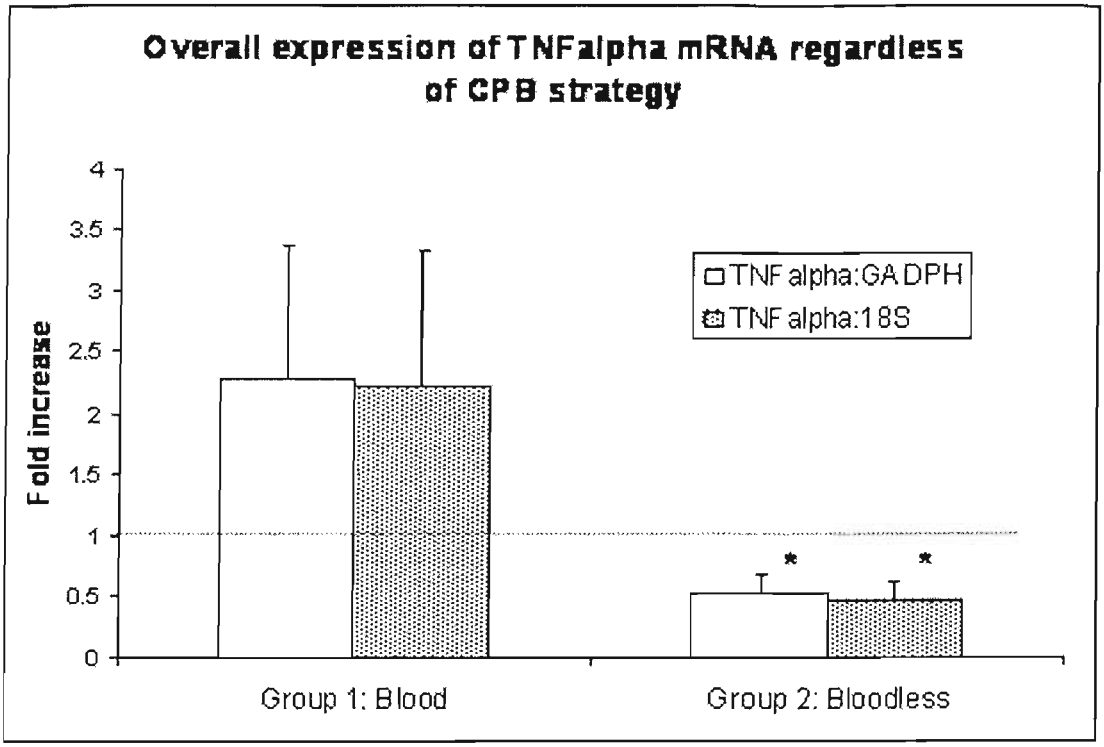


Figure 5.9 Intra-cerebral TNFα mRNA expression, regardless of deep hypothermic CPB strategy adopted. $p<0.05$ compared with animals exposed to CPB using a blood prime.

use of blood generated significantly elevated levels of intra-cerebral TNFα mRNA (2.3 ±2.5 and 2.2 ±2.5 vs. 0.5 ±.34 and 0.5 ±.31 fold, $p<0.05$) (figure 5.9).

5.6 Conclusions

A huge discrepancy exists between the size of the extracorporeal circuit and intravascular blood volume in neonatal CPB. This ratio determines the degree of haemodilution resulting from bypass using a bloodless prime, and consequently neonates exposed to conventional bypass receive a relatively large proportion of allogeneic blood. This frequently amounts to a total body transfusion and consequently, compared to any other group, children under 4 months old receive the highest number of blood components during CPB[204].

In the late 1990s, whole fresh blood was frequently preferred for infant CPB due to suggestions that the non-red blood cell (RBC) component may confer advantages in terms of coagulation[218, 219]. This assertion has not been proved correct. In fact a double-blind randomised study of 200 infants confirmed that the use of whole blood during CPB conferred no advantage in terms of post-operative blood use[202]. The same study also demonstrated that whole blood resulted in greater fluid sequestration, later ICU discharge, and longer hospital stay. This was unexpected as reconstituted blood (packed RBCs and fresh frozen plasma) was considered by many to be more inflammatory[218]. Although the use of packed RBCs alone has not been directly compared to an asanguinous paediatric circuit, studies in adult CPB have suggested that the elimination of the non RBC component (i.e. plasma and leukocyte depletion) does not abrogate the inflammatory side-effects of transfusion. Interleukin-6 levels are significantly higher and the post-operative clinical course significantly worse in patients who have received packed RBCs during CPB[203]. Furthermore, the supernatant from packed RBCs is capable of instigating acute lung injury in animal models[220].

The possibility of completely bloodless prime in neonatal CPB is therefore attractive. Several groups have begun to describe systems for reducing or even eliminating blood use in clinical infant CPB[204, 205, 221-223]. Most notably Merkle et al.[224] have recently described a series of infants as small as 3.4kg placed on CPB with a crystalloid prime of 190ml, all of whom recovered uneventfully. Our efforts have focused on greatly reducing the prime volume by using the smallest available oxygenators, minimising line length and eliminating ancillary equipment such as filters and cardioplegia circuits. Though this would have safety implications in the clinical arena, it has allowed us to characterise the benefits conferred by eliminating blood use. Piglets have a lower baseline haematocrit (25-30%) than humans, but haemodilution in this study was limited to post-CPB levels of 22%. The threshold for "unacceptable" haematocrit is not clearly defined, but several studies indicate that oxygen delivery, tissue blood flow and clinical outcome are only severely affected at levels below 14-17%[225, 226].

This study is the first to examine experimental no-reflow in a neonatal model that avoids the use of donor blood. All previous studies have required large volumes of allogeneic whole blood and have repeatedly demonstrated a significant cerebral no-reflow response following DHCA[120, 227]. This present study suggests a detachment from the usual pathophysiology of no-reflow through the use of bloodless prime, and we have instead observed a mild hyperaemic response in the early reperfusion period. The mechanisms behind these observations are unclear. The small differences in haematocrit between groups (22 vs 28%), and a corresponding fall in CBF as a proportion of total cardiac output suggest that it is unlikely to be related solely to the haemodilution necessarily associated with a bloodless prime. In fact, other groups have studied the influence of haemodilution during neonatal CPB, and report significant elevations in CBF only when the haematocrit falls below 15%[122].

The fact that cerebral no-reflow probably has an inflammatory component makes it attractive to speculate that our observations may be attributed to the lesser inflammatory response associated with bloodless prime CPB. This notion is supported by the demonstration of improved cerebral no-reflow through the targeting of individual components of the inflammatory response. Leukocyte filtration[118], platelet activating factor antagonism[64] and steroid administration[71] all improve CBF following DHCA. Very recently, the inhibition of platelet activation through glycoprotein IIb/IIIa antagonism has been shown to increase cerebral microvascular flow following DHCA, presumably through a reduction in platelet plugging[142]. Our interpretation of this present study is therefore that the elimination of the numerous stimuli contained within whole allogeneic blood may result in the release from the inflammatory mechanisms behind no-reflow.

In non-CPB piglet studies of global cerebral ischaemia, no pre-existing inflammatory response is present, and a hyperaemic response early in the reperfusion period is consistently seen[130]. This is then followed by a more sustained depression of cerebral blood flow (no-reflow) [228]. It may be that a similar phenomenon is occurring in this present model, but an extended survival period would be required to investigate this. Ischaemia – either total or partial – is the common denominator in models exhibiting no-reflow. Although regional cerebral perfusion abnormalities occur during DHCLF, this is less consistently the case[214, 215]. The extreme variability in the CBF response seen in this study following DHCLF probably reflects this, and our choice to use a fixed low-flow rate that is independent of pressure[229].

This study confirms previous reports from our laboratory that a bloodless prime appears to be less inflammatory[61]. This difference is most pronounced when using DHCLF, probably due to the longer dynamic exposure to the extracorporeal circuit during continuous perfusion techniques. We have previously reported DHCLF as being more

inflammatory than DHCA[62]. This is one major expense associated with adopting continuous perfusion techniques in order to avoid any period of circulatory arrest. Cerebral ischaemia itself generates TNFα, and it is interesting that the group exposed to neither blood during CPB or cerebral ischaemia (bloodless DHCLF) yield the lowest levels of both systemic circulating TNFα, and intra-cerebral TNFα mRNA. It may be, therefore, that the inflammatory consequences of continuous perfusion techniques can be offset by using a bloodless prime whilst simultaneously avoiding periods of cerebral ischaemia.

TNFα is a useful experimental inflammatory marker because of its role as an apical cytokine capable of activating almost all arms of the downstream innate immune system. The importance of the inflammatory response – but TNFα in particular - in cerebral injury is now beginning to be recognised. In fact, TNFα (and IL-1β) can itself initiate neuronal apoptosis in the absence of ischaemia in neonatal models of perinatal brain injury[230]. In both global and regional stroke models TNFα amplifies neuronal loss[39], and its antagonism is protective[231]. The magnitude of the inflammatory infiltrate following stroke[232], peri-natal brain injury[233] and sub-arachnoid haemorrhage[234] is directly linked to poor outcome. In conclusion, in all types of neuronal injury, reducing the cerebral exposure to TNFα is beneficial, and therefore the demonstration here that deep hypothermic CPB using a bloodless prime leads to significantly less local production of TNFα in the brain is highly relevant.

There are two principal drawbacks with this study. First, we have only examined a brief window in the early post-CPB period. It would be extremely interesting to investigate a series of time-points in the post-operative period. In this study it was not possible to examine several time-points because of progressive haemodilution occurring with every microsphere data point. Furthermore, longer survival in our model would not be possible without post-operative blood administration. Secondly, we have

compared the use of fresh whole blood with an asanguinous prime. This does not address the role of packed RBCs in exacerbating CPB-induced inflammation, and we can make no conclusions from this study to specifically avoid the use of leukocyte-depleted blood, the predominant form of RBCs now in clinical practice.

This study represents our continued pursuit for more routine elimination of blood products in neonatal CPB. This is the first examination of the cerebral recovery following deep hypothermic CPB strategies using a bloodless prime. Cerebral no-reflow is a marker of injury related to the duration of DHCA that correlates with poor outcome. It is a phenomenon consistently seen following experimental and clinical DHCA. This is the first study to demonstrate the capability of the brain to exhibit a mild hyperaemic response in the early stages following DHCA, and we believe this is attributed to the reduced inflammation associated with a bloodless prime. Attempts to avoid no-reflow through DHCLF have been associated with exaggerated inflammatory responses in experimental models. The analysis of circulating and intra-cerebral TNFα in this study suggests that DHCLF in conjunction with a bloodless prime might offer advantages through avoiding ischaemia, no-reflow and the detrimental inflammatory response.

5.7 Technical pursuits of miniaturisation for the future

The main obstacle in using an asanguinous prime is unacceptable haemodilution. Major haemodilution is not only a problem for red cell-dependent gas transport, but also for platelet and humeral factor-dependent coagulation and protein-dependent intravascular oncotic pressure[196, 197]. Although higher haematocrits are preferred[122, 235], the threshold for "unacceptable" haematocrit is a matter of controversy, although most clinical studies implicate a value of between 17 and 20%[225, 226]. Miniaturisation is the only current strategy by which haemodilution can be limited when blood use is

avoided. This is not a new concept, and direct attempts to miniaturise date as far back as 1972[236]. In our most recent animal model (lacking major ancillary equipment, including cardioplegia circuit and line filters), a prime volume of only 60ml allows implementation of full neonatal piglet ($\geq$2kg) CPB while maintaining a haematocrit above 18% (figure 2). It should be noted that piglets have a haematocrit below that of humans (normal ~30%). Recently, successful CPB has been reported in a series of human infants between 3.4 and 6.0kg using asanguinous prime with a volume as low as 190 mL[224]. The mean haematocrit on CPB was 23% (range 17-29%) and all nine patients recovered uneventfully. In order to capitalise further on these considerable achievements, a systematic approach needs to be adopted to all CPB components, including tubing, pumps, oxygenator/heat exchanger structures, cardiotomy suction, and filters.

Tubing length and diameter are two important factors when trying to minimise circuit priming volume. Down-sizing from 1/4" to 3/16" tubing almost halves the volume to 1.73ml per 10cm length. Some centres apparently even employ 1/8" tubing for arterial lines in very small neonates with a volume of 0.75 mL per 10 cm[224]. Tubing length can be significantly reduced by repositioning the venous reservoir and arterial roller pump console closer to patient, as in our model (figure 2). Alternatively, a conventional console with a mast-mounted arterial roller pump has resulted in a 29% reduction of priming volume[237]. Suction and venting lines can hold a substantial volume which can impact on the dynamic priming of the extracorporeal circuit as it needs to be replaced during CPB. Poiseuille's Law states that flow is proportional to the fourth power of radius, and therefore small decreases in diameter can drastically reduce flow. Drainage may therefore need to be encouraged through the use of kinetic-assisted venous return (KAVD) or vacuum-assisted venous drainage (VAVD). These techniques introduce potential risks of air aspiration should venous return become momentarily insufficient. However, with the use of automated reservoir sensor levels,

this risk should be minimised. We have routinely used VAVD both clinically and in our experimental model at -20mmHg without untoward effects.

The arterial pump in our experimental circuit was a COBE™ roller pump with 3/8" tubing and 40cm boot length, yielding a pump prime of 6ml. Theoretically a shorter raceway would allow for further reductions in tubing length, but the higher revolutions per minute would make hand-cranking more difficult in the event of power failure. Although centrifugal pumps are used in paediatric patients, none are available with 1/4" or 3/16" connectors, precluding their use in miniaturised circuits.

The priming volume of oxygenators has been tremendously reduced since the screen-type oxygenator was used for the first successful open heart surgery in 1953. Current oxygenators for neonatal perfusion have static priming volumes of 43 mL (Capiox RX 05®, Terumo, Japan), 52 mL (Safe Micro™, Polystan, Denmark) and 60 mL (Lilliput I™, Dideco, Italy). The Capiox RX 05® is currently under evaluation[238, 239], and is the oxygenator we currently use in our experimental circuit (figure 2). Recently, Fuji Systems successfully developed a thin silicone rubber hollow-fibber oxygenator with no plasma leakage, better blood compatibility, low blood prime, and low flow resistance in the ex vivo studies[240, 241]. Silicon is generally perceived to be more biocompatible than polyethylene or plastic.

An arterial line filter is a safety device used in a high percentage of paediatric patients[242]. Some departments, however, have eliminated arterial line filters since they have priming volumes comparable to oxygenator modules[224] (Dideco D 736 Newborn 40 mL, and Terumo Capiox AF02 40 mL). However, removal of arterial filter would increase the chances of micro-emboli and sacrifice the bubble purge line, which is usually connected from filter to reservoir, necessitating an additional bubble

sensor filter. Potential volume reductions may also be possible for other integral parts of neonatal circuits, including haemofilters and cardioplegia systems and need to be targeted by the biomedical corporations.

5.8 Summary

Infant CPB has advanced hugely since its introduction in the middle of the last century. Among the technical leaps that have occurred is the replacement of large membrane oxygenators with bubble and then smaller hollow fibre units, the development of cardioplegia and the introduction of novel strategies to reduce or even eliminate cerebral ischaemia. Considerable energy has been spent delineating optimal CPB conditions, including the management of arterial pH, temperature, haematocrit and ultrafiltration, through the use of animal models[8, 243, 244]. The importance of systemic inflammation is now beginning to be appreciated, in particular its impact on post-ischaemic reperfusion and the progression of ischaemic injury. Several strategies for attenuating the inflammatory processes triggered by CPB have proven beneficial and entered the clinical arena, including leukocyte filters[118], steroid administration[71] and circuit coating[239]. Evidence is mounting to implicate allogeneic blood – both whole and leukocyte-depleted - as a significant instigator of systemic inflammation. Our experimental models examining the impact of asanguinous primes in neonatal CPB have supported this concept. Post-operative advantages have been demonstrated in right ventricular function, pulmonary compliance, alveolar gas-exchange, recovery of cerebral perfusion and the inflammatory cytokine load[61]. Clinical attempts to reduce priming volume with conventional equipment have also been encouraging. Recently, successful CPB using completely asanguinous prime has been reported in a series of patients as small as 3.4kg, confirming that completely asanguinous neonatal CPB – although challenging - is currently feasible[224]. Continued miniaturisation, especially of the ancillary equipment, may render asanguinous neonatal CPB a more routine possibility in the future.

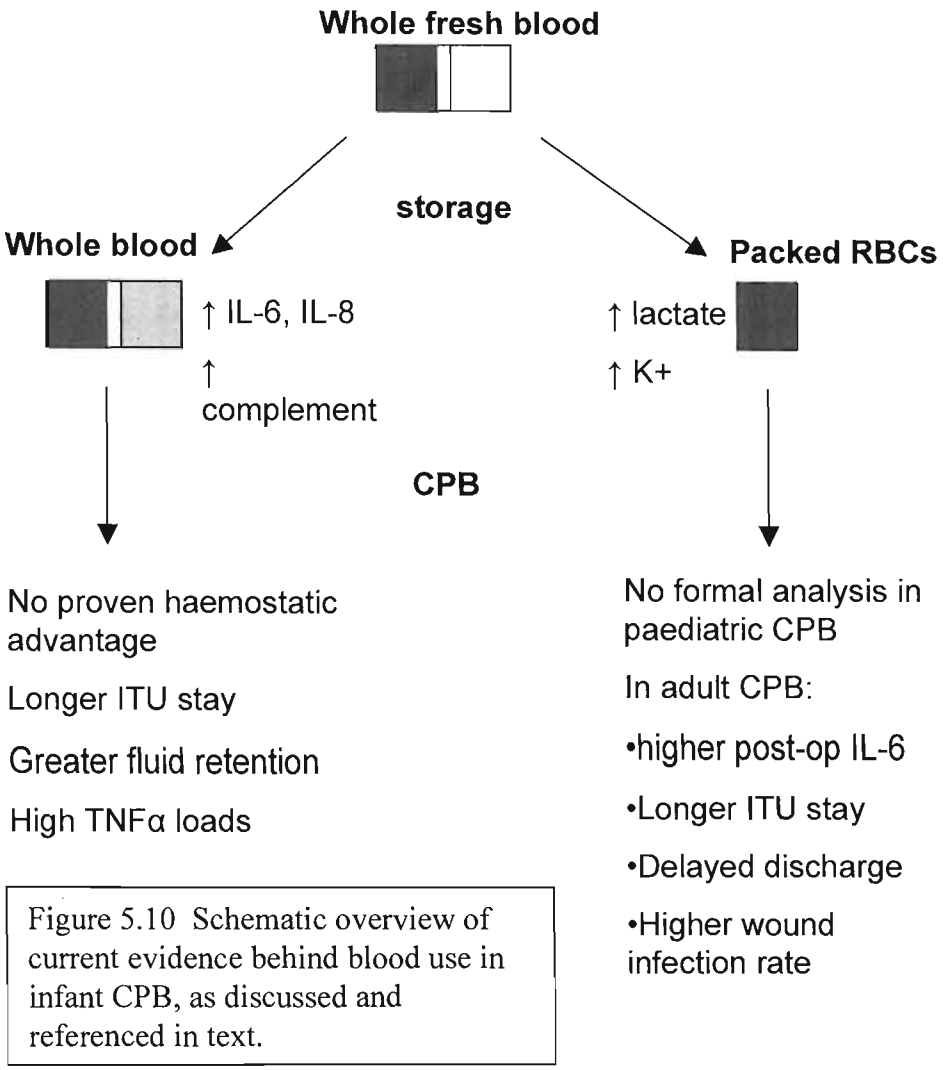
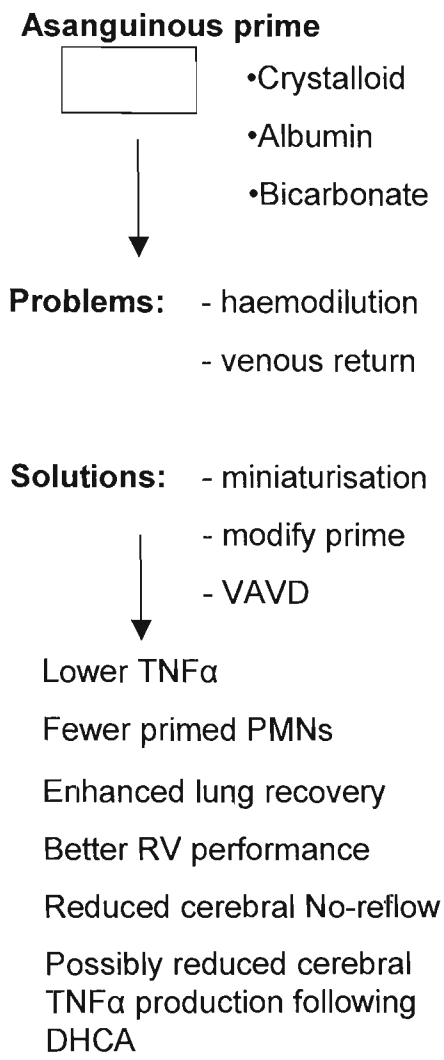


Figure 5.10 Schematic overview of current evidence behind blood use in infant CPB, as discussed and referenced in text.



Chapter 6

Establishing a Reperfusion Model of Histological Injury following DHCA

6.1 Abstract

Objectives: Although several physiological and metabolic parameters have been linked with functional outcome, quantifiable models of histological injury remain the gold standard for assessing neuroprotective strategies. Histological models are notoriously difficult in CPB research because in amplifying the cerebral ischaemic insult, the systemic consequences make survival for adequate durations increasingly difficult. In order to study the impact of oxygenation and preconditioning strategies on neurological outcome from DHCA, the aim was to introduce a quantifiable model of neuronal injury to the laboratory.

Methods: In a series of stepwise (unrandomised) experiments, neonatal piglets were placed on CPB and exposed to increasingly provocative DHCA-reperfusion strategies. This commenced with one hour DHCA and 2 hrs reperfusion, increased to 90 minutes DHCA and 8 hrs reperfusion and culminated in 2 hrs DHCA followed by 20 hrs reperfusion.

Results: The only experimental CPB strategy that elicited any discernible histological evidence of ischaemic injury was 2hrs DHCA followed by 20 hrs reperfusion. Using a novel technique for myocardial protection, full intensive care post-operative support and monitoring, a model was established whereby animals could be routinely placed through this strategy to yield a reproducible and quantifiable model of ischaemic cerebral injury. Histological damage was assessed by H&E light microscopy and Fluoro-Jade™ histochemical staining.

Conclusions: Deep hypothermia confers enormously powerful protection, such that yielding detectable injury requires inordinately lengthy durations of DHCA. Others have described evidence of neuronal injury following much lesser protocols. This discrepancy is likely due to differences in the post-ischaemic recovery between models. The optimal post-operative management (and myocardial protection) appears to mandate lengthier durations of ischaemia in order to elicit histological injury. This serves to emphasis the importance of peri-operative factors in the progression of neuronal injury following CPB.

6.2 Introduction

Differences in the acute physiological consequences of contrasting perfusion strategies have provided much of the recent impetus for the design of novel CPB techniques. For example, no-reflow as a consequence of DHCA has been used as a marker neurological threat[120], and its absence following continuous perfusion an indicator of cerebral well-being[171]. Similarly, greater levels of oxygen extraction following hypothermic CPB have been associated with superior neuronal recovery[71]. A causal relationship between either of these commonly cited parameters and either pathological or functional outcome has, however, never been shown. Furthermore, acute changes in cerebral blood flow of tissue oxygen extraction resolve within hours[245], and the few functional studies have demonstrated no differences between groups at later time points.

In studies of the brain, histopathological change is still one of the most reliable and important parameters by which ischaemic damage and therapeutic effects are evaluated. Its demonstration irrefutably implies a detrimental insult, and more importantly, the severity of histological damage correlates with functional recovery in numerous animal models. The relative paucity of large mammal models studying histological brain injury following cardiopulmonary bypass is due to the considerable cost and technical difficulty in supporting animals for the duration of the recovery period. Furthermore, in an effort to maintain a close resemblance to the clinical conditions, these models invariably involve ischaemia at deep hypothermic temperatures. In order to overcome the robust protection conferred by deep hypothermia, lengthy durations of experimental ischaemia are therefore necessary, which negatively impact on animal survival, especially as cardioplegia is not frequently used.

Accompanied with recent evidence emerging from stroke and perinatal brain injury research[112, 147], a general consensus is emerging that much of the injury sustained is not occurring during periods of DHCA, but instead during the early reperfusion period. The concept of reperfusion injury is widespread, but little researched in the context of cerebral recovery following DHCA, and current intensive care strategies focus principally on systemic, pulmonary and myocardial haemodynamics, rather than a tailored approach to cerebral recovery. In fact, the key factors during the reperfusion period that influence neurological recovery following neonatal CPB have not been delineated.

One of the drawbacks in the investigation of neuroprotective strategies is that no simple indices of injury exist (as with Troponin in myocardial ischaemia, or creatinine with renal function). Attempts have been made to identify brain-specific serum markers, including S100β and neuro-specific enolase amongst others[246], but these lack specificity for cerebral ischaemia, and a relationship with pathological injury has not been clearly established[247]. It is for this reason that the search for neuroprotective strategies ideally requires a model of histological damage.

6.3 Rationale behind experimental protocol design

Experimental models of brain injury invariably require 1) an exaggerated H-I stimulus; 2) a period of adequate reperfusion; 3) characterisation of regional vulnerability; 4) methods of histological analysis; and 5) quantification.

Several models of histological brain injury following experimental DHCA have been described, in species including rat, rabbit, dog, sheep and pig. The advantage of larger mammals (dog, sheep, pig) is their closer phylogenetic similarity with humans, and the possibility of studying neonates due to their size. Piglets are especially appealing as

swine are generally perceived as having the closest vascular physiology and cerebrovascular anatomy compared to humans, of all the non-primate large mammals[248].

The previously described reperfusion piglet models of DHCA are summarised in table 6.2. A model of piglet bypass that involved any significant survival period following CPB was not previously in place in our laboratory. In fact, separation from CPB, using "as required" boluses of adrenaline as achieved only the previous year, and therefore the laboratory had neither the experience or equipment for prolonged wean from CPB.

A series of exploratory studies was therefore embarked upon with the aim of introducing an appropriate reperfusion model of CPB that could be used for both the study of the reperfusion period following clinical CPB, and neuroprotective strategies for DHCA. The ideal criteria for such a model would include:

- **A clinically relevant CPB technique**
- **Clinically relevant peri-operative management**
- **DHCA (i.e. not ischaemia at normothermia/moderate hypothermia)**
- **A stable reperfusion period that could be closely monitored and easily manipulated**
- **An adequate reperfusion period for cerebral injury to become morphologically apparent**
- **Injury to relevant regional areas**
- **A technique for quantification of injury**

6.3.1 Clinically relevant CPB technique

Though closed-chest CPB[122] is considerably easier in terms of eliminating the significant morbidity associated with sternotomy, it presents several difficulties. First, it offers no method of assessing myocardial well-being, nor any easy method for myocardial protection. Although reports exist of snaring the head and neck vessels via a mini-thoracotomy and then infusing a cardioplegia-like solution into the aortic route[169], this appears cumbersome. Furthermore, this technique cannot protect against left ventricular (LV) dilatation. LV distension was noted to be a particular problem with deep hypothermic strategies in preliminary studies, and was presumed to be either due to incompetence (perhaps age-related) of the aortic valve, or related to low-flow techniques being unable to "trip" the valve. The experience resulted in the routine insertion of LV vents in *all* experimental subjects, which would not be possible with closed-chest CPB.

In addition it was clear (during studies performed in chapter 5) that even at 18°C, the myocardium often retained some activity, which would only subside with the application of pericardial slush, a procedure not possible with closed-chest CPB.

For a relevant neonatal model, the use of the smallest piglets possible is necessary. This precludes the use of the femoral artery as a cannulation site, as it is too small in piglets under 4kg for cannulation with a 6Fr CPB cannula. Using the carotid artery is not ideal in experiments where both cerebral hemispheres would be harvested and processed for tissue analysis.

It was therefore decided that a model involving open-chested bypass, with close observation and protection of the myocardium would be most suitable.

6.3.2 Clinically relevant peri-operative management

In order for any such model to have relevance to clinical intensive care management, cardio-respiratory support should closely resemble that in a human paediatric intensive care. This should include similar anaesthetic, inotropic, fluid and organ-support management to that used clinically. A laboratory intensive care environment was therefore created with all the available necessary equipment.

6.3.3 The use of DHCA

Although in modern surgical practice, DHCA fortunately rarely exceeds 60 minutes, more extended periods are typically necessary for generating the amplified injury that is desirable for research purposes. This does not imply that DHCA for one hour or less is free from injury as previously suggested[249]. Instead, in contrast to stroke and peri-natal brain injury, neurological deficit from CPB is recognised to be extremely subtle, and likely is related to tiny proportions of neuron loss by apoptosis[121] perhaps over considerable time. Low levels of apoptosis are extremely difficult to detect and quantify experimentally. In order to study DHCA, it is necessary to instead amplify the ischaemic challenge to provoke detectable injury (apoptotic or necrotic) to an extent that can be easily and reproducibly identified and quantified. The investigation therefore began with a minimum of one hour of DHCA at 18°C, in accordance with other published models.

6.3.4 Stable reperfusion period

Ensuring stability throughout the early reperfusion period necessitates continuous support and monitoring of cardio-respiratory function. Many survival models[250] involve early extubation (and therefore recovery from anaesthesia). Although closed-chest rodent experiments can combine early extubation with continued invasive blood pressure monitoring (via tunnelled femoral artery catheters) and oxygen therapy (with

oxygen incubators), this is much more difficult with larger mammals and requires either physical restraints or the manual application of oxygen masks. **It was therefore decided that throughout the reperfusion period, piglets would remain anaesthetised, intubated, ventilated and with full *continuous* invasive haemodynamic support and monitoring.** This would allow identification of even brief periods of instability, numerous options for intervention, and allows the reperfusion conditions to be easily studied. Furthermore, this scenario closely mimics that of post-operative neonatal management. Very few reports detailing "intensive care"-type management for the study of bypass-related neurological injury exist[122].

6.3.5 Adequate reperfusion period

The precise temporal profile of morphological injury in the brain following DHCA is unclear. In severe unilateral rodent stroke models, histological injury on haematoxylin and eosin can occasionally be identified as early as 2 hrs of reperfusion[124], though beyond 12 hrs, the injury is greatly amplified and is maximal at 24hrs. Piglet survival models following DHCA have also reported injury to be detectable as early as under 2 hrs[251], increasing in severity to 24hrs[121]. The considerable technical difficulties in managing ventilated large mammals overnight mandated that shorter reperfusion periods be investigated first, and **therefore pilot studies were performed with increasing reperfusion periods, beginning with 2hrs.**

6.3.6 Histological Injury

The **regional vulnerability** and **histological injury** would be assessed through serial sagittal coronal sections using routine staining techniques as described in chapter 4. Irreversibly degenerating neurons can be identified on H&E with reproducible ease by their eosinophilic cytoplasm, clumped chromatin, fragmented nucleus and apoptotic

bodies, and is the ideal method for quantifying injury. More elaborate stains are helpful if injury is ambiguous or for investigating the intracellular mechanisms involved.

From the working model of acute neonatal piglet CPB, it was therefore the aim to introduce a reproducible model of CPB with DHCA that would generate neurological injury after a defined period of reperfusion. This model would be an open-chested aorto-right atrial model with periods of deep hypothermic circulatory arrest followed by a stable period of controlled reperfusion whilst ventilated and anaesthetised.

6.4 Experimental Design and Methods

A series of experiments was undertaken in a sequential (i.e.un-randomised) fashion with progressively more severe ischaemia or lengthening reperfusion until significant injury was detected.

6.5 Experiment 1

Group A 2 Sham piglets, with no surgery. Perfusion-fixed only

Group B 2 Piglets 1hr DHCA at 18°C, 2 hrs warm reperfusion

Group C 2 Piglets 2 hrs DHCA at 18°C, 2 hrs warm reperfusion

Group D 2 Piglets 1 hr DHCA at 18°C, 8 hrs warm reperfusion

Group E 2 Piglets 90mins DHCA at 18°C, 8 hrs warm reperfusion

Surgical preparation was as described in chapter 4. DHCA was undertaken without specific myocardial protection other than pericardial slush. Separation from CPB was

performed using noradrenaline infusions as described. Hourly blood gas and haemodynamic recordings were made. At the end of the protocol, piglets were perfusion fixed and the whole brain harvested intact, followed by further immersion fixation in 4% paraformaldehyde at 4°C for 3-4 days before being sectioned, paraffin-embedded and processed for haematoxylin and eosin light microscopy. Slides were examined jointly with a neuropathologist with a special interest and research program directed towards hypoxic brain injury. Careful examination was made of several brain areas noted by others to be susceptible to injury in neonatal piglets, including **neocortex, basal ganglia, hippocampus and cerebellum.**

6.5.1 Results

There were no significant differences between perioperative variables. Representative sections from sham animals are shown in figures 6.1 – 6.5, to provide an indication of normal cortex, basal ganglia, hippocampus and cerebellum. Numerous scattered hyperchromatic neurons were noted in all sections of both controls (figure 6.5). These are a common occurrence in normal perfusion-fixed brains[124] and are considered to be artefact related to the perfusion-fixing process[1].

In no animal from any of groups A-E were neurons identified that would be deemed ischaemic, necrotic or apoptotic in the opinion of a trained specialist in ischaemic neuropathology. This was supported by the lack of any objective differences between sections when examined blindly, including in the number or distribution of these hyperchromatic cells considered to be artefact.

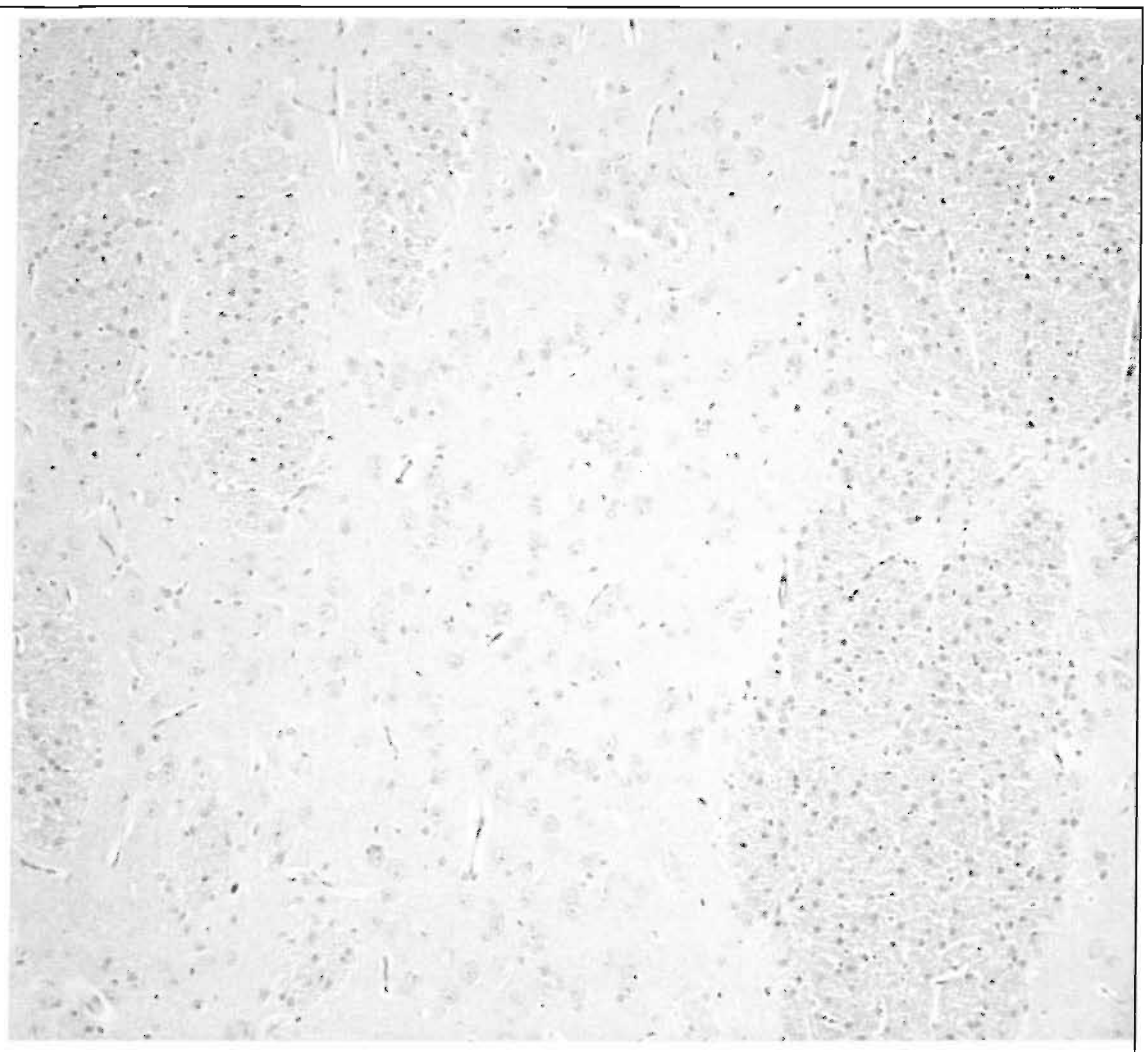


Figure 6.1 Section of the caudate nucleus showing normal neurons.

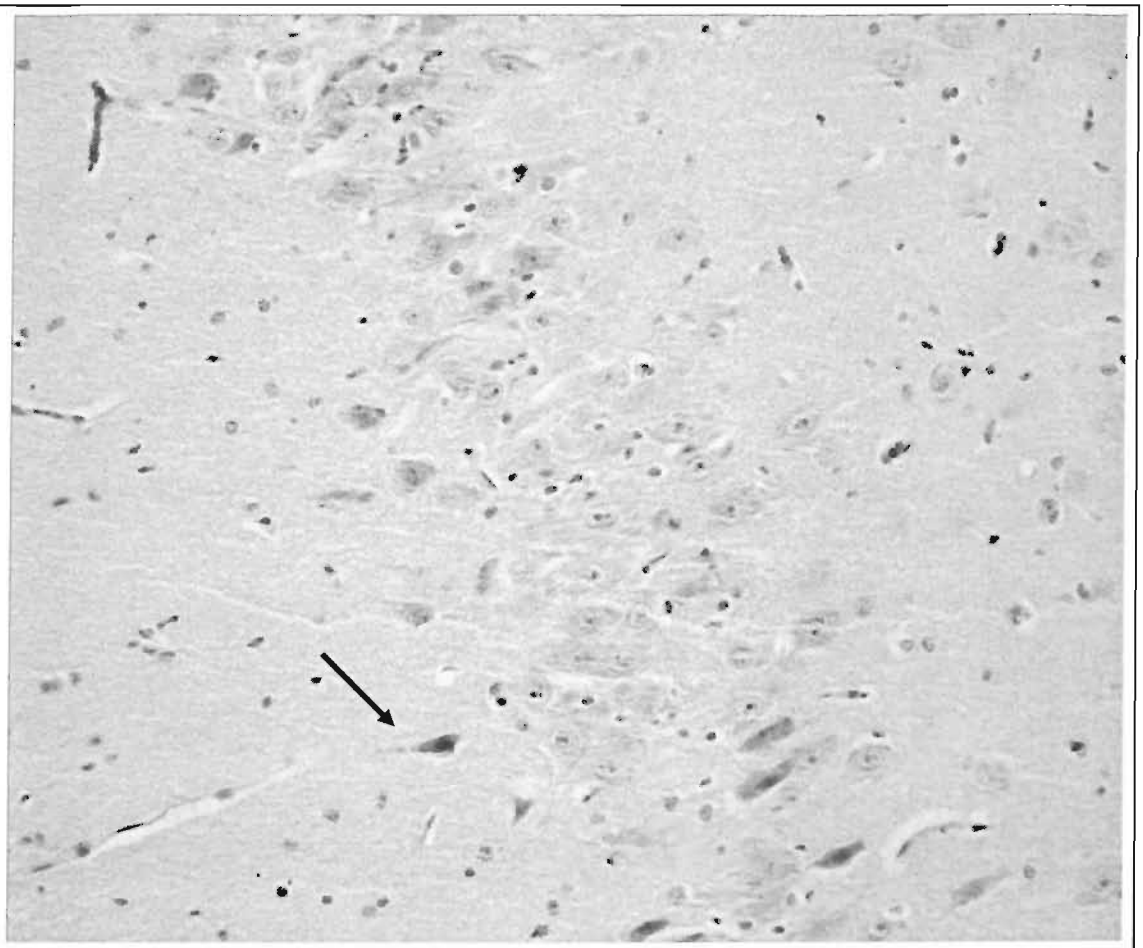


Figure 6.2 A representative slide of neocortex, showing neurons indistinguishable from those not subjected to CPB or ischaemia. Arrowheads are "dark" neurons which are artefact and found in equal numbers in control brains[1].

