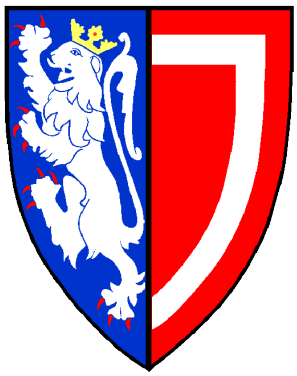
Improving the outcomes of kidney transplantation from deceased organ donors



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“Science is a way of life. Science is a perspective. Science is the process that takes us from confusion to understanding in a manner that's precise, predictive and reliable - a transformation, for those lucky enough to experience it, that is empowering and emotional”

Brian Greene

Forward

This thesis is the culmination of work performed during my DPhil at Oxford University from 2012-2015. The thesis structure is different to that of other Oxford theses by having some Dutch influences in the structure and organisation. There are seven Chapters, the first of which is an introductory Chapter and provides an overview of the relevant literature and sets the scene for the research conducted. The second Chapter provides a rationale for the experimental approach used. The third Chapter, although brief, describes some of the pain staking work performed to establish the animal models used in the remainder of the thesis and Chapters 4-6 describe the main experimental results. Each results Chapter contains an introduction, supplementing the description in the overarching introduction, and the methods used within the Chapter. The final Chapter brings together the thesis, highlighting the main conclusions and future avenues for research.

Abstract

This thesis sought to improve our understanding of how kidneys become injured as a consequence of organ donation, with the aim of improving the outcomes of transplantation. Every year, hundreds of patients on the waiting list die whilst awaiting a kidney transplant. With an ever-increasing demand for suitable organs, supply cannot keep up with the pressures on the transplant waiting list. As a consequence the transplant community are forced to use organs that previously would not have been considered suitable for transplant, including from older donors with additional comorbidities. This thesis aimed to develop an understanding as to how the kidney becomes injured during the donation process, identifying which key cellular homeostatic processes are disturbed as a consequence of donation.

The thesis outlines the experimental development of rodent models of organ donation replicating the donation process for donation after brain death (DBD) and donation after circulatory death (DCD) donors and also the development of a kidney ischaemia reperfusion injury (IRI) model. Proteomics was subsequently used to identifying global protein alterations in the kidney as a consequence of brain death and ischemia reperfusion injury using bioinformatics tools to identify involvement of cellular pathways. The results indicated alterations in mitochondrial function and metabolic homeostasis occurring following brain death.

Alterations in cellular metabolism and mitochondrial function were then confirmed using metabolomics and mitochondrial functional assays. I subsequently evaluated how

alterations in cellular hypoxia and the hypoxia inducible factor system is altered in the brain dead organ donor kidney and aimed to target this system as a means of conditioning the brain dead organ donor to prevent mitochondrial and metabolic mediated injury to kidney cells following brain death. This involved exploring the role of prolyl hydroxylase inhibitors, including dimethyloxalylglycine, on mitochondrial function and whether this could be a therapeutic target in organ donation.

This thesis provides important insights into the mechanism of injury of kidneys following brain death, providing evidence that even before procurement and preservation in the DBD donor alterations in mitochondrial function and metabolic homeostasis occur. I provide preliminary data on the use of prolyl hydroxylase inhibitors in altering mitochondrial function. I also outline my involvement in other ongoing projects in organ donation and machine perfusion that also aim to improve the outcomes of deceased donor kidney and liver transplantation.

Work done in collaboration

Some of the work performed in this thesis has been performed in collaboration with others. Mass spectroscopy was performed with Dr H Huang (Oxford) and Dr A Donna (Imperial). The electron microscopy was performed with Prof D Furgeson (Oxford). I gained experience in the brain death animal model with Ms P Ottens, Dr R Robelledo and Prof H Leuvenink (Groningen). The development of the mitochondrial assays in the laboratory was done with the assistance, knowledge and advice of Dr L LoFaro and Dr K Morten (Oxford). The development of the QUOD biobank and COPE project was done in collaboration with a large number of collaborators (www.nds.ox.ac.uk/research/quod, www.nds.ox.ac.uk/research/cope).

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Abbreviations

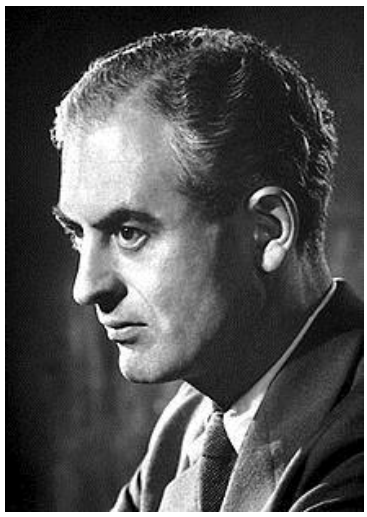
Acronym	Full name
2-OG	2-oxalogluterate
ADH	Adennosine diphosphate
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATP	Adenosine Triphposphate
Bax	Bcl-2-like protein 4
BBB	Blood brain barrier
BCA	Bicinchoninic acid assay
BCL2	B-cell lymphoma 2
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
CAD	C-terminal activation domain
CoA	Coenzyme A
CS	Citrate synthase
DAMPS	Danger associated molecular pattern
DBD	Donation after brain death
DC	Dendritic cell
DCD	Donation after circulatory death
DGF	Delayed graft function
DNA	Deoxyribonucleic acid
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
ECD	Extended criteria donor
EDTA	Ethylenediaminetetraacetic acid
EPO	Erythropoietin
ETC	Electron transport chain
FADH	Flavin adenine dinucleotide
FDR	False discovery rate
FH	Fumarate hydratase
FOXO	Forkhead box O3
GFR	Glomerular filtration rate
HIF	Hypoxia inducible factor
HMP	Hypothermic machine perfusion
HO-1	Heme-oxygenase 1
ICAM-1	Intercellular Adhesion Molecule 1
IGF	Insulin-like growth factor
IL	Interleukine
IP	Immunoprecipitation
IPA	Ingenuity pathway analysis
IRI	Ischaemia reperfusion injury
KO	Knock-out
MAP	Mean arterial pressures
MCP	Monocyte chemoattractant protein-1

MHC	Major histocompatibility
MMP9	Matrix metallopeptidase 9
mPTP	1-methyl-4-phenul-1,2,3,6-tetrahydropyridine
MTOR	Mechanistic target of rapamycin
NADH	Nicotinamide adenine dinucleotide
NMA	N-Methylacetamide
NPE	Neurogenic pulmonary oedema
NRf2	basic leucine zipper (bZIP)
NRP	Normothermic regional perfusion
PAMPS	Pathogen associated molecular patterns
PEEP	Positive end expiratory pressure
PPARGC1A	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PHD	Prolyl hydroxylase
RCR	Respiratory control ratio
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SAH	Subarachnoid haemorrhage
SIRS	Systemic inflammatory response syndrome
STAT3	Signal transducer and activator of transcription 3
SVR	Systemic vascular resistance
TCA	Tricarboxylic acid cycle
TEM	Transmission electron microscopy
TGF-1B	Transforming growth factor beta 1
TLR	Toll like receptor
TNFa	Tumour necrosis factor alpha
TRH	Thyroid releasing hormone
TSH	Thyroid stimulating hormone
TSP-1	Thrombospondin 1
UNOS	United Network for Organ Sharing
VCAM	Vascular cell adhesion molecule

Chapter 1: Introduction: The changing face of deceased organ donation

Kidney transplantation is not a new phenomenon; some of the first descriptions of human kidney transplant were described by the Ukrainian surgeon Yuri Voronoy in the 1930's (1). His first technically successful kidney transplant was performed in 1933 in a 26-year old female who was in an ureamic coma after suffering from mercury poisoning. The donor was a 60 year-old male who had suffered a base of skull fracture and had died six hours earlier. The transplant was ABO incompatible and performed into the thigh and unsurprising failed with no urine production.

Subsequently, sporadic attempts at kidney transplant were attempted in the ensuing 15 years without immunosuppression (2-6). Following the discoveries of Gibson and Medawar the basis for immunuo-biology in transplantation was established and subsequent research carried out into mechanisms of rejection and immunosuppression (7). In 1954, Joseph Murray performed the first successful kidney transplant between identical twins at the Brigham Hospital in Boston (6). Today, kidney transplantation is the treatment of choice for patients with end stage renal disease and with effective immunosuppression over 3,000 kidney transplants are performed annually in the UK (8).



Yuri Vonoroy Ukranian Surgeon Peter Medawar Immunologist

Figure 1. Pioneers in kidney transplantation Yuri Vonoroy a Ukranian Surgeon who pioneered the basic surgical techniques and attempted to perform some of the first kidney transplants in humans. Peter Medawar a founding father of immunology who paved the way for our understanding of the importance of the immune system in transplantation.

Kidney transplantation has now become a victim of its own success however, with a major disparity existing between the number of patients on the kidney transplant waiting list versus the number of transplants actually performed (8). Over 5,800 patients are still waiting on the transplant waiting list in the UK for a kidney. In five years time, 16% of these patients will still be on the waiting list [extracted from NHSBT data 2010-2015]. Thus there is an urgent need to address the organ deficit, a fact that has forced many transplant centres to use organs that previously would not have been considered suitable for transplantation (9). For example, transplant units are increasingly turning to deceased donors who have been declared dead by cardiovascular criteria (donation after circulatory death: DCD), and also older deceased donors with additional co-morbidities such as diabetes or hypertension; the so called extended criteria donors (ECDs) (10-18). However, kidneys from these donors are known to have both poorer short and long term outcomes, and have associated

significant socio-economic costs, compared to standard criteria brain dead organ donors (DBDs) or living donors (LDs) (19). Saidi et al. demonstrated this by following up ECD and DCD donors for a median of 50 months and comparing them to standard criteria donors (SCD). The authors demonstrated that ECD and DCD kidney recipients had a higher incidence of delayed graft function (DGF), took longer to reduce the serum creatinine levels to below 3mg/dl, had longer durations of hospital stays and more readmissions (19). These factors translated into poorer outcomes and increased costs associated with these poorer quality kidneys when compared to SCD kidney transplants.

The total number of DBD donors has remained broadly static over the last decade (8). However, the proportion of ECD donors has increased, with the deceased donor profiles in both the UK and USA showing that donors have become older with an increased body mass index (8, 20). For example in the last decade we have seen that the proportion of ECD donors older than 60 years old has increased from 14% to 35% (21). Kidneys from these older donors tolerate cold preservation less well and have poorer outcomes (9, 22), however authors have demonstrated that compared to continued haemodialysis, kidney transplantation using these higher risk kidneys continues to offer a survival advantage (23). This has led some centres to pursue older kidneys for older recipient programmes (24, 25). However, in an aging population is it appropriate to be discriminating based on age, or guessing the longevity of a patient's life? Fundamentally we need to improve the outcomes of transplantation for these patients regardless of the donor type.

Hypertension, diabetes and obesity are epidemics also affecting the donor population. The number of obese donors with a BMI over 30 kg/m^2 has increased from 14% to 23% in the UK over the last decade (21). Donor BMI has been associated as an independent risk factor for developing delayed graft function (DGF), and in DCD donors extreme obesity (BMI >45 kg/m^2) has been associated with poorer 1-year graft survival rates (25-27).

The use of ECD kidneys from donors with hypertension or a terminal serum creatinine of >1.5 mg/dl or who have suffered a cerebrovascular accident has also increased (16-19, 28). This has an associated effect on both short and long-term outcomes, with ECD kidney graft survival offering 5.1 added life years compared to a standard criteria donor kidney with 10 added life years. There is a greater than 70% risk of kidney failure overall from an ECD donor transplant compared to a kidney from a 35 year old male donor who has been involved in a road traffic accident (29, 30).

Although many of these factors in ECD transplantation have clear effects on the outcomes, the cumulative impact of demographic, social and physiological features of the donor and how they correlate with longer-term outcomes has not been fully delineated. For example to what degree does the impact of obesity, with diabetes and a smoking history have on the survivability of an organ? Does the impact of smoking 15 cigarettes per day compared to 20 matter? Simply, the cumulative effects of these types of risk factors have not been calculated or how risk factors interact with each other has not been fully determined. The application of big data and data mining tools may help with these types of determinations to identify risk associated with

transplantation; these types of approaches have been used in diabetes and cancer (31, 32).

As illustrated in Figure 2, DCD donors are an increasingly utilised source for kidney transplant [NHSBT]. In the UK, for example, in 2003 there were 1.1 DCD donors per million of the population (pmp), this has now increased to over 7 DCD donors pmp (21). In the USA a less substantial increase has been observed (20).

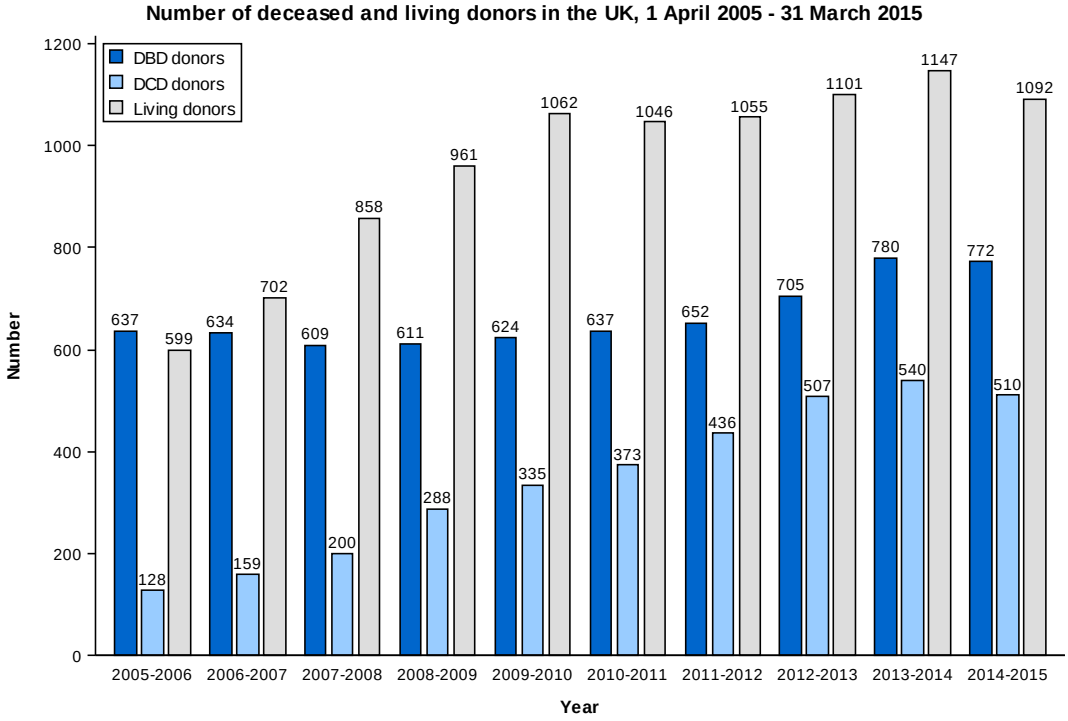


Figure 2. NHSBT Activity report 2005-2015. Increasing numbers of living donation, relatively stable numbers of DBD donors and a major increase in the number of DCD donors.

Table 1 outlines the characterisation of the different DCD donor types. Predominantly in the UK, type III DCD donors are the most frequently utilised. However, increasingly organs from uncontrolled DCD donors (Maastricht I/II/V) donors are being procured for transplant, particularly in Europe (33). In most circumstances this involves the use of hypothermic or normothermic *in-situ* regional perfusion in the donor who has had a witnessed cardiac arrest either in or outside the hospital setting. Kidneys obtained from these so-called uncontrolled DCD donors have comparable long-term outcomes, but have a greater incidence of DGF (34). Gagandeep and colleagues retrospectively evaluated the outcomes of 216 uncontrolled DCD transplants and compared it to 1814 controlled DCD transplants and transplants from brain dead donors (34). They reported similar long-term outcomes between groups but an increased incidence of DGF in the uncontrolled group. However, they did not account for kidneys that were discarded or deemed too high risk in the uncontrolled group, a limitation the authors recognised.

Table 1. The Modified Maastricht Classification of DCD. Taken from 'New classification of donation after circulatory death donors definitions and terminology' (35).

Category I. *Uncontrolled*	**Found Dead** *IA. Out-of-hospital* *IB. In-hospital*	**Sudden unexpected CA* without any** attempt of resuscitation by a medical team**; WIT to be considered according to National recommendations in place; reference to in- or out-of-hospital (IH-OH) setting**
Category II. *Uncontrolled*	**Witnessed Cardiac Arrest** *IIA. Out-of-hospital* *IIB. In-hospital*	Sudden unexpected irreversible CA with **unsuccessful resuscitation by a medical team**; reference to in- or out-of-hospital (IH-OH) setting
Category III. *Controlled*	**Withdrawal of life-sustaining therapy**	Planned withdrawal of life-sustaining therapy**; expected CA
Category IV. *Uncontrolled* *Controlled*	**Cardiac arrest while brain dead**	Sudden CA after brain death diagnosis during donor management but prior to organ procurement.

*CA: circulatory arrest

**: This category mainly refers to the decision to withdrawn life-sustaining therapies. Legislation in some countries allows euthanasia (medically-assisted CA) and subsequent organ donation described as the fifth category.

Despite these factors, the continued organ shortage has meant that we depend increasingly on ECD and DCD kidneys that have poorer outcomes. The aim of this thesis was to further develop our understanding of how organs become injured during the deceased donor donation process and to determine what can be done to improve the outcomes of deceased donor transplantation. This work will focus on kidney transplantation, but also address some aspects of liver transplantation. Initially I explore what is known about the mechanisms of injury to organs from DBD and DCD donors and then focus on the injury mechanisms in DBD donors specifically.

The DBD donor

Traditionally DBD donors were young men who had been involved in a road traffic accident and had sustained major cerebral injury leading to brain death. Due to the welcome changes in road safety, but also management of trauma patients, these patients make up a smaller proportion of the DBD donor cohort (3, 21, 36, 37). Instead, we are seeing older patients who have had major intra-cerebral bleeds being diagnosed as brain dead. In both scenarios, haemorrhage leads to a raised intra-cranial pressure and eventually results in coning and destruction of the brain stem. This leads to the Cushing's reflex, characterised by a period of hypertension, irregular breathing and a reduced heart rate (38-40).

The "autonomic storm" ensues whereby there is activation of both the sympathetic and parasympathetic systems. Initially the sympathetic component predominates causing hypertension; and activation of the baro-receptors in response. Brain herniation also causing stimulation of the vagus nerve, leading to a paradoxical

decrease in heart rate (41, 42). The parasympathetic response then predominates leading to a period of hypotension, followed by correction of mean arterial pressures.

In the ensuing period, donors are typically haemodynamically unstable, often requiring inotropic support and are ventilator dependent. In the clinical setting brain death testing defines the diagnosis of brain death, rather than characteristic changes in physiological parameters. To date, most research in brain dead donors has focused on traumatic brain injury, due to this in the past being the main mechanism of injury to organ donors (43). Animal models of brain injury have shown less haemodynamic instability with gradually increasing intracranial pressures, presumably as a consequence of the partial and gradual dysfunction of the autonomic nervous system (43).

Work performed in the 1980's illustrated that kidneys from brain dead donors had poorer short and long-term outcomes, despite better Human Leukocyte Antigen (HLA) matching, when compared to less well matched living donors (39, 40). This has led to further work to identify the mechanisms behind how brain death itself leads to kidney injury. Rodent and large animal models using haemorrhagic trauma or brain displacement by raising intracranial pressures, have allowed researchers to investigate how the process of brain death can lead to kidney injury (44).

Previous work has shown that this multifactorial impact of cerebral injury and brain death leads to disturbance of a number of homeostatic mechanisms including haemodynamic instability, alterations in hormonal balance, release of chemokines,

effects on the heart, lung and immunological system (45, 46). The consequence of deregulation of these systems is inflammatory cell infiltration into organs, the expression of danger associated molecular patterns (DAMPs) and metabolic disturbances including depletion of adenosine triphosphate (ATP) (46, 47). The next sections discuss each of these aspects in greater detail.

The effect of brain death on hormonal dysregulation

Hypothalamic-pituitary axis dysfunction has been well described in brain death (47-57). However, the degree of disturbance of the axis varies considerably between brain dead donors. For example, some studies have illustrated preserved histological appearances of the hypothalamus in brain dead donors (50). Pituitary dysfunction also commonly occurs but with variability. For example, diabetes insipidus is a common finding in brain dead patients, owing to a cessation in vasopressin production, a posterior pituitary hormone. This, however, is not a universally found phenomenon even in donors with low vasopressin levels. Anterior pituitary hormone deficiency also occurs in brain dead donors, in part due to the effects observed on the hypothalamus. Thyroid hormone dysregulation is commonly found either due to a deficiency in thyroid stimulating hormone (TSH) production, a lack of thyroid releasing hormone (TRH), or due to a sick euthyroid phenonmenon. Similarly, cortisol levels have been found to be variable between brain dead donors (50).

In part, the variability of the hormonal changes observed is likely to be due to different degrees and rates of hormonal axial destruction. In animal models of cerebral injury,

deficiencies in T3/T4 are commonly found, however in the clinical trial setting, mixed results have been found in those donor who are treated with thyroid replacement therapy against those which have not been treated (55, 58, 59). This is likely to be reflecting the different degrees of axial disturbance. For example donors who have a sick euthyroid type of appearance may not necessarily benefit from thyroid replacement. Nivitosky and colleagues have previously explored the benefits of hormone replacement therapies in donors. In their most recent publication, they reported the outcomes of transplants from over 60,000 donors reporting the optimum combination of hormone replacement therapies includes thyroid hormone replacement, steroid replacement and insulin which led to the significantly more organs being utilised from the donor (60). They demonstrated this could improve utilisation of all organ types except for the liver. T3/T4 therapy has been associated with improved graft survival at one year with no detrimental effects, the authors advocating that administration of thyroid hormone replacement therapy is of no harm and could be of benefit. Their recommendations are yet to be introduced in all national protocols.

In experimental studies, Rebolledo and colleagues have suggested that administration of T3 as a preconditioning agent prior to the induction of brain death can reduce hepatic injury as measured by alanine transaminase/ aspartate transaminase (ALT/AST) levels and reduce rates of apoptosis as measured by measuring Bcl2 associated X protein (BAX) and B cell lymphoma-2 protein (Bcl-2) (61). Although the authors did not demonstrate how thyroid hormones are likely to be having an anti-apoptotic effect, they demonstrated an effect on reducing oxidative stress markers including heme-

oxygenase 1 (HO-1), Nuclear factor (erythroid-derived 2)-like (Nrf2) and signal transducer and activator of transcription 3 (STAT3).

The actions of steroid replacement are likely to be complex. Studies have routinely shown that in the brain death donor, haemodynamic instability is improved by the administration of steroids (62). Pinsard and colleagues performed a prospective trial of low dose steroids in DBD donors and demonstrated improved haemodynamic status, although no significant effect on transplant outcomes were demonstrated (63). Others have also failed to conclusively demonstrate a benefit on transplant outcomes despite effects on improving haemodynamic stability. Part of our own research has aimed to explore why there remains a discrepancy in the studies evaluating the beneficial effects of steroid replacement (64). We studied the benefit of steroid pre-treatment using a brain dead organ model and failed to demonstrate a consistent benefit across organ types. The effects of steroids on altering metabolism, haemodynamics and inflammatory status of the donor render this a difficult area in which to dissect the mechanisms of action. At present, there is no clear evidence for giving steroid therapy. However, there is little probability of harm occurring as a consequence of administration and in most clinical scenarios steroids are administered as treatment for raised intracranial pressures (60).

The effect of brain death on cardiovascular dysregulation

During brain death the complex interplay between disturbances on hormonal, inflammatory and pulmonary function makes determining the independent effect on

the heart challenging (43). The autonomic changes result in alterations in cardiac output by manipulating both cardiac contractility and also peripheral vascular resistance.

In experimental models using rodents, researchers have demonstrated that cardiac contractility following brain death falls to approximately 50%, as measured by cardiac catheterization. However, on explanting the organs and performing *ex-situ* Langendorf perfusion, contractility can be recovered (65). During the brain death phase the contractility is influenced by adrenaline release both systemically and locally, initially driving the blood pressure, but subsiding as the brain stem is destroyed. The catecholamines result in myonecrosis and calcium uptake (46). Wilhelm and colleagues have previously described the alterations in the histological appearances of hearts following brain death, with progressive inflammatory cell infiltration, expression of adhesion molecules and expression of MHC class II antigens (66). They postulate that these alterations result in increased incidence of rejection in DBD donor transplant and leads to cardiac vasculopathy.

Catecholamines and brain stem destruction also results in alterations in peripheral vascular resistance, initially this increases, contributing to the initial phase of hypertension seen as part of the Cushing's reflex (46). As this subsides, tissue perfusion increases and oxygen consumption in peripheral tissues rises. The overall effect results in a hyperdynamic, labile circulation and therefore periods of hypotension may occur. However, the actual degree of cardiac dysfunction resulting in haemodynamic instability may be minimal i.e. the haemodynamic instability is autonomically driven.

Herijgers and colleagues explored this question by using a rodent model of brain death using rapid inflation of an intra-cranally placed balloon, this resulted in profound haemodynamic instability (67-69). The authors examined the histology of the hearts and also the contractile reserve by administration of increasing dosages of adrenaline in a Langendorff system. Overall they concluded that myocardial damage, if found, contributes minimally to the haemodynamic profiles observed following brain death, as the contractile response to adrenaline and histological evaluation was grossly normal even with extended periods of brain death. Others have concluded similarly that preload is the major cause of haemodynamic instability following brain death. Szabo and colleagues demonstrated that following brain death there is a 40% reduction in systemic vascular resistance (SVR) over time, which results in reduced coronary perfusion (70, 71). The authors have shown using a cross-circulated canine model, that if the pre-load can be maintained, much of the cardiac dysfunction can be attenuated (72). This is not a universally observed phenomenon however, as others have shown that brain death induction can lead to myocardial injury (73-75). Why there remains a discrepancy between studies could be related to the different speed of onset of brain death in the experimental models used. For example it has been shown that rapid inflation of the balloon intracranially will result in a 750 fold increase in adrenaline and 400 fold increase of noradrenaline, whereas slow inflation results in 175 and 40 fold increase respectively for adrenaline and nor-adrenaline (67). Rapid balloon dilation may also have more of a profound effect on the systemic vascular resistance (SVR).

Attempts to attenuate the haemodynamic instability observed following brain death have been tried. Avoiding hypotension and maintaining haemodynamic parameters

with strict control improves the outcomes of transplantation. This does not completely attenuate the injury occurring to organs at the histological and cellular level (76-79). Approximately 80% of brain dead donors have severe prolonged hypotension. The mainstay of management of these patients is fluid resuscitation and maintaining red blood cell concentrations above 100g/l or a haematocrit of greater than 30%. The use of colloids at the expense of renal function is debated (80, 81). If cardiac output continues to not be maintained, inotropes can be used, with dopamine remaining as the first line choice. Noradrenaline and adrenaline use have been linked to higher rates of cardiac dysfunction and some centres will not retrieve hearts on the basis of high catecholamine use (80, 81). Hormone replacement, as described previously may also improve haemodynamics. Throughout donor management monitoring cardiac output and fluid balance is achieved through placement of pulmonary catheters, oesophageal Doppler, central lines etc. Overall, the effect of brain death on cardiac output is largely influenced by changes in SVR affecting pre-load and after-load. Maintaining coronary perfusion is key to avoid cardiac injury, but maintaining blood pressures within normal parameters is not sufficient to avoid cellular and histological injury to cardiac cells and peripheral organs.

The effect of brain death on pulmonary gas exchange

One of the key issues in brain dead organ donors is that there is a high incidence of aspiration, lung contusion and pneumonia. Occasionally, the donors will develop neurogenic pulmonary oedema (NPE), directly as a consequence of the brain death (82, 83). In patients who develop sudden onset brain death, 90% will develop pulmonary

oedema, often documented prior to the official diagnosis of brain death being declared. The main mechanism for the development of NPE is the sympathetic storm in its initial phase, resulting in high SVR resulting in increased afterload and left ventricular and atrial pressures. This results in higher hydrostatic pressures and damage to endothelium. α-adrenergic stimulation also results in increased permeability of the pulmonary capillaries. Many of the effects of this on causing NPE can be attenuated by spinal cord transection (46).

In a review by McKeown et al. they reported that the main issues surrounding tissue oxygenation and lung function in the brain dead organ donor is due to ventilator associated lung issues (80). UNOS advises to maintain a tidal volume of 10-12ml/kg with a peek expiratory pressure (PEEP) of 5cmH20 (81). Lower tidal volumes and PEEPs are now used in some centres as a lung protection strategy (80).

The effect of brain death on immunological dysregulation

Brain death leads almost invariably to the development of a systemic inflammatory response syndrome (SIRS) in donors. This is defined by the production and release of pro-inflammatory mediators such as tumor necrosis factor α (TNFα), *Interleukin (*IL)-6, IL-8 and IL-1β (84-87). The expression of adhesion molecules such as ICAM-1 and MCP facilitate the rapid accumulation of leukocyte populations into tissues. Overall, the expression of major histocompatibility class (MHC) class I and II are increased resulting in increased allograft immunogenicity (88-90). How the orchestration of the inflammatory response is started remains not entirely understood. The cerebral injury

itself, the ischaemia reperfusion injury (IRI) type phenomenon during coning, and even translocation of microbes from the intestine could be responsible for commencing the immune response (91). These are discussed in further detail in the subsequent sub-Chapters.

Brain injury leads to a pro-inflammatory state in the brain dead organ donor

Injection of TNFα into the brain will result in recruitment of inflammatory cells into end organs (92, 93, 94). The mechanisms for how brain injury leads to this response could occur primarily through the trauma itself leading to disturbance of the blood brain barrier (BBB), alternatively it could be that raised intra-cranial pressure leads to brain ischaemia. Regardless of the mechanism, cytokinaemia almost invariably occurs in the brain dead donor, with cytokine up-regulation occurring often prior to the declaration of brain death(46).

Matrix metallopeptidase 9 (MMP9) has been identified as an important mediator of the brain death induced inflammatory response (95-97). MMP9 breaks down the extracellular proteins including endothelial tight junctions and basal lamina proteins. Inhibition of MMP9 leads to reduced brain oedema. The overall loss of the BBB leads to bidirectional inflammatory response. The degree of the cytokine response is different between different modes and speeds of onset of brain death. This is seen, for example in stroke, when the intracranial pressure (ICP) rises slowly there is a predominant TH2 response, whereas traumatic brain injury leads to a predomination of a TH1 response (98, 99).

In addition to the release of pro-inflammatory cytokines, the inflammatory response is balanced by the production of anti-inflammatory cytokines such as the TNFα receptors P55 and P75, as well as IL-10 (98, 99).

The pro-inflammatory IL-6 response occurs notably following brain death and occurs as a consequence of disturbance of the BBB (100). However, in addition to the early response being mediated by glial cells, later in the response cascade IL-6 could act as an anti-inflammatory mediator, this could in part be mediated through NG-monomethyl-arginine (NMA) and activation of IL-6 receptors (99). Pratschke's group have recently reported on a rodent study where the application of an IL-6 monoclonal antibody could reduce some of the early and late inflammatory cytokine responses from brain dead donors (101). However, the authors did not detect a notable difference in longer-term outcomes at five months from when the kidneys were transplanted.

Endothelin-1 (ET-1), a small polypeptide family member, is released from the brain following subarachnoid haemorrhage (SAH), stroke or traumatic brain injury (102-105). ET-1 is a potent vasoconstrictor but also is pro-inflammatory, partly through stimulating reactive oxygen species production and activating transcription factors such as NfκB. Chatfield and others explored the jugulo-arterial ET-1 gradient after severe traumatic brain injury (106, 107). Their results indicate a surge of ET-1 release following brain trauma.

In addition to the cytokine release the disruption of the autonomic nervous system following brain death can also lead to modulation of the inflammatory response.

Activation of the vagus nerve for example, typically results in down regulation of the inflammatory response (108). Using a brain death model, Hoeger and colleagues have attempted to attenuate this inflammatory process by vagal nerve stimulation (108). Brain dead rats had bipolar electrodes connected to stimulate the vagus nerve applying a constant voltage. The results indicated not only reduced levels of circulating cytokines such as TNFα but also improved outcomes when kidneys from this cohort of donors were transplanted in comparison to controls.