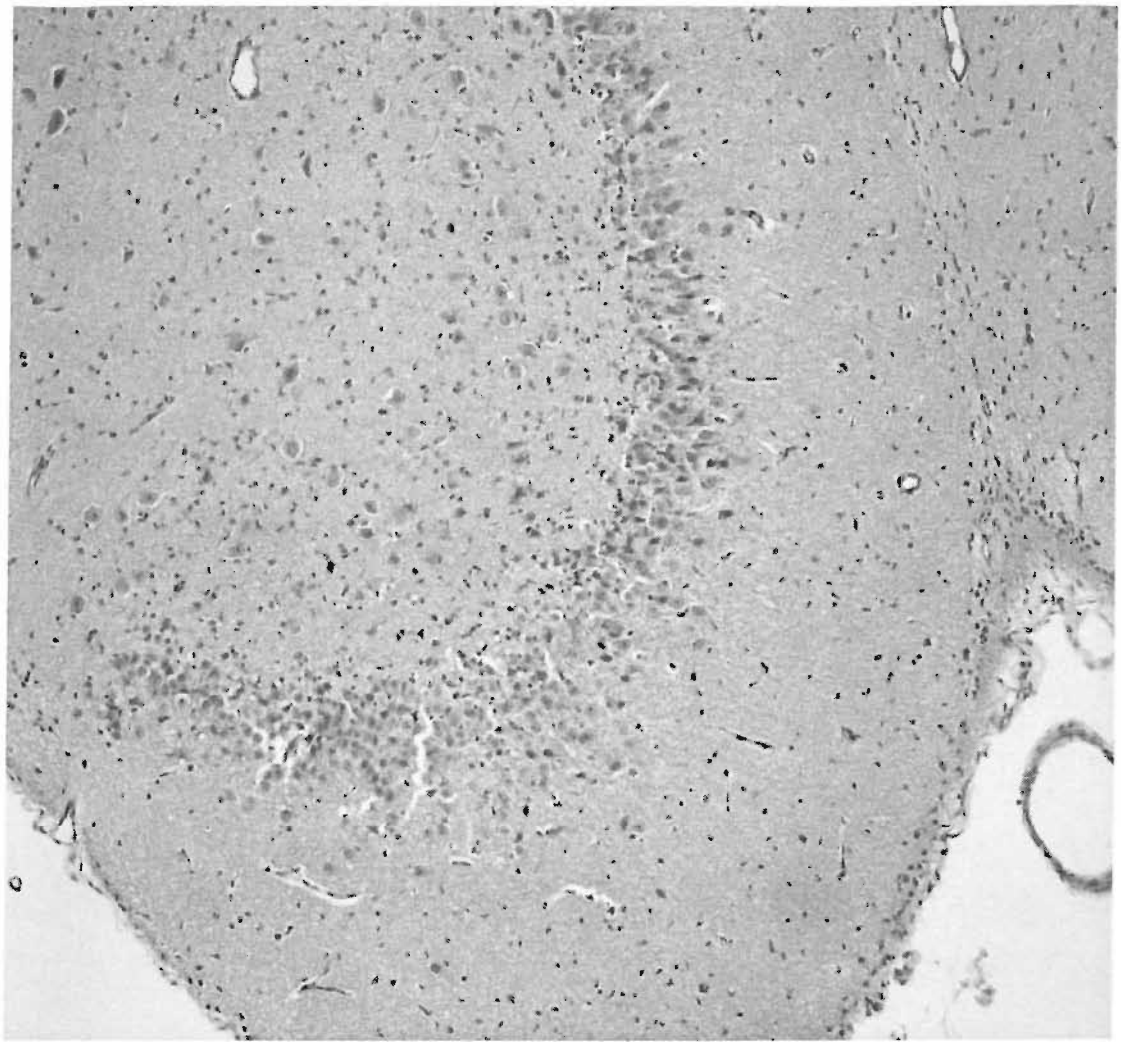


Figure 6.3 Section of hippocampus (dentate gyrus). No evidence of ischaemia is present and the slides were indistinguishable from control animals

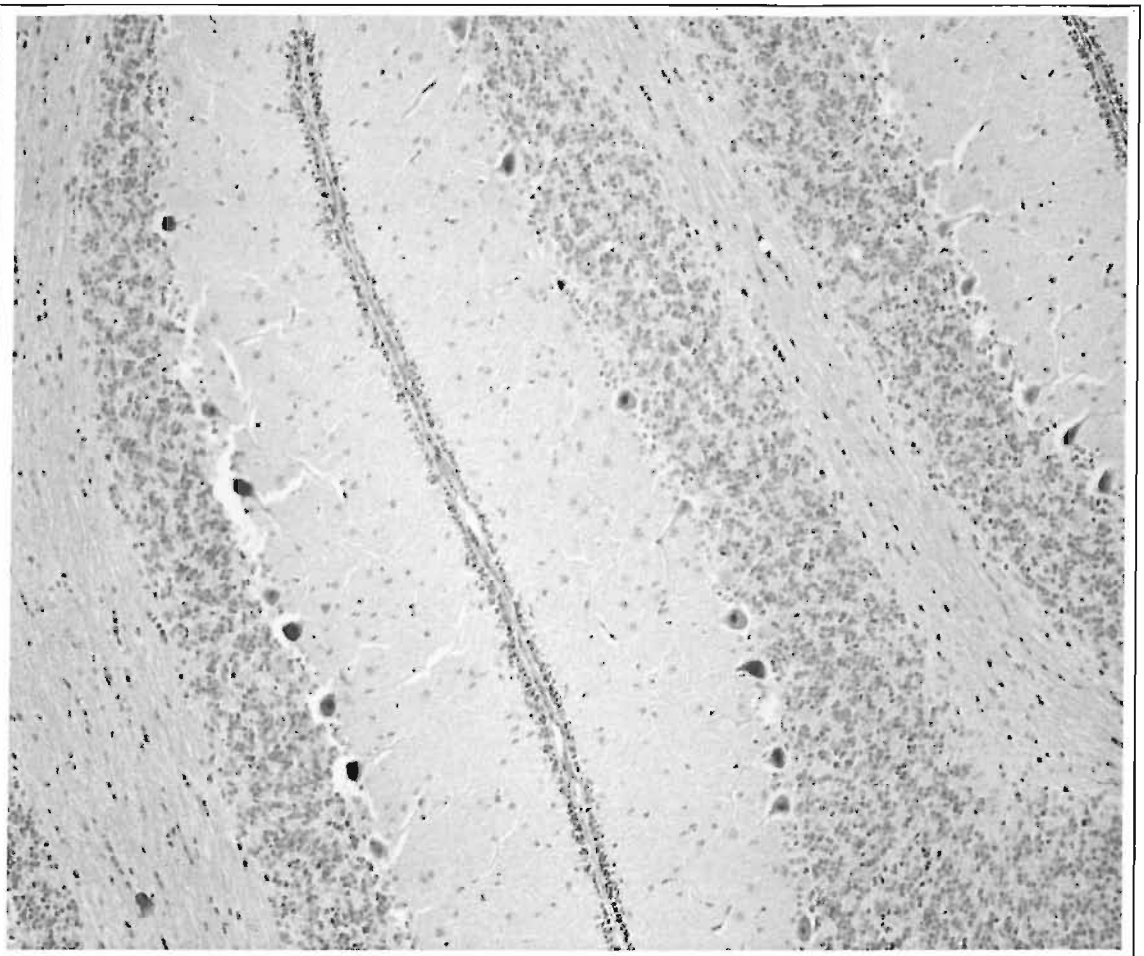


Figure 6.4 Section of normal cerebellum. The "dark" neurons raise suspicion at first glance. They are equally found in non-ischaemic and uninstrumented animals[1].

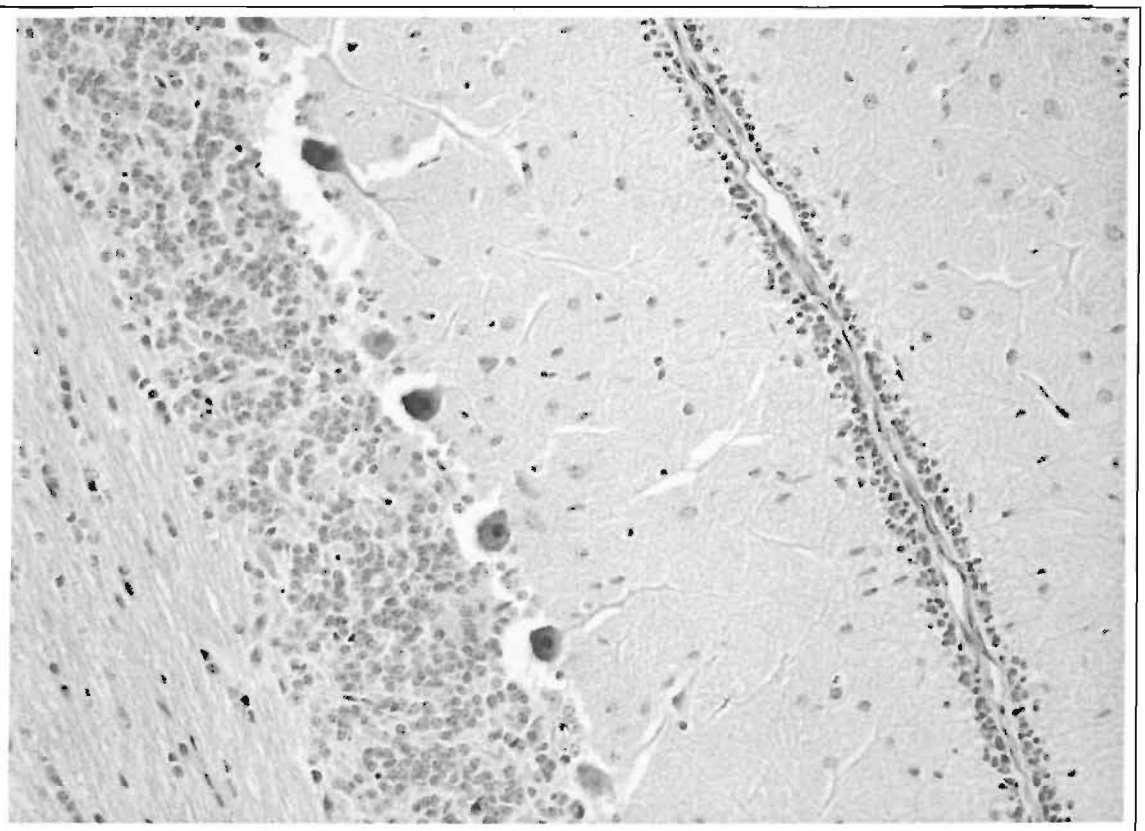


Figure 6.5 Examination under higher power. These "dark" neurons have clearly distinct nuclei and nucleoli. They are believed to represent artefact resulting from the tissue processing steps, and should *not* considered to reflect injury. They occur in control animals and experimental animals, in all regions of the brain[1].

6.6 Experiment 2

Group F 2 Piglets 90mins circulatory arrest at 25°C, 8 hrs warm reperfusion

Group G 2 Piglets 2 hrs Common Carotid Artery Occlusion at 25°C with 8 hrs reperfusion

Without formal myocardial protective mechanisms, increasing durations of cerebral ischaemia are at the expense of increasing myocardial ischaemia and therefore deteriorating haemodynamic recovery following CPB. Although weaning following 90 minutes of total circulatory arrest was possible with repeating pericardial slush, attempts with longer periods of circulatory arrest were unsuccessful and therefore abandoned. Therefore, attempts to harness histological brain injury were reluctantly made by instead increasing the temperature of cerebral ischaemia to 25°C (moderate hypothermia).

A common technique for eliciting experimental global cerebral H-I is to isolate the cerebral circulation through bilateral common carotid occlusion. With the remainder of the systemic circulation intact, ischaemic embarrassment on the myocardium and systemic tissue beds is therefore limited. This was a possible route by which the ischaemic period could be

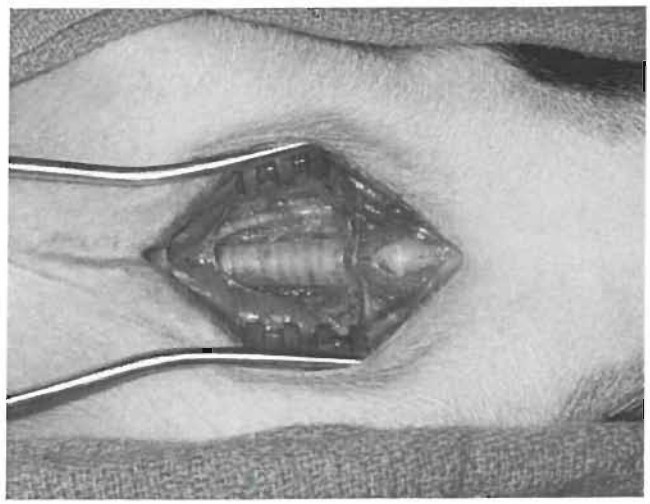


Figure 6.6 Common carotid artery skeletonized in preparation for occlusion

increased without compromising survival. The common carotid arteries are easily exposed during the surgical tracheostomy (figure 6.6), and therefore, in **experimental group G**, bilateral common carotid artery occlusion was undertaken at 25°C for a 2 hour period followed by survival to 8 hrs.

6.6.1 Results

There perioperative variables were not different between the groups. Thorough examination of cerebral neocortex, basal ganglia, hippocampus and cerebellum revealed no evidence is ischaemic injured neurons in any of the sections. It was not possible to differentiate the experimental groups from control slides.

6.7 Experiment 3

Group H Piglets 1 hr DHCA at 18°C, 20 hrs warm reperfusion

Group I Piglets 2 hrs DHCA at 18°C, 20 hrs warm reperfusion

6.7.1 Technique for myocardial protection

As is the case in clinical cardiac surgical practice, the principal determinants of outcome following periods of experimental CPB include myocardial function, systemic inflammation and bleeding. The need to amplify ischaemic insults sustained during CPB by using lengthy periods of DHCA exacerbates not only the systemic inflammatory response, but also exposes the myocardium to increasing durations of uninterrupted ischaemia. In acute experiments attempts are made to minimise this by frequently packing the pericardium with ice, but nevertheless some activity usually remains. Although 90 minutes of myocardial ischaemia was tolerated such that

functional recovery to 8 hrs was possible, it was felt that longer durations of ischaemia would not be possible without a method of formal myocardial protection. In fact, the concept of avoiding any myocardial ischaemia and thus removing any component of haemodynamic insufficiency from the model is extremely appealing. This is one of the major limitations of the reports describing cerebral outcome following closed-chest piglet CPB[122].

The two further components of early outcome – inflammation and bleeding – influenced the decisions in how to progress further towards greater ischaemia with longer reperfusion periods. One option was to continue working at less profound hypothermia (25°C or higher). This was less appealing for a number of reasons, but principally that the concept behind the use of neonatal piglets is that they represent the closest model to human neonates and therefore to remain with more clinically relevant hypothermic temperatures (below 20°C) was preferable. In addition, though raising the temperature of global ischaemia would indeed exacerbate the cerebral impact, the worsening systemic lactic acidosis might make prolonged survival increasingly difficult. It was for this reason that the goal was to remain at deep hypothermia and instead press for significantly longer reperfusion.

In introducing a method of myocardial protection, consideration was given to administering formal cardioplegia. In fact, initial attempts were made (un-presented data) to instil oxygenated blood from the CPB pump with supplemented by K^+ (20mmol/l) via an additional cardioplegia cannula in the aortic route after application of an ascending aortic cross-clamp. This process was extremely cumbersome, not least because of the posteriorly lying aorta in the piglet pericardium. Furthermore, this additional aortic puncture added to problems with haemostasis at the end of the procedure.

It was during the experimentation with this system of cardioplegia that the concept arose of using the arterial CPB cannula for the continuous delivery of oxygenated blood to the coronary circulation, and instead isolating the *entire* systemic circulation from the CPB during periods of circulatory arrest. Such a system would involve the application of a cross-clamp *distal* to the aortic cannula and then running the pump at extremely low rates to perfuse the coronary arteries (figure 6.7). Essential to this technique would be the elimination of any collateral or retrograde flow, and closely monitoring the perfusion pressure of the coronary flow to prevent excessive pressures causing myocardial barotrauma. The former was achieved through concomitant occlusion of the SVC and IVC (figure 6.8) to prevent any risk of retrograde flow to the head, and closely monitoring the arterial blood pressure in the thoracic aorta. Any elevation in thoracic blood pressure would indicate collateral flow around or beneath the clamp, due its sub-optimal positioning. Similarly the persistent thoracic

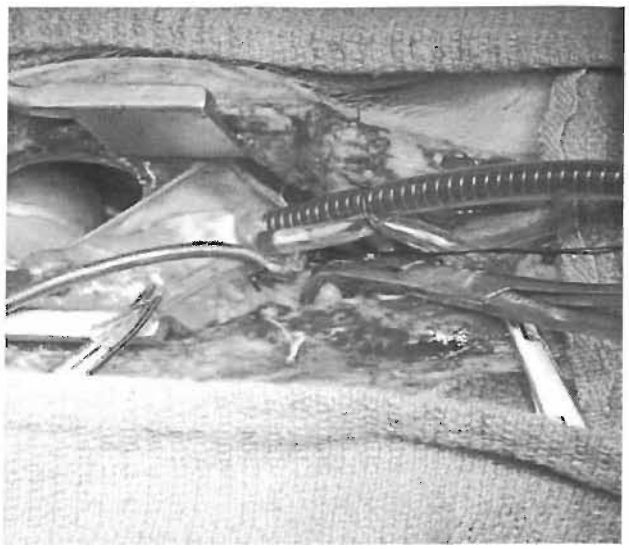


Figure 6.7 The aortic cross-clamp is distal to the arterial cannula and is also occluding the SVC.

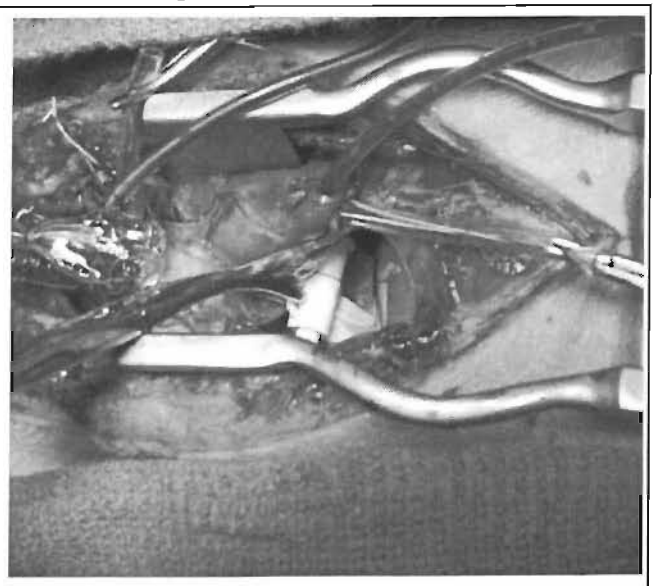


Figure 6.8 The plastic clip is occluding the IVC. Aslo visible is the LV vent.

aortic blood pressure approximately 0mmHg serves to reassure that there is no possibility of antegrade flow through the cerebral circulation. Regulation of the coronary perfusion is by means of a pressure transducer in the arterial CPB line. At low flow rates through DLP cannulae, the pressure gradient across the cannula is lost, such that line pressure approaches aortic pressure. A mean of 30-50mmHg was ideal in providing visible myocardial perfusion for the entire duration of circulatory arrest using. This system was extremely successful in optimizing haemodynamic recovery following CPB, such that the use of myocardial inotropes (adrenaline or dopamine) was eliminated in the entire project thereafter. Any rise in thoracic aortic blood pressure (occurred once) dictated a breach from protocol and elimination of the animal from the study.

6.7.1a Prolonged survival

Having decided that prolonged reperfusion periods were necessary and that this would be performed under full general anaesthesia and ventilation, an intensive care environment was necessary to ensure optimal survival conditions.

Following wean from CPB, protamine was administered (1ml per 1000iu heparin used) and the animals decannulated. Cannulation sites were oversewn using pledgeted 5/0 prolene and haemostasis ensured. After inserting pleural and pericardial chest drains the sternum was approximated with 1/0 catgut and the skin then closed with 2/0 vicryl.

A mini-laparotomy was performed to allow surgical insertion of a bladder catheter, and all peripheral wounds were closed to reduced heat loss (figure 6.9)

The animal was then supported ventilated and anaesthetised (intravenous propofol 3-6ml/hr) for the duration of the reperfusion period, with invasive monitoring. Continuous observation was required for the entire duration of the reperfusion period.

Serial blood gas and haemodynamic measurements were used to ensure stability. A heat lamp was used to maintain a normothermic core temperature (figure 6.10).

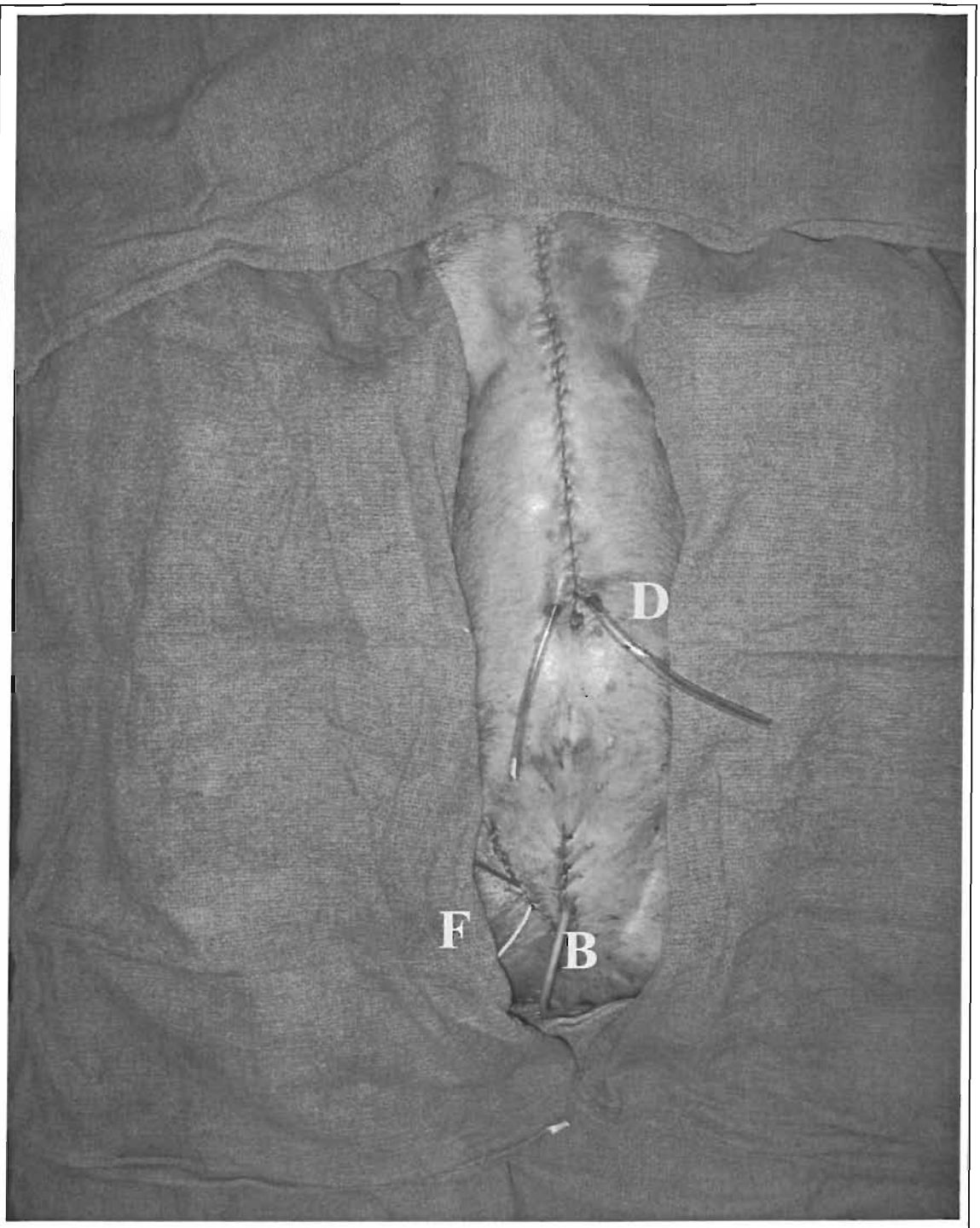


Figure 6.9 The piglet weaned off CPB with all wounds closed. Two pleural drains are in place (D). A bladder catheter has been inserted (B), and the arterial line and femoral line (administereing propofol) are visible (F).

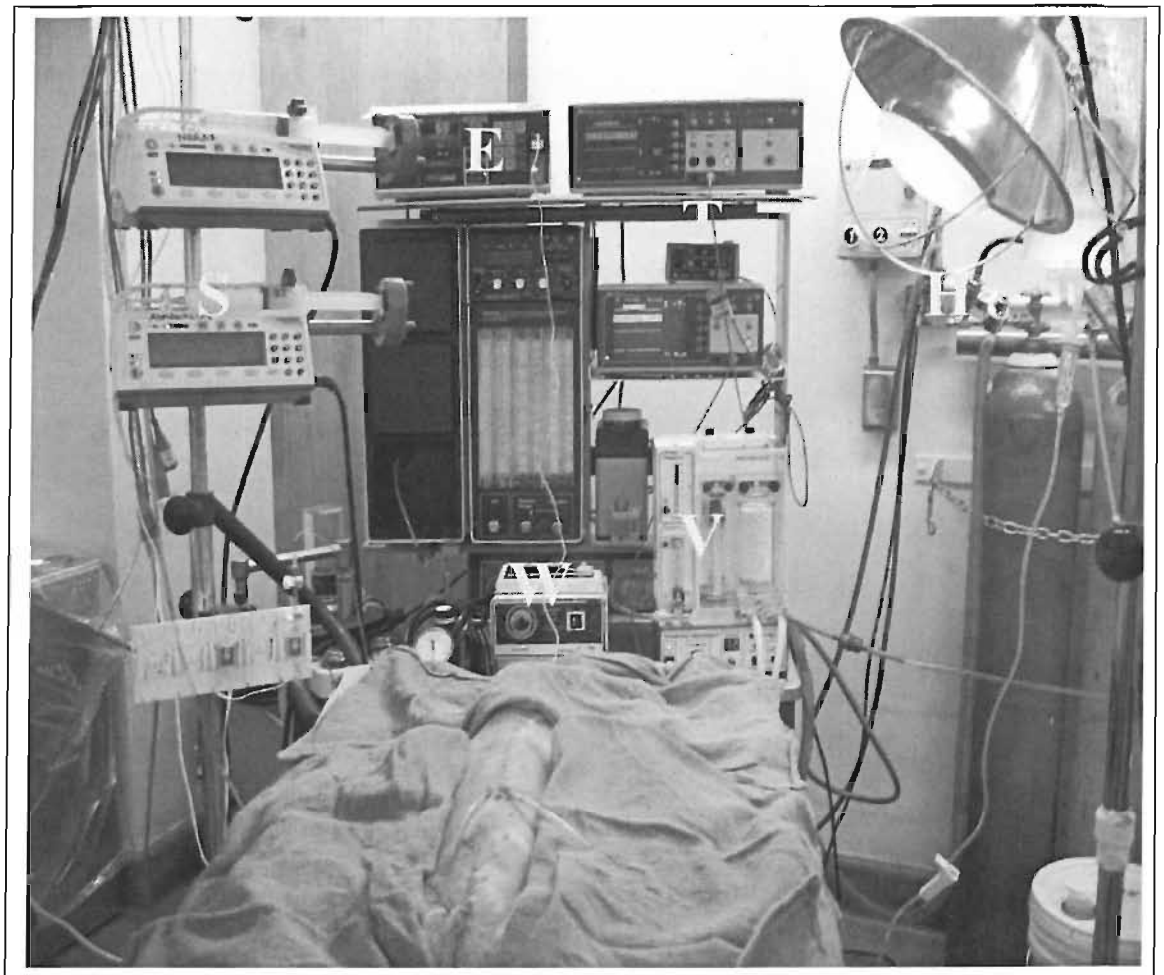


Figure 6.10 Laboratory intensive care set up. The piglet is managed in this fashion for 20hrs. This requires continuous supervision. The experiments therefore take 28hrs in total until completion. H = heat lamp, S = syringe drivers (propofol and noradrenaline), W=water blanket, V=ventilator, T=temperature monitors, E=end-tidal CO2 monitor.

6.7.2 Results

There were no significant differences between the perioperative variables amongst the groups. Both sets of animals had an unremarkable post-operative period.

In all sections from group H slides were indistinguishable from control slides. In contrast, **2 hours of uninterrupted total circulatory arrest at 18°C with 20 hrs reperfusion (group I) were the only CPB-reperfusion conditions in this entire project, that elicited any detectable neurological injury on histological examination by haematoxylin & eosin or Fluoro-Jade™ (figures 6.11 – 6.15)**

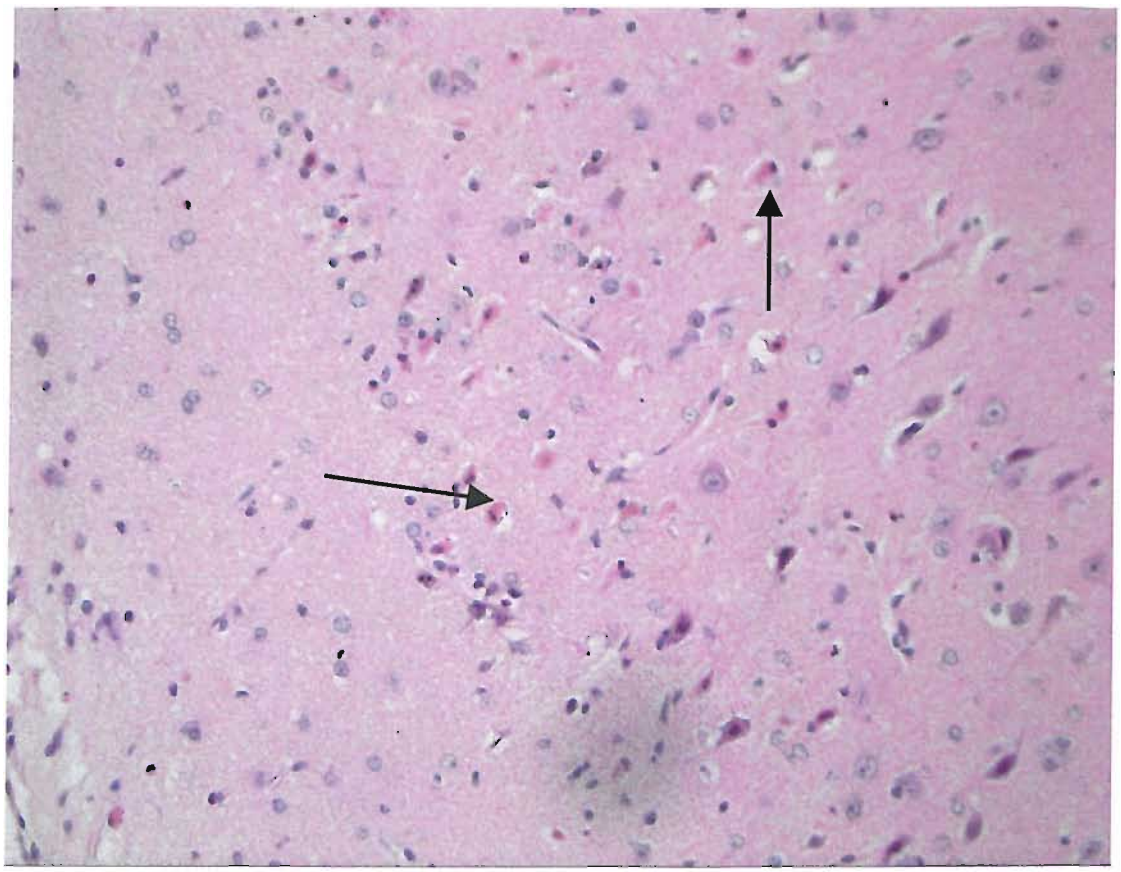


Figure 6.11 Neocortex from an animal exposed to 2hrs DHCA and 20hrs reperfusion (group I), showing several scattered ischaemic neurons (arrows). These are pink (eosinophilic), with hyperchromatic and fragmented nuclei. This represents irretrievable damage.

Figure 6.12a and b *(following two pages).* Figure of a mammalian hippocampus, showing the subregions. Ammon's horn is divided into regions based on cellular types (CA (*cornus ammonis)* 1-4). CA1 are the largest hippocampal neurons and comprise the *outflow*. They are deemed to be particularly sensitive to ischaemia. The dentate gyrus is also shown.

Below is a low power view of a piglet hippocampus showing the dentate gyrus and ammon's horn regions.

The page following (figure 6.13a and b) illustrates the superficial end of the dentate gyrus and CA4 neurons that are especially vulnerable in this model (green)

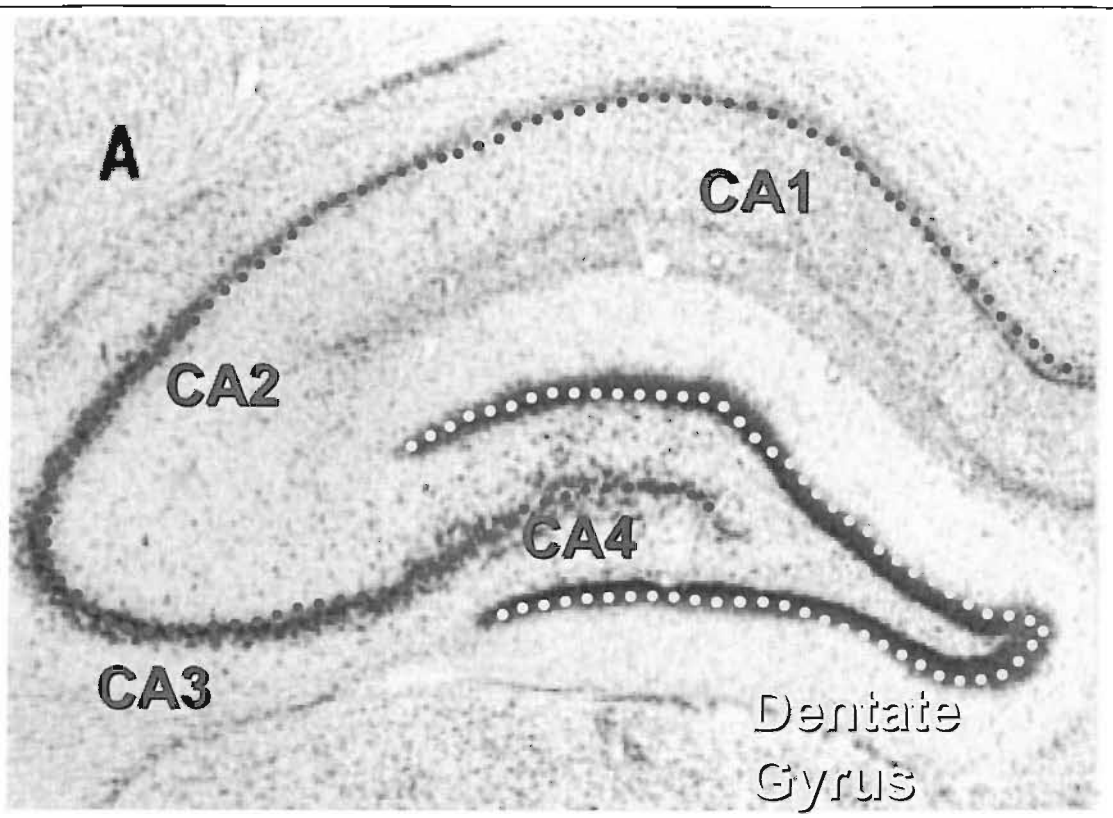


Figure 6.12a

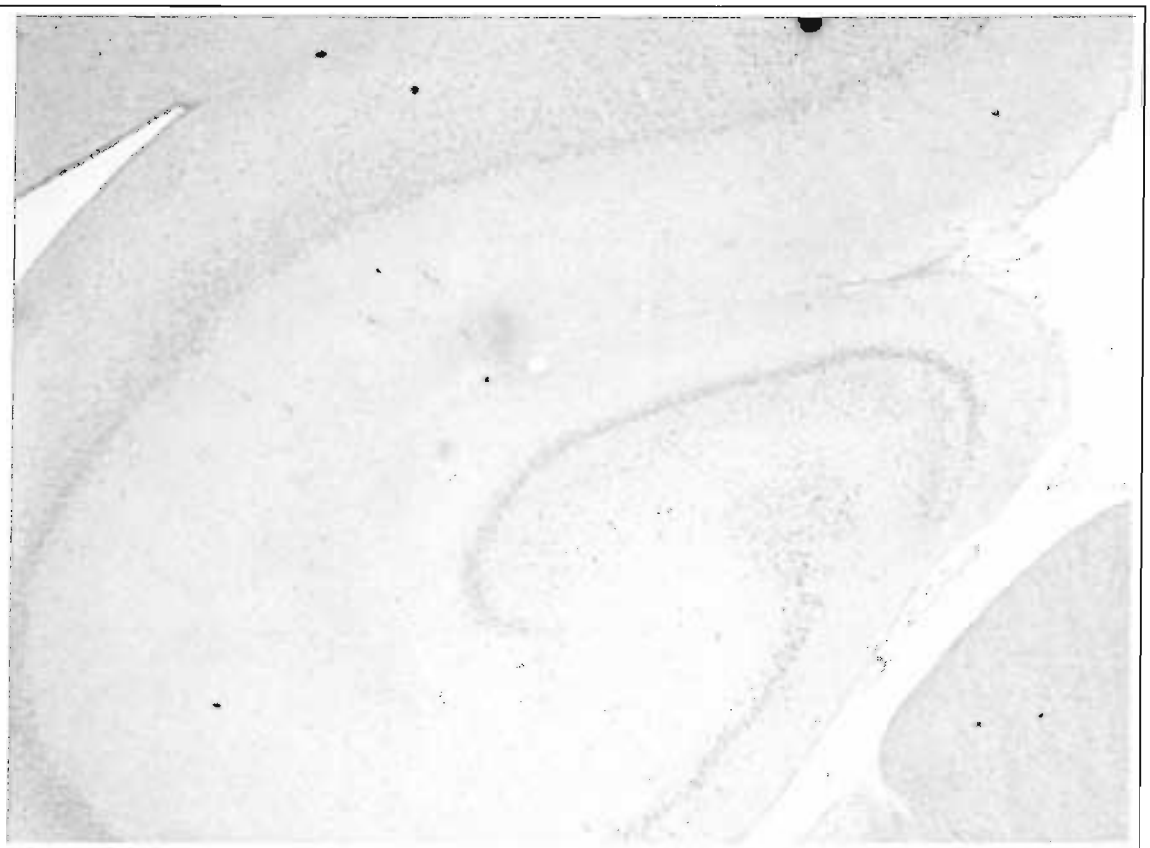


Figure 6.12b

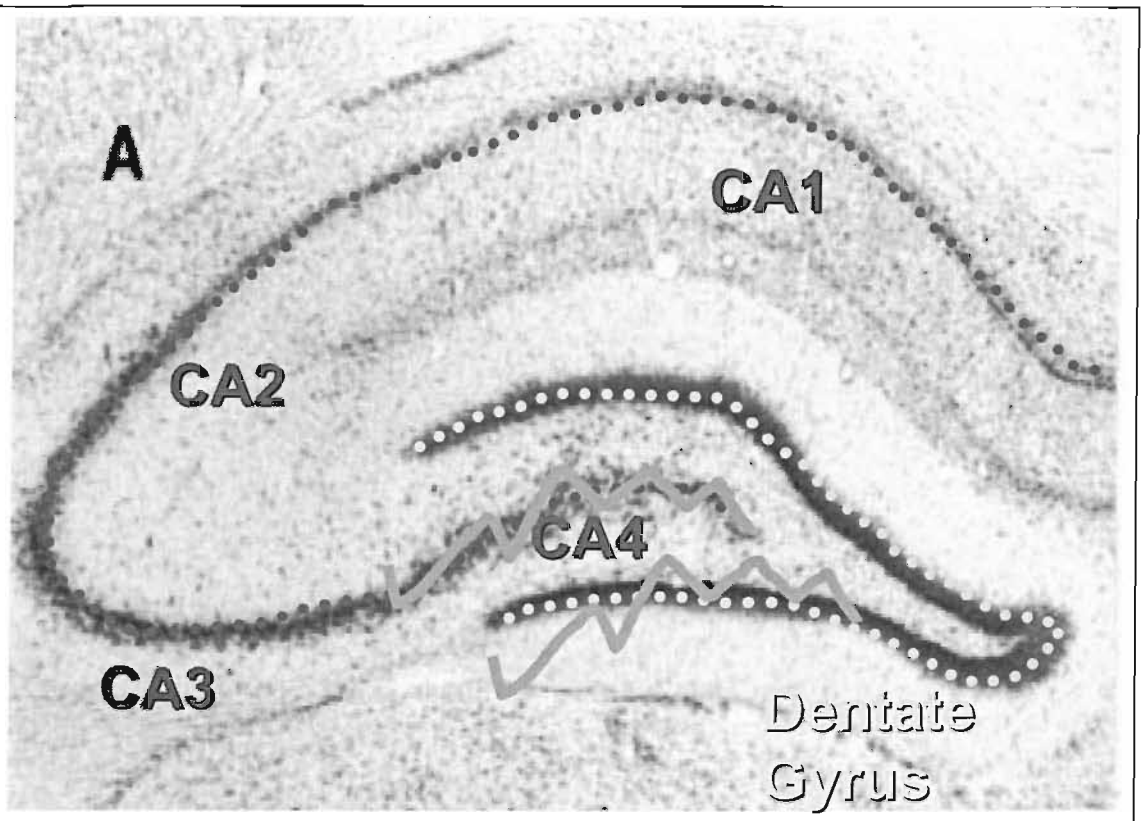


Figure 13a

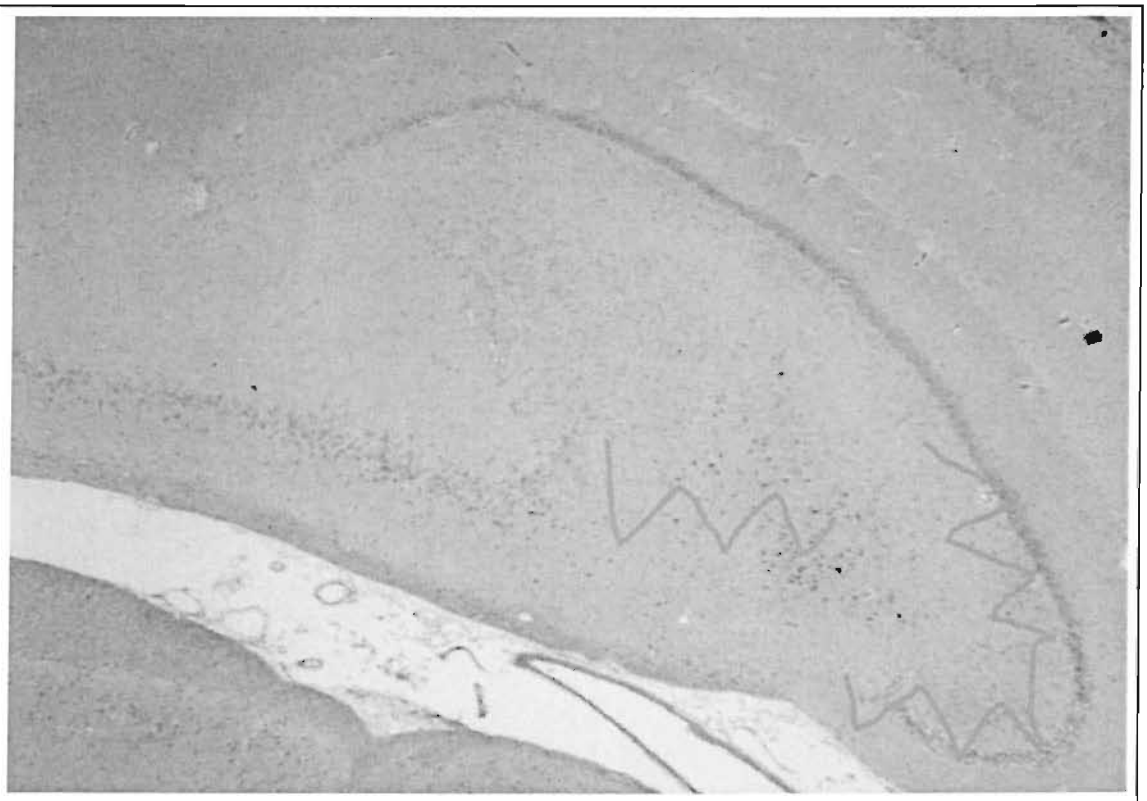


Figure 13b

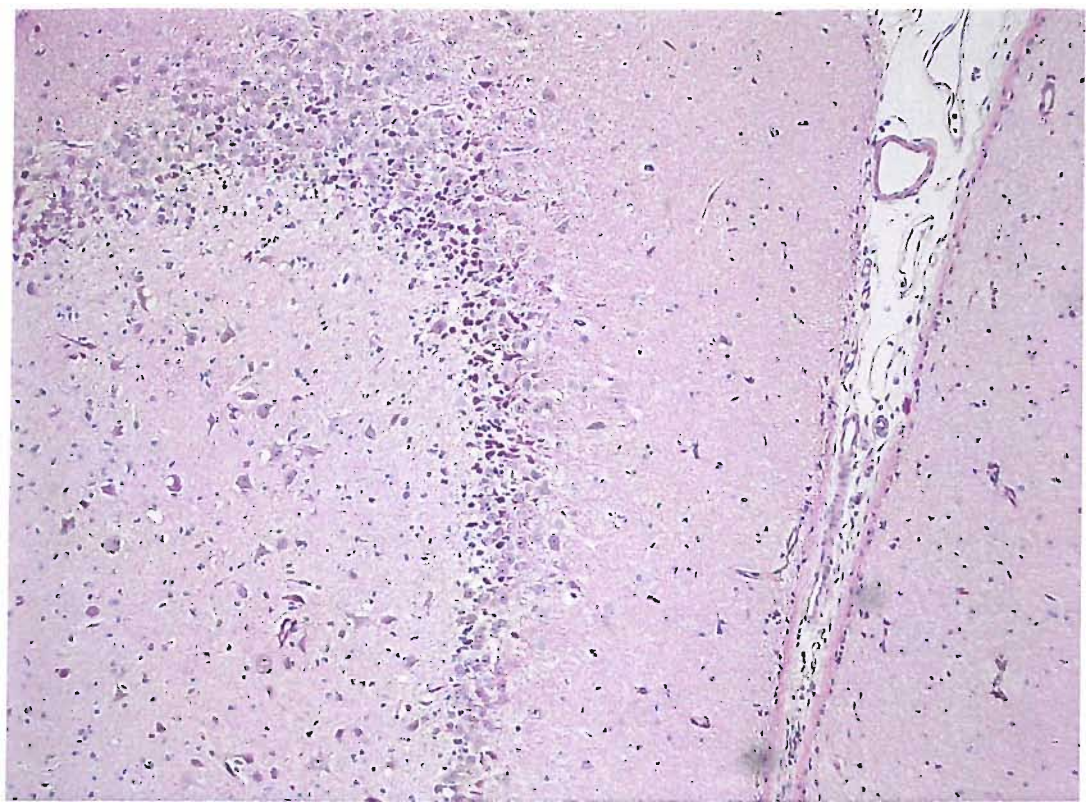


Figure 6.14 Normal dentate gyrus

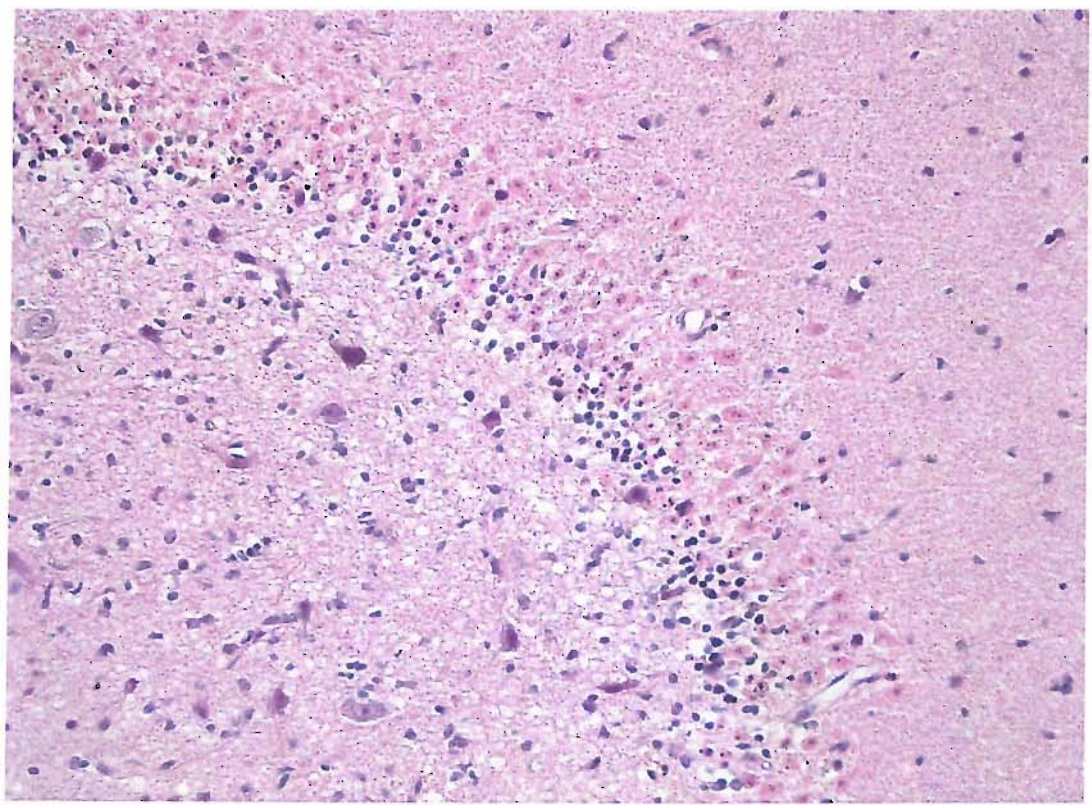


Figure 6.15 Severely injured dentate gyrus littered with ischaemic neurons (group I).