Local inflammatory response in end organs

The systemic inflammatory response is found concurrently with local inflammatory responses. In the kidney for example, expression of VCAM-1 and ICAM-1 is more pronounced in the brain dead organ donor compared to living donors (88). Neutrophils, macrophages and other leukocytes are more predominantly found in organs from brain dead organ donors in comparison to controls (40). The systemic and local effects of brain death also lead to the disruption of the intestinal mucosal barrier as previously mentioned, resulting in translocation of bacteria and bacterial toxins into the blood stream, followed by further enhancement of the inflammatory response. Koudstaal et al. demonstrated this phenomenon using a rodent model of brain death, evaluating the level of LPS binding protein, serum endotoxin and monocyte chemotractant protein (MCP) in the serum of DBD donors (91).

The expression of Toll-like receptors (TLR) has been of particular interest in organ donors and in the realms of IRI. TLRs bind danger or pathogen associated molecular

patterns (DAMPS, PAMS). A wide variety of cell types express TLRs including endothelial cells, epithelial cells, leukocytes and dendritic cells (109-112). LPS, haeme-oxygenase and flagellin are examples of molecular patterns that activate TLRs. In the kidney TLR are predominantly expressed on tubular epithelial cells and mesangial cells. Knock out studies of TLR2 and 4 have shown some ability to be able to attenuate ischaemic injury to kidneys (113). In brain dead organ donors TLR4 and High motility group box-1 (HMGB1) is noted to be higher (100). Although therapeutic manipulation of TLRs has not been attempted in the brain dead organ donor, the ability to predict donor outcomes based on TLR genotypes remains controversial and has not been fully validated (112, 114). Ellet et al. found an association between the genotype of TLR4 and the outcomes of transplant, a finding not supported by others (113).

The interaction of TLR with the complement pathway is of interest. A review by Damman and colleagues reports on the cross-talk between the two systems and how this may be used as a therapeutic target (100). Complement deposition itself is known to occur in the kidney of brain dead organ donors with the same group demonstrating increased C3 deposition following brain death in both animal models and also in human tissues. Pratt and colleagues have demonstrated that C3 deposition occurs in the kidneys of brain dead donors and this is detrimental for kidney survival (115, 116). Complement deficient kidneys, in a mouse model, have been shown to be able to survive for >100 days compared to wild-type mice. Preliminary studies have shown some benefit of administration of complement inhibitors such as Soluble Complement Receptor 1 (SCR1) (117).

The DCD donor

DCD donors, like DBD donors, usually have some degree of brain injury, but this is insufficient to lead to coning and the physiological disturbances observed in brain death. In this patient cohort, medical treatment continuity has been deemed futile and the decision to withdraw treatment has been made. When treatment is withdrawn this results in gradual death; the so-called agonal phase, during which time the organs are subjected to warm ischaemic injury. Functional warm ischaemia is defined as the period when the systolic blood pressure is below 50mmHg (33, 118, 119). In general, younger donors are typically more resilient to lower systolic pressures, owing to better cardiac function (22). Hence the time to complete cessation of circulation is usually longer in these donors.

Warm ischaemia is known to be particularly detrimental for organ function and cell survival. During ischaemia cells are depleted of nutrient and oxygen leading to ATP depletion. This results in dysfunction of cellular Na/K ATP dependent pumps, resulting in cell swelling. The presence of free adenosine also results in free radical release. This ischaemic injury is sufficient to result in a substantial cellular and immune cell response. Tullius et al. demonstrated using a rodent kidney transplantation model that increasing warm ischaemic times were less well tolerated by kidneys from older donors resulting in poorer outcomes of transplantation (120). More recently their group has demonstrated that this is in part through an enhanced immune response to older donor kidneys, higher rates of apoptosis and lower proliferative capacity. Their work

has demonstrated that B Cells are likely to play a key role in mediating the innate response to warm ischaemic injury (121).

The cumulative result of this injury is also reflected by histological changes including tubular atrophy and fibrosis (122). The same group reported that younger kidneys have the ability to also activate cytoprotective pathways, which have commonly been proposed as being the basis for how ischaemic preconditioning confers protection (37, 121).

Although organs from DCD donors have poorer short and long term outcomes compared with DBD donors, the importance of the effect of donation related injury and the injury cascade in DBD donation on kidney function and survival is highlighted by the fact that DBD donors that develop DGF have associated poorer long-term outcomes, when compared to DCD donor kidneys that develop DGF(9, 22, 123). Authors have speculated that this difference is in part due to the mechanisms of injury to the kidney seen in the brain dead donor compared to DCD donors.

The effects of cold ischaemic injury and reperfusion

Regardless of donor type, donor age has a significant effect on the ability of a kidney to withstand cold ischaemic damage (22). Summers et al. recently showed that 3-year graft survival was comparable between age matched DCD and DBD donors (22). However DCD donor kidneys had less ability to tolerate prolonged cold ischaemic injury (>24 hours), which was associated with poorer graft outcomes. Others have also shown

a strong association between ECD kidneys exposed to prolonged cold ischaemic times (>15 hours) being associated with higher DGF rates (124).

Cold ischaemia, like warm ischaemia, results in depletion of ATP stores, although metabolism is not completely halted by cooling the organ on ice (125). It is estimated that metabolic rates reduce during cold preservation (at 5^{o}C) to 10% of the basal rate (126), but despite this mitochondrial dysfunction, measured at the respiratory chain level and oxidative stress persists (127).

Following reperfusion of both DBD and DCD kidneys, reactive oxygen species (ROS) production is accelerated at the cellular level and results in activation of an inflammatory cascade which results in endothelial adhesion molecule expression, release of pro-inflammatory cytokines and the infiltration of inflammatory cells into the organ (128-133). A second or late phase of reperfusion injury typically occurs 6-48 hours after the initial injury and results in further injury to the kidney measured by histological evaluation.

ROS production leads to damage to cellular components including lipids, proteins and DNA, increasing the cellular metabolic demands. This leads to dysregulation of signaling pathways, alterations in the redox state and mitochondrial injury leading to Ca^{2+} release and the activation of cell death pathways including the formation of ATP dependent apoptosome (128). In the absence of ATP, apoptosomes can no longer form and cell necrosis ensues; a fate with significant inflammatory repercussions (45, 89, 90).

Why are older donor kidneys more susceptible to pre-donation injury?

The likely reason why older kidneys, whether they be from DBD or DCD donors, are less able to tolerate pre-implantation injury is due to a number of anatomical and functional changes observed in the kidneys as they age. Indeed, with the exception of the lung, it is thought that the changes observed in the kidney are the most dramatic changes observed of any organ system (134).

Age results in a decline in kidney weight by approximately one quarter comparing young adult kidneys to those in the ninth decade of life. This is predominantly due to the loss of cortical tissue and number of glomeruli with relative sparing of the medulla (135). The afferent and efferent arterioles also begin to atrophy with eventual global sclerosis. The proximal tubules will shorten with age, as does the glomerular size. This structural change is observed with a functional deterioration as recognised by the early work of Alvin and Lewis reported in 1938, who demonstrated a urea clearance rate declining with age (136). Similarly, the creatinine excretion rate, electrolyte absorption capacity and renal functional reserve also decline over time(134). Cardiovascular disease including hypertension contributes to the accelerated development of histological changes which also contribute to a decline in kidney function.

At the cellular level aging is characterised by both a low level of chronic inflammation and also cellular senescence; a state of cellular growth arrest. This arrest can be induced by a number of stressors including oxidative stress, telomere shortening and

oncogene mutations which likely activate p53, p21 and p16 pathways resulting in growth arrest (137). In addition, Westhoff et al. compared first generation to fourth generation telomerase deficient mice and demonstrated reduced regenerative capacity in relation to short telomere lengths in response to IRI (138). Interestingly others have also demonstrated that the elimination of senescent cells delays the progression of age related diseases (139).

Senescence also leads to chronic inflammation, a hallmark of aging cells. In addition aging results in a reduced ability to clear self-antigens. Recent reports suggest that older endothelial cells express increased numbers of cell trafficking markers including VCAM-1 and MCP-1, increasing the immunogenicity of these older donor organs. In addition, age also results in epigenetic changes that may increase the immunogenicity of DNA. The ability of donor derived dendritic (DC) cells to stimulate an immune response is also enhanced in the aged allograft (140-143).

Not only are older cells more immunogenic, but their ability to activate cellular protective pathways are blunted. Heat shock proteins (HSPs), are molecular chaperones and involved in protein homeostasis . Van Dulleman et al. recently demonstrated using a rodent model of brain death that in comparison to living donors HSPs including HSP70 and HO-1 are significantly up-regulated in the kidney (144). In addition, HO-1 has been demonstrated as a promising therapeutic agent in models of IRI (145-149). The ability of cells to produce HSP however declines with age, and could in part be responsible for the susceptibility of these donor organs.

Age also impacts on the cellular ability to regulate autophagy and mitophagy. This was recently summarised by Fougeray et al. in *Nature Reviews Nephrology (150)*. Authors have demonstrated that specific deletions of Atg genes in murine proximal tubules or podocytes were associated with greater incidence of tubular atrophy, apoptosis and autophagy (151). This was associated with poorer renal function and also increased sensitivity to ischaemic or toxic stress. The authors did not demonstrate a similar effect of gene deletion in the distal tubules and suggested that this was due to high-energy demands and mitochondrial dependence of the proximal tubules. Similarly, using models of liver IRI, Wang and colleagues demonstrated decreased levels of autophagy related proteins were present in older livers then which had increased susceptibility to IRI (152).

Aging also affects a number of nutrient signaling pathways including the Insulin-like growth Factor-1 (IGF-1), AKT also known as Protein kinase B (PKB), mechanistic target of rapamycin (mTOR) and FOXO transcription factors (150). Consistent with this, low-caloric diets have been able to modify these transcriptional and also epigenetic changes resulting in promotion of $BNIP_3$ mediated mitophagy. Calorific restriction could also have renoprotective affects through inhibition of mTOR, which has shown to be beneficial in numerous models of IRI (153, 154). Ultimately the induced mitophagy/autophagy will increase the clearance of damaged mitochondria and cells, injured by excessive ROS production.

In summary, these cellular changes result in reduced capacity of aged cells to respond to metabolic, hypoxic and inflammatory challenges, typically observed during organ

donation and during warm and cold ischaemia (155). Therapies targeted at improving outcomes in donors or during preservation will need to address the susceptibility of older kidneys to withstand injury through multiple mechanisms including innate and adaptive immunity.

Why do kidneys from donors with a higher body mass index have poorer outcomes?

Dissecting how major obesity acts as a risk factor for poorer outcomes in deceased kidney donation is challenging, as often patients with significant obesity suffer with metabolic syndrome, i.e. insulin resistance, hyper-insulineamia and dyslipidaemia. However with the continuing epidemic of obesity in developed countries, it is likely we will increasingly be seeing donors with higher BMIs. Therefore understanding how obesity affects kidney function and the subsequent susceptibility of these kidneys for donation and preservation injury is of importance.

Henegar et al. have previously shown using an obese dog model, that in comparison with lean and normally fed dogs, obese dog kidneys had increased diameter of the Bowmann's space, increased mesangial matrix, thickening of the glomerular and tubular basement membranes (156-158). Others have also demonstrated obesity resulting in progressive glomerulosclerosis and also high inter-glomerular pressures(159). Hyperinsulinaemia has also independently been associated with glomerular hypertrophy. These structural changes are associated with poorer renal function (160).

Others have shown that the kidneys from obese animal models are more susceptible to IRI. Recently Jongbloed et al. reported at the *World Transplant Congress* that obese mice, fasted prior to application of IRI were more tolerant to the kidney injury. Supporting the notion that switching to a ketotic diet and modulation of nutrient signaling pathways could also be of benefit in the obese donor (161).

Authors have demonstrated that in nutrient rich environments autophagy is also blunted through increased mTOR activity. In multiple studies application of mTOR inhibitors has been shown to improve the outcomes of kidney transplantation, in part through modulation of autophagy (162). Therefore one can hypothesize that mTOR inhibitors may be of particular use in obese donors although this has yet to be proven experimentally.

Ischaemic preconditioning (IP), the application of brief periods of ischaemia prior to prolonged ischaemic periods is known to confer cellular protection. This is in part through modulation of autophagy, but also up-regulation of cyto-protective pathways (128). The conferred protection of IP, in the cardiac IRI setting, is lost in diabetic and obese mice, which could in part be through modulation of the mTOR activity in energy rich environments (162).

Ex-situ protection of vulnerable kidney allografts

Previous randomised controlled trials have investigated the benefits of hypothermic machine perfusion (HMP) of the kidney. The European machine perfusion trial demonstrated an improvement in DGF and 1 year survival rates for kidneys from both DBD and DCDs with a faster post transplant reduction in serum creatinine in the machine perfused kidneys (123). However, this result that was not replicated in a UK trial which explored the benefits of pre-implantation HMP, the trial focusing on DCD donors. The trial did not demonstrate an effect on improvements of post transplant DGF or graft survival (163). The difference in trial design is likely to account for the differences observed between these two trials.

When specifically looking at the >65 year old donors the European trial was able to demonstrate a reduction in the rate of never functioning kidneys and also improved longer term survival of kidneys that developed DGF (164). More recently Hosgood and Nicholson have been exploring the use of normothermic *ex-situ* machine perfusion of the kidney with promising results in a pre-clinical pig model and using discarded human kidneys (165). It is very possible that normothermic machine perfusion is likely to be of greater clinical value in older donors. Indeed the combined used of normothermic regional perfusion and normothermic *ex-situ* kidney machine perfusion in DCD donors is likely to have the greatest clinical benefit for susceptible donor kidneys and has already been investigated with regards to liver transplantation in the preclinical setting with promising results (166).

Oxygenated machine perfusion of the kidney could also provide additional metabolic support for vulnerable kidneys during *ex-situ* preservation. A number of promising experimental studies, which have shown benefits of oxygenated and also pre-implantation reconditioning of susceptible kidneys this has lead to two major phase 3 European Multi-centre trials being completed as part of the Consortium for Organ Preservation in Europe (COPE) initiative (119). The first trial investigates the benefits of at least two hours of pre-implantation reconditioning with oxygenated machine perfusion against continued cold static storage for kidneys from ECD donors and the second study investigates continuous machine perfusion with or without oxygen for DCD III donor kidneys. The results of these trials will have important implications for the use and *ex-situ* evaluation of these vulnerable kidneys.

Summary

This introductory Chapter has provided an overview on the mechanisms of injury to donor organs from deceased donors. Transplantation has been a victim of its own success, an increasing disease burden and also expansion of the criteria for transplantation has meant there is an increasing requirement for organs for transplant. This has resulted in higher risk donor organs, including older donors or those with a higher BMI being used. The rationale (Chapter 2) reflects on some of the remaining unanswered questions in transplantation and sets out how I attempt to answer some of these in this thesis.

Chapter 2: Rationale

In the introduction I outlined some of the challenges in organ donation, particularly with respects to kidney transplantation. To begin to address the organ deficit and increase the number of available organs, we could increase the number of organ donors i.e. improve supply, or alternatively prevent the discard of organs.

Supply in the UK could be improved through supporting legislative change to result in an "opt-out" system. Alternatively, change could occur by preventing family members from overturning the decision for donation (167-169). In recent years we have seen a surge of public awareness campaigns regarding organ transplantation, this has resulted in more registered organ donors. Interestingly the number of times relatives overturn the decision for organ donation has remained broadly static however (170, 171). The Welsh government has recently trialed an op-out system whereby people are given the choice to either donate their organs or not. Not indicating a preference will result in the assumption that the person on his or her demise will donate their organs. In the UK over 19 million people are on the organ donor register. However, most will die in circumstances that will prevent the donation of organs (170).

In their recent position document "Taking Organ Transplantation to 2020", NHS Blood and Transplant have highlighted their aims for improving organ donation including reviewing the impact of the Welsh governments interventions on the outcomes on the number of donors available for transplant (172). Although these approaches will potentially increase the number of organs made available, it will not change the necessity of higher risk organ donor usage. The other major way to increase the number of suitable organs is to prevent *discard*. This could be through:

- Understanding how organs become damaged during the donation process and developing *in-situ* strategies to prevent this.
- Developing preservation technologies to improve *ex-situ* assessment techniques and develop ways to resuscitate damaged organs.
- Improving surgical techniques and minimise reperfusion injury to organs.
- Developing tools to define what constitutes a suitable organ for transplantation e.g. clinical or molecular algorithms for determining the outcomes of a transplant.

In order to better preserve organs and also understand the implications on injury pathways we first need to understand how donor organs become injured during the donation process. As highlighted in the introduction, little research has focused on the combined milieu of cellular changes that occur in the donor organs. Most studies reported outline changes to single pathways or targets but do not take into consideration the complex interplay of multiple homeostatic mechanisms which are disturbed at the cellular and tissue level as well as systemically. This is the focus of this thesis.

In order to study the effect of the donor on organ injury I developed models of organ donation, to avoid donor heterogeneity and validate events at the cellular level in a controlled manner. In Chapter *3* I outline the establishment, optimisation and validation of rodent models of organ donation. The Chapter focuses on DBD, DCD and renal IRI models to provide access to the whole range of different donor types allowing animal models to be used for determining the pathways of injury and repair. I outline

the variables for the different model types and perform experiments to validate these models against results of other published literature. The brain death model developed in this Chapter, forms the basis for the further research work.

In Chapter 4, I aim to begin to use the DBD model to determine the key cellular processes that are disturbed as a consequence of brain death in the kidney using proteomics. This approach has been used previously when integrative –omics techniques have been used in the field of systems biology, allowing the combination of experimental modeling with computational biology (173-177). This has been used in the field of cell death pathways. Initially, cell death was simply attributed to apoptosis or necrosis (178-180). Today, our understanding of cell death pathways is more complex with the realisation that there are a huge network of intracellular signaling pathways that interact and involve mitochondrial signaling pathways, the generation of apoptosomes and external signals that can modulate the type of cell death encountered. Understanding how these pathways are linked has led to the development of potential treatment options for a number of disease processes including Alzheimer's and Parkinson's disease. Using a similar approach in kidney cells may allow us to further dissect cellular pathways that are disturbed in the kidneys of organ donors. This may be of relevance to develop new strategies to improve the outcomes of kidney transplantation.

Non-biased shot-gun proteomics was used to survey changes in the protein make-up of kidneys from brain dead organ donors compared to sham controls. To further localise the cellular injury I concentrated on cortical segments of tissue and used identified

proteins to further the sub-cellular localisation. To draw a parallel to the injury occurring during renal IRI, I performed experiments to explore the alterations in the proteomic profile of kidneys exposed to IRI.

In Chapter 5, I further investigated the affects of brain death on mitochondrial function and metabolism aiming to validate the findings in Chapter 4. I used 1H-Nuclear magnetic resonance (NMR) spectroscopy and metabolomics to identify alterations to key metabolic pathways and a range of mitochondrial functional assays including generation of ROS intermediaries.

Chapter 6 explores the intrinsic relationship between the alterations at the cellular level in mitochondrial function and metabolism with alternations in the hypoxia inducible factor (HIF) pathway. The HIF pathway is the intrinsic cellular response mechanism to hypoxia for all mammalian cells. The HIF pathway targets a number of important pathways altered as a consequence of brain death including metabolism, mitochondria and many of the pathways highlighted in Chapter 1. Chapter 6 aims to explore how the HIF pathway is altered following brain death and I begin to attempt to characterise the hypoxia response of different sub-cellular components of the kidney. I begin to attempt to modulate the HIF machinery in the brain dead organ donor using prolyl hydroxylase inhibitors.

In Chapter *7* I conclude the findings of the research and define future avenues. I also highlight a number of the collaborative projects I have been involved with during my DPhil including the establishment of a national bio-bank of organ donor samples, which

aims to translate some of the findings made in pre-clinical models into human samples. The bio-bank also aims to facilitate biomarker research. I also describe the establishment of a series of clinical trials as part of the Consortium on Organ Preservation in Europe (COPE) that aims to expand the use of machine preservation to protect and preserve organs.

Chapter 3: Establishing models of organ donation

Introduction

During organ donation the injury an organ is exposed to during the donor management, retrieval and preservation phase renders studying the effects of organ donation on cellular pathways a complex process (128, 181-184). Especially if we consider the multiple biological systems that are affected as a consequence of donation related injury as illustrated in the introductory Chapter (28, 183, 185, 186).

There remains a balance between designing experiments that allow for highly controlled circumstances, reducing variability and allowing the researcher to draw conclusions from low numbers, and those studies which allow the consideration of the multifactorial impact of pre-morbid conditions and disease processes that can affect the quality of an organ (22). For example age and BMI we have already seen have an important impact on the pre-donation quality of an organ, but also the organ's susceptibility to injury during organ donation and preservation (22).

To produce well-controlled experiments that allow us to dissect pathways of injury to an organ, and possibly leading to potential therapeutic targets, there is the need to use highly regulated, relevant, robust and reproducible models of injury such as small animal models (187). In the field of organ donation a number of different models have been described including models of donation after brain death (DBD), donation after circulatory death (DCD) and living donation (LD) donor models. In addition, models of organ ischaemia reperfusion injury (IRI) have been used to allow researchers to specifically explore the impact of oxygen and nutrient deprivation on cellular processes accompanied by the period of reperfusion (188). The duration of the ischaemic and

reperfusion period reveals different magnitudes of injury and differential response mechanisms e.g. sequential recruitment of inflammatory cells into tissues or the immediate and late gene transcriptional responses (128, 132, 189). For example in the kidney following a short period of ischaemia, hypoxia responsive genes are immediately activated and then 24 hours later there is a delayed transcriptional response (36).

The type of hypotheses being tested will determine which type of model will be used for experimental purposes. Table 1 describes the models of organ donation in further detail, considerations for their use and their relative merits.

A number of groups have developed large animal models of organ donation, usually in the context of the organs being used for subsequent transplantation experiments. The attraction of this is that the transplant procedures can be performed by clinically trained surgeons and arguably the translatability of some of the findings, for example with regards to machine preservation, are more readily made to the clinical setting (190). However, these experiments are expensive and the laboratory tools available to unravel pathways of injury and repair are less well developed for large animals. The subsequent sub-Chapters focus on models of organ donation specifically DBD, DCD and IRI models.

Table 1. Models of organ donation and their benefits and drawbacks.

Model	Type of donation	Benefit	Drawback
Brain death (BD)	After confirmation of death based on neurological criteria following destruction of the brain stem	Relevant small animal models exist. Most common form of deceased donation. Opportunity to intervene in the donor.	Difficult to obtain adequate control.
Donor after circulatory death (DCD)	Cessation of circulation induced by administration of agent, overdose of anesthetic or cervical dislocation	Easy model to replicate. Relevant as increasing number of DCD donors are being used worldwide.	For full assessment requires use of a separate reperfusion model.
Ischaemia reperfusion injury (IRI)	Induction of ischaemia to an organ by application of a clip to the arterial inflow or to both artery and vein	Experimental parameters are easily controlled. Includes reperfusion phase.	Does not take into consideration allogeneic immunological responses.

Donation after brain death (DBD) model

The thesis introduction describes some of our current knowledge and understanding of how DBD donor organs sustain injury during the brain death (BD) process. The animal studies described used variations of the BD model with most choosing to use rats. With improvements in the technical management of these models some pioneering groups have now established mouse models of BD, allowing for the mechanistic dissection of molecular pathways using genetically modified mice. Most of these small animal models however are only limited to 1-3 hours of post BD induction period, due to the haemodynamic instability.

Virtually all described models of BD involve anaesthetising the rodent using an inhalation volatile anaesthetic, to allow easy control of depth of anaesthesia (78). Subsequently arterial and venous access is obtained to allow invasive blood pressure monitoring and to allow colloid administration. Induction of BD is typically performed by either application of traumatic injury to the rodent (e.g biomechanical force) or by application of ischaemia or raising intracranial pressures through inflation of an intra-cranially placed balloon (78). Some research centres report placing the balloon extadurally and some report placing the balloon subdurally. Extradural placement allows for a more controlled BD induction, whereas subdural placement replicates an intracranial haemorrhage. Regardless of the technique used loss of brain stem function remains the aim of this application and is tested as in the clinical situation by ensuring the absence of reflexes, a positive apnoea test and in some circumstances loss of EEG signals.

The induction rate of BD also varies with some studies using “fast” induction models, which aim to replicate traumatic brain injury and others using “slow” induction replicating haemorrhagic stroke or progressively rising intracranial pressures e.g. due to hypoxic brain injury (40). Over the last 5-10 years an increasing number of publications report “slow” induction BD models when exploring the impact of brain injury on organs for donation, reflecting the increasing clinical shift from traumatic brain injury to haemorrhagic stroke in the DBD donor cohort (191-195).

The duration of the BD induction varies between studies (38, 78, 91, 100, 117, 181, 182, 185, 194-208). Most report the haemodynamic instability of the donor during the post BD induction phase, with more haemodynamic instability occurring with those donors who have a more rapid induction of BD. The strain of the rodent and the ventilatory compliancy of the lungs of that species may to some degree contribute to the stability of the rodent post BD induction, although other factors such as the mechanism for BD induction, haemodynamic resuscitation and the use of vasopressors will also have impact.

A summary of experimental procedures for performing BD experiments in animal models are summarised in table 2.

Table 2. Active groups using models of BD.

Group	Rodent	Method of BD induction	Duration of BD maintenance	References
Surgery Research Laboratory, Department of Surgery, University Medical Center Groningen, University of Groningen, The Netherlands.	Rat model Fisher F344	16µl per minute injected, usually over an average of 30 min.	4-6 hours	(38, 62, 78, 91, 100, 117, 190, 196, 197, 202-205, 207, 209-211)
Department of Cardiac Surgery, University of Heidelberg, Heidelberg, Germany.	Rats	Subdurally placed 4F Fogharty balloon catheter. Sham operated animals serve as controls.	Up to 5 hours	(200, 201)
Department of Anaesthesiology and Intensive Care, Aarhus University Hospital, Aarhus, Denmark.	Rat model	Inflation of intra-cranially placed balloon catheter, 20µl injected every 5 min.	Up to 4-9 hours	(206, 208)
	Pig	22F Foley catheter placed in the intracranial space and inflated with 1cc of saline every minute.	Up to 4 hours	(205)
Department of Hepatobiliary Surgery & Hepatobiliary Surgical Institute, Chinese PLA General Hospital, Beijing 100853, China.	Mouse model C57BL/6	2F balloon catheter injected with 6ul per minute, induction over 20 minutes. Sham operated animals serve as controls.	2-4 hours	(212)

Laboratory for Experimental Thoracic Surgery, Katholieke Universiteit Leuven, Leuven, Belgium.	Mice	Subdurally placed Fogharty catheter. Sham operated animals serve as controls.	Up to 6 hours	(213)
Department of Cardiothoracic Surgery, Alfred Hospital, Monash University, Melbourne, Australia.	Rats	Rapid balloon inflation using 3F Fogharty balloon catheter. Sham operated animals serving as controls.	Up to 3 hours	(214)
Department of Surgery, First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China.	Rats and mini-pigs	Rapid and slow inflation models. Sham serve as controls	Rats: 9 hours Mini-pigs: 24 hours	(206, 208, 215)
Department of Anaesthesiology, University Medical Centre Mannheim, Heidelberg University, Heidelberg, Germany.	Rats	Subdurally placed Fogharty catheter. Sham operated animals serve as controls.	Up to 5 hours	(201, 216, 217)
Department of Neuroscience, Section of Neurosurgery, Uppsala University, 751 85 Uppsala, Sweden.	Pig	Epidural catheter inflated. Sham operated animals serve as controls.	Up to 3 hours	(44)
Department of Cardiovascular Surgery, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan.	Dogs	Rapid inflation of balloon catheter.	1 hour	(218)

Department of Thoracic Surgery, Ankara Numune Education and Research Hospital, Ankara, Turkey.	Rats	Weight drop to the head in an anaesthetised rodent.	24 hours	(219)
Centre de Recherches Chirurgicales Henri Mondor, URA CNRS 1431, France.	Rabbit	Subdural balloon inflation. Sham served as controls.	1.5 hours	(198)

Donation after circulatory death (DCD) model

Donation after Circulatory Death (DCD) donors are those who are declared dead by cardiovascular rather than neurological criteria as discussed in the introduction (220).

As there has become an increasing dependency on DCD donors in many countries, there has been renewed interest in the effects of the process of DCD donation on pre-retrieval donation injury. Some centres choose to use IRI as a surrogate for DCD donation, although this does not take into consideration the impact of multi-organ functional ischaemia and the associated inflammatory, metabolic and other parameters that are altered as a consequence of organ ischaemia (128). In addition virtually all DCD donors will have experienced some degree of cerebral injury, haemorrhagic or hypoxia related. This is important, but few studies have evaluated the importance of cerebral injury plus warm ischemia in the DCD context.

The type of model used will depend to some degree as to the type of Maastricht donor the model is trying to replicate. Worldwide and in the UK the most common type of DCD donor are category III donors, i.e. those patients in whom medical treatment continuity is futile and the decision made to withdraw support (NHSBT 2013-14 statistics). The types of models which have been used in DCD donation are summarised in Table 3.

Table 3. Models for DCD donation; benefits and drawbacks.

Method of cessation of circulation	Benefit	Drawback
IV injection of KCL	Rapid, well controlled cessation of circulation.	No agonal phase and functional warm ischaemic phase.
Cervical dislocation	Sudden cessation of ventilation, agonal phase occurs. Schedule 1 kill.	Manual traumatic injury may cause intra-abdominal haemorrhage
Manual compression of the heart	Simulates an agonal phase	Varying lengths of agonal phase
Ventilatory support withdrawal following anaesthesia and administration of a muscle relaxant	Closely mimics the clinical situation where ventilation is withdrawn.	Use of muscle relaxant not approved by some animal committees due to not being able to monitor rodent movement.

Ischemia reperfusion injury (IRI) model

The purpose of an IRI model in transplantation is to act as a surrogate for exposing an organ to the detrimental effects of warm ischaemia and then allowing the assessment of the reperfusion effects in the same animal. The model allows for the study of the detrimental effects of isolated ischaemia whilst the researcher may determine the length of the exposure and the follow-up period. The use of one kidney as a control or part of a liver (isolating lobes) allows for the use of an endogenous control and avoids the need for additional sham experiments. This however, does not take into consideration potential remote preconditioning effects (221, 222). Although useful, this model does not allow organs to be exposed to periods of cold ischaemia.

To circumvent this problem some researchers use *ex-situ* reperfusion whereby an organ is exposed to period of warm ischaemia typically in the donor and then the organ explanted and exposed to a period of cold preservation before being placed on an artificial circuit which reperfuses the organ with blood at 37^{o}C (223, 224). This does not fully replicate the environment of transplant however, including the host response. The importance of consideration of the reperfusion phase is due to the majority of the injury response occuring after reperfusion.

A number of different IRI models have been used in both kidney and liver transplantation. In kidney transplantation a range of approaches, either explanting the non-ischeamic kidney or using the contralateral kidney as a control have been described. The duration of application of ischaemia varies between a few minutes to 60 minutes in larger animal models. The degree of ischaemia applied in any given

experiment will determine in part the effects seen on reperfusion and the resilience of an organ to ischeamic injury. For example the liver is less resilient to ischaemic injury than the kidney (128, 225),.

The duration of reperfusion will also determine the effects seen e.g. with a short reperfusion phase some of the delayed responses observed may not be seen (36, 128). A range of studies and models have been described in both kidney and liver transplantation using variations of these protocols (226-231). The number of studies and permutations of the model are too numerous to describe. Figure 1 outlines the different effects observed with different durations of ischaemia and reperfusion.

The aim of this Chapter was to establish and validate rodent models of BD, IRI and DCD donation with the aim of subsequently using these models in experiments

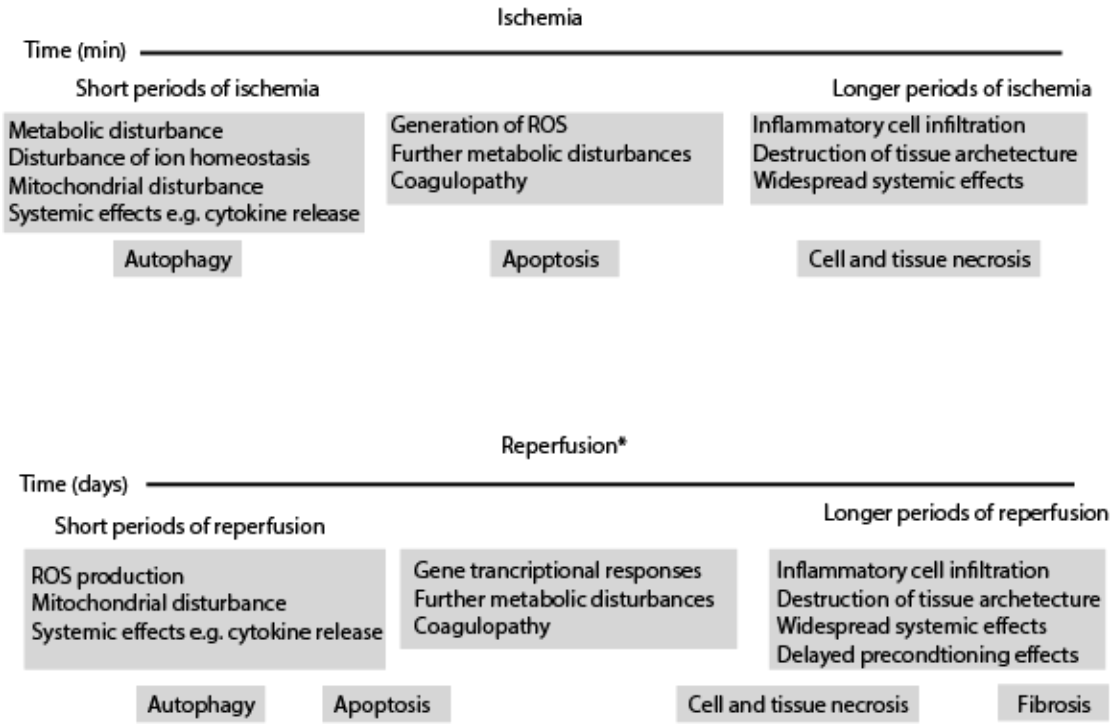


Figure 1. Effect of IRI on cellular and molecular processes. Differential effects of varying durations of ischaemia and reperfusion injury on cellular processes. * responses may be cell and organ specific.

Methods

Animal welfare

All studies were carried out using inbred Fischer F344 male rats (250-300g, Harlan, UK). Animals received free access to food and water in compliance with the Home Office guidance for the care and welfare of animals. Rats were acclimatised for at least one week prior to experimentation.

Brain death model

To learn the BD model I approached the University of Groningen who had published significantly in this area over the last 10 years. I decided to use a slow brain dead induction model, aiming to replicate intracranial haemorrhage and aiming for a 4-hour post brain dead induction period. Training in the model and management of the BD rodent was performed with P.J Ottens and Prof H.G.D. Leuvenink. Subsequently, the model was established in Oxford.

Animals were anaesthetised using isoflurane with O_2. Cannulae were inserted into the femoral artery for blood pressure monitoring and femoral vein for colloid administration. Lines were kept patent with heparin/saline (10iu/ml). Animals were intubated via a tracheostomy and ventilated throughout the experiment (oxygen saturations 100%, FiO2 of 2l/min). Protective ventilator strategies were employed to ensure that minimal traumatic lung injury occurred to prevent confounding results with peak inspiratory pressures of 8-10mmHg and peak expiratory pressures (PEEP) of 3mmHg. Respiratory rates between 80-120 were maintained to maintain to keep the

end-tidal CO_2 measurements between 28-30mmHg (personal communication R. Robelledo and M. Hottenrott).

Through a fronto-lateral borehole in the skull, a 4F-Fogarty catheter (Edwards Lifesciences, Irvine, CA) was placed subdurally and slowly inflated (16 µL/min) with saline. BD was confirmed by the absence of corneal and pupillary reflexes and a positive apnoea test. This occurred 23-35 mins after starting balloon inflation. Following confirmation of BD, anaesthesia was terminated.

Mean arterial pressures (MAP) were maintained between 80-120mmHg with voluven (6% hydroxyethyl starch). Animals were maintained in the BD state for four hours before laparotomy, flushing of organs with 0.9% saline at 4^{o}C followed by organ procurement. 0.2ml 0.9% saline maintenance fluid per hour was administered i.v.

Alterations to the BD model were made in light of preliminary experiments including:

(1) Temperature was kept at 37^{o}C with an auto-regulated heat mat
(2) Switching from a pressure to volume control ventilator
(3) Establishment of end-tidal CO2 monitoring
(4) Establishment of blood gas analysis. 0.1ml was taken from the rat and replaced with an equal volume of 0.9% saline. 0.1ml blood in the line was discarded to ensure no line contamination.

Physiological parameters were maintained within the following boundaries:

- Mean Arterial Blood Pressure: 80-120mmHg

- Oxygen saturations: >95%
- Respiratory rate: 80-120 breaths/min
- End-tidal CO_2: 28-35mmHg
- Core body temperature: 36.5-37.5^{o}C

Validation of the BD model

Once the model was established with robust physiological parameters, I aimed to validate the model using biochemical and histological analysis. I obtained samples from rodents after 1, 2 and 4 hours of BD and compared these to sham control (n=3 per group). An additional experiment was performed comparing the kidneys of long sham controls (sham controlled animal, anaesthetised for 4 hours after a simulated BD induction (no inflation of the intracranially placed balloon catheter) lasting 30 min) against short sham and BD animals. This was done to evaluate if there was a functional effect of different sham controls compared to each other and brain death.

DCD model

I established a reproducible model of DCD III donation, which would allow for an agonal phase, during which the heart would continue to beat, but ventilation stops. I decided to establish this model using cervical dislocation.

Animals were anesthetised using iso-flurane with O_2. Cessation of ventilation was induced by cervical dislocation, confirmed by palpating a gap in the cervical spine and an absence of ventilatory effort. Following confirmation of cessation of circulation (by

cardiac palpation) and 30 min of warm ischaemia a laparotomy was performed and organs flushed with 0.9% saline (4^{o}C).

Tissues samples from the healthy "living donor" controls were also taken. This was a healthy rodent who was anaesthetised and then organs procured aiming to simulate a living donor in the transplant setting (n=3).

IRI kidney model

I established a model, which would be comparable to the BD model in terms of duration of effects whilst also allowing the evaluation of the reperfusion phenomenon, minimising the use of animals and allowing the extrapolation of meaningful results. I decided to establish a model in which the left kidney would be exposed to warm ischaemia and the right act as an endogenous control (n=4).

Animals were anaesthetised using isoflurane with O_2. Temperature was regulated using an auto-regulated heat mat. A mini-laparotomy was performed and the renal pedicle dissected. Graded pressure clamp was applied to the artery (Fine Science Tools) and the kidney noted to change colour turning purple. Any kidney in which colour alteration was not noted was excluded from the study. The renal pedicle of the right kidney was also dissected but the clamp not applied. Heparin (50units/kg in 0.2ml into the tail vein) was administered 5 min before the application of the clamp to prevent renal thrombosis. The abdominal cavity was closed during the application of ischaemia (after

clip application) and the wound covered with a wet gauze. The rat was kept anaesthetised during the period of warm ischaemia.

After 45 min of warm ischaemia the clamp was released and reperfusion of the kidney macroscopically noted. The abdominal wound was closed and intra-peritoneal and wound bupivacaine (0.25%) was applied. The rat was recovered from anaesthesia in warmed housing, transferred to the normal habitat and maintained with free access to food and water. 4 hours of reperfusion was allowed before re-anaesthetising the rat and termination of the experiment by laparotomy and flushing of the abdominal organs.

An additional healthy control was used for experimentation purposes.

Organ, blood and urine procurement

Urine samples were taken directly from the bladder. Blood samples were taken by cannulating the aorta. Following aspiration of blood the organs were flushed with cold 0.9% saline (4^{o}C). Anterior hemi-sections of kidneys were snap frozen and the posterior hemi-section fixed with formalin for 24 hours before transfer to 70% ethanol. The left lobe of the liver was taken and segments frozen and fixed as described. Snap frozen tissues were pulverized and aliquotted for protein and mRNA analysis.

Blood samples were placed into EDTA or heparin tubes and spun at 3000g for 15 min. Urine samples were spun at 3000g for 15 min also.

Biochemistry

Blood gas analysis was performed using the iSTAT blood analyser system (G4+, Abbott, Berkshire, UK) and serum biochemistry analysis performed on plasma samples sent to MRC Harwell (Oxford, UK).

Haematoxylin and Eosin staining

H+E staining was performed as previously described (232). In brief 4µm formalin fixed paraffin embedded samples were cut and stained with haematoxylin and eosin.

Cytokine

IL-6 quantification was performed (R&D systems , UK, anti-rat). In brief 100µl of plasma samples were added to the well. 200µl of (Phycoerythrin) PE-conjugate was added and incubated at room temperature for 2 hours. Conjugate was washed off and the 200µl of substrate was added and the plate protected from light for 20 min. 50µl of stop solution was added and the plate read at 450nm, with the wavelength correction set to 540nm. Results were compared to a standard curve to allow the establishment of absolute values.

Immunoblotting

Western blotting was performed as previously described (233). Antibody incubation with HO-1 (Abcam, UK) antibody was performed overnight at 1:1000 antibody dilution. After washing the membrane a secondary anti-rabbit HRP conjugated antibody was

added for 1 hour (1:1000, Sigma). Blots were developed on Kodak film using high sensitivity substrate. Image J was used to perform relative quantification.

RNA isolation and semi quantitative RT-PCR

Total RNA was isolated from snap frozen tissue (10-30mg) using RNeasy© MiniKit (Qiagen) isolation kit. The GeneAmp © PCR Sytem9700 thermal cycler was used for the cDNA reverse transcription. An Applied Biosystem qPCR machine was used to run the experiment. The primer sequences were:

HMOX-1: Forward: 5'- CCTGGTTCAAGATACTACCTC, Reverse: 5'-ACATGAGACAGAGTTCACAG

NFkB: Forward: 5'-AAAAACGACCTAGAGATTG, Reverse: 5'-ACATCCTTCCTTGTCTTC

TNF-a: Forward: 5'- AGTTACGACATAGACATAG, Reverse: 5'-AGATTCTACCCTGTCTAC

β Actin: Forward: 5'-CAATATGTGGAGCAACTGTG, Reverse: 5'-AGTAGGTGAAGATGAAGAAGAG

Relative quantitation of gene expression was performed using β actin as the control.

Statistical analysis

For statistical evaluation of the data a Kruskal-Wallis (non-parametric) comparison of multiple groups was performed (Graph-pad Prism 2014). Significance was set at a value of P=0.05 and all graphs report results as mean± standard deviation.

Results

Brain death model

Figure 3 a illustrates the learning curve for the model. In the first 7 rodent experiments performed the mean duration of surgical preparation was 98min (+/- 18 min). All 7 rodents died or had systolic pressures below 40mmHg that could not be rescued with fluid administration. Autopsies following cessation of the experiment revealed pulmonary oedema (n=1), myocardial infarction (n=2) and pulmonary embolism (n=1) as being possible reasons. The cause for low MAP or death could not be confidently attributed in 4 cases.

Following modifications to the model including introduction of a homoeothermic auto-regulating temperature blanket system, I was able to take the next cohort of rodents to 1-1.5 hours after BD and reduce the surgical preparation time down to 85min (+/- 9min). In addition the positioning of the balloon catheter was altered to ensure full destruction of the brain stem and the ventilator protocol switched to a 'lung protective' low pressure model. The ventilator was replaced with a newer more finely controllable volume controlled ventilator. With further alterations including switching the ventilation system from a pressure to volume controlled system I was able to maintain circulation in the rodents for 4 hours after BD.

Figure 3 b illustrates the final set up of the rodent including the vascular lines, the Fogharty balloon catheter, O_2 saturations probe and the rectal thermometer linked to the blanket system. Using this defined set up I performed a series of experiments to

validate the above model using rodents kept within strict physiological parameters as defined in the methods.

Figure 4 illustrates the MAPs after BD induction for the 1, 2 and 4 hour post BD groups. Table 4 illustrates the average surgical times, amount of colloid administered, fluid and adrenaline received. The table illustrates the basic biochemical features between the groups. None of these factors varied significantly between the sham and the BD groups.

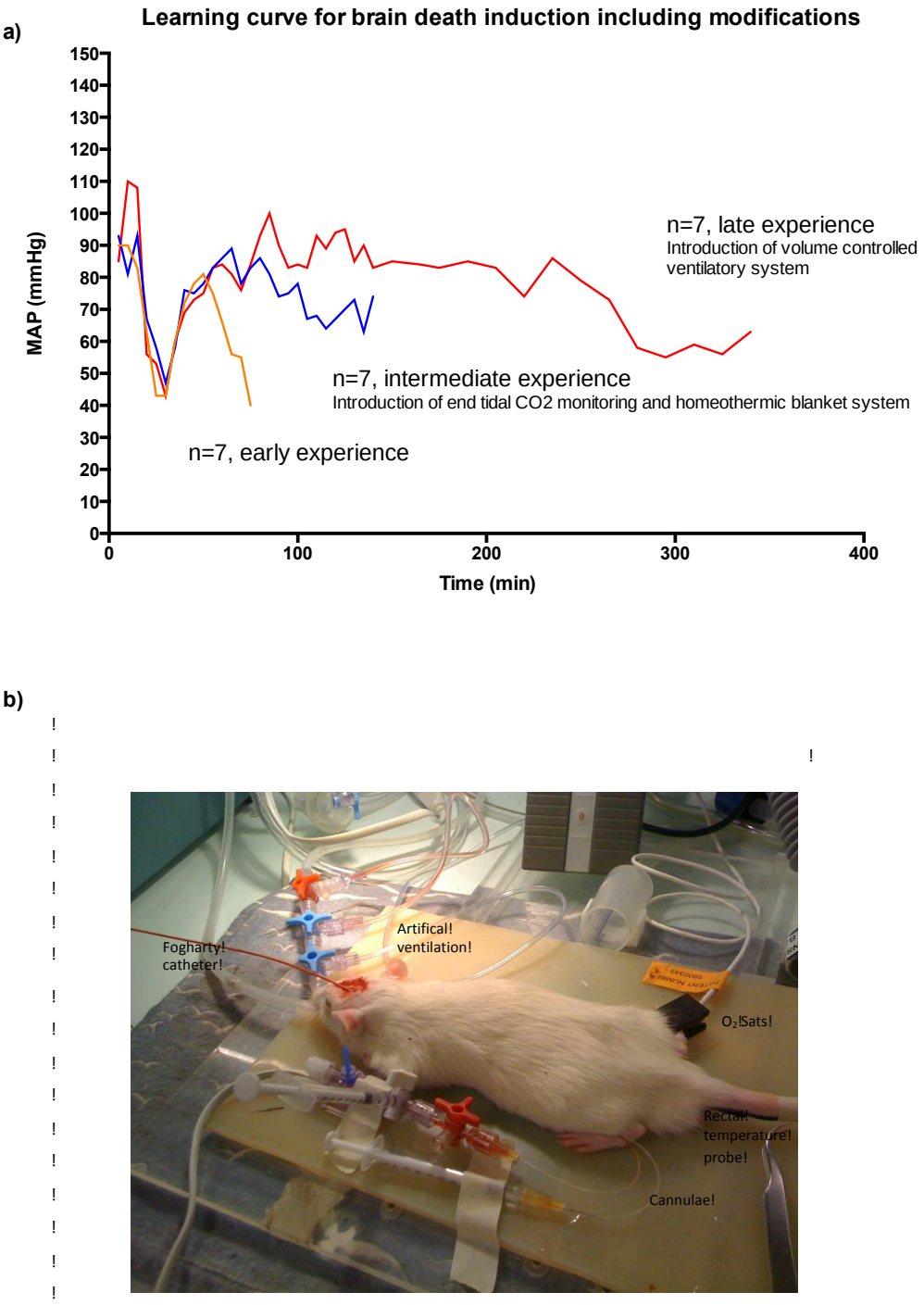


Figure 3. Establishment of the BD model. a) 21 BD animal experiments were performed, separated into early, intermediate and later experiences. Modifications to the protocol included introduction of volume controlled ventilation, end tidal CO2 monitoring and homoeothermic blanket system. b) Final set up of the system including the balloon catheter, ventilator, O2 saturation probe and vascular cannulas.

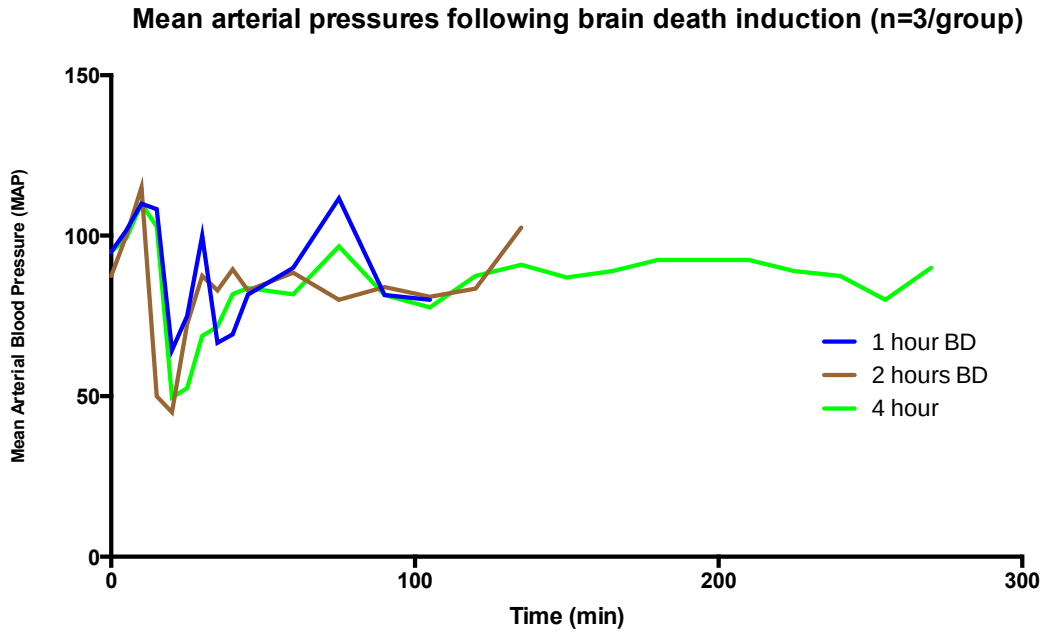


Figure 4. Mean arterial pressures following BD induction in pilot experiments to validate the model. Models carried on to 1, 2 and 4 hours after BD (n=2 per group).

Table 4. Biochemical parameters comparing the control against BD group at different durations. n/s: non significant, n/a non applicable n=3/group

Parameter	Control	Brain death group (hrs)			Significance
		1	2	4	
Weight (g)	283	293	278	287	n/s
Time to BD induction (min)	N/A	32	28	31	n/s
Surgery time (min)	n/a	72	64	70	n/s
Volume received (ml)	n/a	1.5	2.7	3.5	n/a
Na (mmol)	139.5	140.5	142	138.5	n/s
K (mmol)	4.90	4.90	4.93	5.03	n/s
Albumin g/dl	32.3	27.75	24.00	18.80	n/s
Bilirubin mg/dl	2.30	0.97	1.13	1.16	n/s
Triglyceride mg/dl	3.59	2.12	3.05	2.90	n/s
PO_2 mmHg	17.4	16.2	15.9	17.1	n/s
PCO_2 mmHg	4.3	4.2	5.1	4.7	n/s

The creatinine and urea following BD showed a progressive and significant increase (Figure 5, P=0.02 and P<0.01). The AST and GLDH also increased following BD in comparison to the sham control but this did not reach significance. The creatinine kinase was significantly higher in the BD group and rose progressively (P=0.01). No significant alterations in the urinary protein concentration were noted. Glutamate dehydrogenase increased non-significantly when comparing the control to the 4 hour BD group.

There was no significant difference in the urine creatinine, although a dramatic decrease in in the creatinine clearance was noted (P=0.01, Figure 6). Plasma IL-6 and kidney HO-1 levels rose progressively following BD and were undetectable in the short sham control group (IL-6, P<0.01).

Histological evaluation of the kidneys and livers from brain dead rodents showed progressive increase in inflammatory cell infiltration when comparing the short sham control against the 4 hour BD group. Progressive infiltration of neutrophils into the glomerulus and cortical areas of the kidney were noted (Figure 7).

Figure 8 illustrates no significant difference in the serum creatinine between the short and long sham controls, compared to a significantly increased creatinine after 4 hours of BD (P=0.03).

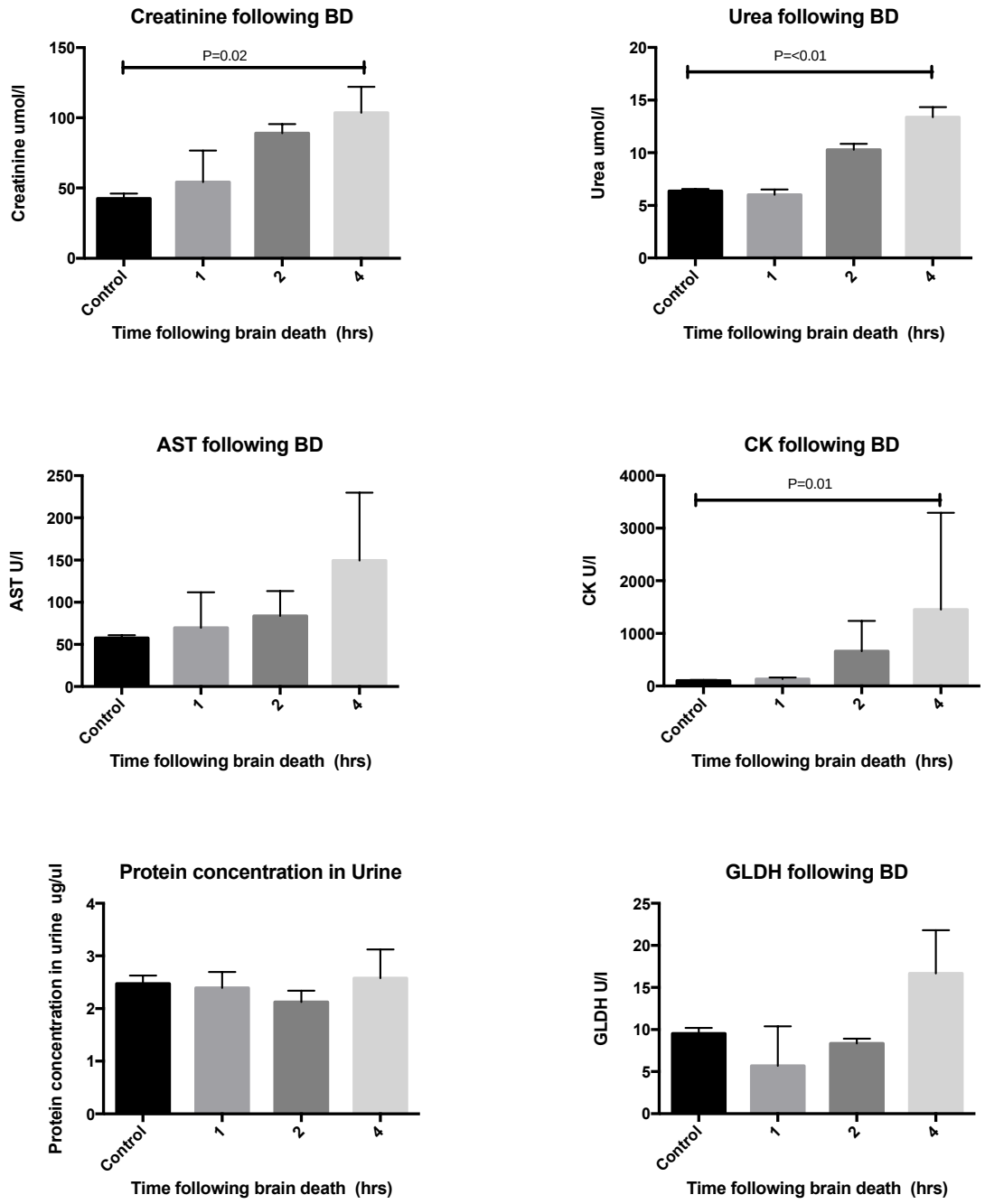


Figure 5. Plasma measurements following BD. Alterations in serum measurements including creatinine, urea, serum aspartate aminotransferase (AST), creatinine kinase (CK), urine protein and glutamate deyhydrogenase (GLDH).

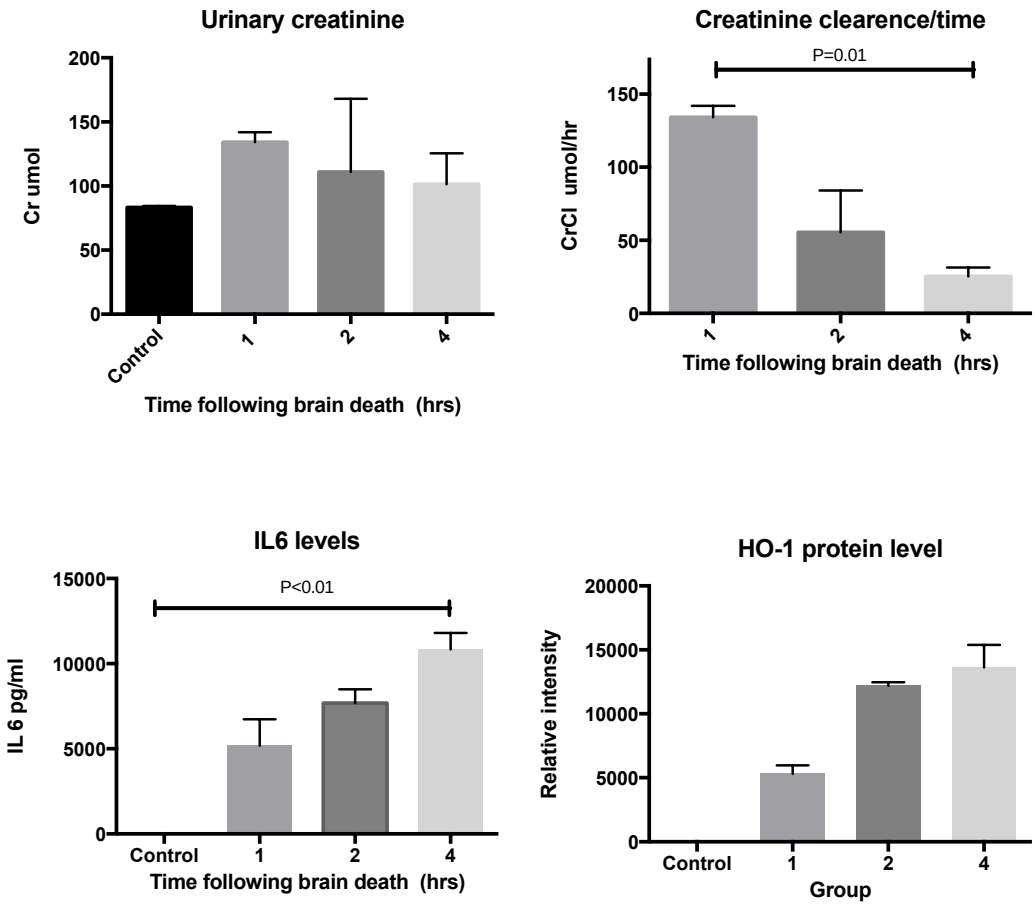


Figure 6. Effects of BD on renal function and inflammation. Summary of urinanry creatinine, creatinine clearance over time and plasma IL6 levels and kidney protein levels of HO-1.

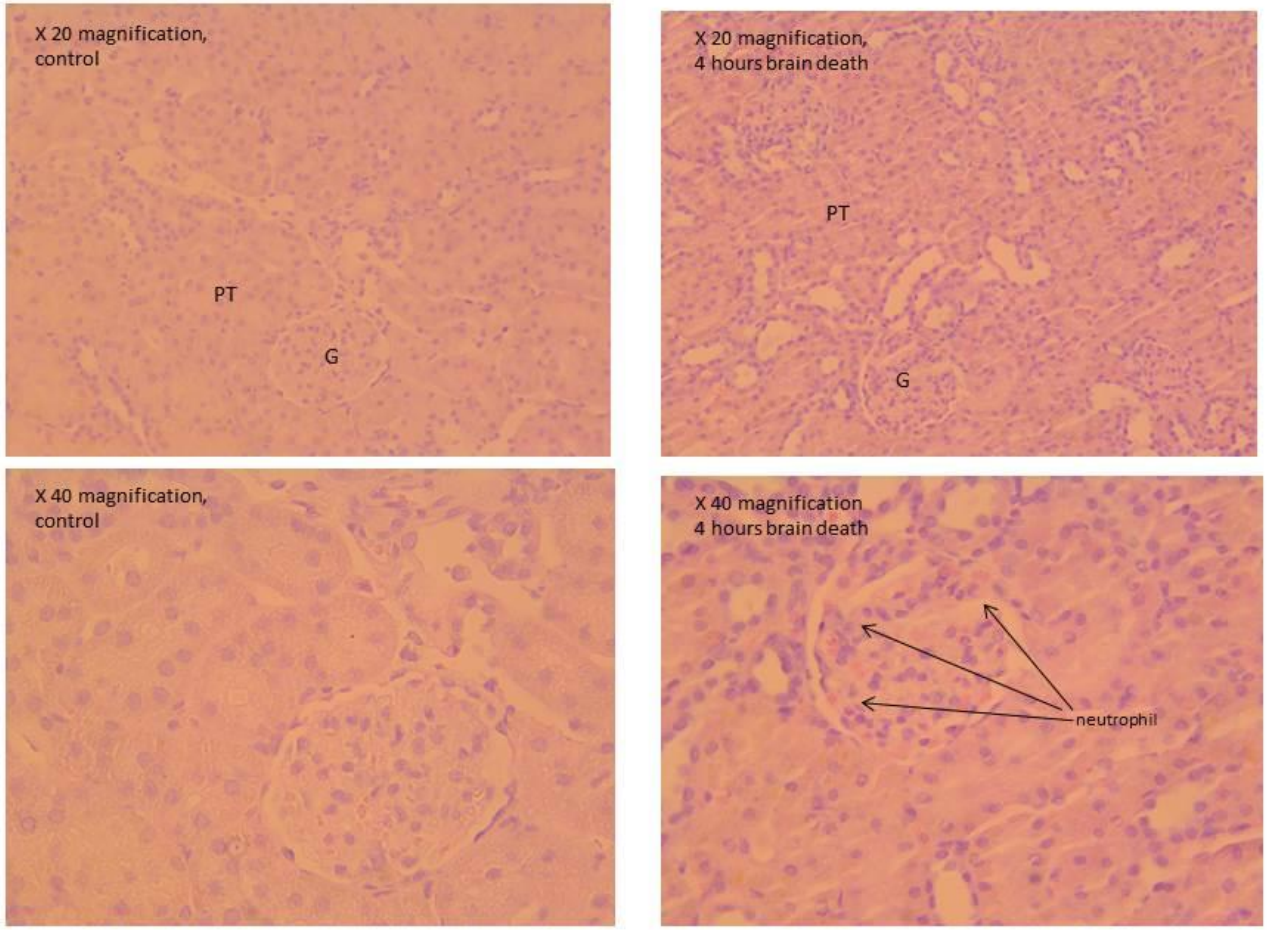


Figure 7. H+E staining of kidney cortical samples from BD rodents. Comparison of H+E staining of renal cortical samples from the kidney of sham control animals and animals exposed to 4 hours of BD, multiple neutrophils are identified infiltrating the glomerulus of the kidney. The neutrophils are predominantly found in association with the glomerulus.

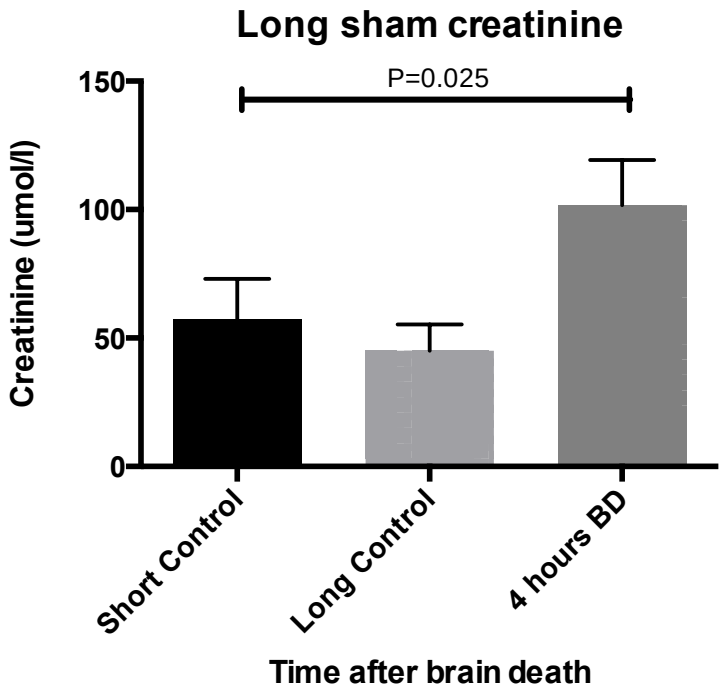


Figure 8. Comparison of BD against short and long sham controls. Comparison of serum creatinine measurements between short, long shams and 4 hour brain dead rodents.