6.8 Histological Injury

As seen in the figures, the ischaemic neurons have a very distinct appearance. Their cytoplasms are prominently eosinophilic, giving rise to a characteristic light pinkish appearance. The normal neurons have easily visible nuclei, containing prominent nucleoli. Nissl substance is also usually apparent in the cytoplasm. By contrast, the ischaemic neurons loose these defining features. The nucleus is obliterated and instead replaced by numerous dark, small fragements and apoptotic bodies (chromatin clumping) (figures 6.11 and 15) ("dead reds"). It should be noted that loosely scattered intensely stained dark neurons occur in all slides including control animals[1] (figures 6.2, 6.4 and 6.5), which may account for false-positive reporting in studies lacking controls. These, although intensely stained, retain the nuclear and cytoplasmic components.

In animals displaying ischaemic injury, the neocortex was only mildly affected. Scattered groups of ischaemic neurons would be identified periodically amongst otherwise unremarkable tissue (figure 6.11).

The basal ganglia were slightly more severely affected, with scattered ischaemic neurons in most high power fields.

The hippocampus was most severely affected, but only in specific sub-regions, and this was a very consistently observed pattern (figure 6.15). The hippocampus is recognised in both adult and neonatal models of cerebral H-I as being particularly sensitive in most species, including humans, sheep, dogs, swine, rabbits and rodents[252]. In addition, the hippocampus is a complex structure with distinct anatomical regions and sub-regions that are preserved across species, and differential susceptibility to injury has

been identified to ischaemia[250]. Detailed examination of the hippocampus was therefore made, and distinct regional vulnerability was also detected in this model.

The injury sustained in this reperfusion model of injury from DHCA occurred in distinct regions. The specific vulnerability of sub-regions of the hippocampus was not necessarily apparent from these preliminary animals in group I. Instead, the following description is based on the subsequent blinded analysis of all animals exposed to this protocol in the remainder of this project. The most severely damaged region was the most lateral part of the dentate gyrus, and with increasing severity, this would extend progressively more medial through the dentate gyrus. The progression of injury then would extend through hippocampal regions CA4 then CA3 (figure 6.13). *In no brains examined throughout this entire project, was injury noted in regions CA2 or CA1.* Furthermore, the deepest part of the dentate gyrus (figure 6.16) was less susceptible than the most superficial part. In fact, selective vulnerability to the dentate gyrus was such a consistent, reproducible and quantifiable observation that it was by far the most sensitive indicator of brain injury. Retrospective blind review of all slides generated by this project indicated that ***lack of injury in the most lateral part of the dentate gyrus almost always dictated that no injury would be found in any other regions of the brain. This could not be said of any other area of brain examined.***

The cerebellum of every animal examined was unremarkable. No ischaemic neurons could be detected by routine light microscopy.

6.9 Quantification of Injury

The hierarchical nature of histological injury in the dentate gyrus and hippocampal CA regions suggested that these regions could be used as a quantifiable scale of injury,

which was devised after having reviewed (blindly) *all histological tissue generated in this entire project.* One problem – distinct from all other experimental models of cerebral ischaemia – with the study of injury following DHCA (exemplified by the negative results in groups A-G) is the extremely mild degree of changes seen. Almost all models involve the exaggeration of injury in order to amplify differences between experimental groups. An example would be the addition of head *warming* during ischaemia in a mouse models of cardiac arrest[253]. The result is tremendous quantities of ischaemic (positive) neurons (up to 80%) in experimental groups. In models of DHCA, injury is so minimal, that conventional methods of quantification are not applicable. Instead, it would be necessary to devise an alternative that would be appropriate to my model, and allow for quantification of severity between groups.

In general, histological grading can be performed either using quantitative or qualitative

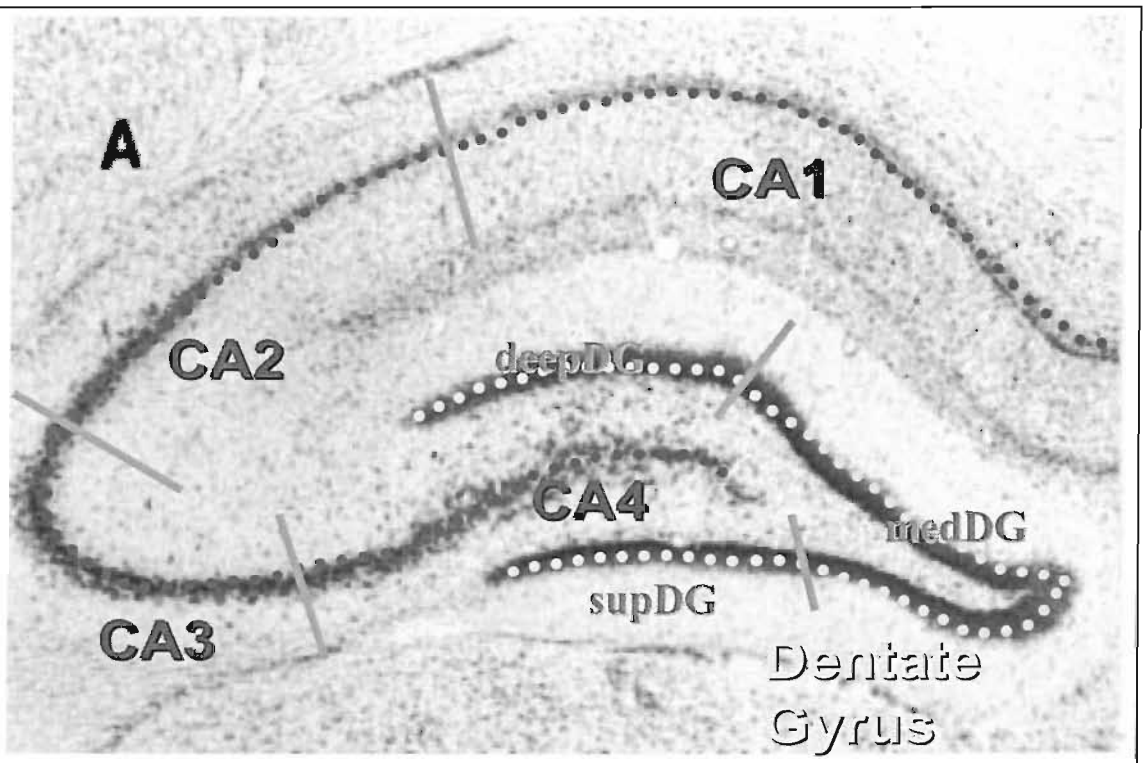


Figure 6.16 Subdivisions of the hippocampus created for subjective regional scoring.

techniques. Advantages of the former include the possible of parametric statistical analysis, greater statistical power and reproducibility, but disadvantages include the painstaking (human) or expensive (analytical counting programs) techniques involved. Instead, blinded semi-quantitative histological staging scores are more common, whereby an overall subjective "score" is given to specific regions. These can then either be considered separately, or in mild models of injury are often summated to provide a total score for each animal[121]. Additional methods include "ranking" experimental subjects according to the subjective opinion of a blinded examiner. Although this may carry power in terms of reproducibility, and can grade any degree of injury severity, its statistical power is greatly reduced, and can be analysed only in a non-parametric fashion.

The consistent pattern of hippocampal injury seen this model of DHCA immediately suggested that a histological severity score might be applied to distinct anatomical hippocampal regions. Subsequent summation would then provide an overall score that could detect even the most mild injury sustained by any given animal. In devising and applying this system, brains from all areas of this project were studied (blinded). The

Histological Score	Morphological Criteria	List of areas studied
0	Entirely normal	Cortex
1	Occasional scattered cells	Basal ganglia
2	Frequent patches of injured cells	Superficial dentate
3	Confluent areas of severe injury	Middle dentate
4	Area completely dominated by injury	Deep dentate
		CA4
		CA3
		CA2
		CA1
		(cerebellum *never positive)*

Table 6.1 Histological scoring system and regions

reproducibility of the regional staining patterns meant that dividing the hippocampus was the most appropriate method (one used by others[251, 252]). Thus the hippocampus was divided up into the respective CA-regions. Furthermore because of the propensity for the superficial edge of the dentate to be more severely affect than the deep edge, the dentate gyrus was divided into thirds, as shown in figure 6.16. Quantification was carried out by two individuals blinded to the experimental protocol. Subjective scores of severity were given for each of the sub regions of the brain listed below. These individual scores could be used for comparison, as well as cumulative scores for the respective brain regions.

6.10 Histochemical staining

6.10.1 Fluoro-Jade™

The aim of using Fluoro-Jade™ was to simplify identification of injured neurons. Staining with Fluoro-Jade™ was technically reproducible and straight-forward, and made identification and quantification extremely simple. The pattern of injury paralleled that of haematoxylin and eosin closely (figures 6.17 – 6.20) but was more sensitive. Its use is especially appealing as an experimental tool because of the reduced subjectivity involved in the identification of "positive" neurons.

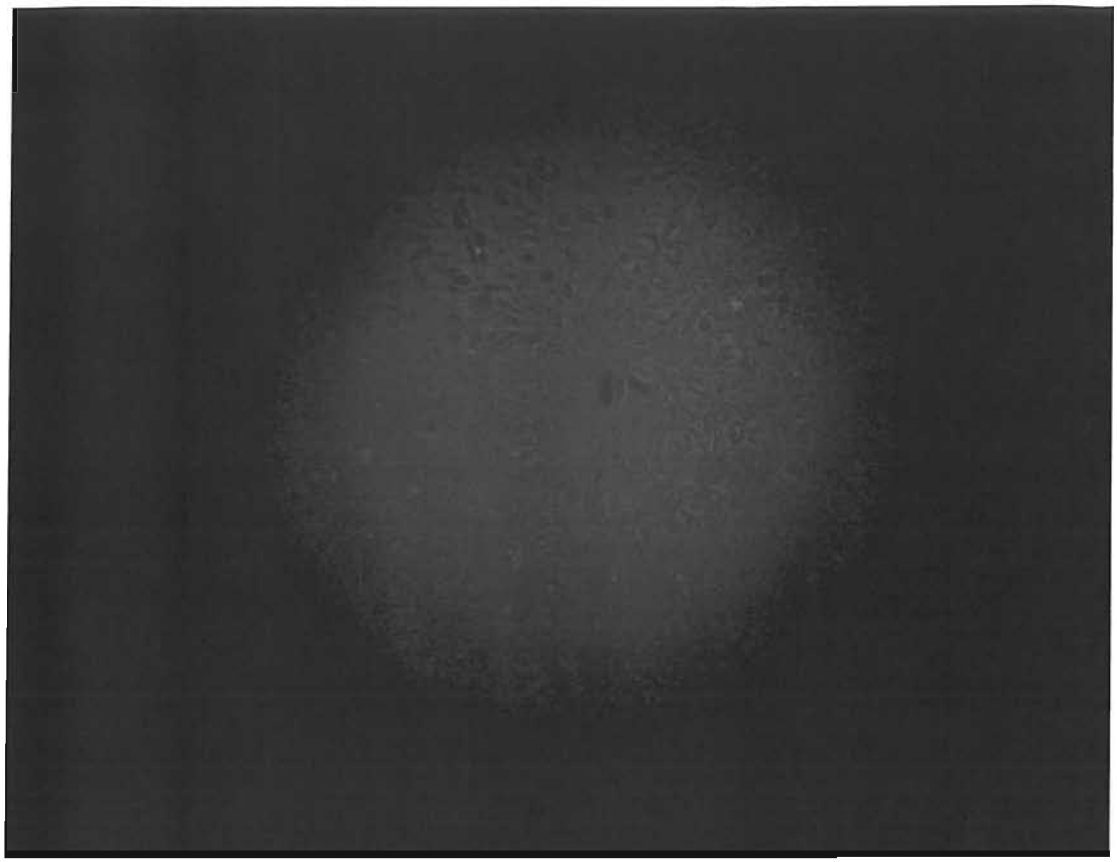


Figure 6.17 Fluoro-Jade™ staining of a control animal's basal ganglia.

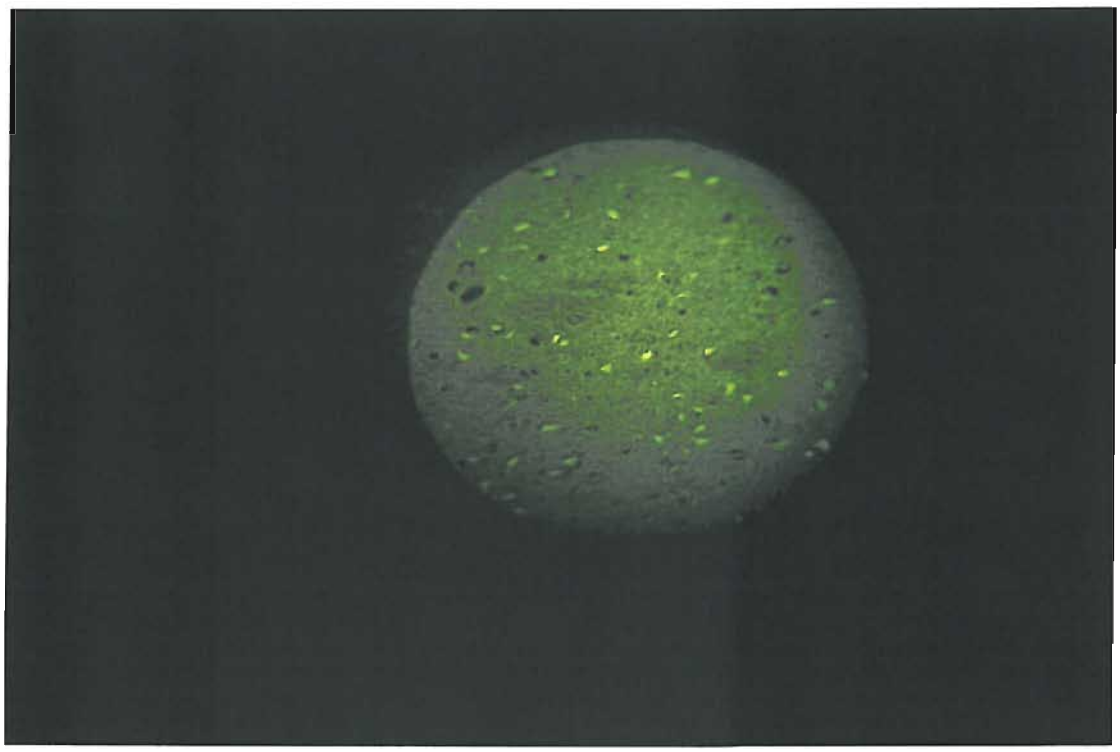


Figure 6.18 Positive staining seen in an ischaemic animal's basal ganglia.

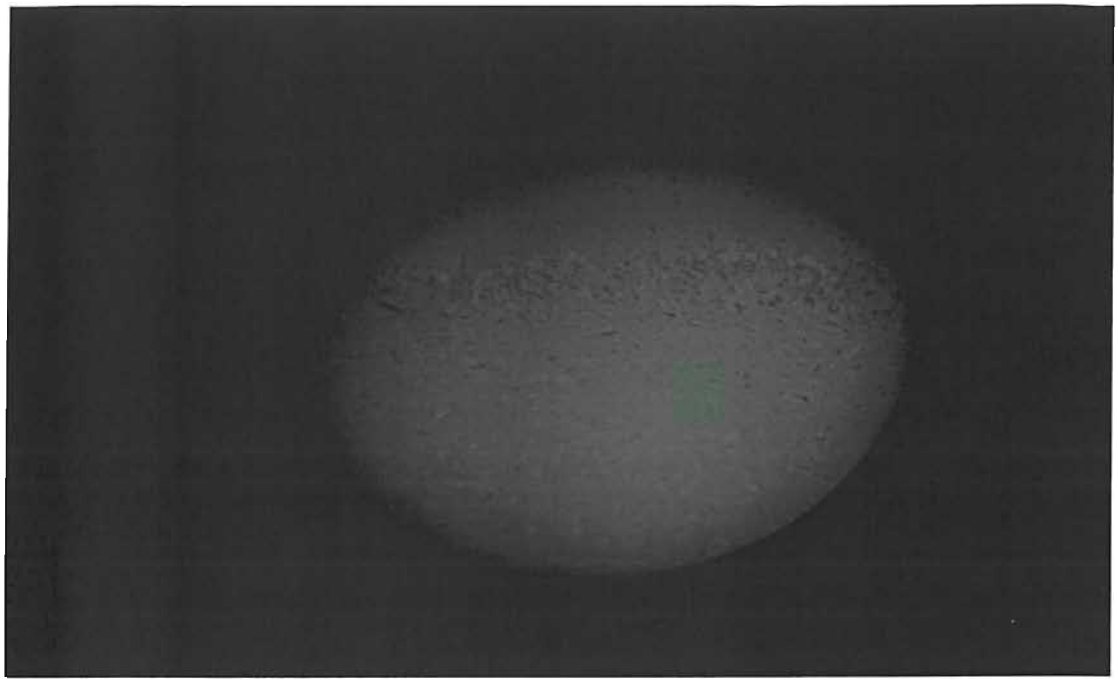


Figure 6.19 Negative Fluoro-Jade™ staining in a control animal's dentate gyrus.

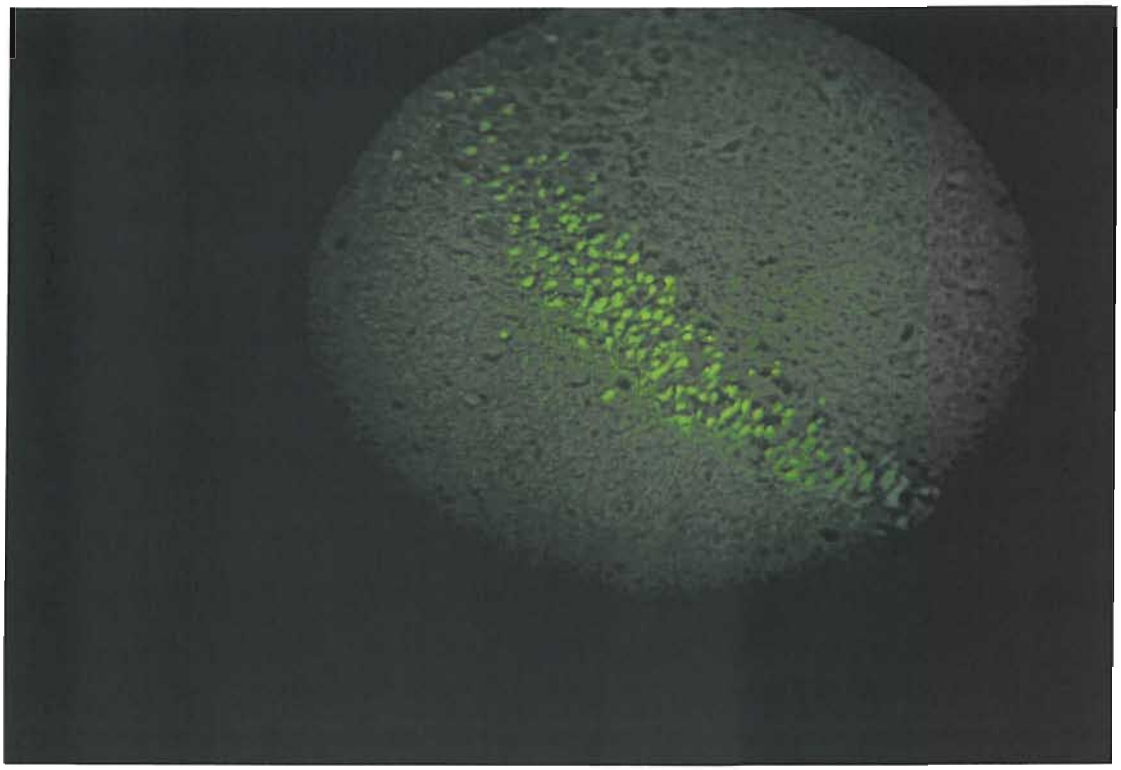


Figure 6.20 Positively stained cells easily visible in the dentate gyrus of an ischaemic animal.

6.10.2 TUNEL and Caspase-3

Despite numerous repeated attempts, it was not possible to generate appropriate staining using two different TUNEL immunohistochemistry kits (Chemicon and Invitrogen), despite control slides (human tonsil) staining appropriately (figure 6.21). A final attempt involving pre-incubation with nuclease (to cause DNA fragmentation and hence a "positive control" with the experimental swine cerebral tissue) also failed to generated staining (figure 6.23). Successful staining of the human tonsil control slides confirms correct function of the TdT reaction conditions, labelling of the fragmented DNA strands and staining using the peroxidase-linked antibody. Because the antigen in TUNEL staining is (generic) nucleic acid, the process is in theory not species-specific, although communication has been received of other laboratories having had difficulty with TUNEL reaction in swine tissue. The subsequent failure to generate staining in swine slides pre-treated with nuclease suggests instead a failure in retrieving antigen upon which the reaction conditions can proceed.

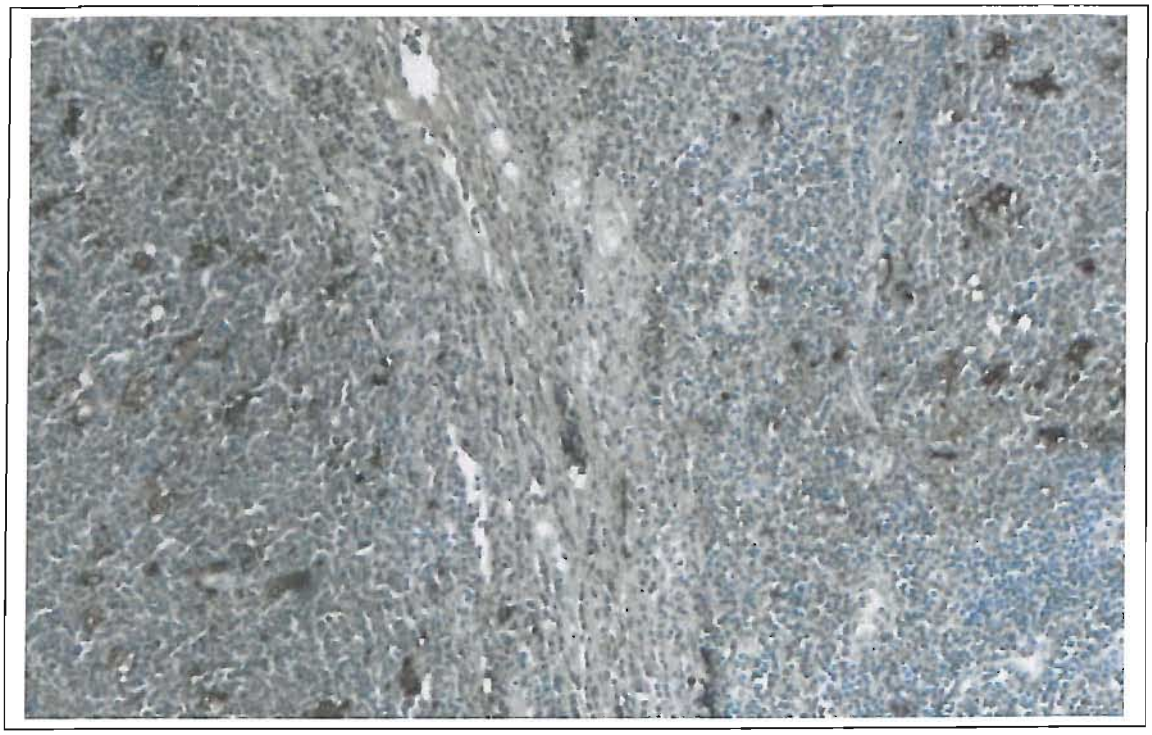
After detailed review of TUNEL literature and discussion with the kit manufacturers, it was concluded that the swine cerebral tissue had been excessively cross-linked in fixative. This would have the effect of being either unable to expose the ends of fragmented DNA or otherwise preventing the action of the TdT enzyme through lack of access to the DNA fragments. This unfortunate conclusion resulted in the need to abandon TUNEL as a potential IHC method for identifying injured neurons from the project. It serves as a reminder that IHC can be frustratingly fickle in terms of substrate and reaction conditions. For future reference the solution would be to avoid cross-linking fixatives altogether for this purpose. Instead, fresh tissue can either be processed frozen and mounted fresh on slides for TUNEL analysis. Alternatively alcohol-based fixation methods (70% ethanol) would preserve integrity of nucleic acid

for TUNEL staining and avoid alteration of tissue structure through cross-linking (section 4.5).

Figure 6.21a and b (*Following page).* Control slides stained with TUNEL. Human tonsil has a high apoptotic rate and positive stained cells are staining apoptotoic lymphocytes, confirming that the IHC technique is working

The page following (figure 6.22 a and b) shows two representative slides from human tonsil stained with TUNEL but replaceing the primary antibody with saline (negative control), confirming specificity for the apoptotic cells..

Figures 6.21a and b:



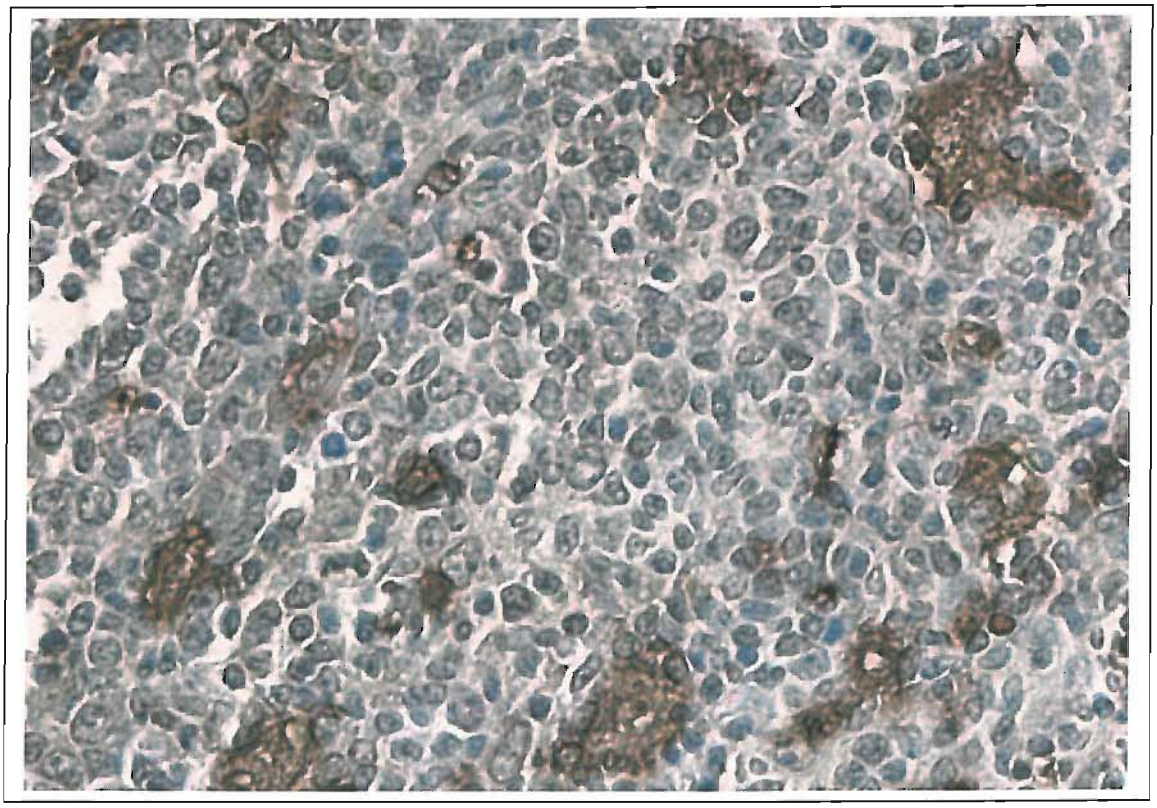
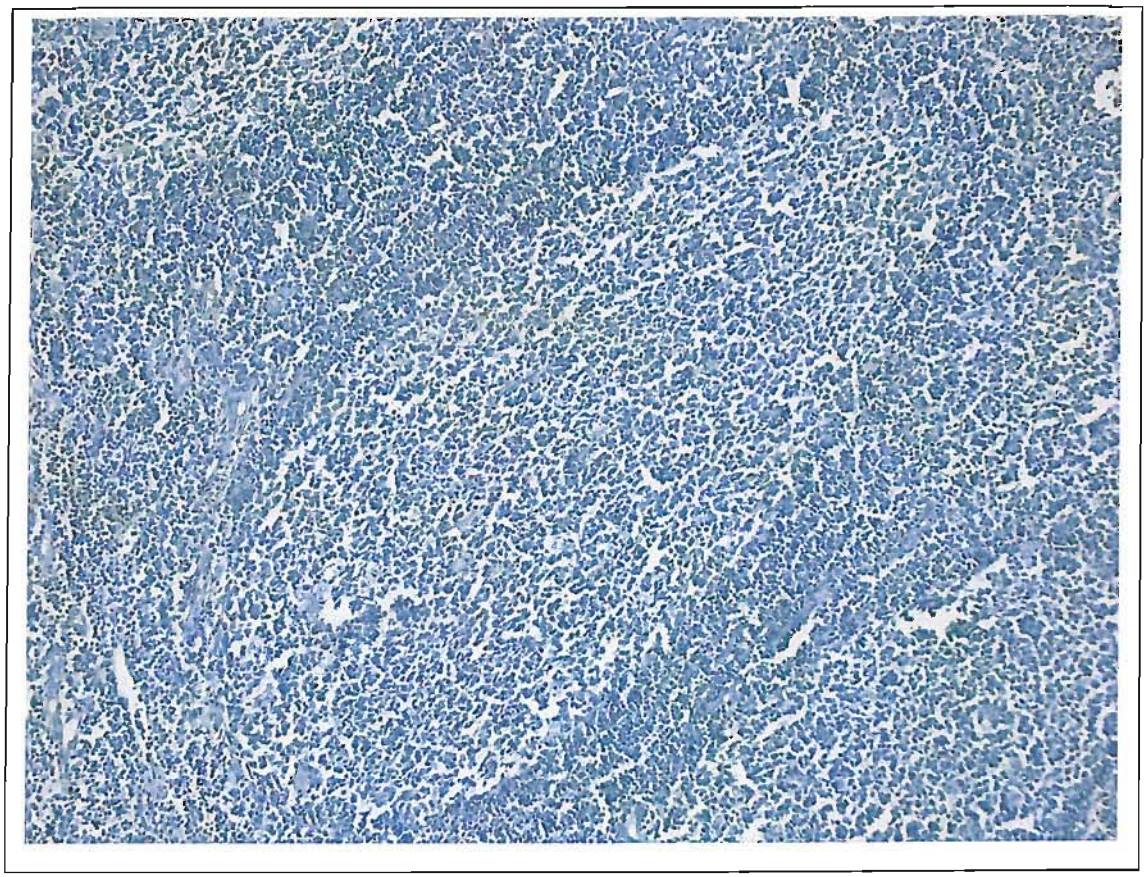
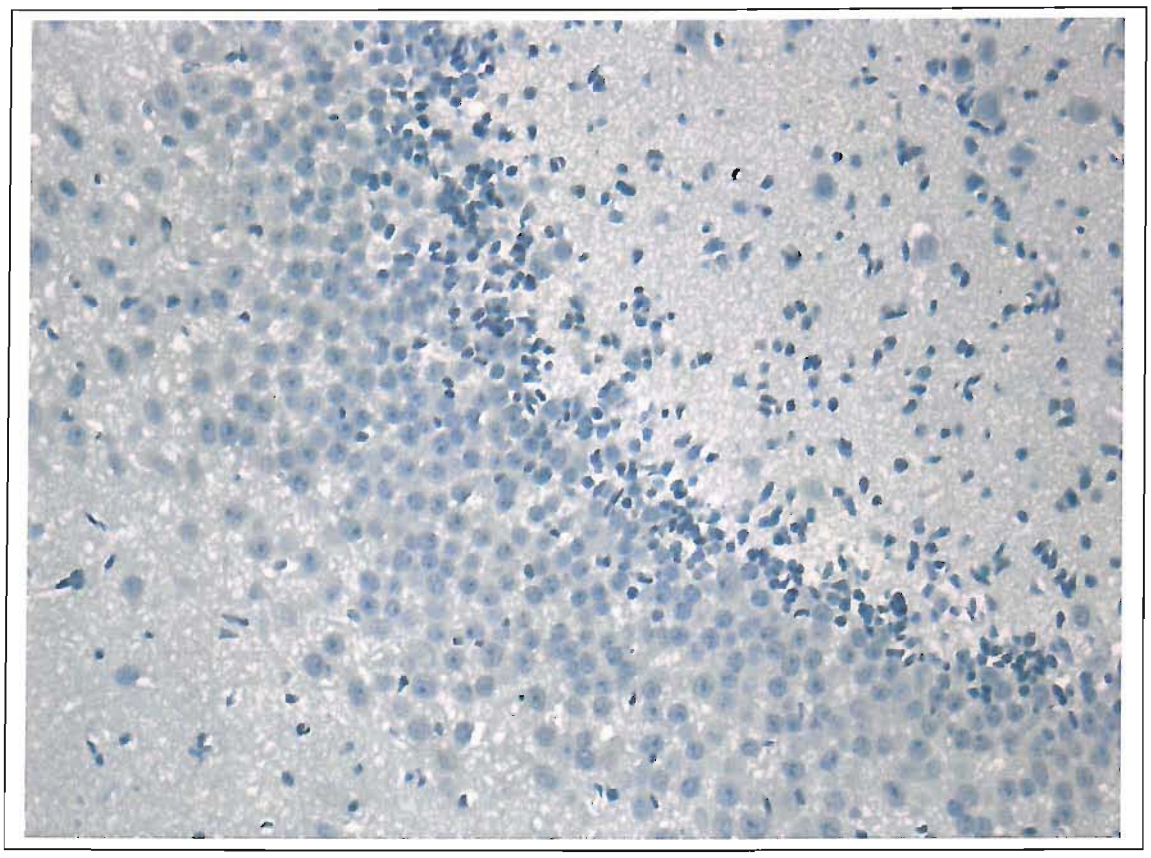


Figure 6.22a and b





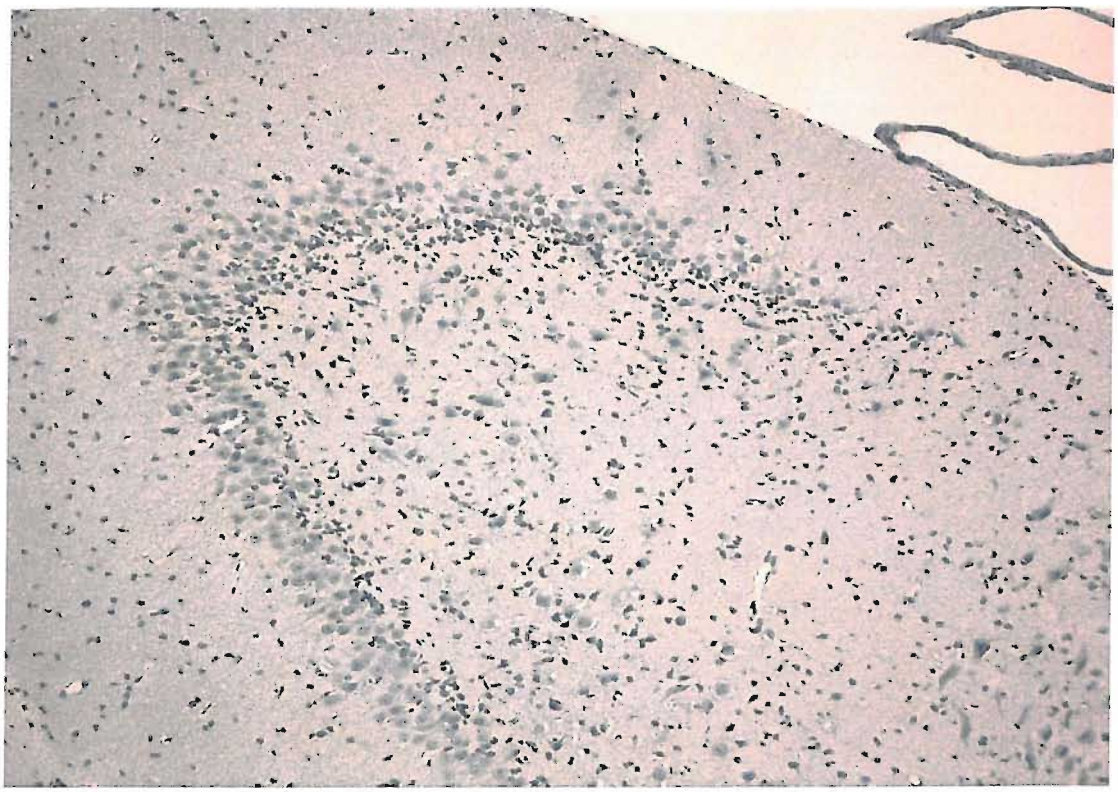


Figure 6.23 The dentate gyrus of an ischaemic animal, stained with TUNEL. No staining was visible after repeated attempts and several kits. This slide has been pre-treated with nuclease, to fragment all the endogenous DNA (and create a positive control from my piglet tissue. The lack of staining indicates a fundamental problem with antigen exposure or retrieval, which was interpreted as over cross-linking of the DNA by formalin.