DCD model

Asystole (measured by absence of palpable cardiac pulsation) occurred 23 seconds (median) following cervical dislocation. Figure 9 illustrates the changes in the inflammatory gene profile following 30 min of warm ischaemia following confirmation of asystole. In comparison to the living donor healthy control there was a significant increase in TNFα ($P<0.01$), but not for HO-1, NFkB or INFA1.

IRI model

Figure 9 illustrates the associated changes in gene expression level of key mediators of IRI in the control, healthy and IRI kidneys. Progressive increase in inflammatory mediators were noted in the ischeamic kidney, significantly increased amounts of NFkB and INFA1 were detected between either the living control or endogenous control and the ischeamic kidney as demonstrated in figure 9.

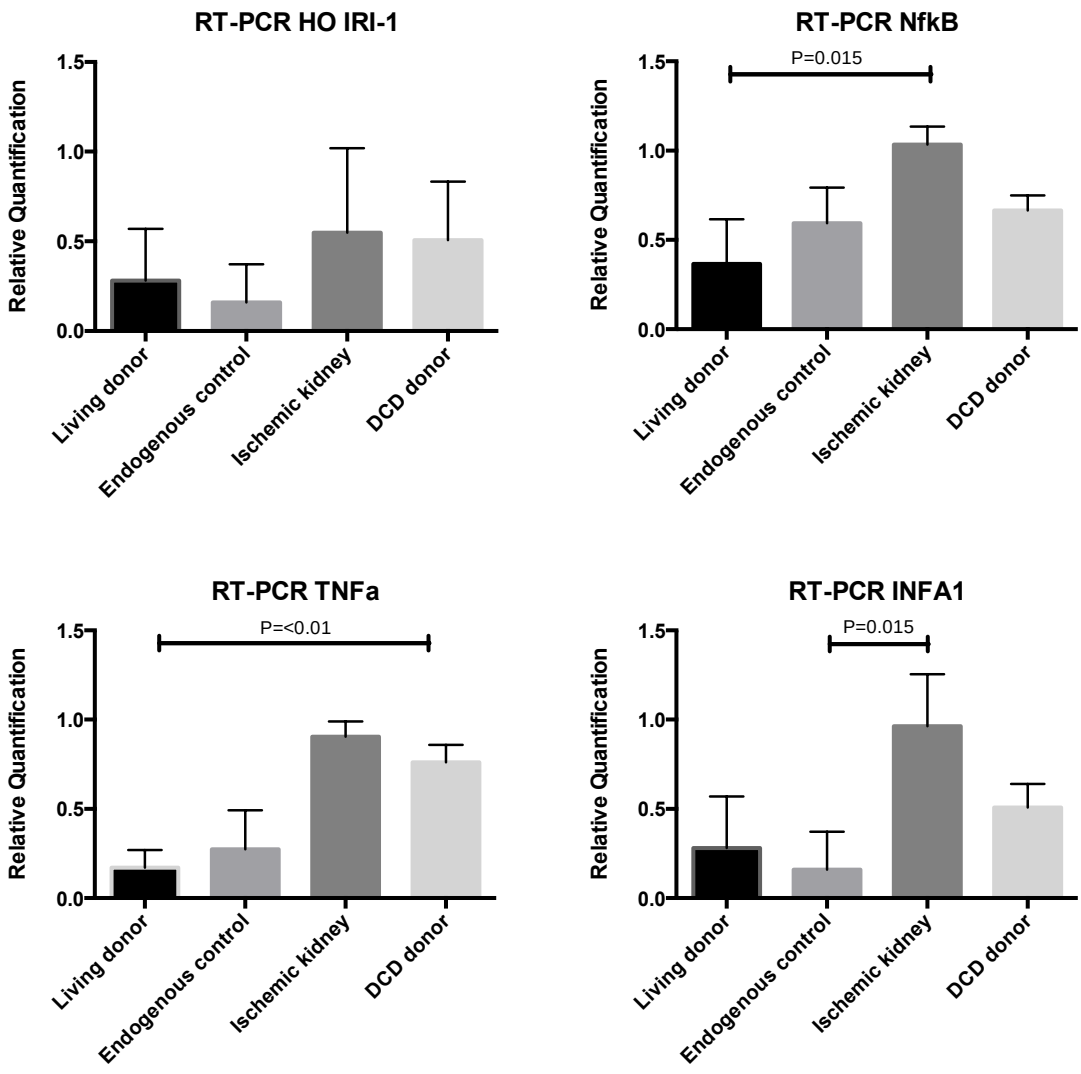


Figure 9. PCR of genes following IRI and DCD donation. Comparison of HO-1, NFkB, TNFa and INFA1 expression in ischaemia reperfusion injury and in DCD donation.

Discussion

Following learning the BD model in the University of Groningen I started to perform experiments in Oxford. As I progressed with the model a number of modifications were made to optimise the experiments and ensure physiological homeostasis. This included ensuring more adequate temperature monitoring and end-tidal CO2 measurements. The inaccuracy of the volume delivered by the pressure-controlled ventilator and the potential ensuing lung injury resulted in switching to a more accurately controlled device and a low pressure ventilatory protocol. This is increasingly being used as a lung protection strategy in intensive care unit setting (234-236). The operative time also decreased substantially and was associated with greater hemodynamic stability following BD induction.

I initially validated the model comparing different durations of BD to a sham control. No differences in basic biochemical parameters or blood oxygen tensions were illustrated in this data noted. A progressive increase in serum creatinine and urea were noted to occur following BD induction. This is comparable to previous published literature that has demonstrated progressive renal and liver dysfunction following BD induction (237). In addition a progressive, but not significant increase in glutamate dehydrogenase (GLDH) was noted an alternative marker of liver injury.

The serum creatinine increase was observed following brain death. If the urinary creatinine was corrected for time there was a dramatic decrease in the creatinine clearance rate. However as the bladder of the rodents was not catheterised we assume the urine aspirated at the end of the procedure was the total urine produced. No

change in urinary protein levels were detected in comparison to controls and on histological evaluation no areas of ischaemic necrosis or gross morphological injury was noted.

Consistent with the previous literature we observed increased serum levels of IL-6, a pro-inflammatory cytokine implicated as being one of the main mediators of BD induced organ dysfunction and at the tissue level observed an increase in a number of markers of cellular stress (87) . This was consistent with the previously published data and also data from the model use at the University of Groningen.

In addition we observed a progressive infiltration of neutrophils into the kidney, mainly focusing around the glomerulus. A number of similar observations have been seen in human brain dead organ donors with progressive recruitment of inflammatory cells, initially neutrophils and then monocytes at longer time periods after BD induction (89). The recruitment of neutrophils to the glomerulus could in part due to the rich vascular network of the glomerulus. This would be compatible with previous studies demonstrating increased expression of inflammatory adhesion molecules in the glomerulus of the kidney following BD (203).

All of the models have a number of limitations as already highlighted. For the BD model one of the major limitations is the absence of an adequate control. The sham experiments are terminated after a simulated period of BD induction, allowing a difference in time to confound the experiments. To ensure that the organ dysfunction observed was not due to a time related confounder I added an additional control

comparing the results of the BD to a long sham. Surgery was performed as previously described, a simulated BD period ensues and then 4 hours of maintenance under anaesthesia. This in itself is not a perfect control as the anaesthesia remains a confounder (238). Reassuringly there was no significant difference between the serum creatinine between the short and long sham controls.

In these sets of experiments inotropic support was not administered to any rodents. The predicted culminative mortality or exclusion from data analysis was 30%. Having discussed the model with collaborators and veterinary specialists I decided to use inotropic support in rodents who are hemodynamically unstable to reduce the rodent loss rate to under 10%. In most other protocols of BD described in Table 2 rodents are supported post BD induction with inotropes. In the clinical situation most brain dead donors would be on inotropic support (personal communication Gerlinde Mendersloot, Consultant intensivist).

The question then occurs why does BD result in organ dysfunction in this highly controlled model where mean arterial pressures and oxygenation are maintained. We can see that inflammatory cells do infiltrate and a number of pro-inflammatory markers are increased following BD. Although we have not evaluated this in the described experiments, complement activation is likely to be of importance also as demonstrated by others (117). What drives this inflammatory process and cell recruitment to an organ following BD is multi-faceted as discussed in the introduction. The systemic release of cytokines could be important in addition to the exposure of organs to ischaemia during the BD induction phase. It remains to be determined what

the effects of BD are on the cellular level of the kidney. One can hypothesise that if we evaluate the cellular response following BD we can target cellular pathways to protect cells from injury in the face of major alterations in biological homeostasis. This could potentially have an impact on the pro-inflammatory state of the organ donor.

Whilst there is a substantial body of literature that suggests there such a pro inflammatory state in the brain dead donor leads to organ dysfunction, some authors are suggesting that increased durations of BD may not necessarily be harmful (239). For example registry data analysis performed by Boffa et al. demonstrated that longer durations of BD were not associated with increased graft dysfunction or poorer 1 year outcomes (240). Indeed the authors speculate that increased durations of BD were in fact protective against cold ischaemia in kidney transplantation and reduced incidence of DGF. Is BD then really so detrimental to organ function?

The data in these studies do not take into consideration unstable donors, in whom organ donation may not proceed, or in whom organ explantation, triggered by donor instability, may happen more quickly. Significant improvements in donor management, particularly with the introduction of the donor care -bundle pathway, which was introduced in 2011, could also be in part responsible for the lack of harm caused by increased durations of BD (241).

Others have speculated that longer durations of BD may also be associated with the activation of protective molecules or pathways such as the production of heat shock proteins, which we have seen in this study also. As outlined in the introduction Van

Duellemen et al. have demonstrated progressive increase in heat shock proteins (HSPs) including HO-1 (204). Whether this stress response can overcome the incurred injury within the time period of DBD donation or indeed actually improve transplant outcomes, as suggested by some authors, seems unlikely. Certainly with this model, in which genetic factors and physiology are matched as close as possible there does seem to be progressive deterioration of organ function following BD. Addressing this pre-procurement injury will be of importance in trying to optimise organs from more vulnerable donors such as older and more obese donors.

As expected the application of the DCD model resulted in progressive inflammation in the kidney. I was unable to establish a reperfusion system to fully evaluate the effects of the DCD warm ischeamic phase, with the expectation that the inflammatory response observed will have been significantly more pronounced following reperfusion, which is perhaps why the inflammatory response observed was not as significant as in the IRI model. The model was simple to use but without a surrogate for reperfusion (either *ex-situ* reperfusion or transplantation) had limited use. Although I contemplated the establishment of a reperfusion system, I decided to focus on the DBD model for the remainder of the thesis.

Each of the DCD models has its own limitations, but essentially this was a rapid, reproducible and cost effective way of inducing cessation of circulation without any harm or discomfort to the rodents.

The results of the IRI study indicated successful macroscopic application of ischaemia to the kidney. The transcriptional gene response observed was consistent with the previous published literature demonstrating activation of a number of key pathways including upregulation of the NFkB, TNFα and INFα (62, 203, 204). The use of the endogenous control was to eliminate the need for additional rodents to be used as a sham control. The limitation of this approach was that is did not allow use to observe a significant alteration in the serum creatinine of the rodent in whom the ischaemia was applied. Secondly there were some non significant inflammatory pathway upregulation in the non- ischeamic endogenous control (TNFa and NFkB) compared to truly healthy controls.

Increasingly in the literature a benefit of remote ischaemic preconditioning (rIP) has been suggested although the exact mechanism for how rIP works has not been fully delineated. Nevertheless rIP is now being evaluated in clinical trials in cardiac, renal and liver transplantation (221, 222, 225, 238). To what degree this effect confounds the use of this model is undetermined in these experiments.

Conclusion

The aim of this Chapter was to establish and validate models of organ donation including BD, DCD and ischaemia reperfusion injury. Fundamentally, what I learnt during this was the limitations of the models. None of them are perfect or replicated the complicated situation that occurs in human donors perfectly. One of the major limitations is ensuring adequate control for experimental purposes. Nevertheless these

are useful tools to begin to understand mechanisms of injury in a well-controlled environment allowing us to isolate individual events/processes.

I became particularly interested in the BD organ donor model, using this as a basis to understand how BD results in organ dysfunction particularly at the cellular level. The reasons for this are that there remains an opportunity to intervene in the brain dead organ donor as highlighted in the rationale.

Chapter 4: Proteomic analysis of kidneys from brain dead donors and following ischaemia reperfusion injury

Introduction

In the previous Chapter, I observed that BD leads to progressive renal failure and a pro-inflammatory state consistent with previously published literature (181-183, 196). No previous study has explored in detail the global changes that occur in cells and cellular homeostasis following BD. This is of relevance to identify how potential organ dysfunction actually occurs, what potential signals are driving an inflammatory response and how these may be mitigated.

In the realms of ischaemia reperfusion injury (IRI), as opposed to BD, the effects have been better characterised (128, 242). A complex interplay of alterations in cellular homeostasis, physiology and inflammation occurs. On a global scale, application of ischaemia results in regional alterations in oxygen tensions in the kidney. Following the application of ischaemia the outer medulla and cortex, containing the proximal tubules, are the most susceptible (243-245). The S3 component of the proximal tubules and thick ascending limb (TAL) are the regions of highest oxygen consumption. Under transient hypoxic conditions alterations occur reducing flow disproportionality to high oxygen demand areas and reducing the glomerular filtration rate (GFR). This is due to a tubuloglomerular feedback mechanism as well as the production of local vasoconstrictive mediators such as antidiuretic hormone (ADH) (246). This results in less filtration and therefore reduced oxygen consumption. However, as reactive oxygen species production increases this shifts the balance towards oxygen consumption driving S3 and TAL activity, resulting in cellular injury (245).

This results in altered cellular function including endothelial barrier dysfunction, followed by the migration of inflammatory cells and expression of adhesion molecules such as ICAM, VCAM, E-selectin and B7-1 (242). We have already seen that part of the inflammatory response to this is mediated by TNFα, which is thought to be largely produced by dendritic cells coordinating the local innate response (247, 248). The role for toll-like receptors and other DAMPs in mediating this response is also important (111, 130, 249-251).

At a cellular level, in these susceptible areas, hypoxia results in altered cellular metabolism. ATP dependent processes shut down, such as Na-K ATPases, membrane depolarisation subsequently occurs and a sustained Ca^{2+} influx through voltage gated Ca^{2+} ion channels occurs resulting in phospholipase and protease activation. This results in cell swelling and eventual necrosis. Cell lysis and the extracellular release of ATP, and other DAMPs further perpetuate the inflammatory response to cell death and leads to the migration of phagocytes into exposed tissues (251).

The role of mitochondria in mediating the response to ischaemia and reperfusion injury has been previously well characterised (184). Ca^{2+} overload in addition to causing activation of phospholipase and proteases also results in increased reactive oxygen species generation (ROS). This occurs indirectly through Ca^{2+} increasing the metabolic rate of cells, causing Cytochrome c dissociation, cardiolipin peroxidation, mitochondria permeability transition pore (mPTP) opening and perpetuating a feed-forward self-amplifying loop by increasing a further surge in cellular Ca^{2+} release (111, 243, 249). The majority of studies in this area have used *in-vitro* or small animal models including

Langendorff isolated heart models. Laboratory techniques for assessment have included measuring ion flux, isolating mitochondria to assess respiratory capacity and also using imaging techniques (243).

Ischaemia is detrimental for cells and the cellular injury is accelerated upon reperfusion. For example 4-17% of cardiac mycoytes are noted to be dead after the application of 1-4 hours of ischaemia. However, following only 3 hours of reperfusion over 70% of cells were noted to be lost. The return to normoxia results in the accelerated production of ROS, which together with dysregulation of Ca^{2+} homeostasis results in further mPTP opening and cellular dysfunction and cell death through mediating autophagy, apoptosis or necrosis (249). Cells respond to hypoxia and ischaemic insults by activating protective pathways (252, 253). The hypoxia inducible factor (HIF) system is known to be the master transcriptional regulator of this response and activates a number of protective pathways (as discussed in Chapter 6).

In BD the cellular implications of alterations in haemodynamic stability and therefore oxygen and nutrient supply to the kidney and the alteration in hormone, coagulatory and complement function are simply not known. We have seen from the introduction that organs from brain dead donors are known to be more “immunogenic”, but what drives this response and is it similar to the effects observed during ischaemia reperfusion injury? (88, 89).

The aim of this Chapter was to define, in a non-biased way, the cellular processes that are disturbed in the kidney following BD using the rodent model described in Chapter

3. To do this I used a “shot-gun” based proteomic approach. Proteomics uses mass spectrometry to identify alterations in the protein profile of cells or groups of cells or other biological materials (e.g. serum/plasma or urine). It is commonly being utilised as a technique for identification of cellular pathways, pathway interactions (as part of systems biology) and biomarker discovery (254-258). In addition, I performed a series of parallel experiments in which I used samples from an IRI experiment to assess the impact of this on the proteomic profile and to allow comparisons between the mechanism of injury in BD and IRI.

Methods

Animal welfare

All studies were carried out using inbred Fischer F344 male rats (250-300g, Harlan, UK). Animals received free access to food and water in compliance with the Home Office guidance for the care and welfare of animals. Rats were acclimatised for at least 1 week prior to experimentation.

Groups

Brain dead animals (n=6) were compared to sham controlled animals (n=6, Figure 1). The sham control used was a short sham, simulating a living donor control. In the IRI experiments kidneys exposed to ischaemia (n=6) were compared to contralateral endogenous (n=6) and healthy controls (n=2, Figure1).

Brain death model and sham control

The BD model was performed as described in Chapter 3. In brief, animals were anaesthetised using isoflurane with O_2. Cannulae were inserted into the femoral artery for blood pressure monitoring and femoral vein for colloid administration. Animals were intubated via a tracheostomy and ventilated throughout the experiment (oxygen saturations 100%). Through a fronto-lateral borehole in the skull a 4-Fogarty catheter (Edwards Lifesciences, Irvine, CA) was placed subdurally and slowly inflated (16 µL/min) with saline. BD was confirmed by the absence of corneal and pupillary reflexes and a positive apnea test. This occurred 23-35 mins after starting balloon inflation. Following confirmation of BD, anaesthesia was terminated.

Mean arterial pressures (MAP) were maintained between 80-120mmHg with voluven (6% hydroxyethyl starch). Animals were maintained in the BD state for four hours before laparotomy, flushing of organs with 0.9% saline at 4^{o}C followed by organ procurement. 0.2ml maintenance fluid per hour was administered i.v.

The short sham control was performed using the same surgical set-up, but no inflation of the intracranially placed balloon catheter. After a simulated 30 min BD induction period the experiment was terminated.

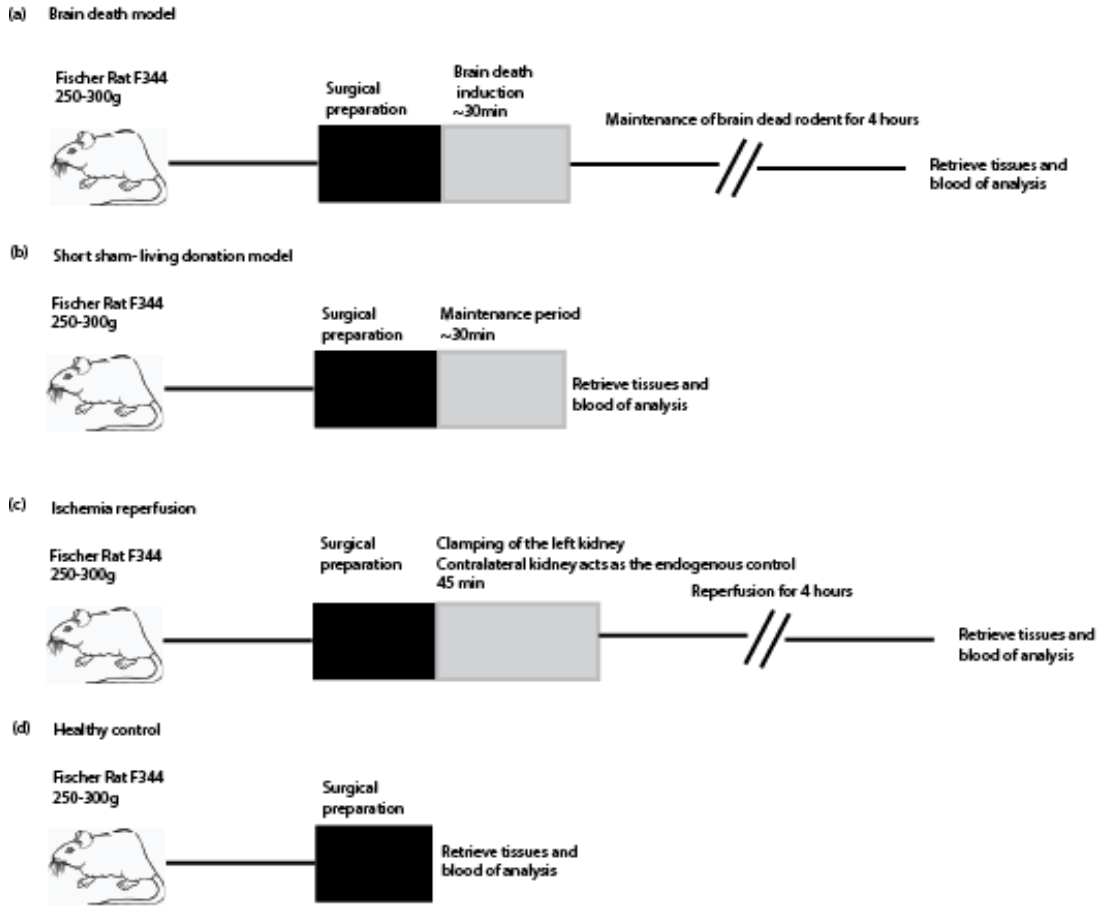


Figure 1: Surgical groups: Comparing the (a) brain death model against (b) the short sham control and the IRI experiments comparing the (c) ischaemic left kidney against the contralateral control from the same rodent and the (d) healthy control.

IRI kidney model

The IRI model was performed as previously described in Chapter 3. In brief, animals were anaesthetised using isoflurane with O_2. Temperature was regulated using an auto-regulated heat mat. A mini-laparotomy was performed and the renal pedicle dissected. Graded pressure clamp was applied to the artery (Fine Science Tools) and the kidney noted to change colour turning purple. The renal pedicle of the right kidney was also dissected but the clamp not applied. Heparin (50units/kg) was applied five min before the application of the clamp to prevent renal thrombosis. The abdominal cavity was closed during the application of ischaemia (after clip application) and the wound covered with a wet gauze. The rodent was kept anaesthetised during the procedure.

After 45 min of warm ischaemia the clamp was released and reperfusion of the kidney macroscopically noted. The abdominal wound was closed and intra-peritoneal and wound bupivacaine (0.25%) was applied. Four hours of reperfusion was allowed before termination of the experiment by laparotomy and flushing of the abdominal organs.

Organ, blood and urine procurement

Urine, blood and tissue samples were procured as described in Chapter 3. Cortical segments of the kidney were dissected under a microscope and were subsequently subjected to sample preparation for mass spectroscopy. The reason I decided to use cortical samples was to reduce the cell type variability and to allow us to look at the proximal tubules which are a site commonly implicated as being susceptible to ischaemic injury as previously described.

Tissue preparation for mass spectroscopy

Kidney cortical samples were dissected, cut into smaller segments (~20mg), washed with cold phosphate-buffered saline and homogenised using 1ml of lysis buffer (8 M urea, 50mM Tris-HCL, pH 8.0, 5mM DTT, 1%SDS, protease (Roche) and phospho protease inhibitor (Sigma, dilution 1:1000)) using an electrical homogenizer for 30 seconds at speed 5 (Ultraturrax T25 using small blade). The samples were subjected to sonication at 20% amplitude three times for 10s to allow DNA breakdown and further lysis of cell membranes. Samples were centrifuged at 2,000g for 5 min at 4^{o}C and protein concentration determined using a BCA assay (Thermo Scientific, UK).

100µg of total protein were added to a 30kDa filter (Millipore) and total tissue lysate was reduced using 20mM of DTT at 37^{o}C for one hour. The DTT was subsequently removed by centrifugation filtration at 14,000g for 20min and the samples alkylated with 100mM iodoacetamide for 45 min in the dark at room temperature. The iodoacetamide was subsequently removed by centrifugation at 14,000g for 20min. The lysate was subjected to buffer exchange using 8M urea and 50 mM NH_4HCO_3 three times. The proteins were cleaved into peptides using trypsin (20ng/ul) added in a ratio of 1:50 (trypsin:sample) and incubated overnight at 37^{o}C. The peptides were then spun down, washed with 0.5 M NaCl and desalted using Sep-Pak C18 cartridge, dried using a SpeedVac and resuspended in buffer A (2% acetonitrile, 0.1% formic acid) to 1µg/ul and run in LC-MS (Orbitrap, Thermo Scientific).

Proteomic data and statistical analysis

Data was analysed using Progenesis LC-MS (Non-linear Dynamics) allowing for automatic alignment of ion chromatograms and ion quantification. The peptides were identified by searching against Swissprot rat database use MS/MS Ion. Protein identification was based on a ≥2 peptide match to a protein in Swissprot rat database using Mascot (Matrix Science London, UK). False discovery rates were calculated using a decoy database (259).

All of the identified protein signatures were subjected to Ingenuity Pathway Analysis (IPA, Qiagen). Canonical pathway interrogation was performed and reported using the -log of the P value calculated using a Fischer's exact test right tailed.

Protein shortlisting was carried out on proteins with at least two fold up- or down-expression in the BD group compared to the short sham, using the Progenesis software system, and using an analysis of variance (ANOVA) test for statistical significance.

Results

Surgical preparation and rodent experiments

Rodent experiments were performed as described in the methods (Figure 1). The mean arterial pressure (MAP) was monitored throughout the procedure. The median time for declaration of BD was 27 min. Mean arterial pressures were kept between 80 and 120mmHg for four hours following BD induction.

The duration of surgery did not significantly vary between the experimental groups (Figure 2, P=0.77). Volumes of fluid administered varied significantly between the short sham in comparison to the long sham and the 4 hour BD group, as expected (Figure 2, P<0.01).

The amount of adrenaline (1:1000) administered to maintain MAP was higher in the BD group in comparison to controls (Figure 2, P= 0.03). A significant increase in serum creatinine was observed in the BD group in comparison to controls (Figure 2, P< 0.01).

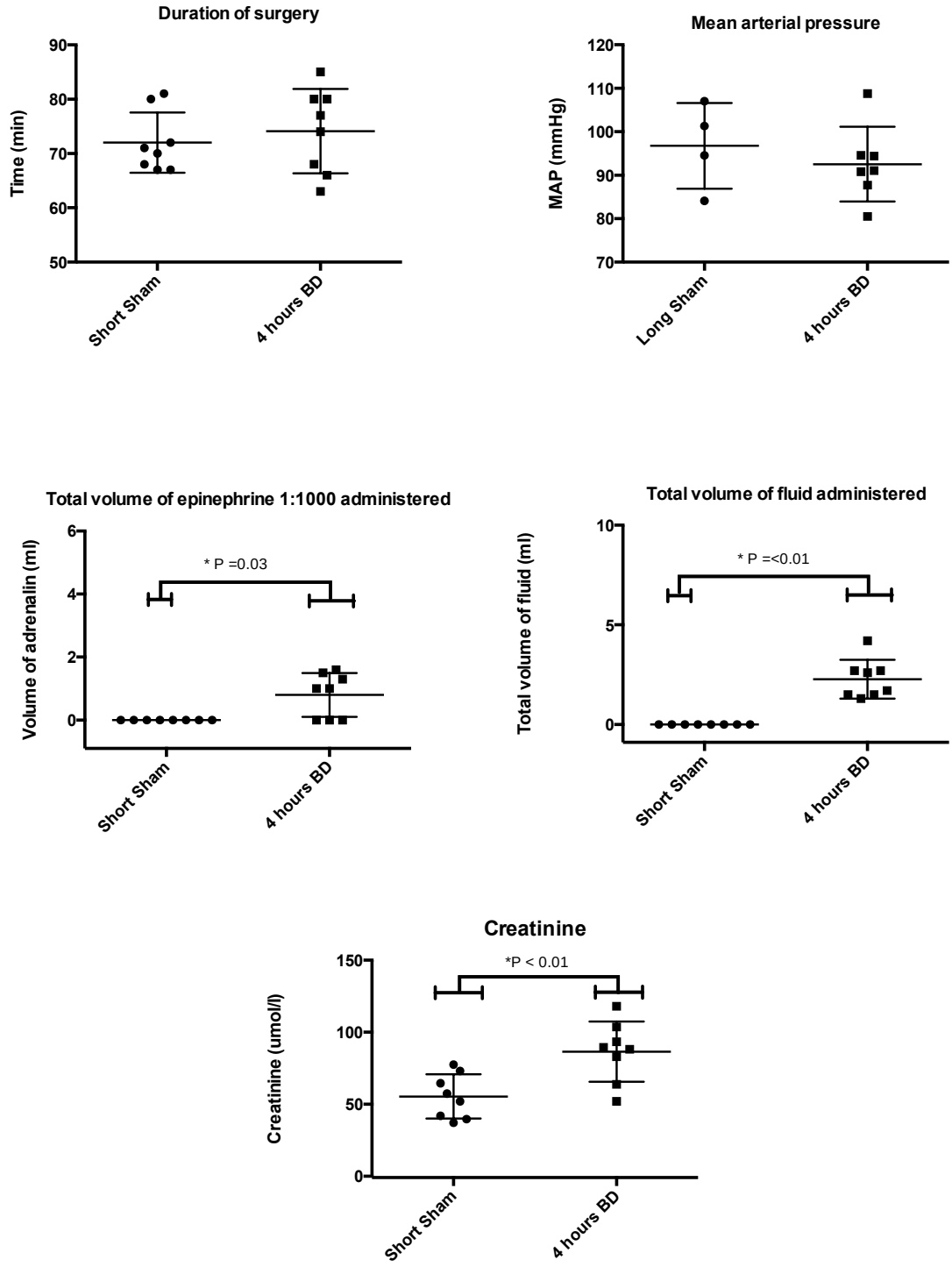


Figure 2: Physiological parameters comparing the short sham group against the 4 hour BD group. Comparing the duration of surgery, mean arterial pressures, epinephrine, total volume of fluid administered and serum creatinine.

Proteomics in brain death

I identified 1434 proteins from the renal cortical samples. The use of a randomised decoy database revealed a false discovery rate (FDR) of 1%.

Principal component analysis (PCA) allowed me to determine the ability of the protein signature to differentiate between groups. A 2D PCA demonstrated that the two populations (SS and BD) could be broadly distinguished based on their protein signatures, with 62.64% of the identified proteins forming the principal component on the x axis and 11.44% on the y axis (Figure 3).

Canonical pathway interrogation revealed mitochondrial and metabolic pathway dysfunctions as predominating in the BD group in comparison to the short sham control (Figure 4). Altered proteins relating to mitochondria are graphically depicted in Figure 5 and include proteins related to the Kreb's cycle, the electron transport chain (ETC) and ATP synthase. In addition, a large number of proteins involved in the generation and deactivation of ROS were also detected. Proximal tubular proteins were found to be significantly altered compared to proteins in other compartments within the cortex ($P<0.01$, table 1) in keeping with this being a major site of change in donor kidneys.

Proteins were further analysed by shortlisting based on having a ≥2-fold increase or decrease relative to the control short sham group and being significantly differentially expressed between the two groups ($P<0.05$), allowing a shortlist of 42 proteins listed in table 2. The majority of the deregulated proteins concerned pathways of oxidative

stress, metabolism and cell signaling. This included enolase and glucose-6-phosophate isomerase, key protein mediators of glycolysis. Decreased carnitine carrier protein and fatty acid binding protein indicative of reduced fatty acid metabolism were also found. A number of inflammatory mediators and cellular structural components were also identified. Overall the protein data suggested a metabolic switch to anaerobic fuel sources and increased oxidative stress.

Pathway interrogation revealed a number of proteins involved in overlapping pathways which were disturbed following BD, including CD163, fatty acid binding protein, heat shock proteins and glucose transporters (Figure 6). A number of central regulators such as IL-6, TNFα, NFkB and MAPK were identified as being able to link the dysregulated pathways.