Caspase-3 was equally problematic, but successful staining was achieved (figure 6.25-26). However, it was extremely weak and required meticulous and enquiring analysis. Use of the technique was initially suggested because its Caspase-3 activation occurs earlier in the intra-cellular chain of events during apoptosis (section 4.6.2a) than DNA fragmentation. The absence of observable histological injury using H&E in Groups A-E raised the suggestion that more sensitive means might reveal injury. Caspase-3 activation might be an example of a very early cellular marker of irretrievable injury. However, the laborious nature of the technique and the extreme paucity of staining made its use unattractive. In fact, the clear injury detectable using H&E and FluoroJade™ in Group I detracted from the usefulness of persisting with Caspase-3 staining. It was not deemed a useful adjunct, and therefore abandoned, if only after considerable time and energy had been dissipated in establishing a successful staining protocol.

Figure 6.24a and b Representative slides of cleaved caspase-3 staining. On the top is a positive control slide from Jac+ve cells (chemically induced apoptosis). The bottom is the same section stained as a negative control.

The page following (figure 6.25a and b) shows extremely faint nuclear staining in ischaemic experimental animals (group H) (arrowheads).

This staining is more prominent in figures 6.26a and b. The stain is nuclear, with the unstained nucleolus prominent. These are cerebellar purkinje cells, which were not identified as ischaemic using routine H&E. This confirms the more sensitive nature of immunohistochemistry as a tool for identifying early ischaemic injury. Reliable and reproducible immunostaining might be a more sensitive endpoint for detecting injury, but because of the considerable problems encountered whilst developing the staining protocol, it had to be abandoned.

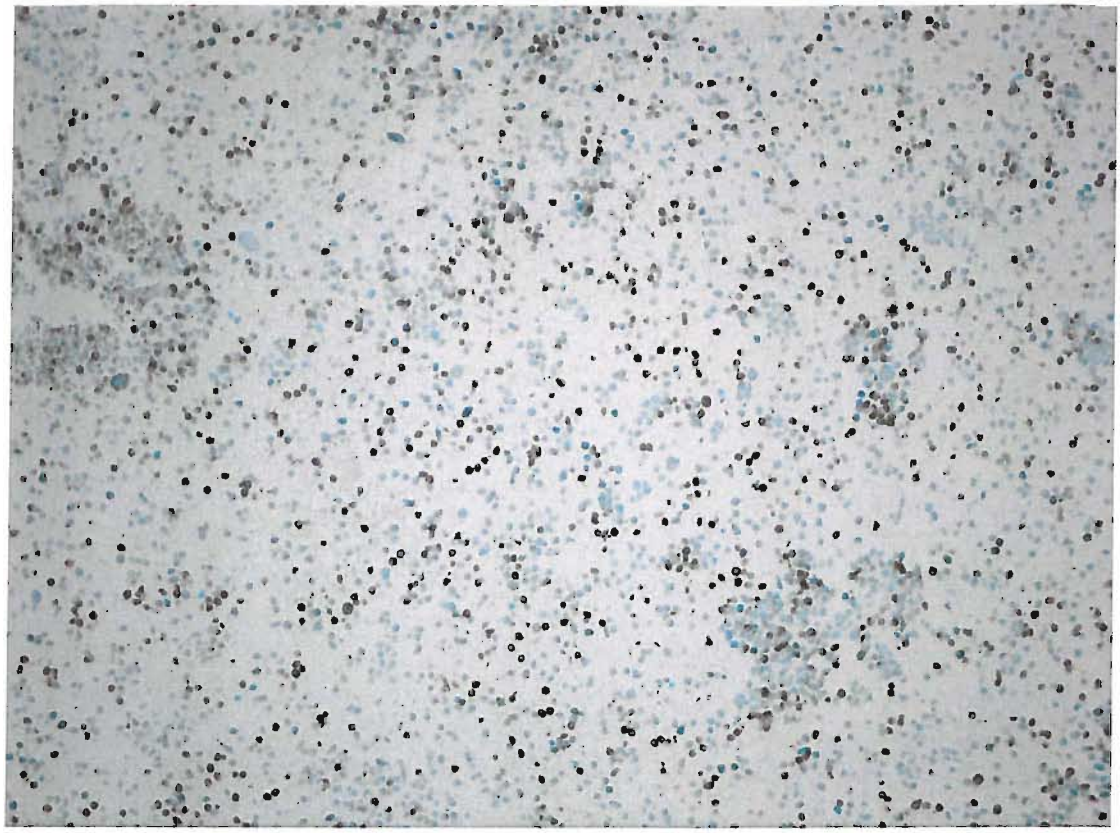


Figure 6.24a

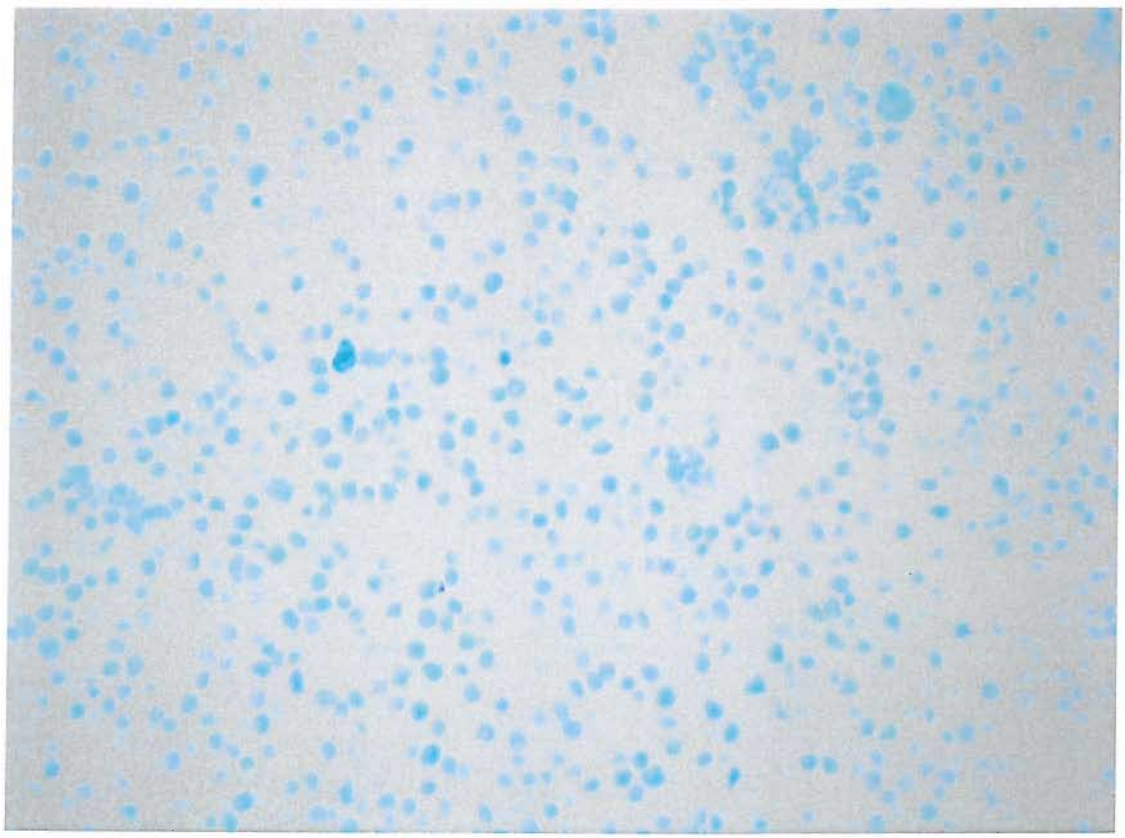


Figure 6.24b

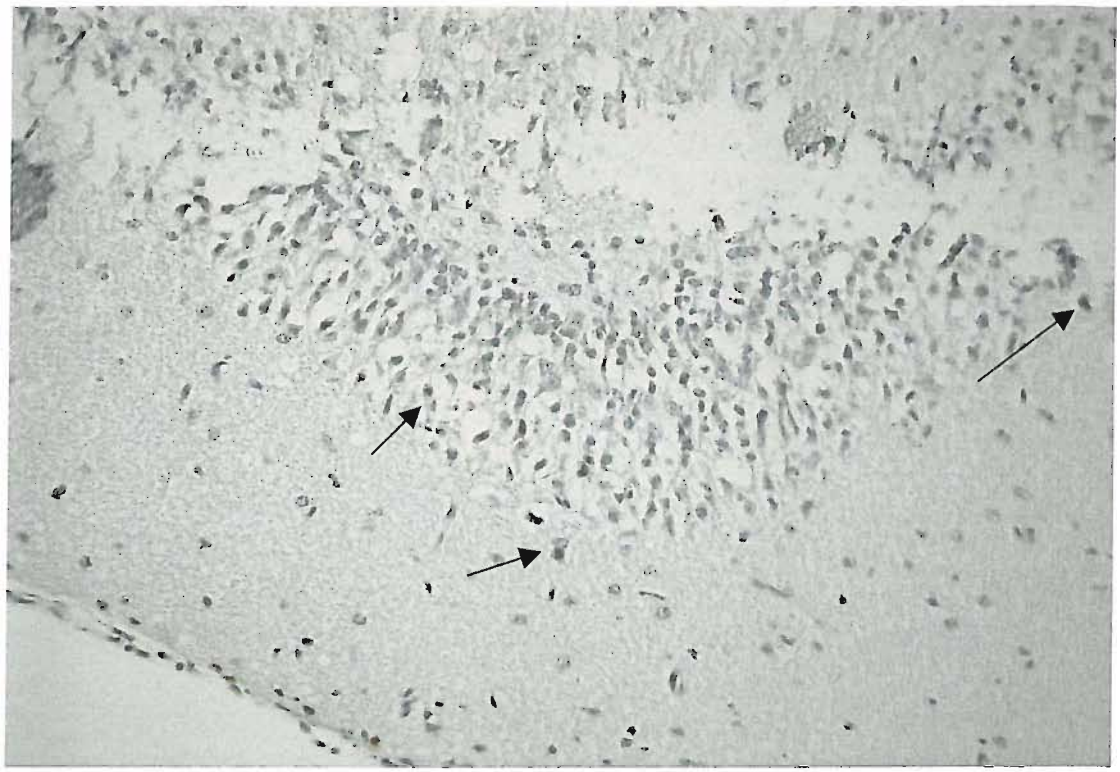


Figure 6.25a

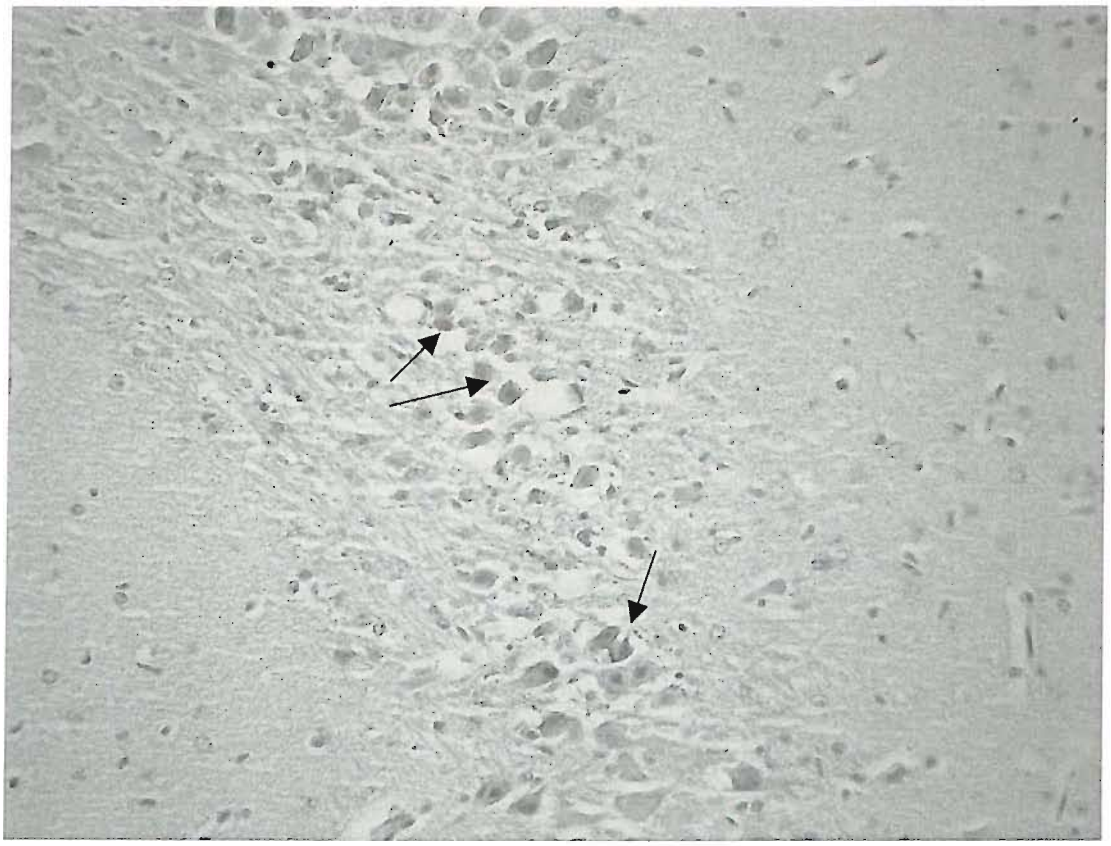


Figure 6.25b

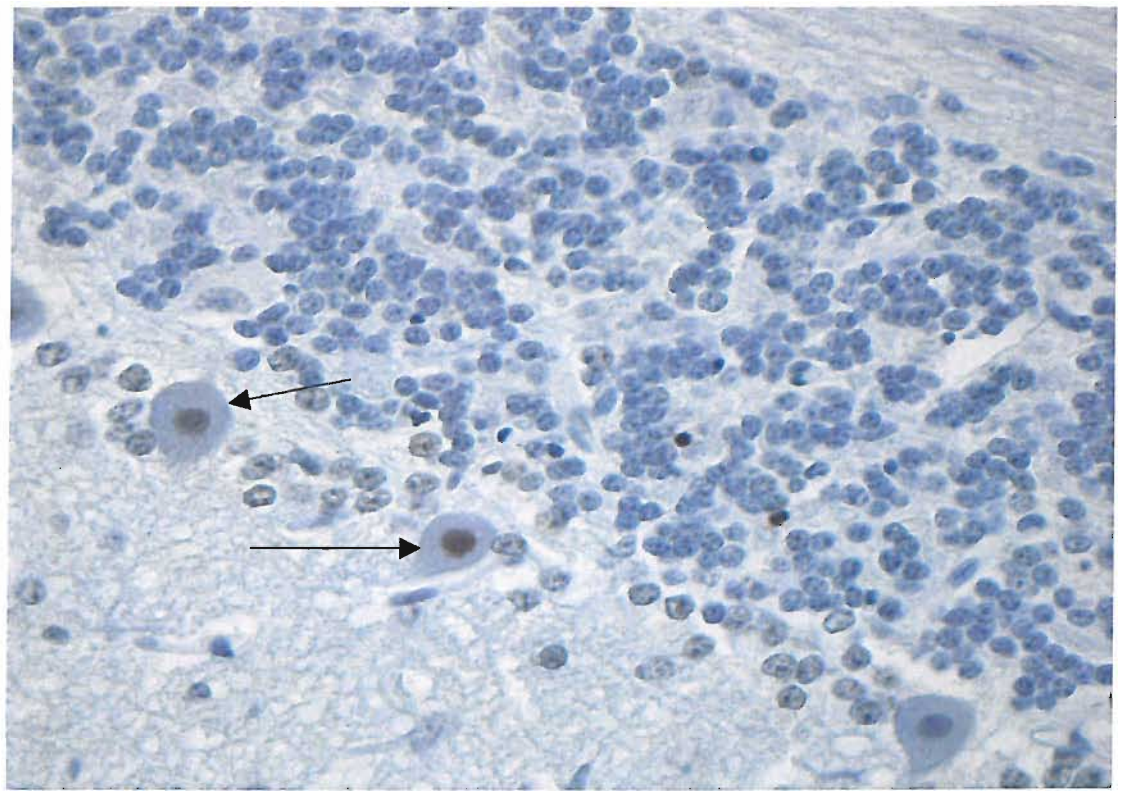


Figure 6.26a

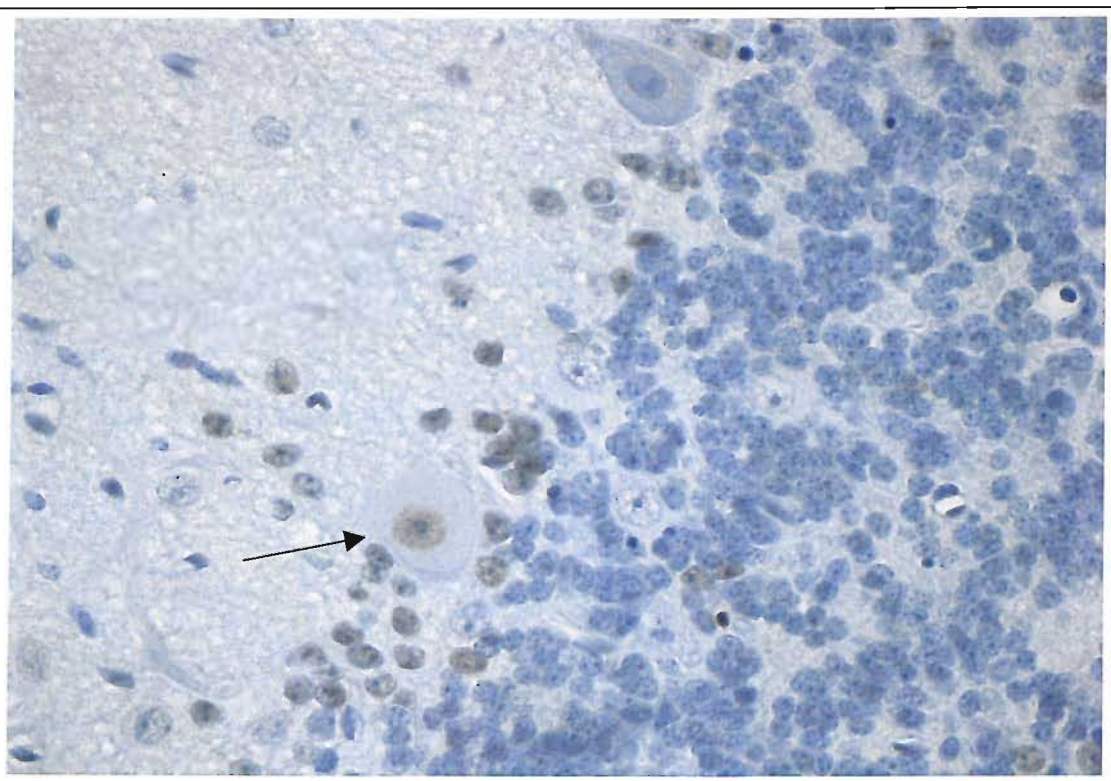


Figure 6.26b

6.11 Conclusions

The most striking revelation from this series of studies is the extraordinary lengths required to elicit injury using deep hypothermic (and, in groups F and G, even with the use of moderate hypothermia) CPB strategies. In fact, with the use of much briefer periods of DHCA in the clinical arena, an immediate supposition is that **no histologically apparent injury would be detectable even after significant periods of reperfusion (20-24hrs).** Hypothermia as a neuroprotective technique is well known in stroke models, and even unintentional drops in temperature of a few degrees from normal can drastically affect the severity of injury sustained. However, the sheer extent of protection such that **no visible injury was seen until a piglet was subjected to as long as 2 hrs uninterrupted DHCA at 18°C** was unexpected.

It is important to emphasise that the corollary is *not* true. The absence of frank histological injury neither implies that no injury has been sustained, nor that a particular technique or strategy is safe. In fact this assertion – that the lack of histologically apparent injury indicates the safety of a defined duration of DHCA – has been made previously, and is unwise[123, 249]. Clinically, a general perception holds that beyond approximately 45 minutes of DHCA, injury is increasingly likely and beyond 1 hour a degree of neurological deficit is almost anticipated. Electron microscopic analysis of piglet brains following 1 hour DHCA with very brief reperfusion periods has confirmed ultrastructural abnormalities, and it is likely that such abnormalities – especially involving dendritic processes – would have functional sequelae. Moreover, the temporal pattern of injury following cerebral ischaemia is remarkably long, with an elevated apoptotic index noted as much as six weeks following the ischaemic insult. This observation is alarming as it suggests that early histological models may not be a true reflection of the injury sustained in the long-term. It will take more complex survival models to address this issue following neonatal DHCA. It is likely that injury

occurring below the threshold for detection would follow similar temporal patterns and contribute to functional deficit.

The duration of reperfusion necessary for the morphological changes to become apparent under light microscopy following H-I have not been clearly established, although occasional reports exist describing histological ischaemic injury as soon as 90 minutes following DHCA in piglets, as early as 2 hrs following H-I in normothermic rat models of stroke[124] and 4 hrs in a rabbit model of DHCA[254]. These reports provided the reasoning for initiating this present series of studies with increasing reperfusion periods from 2hrs. Although 2hrs DHCA generated injury after 20hrs reperfusion, this same ischaemic period was not examined at 8hrs, however given that other animal models have described increasing injury to a maximum at 24hrs reperfusion it is likely that any injury seen at 8hrs would have been very mild. Furthermore, these studies might also suggest that the window examined at 20hrs reperfusion might be the most severe (and therefore the most clinically relevant) window for study.

This present study therefore contrasts markedly with numerous other reported investigations which describe histological injury after briefer periods of DHCA and significantly reduced durations of reperfusion. This was a cause of considerable frustration and concern, until a detailed analysis of the experimental methodology used in other reports was undertaken. Almost all of the previous reports have either firstly not included the pertinent controls (instrumented but no CPB, or continuous full-flow CPB at normothermia or hypothermia) for histological comparison, or secondly have involved experimentally "unmonitored" periods of reperfusion (table 6.2). For example by including surgical sham groups (control animals not subjected to CPB), significant histological artifact is revealed (figure 6.5), unrelated to ischaemic exposure[1]. This issue severely compromises the interpretation of studies lacking controls. Almost all of

the studies listed in table 6.2 detected "ischaemic changes" in experimental controls (lacking periods of ischaemia), validating this point.

Importantly, after blinded review of the histology from every animal involved in this thesis, not a single animal exposed to less than 2 hrs DHCA was identified as harbouring ischaemic neuronal injury. This contradicts the findings of many other investigators who report ischaemic injury more brief durations of DHCA (and more brief reperfusion periods). The explanation for this discrepancy almost certainly lies in the *nature of the reperfusion period.* Virtually all other groups reporting histological injury following lesser ischaemic or reperfusion periods have used protocols that involve recovering the animal early after CPB, therefore simultaneously surrendering invasive monitoring, oxygen supplementation, inotropic support (and note that the use of myocardial protection was rare) and blood gas analysis. Therefore the animals were instead fending for themselves after having suffered a lengthy duration of

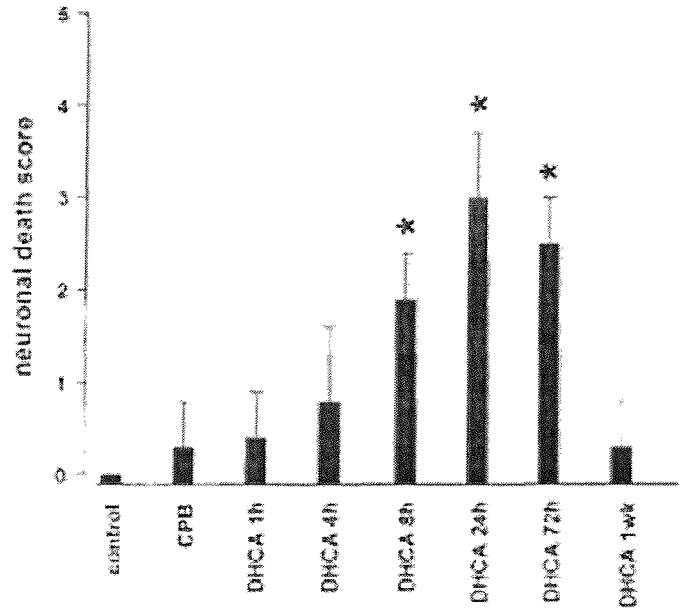


Figure 6.27 Figure illustrating the importance of the management. The authors concluded that injury became apparent after 8 hrs reperfusion. Infact, all the animals surviving 8hrs or more were extubated and woken immediately (and therefore had no support. It is likely these animals (the ones with injury) therefore were haemodyanimcally unstable and possibly hypoxic. [Ditsworth et al 2003, ref 121]

ischaemic CPB with no myocardial protection. In one notable paper – the first to describe the regional pattern of neurological injury following DHCA – this concept is illustrated well. Investigating the temporal nature of neurological injury following DHCA, Ditsworth et al examined groups of piglets reperfused for different durations following DHCA. The results appear to confirm that increasingly lengthy periods of reperfusion result in increasing histological injury (maximal approximately 24hrs following DHCA, figure 6.27)[121]. Their experimental methodology, however, does not necessarily allow this conclusion. The animals reperfused for brief periods were all supported anesthetized and monitored before perfusion-fixation. Animals allowed to reperuse for longer (8, 24, 72hrs) were recovered and extubated *immediately following CPB,* and thereafter *not* supported or monitored. These latter groups were the ones who demonstrated significantly greater histological injury patterns[121].

The only group who used a model of continuous invasive monitoring and support throughout the post-operative period, also report similarly low levels of neurological injury[102]. **Overall, this serves to highlight the importance of the reperfusion conditions in the progression of neurological injury following periods of DHCA. It appears that optimal post-ischaemic management greatly reduces the subsequent magnitude of the ischaemic lesion. Because this period can be clinically manipulated, it is likely to be of considerable therapeutic importance.**

The mammalian brain exhibits regional vulnerability to ischaemia. Anatomical sub-regions deemed to be especially susceptible include the CA1 neurons of the hippocampus and the Purkinje neurons of the cerebellum. These both represent the large efferent neurons from these structures, and their size is believed to reflect a metabolic vulnerability. It is therefore surprising that no ischaemic neurons were identified in either of these areas in any brains examined during the course of this project. The explanation for this appears to relate to the temporal nature of neuronal

loss following cerebral ischaemia. Human tissue rarely presents to the pathologist within a day of the ischaemic insult, because the analysis of central nervous system autopsy material usually follows a lengthy period of intensive care therapy. By contrast, experimental models allow examination earlier in the pathogenesis of the injury. Studies examining the temporal nature of injury in rat models of cerebral ischaemia reveal that at 24hrs, the predominant hippocampal areas of vulnerability are indeed the dentate gyrus and CA3/4, as observed in our model, whereas at one week, the relative contribution of CA1 neurons increases greatly. This predominant pattern of the dentate gyrus vulnerability is also described in piglet DHCA models, confirming that ours is not a unique finding.

Fluoro-Jade™ has been an extremely useful adjunct to the identification and quantification of injury in our model. Remarkably, only a handful of reports exist as to its use[190, 255, 256], and all relate to the identification of neuronal ischaemia. Interestingly, in our institution preliminary studies have indicated that its use may extend to renal tubular ischaemic injury – which has not previously been reported. Although frustrating that the more subtle immunohistochemical techniques have not proved fruitful, the effectiveness of the H&E and Fluoro-Jade™ stains in reproducibly identifying ischaemic injured neurons rendered the need to pursue these techniques unnecessary.

The introduction of an exaggerated model of DHCA with a closely controlled 20hr reperfusion period presents several advantages. Firstly, the use of maintenance anaesthesia with ventilation and full invasive monitoring is highly relevant to the clinical arena. Second, the continuous haemodynamic, pulse-oximetry and end-tidal CO2 information ensures that therapy is maximal throughout the experiment and that periods of inadequate oxygenation or perfusion are eliminated. This is not possible with any model that involves early extubation and recovery from

anaesthesia. Lastly, this system will allow controlled manipulation of the period of reperfusion for further study of the important factors involved in progression of injury.

Dean 99 Piglets	19°C 90mins DHCA	Neck	6 hrs, 2 or 7 days	Woken immediately	No	Yes	Con + Surg + FF	Controls no injury 6hrs Injury seen at 6 hrs, max at 2 days
Kin 99 Adult Dogs	**15°C** 60 or 90mins DHCA	Fem-Fem	72 hrs	Woken immediately	No	Yes	FF	Conclude 60mins to be "max safe"
Tseng 97 Adult Dogs	18°C 120mins DHCA	Fem-Fem	8 hrs GA vent		No	Yes	No	
Midulla 94 30kg Pigs	20°C 90 mins DHCA	N/K	7 days	Woken immediately	N/K	Yes		
Fessatidis 94 Piglets	15°C 70-120 mins DHCA	N/K	6 hrs		N/K			
Gillinov 93 Adult Dogs	18°C 120 mins DHCA	Fem-Fem	3 days	20hrs then woken	N/K	All		

Table 6.2 Summary of the major experimental CPB models of histological brain injury in use. Produced from review of the 397 results of Medline search including the terms "histopathology, brain, cardiopulmonary bypass." Note the lack of myocardial protection strategies used by most groups, despite the long durations of circulatory arrest. In addition, most groups recover the animals immediately (with no monitoring or support). These are major confounding factors when interpreting their data which suggests histological injury using their protocols, even as soon as 60 minutes following the ischaemic period. Furthermore many of the groups lack experimental histological controls (continuous perfusion or no CPB) and therefore much of the "injury" may be artefact[1]. The closest experimental correlate to the protocol developed here is described by Jonas' group (Shum-Tim, Hagano), where the animals are supported ventilated for 24hrs before being recovered. They report very low levels of injury in regions that closely parallel the data described in this chapter.

Author	Protocol	CPB	Reperfusion	Recovery	Myocardial protection	Injury	Controls	Comments
Loepke 05 Piglets	22°C 150mins DHCA	Neck	2-9 days	Woken immediately	No	Yes	Cold FF (no injury)	Cold FF controls (no injury)
Mahan 05 Piglets	16°C 60 mins DHCA	FA-RA	6 hrs	GA, but unsupported	No	Yes all groups	Cold FF (injury)	Cold FF controls (injury)
Myung 04 Piglets	18°C 90mins DHCA	Neck	1,3,7 days	Woken immediately	Arch snug	Yes		25% animals died!
Ye 96 30kg Pigs	15°C 120mins DHCA	Ao-RA	60 mins	Remained on CPB	No	Yes	GA alone	No injury in controls
Ditsworth 03 Piglets	19°C 90mins DHCA	Neck	1,4,8,14,72 hrs + 7 days	**Either immediate recovery or GA + ventilated**	No	Yes	GA/FF	**Injury in those recovered immediately, but not those managed and supported under GA (figure 6.27)**
Shum-Tim 98 10kg Piglets	15°C 100mins DHCA	Fem - RA	24 hrs GA then to day 4	24 hrs GA then woken	No	Yes	No	**Excellent protocol Correlates with current model presented in chapter 6. Very little injury seen**
Hagino 05 9kg Pigs	15°C 120mins Low Flow	Fem - RA	12 hrs GA then to day 4	12 hrs GA then woken	No	No	No	**Excellent protocol Correlates with our findings.**
Romsi 02 10kg Piglets	20°C 75mins DHCA	Mini thoracotomy	7 Days	18 hrs GA but no support	Yes	Yes	No	50% died

Author	Model overview	Findings	Summary
Lee 00 **J Cereb Blood Flow Met**	Adult Dogs Intra-ventricular infusion 20 mins cerebral perfusion pressure only 10mmHg Survival either day 1 or 7 H&E, TUNEL	Day 1 DG >> CA1 Day 7 CA1 injury proportionally increased. CA1 death predominantly necrosis DG death largely apoptois	Temporal difference between DG and CA cell death Perhaps related to different mechanisms
Bottinger 99 **Mol Brain Res**	Cardiocirculatory arrest model in mice Ventricular fibrillation 5 mins	Delayed CA1 death TUNEL>>H&E cell death IE gene expression relatively specific to CA1 and DG	Delayed CA1 death Differences in regional expression of IE genes within hippo
Bottiger 98	Rat 10 mins global H-I	Delayed CA1 death Temporal and differences in TUNEL staining compared to thalamus c-jun/hsp70 associated with CA1 death but not thalamus	Delayed CA1 death Supports notion of differential cell death mechanisms between areas

Table 6.3 Summary of studies implicating differential injury between hippocampal sub-regions relative to reperfusion time. The "typical" hippocampal injury witnessed following human global ischaemic brain injury is associated with CA1 vulnerability in particular. The surprisingly lack of CA1-2 injury observed in the present model is perhaps explained by this data: early following reperfusion, a particular vulnerability is observed in the dentate gyrus (as seen here). CA1 susceptibility becomes proportionately more prominent with time. This regional temporal vulnerability has not yet been addressed and is clearly a separate area for investigation to target in the future.

Chapter 7

Hypoxaemic Reperfusion Exacerbates the Neurological Injury Sustained during Neonatal DHCA: A Model of Cyanotic Surgical Repair

7.1 Abstract

Objectives: Deep hypothermic circulatory arrest is frequently used in infants undergoing the Norwood procedure. These infants are necessarily hypoxaemic after separation from CPB. Considerable energy has been spent characterising the physiological and histological consequences of DHCA, but these have largely focused on a normoxaemic period of reperfusion. Furthermore, evidence has accumulated to suggest that the cerebral vascular autoregulatory mechanisms are dysfunctional following DHCA. In particular, the vasodilatation that elevates CBF in response to hypoxaemia is absent. This study therefore aimed to investigate whether post-CPB hypoxaemia exacerbates brain injury resulting from DHCA.

Methods: Twelve neonatal piglets were subjected to 2 hrs DHCA and then separated from CPB. They were then randomised to either normoxaemia (n=5) or hypoxaemia (n=7). The normoxaemic animals were managed with normal oxygen tensions. The hypoxaemic group were managed with arterial PaO_2 of 40-50mmHg for the duration of reperfusion. Following a 20hr period of warm reperfusion, the animals were perfusion fixed and the brain analysed for histological evidence of injury. Three additional non-ischaemic control animals were exposed to 2hrs deep hypothermic continuous CPB and managed post-operatively with the hypoxaemic group.

Results: All animals survived the protocol. Post-operative parameters - including mean arterial pressure, acid-base status, inotrope requirements and arterial $PaCO_2$ – were similar. None of the non-ischaemic control animals had any evidence of ischaemia. The five normoxaemic animals had moderate injury (total score 7.25±4.5). Of the 7 hypoxaemic animals, three were brain dead, as evidenced by lack of reflexes and gross cerebral ooedema. The remaining 4 had severe ischaemic histological changes (score 14.5±1.5, $p<0.05$).

Conclusions: Cerebrovascular autoregulation in response to hypoxaemia is lost following DHCA. This lack of compensation has serious implications for the cerebral recovery following cyanotic staged repairs using DHCA. Hypoxaemic infants therefore represent a subset of patients in whom DHCA should preferentially be avoided.

7.2 Introduction

Blood flow to the brain is influenced by several powerful autoregulatory mechanisms. Under normal circumstances, control is exerted predominantly through the accumulation of products of metabolism (carbon dioxide and acidotic anaerobic products). The absence of oxygen in the usual control of CBF autoregulation reflects the fact that under normal conditions arterial blood contains significantly more oxygen than required. However, if arterial oxygen tensions should fall severely low (40-60mmHg) then the most potent trigger for enhancing CBF is triggered: the hypoxic CBF response. This is an evolutionary preserved reflex mechanism, mediated through hypoxic chemoreceptors receptors in the wall of the carotid sinus and aortic arch. These chemosensitive areas are extremely vascular-rich, and are therefore exposed almost exclusively to arterial blood. These arterial chemoreceptors are especially sensitive within the range of 30-60mmHg and, via the vagus and Hering's (glossopharyngeal) nerves, powerfully stimulate both the respiratory and vasomotor centres in the brain stem. Life-threatening hypoxia therefore results in a powerful activation of systems to augment oxygenation of blood and its subsequent delivery to the brain[257, 258]. As a result, near-normal energy states can be maintained within the brain despite exposure to severe hypoxaemia[259].

Cerebral blood flow control is dysregulated following periods of DHCA[260]. DHCA results in physical no-reflow[261], and an imbalance in the vasoconstrictor-vasodilator axis[134]. Endothelin-1[262] and thromboxane A_2[57] levels are elevated and production of NO deficient[263]. Consequently, neither the response to increased $PaCO_2$ tensions, nor high H^+ result in the usual degree of cerebral vasodilatation. Recently, the response to hypoxia has been examined, and it has become evident that the hypoxic CBF response is disrupted or absent following DHCA[3]. In fact, whereas increasing hypoxia below 60mmHg typically results in corresponding large increases in CBF, following DHCA the exact opposite is true. Increasingly severe hypoxia below

approximately 60mmHg results in progressively *depressed* CBF. Therefore at a time when the normal brain would be increasing oxygen delivery to ensure adequate tissue provision, oxygen delivery is instead decreased further, potentiating the risk of ischaemic injury[3].

Systemic cyanosis is an inexorable component of congenital heart disease. Pre-operatively this is due to mixing lesions or pulmonary dysfunction. Both intentional and unintentional post-operative hypoxaemia are also common. The successful application of the systemic-pulmonary artery shunt in 1945 by Blalock Taussig in a child with right ventricular outflow tract obstruction heralded the birth of staged congenital repair. In an era where even the most unlikely lesions can now be successfully corrected through to adulthood (e.g. hypoplastic left heart syndrome, HLHS) post-operative cyanosis is therefore increasingly common. For example, following the first stage of a Norwood conversion for HLHS, *arterial* oxygen tensions are typically in the range 25-50mmHg. In fact, oxygen tensions exceeding this might be construed as a sub-optimal repair with too great a shunt size at the expense of the systemic perfusion. Hypoxic ventilation strategies, used to adjust the pulmonary vascular resistance to balance the pulmonary and systemic circulations in single ventricle physiology, may further exacerbate the reduced arterial oxygen tensions.

Unfortunately, it is exactly this group of patients that are among the most likely to be repaired under periods of DHCA (see figure 3.6). DHCA has been implicated as an aetiological component of neurological deficit following congenital repair[107, 116]. However the contribution of the early period of reperfusion has been seriously overlooked until very recently[128, 264]. Its impact on the progression of injury may be of greater significance than the ischaemic period *per se*, and in all areas of cardiovascular medicine the reperfusion period is subject to intense research.

Because DHCA has been demonstrated to disrupt the normal CBF autoregulatory mechanisms and because (intentional) post-operative hypoxaemia is common following DHCA, the hypothesis was made that neuronal injury would be more severe following periods of DHCA with superimposed hypoxaemic reperfusion. This study therefore aimed to investigate the histological impact of hypoxaemic reperfusion on brain injury following DHCA. Neonatal piglets were therefore placed on CPB and subjected to 2 hrs DHCA. After separating them from CPB, the arterial oxygen tensions were lowered to 40-50mmHg in the experimental group, but maintained normoxaemic in the control group. The brains were subsequently examined and quantified for evidence of ischaemic injury.

7.3 Experimental Design and Methods

Neonatal piglets (3-5kg) were placed on CPB and subjected to 2 hrs DHCA at 18°C before rewarming and separating from CPB. The animals were then randomised to a strategy of either: group 1 (n=5), normoxaemic throughout the reperfusion period (100-300mmHg); or group 2 (n=7), hypoxaemic (40-50mmHg) throughout the reperfusion period. A "hypoxaemic control" group 3 (n=3) were placed on CPB, cooled to 18°C and subjected to 2 hrs deep hypothermic full-flow (uninterrupted) CPB (DHFF) before being rewarmed and separated from CPB. Following separation from CPB, group 3 were managed hypoxaemic (40-50mmHg) throughout the reperfusion period.

7.3.1 Surgical protocol and post-operative management

The surgical protocol and post-operative management is described in chapter 6. To summarise, under general anaesthetic, animals underwent surgical tracheostomy and insertion of femoral vascular monitoring lines. Following median sternotomy, they were placed on aorto-right atrial CPB using a conventional blood prime and perfusion

cooled to 18°C using a pH-stat strategy. In groups 1 and 2, CPB was then ceased, the circulating volume drained into the reservoir and the arterial line clamped for 120 minutes. During the circulatory arrest period the coronary circulation was isolated and perfused to provide myocardial protection as described in chapter 6. In group 3, full-flow CPB was instead continued uninterrupted for 120 minutes. Animals were then rewarmed and separated from CPB.

Post-operative management included vigorous rehydration with either blood or saline according to the haematocrit, and noradrenaline infusion as necessary to maintain a mean arterial blood pressure above 50mmHg. No bicarbonate was administered following wean from CPB, and serial blood gas analysis confirmed correction of the acidosis that necessarily occurs in the early reperfusion period in all animals exposed to long DHCA durations. The animals remained ventilated under general anaesthesia (intravenous propofol 3-6ml/hr) until the end of the experiment.

Post-operative arterial oxygen tensions

Animals in group 1 were ventilated in the usual fashion aiming to maintain arterial oxygen tensions between 100-250mmHg, by adjusting the inspired oxygen concentration accordingly. Following separation from CPB, target arterial oxygen tensions (40-50mmHg) were achieved in group 2, by lowering the inspired oxygen concentration towards room air. Blood gas analysis was performed repeatedly to ensure closely controlled levels. Control animals in group 3 (deep hypothermic CPB, but no ischaemia) typically required single-lung ventilation with room air to achieve the target arterial oxygen tensions. Single lung ventilation was performed by replacing the endotracheal tube with an under-sized tube and advancing it to enter a main bronchus. Single lung ventilation could be confirmed by directly examining lung expansion through the open pleura.

7.3.2 Histological analysis

At the conclusion of the 20hr reperfusion period, the animals were re-cannulated, euthanised and perfusion-fixed as described in chapter 6. The brains were harvested and processed and subsequently scored histologically using H&E and Fluoro-Jade™ as described.

7.4 Results

7.4.1 General parameters

The pre-CPB piglet variables and CPB details are detailed in tables 1 and 2. All parameters were similar and no significant differences were observed between the groups.