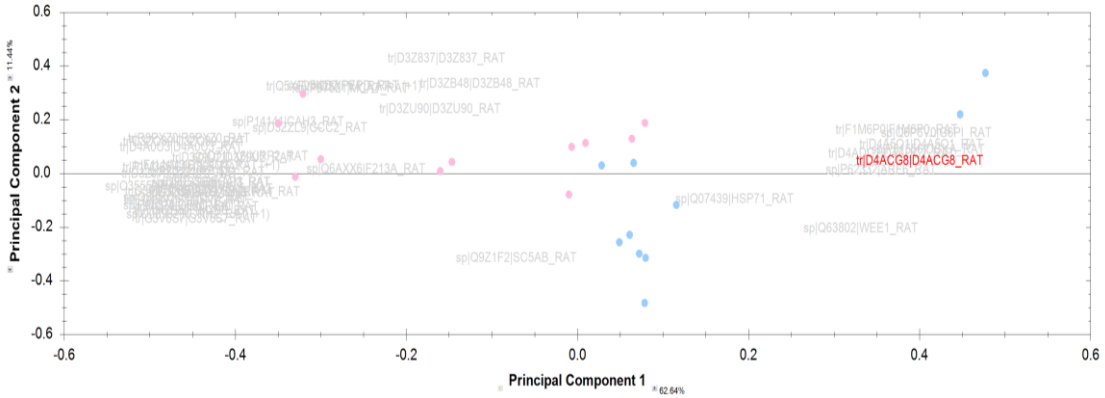


Figure 3: Principle component analysis (PCA) between the BD (red) and short sham (blue) groups. The grey identifiers represent individual protein ID's (red protein ID highlighted for illustrative purposes). Two clear groups emerge, differentiating between the short sham and BD group.

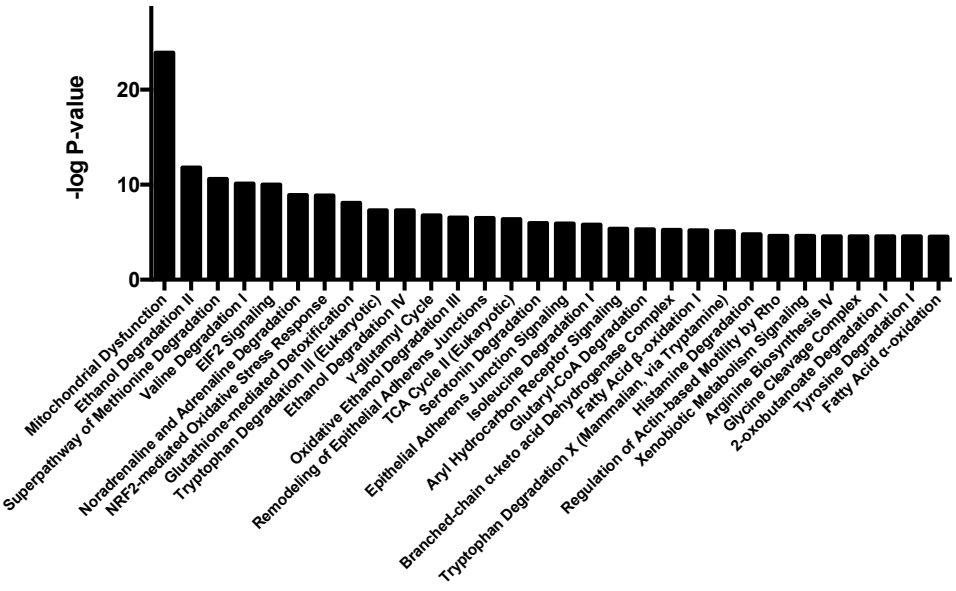


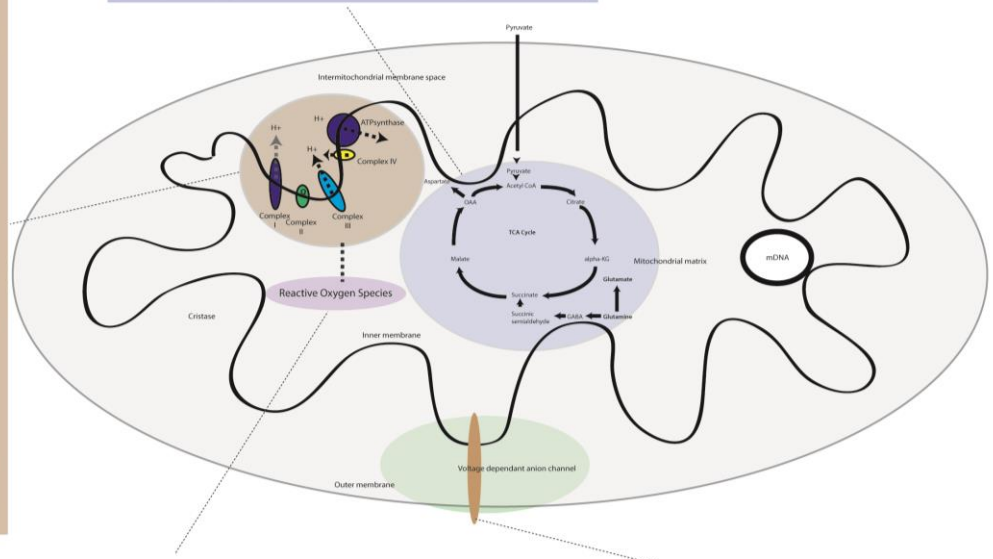
Figure 4: Pathway analysis of differentially regulated proteins following BD. Top 30 canonical pathway disturbances following 4 hours of BD in comparison to short sham controls (IPA analysis). Mitochondrial dysfunction and metabolic changes predominate the canonical pathways that are disturbed following BD.

Electron Transport Chain related proteins

Symbol	Gene name	Fold change
ATP5A1	ATP synthase	1.114
ATP5B	ATP synthase	1.133
ATP5C1	ATP synthase	1.29
ATP5D	ATP synthase	1.401
ATP5F1	ATP synthase	1.211
ATP5H	ATP synthase	1.302
ATP5J	ATP synthase	1.293
ATP5O	ATP synthase	1.22
COX4I1	Cytochrome c oxidase subunit IV isoform	1.243
COX41	Cytochrome c oxidase subunit Vb	2.029
COX6c	Cytochrome c oxidase subunit Vic	1.59
CYB5A	Cytochrome b5 type A (microsomal)	1.29
MT-CO1	Cytochrome c oxidase subunit I	1.14
MT-CO2	Cytochrome c oxidase subunit II	1.099
MT-ND1	NADH dehydrogenase, subunit 1 (complex I)	1.284
MT-ND4	NADH dehydrogenase, subunit 4 (complex I)	1.043
MT-ND5	NADH dehydrogenase, subunit 5 (complex I)	1.365
NDUFA3	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 3	1.594
NDUFA4	NADH dehydrogenase mitochodnrial complex associated	1.207
NDUFA5	NADH dehydrogenase (unquinone) 1 alpha subcomplex 5	1.284
NDUFA7	NADH dehydrogenase (unquinone) 1 alpha subcomplex 7	1.152
NDUFA9	NADH dehydrogenase (unquinone) 1 alpha subcomplex 9	1.268
NDUFA10	NADH dehydrogenase (unquinone) 1 alpha subcomplex 10	1.088
NDUFA11	NADH dehydrogenase (unquinone) 1 alpha subcomplex 11	1.18
NDUFA12	NADH dehydrogenase (unquinone) 1 alpha subcomplex 12	1.001
NDUFA13	NADH dehydrogenase (unquinone) 1 alpha subcomplex 13	1.013
NDUFAB1	NADH dehydrogenase (unquinone) 1 alpha/beta subcomplex 1	1.866
NDUFB5	NADH dehydrogenase (unquinone) 1 beta subcomplex 5	1.103
NDUFB8	NADH dehydrogenase (unquinone) 4 beta subcomplex 8	1.142
NDUFB9	NADH dehydrogenase (unquinone) 5 beta subcomplex 9	2.393
NDUFS1	NADH dehydrogenase (ubiquinone) Fe-S protein 1	1.049
NDUFS2	NADH dehydrogenase (ubiquininone) Fe-S protein 2	1.275
NDUFS8	NADH dehydrogenase (ubiquininone) Fe-S protein 8	2.023
NDUFV1	NADH dehydrogenase (ubiquininone) flavoprotein	1.005

Mitochondrial metbaolism related proteins

Symbol	Gene name	Fold change
ACO1	Aconitase 1	1.263
OGDH	Oxalogluterase (alpha ketogluterate) dehydrogenase	1.029
SDHA	Succinate dehydrogenase complex, subunit A, flavoprotein	1.167
SDHC	Succinate dehydrogenase complex, subunit C, integral membrane	1.142
XDU	Xanthine dehydrogenase	1.412



Generation of reactive oxygen species

Symbol	Gene name	Fold change
CAT	Catalase	1.407
GPX4	Glutathione peroxidase 4	1.132
GSR	Glutathione reductase	1.064
HSD17B10	Hydrosteroid (17-beta) dehydrogenase	1.291
PARDX3	Peroxiredoxin 3	1.091
PRDX5	Peroxiredoxin 5	1.091
SOD2	Superoxide dismutase 2, mitohchondrial	1.211
UQCRQ	Ubiquino-cytochrome c reductase, complex III, subunit VII	1.157
VDAC1	Voltage dependant anion channel 1	1.256
VDAC2	Voltage -dependent anion channel 2	1.2
VDAC3	Voltage -dependent anion channel 3	1.016
XDU	Voltage dependent anion channel 3	1.412

Structural proteins, ion channels and protein handling

Symbol	Gene name	Fold change
FIS1	Mitochondrial fission 1 outer	1.142
PARK7	Parkinson protein 7	1.421
VDAC1	Voltage dependant anion channel 1	1.256
VDAC2	Voltage -dependent anion channel 2	1.2
VDAC3	Voltage -dependent anion channel 3	1.016

Figure 5: Mitochondrial related proteins altered following brain death. Schematic of a mitochondrion and summary of the mitochondrial related proteins altered following brain death vs short sham,IPA (Qiagen)). Succinate dehydrogenase is also known as complex II, part of the electron transport chain.

Table 1: Proteins altered in renal compartments. A high number of significantly altered (P<0.05) proteins concerning several mechanisms were found in the proximal tubular component of the kidney compared to the glomerulus and nephron.

Disease or function association	P value	Proteins
Damage to proximal tubular toxicity	2.67E-10	ACAT1: Acetyl-CoA acyltransferase 1 CRYM: Crystallin FABP3: Fatty acid binding protein 3 GSR: Glutathione reductase GSS: Glutathione S-transferase GSTM1: Glutathione S-transferase GSTP1: Glutathione S-transferase HAGH: Hydroxyacylglutathione hydrolase HSP90AA1: Heat shock protein 90kDa alpha (cytosolic), LYZ: Lysozyme PECR: Peroxisomal trans-2-enoyl-CoA reductase RGN: Regucalcin SCL15A2: Solute carrier family 15 (oligopeptide transporter), member 2 SLC16A7: Solute carrier family 16 (monocarboxylate transporter), member 7 SLC22A6: Solute carrier family 22 (organic anion transporter), member 6 SLC25A11: Solute carrier family 25 (mitochondrial carrier; oxoglutarate carrier), member 11 SLC27A2: Solute carrier family 27 (fatty acid transporter), member 2 Slco1a1: Solute carrier organic anion transporter family, member 1a1 TXNRD1: Thioredoxin reductase 1 YBX1: Y box binding protein 1
Damage to nephron	0.02	CAT: Catalase REN: Renin
Damage to renal glomerulus	0.16	REN: Renin

Table 2: Identified proteins up or down-regulated in the brain death group in comparison to the short sham. Short-listed proteins were identified based on a ≥2 peptide sequence homology (P<0.05). Protein subcellular localizations and function were collated using the Uniprot database (www.uniprot.org) or the European Bioinformatics Gene Ontology database.

Protein description and gene name	Fold change of protein BD-Short Sham	Subcellular localization	Broad class	Role
Heat shock 70 kDa protein 1A/1B GN=Hspa1a	4.42	Cytoplasm	Stress response chaperon protein protein	Chaperon protein, prevent protein aggregation
Wee1-like protein kinase GN=Wee1	3.44	Nuclear	Cell cycle	Tyrosine kinase, belonging to the Ser/Thr family of protein kinases, coordinates transition between DNA replication and mitosis
Protein Pric285 GN=Pric285	3.11	Nuclear	Trascriptional regulation	Nuclear transcriptional activator for the peroxisome proliferator activated receptor alpha.
ADP-ribosylation factor 6 GN=Arf6	2.73	Cytoplasm, membrane	Cell structure	GTP binding protein that regulates endocytic recycling and cytoskeletal remodelling
Fucose mutarotase GN=Fuom	2.67	Cytoplasm	Metabolism	Involved in the Interconvertion between alpha- and beta-L-fucoses. GDP-L-fucose formed either by the de novo or salvage pathways is transported into the endoplasmic reticulum, where it serves as a substrate for N- and O-glycosylations by fucosyltransferases
Enolase (Fragment)	2.67	Cytoplasm	Metabolism	2-phospho-D-glycerate = phosphoenolpyruvate + H2O
Glucose-6-phosphate isomerase GN=Gpi	2.44	Cytoplasm	Metabolism	Glycolytic enzyme
Sodium/myo-inositol cotransporter 2 GN=Slc5a11	2.33	Membrane	Cell signaling	Sodium-dependent cotransport of myo-inositol (MI) with a Na^+. Also can transport D-chiro-inositol (DCI) but not L-fructose. Exhibits stereospecific cotransport of both D-glucose and D-xylose. May induce apoptosis through the TNF-alpha, PDCD1 pathway.
Diacylglycerol kinase alpha GN=Dgka	2.32	Cytoplasm, membrane	Cell signaling	Upon cell stimulation converts the second messenger diacylglycerol into phosphatidate, initiating the resynthesis of phosphatidylinositols and attenuating protein kinase C activity.
Phosphatidylinositol 3-kinase catalytic delta polypeptide (Predicted) GN=Pik3cd	2.23	Cytoplasm	Cell Signaling	Generates phosphatidylinositol 3,4,5-trisphosphat (PIP3) which activates signaling cascades involved in cell growth, survival, proliferation, motility and morphology it also mediates the immune responses.
Uncharacterized protein	-2.04	N/A	Unknown	Unknown
GRIP and coiled-coil domain-containing protein 2 GN=Gcc2	-2.07	Membrane	Protein handling	Tethers transport vesicles to the trans-Golgi network (TGN) and regulates vesicular transport between the endosomes and the Golgi.
Xaa-Pro dipeptidase GN=Pepd	-2.25	Extracellular vesicular exosome	Metabolism	Splits dipeptides with a prolyl or hydroxyprolyl residue in the C-terminal position. Plays an important role in collagen metabolism because the high level of iminoacids in collagen.
Uncharacterized protein GN=Fer1l5	-2.25	N/A	Unknown	Unknown
G-protein coupled receptor family C group 5 member C GN=Gprc5c	-2.32	Cell membrane and intracellular membranes	Cell signaling	G-protein coupled receptor
Kinesin family member 21B (Predicted) GN=Kif21b	-2.35	Associated with Kinesin/cytoskeleton	Cell structure	ATPase family member

Protein description and gene name	Fold change of protein BD-Short Sham	Subcellular localization	Broad class	Role
Hook homolog 1 (Drosophila) (Predicted) GN=Hook1	-2.52	Cytoskeleton	Protein handling	Endosome and lysosome transportation
Leucine-rich repeat transmembrane neuronal protein 4 GN=Lrrtm4	-2.90	Cell membrane	Cell structure	Maintenance of vertebral nervous system
cAMP-dependent protein kinase type II-beta regulatory subunit GN=Prkar2b	-2.96	Cytoplasm, cell membrane	Cell signaling/metabolism	Regulatory subunit of the cAMP-dependent protein kinases involved in cAMP signaling in cells. Type II regulatory chains mediate membrane association by binding to anchoring proteins, including the MAP2 kinase.
Redox-regulatory protein FAM213A GN=Fam213a	-3.04	Cytoplasm	Cell signaling	Involved in redox regulation of the cell. Acts as an antioxidant. Inhibits TNFSF11-induced NFkB1 and JUN activation and osteoclast differentiation. May affect bone resorption and help to maintain bone mass (By similarity).
Protein Mpnd GN=Mpnd	-3.11	N/A	Protein handling	Possible protease
Transferrin receptor protein 1 GN=Tfrc	-3.15	Cell membrane	Metabolism	Iron uptake via receptor mediated endocytosis
Sodium channel protein type 10 subunit alpha GN=Scn10a	-3.28	Membranes	Cell signaling	Voltage-dependent sodium ion permeability of excitable membranes
Protein Klk13 GN=Klk13	-3.51	Extracellular and secretory granules	Protein handling	Catalysis of the hydrolysis of internal, alpha-peptide bonds in a polypeptide chain by a catalytic mechanism that involves a catalytic triad consisting of a serine nucleophile that is activated by a proton relay involving an acidic residue (e.g. aspartate or glutamate) and a basic residue (usually histidine).
Mitochondrial carnitine/acylcarnitine carrier protein GN=Slc25a20	-3.76	Mitochondrial inner membrane	Metabolism	Mediates the transport of acylcarnitines of different length across the mitochondrial inner membrane from the cytosol to the mitochondrial matrix for their oxidation by the mitochondrial fatty acid-oxidation pathway.
Potassium voltage-gated channel subfamily H member 2 GN=Kcnh2	-3.78	Cell membrane	Cell signaling	Pore-forming (alpha) subunit of voltage-gated inwardly rectifying potassium channel.
Basal cell adhesion molecule GN=Bcam	-3.88	Membrane	Cell signaling	Laminin alpha-5 receptor. May mediate intracellular signaling
Glucose-6-phosphate 1-dehydrogenase GN=G6pdx	-3.93	Cytoplasm	Metabolism	Catalyzes the rate-limiting step of the oxidative pentose-phosphate pathway. The main function of this enzyme is to provide reducing power (NADPH) and pentose phosphates for fatty acid and nucleic acid synthesis.
Macrophage stimulating 1 (Hepatocyte growth factor-like) (Precursor) GN=Mst1	-4.25	Extracellular vesicular exosome	Protein handling	Catalysis of the hydrolysis of internal, alpha-peptide bonds in a polypeptide chain
Protein Ube3a GN=Ube3a	-4.27	Nucleus	Protein handling	E3 ubiquitin-protein ligase which accepts ubiquitin from an E2 ubiquitin-conjugating enzyme in the form of a thioester and transfers it to its substrate
CD163 antigen (Predicted) GN=Cd163	-4.34	Cell membrane	Metabolism	Combining with any modified low-density lipoprotein (LDL) or other polyanionic ligand and delivering the ligand into the cell via endocytosis.
CDC42 binding protein kinase gamma (DMPK-like) (Predicted) GN=Cdc42bpg	-4.44	Cytoskeleton	Cell structure	May act as a downstream effector of CDC42 in cytoskeletal reorganization.
Rab GTPase-binding effector protein 1 GN=Rabep1	-5.56	Cytoplasm, early endosome	Protein handling	Involved in endocytic membrane fusion and membrane trafficking of recycling endosomes.
Interleukin 5 receptor, alpha GN=Il5ra	-7.22	Cell membrane	Cell signaling	This is the receptor for interleukin-5. The alpha chain binds to IL5.
Carbonic anhydrase 3 GN=Ca3	-7.49	Cytoplasm	Metabolism	Reversible hydration of carbon dioxide.

Protein description and gene name	Fold change of protein BD-Short Sham	Subcellular localization	Broad class	Role
Protein Ints9 (Fragment) GN=Ints9	-8.47	Cytoplasm	Transcriptional regulation	Any process involved in the conversion of a primary small nuclear RNA (snRNA) transcript into a mature snRNA molecule.
Protein Zfp638 GN=Zfp638	-9.54	Nucleus	RNA processing	Nucleotide and zinc ion binding.
Fabp4 protein GN=Fabp4	-13.24	Cytoplasm	Metabolism	Lipid transport protein in adipocytes. Binds both long chain fatty acids and retinoic acid. Delivers long-chain fatty acids and retinoic acid to their cognate receptors in the nucleus (By similarity).
E1A binding protein p400, isoform CRA_a GN=Ep400	-22.04	Histone complex, nuclear speck, swr1 complex	Transcriptional regulation	ATP and DNA binding
Protein Ank3 GN=Ank3	-38.56	Cytoplasm, cytoskeleton ,cell junction	Cell signaling	Membrane-cytoskeleton linker. May participate in the maintenance/targeting of ion channels and cell adhesion molecules

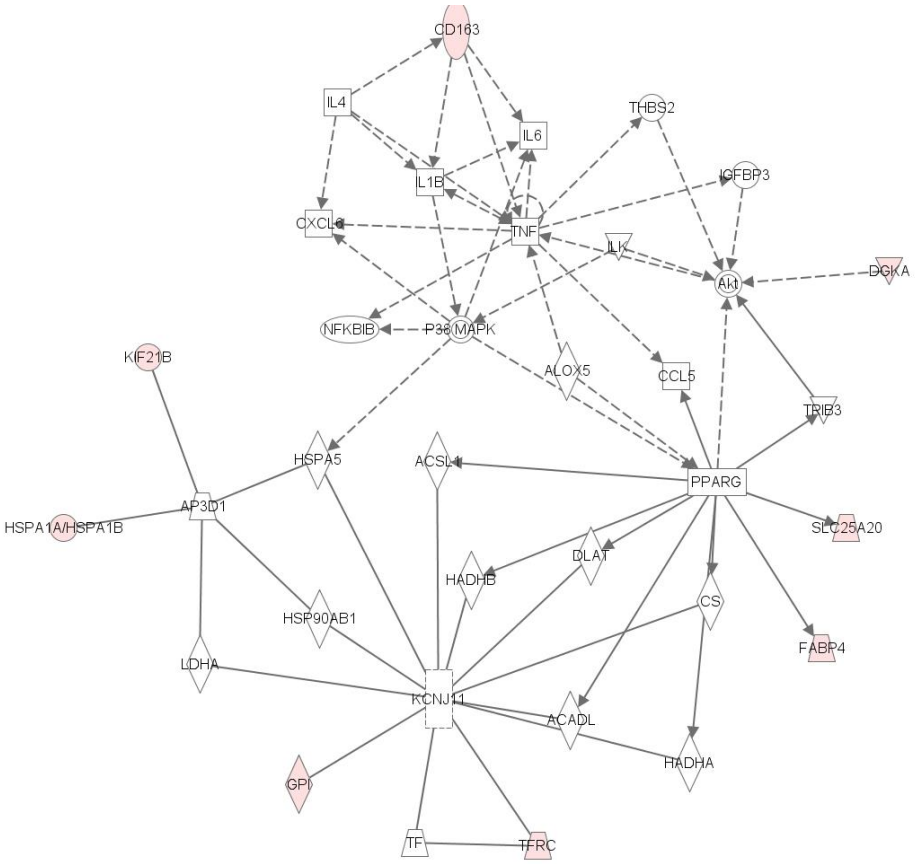


Figure 6: Pathway interactions. Red signify up-regulated in BD in comparison to controls and potentially linking regulators and direction of cellular interaction. A number of cellular mediators related to inflammation and the cellular response have been identified including TNF, MAPK, Akt and IL6 amongst others. The individual proteins identified were predominantly metabolic related proteins these interactions demonstrating the overlap between metabolic and inflammatory pathways.

Proteomics in ischaemia reperfusion injury

Similar to the BD model, the PCA demonstrated that two distinct populations of proteins were discernible when comparing the IRI ischaemic kidney to the endogenous control with 57.78% of the proteins forming the principal component on the X axis and 8.15% on the Y axis. The 65 significantly dysregulated proteins identified between the IRI ischaemic kidney and control groups (Appendix 1). Top canonical pathways that were dysregulated included mitochondrial dysfunction, ethanol degradation, superpathway of methionine degradation, valine degradation and EIF2 signaling (Table 3).

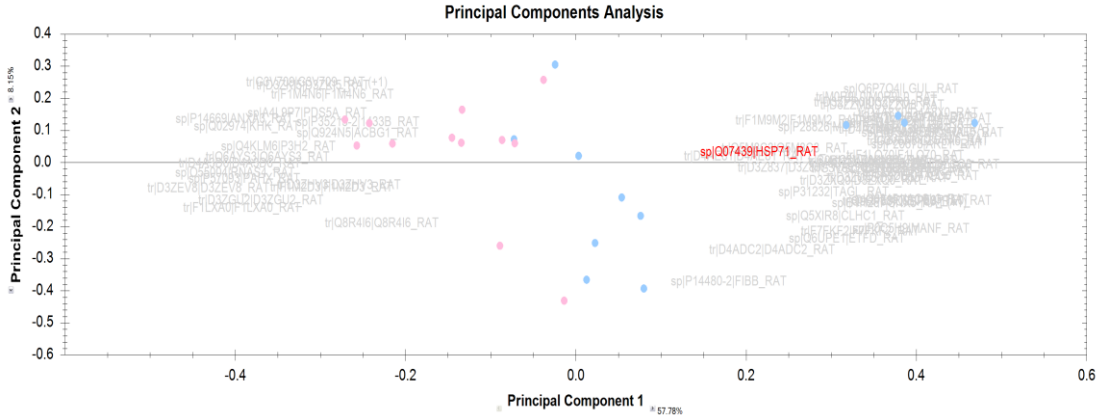


Figure 7: Principal Component Analysis (PCA) of ischaemic kidneys against endogenous controls. Pink: ischaemic kidney, Blue: endogenous control

Table 3: Top altered canonical pathways in the ischaemic injured kidney in comparison to endogenous controls. Mitochondrial dysfunction, metabolic pathway and inflammatory pathway signaling predominated.

Canonical pathway	P value	Proteins
Mitochondrial dysfunction	1.73E-24	45/144 (0.312)
Ethanol degradation II	1.6 E-12	15/30 (0.5)
Superpathway of methionine degradation	2.98 E-11	14/30 (0.467)
Valine degradation I	8.75 E-11	11/18 (0.611)
EIF2 signaling	1.09 E-10	31/168 (0.185)

Discussion

The results of the comparison between the BD and sham control animals demonstrated, consistent with Chapter 3, progressive kidney dysfunction as measured by serum creatinine levels.

The number of total proteins identified from the BD and IRI experiments were comparable. Although an increased number of significantly differentially expressed proteins i.e. proteins which were significantly different between groups, were identified in the IRI experiments.

The PCA demonstrated two distinct populations of proteins that could be separated using the majority of identified proteins, when comparing the BD against control groups. Suggesting that the protein signature is altered and unique in the BD animal. A similar result was observed in the IRI group when we compared the kidney exposed to ischaemia to the contralateral endogenous control.

Canonical pathway interrogation revealed that mitochondrial dysfunction occurs in the cortex of the kidney following BD. Interrogation of the alterations in the mitochondrial proteins revealed this predominantly related to alterations in the electron transport chain (ETC) activity, but also ROS generation, ion channel homeostasis and the intrinsic relationship between mitochondria and altered metabolism (Figure 5). The similarities between these generic canonical pathway alterations and those observed in IRI was consistent e.g. alterations in mitochondrial function (table 3).

Alterations in methionine and valine metabolism were noted in the canonical pathway interrogation, both are essential branch chain amino acids that can act as an alternative source of energy or used for biosynthetic purposes (260). Similarly, degradation of other amino acids and alteration of fatty acid oxidation pathways following BD were suggested by the results, in addition to alterations in the TCA cycle and ethanol degradation.

When exploring the compartments of the kidney where the protein alteration occurred following BD, the proximal tubules out-weighed the other cellular subsets of the cortex e.g. glomerulus, consistent with previous knowledge based on IRI. The central regulators already mentioned in the introduction and Chapter 3, were identified based on pathway prediction and included IL6, TNFα, ion channels amongst others.

I therefore demonstrated that following BD we have alterations in the protein signature of the cortex of the kidney, with the suggestion that we observe altered mitochondrial function and metabolism as central etiological factors. It appears altered ETC activity and mitochondrial related metabolism may be of particular importance, with BD driving a catabolic state. I can also infer that there are similarities in the injury process observed in the renal cortex of brain dead rats when compared to the cortex of kidneys exposed to 45min of warm ischaemia and 4 hours of reperfusion.

Why do we observe alterations in mitochondrial function and metabolism following BD? This will likely to be due to a multifactorial insult on the kidney. The period of hypertension followed by hypotension and then normalisation of the MAP essentially

exposes the kidney to a period of ischaemia followed by reperfusion, which may in itself be sufficient to cause the injury. Prastchke et al. suggest that the catecholamine storm and relative hypotension occurring during BD can cause an IRI type injury to kidneys even before organ procurement. The authors in the same article speculated this was in part the reason why living donor kidneys, regardless of the HLA miss-match have better outcomes compared to DBD donors (261). These experiments support this argument by also illustrating that the proximal tubular proteins are specifically altered in BD.

My own impression is that the situation is likely to be more complex in the clinical setting with alterations in hormone status (including the catecholamine storm and possibly exogenous adrenaline) for example, adding to the cellular stress observed. The complex interaction of metabolic, structural, enzyme and inflammatory pathways observed in the canonical pathway proteomic data reflects this.

Another explanation for how or why there seemed to be such a similarity between the BD and IRI data relates to cellular composition and the bioinformatics analysis performed. Mitochondria are one of the most populous organelles in kidney cells, which are made from a wide variety of different protein types (262). Therefore it is possible that the weight of involvement of the mitochondria is biased by the number of mitochondria and also the shear number of different proteins found within a mitochondrion. Secondly the bioinformatics software tool predicts pathway involvement based on the known literature, drawing on predominantly cancer based research including cell culture based models. The applicability of drawing these

comparisons with experiments in the organ donation field may undermine the results and require further validation to prove the association of BD with mitochondrial dysfunction and alterations in metabolism.

One of the other underlying difficulties with this research is that I have only looked at the four hour BD animal and the four hour post IRI. It could be that following longer, or shorter periods after BD other cellular features predominate e.g. alterations in other cell signaling pathways. In addition, the approach weights contributions of pathways or organelles based on protein number, some low expressed proteins (e.g. enzymes and transcription factors) have significant cellular functions, and small differences in their expression may have significant effects on cellular homeostasis.

Based on the proteomic data we can also predict that alterations in metabolism may shift away from oxidative phosphorylation in the presence of mitochondrial injury or dysregulation and potential hypoxia. Under cell stress conditions a catabolic state may occur, attempting to support cellular metabolism. Understanding the underlying metabolic disturbances in the kidney following BD will be an important component to confirm the hypotheses generated as a result of this Chapter. The intrinsic relationship between alterations in metabolism with mitochondrial function/dysfunction need to be investigated further.

Conclusion

I have demonstrated that following BD, we observe a number of alterations in the protein signature of the renal cortex are observed. This suggests alterations in mitochondrial function and altered metabolism. I have shown a number of similarities of the alterations of the protein signature to that observed following subjecting a kidney to ischaemia reperfusion injury and suggest that the alterations may be due to underlying ischaemic injury during the BD induction phase. I speculate that other factors are likely to be involved in causing this injury but recognise the difficulty in definitively proving this. I hypothesize that owing to mitochondrial dysfunction the kidney may become less dependent on oxidative phosphorylation as the main route to maintain cellular energy.

Chapter 5: Investigating mitochondrial dysfunction and metabolic disturbance in the brain dead organ donor

Introduction

In the previous Chapter I observed that alterations in the protein signature of kidneys from brain dead rodents suggests that major alterations in mitochondrial function and metabolism occur. I recognised that determining how kidneys are injured may be difficult due to the multifactorial disturbance in homeostatic mechanisms which occur following BD, although the injury may be similar to that encountered following ischaemia reperfusion injury (IRI). In this Chapter, I further explore how metabolism is altered in the kidney of brain dead rodents and link this to the protein alterations identified in Chapter 4. I attempt to validate alterations in the mitochondria by investigating the impact of BD on mitochondrial structure and function within the kidney.

It is well known that in the face of cellular stress, e.g. from hypoxia or ischaemia, one of the first changes to occur is in the metabolic status (131, 263). ATP has a very short half-life and ischaemia can result in rapid ATP depletion. The protein data from Chapter 4 suggested that in the brain dead kidney, mitochondrial complex dysfunction occurs and an increased dependency on anaerobic metabolism.

Mitochondria are endosymbiotic organelles which are responsible not only for energy production and metabolism but also for mediating cell signaling cascades, apoptosis and have a role in mediating a cell's response to hypoxia (264-275). They are unique in that as organelles they have their own DNA, comprising of over 16,000 base pairs encoding 36 different genes (276). They are typically responsible for producing over 90% of the cell's ATP and house the enzymes required for the TCA cycle, metabolising

incoming pyruvate produced as a consequence of glycolysis, producing reduced nicotinamide adenine dinucleotide (NADH), or flavin adenosine dinucleotide (FADH) and the hydrogen ions required for establishing the proton gradient formed as part of the electron transport chain (ETC). Complexes (I-IV) generate the proton gradient that is then used to produce ATP through ATP synthase (complex V).