Table 7.1 General piglet experimental parameters

	Group 1 Normoxic	**Group 2 Hypoxaemic**	**Group 3 Non-ischaemic Control**	***p value***
Weight (kg)	**2.8 ± .18**	**3.3 ± .16**	**2.9 ± .24**	***.11***
Haematocrit	**29 ± 1.9**	**27 ± 1.2**	**26 ± .82**	***.57***
pH	**7.36 ± .04**	**7.49 ± .04**	**7.49 ± .07**	***.07***
Base deficit	**-5.3 ± 2.1**	**0.0 ± 2.9**	**-0.3 ± 1.8**	***.18***
PaO_2 (mmHg)	**245 ± 39**	**315 ± 42**	**314 ± 72**	***.28***
$PaCO_2$ (mmHg)	**32.8 ± 5.2**	**25 ± 1.4**	**29 ± 4.6**	***.18***
MAP (mmHg)	**42 ± 1.3**	**48 ± 2.5**	**44.4.0**	***.12***
Heart rate/min	**115 ± 5.7**	**119 ± 8.7**	**113 ± 12**	***.75***
Core temp (°C)	**37.3 ± .32**	**36.3 ± .13**	**36.6 ± .2**	***.14***

Table 7.2. Parameters during CPB strategy

	Group 1 Normoxaemic	Group 2 Hypoxaemic	Group 3 Non-ischaemic Control	*p value*
Cooling duration (mins)	**27.5 ± 2.5**	**28.7 ± .9**	**26.6 ± 1.7**	*.67*
Rewarm duration (mins)	**52.5 ± 5.1**	**55.0 ± 2.2**	**41.7 ± 1.7**	*.64*
Total CPB duration (mins)	**201 ± 4.5**	**205 ± 2.4**	**188 ± 3.3**	*.51*
Base deficit at 25°C during rewarm	**-14 ± 2.2**	**-10.6 ± 1.9**	**-4.4 ± .9**	*.29*
H_2CO_3 required during CPB (ml)	**20.5 ± 2.0**	**18.0 ± 1.7**	**3.5 ± 2.8**	*.39*
pH at wean	**7.41 ± .03**	**7.45 ± .03**	**7.50 ± .08**	*.43*
Base deficit at wean	**-3.2 ± 1.9**	**-2.8 ± 1.7**	**-2.9 ± 1.4**	*.86*
PaO_2 at wean	**316 ± 48**	**241 ± 38**	**274 ± 24**	*.26*
$PaCO_2$ at wean	**29.3 ± 3.8**	**27 ± 2.6**	**24 ± .63**	*.63*
Haematocrit at wean	**34 ± 1.7**	**30 ± 2.5**	**31 ± 3.7**	*.28*

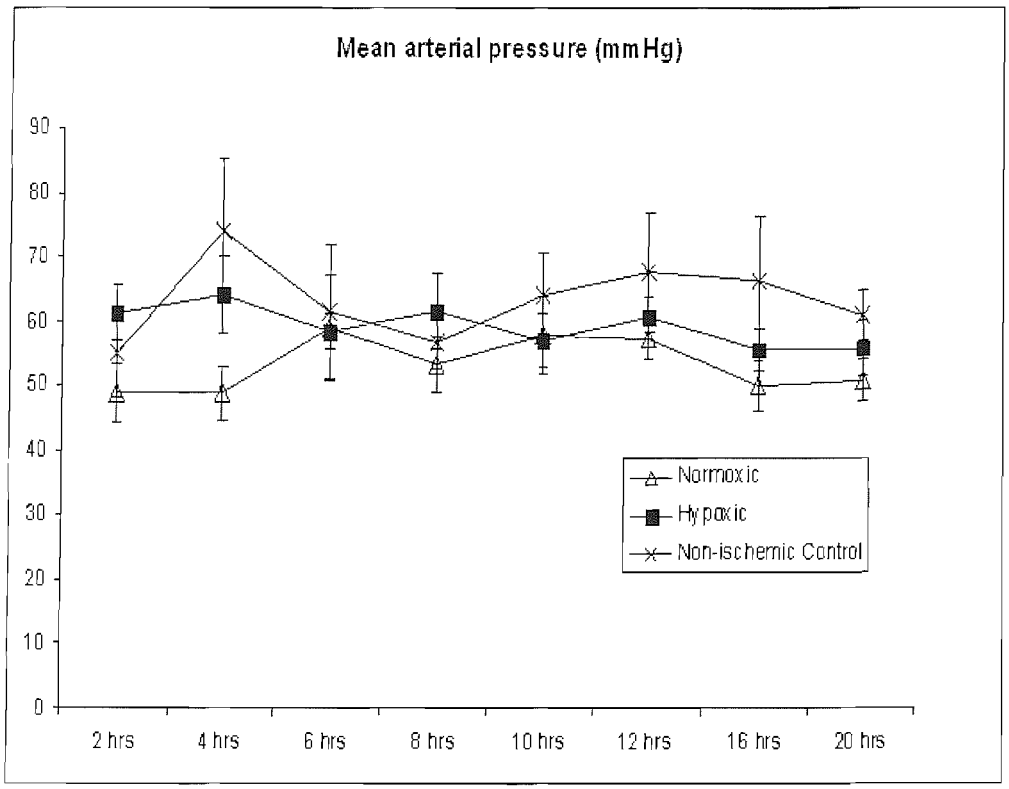


Figure 7.1 Mean arterial blood pressure during the reperfusion period. Differences were not significant between the groups.

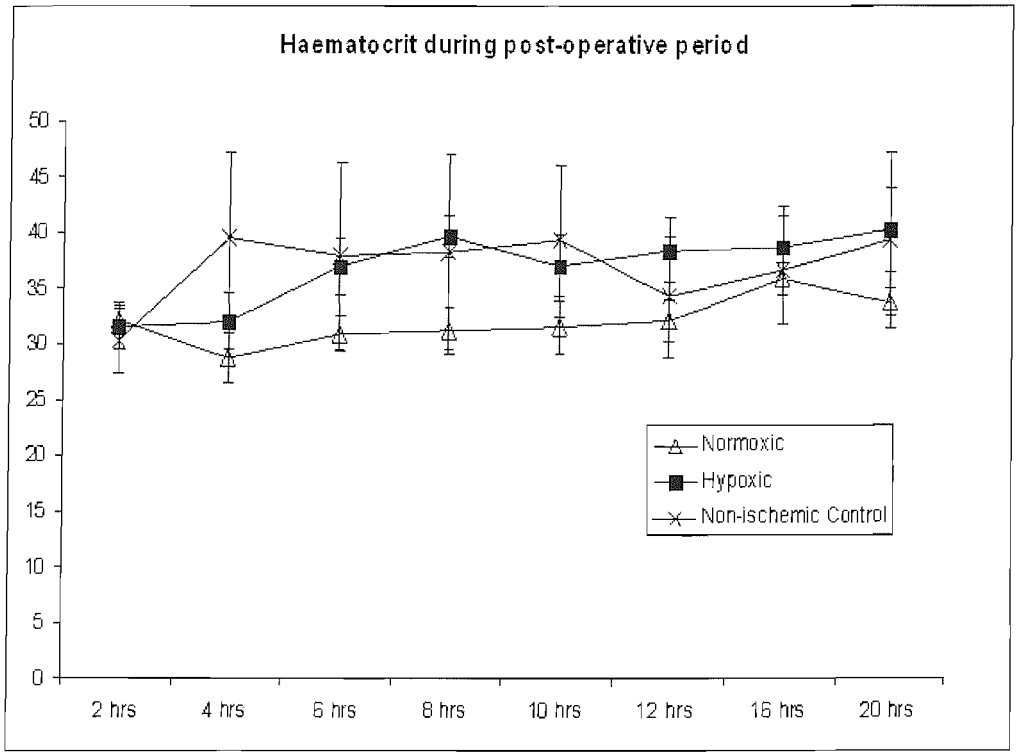


Figure 7.2 Haematocrits during the post-operative period. Differences were not significant between the groups.

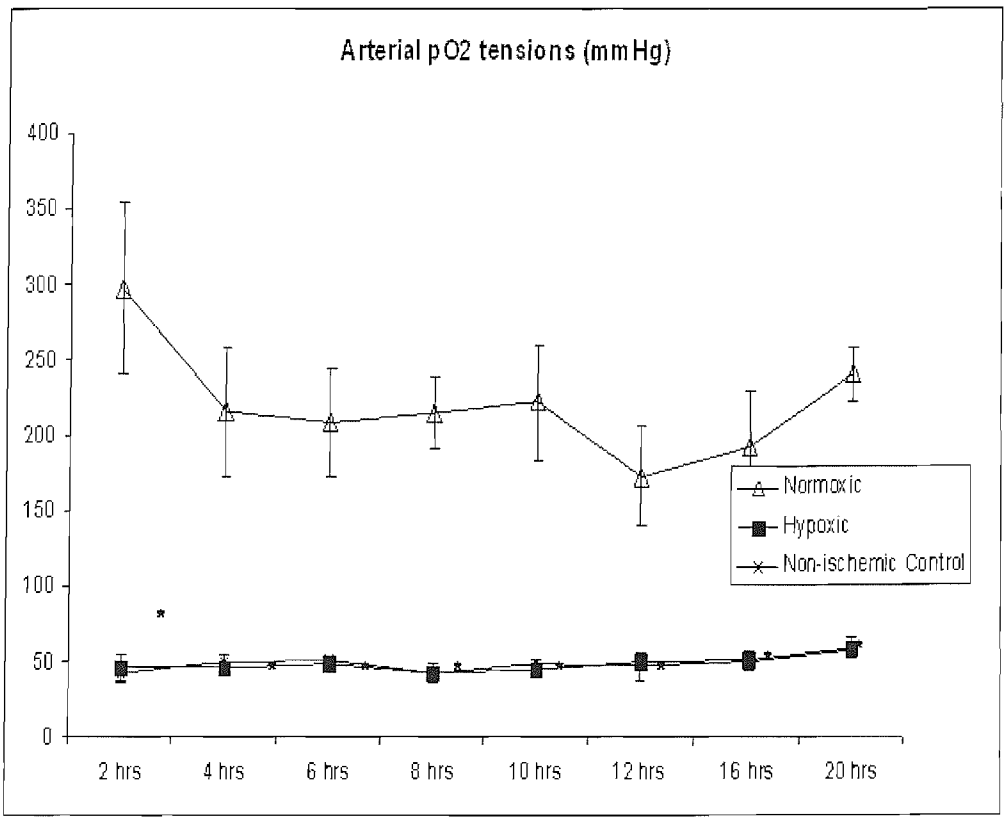


Figure 7.3 Arterial PaO_2 tensions (mmHg) during the post-CPB period of reperfusion. Differences were significant compared to the normoxic group at every time point ($p<0.01$*)

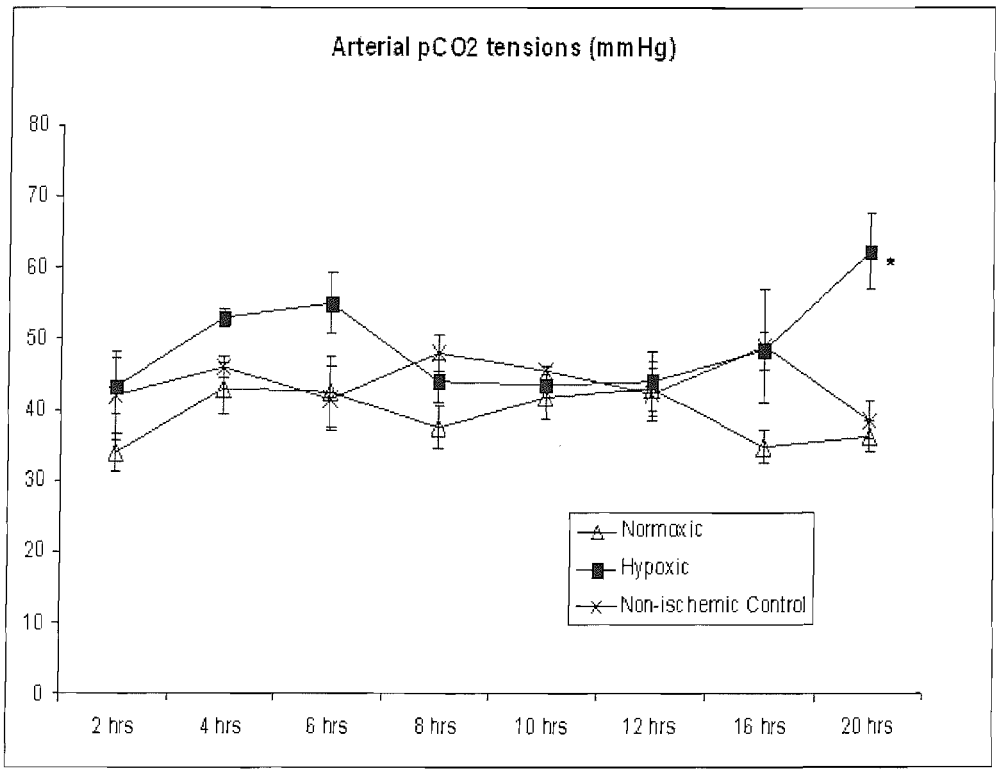


Figure 7.4 Arterial $PaCO_2$ tensions between the groups during the post-operative reperfusion period. Differences were only significant between the normoxic and hypoxic groups at the final timepoint (*$p<0.05$)

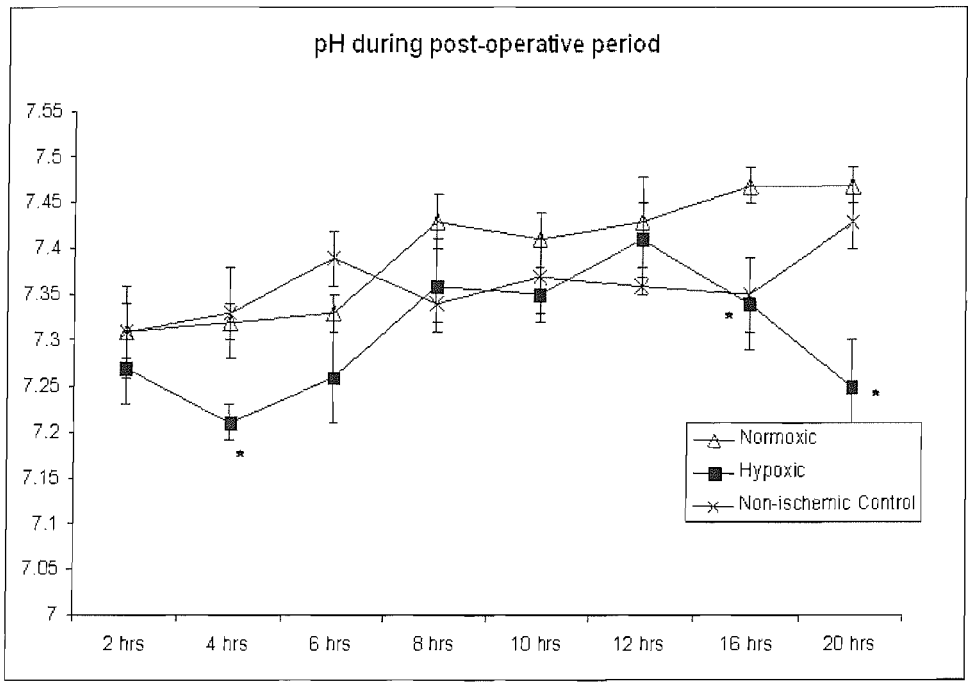


Figure 7.5 Arterial pH during the post-CPB period of reperfusion. Levels were never below 7.2, but differences were significant between the hypoxaemic and normoxaemic groups at three timepoints (* $p<.05$)

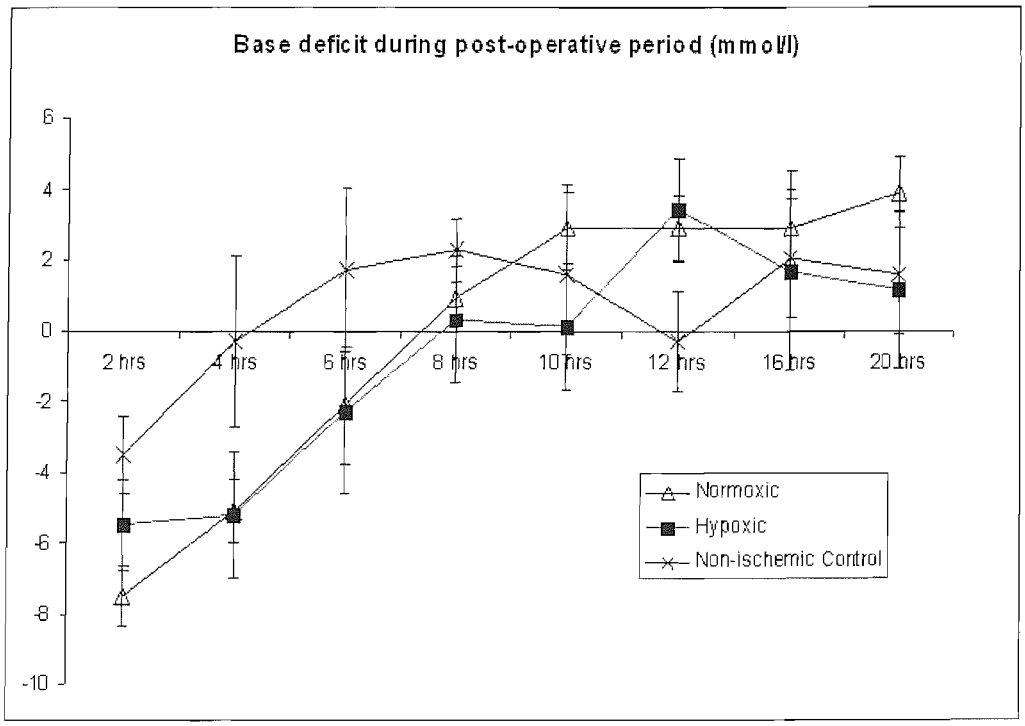


Figure 7.6 Arterial base deficit (mmol/l) during the post-CPB period of reperfusion. Differences were not significant between the groups at any timepoint.

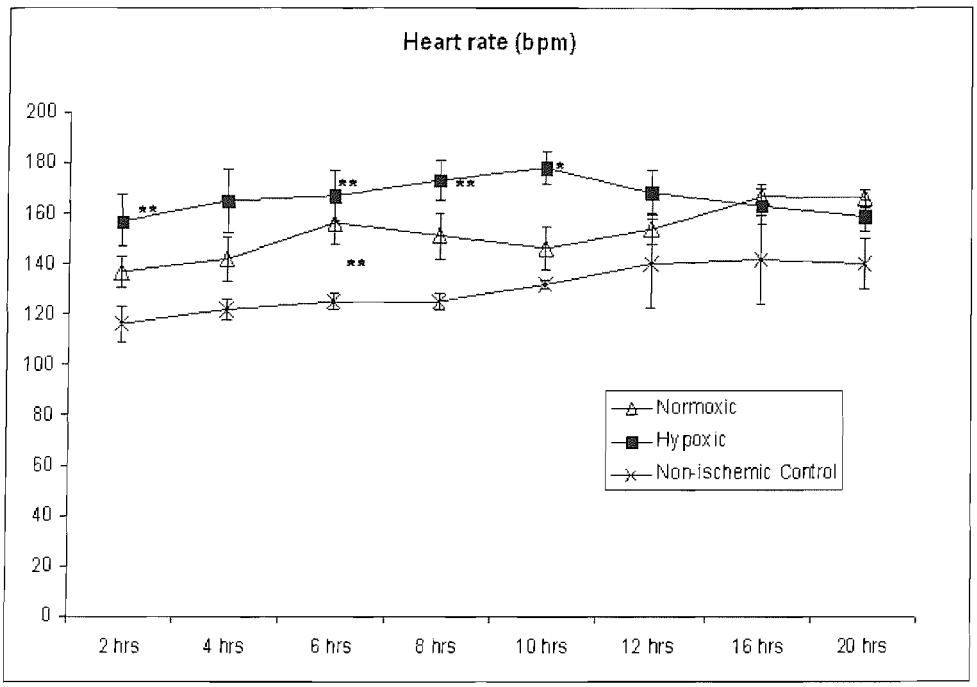


Figure 7.7 Post-CPB heart rate (beats/min). Differences were significant between the hypoxaemic and non-ischaemic controls at several time-points (**p<.05). Differences were only significant between the hypoxaemic and normoxaemic groups at one time-point (*p<.05)

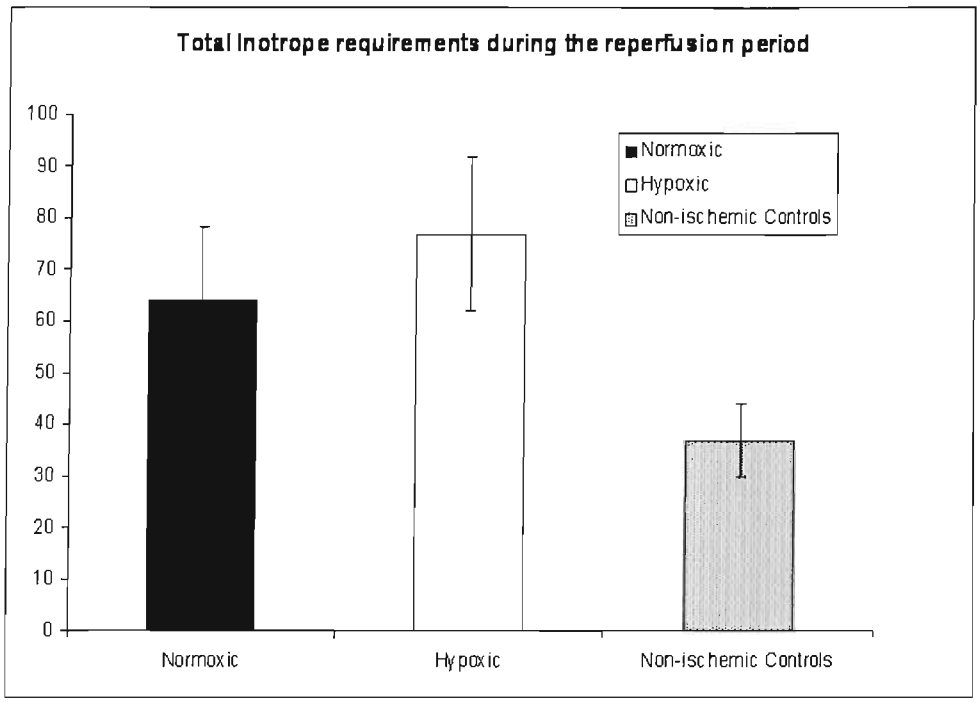


Figure 7.8 Total inotrope requirements (mg/kg) for the duration of the post-CPB period. Differences were not significant (n=only 3 in the non-ischaemic group).

Following wean from CPB, the post-CPB course was closely monitored and graphical illustration of the post-CPB period is provided in figures 7.1 – 7.8. An important feature is that the haemodynamic profiles are no different, indicated especially by the acid-base profile: **no bicarbonate was administered following wean from CPB.** Following the extended period of whole-body ischaemia, the piglets necessarily develop a metabolic acidosis during the early reperfusion period, which was corrected by ensuring optimal systemic perfusion pressures and adequate diuresis. Because exogenous bicarbonate was not administered, the normalisation of acid-base is therefore a useful indicator of systemic well-being.

Systemic perfusion pressures and total inotrope requirements were not different in the experimental group – mirrored by similar total inotrope requirements – indicates that oxygen availability appears adequate for myocardial support. This also implies that availability should also be adequate for remote tissue requirements. The purpose of the non-ischaemic control group was to confirm that the level of hypoxaemia induced during the course of the experiment did not fall below the threshold for adequate systemic metabolic function.

Arterial carbon dioxide tensions (a powerful cerebral vasodilator) were higher in the hypoxic group, although not greatly, and was likely a reflection of the methodology in rendering the hypoxaemic. Importantly, the slight hypercarbia in the experimental group would be considered protective in encouraging enhanced cerebral perfusion, and therefore does not serve to counter our conclusions from this study.

The haematocrits in the two hypoxaemic groups were higher than the normoxic group at most experimental time-points. This was not a pre-determined component of the experimental design. Because oxygen delivery is in part related to the oxygen binding

capacity of the blood, attention was directed towards ensuring that the haematocrit in the hypoxaemic groups did not fall to levels lower than the normoxic group. In fact, the converse was true: group 2 (hypoxaemic) actually had higher haematocrits that the remaining groups in the post-operative period. These higher haematocrits do not detract from the conclusions drawn from this study, because they would typically be construed as protective against the ischaemic effects of hypoxaemia.

7.4.2 Cerebral viability

During the course of the experiment, it became apparent that 3 of the hypoxaemic animals had absent respiratory and corneal reflexes, despite reducing the level of anaesthesia. Following perfusion fixation and brain retrieval it was evident that these animals were brain-dead, as evidenced by the onset of liquefactive necrosis. In fact, the cerebral ooedema was so marked that perfusion-fixation was not generally possible in these animals. This was a process that did not occur in any other animal throughout the series of experiments reported in this thesis. It indicates a failure in the compensatory mechanisms that normally regulate intra-cerebral homeostasis, presumeably as a result of the excessively severe insult that reperfusion hypoxaemia conveys upon an ischemic injury. Although the tissues of these animals were processed for histological analysis, the sections were of poor quality and were not amenable to the usual methods of examination or quanitification. Nevertheless, this is an important experimental finding, because these animals clearly represent the extreme end of the spectrum of cerebral injury, despite not being amenable to histological quantification.

7.4.2a Histological scores of injury

Histological scores for the experimental animals is given in table 7.3. All slides were examined blindly. No injury was observed in either group in the cerebellum. No injury was noted in any animal not exposed to DHCA regardless of post-CPB hypoxaemia (hypoxaemic controls). The hippocampal scores of histological injury using H&E were worse in the hypoxaemic animals (9.5 ± 2.7 vs 4.7 ± 2.8, $p<0.05$) as were the total cumulative scores of histological brain injury for these animals (14.5 ± 4.1 vs 7.2 ± 4.5, $p<0.05$). FluoroJade™ quantification of injury revealed the same differences for hippocampal (9.25 ± 1.7 vs 3.75 ± 3.8, $p<0.05$) and overall cumulative brain injury (14.3 ± 2.5 vs 6.3 ± 5.5, $p<0.05$) (figures 7.9-7.12).

Animal	Cortex	Basal Ganglia	Cerebellum	Hippocampus								Total
				CA1	CA2	CA3	CA4	DG sup	DG med	DG deep	Total	
Normoxic												
1	1	0	0	0	0	0	2	3	2	0	7	8
2	1	1	0	0	0	1	1	2	1	0	5	7
3	1	0	0	0	0	0	0	1	0	0	1	2
4	1	1	0	0	0	0	2	2	1	0	5	7
5	2	3	0	0	0	0	1	4	3	0	8	13
6												
Mean	1.3	1.3	0	0	0	0.25	1	2.2	1.3	0	4.7	7.3
Hypoxic												
1	3	4	0	0	0	2	3	4	3	0	12	19
2												**Dead**
3												**Dead**
4	2	2	0	0	0	1	3	4	3	0	11	15
5												**Dead**
6	3	3	0	0	0	1	2	4	2	0	9	15
7	1	1	0	0	0	0	0	4	2	0	6	9
Mean	2.3	2.8	0	0	0	1	2	4	2.5	0	9.5 *	14.5 *
Non-ischaemic Control												
1	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0
Mean	0	0	0	0	0	0	0	0	0	0	0	0

Table 7.3 Regional histological scores (H&E) in the experimental piglets. * $p<0.05$ compared with corresponding score in normoxaemic animals.

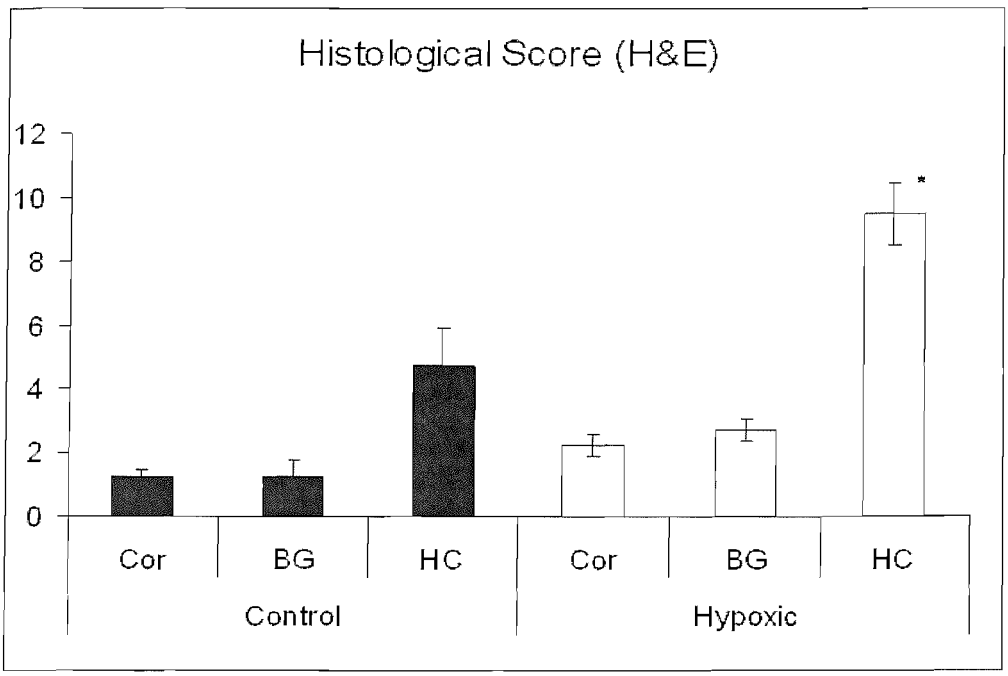


Figure 7.9 Histological scores for the Cortex (Cor), basal ganglia (BG) and hippocampus (HC) for group 1 (normoxaemic) and group 2 (hypoxaemic). * $p<0.05$

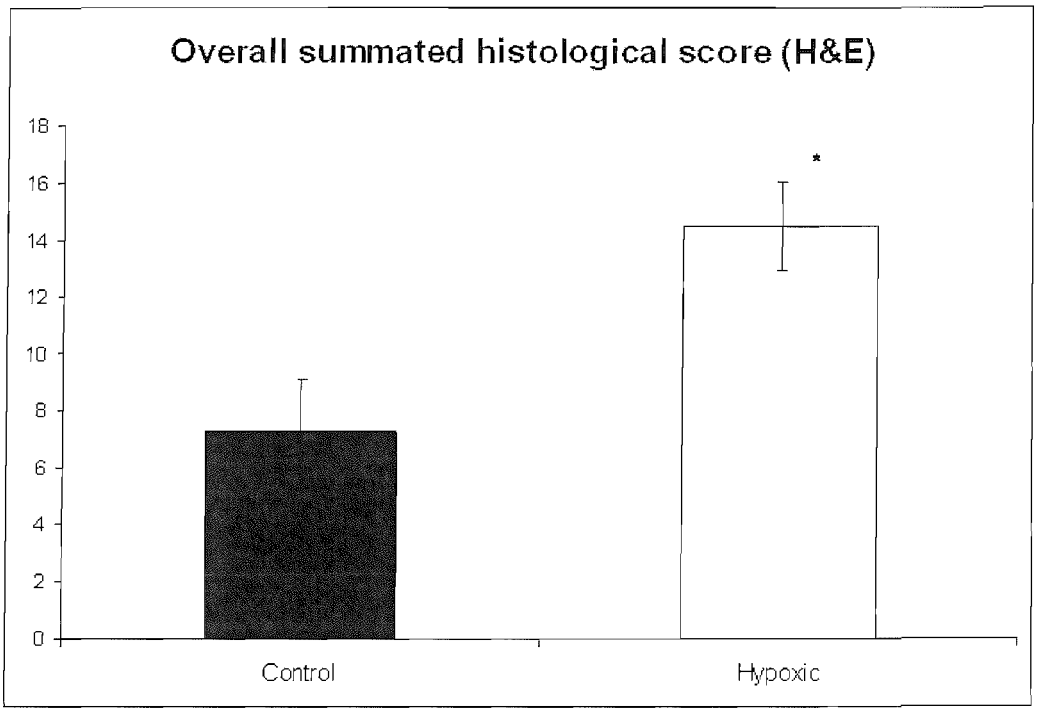


Figure 7.10 Total cumulative histological scores for experimental groups 1 (normoxaemic) and 2 (hypoxaemic). (Cortex + basal ganglia + hippocampus).
* $p<0.05$

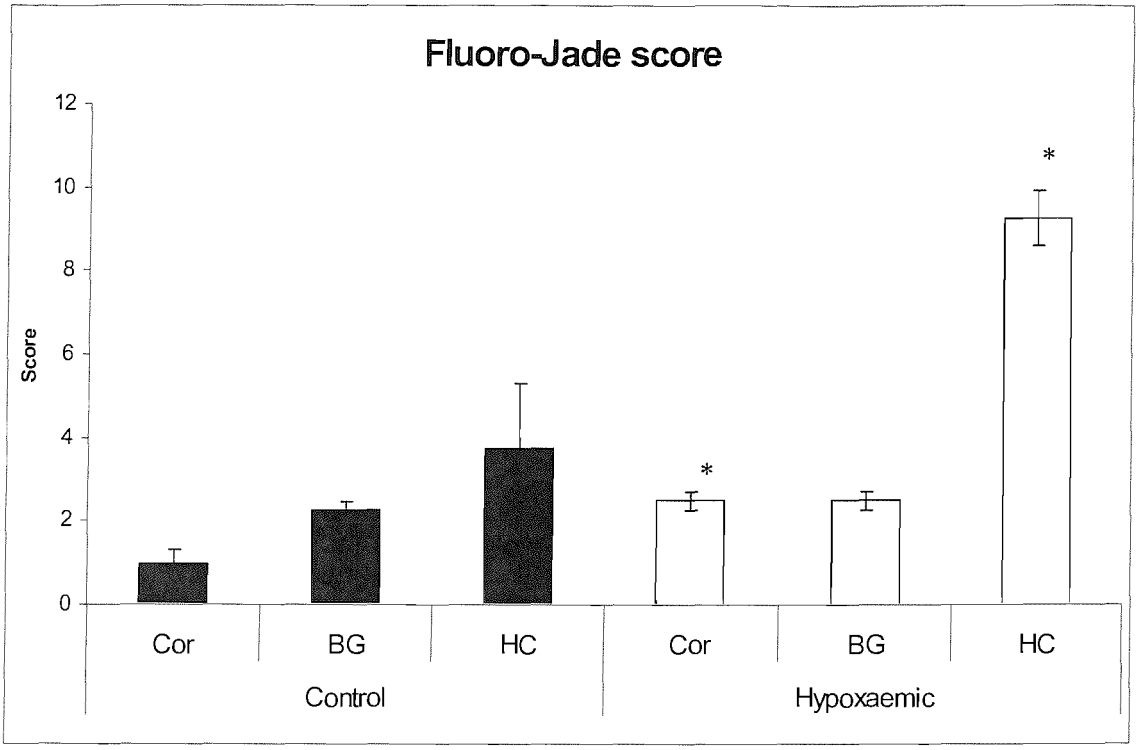


Figure 7.11 Fluoro-Jade™ score of injury for the cortex (Cor), basal ganglia (BG and hippocampus (HC) for experimental groups 1 (normoxaemic) and 2 (hypoxaemic). *$p<0.05$

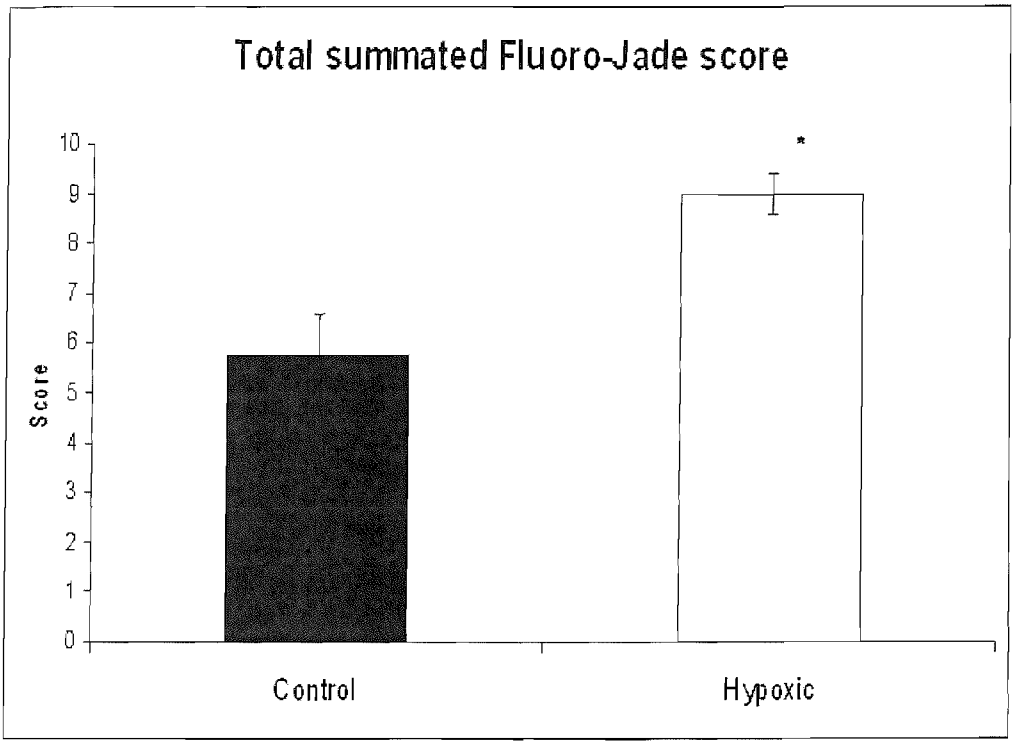


Figure 7.12 Cumulative scores using Fluoro-Jade™ histochemical staining to identify ischaemically injured neurons for experimental groups 1 (normoxaemic) and 2 (hypoxaemic). (Cortex + basal ganglia + hippocampus) *p<.005

7.5 Conclusions

This study confirms that post-operative hypoxaemia in the range commonly encountered following congenital repair significantly exacerbates neuronal loss from ischaemic injury sustained during periods of DHCA. It serves to highlight the notion that the early reperfusion period can critically affect the progression of neuronal injury following DHCA, and that certain sub-groups of patients may represent different categories of risk following exposure to DHCA.

Cerebral autoregulation is disrupted by periods of ischaemia. However controversy exists as to the selective nature of the loss of autoregulation to different stimuli. In the context of normothermic global cerebral ischaemia, Leffler et al discovered that global ischaemia in piglets abrogates the usual cerebral vasodilation that usually accompanies hypercapnia and hypoxaemia[265]. Further studies indicated that the mechanisms were selective in that arteriolar dilatation to hypotension was absent, but the vasoconstriction response to adrenaline remained intact. The response to hypoxia is less clearly defined, and Armstead et al reported an intact dilatory response to hypoxaemia following normothermic global ischaemia[266]. In the context of DHCA, the responses are additionally affected by the contribution of deep hypothermia. Although deep hypothermia is deemed to be protective to ischaemia by conserving high-energy phosphate consumption and decreasing products of lipid peroxidation and excitatory amino acids, it itself blunts the microvascular responses to both hypercapnia and hypoxaemia as determined by near infra-red spectroscopy[260]. Tsui et al examined the CBF response following normothermic CPB, and demonstrated a blunted CBF response to progressive hypoxaemia compared to instrumented controls, suggesting that CPB itself deleteriously affects cerebral autoregulation, perhaps through the

inflammatory consequences of CPB[3]. Reports detailing the cerebral response to hypoxaemia following DHCA are conflicting. Although O'Rourke and colleagues demonstrated an intact cerebral vasodilatation response to hypoxaemia, direct quantification of CBF following DHCA and progressive hypoxaemia clearly demonstrated a progressively reduced CBF whilst controlling a constant overall cardiac output[260]. This observed inability of the cerebral vasculature to augment its blood flow is consistent with the repeated demonstration of cerebral no-reflow following DHCA[120, 261]. The interpretation of the present study is that an abnormal response to progressive hypoxia underlies the more extensive progression of injury witnessed in the experimental hypoxaemic animals.

The severe progression of injury sustained following hypoxaemic reperfusion was exemplified by the demonstration that 3 of the experimental animals were clinically

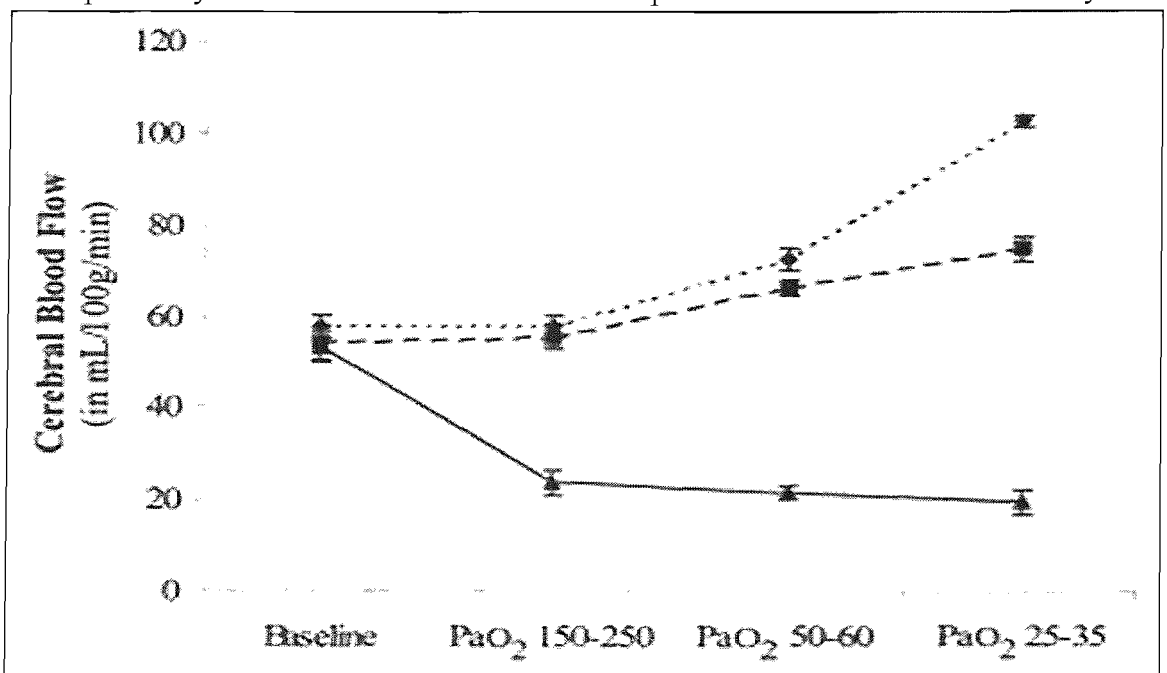


Figure 7.13 Taken from Tsui et al[3]. Animals following surgery were subjected to worsening hypoxaemia. The top line (diamonds) are controls (no CPB). The middle line (squares) represents animals subjected to normothermic continuous CPB. Both these groups display a hyperaemic cerebral response with worsening hypoxaemia. The lower line (triangles) group – DHCA – exhibit worsening CBF with progressive hypoxaemia.

brain dead with severely decompensated cerebral ooedema. No other animal in this entire project had a similar outcome, and it implies the extreme nature of the injury imposed. Although the tissue was not able to be processed and assessed histologically, this does not detract from its significance, because they represent instead the most extreme end of the injury spectrum. It is likely that in these animals, initial cerebral compensatory mechanisms were overwhelmed by the injury sustained and that therefore cerebral ooedema became progressively more severe until viability could not be sustained.

A problematic feature of cyanotic animal models is their relevance to the clinical scenario. One criticism with this model may be that the hypoxaemia is abrupt and therefore excludes the possibility for compensatory mechanisms (polycythaemia, neovascularisation, intra-cellular augmentation of metabolic pathways) to become apparent. This is a problem that is extremely difficult to overcome without complex pre-operative incubation of animals (perhaps from birth) in enclosed chambers of sub-physiological oxygen. Certainly in rat experimentation this has been performed to create models of cyanotic heart disease[267]. These rat models have suggested that the neonatal brain is capable of adapting to profound cyanosis in this post-natal window, such that no histological evidence of injury is sustained. The notion that abrupt cyanosis in the peri-natal period can be tolerated in human neonates is supported by magnetic resonance imaging and functional studies suggesting no difference in cerebral injury between cyanotic and acyanotic infants. The control group in this study was used to exclude the possibility that hypoxaemia *per se* in the region of 40-50mmHg is a significant instigator of ischaemic neuronal loss. The lack of any discernible ischaemic injury suggest that following deep hypothermic full-flow CPB, the brain is capable of extracting sufficient oxygen to prevent demonstrable cell loss. This assertion supports the demonstration that both normal brains not exposed to CPB, and those exposed to

normothermic full-flow CPB augment their blood flow in response to progressive hypoxaemia[3].

The abrupt introduction of post-operative hypoxaemia in this model is not necessarily irrelevant. Cyanosis typically develops pre-operatively in infants either immediately at birth, rapidly on closure of the ductus arteriosus, or later and more chronically with the emergence of a right-to-left shunt. The two former scenarios may occur rapidly and require prompt surgical intervention before significant compensatory mechanisms have emerged. One important compensatory mechanism is an augmentation of the blood oxygen carrying capacity through polycythaemia, and cyanotic patients are usually managed with elevated haematocrits. It was important to ensure during this study that the post-operative haematocrits in the experimental group were not lower than the control group, and in fact the converse was true.

Perhaps the most pertinent question that is raised by this study is whether a particular window exists in which augmenting cerebral oxygen delivery has a protective effect in reducing the progression of neuronal loss. Previous studies[3] have suggested that the exaggeration of injury from hypoxaemia is due to the loss of the cerebral hypoxic autoregulatory mechanisms following periods of DHCA[260, 265]. This dysregulation is linked in aetiology to cerebral no-reflow. Cerebral no-reflow is a phenomenon that causes sustained depression of post-ischaemic CBF for a window of several hours following DHCA. It is likely, therefore, that hypoxaemia during this early post-CPB period is responsible for the bulk of additional injury demonstrated in this study. This window – perhaps only the first 8 hrs[268] – may therefore be the period during which artificial enhancement of oxygen delivery could abrogate the amplification of injury by post-bypass hypoxaemia. This would be an interesting study for the future, as several clinical means exist that could temporarily augment cerebral oxygen delivery for brief periods, including oxygenating techniques (extra-corporeal membrane oxygenation

(ECMO)), or techniques to enhance cardiac output (ventricular assist devices (VAD), inotropes).

The potential for augmenting delivery of oxygen to the brain has not been addressed in this study. In this present model, satisfactory overall haemodynamic status of the animals was inferred from the adequate systemic blood pressures, improving acid-base status during the reperfusion period, and the similar inotrope requirements. Direct cardiac output measurements were not made because this involves the application of pulmonary artery flow probes which became problematic upon chest closure in preliminary studies, due to the propensity for kinking the right ventricular outflow tract and compromising cardiac function. An interesting avenue for future studies is to consider the potential for directly augmenting overall cardiac output (tissue oxygen delivery) in hypoxaemic animals following DHCA to investigate whether this satisfactorily compensates for lower arterial oxygen content[269].

Overall, this study serves to emphasise that the period of reperfusion following DHCA is crucial to the progression of neurological injury sustained. The current application of DHCA without specific consideration for the conditions of reperfusion clearly has implications for subsequent outcome. It may be that either DHCA should preferentially be avoided in staged cyanotic congenital repairs, or otherwise the technique be used in the knowledge that duration of ischaemia likely has greater importance than in other patient populations. Furthermore, attempts to augment cerebral oxygen delivery by ensuring optimum perfusion pressures, above normal haematocrits and perhaps temporary methods of artificial oxygenation may need to be aggressively pursued in order to minimise neurological consequences.

Chapter 8

The use of Lipopolysaccharide to Precondition the Brain against Injury sustained during DHCA

8.1 Abstract

Objectives: Delayed preconditioning genetically reprograms the response to ischaemic injury. Subclinical bacterial lipopolysaccharide (LPS) acts through preconditioning, powerfully protecting against experimental stroke. We investigated the potential for LPS to protect against cardiopulmonary bypass (CPB) related brain injury.

Methods: Neonatal piglets were blindly and randomly preconditioned with LPS (n=6) or saline (n=6). Three days later, they experienced 2hrs uninterrupted deep hypothermic circulatory arrest (DHCA) before being weaned and supported anaesthetised for 20hrs in a laboratory intensive care setting. Controls included CPB without DHCA (n=3) and instrumented with no CPB (n=3). Brain injury was quantified using light and fluorescent microscopy (Fluoro-Jade™).

Results: All animals were clinically indistinguishable prior to surgery. Peri- and post-operative parameters between experimental groups were similar. No control animal scored falsely positive. Histological scores were 0.33±0.21, 0.66±0.42 and 0.5±0.24 in the cortex, basal ganglia and hippocampus respectively, in the LPS animals but significantly worse in saline animals (1.33±0.21($p<0.01$); 1.66±0.33,($p=0.09$); and 6.0±1.5,($p<0.01$)). Two LPS brains were histologically indistinguishable from controls.

Conclusions: This is the first report of successful delayed preconditioning against CPB brain injury. Because LPS preconditioning is a systemic phenomenon offering proven protection against myocardial, hepatic and pulmonary injury, this technique offers enormous potential for protecting against systemic neonatal CPB-injury.

8.2 Background

The search for pharmacological methods for neuroprotection have largely been driven by the huge burden (adult) stroke imposes on healthcare in developed countries. Stroke is the third leading cause of death and the leading cause of disability in the West. Of approximately 750,000 people afflicted in the US, 158,000 die annually. Health care costs for this disease exceed 45 billion dollars annually[270-272]. Because of this, enormous energy is being spent understanding potential mechanisms for limiting injury sustained during stroke. These efforts have benefited the research programs aimed at improving outcome from other causes of cerebral ischaemia by providing greater understanding of the generic mechanisms behind brain injury and neuroprotection.

A significant problem with neuroprotective strategies in adult stroke is the unpredictable nature of the disease and lack of a well-defined pre-aura. This typically precludes the administration of agents in the pre-ischaemic window. In contrast, neurological complications of both adult and congenital cardiac surgery are ideally suited to pre-emptive neuroprotection. **Cardiac surgery involving periods of circulatory arrest is the only circumstance in which a (controlled) severe global ischaemic hit is administered to the brain in a premeditated fashion.** Numerous additional clinical scenarios exist in which neuroprotection against the *risk* of a subsequent stroke exist, including carotid, coronary and valve surgery. Such patient populations motivate research for pre-emptive neuroprotective strategies.

New approaches to cerebral neuroprotection are needed. Decades of research investigating stroke pathogenesis and treatment have revealed robust neuroprotective treatments in the laboratory, however, all have failed to translate into treatments for patients[273, 274]. The bulk of this work has centred on drug discovery aimed at blockade of "excitotoxicity" whereby the extracellular concentrations of the excitatory

neurotransmitter glutamate are increased in ischaemic brain. Glutamate binding to its postsynaptic receptors opens calcium channels. Toxic intracellular calcium concentrations accumulate and cell death results by apoptotic or necrotic mechanisms depending on ATP availability. The failure of a pharmacologic approach to induce neuroprotection in humans may be due to trial design, dose response or time window issues of selected compounds or side effects of study agents, all of which might be corrected in another clinical trial. However all neuroprotective trials over 25 years have been negative while the potential for salvage of ischaemic brain has been confirmed by trials of thrombolytics and anti-platelet agents. A search for alternative and novel strategies for introducing neuroprotection is therefore warranted.

8.3 The concept of cerebral preconditioning

Preconditioning is a concept whereby brief exposure to a harmful stimulus in a dose below the threshold for tissue injury provides robust protection, or tolerance, against the injurious effects of a subsequent more severe insult (figure 8.1). This interesting notion probably reflects an evolutionary protective mechanism and has long been recognised in the context of myocardial and cerebral ischaemia models, where a preconditioning stimulus can reduce infarct size by as much as 50%[275-277]. The primary stimulus can include a wide variety of insults, including

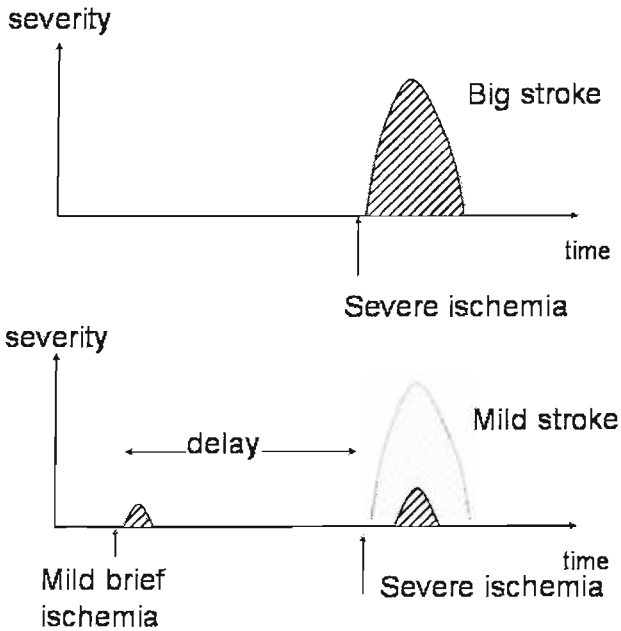


Figure 8.1 Concept of preconditioning.

hypoxia/ischaemia, brief episodes of seizure, toxins or noxious drugs, but to successfully precondition, they must all be given in a brief, low dose.

In elucidating the mechanisms underlying preconditioning, it has become apparent that two main types exist. *Early preconditioning* occurs minutes to hours following the preconditioning stimulus, and requires no *de novo* protein synthesis. It probably involves post-translational processing, but the involvement of circulating mediators is suggested by the intriguing demonstration of "remote preconditioning" whereby brief cycles of limb ischaemia induces almost immediate tolerance to experimental myocardial infarction[278, 279] and acute lung injury[280]. Early CNS preconditioning might have several features in common with myocardial preconditioning, including the role of nitric oxide[281], adenosine A1 receptors[282] and K-ATP channels[282].

D*elayed (late) preconditioning* by contrast appears to be an active process dependent on protein synthesis. It powerfully preconditions over a larger window (between 1-7 days following the preconditioning stimulus) and therefore represents an extremely attractive therapeutic concept. Certainly in the brain, (delayed) ischaemic tolerance appears to involve an endogenous program of neuroprotection[283, 284]. Triggering this program sets into motion a complex cascade of signalling events leading to new synthesis of proteins[284, 285] that ultimately re-programs the cellular response to subsequent injury. The sequence of events that leads to ischaemic tolerance is only partially known[283], although evidence is emerging that **diverse stimuli** that trigger preconditioning may share a **common pathway** that confers neuroprotection[281, 286-288]. Thus, identifying the central mediators involved in preconditioning may define essential pathways responsible for this important cellular response program and thereby provide novel therapeutic strategies in ischaemic brain injury.

Among the diverse stimuli than can trigger ischaemic tolerance in the brain are non-injurious ischaemia, cortical spreading depression and brief episodes of seizure. Endotoxin (bacterial lipopolysaccharide, LPS)[276, 289] given systemically also induces tolerance to ischaemic injury, a further example of cross-tolerance, where a non-lethal stress confers tolerance to a subsequent stress that is different in nature from the first. Low dose endotoxin also confers protection against subsequent exposure to lethal doses of endotoxin—an example of homologous tolerance (figure 8.2). LPS has now been shown to confer ischaemic tolerance in several systems including the liver, myocardium and brain[276, 290, 291], and it appears to share several hallmark features with delayed *ischaemic preconditioning* in the brain[276], suggesting that central pathways may link the numerous primary preconditioning stimuli. **Central to the concept of preconditioning – LPS or otherwise – is that the preconditioning stimulus must be applied in a low, brief dose, below the threshold for instigating tissue injury within the target organ. However, LPS preconditioning does elicit a mild (subclinical) systemic inflammatory response, which may be a necessary pre-requisite to successful induction of tolerance.**

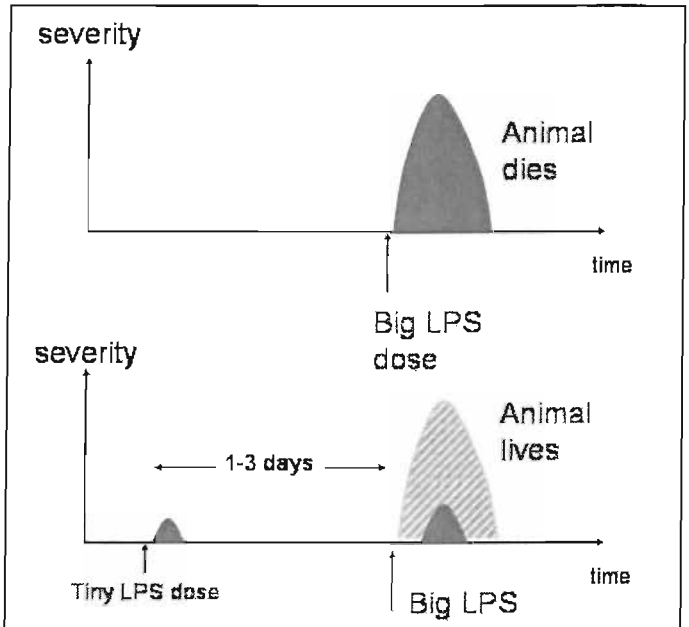


Figure 8.2 Concept of LPS preconditioning.

8.4 The role of inflammation in preconditioning

The activation of inflammatory pathways plays an important role in LPS-induced ischaemic tolerance. TNFα and its downstream signalling mediator, ceramide are critical effectors of LPS-induced neuroprotection[292, 293]. An essential role for TNFα in LPS preconditioning has been shown in studies where LPS treatment was accompanied by concurrent neutralization of TNFα in the circulation (with a soluble TNFα receptor). Blockade of TNFα reversed the protective effect of LPS preconditioning[276]. Thus, proximal members of the TNFα pathway, namely TNFα and its receptors, TNFR1 (p55) and TNFR2 (p75) as well as sphingomyelin-based second messengers such as ceramide are likely mediators of the protective effects of TNFα in LPS preconditioning. TNFα-activation of NF-κB may also be involved, as inflammatory molecules regulated by NF-κB, such as superoxide dismutase (SOD) are known to be important in LPS preconditioning[285]. In fact, exogenous TNFα can itself induce ischaemic tolerance, a process that involves ceramide in its downstream execution[292, 294].

8.4.1 The dual role of TNFα

TNFα is also a potent *cytotoxic* molecule. TNFα as a mediator of ischaemic damage in stroke is therefore something of a paradox. It appears that the TNFα-signaling axis is a finely balanced cascade that can lead to both cell survival and apoptosis (reviewed in [295]). The outcome of TNFα-signaling in the setting of injury may be determined by factors such as the context of the receptor complex, the type of cell and its activation state[296]. TNFα appears to affect initial events in stroke as well as the progression of inflammation and apoptosis in response to injury. Collectively, animal models of cerebral ischaemia support a damaging role for TNFα by the fact that blockade of TNFα in the brain improves stroke outcome[292], while administration of TNFα exacerbates neuronal damage in ischaemia[297]. Less clear are cerebral ischaemia studies in TNFα-receptor knockout mice that suggest a cytoprotective role for TNFα[298]. It has been

suggested that lack of both TNFα receptors in this setting may create a disrupted state of homeostasis due to chronic lack of TNFα-induced negative feedback signaling[295]. Overall, current understanding suggests that TNFα may masquerade as cytoprotective in preconditioning doses or via receptor-signalling feedback loops, but is irrevocably detrimental to the initiation and progression of neuronal ischaemia in larger "inflammatory" doses.

8.5 Cerebral preconditioning in cardiac surgery

The concept of neuroprotection through preconditioning has been recognised for over 15 years. It seems extraordinary therefore that the first attempt to precondition against neuronal injury sustained during DHCA was reported in the literature only one year ago[254]. In this study, the investigators used a rabbit model of ischaemic brain injury during prolonged DHCA to examine the potential for **acute** ischaemic preconditioning as a neuroprotective strategy. The preconditioning stimulus was three cycles of brief (2 minutes) bilateral common carotid artery occlusion and simulataneous ventilation with sub-physiological oxygen, and was administered 15 minutes before commencing CPB. This study revealed a significantly lower hippocampal apoptotic index (using TUNEL and Caspase-3). The investigators decided to use products of the Immediate-Early (IE) genes *c-fos* and *c-jun* as markers of tissue injury and revealed higher expression levels following acute ischaemic preconditioning, which was interpreted as indicative of protection. Despite their assertions, the profile of IE gene products in ischaemic injury is not clearly delineated[299, 300].

Delayed preconditioning is more potent, it preconditions for a significantly longer window (between 7-10 days), and is more effective at remote organs and tissue beds. It is far more attractive as a preconditioning agent. The potential exists, to powerfully protect multiple organs against numerous harmful peri-operative stimuli.

Furthermore, the duration of this protection should continue for at least 7 days. Perhaps most exciting in the context of cardiac surgery is the observation that following LPS preconditioning, both circulating leukocytes and resident tissue macrophages (and glia) are *refractory to activation.* The activation status of platelets has not been examined to date. This immunomodulatory phenomenon is perhaps exemplified by the very recent demonstration that not only is the TNFα load reduced after preconditioning, but that neurons are in fact *resistant* to exogenous TNFα: the entire inflammatory response is altered by LPS preconditioning[301]. This re-programming does not occur in acute preconditioning.

8.6 Aims

Neuroprotection through LPS preconditioning has now been demonstrated against numerous stimuli in several species, including the mouse, rat, guinea pig, sheep, dog, rabbit and goat. No experimental reports exist to describe either successful delayed cerebral preconditioning in piglets, or delayed preconditioning against brain injury sustained during CPB. The aim was therefore to examine the potential for LPS preconditioning to confer neuroprotection against DHCA during neonatal CPB. The hypothesis was that neurons preconditioned with LPS would be resistant to ischaemic injury sustained during DHCA. This hypothesis was tested in the newly established model of histological brain injury during CPB described in chapter 6, because successful preconditioning can only irrefutably be confirmed by quantifying tissue or cellular loss.

8.7 Experimental approach

A full dose-response experiment for LPS preconditioning in the neonatal pig would require a regional stroke model and lengthy series of studies. It was therefore instead decided that a dose extrapolation from other species could be entertained. The first

animal to be inoculated received 0.2mg/kg intravenously, which is the direct dose correlate in the mouse. Shortly after injection is became clear that the animal was systemically unwell. Though we chose (under veterinary guidance) not to euthanise – and the animal survived – recovery was slow and lengthy. A detailed review of LPS (and derivatives of) in all published species was undertaken. This revealed two general dose brackets: one for rodents (approximately 200-500μg/kg) and one approximately an order of magnitude lower in higher mammals (10-35μg/kg). We therefore empirically adopted a dose of 20μg/kg intravenously, 72hrs prior to surgery.

8.8 Experimental design

Twelve neonatal piglets were subjected to intravenous inoculation randomly and blindly with either normal saline (control group) or 20μg/kg E Coli bacterial lipopolysaccharide (serotype 0111:B4, lot no. 104K4109, Sigma-Aldrich Co., USA) (LPS group) (figure 8.3). Inoculation was performed under brief general anaesthesia with 1% isofluorane, and the animals were allowed to recover before being returned to their housing. They were observed closely for the first 24 hrs for signs of ill, including sickness, lethargy or sepsis. None of the experimental animals displayed signs of ill-health and all were indistinguishable in the pre-operative period. Three days (72hrs) later, animals were placed on CPB and subjected to 2 hours DHCA at 18°C. Following re-warming and separation from CPB, animals were reperfused for a further 20hrs before being perfusion-fixed and brain tissue extracted for histological and Fluoro-Jade™ scoring of neurological injury.

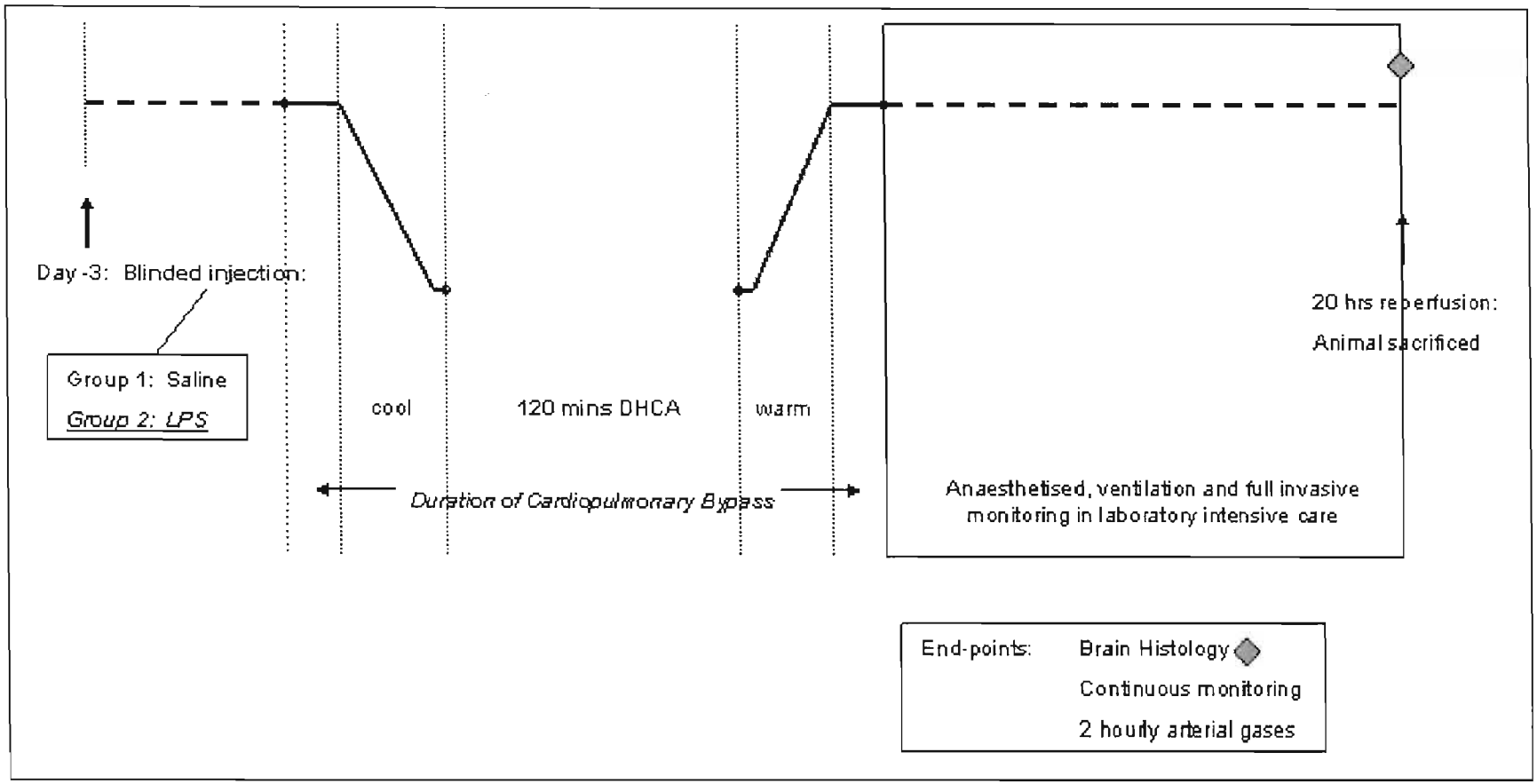


Figure 8.3 Schematic representation of the experimental protocol. Separate controls were used to generate histological controls for the blinded evaluation of injury (see text). Animals were randomised to receiving blinded inoculation with either blank (saline) or bacterial LPS 72 hrs prior to surgery. They were then placed on Ao-RA CPB and subjected to 2hrs uninterrupted DHCA before being re-warmed and separated from CPB. They were then supported for 20hrs with full ITU support antil they were sacrificed and immediately perfusion-fixed for histological analysis of brain injury.

8.8.1 Surgical protocol and post-operative management

The surgical protocol and post-operative management is described in chapters 4 and 6. To summarise, under general anaesthetic, animals were instrumented with surgical tracheostomy and femoral vascular monitoring lines. Following median sternotomy, they were placed on aorto-right atrial CPB using a conventional blood prime and perfusion cooled to 18°C using a pH-stat strategy. CPB was then ceased, the circulating volume drained into the reservoir and the arterial line clamped for 120 minutes. During the circulatory arrest period the coronary circulation was isolated and perfused to provide myocardial protection as described in chapter 6. Animals were then rewarmed and separated from CPB.

Post-operative management included vigorous rehydration with either blood or saline according to the haematocrit, and noradrenaline infusion as necessary to maintain a mean arterial blood pressure above 50mmHg. No bicarbonate was administered following wean, and serial blood gas analysis confirmed correction of the acidosis that necessarily occurs in the early reperfusion period in all our animals exposed to long DHCA durations. Animals remained ventilated under general anaesthesia (IV propofol 3-6ml/hr) until the end of the experiment.

8.8.2 Histological analysis

At the conclusion of a 20hr reperfusion period (measured from the start of rewarming), the animals were re-cannulated and euthanised, irrigated and perfusion-fixed as described in chapter 6. Brains were harvested and scored histologically using H&E and Fluoro-Jade™ as described in chapter 6.

8.9 Results

8.9.1a General parameters

The pre-operative and CPB parameters are given in tables 8.1 and 8.2. Differences were not significant between the groups.

	Control	LPS	*p* value
Weight (kg)	**2.8 ± .18**	**3.3 ± .50**	**.43**
Haematocrit (%)	**28.5 ± 1.9**	**27.8 ± 1.9**	**.81**
pH	**7.36 ± .04**	**7.39 ± .64**	**.64**
Base deficit	**-5.3 ± 2.1**	**-2.0 ± 2.4**	**.33**
PaO_2	**245 ± 39**	**308 ± 47**	**.35**
$PaCO_2$	**33 ± 5.1**	**36 ± 3.3**	**.68**
MAP (mmHg)	**45.0 ± 2.3**	**50.8 ± 4.4**	**.30**
Heart rate/min	**117 ± 5.4**	**131.±.1**	**.08**
Core temp (°C)	**37.3 ± .32**	**36.7 ± .58**	**.54**

Table 8.1 General piglet parameters

	Control	LPS	*p* value
Cooling duration	**27.5 ± 2.5**	**25.8 ± 2.0**	**.61**
Rewarm duration	**52.5 ± 5.1**	**50.0 ± 4.1**	**.71**
Total CPB duration	**201 ± 4.6**	**195 ± 4.6**	**.38**
Base deficit at 25°C during rewarm	**-13.8 ± 2.2**	**-11 ± 1.3**	**.33**
HCO3 (ml)	**21 ± 2.0**	**17 ± .3**	**.31**
pH at wean	**7.41 ± .03**	**7.42 ± .05**	**.95**
Base deficit at wean	**-3.2 ± 1.9**	**-3.7 ± 2.0**	**.86**
PaO_2 at wean	**316 ± 48**	**245 ± 56**	**.38**
$PaCO_2$ at wean	**29 ± 3.7**	**28 ± 4.6**	**.85**
Haematocrit at wean	**34 ± 1.7**	**32 ± 2.5**	**.65**

Table 8.2 CPB parameters

8.9.1b Post-operative management

Haemodynamic and blood gas data during the re-perfusion period following CPB is provided in figures 8.4 – 8.6. No bicarbonate was given following wean from CPB, so that the acid-base status can be used comparatively to provide an indication of systemic circulatory well-being following the recovery from DHCA.

An acidosis develops in the initial few hours following wean from CPB, likely a reflection of the systemic tissue reperfusion and clearance of products of anaerobic metabolism, in concert with poor renal plasma clearance. The continued correction of the acid-base status (figure 8.8-9) importantly indicates that the systemic circulation is adequate to correct this state of metabolic deficiency.

Arterial pH and $PaCO_2$ (usually powerful stimuli for cerebral vasodilatation) (figures 8.6 and 8.10) were no different between the groups.

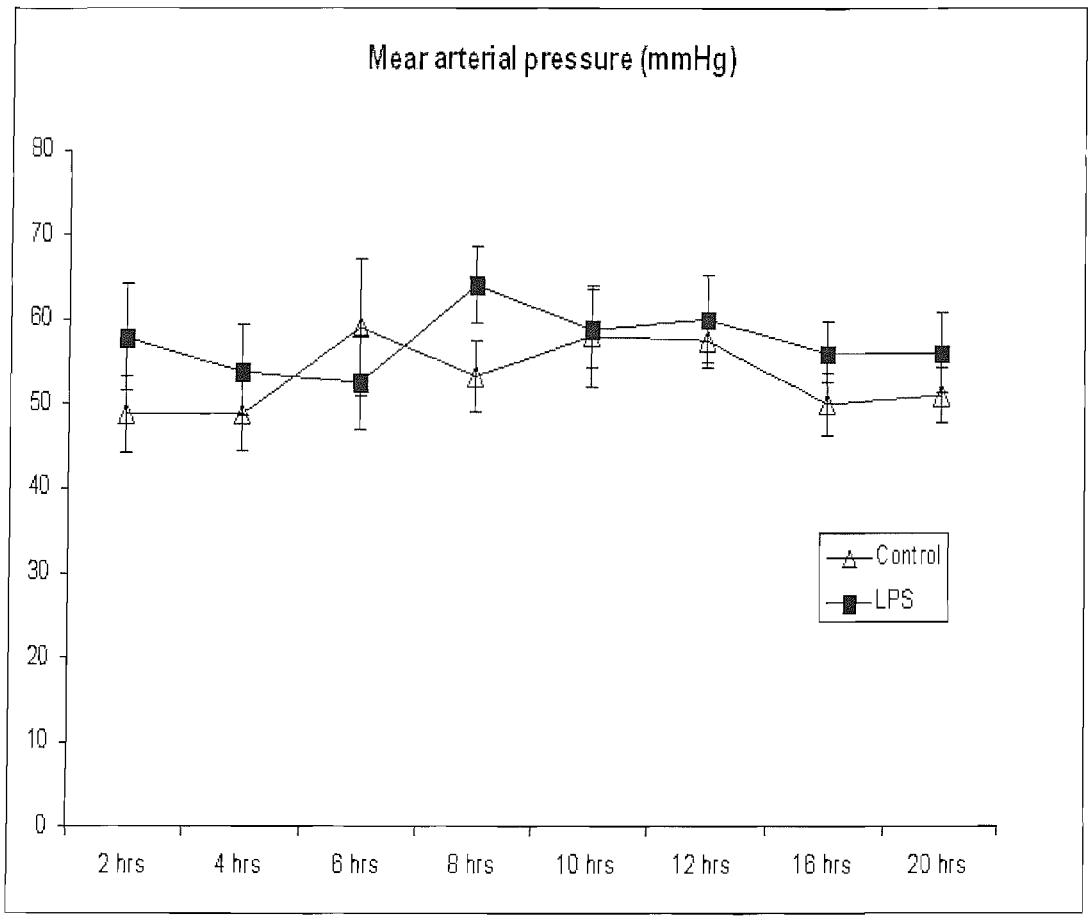


Figure 8.4 Mean arterial pressure during the post-CPB period of reperfusion. Differences were not significant between the groups at any timepoint.

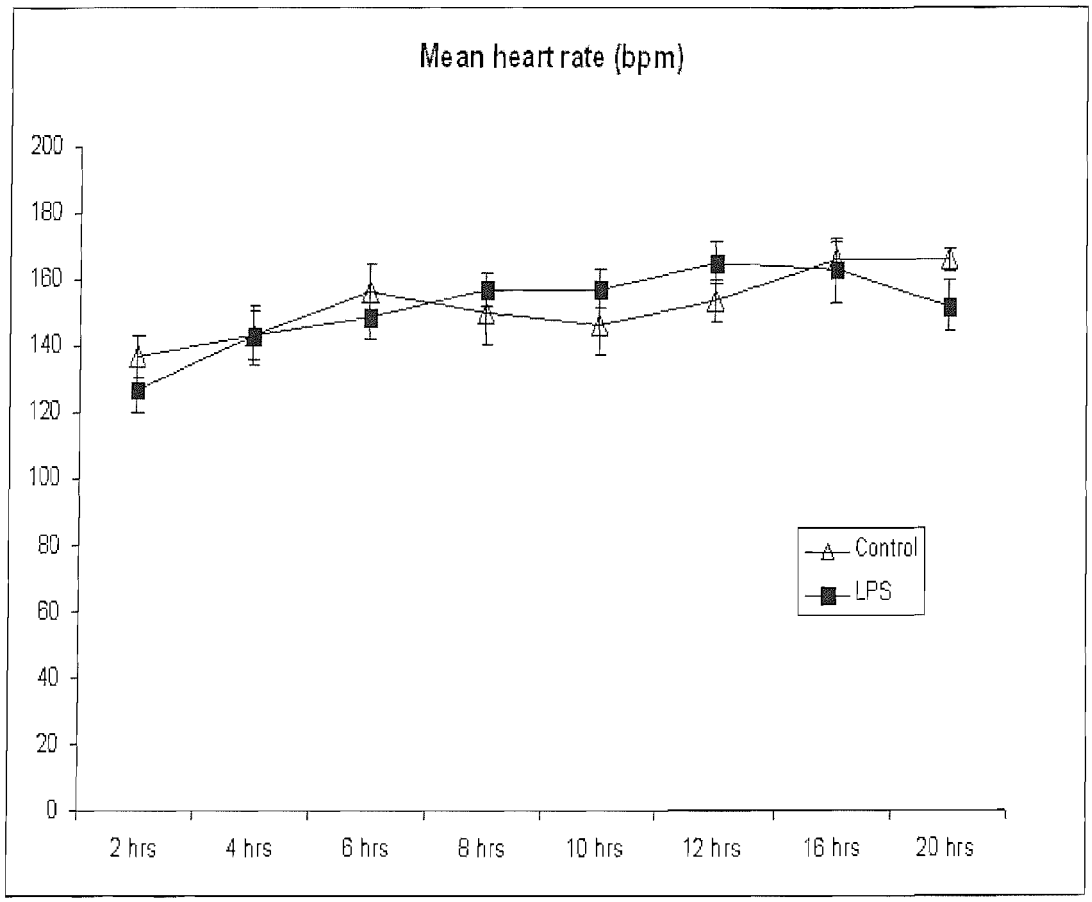


Figure 8.5 Mean heart rate during the post-CPB period of reperfusion. Differences were not significant between the groups at any time point.

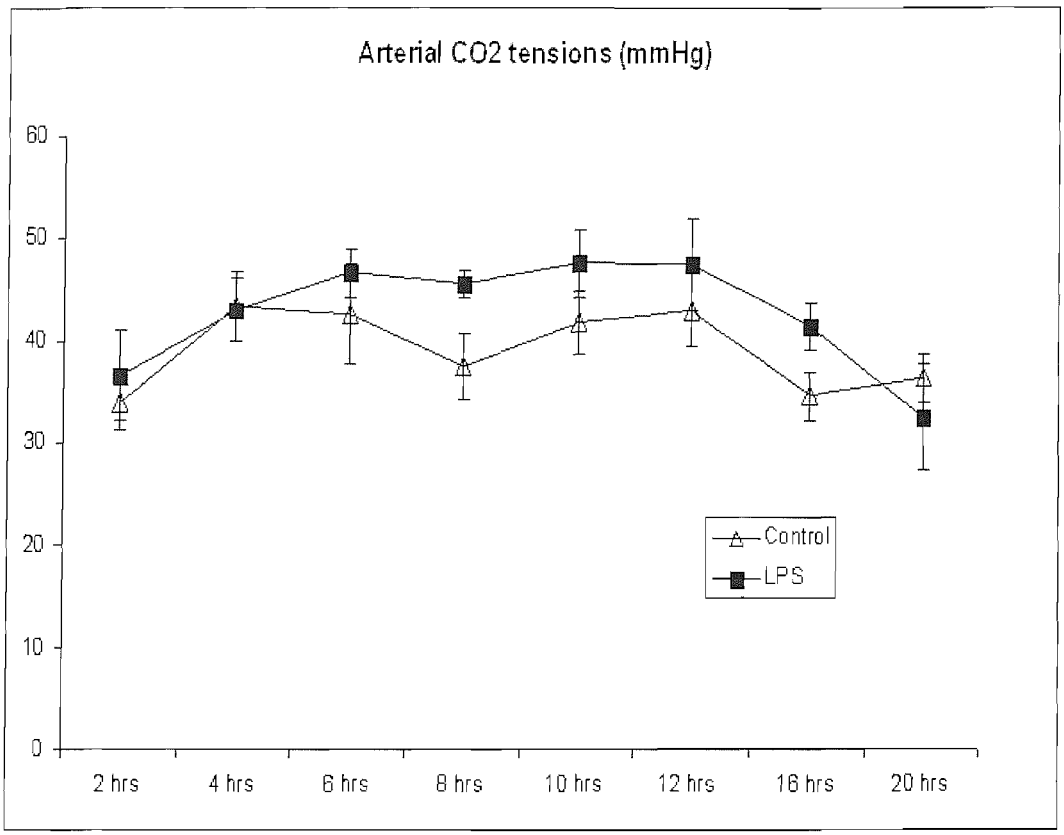


Figure 8.6 Arterial $PaCO_2$ during the post-CPB period of reperfusion. Differences were not significant between the groups at any time point.

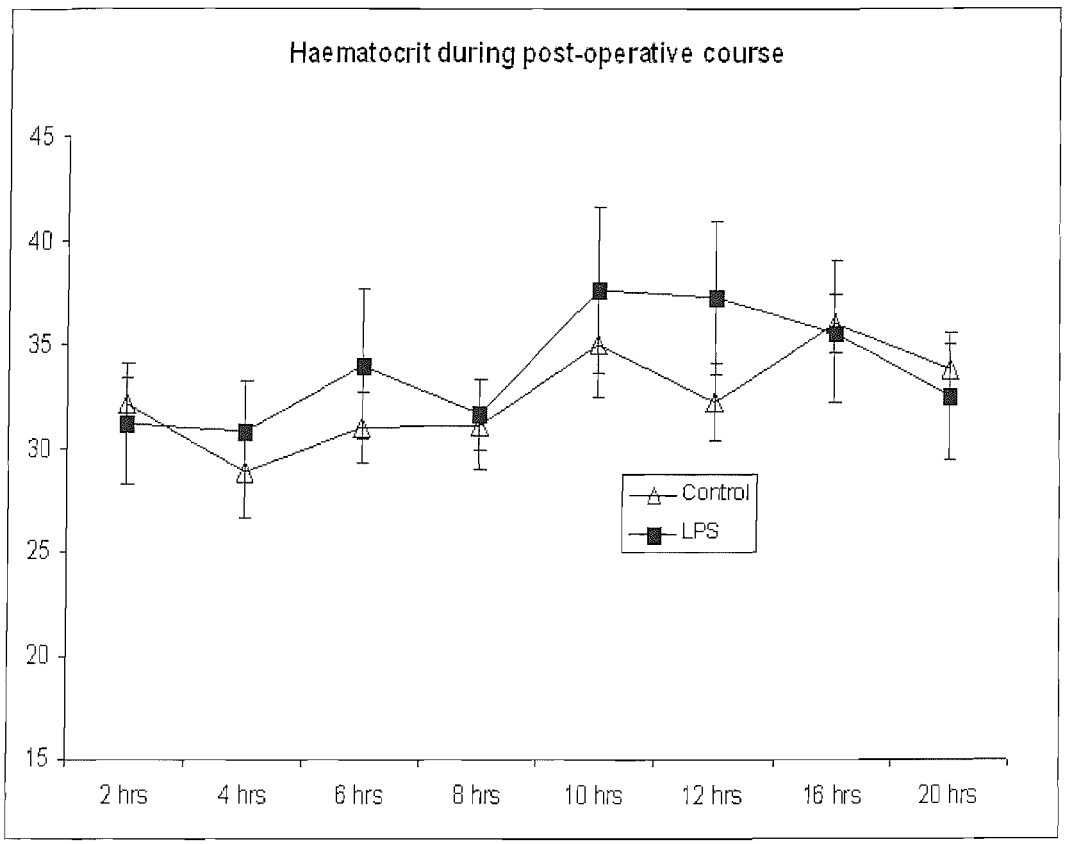


Figure 8.7 Haematocrit during the post-CPB period of reperfusion. Differences were not significant between the groups at any timepoint.

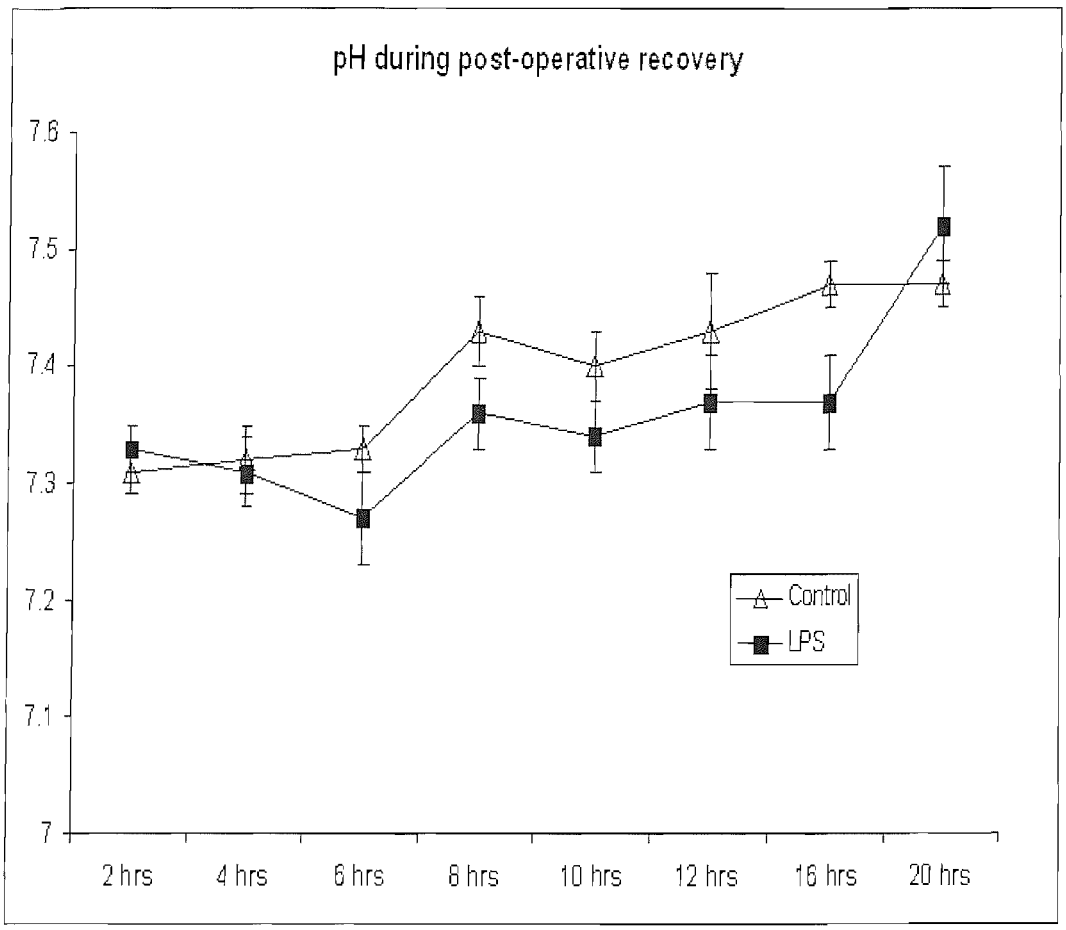


Figure 8.8 Arterial pH during the post-CPB period of reperfusion. Differences were not significant at any timpoint.