Under normal metabolic conditions, 0.4-4% of consumed oxygen is converted into superoxide radicals by leakage from the ETC (277). Under normal conditions mitochondria have the required capacity to handle these free radicals by using thioredoxin reductase/thioredoxin/peroxiredoxin-3,5 system, glutathione peroxidase (GPx) and glutathione (GSH). The production of superoxide free radicals will result in mild uncoupling and result in a negative feedback loop, which aims to decrease free radical production. Furthermore mitochondria permeability transition pores (mPTP) open to increase the electrochemical gradient within the cells and accelerate the consumption of oxygen with the aim of preventing further ROS production. When this system is overwhelmed however this can result in rapid mitochondrial injury (128). This includes damage to mitochondrial DNA, mitochondrial structural enzymes and if radicals are released from the mitochondria other cellular components can become injured activating cell death pathways including apoptosis (242, 272, 278, 279).

As organelles mitochondria are constantly undergoing fusion and fission, a process required to maintain mitochondrial integrity (280). Excessive fission or reduced fusion will be detrimental to mitochondrial function and result in the release of Cytochrome C, opening of MPTPs, release of pro-apoptotic mediators such as BNIP3 and eventual cell

destruction. The relationship, triggers and abilities of cells to clear damaged mitochondria (mitophagy) and how this alters the balance of autophagy, apoptosis and cell necrosis remains an area for investigation (281-284).

The data from Chapter 4 suggested increased amounts of ROS occurred in the kidneys of brain dead rats, based on the protein alteration, possible modified complex activity and concurrent alterations in the protein make-up of enzymes forming the TCA cycle (amongst other metabolic protein alterations). Thus suggesting that mitochondrial function is altered following BD.

We observed that within the cortex the proximal tubules, the majority of altered proteins occurred. As an organ the kidney is mitochondrially rich, the proximal tubules have a particularly high density of mitochondria and under basal conditions are heavily dependent on aerobic metabolism. This is in contrast to the distal tubules where anaerobic metabolism occurs more readily, partly because of mitochondria in the proximal tubules existing in a more highly oxygen dependent state and the inability of the proximal tubules to synthesise glutathione, which has to be extrinsically taken up. Thus the renal proximal tubular endothelial cells are particularly vulnerable to hypoxic injury as evident in the realms of renal IRI and the development of renal fibrosis which often is most prominent in the proximal tubules (133) .

During periods of hypoxia or ischaemia, mitochondrial fragmentation is noted to occur as an early event (285). This results in reduced rates of transcription and translation of oxidative phosphorylation related genes and results in a down regulation of

peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1 α), a regulator of mtDNA replication. Nitric oxide pathway signaling is also altered, resulting in a reduced microcirculation at times of severe oxidative stress, further perpetuating the damaging effects of hypoxia.

The purpose of the work described in this Chapter is to identify how the metabolic profiles of the kidney changes with BD in comparison to controls. I explored whether the metabolomic changes were similar to those observed in a renal model of IRI. I also aimed to support the hypothesis that mitochondrial dysfunction occurs following BD by characterising the effects of BD on the ability of mitochondria to consume oxygen and relate this to mitochondrial complex activity and structural alterations characterised using electron microscopy. To establish whether the altered mitochondrial activity is related to ROS production I characterised the effects of BD on surrogate markers of ROS related injury.

Methods

Animal welfare

All studies were carried out using inbred Fischer F344 male rats (250-300g, Harlan, UK). Animals received free access to food and water in compliance with the Home Office guidance for the care and welfare of animals. Rats were acclimatised for at least one week prior to experimentation.

Groups and experimental procedure

Brain dead animals (n=6) were compared to short sham controlled animals (n=6). In the IRI experiments kidneys exposed to ischaemia (6) were compared to endogenous (n=6) and healthy controls (n=2). BD and ischemia reperfusion injury were performed using the protocols described in Chapter 3 and 4. To further validate the findings in the BD animals and to add security to the data interpretation I introduced a long sham control, whereby following a simulated BD induction, rodents are maintained in an anaesthetised state for four hours (n=4).

Preparation of tissues for NMR-spectroscopy

NMR spectroscopy was performed on cortical samples to identify alterations to the metabolome. Figure 1 summarises the steps for metabolite extraction (286). 30-60mg of cortical samples were placed in 1.5ml of water and ethanol (1:1) and frozen on dry ice prior to loading into a bead beater and subjected to homogenisation (4 cycles 6500 Hz; 40 sec, separated by cooling on ice). Samples were then centrifuged 13,000rpm for 20 minutes at 4^{o}C. The supernatant, which was composed of aqueous metabolites, was

removed and the protein precipitated by the addition of MeOH and water (5:1). Samples were vortexed using a multimixer for 3 min and kept at -20^{o}C for 12 hours. Samples were then centrifuged at 13,000rpm for 20 min at 4^{o}C, SpeedVac dried overnight and then subjected to 1H- NMR analysis.

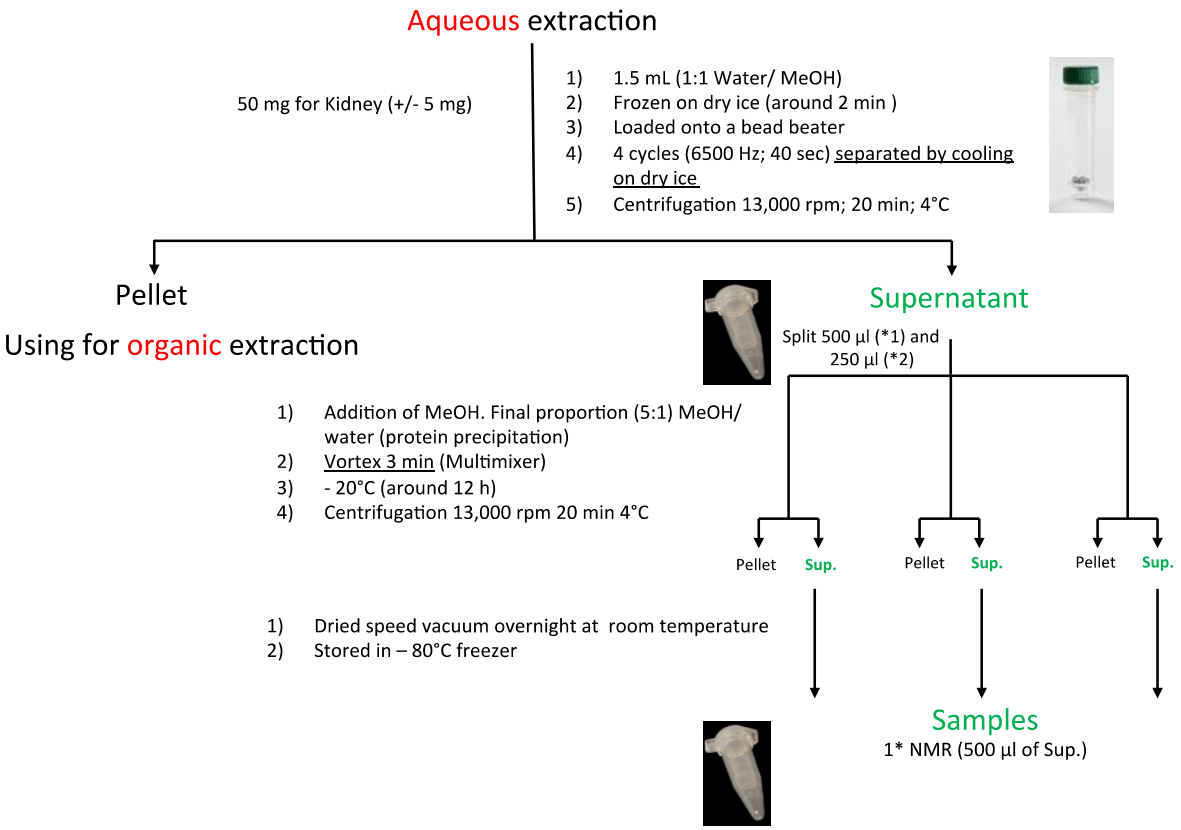


Figure 1: Summary of inorganic metaboome sample preparation for 1H-NMR spectroscopy for kidney samples.

Metabolomic data and statistical analysis

NMR spectral peaks were compared to a library of known metabolites (286). The normalised intensity of the spectra between the two groups was compared and overlaid onto known metabolic pathways. An orthogonal projection of latent structure (OPLS) analysis was performed to graphically demonstrate separation of the groups based on metabolic profile and variability (287-289). Comparison between groups of specific metabolites was performed using a Mann-Whitney non-parametric test (Prism 6, Graphpad software, $P<0.05$).

Mitochondrial isolation

Mitochondrial isolation was performed using a previously published protocol (290). In brief, a hemi section of a kidney was obtained and stored in an ice cold isolation buffer (IBc, 10ml of 0.1M Tris-MOPS,1ml of EGTA/Tris to 20ml of 1M sucrose, final volume adjusted to 100ml with distilled water. Adjust pH to 7.4 with Tris buffer). 4-5 washes were performed to ensure that the kidney was completely devoid of blood. The kidney was minced into small segments using scissors and homogenised using a Teflon pestle (1,600 rpm) with 5-6 strokes. The homogenate was centrifuged at 600g for 10min at 4^{o}C. The supernatant was further centrifuged for 10 min at 4^{o}C at 7,000g and the pellet washed with 5ml of cold IBc. The supernatant was then centrifuged (7,000g for 10 min at 4^{o}C). The supernatant was discarded and he pellet re-suspended in a small volume of IBc. Protein quantification was performed using a bicinchoninic acid assay (BCA) assay (Thermos Scientific) and concentration adjusted to 1mg/ml.

Clarke Electrode O_2 consumption activity

O_2 consumption assays were performed as previously described (290). In brief, the electrode was calibrated and 1ml of experimental buffer (EBc) was added following equilibration of the buffer in a water bath (30^{o}C). Mitochondria were added to the electrode to obtain a final concentration of 1mg/ml and state 1 respiration recorded. Succinate (5mM) was added to obtain the state 2 respiration and ADP (100uM) was added to determine state 3. Following establishment of state 4, state 5 was induced by adding 60nM of FCCP.

The respiratory control ratio (RCR) was calculated by dividing the state 3 by state 4 respiration.

Mitochondrial electron transport chain complexes activity assays

Mitochondrial isolates were prepared from dissected hemi-section of the left posterior portion of the kidney samples as previously described (291). Mitochondrial complex activity assays were performed by normalising values to citrate synthase activity using aliquots from the frozen samples. Assays were performed on a microplate reader (BMG Labtech) using previously published protocols (292). In brief:

1. Citrate synthase (CS): CS forms citrate and CoA from the reaction between oxaloacetate and acetyl-CoA. CS activity was measured through the reaction of freeCoA with 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) using a spectrophotometric assay (BMG Labtech Fluostar Omega). DTNB consumption was calculated at 412 nm using the Beer-Lambert Law (ε DTNB at 412 nm=

14,150 M^{-1} cm^{-1}). 10µl of samples were plated (in triplicate) into a 96 well plate. 250µl of reaction mixture (100mM Tris buffer, pH 8.0, 0.1% (v/v) triton X-100, 0.1mM acetyl CoA, 0.2mM DTNB) and 5µl of the substrate oxaloacetate (20mM) were injected into each well. Absorbance measurements were taken at 412nm for 3 min. Rates of DTNB consumption were calculated as initial linear rates, corrected against blank controls and normalised against protein concentration.

2. Complex I (NADH:ubiquinone oxidoreductase): Complex I activity is calculated through the detection of NADH consumption (decrease in absorbance at 340nm), following reduction of Coenzyme Q1. 30µl of samples were plated (in triplicate) into a 96 well plate and 230µl of reaction mixture added (25mM potassium phosphate buffer, pH7.2, 10mM $MgCl_2$, 1mM KCN, 2.4mg/ml fat free BSA, 0.2mM NADH). Activities were corrected against samples incubated with 2µl of the complex I inhibitor rotenone (25µM final concentration). 10µL of CoQ1 (50µM final concentration) were also added to the wells to start the reaction and absorbance measurements were taken at 340nm for 3 min. Rates of NADH consumption were calculated as initial linear rates, corrected against blank controls and normalised against CS activity.

3. Complex II/III (succinate dehydrogenase/coenzyme Q:cytochrome c – oxidoreductase): Complex II/III activity was detected through the reduction of cytochrome c (increase in absorbance at 550nm). 30µl of samples were plated (in triplicate) into a 96 well plate and 230µl of reaction mixture were injected (100mM potassium phosphate buffer, pH 7.4, 100µM cytochrome c, 0.3mM EDTA, 1mM KCN). Activities were corrected against samples incubated with 5µl of the complex III inhibitor Antimycin A (10µg/ml final concentration). 20µl of

succinate (1mM final concentration) were injected to start the reaction and used as substrate for the study. The rates of reduction of cytochrome c were recorded for 3 min, corrected against blank controls and normalised against CS activity.

4. Complex IV (cytochrome c oxidase): Complex IV activity is recorded by measuring the oxidation of reduced cytochrome c (decrease in absorbance at 550nm). Reduced cytochrome c was prepared by adding ascorbic acid crystals to oxidized cytochrome c solutions and purified using a desalting column and water as eluent. 30µl of sample were plated (in triplicate) into a 96 well plate and 220µl of reaction mixture were injected (10mM potassium phosphate buffer pH 7.0, 25 µM reduced cytochrome c). Absorbance measurements were taken at 550nm for 3 min. After 3 min, 10µl ferricyanide were added in each well (1mM final concentration) to block complex activity and subsequent measurements were taken for 98 sec. Complex IV activity was expressed as rate constant for the reaction (K/s/mg protein), corrected against blank controls and normalised against CS activity.

Transmission electron microscopy

Kidney segments were prepared for transmission electron microscopy (TEM). One micrometre sections were stained with Azure A and examined by light microscopy to identify areas of interest. Thin sections (80 nm) of suitable areas were cut and stained with uranyl acetate and lead citrate prior to examination under electron microscopy (Jeol 1200EX, Japan). Mitochondrial morphology was assessed qualitatively.

RNA isolation and semi quantitative RT-PCR

Total RNA was isolated from snap frozen tissue (10-30mg) using RNeasy© MiniKit (Qiagen) isolation kit. The GeneAmp © PCR Sytem9700 thermal cycler was used for the cDNA reverse transcription. An Applied Biosystem qPCR machine was used to run the experiment. The primer sequences were:

HMOX-1: Forward: 5'- CCTGGTTCAAGATACTACCTC, Reverse: 5'-

ACATGAGACAGAGTTCACAG

NFkB: Forward: 5'-AAAAACGACCTAGAGATTG, reverse: 5'-ACATCCTTCCTTGTCTTC

β Actin: Forward: 5'-CAATATGTGGAGCAACTGTG, Reverse: 5'-

AGTAGGTGAAGATGAAGAAGAG

Relative quantitation of gene expression was performed using β actin as the control.

Aconitase activity

Mitochondrial aconitase activity was characterised using a spectrophotometric assay as previously described (293). Activity levels were normalised for mitochondrial protein content.

ATP assay

An ATP luciferase based assay was performed based on the metabolomics findings to evaluate the effects of BD on ATP levels. This was performed subsequent to the initial

series of BD animals, using fresh tissues to avoid potential degradation effects. The pilot data is shown (n=3/group).

The assay was performed as previously described using the Entliten kit (Promega (294)). In brief tissues were homogenised using a Teflon pestle (1600 rpm) with 6-8 strokes in 0.5% trichloroacetic acid (TCA). The TCA is neutralised following extraction by the addition of Tris-acetate to adjust the pH to 7.75. The assay was performed by adding 0.1ml of the mixture to each well and read on a microplate reader (BMG biotech). Samples were assayed in triplicate, corrected against blank samples and compared to an established standard curve. Levels are expressed in nmol/g.

Serum biochemistry parameters

Blood gas analysis was performed using the iSTAT blood analyser system (G4+) and serum biochemistry analysis performed on plasma samples sent to MRC Harwell.

Statistical analysis

For statistical evaluation of the data (excluding metabolomics bioinformatics) a Kruskal-Wallis (non-parametric) comparison of multiple groups was performed (Graph-pad Prism 2014). For comparison of two groups a non-parametric T-test was performed (Mann-Whitney). Significance was set at a value of P=0.05 and all graphs report results as mean± standard deviation.

Results

Metabolomics

To assess further the significance of the proteomic results the metabolome of kidneys from brain dead rats were evaluated using 1H-NMR spectroscopy. Figure 2 a) depicts the loading plot of the predictive component of the orthagonal partial least squares (OPLS) model constructed from the 1H-NMR spectra observed from kidney extracts. The peaks correspond to metabolites that are subsequently assigned using a previously established library of in house references and online resources. Overall 29 metabolites were identified, including amino acids, substrates and intermediates of glycolysis and the Kreb's cycle (table 1). The abundance of 12 metabolites was significantly different between the BD and short sham controls leading to a highly predictive model of discrimination between BD and short sham controls. Figure 2 b) shows an OPLS plot that allows differentiation between the BD and short sham groups based on the metabolic profile.

In agreement with the proteomic data indicating increased anaerobic glycolysis, the metabolite with the largest scale increase was lactate in the BD group compared with the short sham control. Succinate, an intermediary of the Kreb's cycle and recently implicated in inducing ROS generation following ischaemia/reperfusion was also increased in the BD group, further reinforcing the presence of dysfunctional mitochondrial metabolism and the potential for oxidative damage.

Figure 3 illustrates a 3D comparison of the metabolomic changes in BD kidney compared to ischaemia reperfusion injury samples and healthy controls. In 3D, based on the metabolomics data we could distinguish BD, from the shams and the IRI from the endogenous controls. Reassuringly the healthy control samples overlapped with the endogenous controls. This data demonstrates the unique signature of metabolites from BD and IRI.

ATP data

To evaluate the effects of BD on ATP levels in the kidney (Figure 4), ATP was isolated from kidneys stored in liquid nitrogen. The results indicated a trend towards a lower amount of ATP in the BD animals in comparison to the short sham controls, but this did not reach statistical significance with the small group size used.

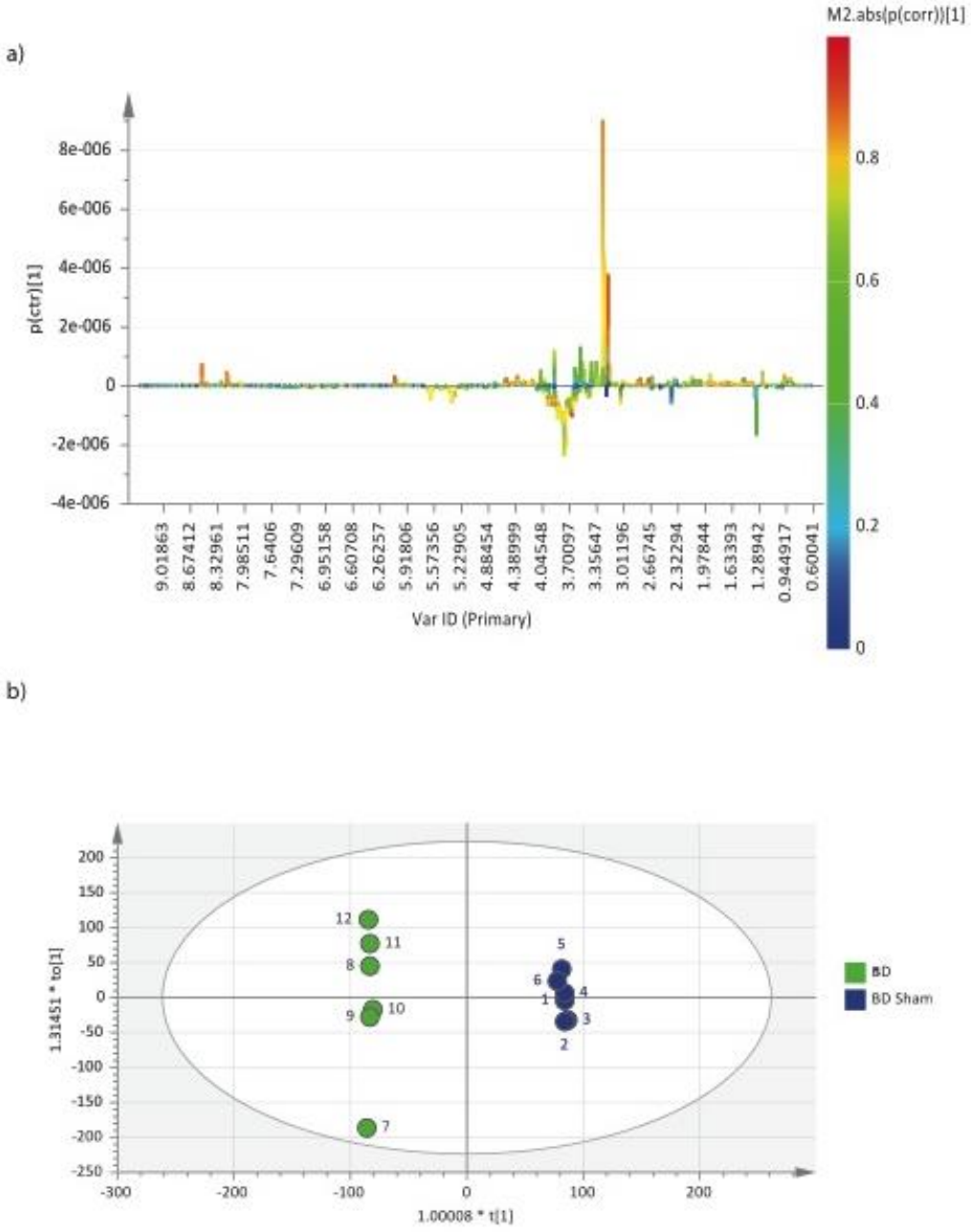


Figure 2: Metabolic analysis of BD kidneys compared to short sham controls. a) 1H-NMR spectra of an aqueous extraction from kidney biopsies, x axis illustrates the chemical shift (ppm) and the y axis the normalised intensity of the metabolites relative to the short sham mean. Peaks correspond to metabolites that are assigned using a library of known metabolites. Peaks above zero were more abundant in the BD animals and those below zero were less abundant. b) Principal component analysis (PCA) of metabolites presented in 2D, blue represent short sham controls and green BD kidney samples. We were able to group the two experimental arms. More variation existed in the BD cohort.

Metabolite	Fold change BD-BD Sham	P value	Pathway/function of metabolite
Lactate*	15.90	*0.04	Produced from pyruvate, generating NAD+
Glutamate	9.45	0.17	Amino acid, aids in disposal of waste nitrogen, can be produced or consumed by the TCA cycle
α-glucose	5.39	0.13	Stereo-isomer of glucose responsible for feeding glycolysis
Taurine	3.44	0.67	Organic acid, forming membrane constituents, acting as antioxidants, involved in osmoregulation and calcium signaling
Betaine*	3.36	*0.04	Organic ion responsible for protection against osmotic stress
Succinate*	2.64	*0.04	Intermediary of the TCA cycle and responsible for donating electrons to the electron transport chain
β-glucose	2.57	0.23	Stereo-isomer of glucose responsible for feeding glycolysis
Citrate	1.80	0.67	Intermediary of the TCA cycle, derives fatty acids
Hippurate*	1.61	*0.02	Carboxylic acid participating in the correction of metabolic acidosis [Ref Dzurik Kidney Int Suppl. 2001 Feb;78:S278-81
Creatinine*	1.56	*0.01	Synthesized in the liver and excreted renaly, serum creatinine is used as an indirect measure of kidney function
Glycine	1.53	0.57	Precursor for protein synthesis
Valine*	1.11	*<0.01	Derivative of pyruvate
Creatine*	1.09	*0.03	Produced in the kidney and liver, part of the phosphocreatine system. Product of the metabolism of creatine allowing the production of ATP.
Alanine	1.05	0.13	Non polar amino acid, formed from pyruvate by the action of alanine amino transferase
3-Hydroxybutyrate*	0.60	*0.02	Precursor of polyesters and used as an energy source during periods of energy depletion
Isoleucine*	0.58	*<0.01	Can be transaminated with α-ketoglutarate and fed into the TCA cycle or be used for gluconeogenesis. Also can enter the TCA cycle via acetyl-CoA
Leucine*	0.46	*0.04	Essential amino acid forming ferritin and other 'buffer' proteins
Acetate	0.17	0.30	Formation of acetyl CoA
Glutamine	0.15	>0.99	Used as an energy source, protein synthesis and regulating acid base status
NAD+	0.08	>0.99	Mediator of redox reactions and co-enzyme to many intracellular enzymes
3-Hydroxyisovalerate	0.06	0.57	A hydroxy monocarboxylic acid anion that is the conjugate base of 3-hydroxyisovaleric acid
Lysine	-0.03	0.79	Gives rise to acetyl-CoA via transamination with α-ketoglutarate
Formate	-0.03	0.80	Energy metabolite used in aerobic and anerobic metabolism as an electron donor. Produced from ethanol is potentially toxic to mitochondria.
UDP - glucose	-0.12	0.79	Precursor for glycogen
Methanol	-0.34	0.57	Intracellular by-product of carbon dioxide
AMP*	-2.51	*<0.01	Building block for ATP synthesis, during catabolism can be converted to urate
myo-Inositol	-2.98	0.68	Carbohydrate, involved as a second messenger
Aspartate*	-3.46	0.02	Metabolite of the urea cycle involved in gluconeogenesis
Trimethylamine N-oxide*	-4.46	*0.02	Protein stabiliser, terminal electron acceptor for anoxic growth

Table 1: Identified metabolites up- or down-regulated in the BD (BD) group in comparison to the short sham control (BD-sham). * Metabolites which were significantly different between BD and BD sham. Descriptions of functions derived from European Bio-informatic Institute database.

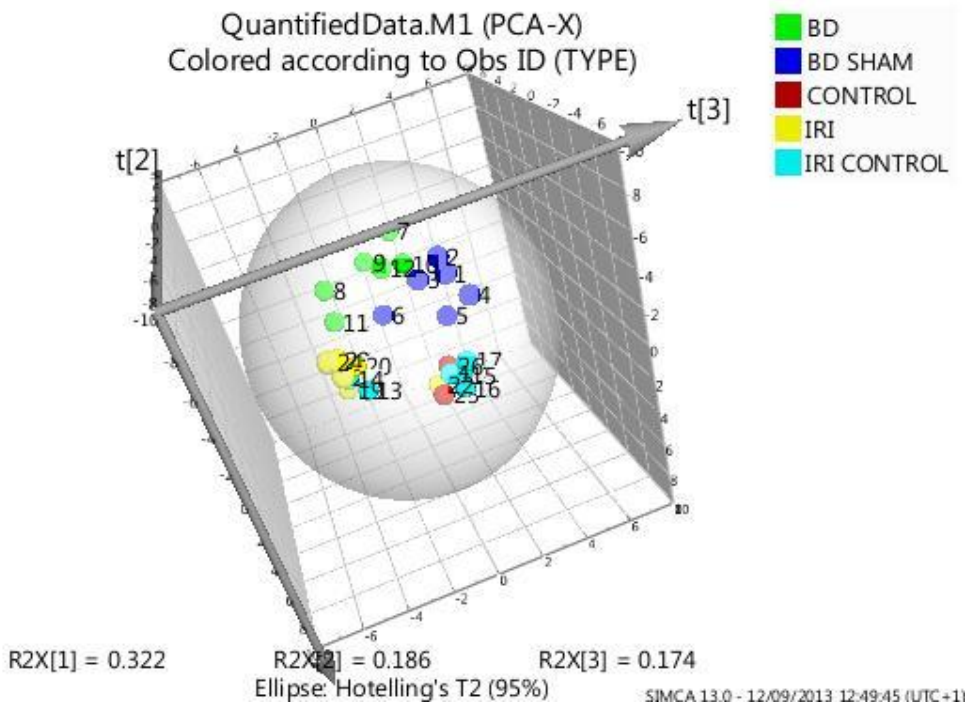


Figure 3: Metabolic analysis of BD kidneys compared to kidneys from ischaemia reperfusion injury experiments and healthy controls. Unique well clustered profiles were detected for BD kidneys, short shams, IRI and endogenous controls. The healthy kidneys overlapped with those of the IRI controls.

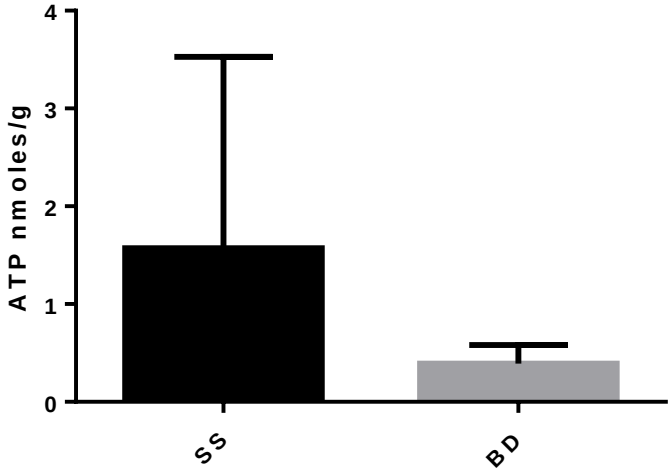
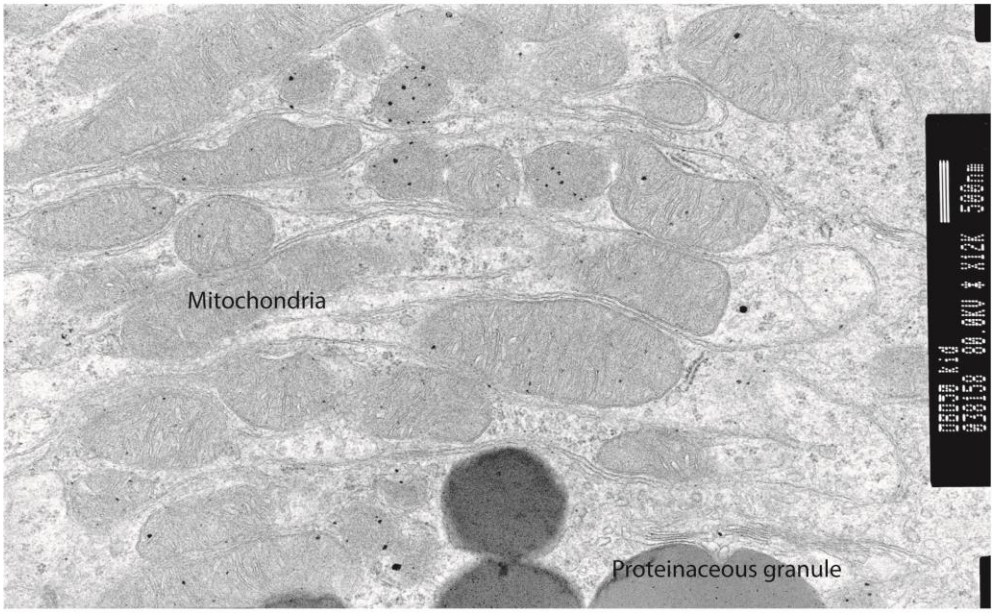


Figure 4: ATP levels comparing short sham against BD kidneys. A trend towards lower levels of ATP were present in the BD animals, this did not reach statistical significance.

Transmission electron microscopy (TEM)

TEM was performed on cortical samples after BD and compared to short sham controls (Figure 5). The gross morphology of the mitochondria in the proximal tubular compartments of the cortex demonstrated greater mitochondrial swelling in the BD group. Increased detachment of cristae was noted in the BD group, in comparison to the short sham controls.

(a) Short sham- living donor



(b) Post 4 hours of brain death

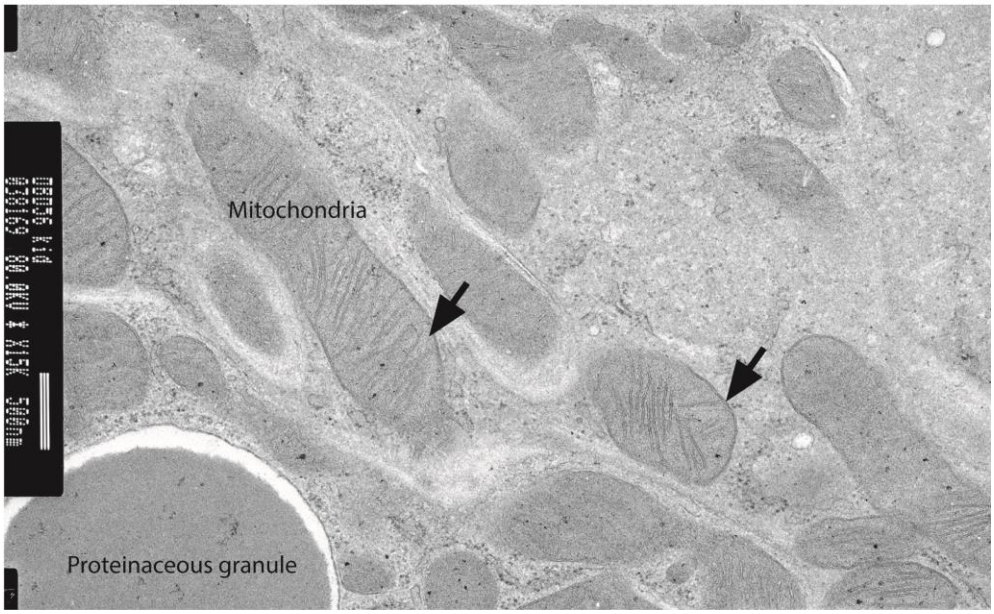


Figure 5: Structural evaluation of the proximal tubular mitochondria. TEM of proximal tubular cell mitochondria (a) X12K short -sham (b) X15K 4 hours post BD. Arrow demonstrates detachment of the cristae.