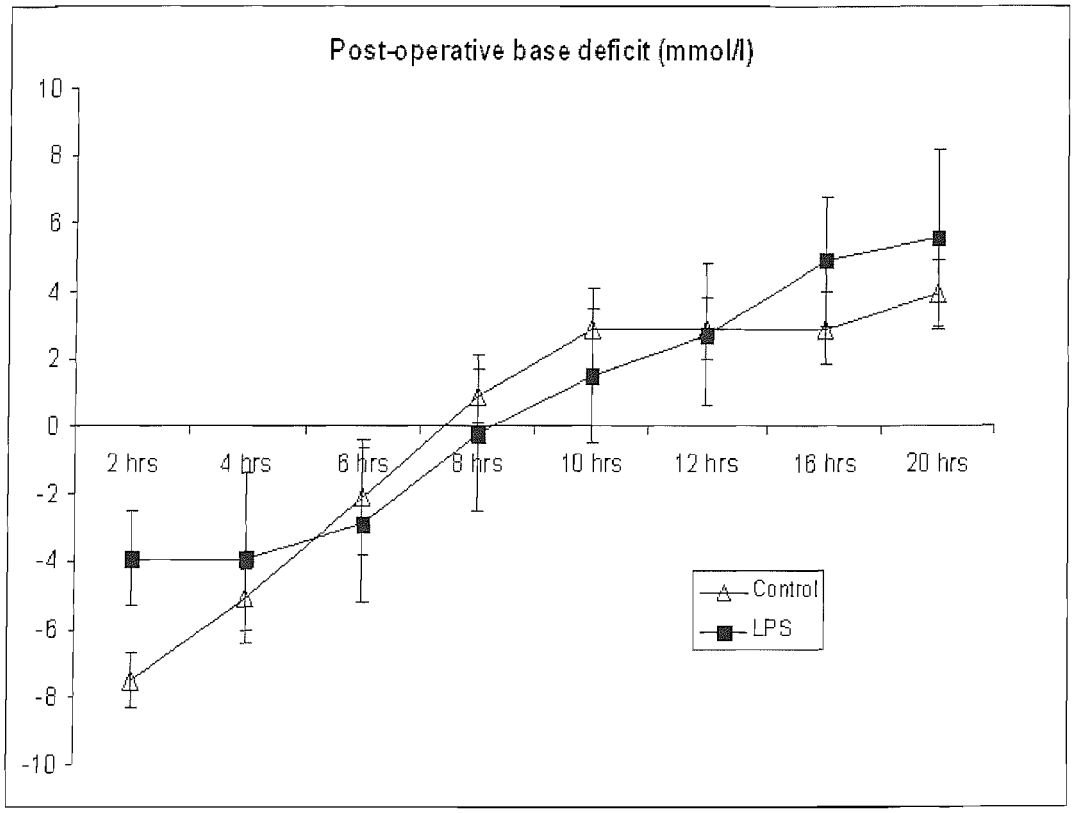


Figure 8.9 Arterial base deficit during the post-CPB period of reperfusion. Differences were not significant between the groups at any timepoint.

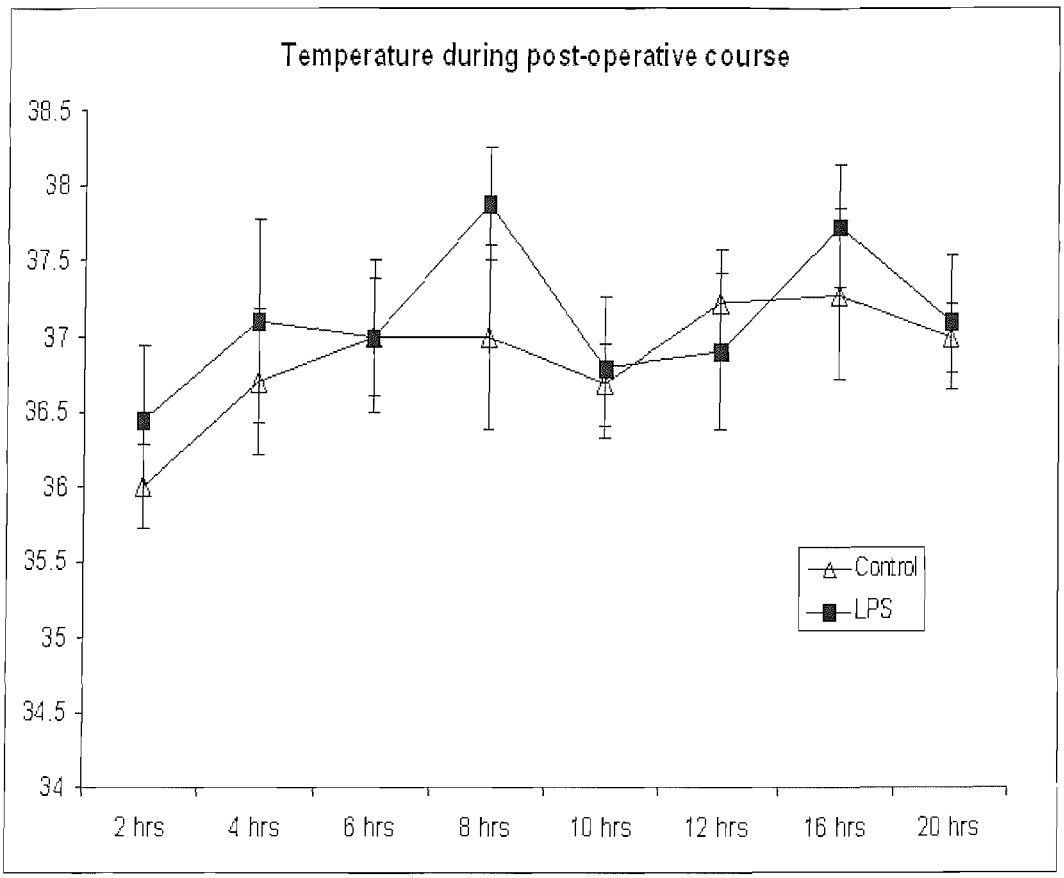


Figure 8.10 Temperature during the post operative period were not different between the groups.

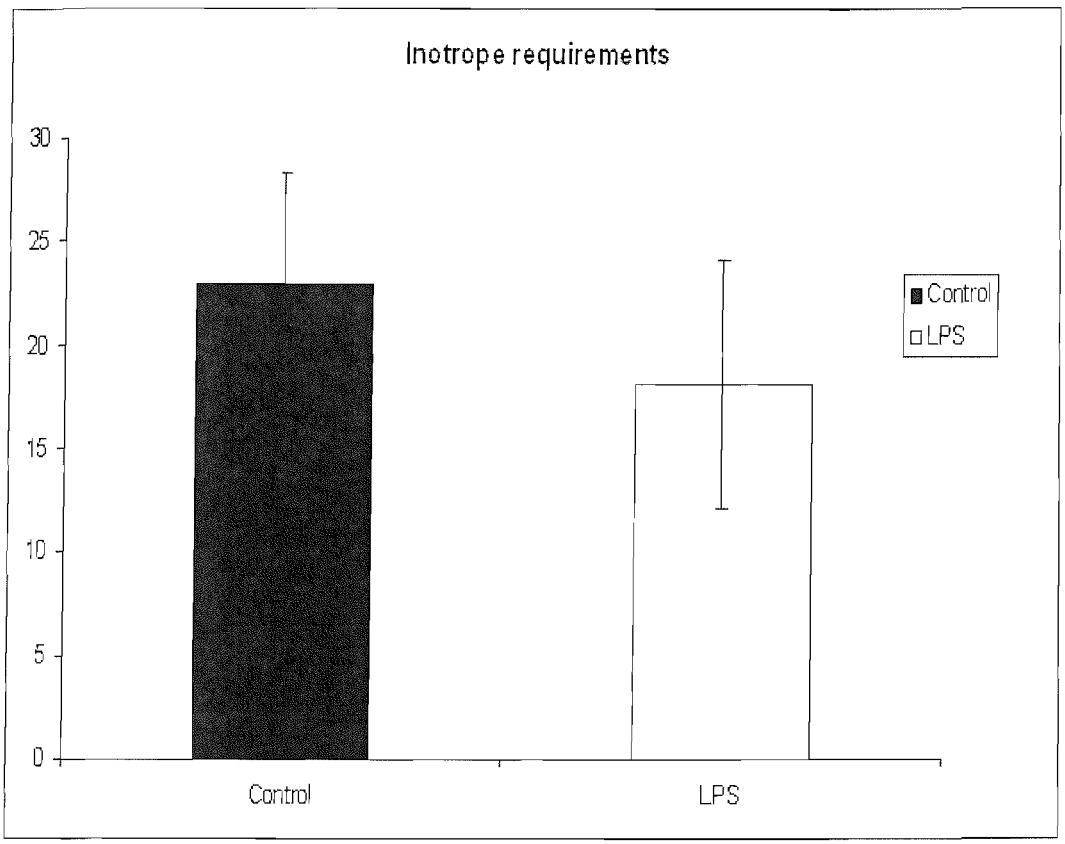


Figure 8.11 Total inotrope requirements (units/ml) during the post-CPB period of reperfusion. Differences were not significant between the groups.

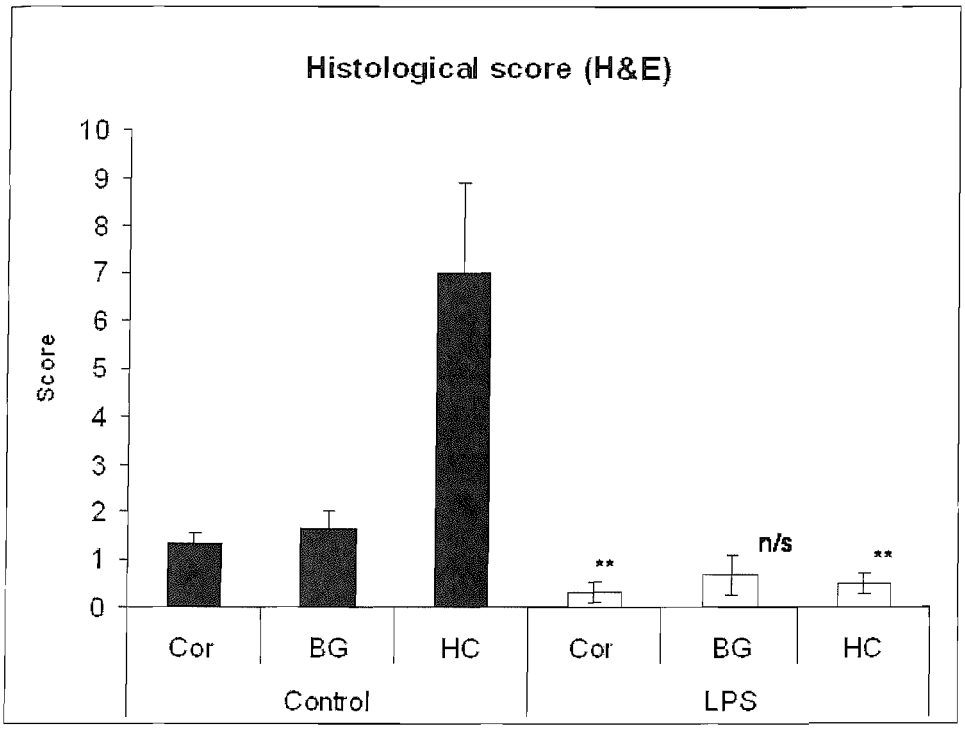


Figure 8.12 Histology scores for the cortex (Cor), basal ganglia (BG and hippocampus (HC) between the two groups. **p<.01, n/s=not significant.

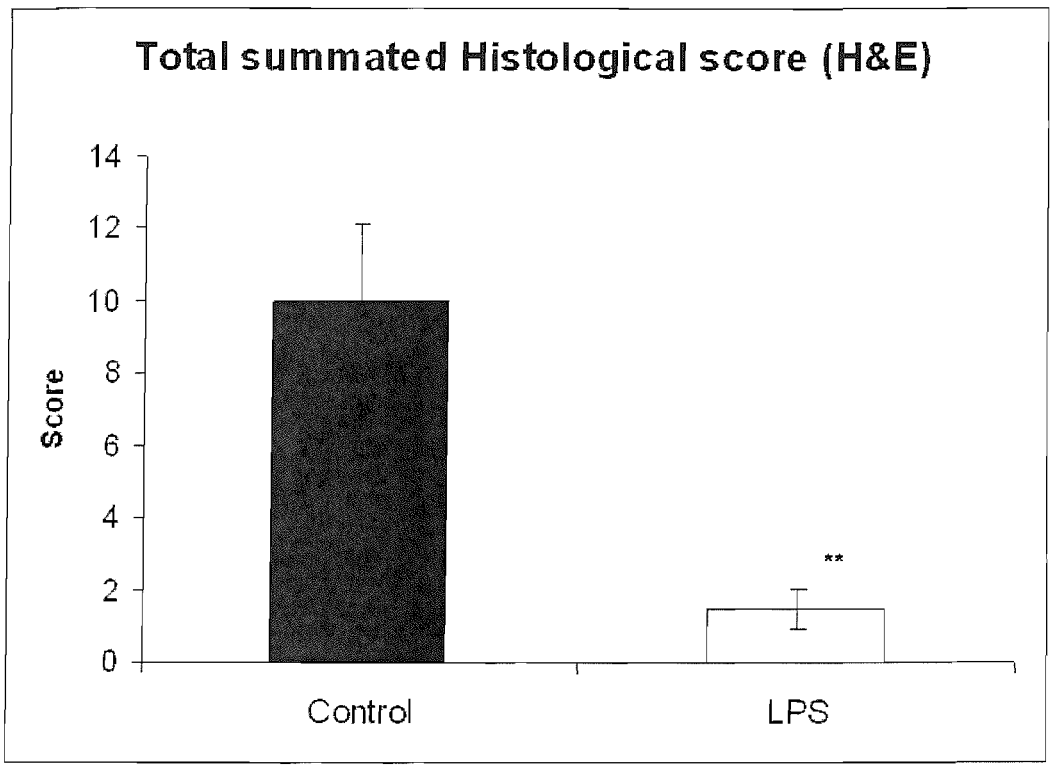


Figure 8.13 Summated histology scores for the two groups. (Cortex + basal ganglia + total hippocampus score) **p<.01

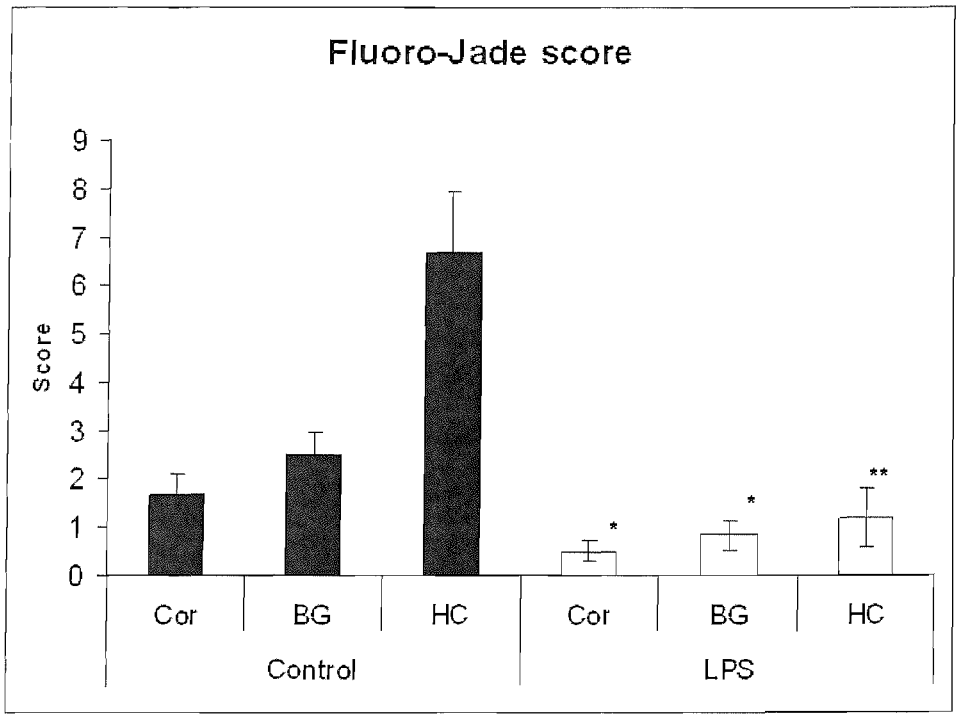


Figure 8.14 Fluoro-Jade™ scores for the cortex (Cor), basal ganglia (BG) and hippocampus (HC). *p<.05, **p<.01

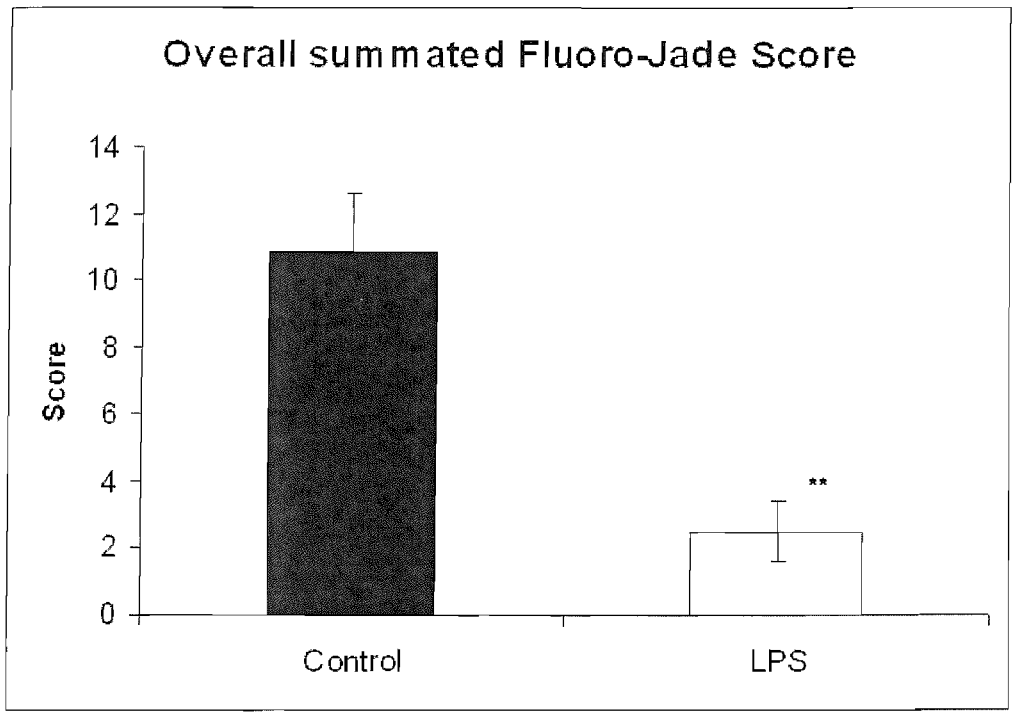


Figure 8.15 Summated Fluoro-Jade™ scores of injury between the experimental groups. (Cortex + basal ganglia + total hippocampal score) **p<.01.

8.9.1c Histological scores of injury

All animals in the control (unpreconditioned) group exhibited histological evidence of irreversible ischaemic cerebral injury in the cortex, basal ganglia and especially the hippocampus (figures 8.12-13). By contrast, some LPS preconditioned animals displayed mild evidence of ischaemic brain injury, the cerebral sections in some animals were indistinguishable from either control animals subjected to CPB with no ischaemia, or controls not subjected to CPB. All slides were examined blindly. No injury was observed in either group in the cerebellum.

Fluoroscopic examination of the slides stained with Fluoro-Fade™ revealed similar differences between the groups (figures 8.14-15). No evidence of injury was observed in either group in the cerebellum.

These results indicate significantly reduced histological injury sustained during experimental DHCA in the animals that had received LPS preconditioning in a random and blinded fashion prior to surgical manipulation.

8.10 Conclusions

Experimental delayed preconditioning probably represents the most powerful neuroprotective strategy short of hypothermia. Its absence from the clinical trials is almost entirely a reflection of the lack of an appropriate clinical situation where a pre-eminent neurological insult can be predicted with a high degree of accuracy. If experimental models were born into practice, either the incidence of neurological deficit following CPB, or the severity of deficit sustained (or perhaps both) would be halved[277]. This study represents the first description of successful neuroprotection

through delayed preconditioning in a model of CPB-related brain injury. As a large mammal model closely mimicking clinical circumstances, it therefore serves as a stepping stone between well understood mechanisms in rodent models, and application in a clinical situation.

8.11 Effector pathways of LPS preconditioning

Very recently, evidence has emerged to link putative effector pathways. One proposed theory that is gathering support involves a central role of Interferon-β, secondary to the TNF-signalling axis. A neuroprotective role of IFNβ is supported by the recent finding that its systemic administration improves stroke outcome in rodents[302] and rabbits[303]. The mitigating role of IFNβ in stroke is primarily ascribed to its anti-inflammatory properties that reduce cell infiltration into the injured brain via regulation of matrix metalloproteinase-9. In addition, IFNβ has been shown to decrease reactive oxygen species, suppress inflammatory cytokines[304], and promote cell survival[305]. These functions could contribute to improved outcomes in stroke injury. The anti-inflammatory effects of IFNβ have long been valued therapeutically in the setting of multiple sclerosis[306] and as such have been approved for clinical use.

IFNs are a family of cytokines comprised of type I (IFNα and IFNβ) and type II IFNs (IFNγ). First characterised based on anti-viral properties, type I IFNs have many immunomodulatory functions, some of which link innate and adaptive immunity[307, 308]. Generally, IFNα/β is associated with anti-inflammatory cytokines[309]. IFNα/β production is induced by many viral infections and relevant to this proposal, by the nonviral, pathogen associated molecule, LPS. Evidence is emerging that in addition to anti-viral effects, type I IFNs inhibit and/or modulate activation of cells in the immune system [310, 311]. The basis for some of IFNαβ's regulatory functions lies in its action as a facilitator of expression of other IFN-inducible proteins known as IFN regulatory

factors (IRFs), that in turn transactivate additional IFN-inducible genes[307]. IRFs constitute a family of transcription factors whose functions in some instances are distinct and independent of one another, while in others, appear to be critically interdependent[312]. Involvement of IRFs in ischaemic brain injury has been shown for IRF1 whose activation worsens stroke outcome, possibly through its known functions in activation of iNOS or enhancement of apoptosis[313].

Recent advances in our understanding of LPS signalling (figure 8.16) have shed light on interactions between IFNs and the LPS receptor, Toll-like receptor-4 (TLR4)[314]. LPS binding to TLR4 and its association with specific intracellular receptor adaptors leads to activation of IRF3 and subsequent IFNβ production[315]. The ability of IRF3 to transactivate IFNβ in this scenario depends on NFκB as well. NF-κB is also activated by TLR signalling, independent of the IRF3 activation pathway. Since NFκB

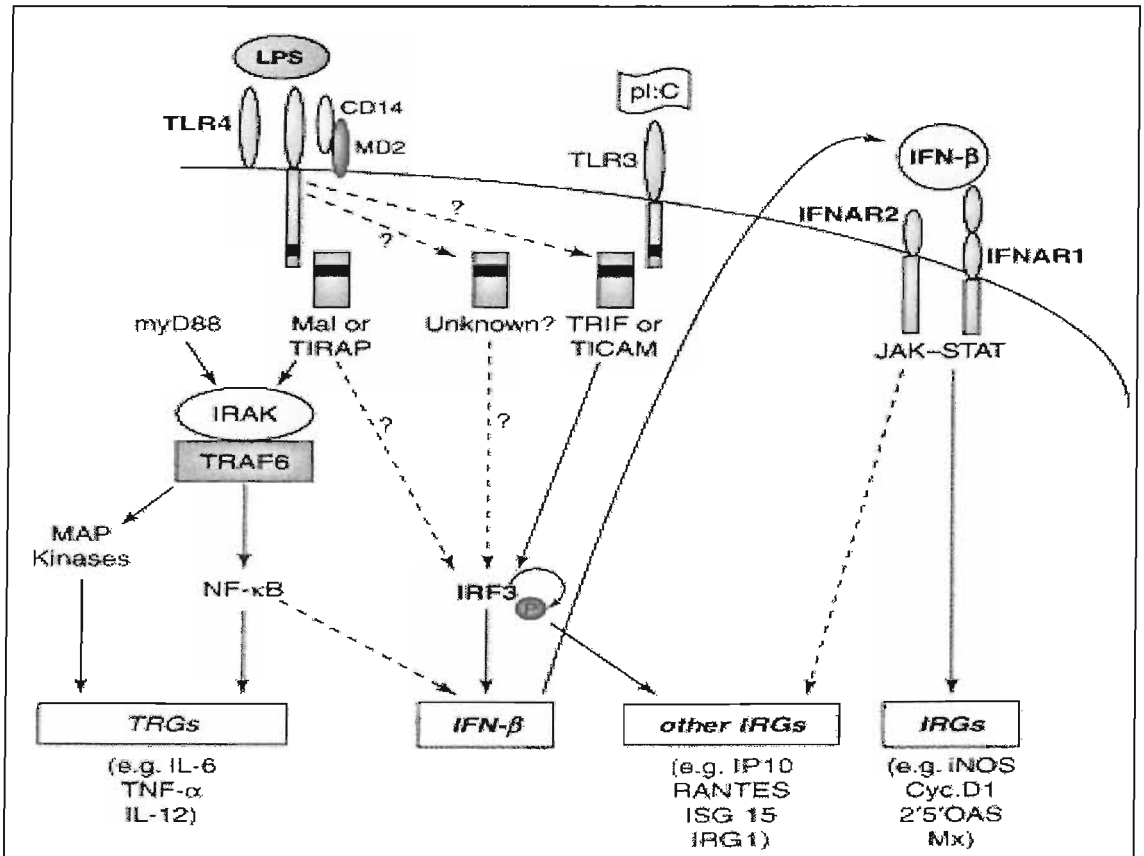


Figure 8.16 Putative signal transduction of LPS via TLR4 receptors. Taken from Hertzog et al. [2]

is required for the establishment of neuroprotection in ischaemic preconditioning models[316], its interaction with IRF3 following TLR4 signalling is of particular interest. It is important to emphasise here that IFR3 can be activated via IFNβ signalling through the IFN type I receptor as well as independently (without IFN type I receptor signalling) via interactions with TLR4.

8.11.1 Reprogramming

Delayed tolerance appears to reflect a fundamental change in the genomic response to injury that shifts the outcome from cell death to cell survival[317]. This change in gene expression in response to ischaemic injury, which is known as genomic reprogramming, was first suggested by gene expression profiles found in animals made tolerant to ischaemic injury by prior preconditioning with a brief period of ischaemia. It was found that ischaemic preconditioning caused pronounced suppression of gene expression in response to ischaemia that is ordinarily injurious. Such suppression contrasted sharply with the upregulation of mRNA in ischaemic injury without prior preconditioning. Specifically the genetic response to injurious ischaemia was characterised by upregulation of a large number of genes related to immune defense strategies. After preconditioning, however, the transcriptional response to ischaemic injury was largely suppressed. These features – including downregulation of metabolic, cell cycle and transmembrane proteins - mimic specific adaptive neuroprotective strategies seen in hibernation and other hypoxia-tolerant states. Thus, as in hibernation, preconditioning elicits endogenous genetic adaptations that confer tolerance to the injurious effects of oxygen deprivation.

8.12 Clinical applications

In reviewing the current theory behind the mechanisms involved in LPS preconditioning, it appears that the initial preconditioning stimulus could be likened to a

"trip" switch, which may be via the TNF-signalling and TLR4 –signalling axes. Stimulation below a particular threshold "trips" the switch in the direction of protection and survival through the production of IFNβ, and subsequent re-programming of the genomic response. Stimulation above a particular threshold, however, "trips" the switch in favour of a harmful and injurious response (typically associated with LPS).

One important prediction, therefore, is that if this process has already been tripped in the direction of injury, then preconditioning is probably not possible. The clinical correlate, for example, is that it is not likely possible to precondition a patient who is haemodynamically unstable with an established systemic inflammatory response syndrome. Successful preconditioning might instead be reserved instead for those "cold" (elective) patients with no active systemic inflammatory compromise.

One concern with preconditioning is that the initial stimulus is a potentially harmful one, albeit in a very low dose. Even though the successful administration of LPS would necessitate that the dose be below the threshold for eliciting a clinically detectable injurious response, and experimentally we observe no signs of ill-health to discern the from control animals receiving blank inoculation, the use of a bacterial agent that is commonly linked with sepsis, shock and death frequently raises concern.

These concerns might be alleviated by more directed searches for appropriate agents. The recent implication of TLRs in the signal transduction of LPS preconditioning suggests that searches for more innocuous ligands for these types of receptors may yield more clinically acceptable preconditioning agents. Several ligands for TLRs are now known. In particular, preliminary studies using an alternative TLR-agonist that is in current human clinical use (for a completely unrelated purpose) has revealed cerfebral preconditioning potency to match LPS in rodent models. There exits the real

possibility, therefore, of identifying an agent that would be acceptable for early human trials in the near future (see chapter 10).

8.13 Summary

Delayed preconditioning represents the most powerful neuroprotective strategy other than hypothermia. LPS preconditioning is one of the best understood examples, and its mechanisms are beginning to be unraveled. The process involves a huge re-programming of the genomic response to injury resulting in a predominance of protective and survival processes. All tissues appear to be affected in this systemic-wide response. The potential for protection against injury – both ischaemic and otherwise – sustained during CPB is huge. Experimental protection is afforded against myocardial, liver and cerebral ischaemia, and examination of the renal effects is underway. Furthermore, LPS preconditioning reduces the inflammatory infiltrate resulting from cerebral ischaemia, and in addition leukocytes are refractory to activation. This study is the first to 1) report successful cerebral protection through LPS preconditioning in neonatal; and 2) demonstrate significant cerebral protection against brain injury sustained during a CPB model.

Chapter 9

The Investigation of Gadolinium Magnetic Resonance Imaging for evaluation of Blood-Brain Barrier Integrity following Neonatal Cardiopulmonary Bypass

9.1 Abstract

Objectives: BBB injury is a common mechanism of widely disparate cerebral pathologies. Its severity is increasingly recognised as an indicator of both pathological and clinical outcome. Despite earlier reports to the contrary, recent studies have implied that uninterrupted normothermic CPB is a stimulus for BBB injury. Gadolinium is a safe intravenous MRI contrast agent that does not appreciably cross the normal BBB. Injury to the BBB characteristically increases its permeability of gadolinium. We therefore hypothesised that BBB injury during CPB might be detected and quantified by using Gd-MRI techniques.

Method: Eleven neonatal piglets were randomly exposed to either 2hrs of normothermic full-flow CPB, 2hrs deep hypothermic full-flow CPB, or 1hr DHCA with 1hr cooling/re-warming. The animals were separated from CPB and transferred to the MRI suite. T1-weighted sequences were performed using a 3Tesla magnet, using sequential inversion times. These were then repeated following delivery of a Gd bolus. After calculation of the specific inversion time of both grey and white matter, differences pre- and post-contrast resulting from parenchymal Gd accumulation were calculated.

Results: The difference in relaxation time in pre-and post-contrast images ($\Delta R1$) was greatest in both grey and white matter following normothermic full flow CPB. The $\Delta R1$ was significantly shorter following deep hypothermic full flow CPB ($p<0.05$), and DHCA was intermediate.

Conclusions: The use of Gd-MRI spectroscopy to evaluate brain injury has not been previously reported. This preliminary study suggests that normothermic continuous perfusion strategies may initiate more ultrastructural injury to the BBB than an identical strategy using deep hypothermia.

9.2 Introduction

A major hinderance in the study or detection of brain injury following neonatal CPB is the lack of sensitive and specific biomarkers of neurological injury. Considerable energy has been spent attempting to identify such markers, and attention has largely been focused on serum proteins that are believed to be brain-specific. Examples of these include neuro-specific enolase and S100B protein[246], of which the latter in particular sparked considerable interest following its initial discovery.

S100B protein is a member of the larger S100 family of small calcium binding proteins. In general, the functions of the S100 proteins are poorly characterised, although evidence is accumulating to implicate certain members of the S100A family in immune function. In contrast to the S100A isoform of the family, S100B is a uniform member consisting of a dimer (S100BB and S100A1B). It appears to a relatively brain-specific intracellular protein, present in glia and schwann cells, although its function is not clear. A role in memory and learning has been suggested by recent studies in mice, including transgenic animals deficient in S100B[318, 319], or those injected with S100B antiserum[320].. Several studies have implicated a pathological role of S100B, for example in Alzheimer's and AIDS[321, 322]. The demonstration of elevated serum levels following stroke and trauma[323], which appear to correlate with both pathological and functional outcome[324] suggested a potential role for the quantification of injury following CPB and DHCA[325, 326]. Although several early reports supported this potential role, the picture has become less clear. It appears S100B is not brain specific, and can be released in response to widely varied stimuli. The high concentrations found in shed mediastinal blood that are then retransfused through cardiotomy suction and autotransfusion have added to the confusion surrounding S100B as a biomarker of brain injury following cardiac surgery[327]. Furthermore, the half-life of S100B is only short (25min) [328] and its serum

concentrations can vary inconsistently[247]. To summarise, therefore, despite considerable excitement about its prospects as a useful biomarker of cerebral well-being following CPB, other indicators of ultrastructural injury are instead warranted.

The BBB consists of endothelial cells (EC), basement membrane and closely wrapped astrocytes. Inter-cellular junctions are tight and passage of both metabolites and toxins is highly regulated, providing an insulated environment for neurons to reside. Early loss of BBB integrity is subject to increasing interest in all aspects of neurological research, as it is a common event - whether cause or effect – in all mechanisms of brain injury. Furthermore the magnitude of BBB disruption has very recently been shown to correlate with greater cellular loss and worse functional outcome in both experimental and clinical cerebral ischaemic injuries[139, 146, 147]. One report has identified EC apoptosis as playing a key role after brain injury. Protection of ECs against apoptosis resulted in less BBB permeability and improved neurological outcome[139]. Latour et al have provided two reports within the past year whereby BBB injury was quantified through the use of (gadolinium) contrast magnetic resonance imaging (by measuring gadolinium CSF accumulation – the FLAIR technique) and permeability was demonstrated to correlate with worsening functional outcome[146, 147].

The recent demonstration that BBB disruption correlates with both histological injury and poor clinical outcome following ischaemic cerebral insults has resulted in several studies aimed at elucidating the fate of the BBB during and after CPB. Despite initial reports to the contrary[329, 330], it now appears that CPB – in the absence of ischaemia – can itself instigate a significant rise in BBB permeability. Albumin is a relatively huge protein, and although it exists in both the intravascular and extravascular spaces, it is compartmentalised, and unable to cross the intact BBB. Cavaglia and colleagues, using fluorescent albumin tracers recently demonstrated that after as little as 15 minutes of normothermic CPB, the BBB is permeable to small amounts of albumin. This

phenomenon is proportional to CPB duration and greatly increased by periods of ischaemia[331]. It is unfortunate that this group did not examine the effects of hypothermic continuous CPB, as the ultrastructural consequences of deep hypothermia (in the absence of ischaemia) are not clearly known.

Magnetic resonance imaging (MRI) has represented a huge leap in the diagnosis and quantification of brain injury. Non-invasive and safe, its use as a conventional (anatomical) diagnostic tool has proved to be reliable in detecting brain damage 1 to 2 weeks following significant ischaemic injury[201, 332]. However, more subtle stucutral damage may not be discernable and indeed conventional MRI has not become a sensitive tool for detecting or quantifying subtle cerebral compromise early following CPB. The concept of MRI spectroscopy, whereby the physical properties of tissue can be delineated in an extremely small field-of-view, instead provides novel methods by which lesions can be identified and quantified. Diffusion-weighted imaging (DWI) for example studies the Brownian motion of water and then calculates the apparent diffusion co-efficients of tissues[333] which are altered especially in ischaemic pathologies[334, 335], where biological barriers may become more permeable. Specific spectroscopic assessment of 1-hydrogen (1H-MRS) can provide detailed analysis of the metabolic alterations following cerebral ischaemia[336] and has even been successfully applied following DHCA during CPB. In addition, T1 and T2 relaxation times following excitation can also be used to study cerebral lesions. For example, unusually short T1 relaxation times identified in periventricular leukomalacia have recently been interpreted as representing early haemorrhagic areas[337].

Gadolinium (Gd) compounds are commonly used contrast agents for magnetic resonance imaging. In fact Gadopentate Dimeglumine was the first MRI contrast agent described when in 1984 it was used for contrast enhancement of intracranial lesions with abnormal vascular beds or those in which breakdown of the BBB was a feature.

Gadolinium chelates (Gd-DTPA) are now in ubiquitous use, and have a proven track record for safety. The properties that render Gd-DPTA a useful contrast agent are related to its high proportion of unpaired electrons. Magnetic resonance imaging works by inducing magnetic moments in protons or unpaired electrons, however most elements have paired electrons and therefore have their magnetic moment cancelled. Gd+++ in its native state has 7 unpaired electrons (more than any other element). Unpaired electrons generate large magnetic moments (657 times greater than a proton) and therefore the 7 unpaired electrons in Gd-DPTA are 10^6 times better at stimulating relaxation than a single proton. Gd chelates injected intravenously are not tissue specific, and will distribute within any extravascular space to which it has access. However an intact BBB is deemed to be (relatively) impermeable to Gd, and therefore although contrast will be seen in the cerebral vasculature, it does *not* normally accumulate in the cerebral parenchyma.

We therefore hypothesised that the use of Gd-DPTA MRI spectroscopy might be a potential technique for identifying and quantifying BBB injury following CPB strategies. In addition to representing a possible experimental tool for identifying and quantifying BBB disruption, it also carries the potential to be applied clinically (in contrast to other experimental techniques for assessing BBB injury). Central to our hypothesis was that Gd-DPTA would accumulate in the cerebral parenchyma in proportion to the degree of BBB disruption. Gd-DPTA reduces (shortens) the T1 relaxation times of target tissues. Therefore, by measuring T1 relaxation times of tissues before and after the administration of Gd-DPTA, a greater change in T1 relaxation time (ΔR1) would reflect greater accumulation of contrast agent within the (extravascular) cerebral parenchyma, and therefore a corresponding greater injury to the BBB.

9.3 Experimental methods

Neonatal piglets were randomly assigned to a CPB strategy of either **2 hrs normothermic full-flow (Warm FF) CPB (n=4), 1 hr deep hypothermic full-flow (Cold FF) CPB (n=3) or 1 hr DHCA (n=4)** (figure 9.1). The total duration of CPB – including cooling and rewarming periods - in all groups was therefore 2 hrs. The surgical protocol was as detailed in chapters 4 and 6. To summarise, under general anaesthetic, animals were instrumented with surgical tracheostomy and femoral vascular monitoring lines. Following median sternotomy, they were placed on aorto-right atrial CPB using a conventional blood prime. The normothermic group remained on full-flow CPB for 2hrs. The remaining groups were perfusion cooled to 18°C using a pH-stat strategy. CPB was then either ceased (DHCA) unterrupted for 1 hr or continued at full-flow for 1 hr (Cold FF). During the circulatory arrest period the coronary circulation was isolated and perfused to provide myocardial protection as described in chapter 6. Animals were then re-warmed, separated from CPB and supported anaesthetised (IV propofol) and ventilated with full invasive monitoring and intensive care support for the duration of the experiment (including transfer and scanning).

9.3.1 Animal transfer

Following separation from CPB, heparin was reversed with protamine (1ml/1000iu heparin) and the animal allowed to stabilise for 30 minutes. Pleural and pericardial drains were inserted and the chest wall approximated with size 1 polypropylene suture. Catheter extensions (15ft) were placed on all invasive lines. The animal was then detached from the ventilator and manually ventilated ("bagged") with 100% oxygen using a portable anaesthetic machine. The animal was transferred to a warmed mattress on a trolley and surrounded with warm bean-bags and blankets (figure 9.2). Battery-

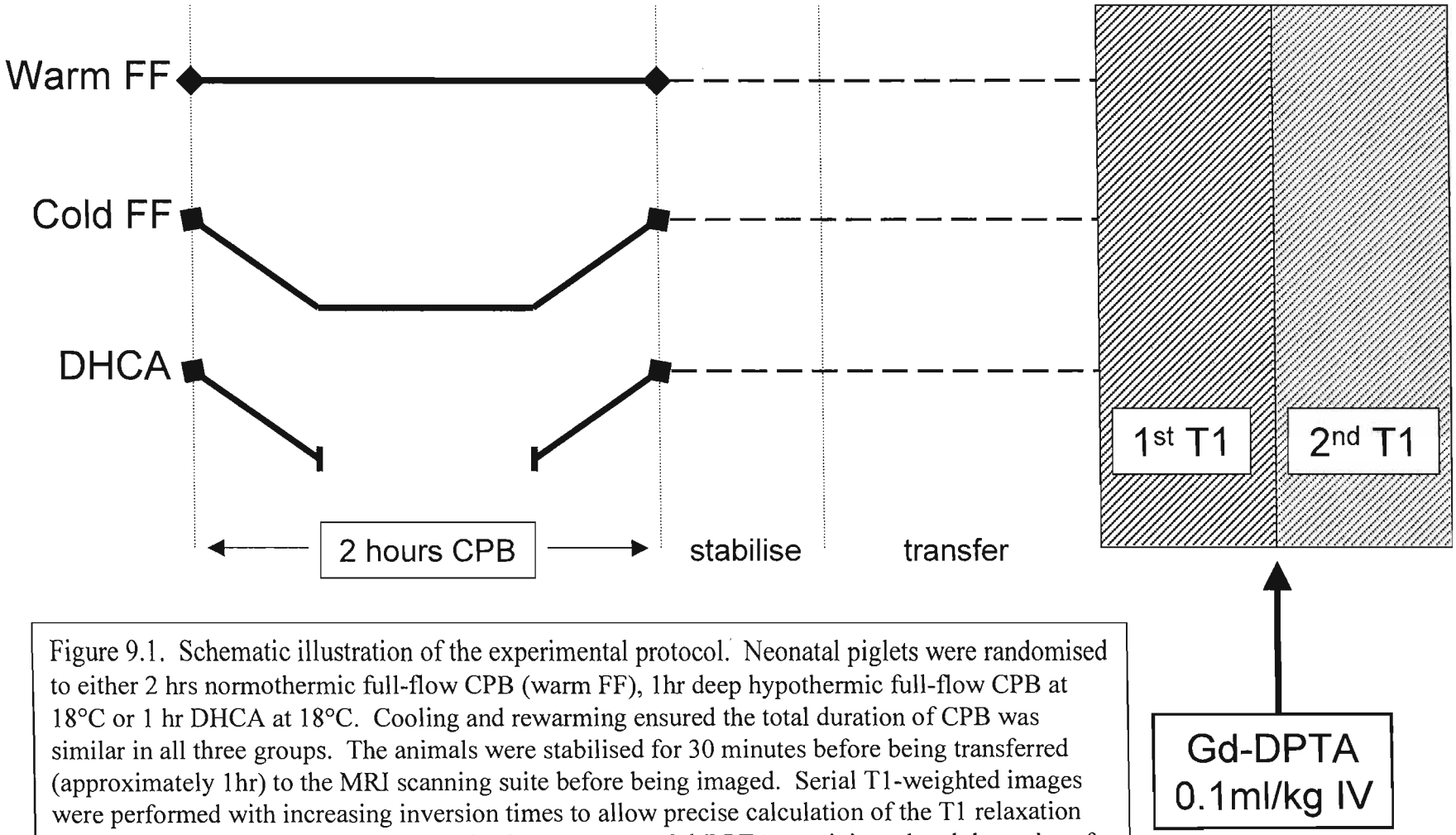


Figure 9.1. Schematic illustration of the experimental protocol. Neonatal piglets were randomised to either 2 hrs normothermic full-flow CPB (warm FF), 1hr deep hypothermic full-flow CPB at 18°C or 1 hr DHCA at 18°C. Cooling and rewarming ensured the total duration of CPB was similar in all three groups. The animals were stabilised for 30 minutes before being transferred (approximately 1hr) to the MRI scanning suite before being imaged. Serial T1-weighted images were performed with increasing inversion times to allow precise calculation of the T1 relaxation time using computer programs. After the first sequence, Gd-DPTA was injected and the series of studies repeated. The difference in T1 relaxation times was therefore due to accumulation of exogenous Gd-DPTA.

operated monitors and syringe drivers allowed continuous administration of intravenous propofol and noradrenaline as necessary while the piglet was transferred from the operating room to a loading bay. A closed heated truck was prepared to receive the animal with incumbent equipment, for relay to the MRI scanning suite located approximately 1 mile away (figure 9.3).