Oxygen consumption assays

Figure 6 illustrates the states of oxygen consumption comparing the BD samples to controls. In kidneys from BD animals all of the states of mitochondrial respiration were lower compared to the control. When evaluating the respiratory control ratio (RCR) a significantly lower level was noted in the BD group (P=0.01). This was suggestive of an increased proton leak and also a less efficient coupling of oxidative phosphorylation to ATP production.

Mitochondrial electron transport chain complexes activity assays

To validate the proteomic and metabolomic data, specifically with regards to alterations in ETC complexes in the BD samples, and to determine the influence on overall mitochondrial function I performed complex activity analysis. This was to further determine the effects observed on the RCR, protein and metabolites on function of the individual complexes of the mitochondria. The purpose of the additional long sham control was to determine the effect of duration of the experiment on the mitochondrial activity and possible mitochondrial dysfunction. Figure 7 illustrates no significant difference between groups on citrate synthase activity, a mitochondrial matrix enzyme against which complex activity is normalised (P=0.85).

Complex I analysis suggested a trend towards increased activity in the 4h BD group, but this did not reach statistical significance (P=0.61). No significant difference was observed in the complex II/III activity (P=0.57). Complex IV activity was significantly higher in the BD group compared to the short sham (P=0.02), however no difference was detected against the long sham control.

Markers of oxidative stress

Proteomic and morphological data indicated increased oxidative damage to the mitochondria. To assess the effect of altered mitochondrial activity and metabolism on markers of oxidative stress, RT-PCR was performed for haem-oxygenase 1 (HO-1) and NFkB, as surrogate markers of oxidative stress. I also evaluated mitochondrial aconitase, a Kreb's cycle enzyme whose activity is down-regulated under oxidative stress conditions.

Figure 8 demonstrates a trend towards increased HO-1 mRNA expression in the 4 hour BD group in comparison to controls, however this did not reach statistical significance. Significantly higher amounts of NFkB expression were noted in the BD group (P=0.01).

		BD (n=5)	Control (n=2)
Mitochondrial state respiration	State 1	0.10473167	1.192152134
	State 2	0.35999689	1.253392857
	State 3	0.62348922	3.361666667
	State 4	0.35644834	1.43984127
	RCR	1.77451247	2.333461359

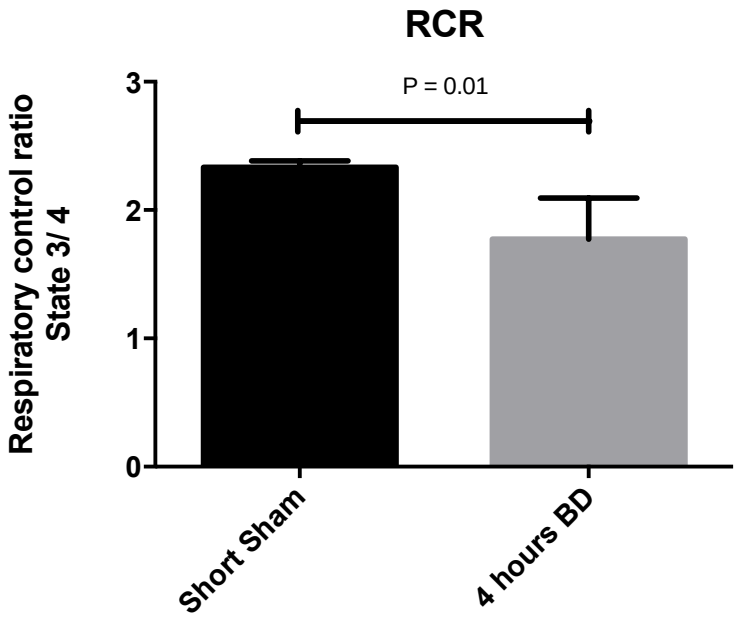


Figure 6: The respiratory state respirations of mitochondria isolated from BD kidneys and short sham controls. The respiratory control ratio was lower in BD animals in comparison to controls. (P=0.01)

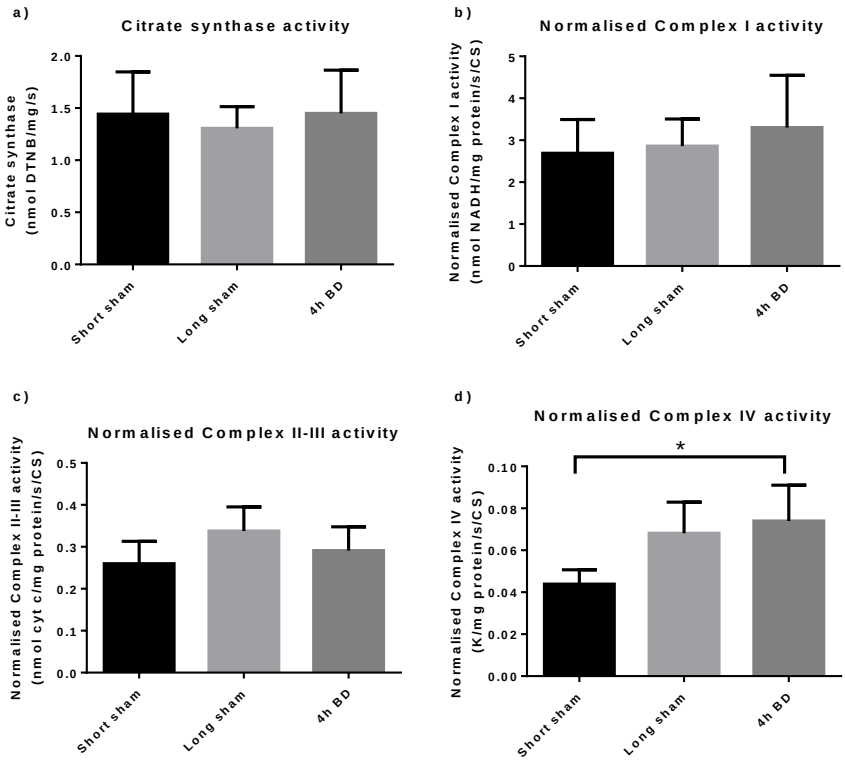


Figure 7: Mitochondrial complex analysis. Citrate synthase activity was measured and did not appear to be significantly different between groups. Normalised complex I activity showed a trend towards increased activity in the BD group but this did not reach significance. Complex II/III analysis demonstrated no significant difference between groups. Complex IV was significantly different between the 4 h BD group in comparison to the short sham (P=0.02). No statistical difference was detected when comparing the long sham.

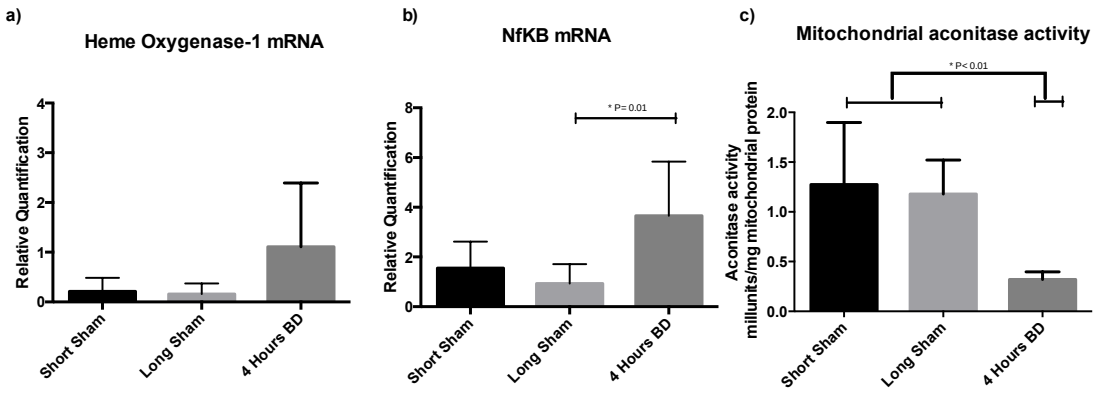


Figure 8: Haem-oxygenase 1, NFkB mRNA expression and mitochondrial aconitase activity. Comparison of HO-1 and NFkB mRNA expression between short, long shams and 4 h after BD. No statistical difference was observed in the expression levels of HO-1 between groups, although there was a trend towards increased HO-1 in the 4 hours BD group. NFkB expression was higher in the BD group compared to the long sham control (P=0.01). Mitochondrial aconitase activity was lower in the BD group compared to both short and long sham (P<0.01).

Discussion

To further validate the proteomic findings, I performed 1H-NMR metabolomic profiling using cortical kidney samples from the brain dead rats. As with the proteomic study, the metabolomic profile of the BD rats demonstrated a distinct signature in comparison to the control group. I identified a number of metabolites that were significantly different between the two groups. These included increased lactate and creatinine, whilst there was a reduction in aspartate and AMP levels. There was also a trend towards an increase in the amount of glucose in the BD group, along with up-regulation of a number of TCA cycle intermediates, including succinate. Combined with the proteomics data we can infer that the kidneys from the BD rats were metabolising mostly anaerobically, in a catabolic state and with an increased dependence on glycolysis (Figure 9) characteristic of adaptation to decreased oxygen availability.

The increased levels of succinate are of particular interest. In a recent letter to *Nature*, Chouchani et al. demonstrated the accumulation of succinate in ischaemic tissues occurs as a consequence of reversal of activity of succinate dehydrogenase, which drives mitochondrial ROS production (295). Inhibition of succinate accumulation was able to ameliorate ischaemic injury when assessed in murine models of myocardial infarction and stroke. Our results demonstrating higher amounts of succinate and induction of succinate dehydrogenase were consistent with these findings, suggesting hypoxic exposure during induction of BD may be of importance. Furthermore, in the context of papillary renal cancer fumarate hydratase (FH) deficiency leads to high succinate levels, succination of Keap1, stabilisation of Nrf2, and induction of stress-response genes including HMOX1(296, 297). Thus, increased succinate may provide a

mechanism to account for the increased oxidative damage identified morphologically and biochemically in the kidneys after BD.

ATP was not identified in the 1H-NMR profiling of the kidneys and was further investigated in a pilot study comparing a short sham against BD kidneys. The kidneys were snap frozen and stored in liquid nitrogen until processing to avoid ATP degradation. The results, although not significant, demonstrated a trend towards reduced ATP level in the BD kidneys. One may consider that if this result is true, it may be at odds with the AMP data, considering the AMP – ADP – ATP axis (298). However if mitochondria and cells are sufficiently damaged AMP/ADP/ATP leak may occur which may result in a drop of both ATP and AMP levels. This test result requires further validation and indeed the investigation of the effects of BD on the ATP/ADP ratio, a more sensitive marker of cellular energetics could be performed (298).

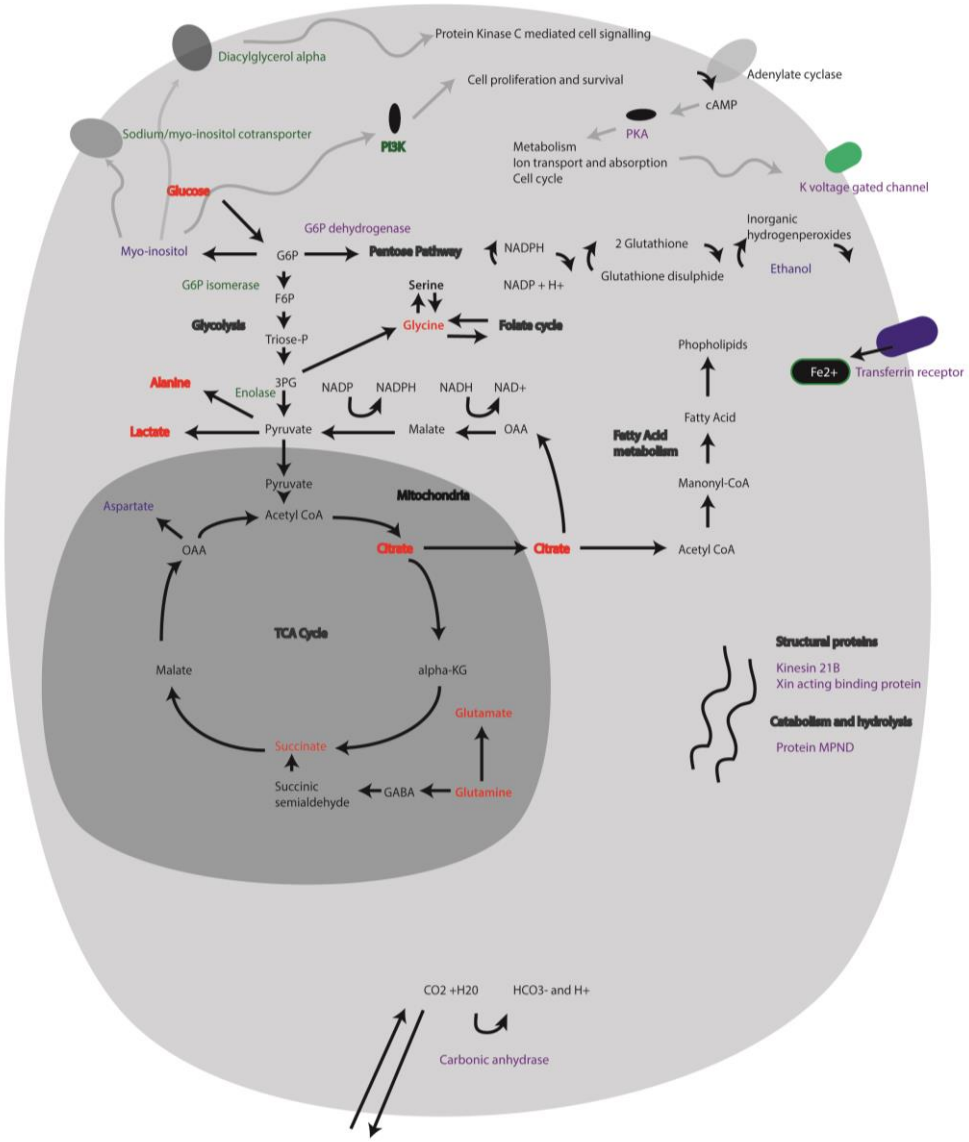


Figure 9: Metabolic and protein disturbances following BD in the renal cortical cells of rat kidneys. Metabolites: Red signifies increased abundance, blue decreased abundance relative to the short sham BD model (* $p<0.05$). Proteins: Green signifies increased protein expression and purple decreased protein expression, all of which were significantly different between BD and controls. Alterations suggest profound metabolic dysregulation in the kidney as a consequence of BD.

In broad terms the metabolic changes observed were actually unique to the injury observed in the IRI model. This is probably due to the fact they are indeed very different models. What was surprising was that we could distinguish the BD shams from the IRI endogenous controls and healthy controls. One may expect these groups to be similar, with little or no metabolic changes. Our results may reflect the nature of the surgery itself in the sham and the length of the surgical procedures, which differs between the groups.

When examining the ultrastructure of the kidneys the site of the most consistent damage was the proximal tubular component of the kidney, consistent with the proteomic data. Mitochondria here demonstrated increased cristae detachment and also features of mitochondrial swelling. No gross differences were detected in the number of apoptosomes between the groups. Although detailed evaluation of the effects of BD on apoptosis will require further investigation.

Thus, evidence exists that metabolism is altered in the kidneys of brain dead rodents and we have histological evidence of altered mitochondrial morphology. The ability of the mitochondria to consume oxygen in all 4 states of mitochondrial respiration was noted to be reduced in BD compared to controls. A significant difference was noted in the RCR, a measure of how well oxidative phosphorylation is coupled to ATP production. Together with the proteomic, histological and metabolomic data, this would concur with the hypothesis that functional alterations in mitochondria occur. This results in a reliance of the cell to produce energy through anaerobic metabolism.

Increased leakiness of the mitochondria may also explain the lower RCR, and also be consistent with evidence of mitochondrial injury and fit with the ROS data.

One of the general points noted was that the RCRs in both groups were low, most research groups will report RCRs between 2-5. This is likely to have been in part related to the extraction technique. Although the extraction technique was the same between the groups, to optimise this data I would have ideally spent more time on optimising the extraction protocol. On reflection, part of the reason why it was low, was because I adapted a protocol for mitochondrial extraction from the liver (which is much better characterised). Homogenisation of the tough kidney tissue is more challenging and it could be that the mitochondrial enrichment was not sufficient to achieve higher basal RCRs. Nevertheless as the extraction protocol was the same between the groups we can infer the difference is due to BD.

When measuring the RCRs the mitochondria, which have been kept at 4^{o}C, are now warmed up to 30^{o}C for the purpose of the assay. Substrates, which could be the rate-limiting step in an ischaemic tissue, are provided exogenously. Therefore, clearly the effect of BD on mitochondria persists after isolation and without the limited substrates. The effect on the mitochondria could therefore be related to altered structure, modifications (e.g. carbonylation) effecting the efficiency of the ETC or otherwise (299). What these potential alterations could be were not examined in detail and may warrant further investigation.

In keeping with activation of the NRF-2 pathway, from the proteomic data, we observed increased levels of surrogate markers of oxidative stress, including heat shock protein 70 at the protein level, NFkB and haem-oxygenase 1 at the mRNA level. This was also consistent with the identification of mitochondrial specific ROS-related proteins and a reduction in mitochondrial aconitase activity. Thus it appears that mitochondrial dysfunction and metabolic stress occurs and this leads to ROS generation which then leads to further injury.

Our mitochondrial complex analysis revealed no changes in the activity of complexes I-III but increased activity of mitochondrial complex IV in the BD group in comparison to the short sham controls. The increased activity of complex IV could be a response to support energy production in the kidneys from BD rodents. The increase in activity of complex IV could occur as a response to relative hypoxia and a subunit switch (COX4-1 to COX4-2), which allows the complex to work more efficiently at lower oxygen tensions, a process mediated by hypoxia inducible factors (300). The lack of effect on complex III, which is thought to mediate the majority of ROS production, could in part be due to the assay evaluating complex II and III together. Dissection of the involvement of these individual complexes may have revealed alterations in complex III activity. The lack of effect on complexes I-III requires further detailed evaluation.

Conclusion

I have demonstrated that metabolic disturbances and alterations in mitochondria occur in the kidney following BD. I hypothesise that targeting these pathways during the BD

period, may help to protect kidneys and allow them to withstand preservation and reperfusion injury, particularly when they come from older donors. Future work needs to be performed to assess the effects of modulation of hypoxia inducible factors in BD and the role of metabolically supporting and profiling donor kidneys for transplantation.

Chapter 6: Hypoxia inducible factors as therapeutic targets in organ donation and transplantation

Introduction

In Chapter 5, we observed that altered metabolism and mitochondrial function occurs during brain death. Can anything be done to preserve the metabolic and mitochondrial status of the organ during or following the diagnosis of brain death? One attractive strategy would be to create a pseudo-hypoxic environment within cells, activating the cells own cellular response mechanisms that could then be used to protect against the ensuing cellular injury.

It is recognised that all mammalian cells have the intrinsic ability to sense oxygen and the capacity to activate protective genes in the face of hypoxia (301). This oxygen sensing capacity was discovered by the demonstration that a hypoxia responsive element (the 3' enhancer of the gene for erythropoietin (EPO)) could regulate transcription of a reporter gene in all cell types tested (302). The proteins that bind to this enhancer sequence were called hypoxia inducible factor (HIF) (303). HIF is now considered to be the *"master regulator"* of the cellular response to hypoxia and regulates several hundred target genes affecting metabolism, the cell cycle and inflammation amongst others. The HIF pathway has been of great research interest as a target for cancer therapies, respiratory diseases and treating anemia.

Better understanding of the role of the HIF pathway in the context of organ donation and transplantation could be of importance in improving the quality of organs from DBD donors, through instigating rescuing therapies in the donor or alternatively during machine preservation.

Overview of the HIF pathway

HIF is a heterodimeric complex that is composed of one of three hypoxia regulated α subunits (HIF1 α, 2 α and 3 α) bound to an oxygen independent β subunit. The rate of HIF1α translation is regulated by an AKT-mToR-dependent pathway. Under normoxic conditions, the α subunits are targeted for degradation by prolyl hydroxylase enzymes (PHD) which hydroxylate specific prolyl residues resulting in HIF degradation by a von Hippel-Lindau, polyubiquitination, proteasome mediated pathway (304) . The transcriptional activity of HIF1α and 2α is also regulated under normoxic conditions by Factor Inhibiting HIF (FIH), which regulates activity of the C-terminus transactivation domain (CAD) by asparaginyl hydroxylation (305). During hypoxia PHD and FIH are inhibited allowing the accumulation of α subunits which heterodimerize with the β subunit and recruit co-activators to hypoxia responsive elements (Figure 1 (306)). This post-translational regulation by PHDs allows a prompt response to hypoxia, HIF1α signaling is also rapidly switched off once normoxia returns. The half-life of HIF1α in normoxia has been shown to be only 5-8min (307).

The different HIF heterodimers have differing expression patterns and non-redundant functions. PHDs are oxygen dependent enzymes that use Fe^{2+} as a co-factor and 2-oxoglutarate (2-OG) as a co-substrate. In humans three isoforms of PHDs (PHD1-3) have been characterised. The intracellular localisation and relative abundance of the PHD isoforms also varies from organ to organ as well as within the cellular architecture of an organ, whereas FIH expression appears to be more homogeneous(308).

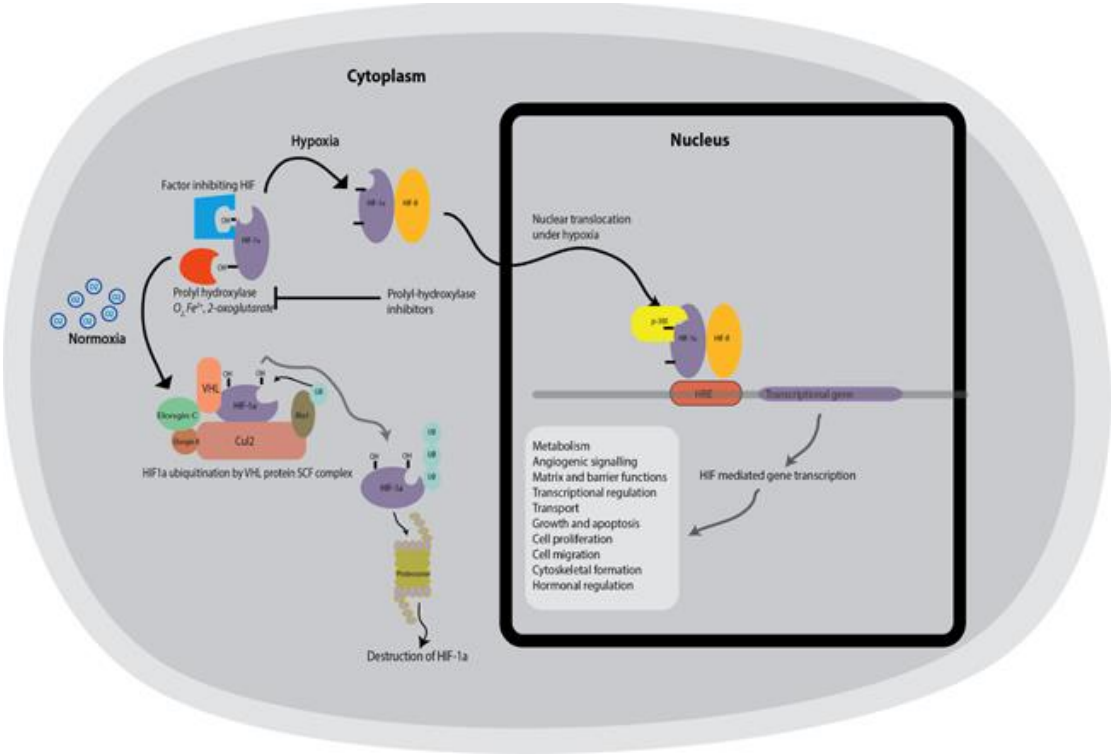


Figure 1. A schematic depiction of the hypoxia-inducible factor-1α (HIF-1α) pathway. HIF1α is a heterodimeric protein made from α and β subunits. Under normoxic conditions, the α subunits are targeted for degradation by prolyl hydroxylase domain (PHD) enzymes, which hydroxylate specific prolyl residues resulting in HIF degradation by a von Hippel-Lindau, polyubiquitination, proteasome-mediated pathway. The transcriptional activity of HIF1α is also regulated under normoxic conditions by factor inhibiting HIF (FIH), which regulates activity of the C-terminus transactivation domain (CAD) by asparaginyl hydroxylation. During hypoxia PHD and FIH are inhibited allowing the accumulation of α subunits that heterodimerize with the β subunit and recruit co-activators to hypoxia responsive elements. This posttranslational regulation by PHDs allows a prompt response to hypoxia, HIF-1α signaling is also rapidly switched off once normoxia returns (309).

The HIF system is subject to a number of complex feedback mechanisms. PHD 2 and 3 enzymes for example, are recognised to be a downstream target of HIF. Other regulatory enzymes and proteins are part of negative and positive feedback loops and are discussed in more detail in a review by Henze et al (310).

HIF targets several cellular processes leading to the reprogramming of cellular energy metabolism. HIF induces the production of target genes including pyruvate dehydrogenase kinase to divert pyruvate away from mitochondrial oxidation. In addition HIF increases anaerobic glycolytic ATP formation by increasing glucose availability by promoting glucose transporter, lactate dehydrogenase and monocarboxylate transporter 4 activity and increasing transcription of a variety of genes encoding most enzymes in the glycolytic pathway (310). Other enzymes including hexokinase and phosphofructokinase are also induced by HIF. The effect of HIF on the metabolic status would be an attractive reason to investigate the manipulation of this pathway in the brain dead organ donor.

In addition to the metabolic effects of HIF, which are relevant to protect against cellular injury following brain death, HIF is also identified to up-regulate cytoprotective molecules, including HO-1, HSPs and erythropoietin (EPO). These molecules act through having anti-inflammatory and anti-apoptotic properties (37). HIF itself is recognised to have both pro- and anti-apoptotic effects, with more severe or prolonged hypoxia resulting in apoptosis as a consequence of the association of HIF with p53 and subsequent Bax expression (311).

HIF is known to be pro-angiogeneic with downstream targets including vascular endothelial growth factor (VEGF) and NOS amongst others (309). Renal IRI triggers the release of VEGF by podocytes and tubular cells resulting in angiogenesis which maintains tissue architecture and organ function. As macrophages and monocytes are recruited to the renal tissue following endothelial cell activation anti-angiogenic factors including transforming growth factor- 1β (TGF-1β) and thrombospondin-1 (TSP-1) are released. This results in pro-fibrotic, anti-angiogenic environment which may contribute to chronic allograft nephropathy (312).

In the wider context of inflammation HIF induces mediators of innate immunity including iNOS, and promotes the release of cytokines including TNFα. HIF appears to have an intrinsic relationship with the innate immune system with cross-talk proposed with the NFkB system, although this has not been a reproducible finding within our own laboratory (313).

Thus the enzyme regulated HIF pathway, has the potential to be exploited as a therapeutic target during transplantation where hypoxia and tissue ischaemia are central aetiological factors. For example in DBD donors, the HIF pathway can be activated early in the injury cascade to protect against some of the detrimental metabolic and mitochondrial effects. However it is likely that not all of the down-stream effectors of HIF are beneficial, and effects are likely to vary over time. The extent to which the HIF systems are activated following these ischaemic periods should be evaluated prior to attempts at therapeutic manipulation.

A number of studies, including clinical trials have evaluated the role of downstream effectors of HIF as pre-conditioning agents. EPO and HO1 up-regulators for example, have undergone extensive evaluation and remain to be established as to whether they are of clinical value (314). Entrainment of the entire transcriptional pathway, that has evolved to provide adaptive protection, should be better than any individual component.

The challenge of HIF in research

Despite a number of studies that implicate HIF or its numerous target genes as conditioning agents, there remain a number of physiological and technical challenges in directly quantifying the responsible effect of this pathway in transplantation. For example HIF expression is dynamic, biphasic, overlaps with other pathways and is subject to a variety of feedback mechanisms. The predominance of post-transcriptional regulation of HIF has rendered protein quantification with western blotting or immunohistochemistry the mainstay of detection. The dynamic regulation of HIF protein levels may be distorted by pre-analytical variability, such as techniques and methods for preservation and sampling of tissues. Furthermore, studies that have concluded an absence of a HIF related effect when quantification is solely at the mRNA level should be approached with caution. In addition, the apparent paradoxical regulation of genes with opposing functions, for example pro- and anti-apoptotic, requires further evaluation, as does the relative contributions of HIF-1α and HIF-2α to gene regulation.

Inhibition of HIF hydroxylases provides a potentially translatable route into manipulating this complex system *in vivo*. However, care will be required when extrapolating studies from one organ system to another. Furthermore, studies demonstrating a benefit of non-selective PHD inhibitors such as cobalt chloride and desferrioxamine may also act via HIF-independent pathways. Currently, the lack of selective pharmacological PHD isoform inhibitors limits studying the differential effects of isoforms to *in-vitro* work and studies using knock out mice. Importantly, such studies have demonstrated that PHD 1, 2 and 3 contribute in a non-redundant fashion to the regulation of both HIF1α and HIF2α(315).

HIF in kidney transplantation

In renal transplantation, acute tubular necrosis (ATN) contributes significantly to the development of delayed graft function (DGF). This has led authors to pursue *ex-vivo* machine preservation in an attempt to reduce the impact of IRI (316).

Authors have investigated both in the *in-vitro* and *in-vivo* settings the effect of stabilising HIF1α on the outcomes of IRI models. Conde et al. exposed a proximal tubular HK-2 cell line to a period of 6 hours of hypoxia and nutrient deprivation to mimic ischaemia followed by re-oxygenation in a complete medium as an analogue of reperfusion (317). They identified HIF1α stabilisation during hypoxia and again following three hours of reperfusion, with lower signals being detected between the two periods. The authors went on to demonstrate the response was mediated by an

Akt/mTOR signaling pathway and the protective effects could be ameliorated by siRNA for HIF1α or YC-1 (a pharmacological inhibitor of HIF1α).

In further studies, in an *in-vivo* rat model, immunohistochemistry was used to demonstrate stabilisation of HIF during ischaemia and between day 3-7 post reperfusion (317). In a correlative study in human post-transplant biopsies, the authors revealed HIF1α stabilisation only in the proximal tubular cells and demonstrated a significant negative correlation with the incidence of acute tubular necrosis (ATN). However, it was not clear whether this effect was due to necrotic cells not having the capacity to stablise HIF.

Others have demonstrated that the application of preconditioning agents can provide resistance against IRI. Zhang et al. applied dimethyloxalylglycine (DMOG), a 2-OG analogue and inhibitor of PHDs and other 2-OG dependent enzymes, 24 hours prior to 30 minutes of warm ischaemia followed by 24 hours of reperfusion of the kidneys in a mouse model (318). They demonstrated stabilisation of HIF1α and its target genes including iNOS, EPO and HO-1 and improved tubulo-interstitial injury in the preconditioned mice. This beneficial effect could be ameliorated by the application of an iNOS inhibitor. Others have demonstrated a similar protective effect using other pharmacological inhibitors of PHDs and heterozygous HIF knockout mice models(319). Indeed it has been suggested that the beneficial effects of many preconditioning agents including isoflurane, EPO and even xenon occur through inducing the HIF pathway and its downstream targets (320). This has largely been based on the detection of increased

HIF expression following administration of the therapies but proof of causation rather than just association is often lacking.

Yang et al. have attributed the protection conferred by repetitive hypoxic preconditioning on rats subjected to renal ischaemia to HIF. Rats subjected to 15 hours of hypoxia (380 Tor for 15 hours a day for 28 days) had increased HIF1α expression and downstream up-regulation of Bcl-2, resulting in the prevention of Bax mitochondrial translocation, cytochrome C release and tubular apoptosis following IRI(321).