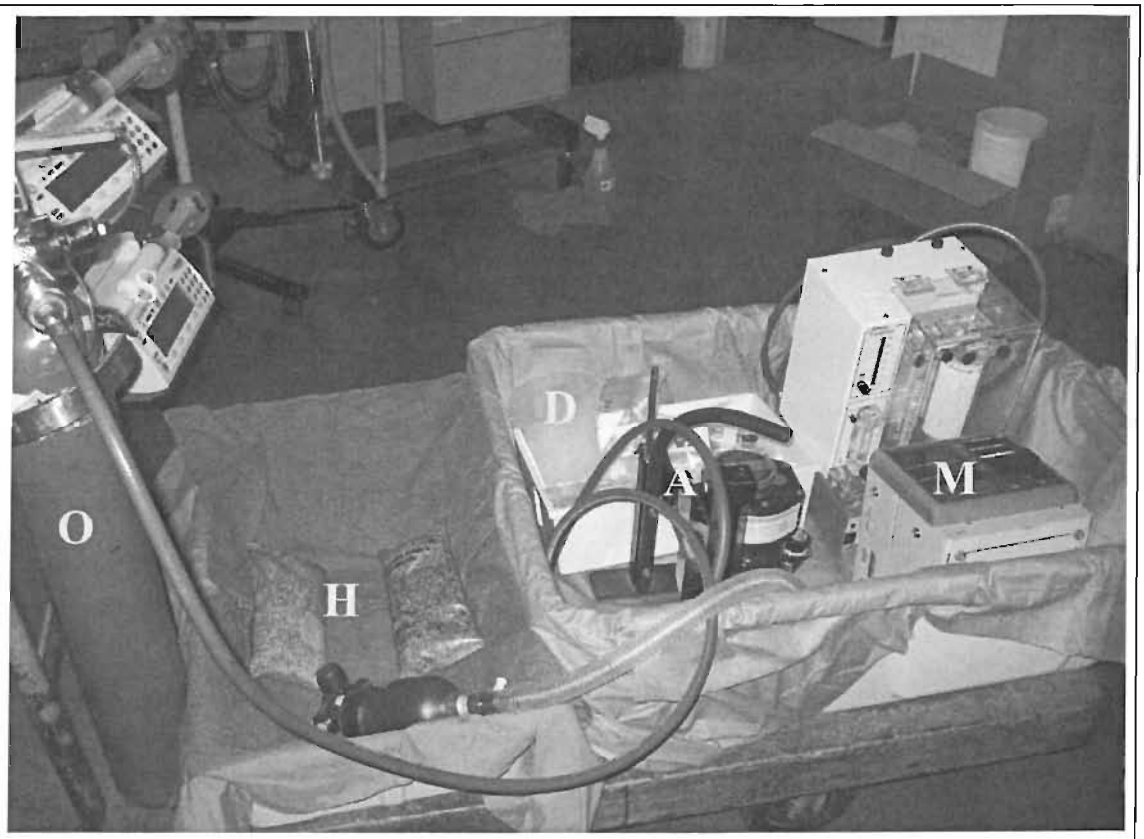


Figure 9.1 Animal transfer trolley, complete with portable oxygen (O), ventilator (V), anaesthetic machine (A), syringe drivers (S), heat pads (H), monitors (M), and emergency drugs (D).

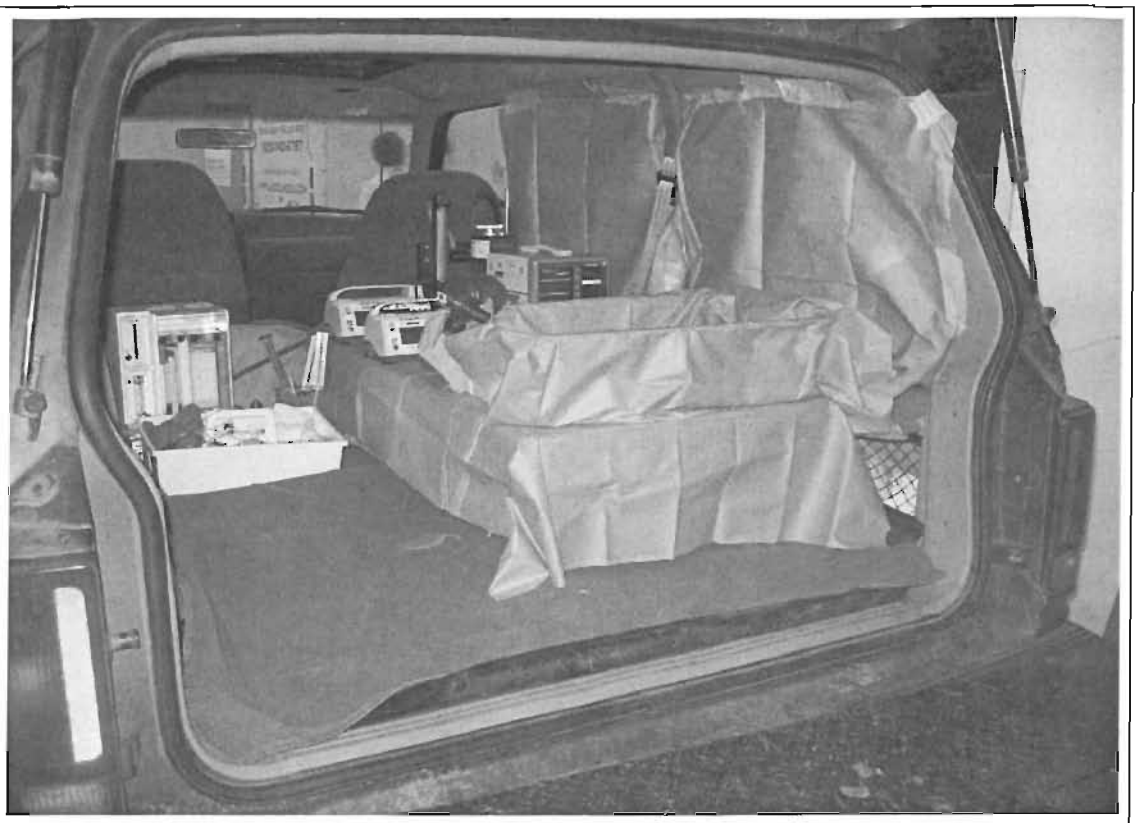


Figure 9.2 Truck prepared for transfer, complete with full mobile intensive care support. The piglet was manually ventilated for the entire duration of the transit, until all the tubing was in place in the scanner. The complete transit was usually undertaken by a team of two.

MRI scanning ventilated subjects poses several hurdles due the risks of ferro-magnetic equipment in the proximity of the magnet. Several deaths have occurred from the accidental introduction of oxygen cannisters which become lethal missiles in the vicinity of powerful magnets. Extreme caution must therefore be taken. Syringe drivers and the monitoring units were therefore placed at the boundary of the suite, immediately visible through the observation screen from the control room. The paediatric ventilator and portable anaesthetic machine were stationed in an annexe room, protected by the iron radiofrequency shields in the scanner's walls. Extended hoses passed through a small tunnel through to the scanning suite and connected the ventilator to the endotracheal tube.

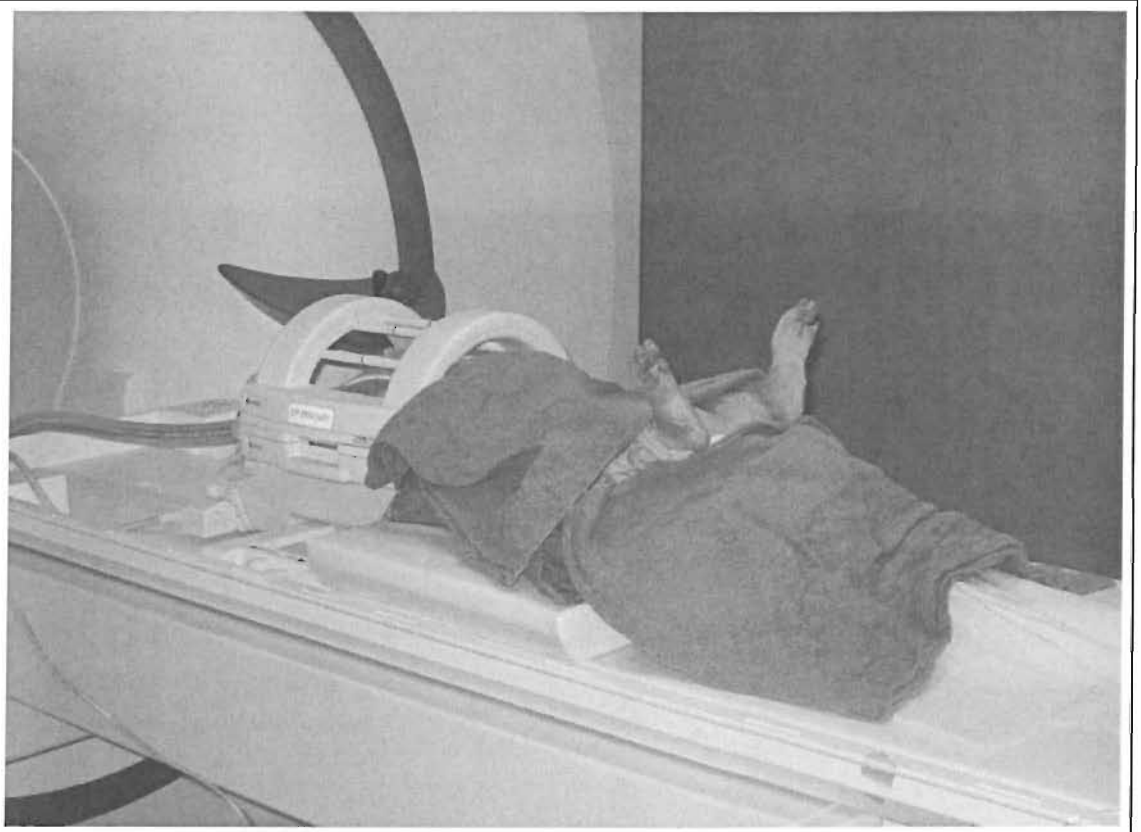


Figure 9.3 A human knee coil was used for the piglet. Warmed blankets and heated bags surrounded the animal. The green ventilator hosing is visible passing through the magnet where it was attached to a hosing extension which passed through the tunnel in the wall to connect to the ventilator and oxygen tank in the annexe. Extended lines are leaving to the right of the picture to syringe drivers situated well away from the magnet.

Figure 9.4 The view from the control room. The monitors were placed immediately opposite the viewing window to allow continuous observation of the piglet's well-being.

The piglet was positioned on the scanning table on a bed of warm blankets and heated bags. A knee coil was positioned around its head and all lines firmly secured in place. Following transfer, a period of 10 minutes was allowed to ensure haemodynamic stability and the head coil was then localised and the piglet inserted into the tunnel.

9.3.2 Magnetic resonance imaging

All magnetic resonance images were performed using a research-dedicated 3 Tesla Siemens scanner, with the animal's head positioned in a human knee coil (figure 9.4-5). Anatomical scout images were acquired in both the horizontal and coronal planes using a fast spin-echo T2-weighted sequence, with a repetition time (TR) of 3sec, echo time (TE) of 80ms, matrix size 130 x 130 and field of view (FOV) of 12x12cm. Slice thickness was 2mm in the horizontal plane and 1mm in the coronal plane. Serial spin-echo T1-weighted images were then taken with progressively lengthening inversion times (TI) of 100, 200, 300, 400, 500, 600, 800, 1000, 1200, 1500 and 2000 msec.

Following this pre-contrast series of images, a bolus of Gd-DPTA (Omniscan™, gadodiamide, GE Healthcare, US) was administered intravenously (0.1mg/kg). A second series of spin-echo T1-weighted images was then performed using the same protocol.

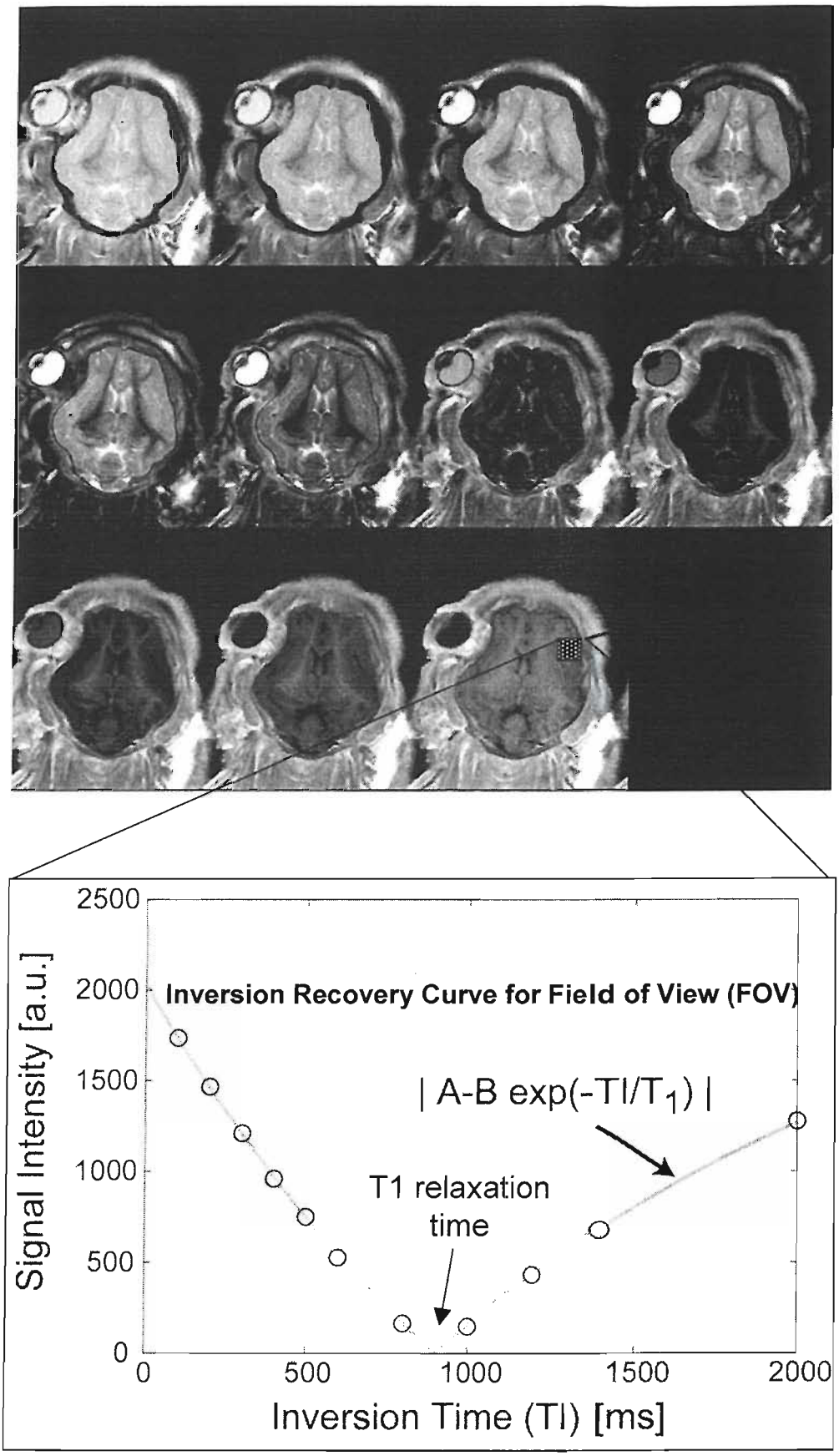
The images from each of the pre- and post-series were subsequently fed into a specifically designed software program to determine the *recovery of inversion* curve (figure 9.6). This yields a specific inversion time for the tissue in the field of view being analysed. Serial calculations were made for white matter, grey matter and skeletal muscle. Gd-DPTA accumulates indiscriminately in skeletal muscle and therefore this reading was used to allow for normalisation of the blood gadolinium concentrations between animals.

Data was interpreted by analysing the change in T1 inversion times pre- and post-contrast ($\Delta R1$). This was then expressed relative to skeletal muscle within each animal.

Figure 9.6 (Overleaf). T1-weighted scanning is performed with sequentially increasing inversion times (TI). When the inversion time equals the T1-relaxation time, the image appears black, hence the differential image intensity of the same brain slices at the different inversion times.

The data from these sequences is processed by a computer program to construct an inversion recovery curve. This can be done for any particular "field of view" in questions within the slice of tissue imaged. The inversion recovery curve can be used to precisely determine the T1 relaxation time in milliseconds.

Following the administration of Gd-DPTA the T1-relaxation time will shift leftwards ("reduce" or "shorten") proportional to the concentration of gadolinium present within the field-of-view. The change in T1-relaxation time ($\Delta R1$) in one field-of-view from *before* Gd-DPTA administration and *after* therefore can be used to interpret the accumulation of gadolinium.


Inversion Recovery Curve for Field of View (FOV)
| A-B exp(-TI/T1) |
T1 relaxation time
Signal Intensity [a.u.]
Inversion Time (TI) [ms]
0
500
1000
1500
2000
2500

9.4 Results

General peri-operative animal parameters are given in table 9.1. There were no significant differences between the groups. In particular, all the animals weaned from CPB without incident and maintained excellent haemodynamic status during the course of the transfer and scan. Despite the complexity involved in transferring large mammals anaesthetised after major surgery, all animals survived transfer and underwent successful imaging.

The relative changes in T1-relaxation times ($\Delta R1$) for cerebral grey matter (GM) and white matter (WM) are illustrated in figures 9.7 and 9.8. The changes were significantly greater following warm FF CPB than cold FF ($p<0.05$ for both GM and WM) and DHCA was intermediate. In cerebral GM, DHCA appeared to trend towards a greater $\Delta R1$, though it was not statistically significant from either continuous perfusion strategy with the animal numbers used. For WM, the $\Delta R1$ following DHCA again tended to be greater than cold FF (not significant), but it was significantly less than warm FF ($p<0.05$).

		Pre-CPB	Post-CPB
Mean Arterial Pressure (mmHg)	Warm FF	49 ± 3.3	60 ± 2.6
	Cold FF	49 ± 7.5	74 ± 13
	DHCA	56 ± 4.0	65 ± 4.5
Haematocrit (%)	Warm FF	24 ± 1.5	28 ± 1.1
	Cold FF	33 ± 2.7	36 ± 3.9
	DHCA	33 ± 2.7	28 ± 1.6
pH	Warm FF	7.45 ± .9	7.34 ± .04
	Cold FF	7.49 ± .02	7.42 ± .06
	DHCA	7.47 ± .04	7.34 ± .05
Base deficit (mmol/l)	Warm FF	1.24 ± .9	-1.5 ± 1.7
	Cold FF	1.3 ± 3.5	-1.9 ± 1.3
	DHCA	0.0 ± .6	-0.82 ± 2.4
PaO_2 (mmHg)	Warm FF	325 ± 33	177 ± 47
	Cold FF	398 ± 53	394 ± 58
	DHCA	254 ± 70	347 ± 46
$PaCO_2$ (mmHg)	Warm FF	34 ± 4.9	43 ± 4.6
	Cold FF	32 ± 2.0	32 ± 5.4
	DHCA	29 ± 3.8	47 ± 7.4

Table 1.

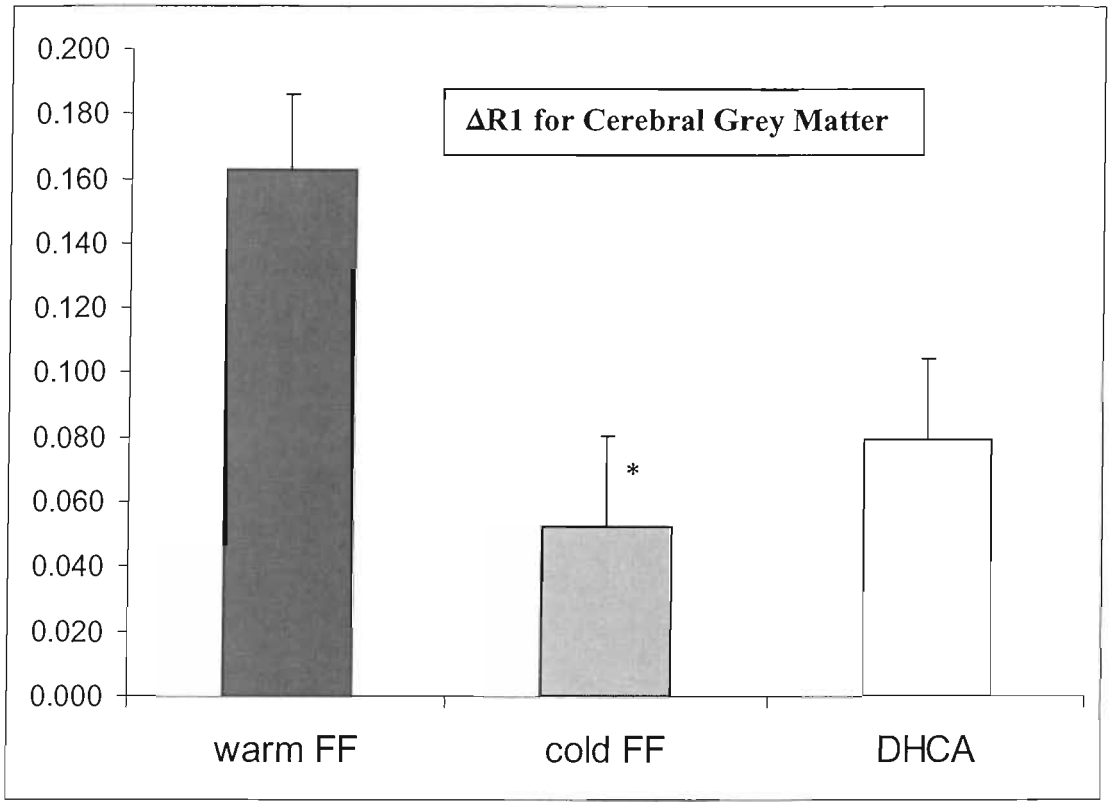


Figure 9.7 Relative change in T1 relaxation times for cerebral grey matter (ΔR1 change/sec).

* $p<0.05$ compared with warm FF CPB strategy. Warm FF CPB exhibits the greatest change in T1 relaxation time following the administration of contrast. The greater reduction in T1 relaxation time following Gd-DPTA administration implies a higher concentration of Gd-DPTA within the GM field of view.

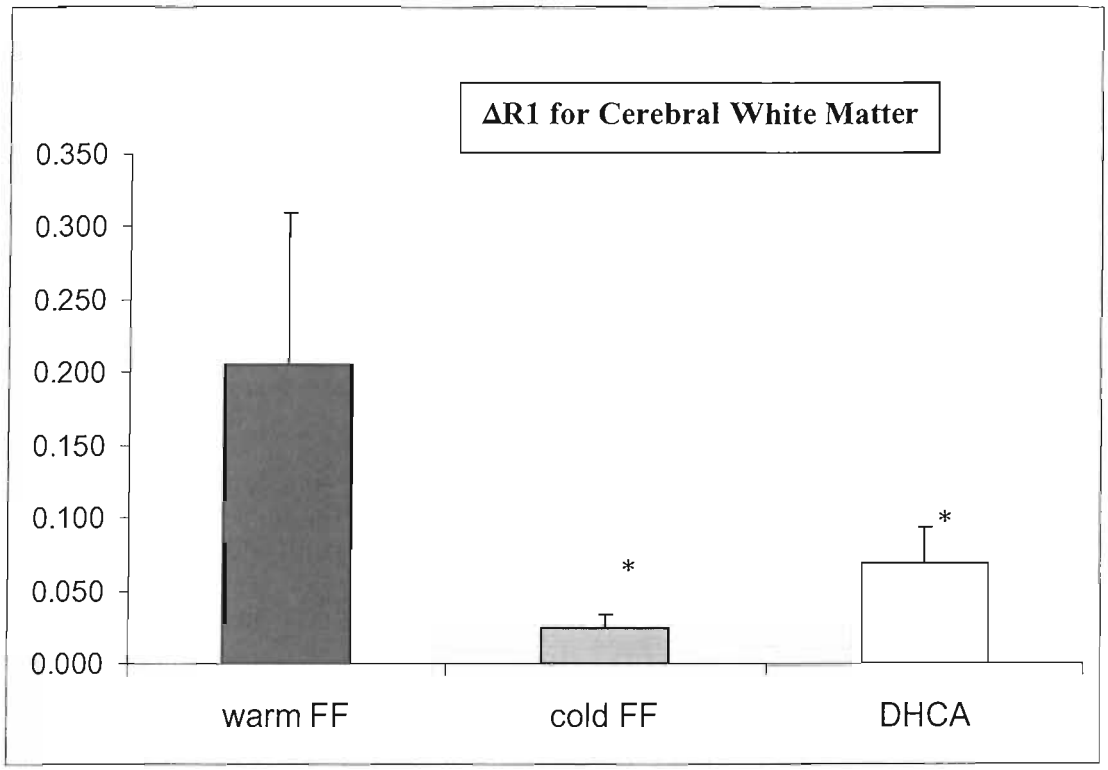


Figure 9.8 Relative change in T1 relaxation times for cerebral white matter (ΔR1 change/sec).

* $p<0.05$ compared with warm FF CPB strategy. The pattern is repeated in cerebral white matter, through the difference is more pronounced. Both DHCA and deep hypothermic CPB display significantly less change in T1 relaxation time following the administration of Gd-DTPA.

9.5 Conclusions

Despite early reports to the contrary[329, 338], this study supports the more recent demonstration by others[331] that normothermic full-flow CPB causes an increase in permeability of the BBB, that rises correspondingly with CPB duration. In fact, this has been one of the inferences for the rise in serum S100B following CPB[339]. The notion that the BBB might be injured following continuous CPB could be explained by the fact that BBB permeability is affected deleteriously by systemic inflammation[340], and antagonists of members of the inflammatory response can reduce its permeability[341]. The BBB is sensitive to numerous other stimuli, including inflammatory pain[342] and inflammatory chemicals[343].

The suggestion that normothermic full-flow CPB disrupts the blood brain barrier is important, because this loss of integrity exposes the neuronal parenchyma to the deleterious systemic inflammatory consequences of CPB. In fact, downstream signalling of intravascular inflammatory mediators to neurons is believed to be critically affected by the composition and structure of the BBB, and the differential expression of inflammatory receptors that can alter depending on the activation status of the BBB[344].

Ischaemia is also a potent stimulus for disruption of the BBB, probably through direct injury to endothelial cells and astrocytes[139, 330]. Animal models consistently demonstrate abnormal BBB permeability following experimental regional or global ischaemia[345] and MRI studies in humans have revealed similar findings. Furthermore, this damage appears to be exacerbated by thrombolytic therapy due to the reperfusion-injury component[147]. Cavaglia and colleagues examined the impact of DHCA and observed significantly greater BBB injury than the normothermic CPB

strategy[331]. However, in their experiment, they used an extremely long DHCA duration (2 hrs), superimposed on the cooling and rewarming periods. In this present study, it was decided to compare the impact of strategies of similar overall duration that might be used in clinical practice. Normothermic full-flow strategies typically have a longer dynamic exposure to CPB, than one involving DHCA (where exposure is absent for the duration of arrest). This may be the reason for our observation of less cortical grey matter BBB injury following a strategy of DHCA than with normothermic CPB. Furthermore it may be that the duration of DHCA is important. The 2 hrs used by Cavaglia and co-workers may exceed a threshold for causing severe injury (perhaps similar to the apparent threshold for histological injury that was encountered in chapter 6)[331]. Studies examining the BBB consequences of much shorter durations of DHCA are clearly warranted in order to establish the profile of this ischaemic injury. It is interesting that when comparing DHCA and DHCLF with comparable overall CPB durations, DHCA tend to instigate a lesser inflammatory response than continuous perfusion[62] (figure 5.7, chapter 5) – an observation attributed to the duration of exposure to dynamic CPB.

The impact of deep hypothermia during continuous CPB on BBB injury has not been examined in detail. The effect of hypothermia on the permeability to albumin during full-flow CPB would be extremely interesting, but these investigators unfortunately did not examine this. Despite assertions to the contrary[181], the inflammatory response resulting from hypothermic continuous full-flow CPB appears to be reduced in most studies[93-95] (although not all[68]). Interestingly this present study suggests the least overall impact on BBB injury resulting from continuous deep hypothermic CPB. This was unexpected, not least because of the greater inflammatory load generated using this technique when compared to DHCA[62]. However, it does lack the period of ischaemia which is known to directly harm the BBB. Interestingly, in animal models of stroke, hypothermia has been shown to inhibit BBB opening[346].

One confusing aspect to BBB injury following experimental ischaemia that has not been addressed in this study is the temporal nature of injury. BBB permeability following injury appears to be a biphasic process, with an early and a late component. The early component occurs within the first 4 hrs, with the second occurring approximately 20hrs later[346, 347]. It was for this reason that transfer to the MRI suite in this study was necessarily prompt. We have therefore attempted to characterise the *early* phase of BBB permeability, and can make no comment on later developments. Experimental observations have led to conflicting reports regarding the relative contributions of each phase to subsequent pathological events[345, 347].

This preliminary study is the first to examine the use T1 relaxation profiles via MRI contrast spectroscopy in an attempt to characterise the response of the cerebral ultrastructure to CPB strategies. Gd-DPTA has been demonstrated to be a useful agent for quantification of early brain injury following stroke[146, 147], encephalopathy[348] and perinatal brain injury[333], both through T1 relaxation and FLAIR imaging (Gd-DPTA accumulation in cerebrospinal fluid), and even through linking it to adhesion molecule substrates[349]. Attempts have also been made to characterise the metabolic state of the brain following infant CPB using MRI spectroscopy for lactate concentrations. Continued efforts to improve these safe and non-invasive techniques for detecting early brain injury following infant CPB would be extremely valuable in the absence of other suitable neurological biomarkers.

Chapter 10

Concluding Remarks

10.1 Avoiding Blood in Neonatal CPB

Evidence to support a central role of the systemic inflammatory response in propagating and amplifying injury following cerebral ischaemia is accumulating inexorably. In fact, inflammation can even initiate neuronal apoptosis independent of ischaemia in models of peri-natal brain injury. Strategies that reduce the systemic and local cerebral inflammatory load are therefore of huge importance in the context of neonatal CPB strategies.

The inflammatory consequences of allogeneic blood transfusion are now beginning to be recognised. Conventional neonatal CPB involves huge exposure to either whole or leukocyte and plasma depleted allogeneic blood, equating to a whole-body transfusion in most circumstances.

The study in chapter 5 confirms the deleterious inflammatory consequences of allogeneic blood transfusion during neonatal CPB, and furthermore links these consequences to the cerebral no-reflow response. Cerebral no-reflow has been associated with poor functional outcome. The use of an entirely bloodless prime in the novel miniaturised CPB circuit was associated with a departure from the typical cerebral no-reflow response observed following DHCA, and instead exhibited a mild reactive hyperaemia, similar to that observed in non-CPB models of global cerebral ischaemia. Importantly, the local cerebral inflammatory load was significantly lower following deep hypothermic CPB using a bloodless prime.

These observations raise numerous questions and areas for future study. Of particular relevance is the role of packed (leukocyte and plasma depleted) red cells, that are more commonly used in clinical CPB. Experimental CPB has made use of fresh whole

allogeneic blood, and the acquisition of depleted swine blood would allow characterisation of the inflammatory load associated with its use in CPB. A second area of interest would be the consequences of *post-operative* transfusion on the recovery of the brain following DHCA. Continued miniaturisation of the integral and ancillary components of infant CPB circuits may allow more routine use of bloodless prime CPB in the clinical arena.

10.2 The impact of post-CPB hypoxaemia

The study in chapter 7 adds to the growing body of evidence that implicates the early reperfusion period following DHCA as crucial in the subsequent progression of injury. The management of oxygenation following cerebral ischaemia is being closely studied in several areas of brain injury including stroke and peri-natal asphyxia. Under particular scrutiny is the impact of exposure to 100% oxygen in fuelling free-radical-mediated reperfusion injury and a consensus is emerging that aggressive re-oxygenation therapies are deleterious in the regard.

Considering that systemic cyanosis is an integral component of congenital heart disease, it is surprising that the impact of post-operative hypoxaemia on progression on cerebral injury has been studied little. The autoregulation of the cerebral vascular bed is known to be disrupted following periods of DHCA (and possibly even deep hypothermic continuous CPB) to several stimuli, including acidosis, hypercarbia and hypoxaemia. The data in chapter 7 confirms that cerebral histological injury is significantly more severe following hypoxaemic reperfusion. Previous studies indicate that the CBF response to hypoxaemia is greatly impaired. The abnormal response to hypoxaemia following DHCA is most likely a significant component of the observations seen in this experiment.

Overall, this chapter suggest that in the same way that pre-operative variables are being identified as risk factors for poor neurological outcome, so too are post-operative factors. It is likely that infants exposed to cyanotic palliative repairs are at greater risk of neurological injury than those receiving normoxaemic repairs, if DHCA is used. The implications of this are that either certain subgroups of patient are ideally suited to continuous perfusion strategies, or that artificial augmentation of oxygen delivery may be beneficial in the early reperfusion period. The timing and nature of such management have yet to be defined and would be the subject of considerable interest and importance for further study. These may include supplemental oxygen therapy, ECMO, or augmentation of cardiac output (ventricular assist devices or pharmacological means). Thus evidence is accumulating to suggest that a calculated approach to cerebral protection is necessary in order to identify particular areas of vulnerability to which management strategies can be targeted.

10.3 LPS preconditioning

The potential to reprogram the entire genomic response prior to severe cerebral injury is hugely exciting. LPS preconditioning as a cerebral protective strategy has been recognised for a decade. However, the data in chapter 8 represents the first successful application of this phenomenon in any clinically applicable model. This is the first demonstration of protective delayed cerebral preconditioning in the context of cardiac surgery, let alone DHCA or neonatal perfusion.

Recently the intracellular mechanisms involved in delayed preconditioning have been examined and the discoveries are extremely exciting. Delayed preconditioning involves a pan-genomic switch, mediated through Toll-like receptors, that trips the entire cellular

response to injury towards a protective one. This is not a blanket suppression of processes (in the way that steroids might be construed to work), but instead an active process that economises metabolic demands, inhibits tissue damaging processes and augments protective and anti-inflammatory pathways. It is a state very close to hibernation and has been described as such. Perhaps of far greater significance to CPB is that delayed preconditioning affects the systemic inflammatory response such that leukocytes and macrophages are refractory to activation. The effect on platelets has not yet been examined. Delineating the activation status of these cellular inflammatory components following delayed preconditioning will clearly be of considerable interest.

A further advantage with the use of LPS preconditioning (over other types) is that it acts systemically on numerous tissue beds. Several organs have been examined by others in non-CPB models, and LPS provides protection against injury in the heart, liver, kidney and against bacterial endotoxaemia/septic shock. Therefore the advantages conferred against cerebral ischaemia may be equally observed in these organs, providing a multi-system mechanism for organ protection.

One reason for the lack of transition from experimental tool to the clinical arena has been the nature of LPS as a potentially harmful endotoxin. Despite the extremely low doses used in preconditioning, this is a psychological – and perhaps ethical - obstacle to its use in humans, especially those in a state of haemodynamic compromise. Recent discoveries regarding the initiation of preconditioning have implicated activation of TLRs as playing a central role. LPS acts via TLR-4, however other ligands have been identified that act via TLR-9 and are currently in clinical use as immune adjuvants in vaccinations. One particular adjuvant has been shown in rodents to offer the most powerful delayed protection to experimental stroke demonstrated to date. Dose-response curves suggest that the therapeutic concentrations should fall significantly short of the concentrations currently in clinical use in humans. These developments

have generated considerable excitement regarding the possiblility of initiating a clinical study in human CPB patients.

A clinical attempt at delayed cerebral preconditioning has not been reported in any context – presumeably due to a lack of suitable agent. The discovery of agents mimicking LPS that already have FDA approval has led to plans for a pilot study in patients undergoing coronary artery bypass operations on CPB. Following a series of non-human primate dose-response studies, patients would be inoculated prior to surgery and assessed for 1) myocardial biomarkers of ischaemic injury; 2) biomarkers of renal injury; 3) circulating cytokine load; 4) activation status of circulating leukocytes by flow cytometry; and 5) detailed neuropsychological assessment for evidence of subtle permanent or temporary deficit ("post-pump syndrome"). This surgical project, involving collaboration with several groups has developed purely from the application of LPS preconditioning described here. I am solely responsible for the design and execution of the clinical arm, for which funding applications ($3million) are under way.

10.4 MRI contrast spectroscopy

The lack of simple, sensitive and specific biomarkers of neurological injury has been a major obstacle in the clinical progress to reduce its incidence and improve its management. This is made more complicated in the neonate due to the developmental immaturity and therefore objective nature of clinical assessment. Furthermore, humans are not fully myelinated at birth, and during the neurological maturation, a process known as neurological plasticity occurs. Therefore, subtle neurological defects are typically detected only in retrospect.

The introduction of MRI has represented a huge milestone in this respect. Not only is it safe and non-invasive, but in addition to the traditional anatomical imaging it can be used for novel characterisation of the physical properties of tissues. This technique – MRI spectroscopy – enables detailed analysis of the chemical composition of tissues, and can therefore be used as a tool for assessing functional properties. This concept has already been used to quantify cerebral biochemical abnormalities following insults, and has recently been used for assessing neurological deficits both pre- and post-surgical repair of congenital cardiac lesions.

Magnetic resonance imaging in large animals is technically demanding and extremely labour intensive. The study in chapter 9 represents a preliminary investigation into the potential for MRI spectroscopy to be used for quantifying the ultrastructural response of the blood-brain barrier to contrasting CPB strategies. The impermeability of the BBB is of fundamental importance in providing a stable homeostatic environment to reside and buffer the parenchyma from systemic insults. Increased permeability resulting from injury to the BBB appears to be a central component of cerebral injury of various aetiologies, and its magnitude correlates with functional outcome. The BBB has been reported to be injured during CPB even without ischaemia, but the nature or significance of this process has not been fully characterised.

We hypothesised that the various insults involved in CPB may differentially affect the integrity of the BBB. We used MRI spectroscopy to quantify the differences in T1 relaxation times before and after the administration of gadolinium. Gadolinium does not readily cross the BBB, and therefore its parenchymal accumulation reflects BBB permeability.

Surprisingly, the greatest contribution of gadolinium to T1 relaxation times was noted following a normothermic full-flow CPB strategy, and the least occurred in association with a deep hypothermic full-flow CPB strategy. Previous studies have confirmed BBB injury resulting from normothermic CPB, but have not included a comparison to deep hypothermic CPB. Advocates of normothermic full flow CPB describe it as a more physiological state. The data in this study suggest that in fact maintaining normothermia may amplify the inflammatory consequences of CPB, a notion support by others investigating the impact of hypothermia. Ischaemia is a known instigator of BBB injury, and DHCA was shown to result in more gadolinium accumulation than deep hypothermic continuous CPB, but less than normothermic full flow CPB.

Overall, this study is the first to use the concept of gadolinium contrast MRI to attempt to quantify ultrastructural damage in the brain following CPB strategies. It therefore represents a preliminary examination and the results suggest that further investigation into the technique is warranted both as an experimental and clinical method of assessing ultrastructural injury. MRI spectroscopy represents probably the most clinically applicable tool for detecting and quantifying neurological injury both before and after cardiac surgery.

10.5 Summary

Since it's birth as a specialty only six decades ago, surgical repair of congenital heart disease has progressively improved at an extraordinary pace. Early mortality rates are now as low as 2% overall in selected units and single ventricle physiologies regularly surviving late into adulthood. Attention is now starting to shift instead towards the long term quality of life. It is disheartening that while the pace of the cardiovascular improvements has been unrelenting, the prognosis following a lengthy period of global

cerebral ischaemia is little different to what it was at the time John Gibbon was spinning cylinders of blood. To address this will take surgical resolve to look outside the box, and work with our intensivist, anaesthetic and perfusion colleagues to ensure that cerebral well-being becomes a priority in decision-making and practice both pre-, peri- and post-operatively. The growing recognition of cerebral injury being initiated as early as *intra-utero* and continuing as late as many weeks or perhaps months following medical intervention for congenital heart defects is fuelling a burst of interest in the mechanisms of neonatal brain injury across the clinical spectra. Inflammatory processes are increasingly being accepted as playing a role possibly as large as ischemia itself. Mechanisms for harnessing these processes are of huge clinical potential, and the concept of genetically directing the responses to these stimuli are extremely appealing.

When moving forward toward the discovery of the unknown, the scientist is like a traveller who reaches higher and higher summits from which he sees in the distance new countries to explore

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