Others have demonstrated that application of the PHD inhibitor FG 4497 6 hours prior to donor nephrectomy in a rodent model could up-regulate HIF1α and downstream effectors which would persist beyond a period of cold storage. The authors went on to demonstrate significant improved graft function and survival in an allogeneic model of renal transplantation, advocating the evaluation of PHD inhibitors in a clinical trial (308). However, it has yet to be established what the effect of PHD inhibition in brain dead organ donors would be.

Biopsies taken during transplantation have demonstrated that the HIF system is activated as a result of the procurement process and correlates with the degree of exposure of kidneys to warm and cold ischaemia (322). Rosenberger et al. demonstrated using high amplification immunohistochemistry in human kidney biopsies, that immediately following engraftment HIF1α was detectable with low HIF detection scores correlating with primary non-function (323). The authors demonstrated HIF1α was detectable both in the renal cortex and also in the medulla

with collecting ducts and glomeruli demonstrating a high signal. 2-week post engraftment biopsies demonstrated widespread HIF1 α induction, the authors attributing the presence of HIF to the effects of post-transplant hyperfiltration, hypertrophy and calcineurin inhibitor induced toxicity. At 3 months HIF expression was no longer detectable except in kidneys that went on to demonstrate clinical and sub-clinical rejection. The authors hypothesised that the increased HIF expression was due to rejection induced hypoxia. However inflammatory cell induction of HIF remains a possibility and the relation of HIF levels with allograft rejection requires further evaluation.

The aim of this Chapter was to characterise alterations to the HIF machinery following brain death induction, utilising the same brain death model, described previously, and then to determine the effects of modulation of HIF on mitochondrial function. The aim of this was to provide evidence for, and then test the therapeutic manipulation of the HIF pathway in the brain dead organ donor. To achieve these aims I:

1. Optimised HIF1α immunoblotting techniques for the rat
2. Established the effects of brain death on hypoxia inducible factors and target genes
3. Evaluated the effects of modulation of the HIF system on mitochondrial function in an attempt to test whether therapeutic manipulation in the brain dead organ donor can improve outcomes.

Methods

Animal welfare

Studies were carried out using inbred Fischer F344 male rats (250-300g, Harlan, UK) or C57B6 mice (25-32g). Animals received free access to food and water in compliance with the Home Office guidance for the care and welfare of animals. Rodents were acclimatised for at least 1 week prior to experimentation.

Groups and experimental procedure

The following experimental groups were formed:

(1) Optimisation of HIF1α immunoblotting techniques. To date, few studies have been published, with reproducibility, identifying HIF1α in *rat* tissues (36). Our labs previous attempts at characterisation at the protein level failed to identify HIF1α in rat liver using immunoblotting (A Sutherland's thesis). This was partly due to lack of antibodies with the necessary sensitivity, but also due to the rapidly degrading nature of HIF. To allow quantification of the effect of brain death on the HIF system, I initially established a robust protocol for HIF1α protein extraction and quantification. To do this I examined the following groups:

   a. Cells:

      i. Negative control: Normoxic normal rat kidney cells (NRK-49) cells

      ii. Positive control: Normal rat kidney cells (NRK-49) cells exposed to 1% O_2 for 24 hours

   b. Tissue:

i. Negative control: Normoxic Fischer rats (n=2)

ii. Positive controls: Fischer rats treated with 40mg/kg of dimethyl oxalylglycine (DMOG), a cell permeant oxoglutarate analogue that inhibits oxoglutarate dependent dioxygenases i.v. (n=2). 40mg/kg was chose based on previous work performed in the laboratory and consistent with the data of others.

The tissues and cells were examined for HIF1α expression using antibodies, of which, a number were tested (table 1). Immunohistochemistry was performed on the tissues to evaluate the subcellular localisation of the antibody in the kidney, using the antibody optimised for western blotting.

Table 1. Antibodies and dilutions tested. All antibodies were tested up to a maximum of overnight incubation.

HIF1a antibody	**Company**	**Dilutions tested**	**Duration of incubation tested**
Cayman10006910	Cayman	1/1000, 1/500, 1/250	Up to overnight (12 hour)
Antibody 241809 (MAB1536)	RnD systems	1/1000, 1/500	Up to overnight (12 hour)
HIF-1α (H-206)	Santa Cruz	1/1000, 1/500	Up to overnight (12 hour)
HIF-1α (Y-15)	Santa Cruz	1/1000, 1/500	Up to overnight (12 hour)
HIF-1α (H1alpha 67)	Santa Cruz	1/1000, 1/500	Up to overnight (12 hour)
NB100-134	Novus	1/1000, 1,500, 1/250	Up to overnight (12 hour)
NB100-105	Novus	1/1000, 1/500	Up to overnight (12 hour)
ab10625	Abcam	1/1000, 1/500	Up to overnight (12 hour)
GTX127309	Genetex	1/1000, 1/500	Up to overnight (12 hour)
HIF-1 alpha Antibody (mgc3)	Thermo antibodies	1/1000, 1/500	Up to overnight (12 hour)

(2) Hypoxia inducible factors following brain death. To identify the effects of modulation of hypoxia inducible factors and target genes during brain death, I compared two controls to the brain death group. Brain death was induced as described in previous Chapters (3, 4 and 5) in male Fischer rats.

    a. Short sham (n=4)
    b. Long sham (n=4)
    c. Brain death for 4 hours (n=6)

Tissues were evaluated for HIF1α expression and activity using western blotting, immunohistochemistry and evaluating the effect on HIF target genes.

(3) Modulation of mitochondrial function by manipulating HIF. Effects of modulation of the HIF system on mitochondrial respiration and function was subsequently assessed. To evaluate this I first tested (a) the effects of administration of DMOG on the mitochondrial function as assessed using a Clarke electrode. I then used an inducible knock down mouse system to evaluate the contribution of HIF to this effect (b) and then aimed to further clarify the effects in a knock out cell line (c). To confirm the effects across organ types I evaluated the effects on the liver and also the kidney.

    a. Testing the effects of administration of a HIF inducing agent on oxygen consumption by mitochondria I obtained from the kidney and liver of mice in two groups at two time points (1 and 4 hours):
        i. Control: Male C57BL/6 mice (n=3)
        ii. Treated: Male C57BL/6 mice treated with DMOG 40mg/kg i.v. (n=3) 1hour prior to harvest

In parallel the effects on target genes of HIF were characterised in the same organs.

b. I further aimed to determine the relative contribution of the HIF1/2α component of the observed effects using a ER Rosa-Cre-tamoxifen inducible knock down system comparing:
    i. Wild-type
    ii. HIF1α Cre DMOG
    iii. HIF1α Cre –'ve DMOG
    iv. HIF2α Cre DMOG
    v. HIF2α Cre –'ve DMOG
c. To further dissect the HIF1α the effects of two PHD inhibitors DMOG and a Molidustat (Produced by the C Schofield laboratory, Oxford) were tested on wild-type and HIF1α knock out cell lines (HCT116) treated at different concentrations.
    i. HCT (+/+) DMOG: 10μM, 100uM, 500uM Bayer:10uM, 50uM, 150uM
    ii. HCT (-/-)DMOG: 10uM, 100uM, 500uM Bayer:10uM, 50uM, 150uM

Cell culture

NRK cells

Cells were cultured in DMEM (20mM glucose media), penicillin/streptomycin (5%), glutamine (5%). After 5 passages cells were incubated in 1% hypoxia for 24 hours. Normoxic cells were prepared at the same density. Cells were harvested in the hypoxia

chamber or in the culture hood, using a urea-SDS buffer and prepared for protein western blotting.

HCT116 cells

HCT116 cells were cultured in a low glucose media (DMEM +5% glucose). Cells achieved 4 passages prior to use. Cells were incubated with DMOG (for concentration please see groups and experimental procedure section).

Preparation of compounds

DMOG was dissolved in 1% DMSO. 40mg/kg was administered to animals (i.v). For the cell lines 10, 100 and 500µM solutions were tested.

Molidustat was prepared in 1% DMSO. For the cell culture work 10, 50 and 150µM was tested.

Inducible knock-downs

Hif-$1^{f/f}$, and Hif-$2^{f/f}$ (where f denotes the floxed allele) conditional knockout and Rosa26Cre^{ERT2} mice have all been described previously and were obtained from these sources (324-327). Each line had been backcrossed with C57BL/6 for at least five generations and was intercrossed to generate littermates of appropriate genotypes (328). $Phd2^{+/-}$, Hif-$1^{+/-}$ and Hif-$2^{+/-}$ mice are as described previously these lines were intercrossed to generate mice that were maintained on a mixed Swiss/129SvEv/C57BL/6 genetic background (326, 329-331). Genotype was determined

by PCR (331).

Tamoxifen administration. Tamoxifen (prepared to 20 mg/ml in corn oil; Sigma, US) was administered by oral gavage to ~6 week old mice at a dose of 2 mg/kg per day for 5 consecutive days as described in a previous publication from our group (332). Recombination was assessed using DNA isolated from ear biopsies obtained 10 days after the first dose of tamoxifen (331).

Mitochondrial isolation

Mitochondrial isolation was performed using a previously protocol, described in Chapter 5. In brief, a hemi section of a kidney was obtained and stored in an ice cold isolation buffer (IBc, 10ml of 0.1M Tris-MOPS,1ml of EGTA/Tris to 20ml of 1M sucrose, final volume adjusted to 100ml with distilled water. Adjust pH to 7.4). 4-5 washes were performed to ensure that the kidney was completely devoid of blood. The kidney was minced into small segments using scissors and homogenised using a Teflon pestle (1,600 rpm, 5-6 strokes). The homogenate was centrifuged at 600g for 10min at 4^{o}C. The supernatant was further centrifuged for 10 min at 4^{o}C at 7,000g and the pellet washed with 5ml of cold IBc. The supernatant was discarded and the pellet re-suspended in a small volume of IBc. Protein quantification was performed using a BCA assay (thermos scientific) and concentration adjusted to 1mg/ml.

Clarke Electrode O_2 consumption activity

O_2 consumption assays were performed as previously described. In brief, the electrode was calibrated and 1ml of experimental buffer (EBc) was added following equilibration

of the buffer in a water bath (30^{o}C). Mitochondria were added to the electrode to obtain a final concentration of 1mg/ml and state 1 respiration recorded. Succinate (5mM) was added to obtain the state 2 respiration and ADP (100 μ M) was added to determine state 3. Following establishment of state 4, state 5 was induced by adding 60nM of Carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (FCCP).

The respiratory control ratio was calculated by dividing the state 3 by state 4 respiration.

Mitochondrial electron transport chain complexes activity assays

Mitochondrial isolates were prepared from dissected hemi-section of left posterior portion of the kidney samples as previously described in Chapter 5. Mitochondrial complex activity assays were performed by normalising values to citrate synthase activity using aliquots from the frozen samples. Assays were performed on a microplate reader (BMG Labtech) using previously published protocols.

RNA isolation and semi quantitative RT-PCR

Total RNA was isolated from snap frozen tissue (10-30mg) using RNeasy© MiniKit (Qiagen) isolation kit. The GeneAmp © PCR Sytem9700 thermal cycler was used for the cDNA reverse transcription. An Applied Biosystem qPCR machine was used to run the experiment. The primer sequences were:

PDK1: Forward: CCAACATCGAGCCACTC, Reverse: CTGGCTGTGACACGGGTAC

GLUT-1: Forward: AGGTCCCCAGCATGTTTACGTT, Reverse:

GGGCTAATAGCGTTCTGATCTG

PHD2: Forward: GTTCAGCCCTCCTAGC, Reverse: ACCACCGTCAGTCTTTA

β Actin: Forward: 5'-CAATATGTGGAGCAACTGTG, Reverse: 5'-

AGTAGGTGAAGATGAAGAAGAG

Relative quantitation of gene expression was performed using β actin as the control.

Western blotting for the HIF machinery

Tissue preparation for western blotting was performed by lysis of snap frozen tissue in a urea-SDS buffer. The protocol was optimised for extraction (supplement). A number of antibodies were surveyed (table 1). The best antibodies i.e. with higher specificity, were taken forward for further use.

Antibody incubation with HIF1 α antibody was performed overnight at 1:1000 antibody dilution. After washing the membrane a secondary anti-rabbit HRP conjugated antibody was added for 1 hour (1:1000, Sigma). After addition of super-signal chemiluminescent substrate light was detected using Kodak abc film (exposure time z seconds) developed using a KLM film processor. Similar approach was used for HIF2 α and PHD1 and PHD2.

Mito-express oxygen consumption assay

Cells were seeded at a density of 50,000 per well and incubated with 10µl of Mitoexpress reaction mixture. 1µl of antimycin A was added to the control wells.

Compounds were added at the described doses for 1 hour and kept in humidified warmed conditions. Wells were sealed with 100µl of pre-warmed mineral oil and the plate read on the plate reader (BMG, UK). Assays were performed in triplicate. Kinetic measurements were made for at least 90min. Mitoexpress lifetime values were plotted against time. Measurements of the oxygen consumption rate could then be made by determining the linear regression of the slope. Values were normalised against a cell viability (*propidium* iodide) assay performed in parallel on separately plated cells, with the same density, treated with the same compounds.

Statistical analysis

For statistical evaluation of the data a Kruskal-Wallis (non-parametric) comparison of multiple groups was performed (Graph-pad Prism 2014). For comparison of two groups a non-parametric T-test was performed (Mann-Whitney). Significance was set at a value of P=0.05 and all graphs report results as mean± standard deviation.

Results

Optimisation of HIF1 α immunoblotting techniques

The 3 antibodies with the best detection for HIF1α are shown in Figure 1. Based on these results the Cayman antibody was used for the remainder of the experiments as it allowed the easiest identification of a band co-migrating with the dominant hypoxia band seen in the cell line at a molecular weight concordant with that expected for HIF1α on an SDS page gel.

Immunohistochemistry was performed on tissue samples procured from the DMOG treated rats (Figure 2). Marked, widespread detection of HIF1α was detected in both the medullary and cortical segments of the kidney. More prominent staining was detected in the medulla. Sparing of the cortical glomeruli was noted to occur. Absence of stain detection was found in the control tissue.

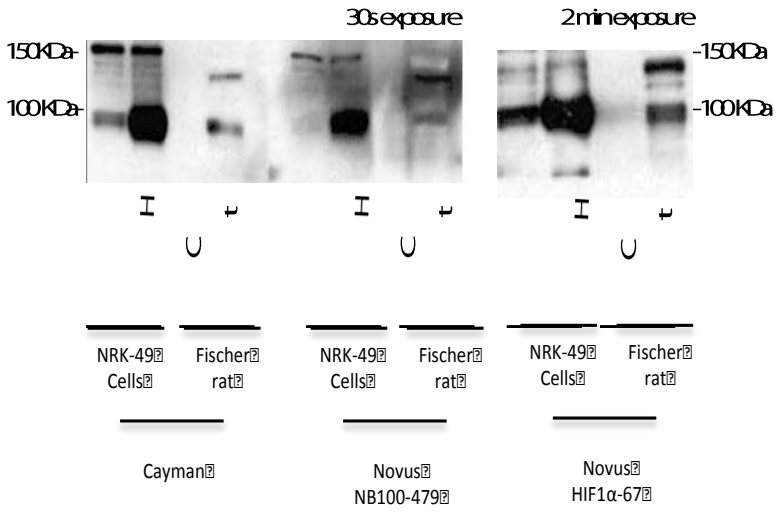


Figure 1. Identification of optimised antibody for HIF1 α detection in rat. 3 of the best results of the antibodies tested are described. Nx: Normoxia, Hx: hypoxia.

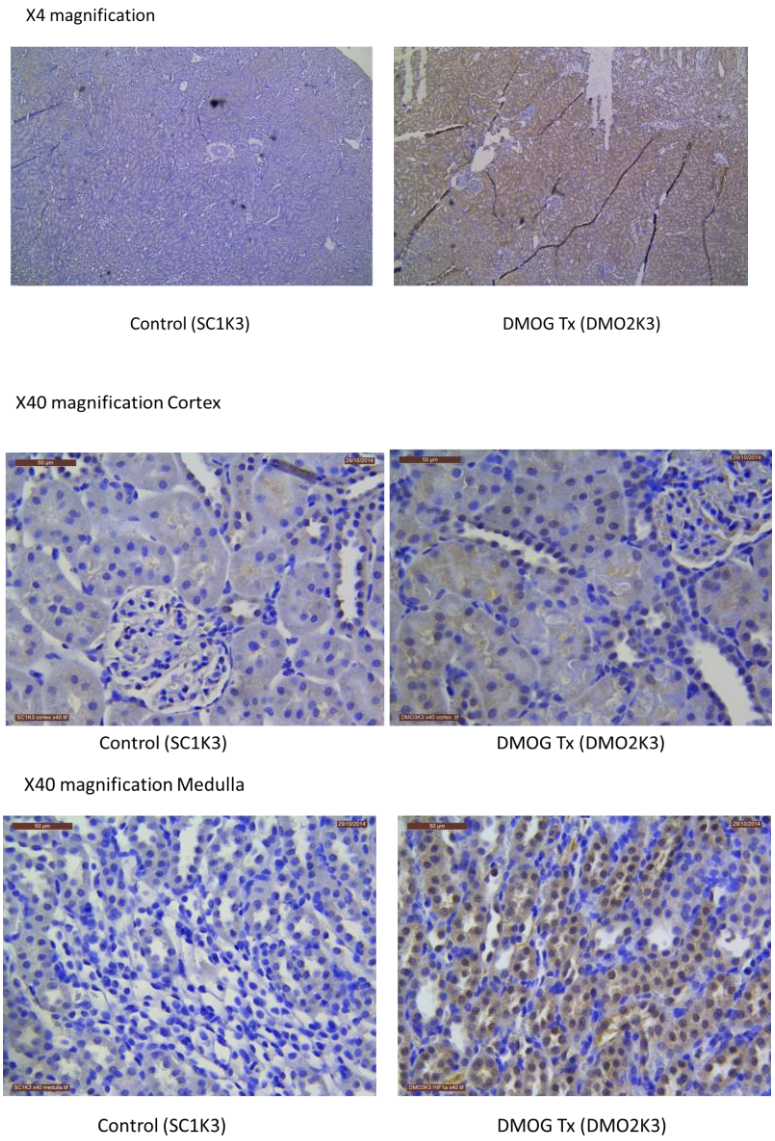


Figure 2. Distribution of HIF1α in the rat kidney following DMOG treatment detected using the Cayman antibody Control: kidney from healthy rat, DMOG: kidney from rodent treated with DMOG. Expression of HIF1α at low magnification demonstrated expression of HIF in both the cortex and medulla of the kidney. However, at higher magnification predominant expression was observed in the medullary segments.

Hypoxia inducible factors following brain death

Figure 3. demonstrates blots illustrating the effect of brain death on HIF1α. The results indicated an increased expression of HIF1α following brain death. Semi-quantification was performed by evaluating band intensity, the difference in expression levels was not statistically significant between the groups however as both in the kidney and liver the standard deviation for the detection of HIF was wide ($P>0.05$). A higher amount of HIF1α was detected in the long sham compared to the short sham control. No statistical significance was detected in alterations in three the mRNA levels of target genes of HIF PDK1, PHD2 and GLUT1 measured by RT-PCR (Figure 4).

When examining the cellular localisation of HIF1α in the brain death animals, it appeared to be predominantly located in the medulla of the kidney (Figure 5/6) In the cortex the proximal tubules were the sites of the predominant HIF1α expression. Both staining of the cytoplasm and nuclear staining were noted to occur. Concordance existed between the western blotting and immunohistochemistry data, demonstrating a higher HIF1α expression level in the long sham, compared to the short sham controls. The level of HIF1α corresponded strongly with the post brain death induction MAPs (Figure 7). The Spearmen's correlation revealed a coefficient of -1, ($p=0.01$).

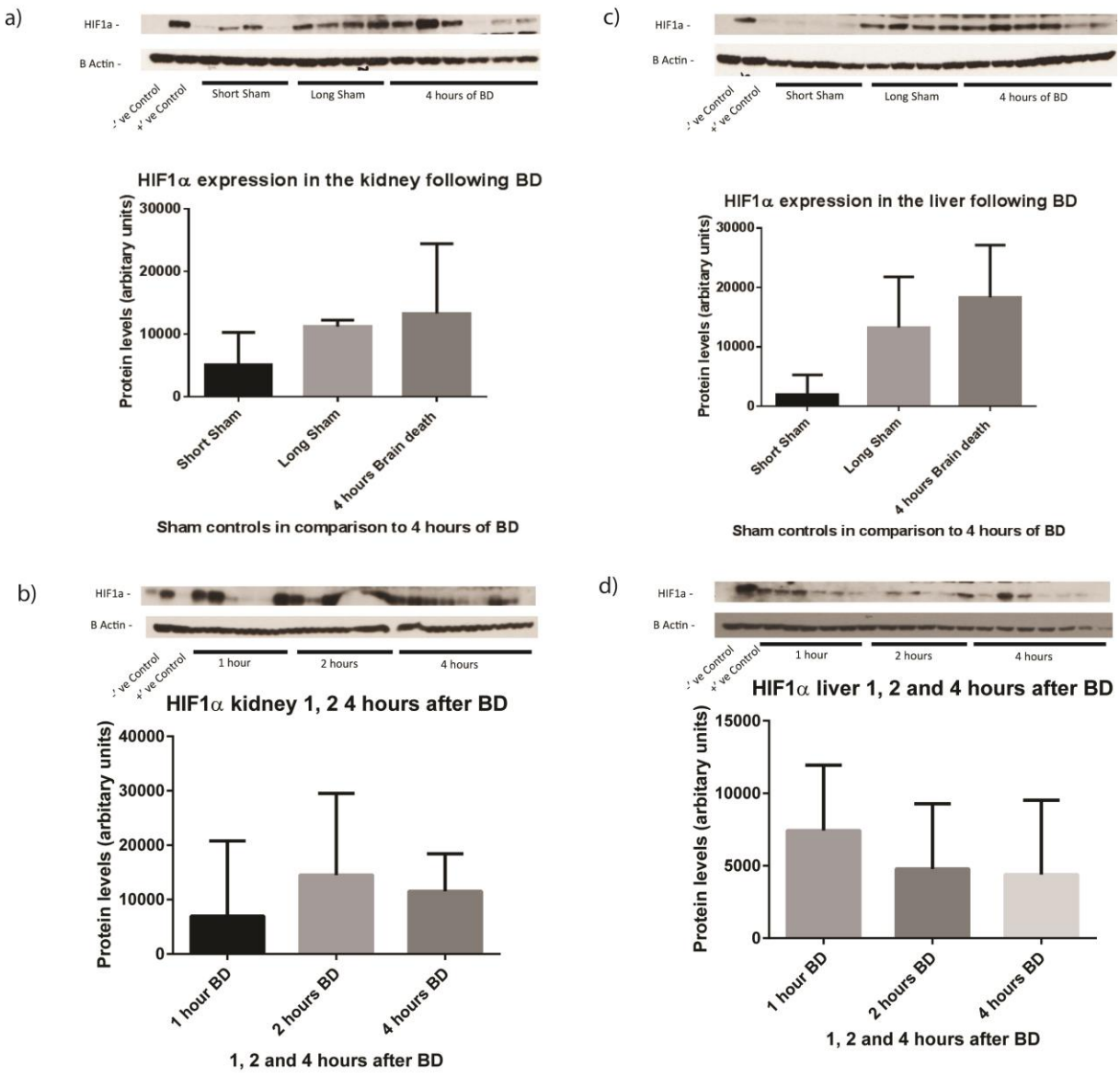


Figure 3. Effects of brain death on HIF1α expression in the kidney and liver and on HIF target genes. a) Illustrates the effect on HIF-1α protein expression after 4 hours of brain death in comparison to sham controls b) No significant difference was noted when comparing 1-4 hours of brain death In the kidney. Similar patterns of HIF1α expression were noted in the liver when comparing the controls to the 4 hour brain death animals (c and d). A different pattern of HIF1α expression, compared to the kidney, was noted when comparing 4 hours of brain death to 1 and 2 hours.

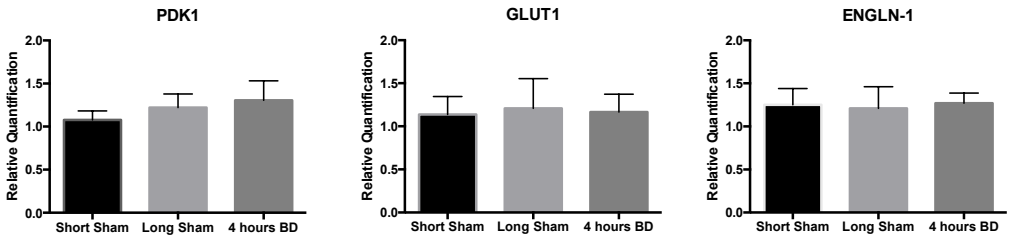
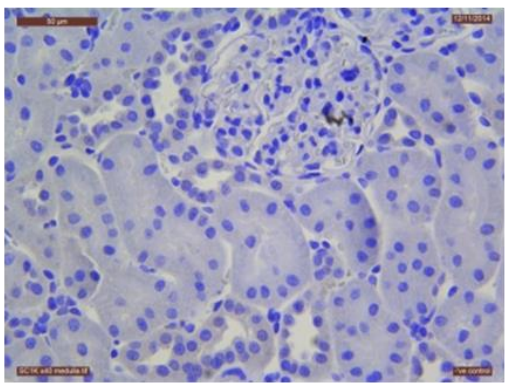
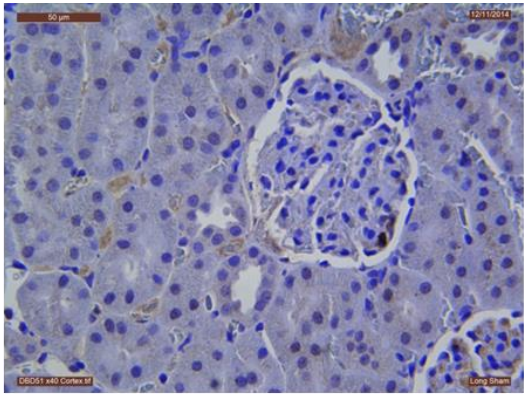


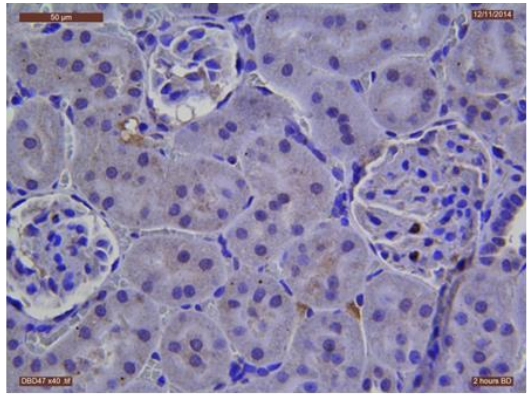
Figure 4. Effect of brain death on HIF target gene expression. No significant alteration in the HIF target gene expression was noted as measured by RT-PCR.



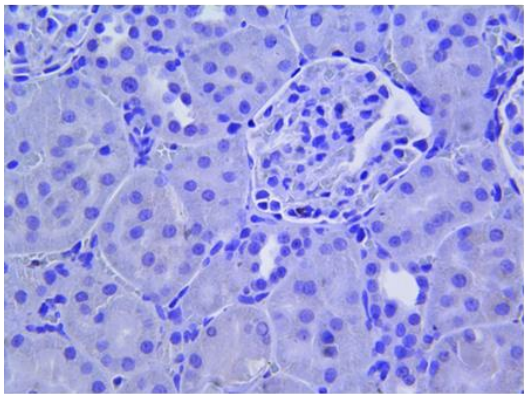
-'ve control



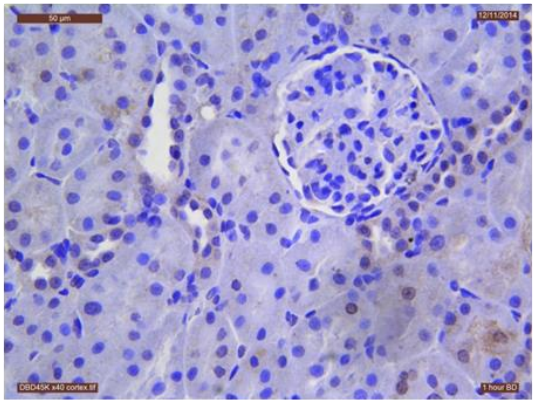
Long sham



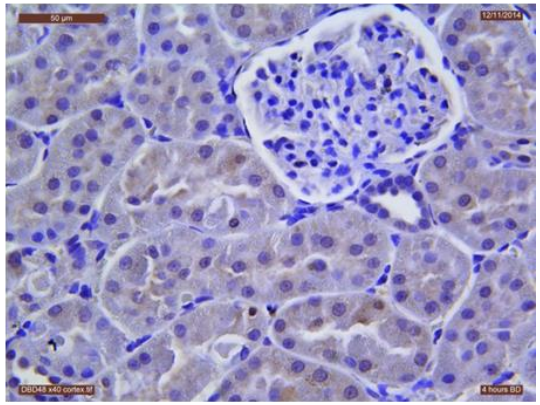
2 hours post BD



Short sham

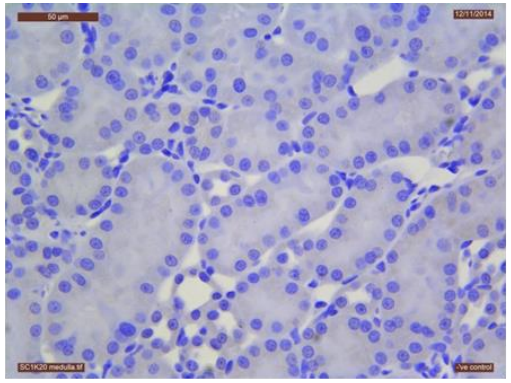


1 hour post BD



4 hours post BD

Figure 5. High powered (x100) examination of the effects of brain death on HIF1α expression in the cortex. Cytoplasmic and nuclear staining noted in the tubules, sparing of the glomeruli is noted. Brown staining regarded as demonstrating positivity.



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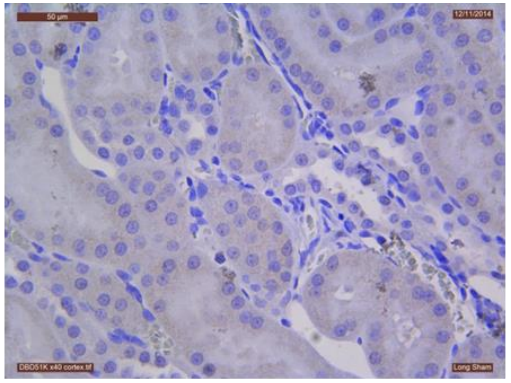
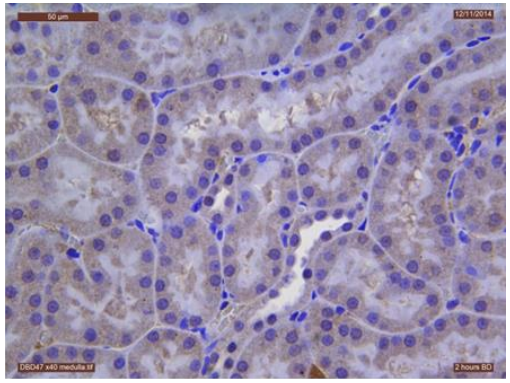
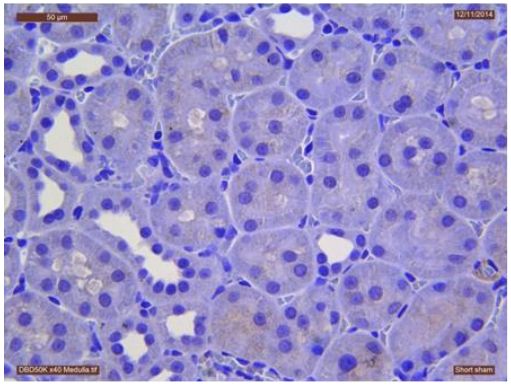


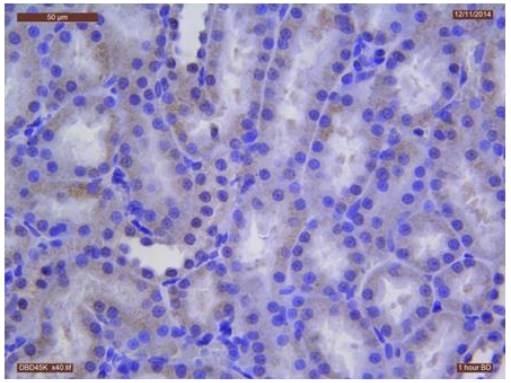
Long sham



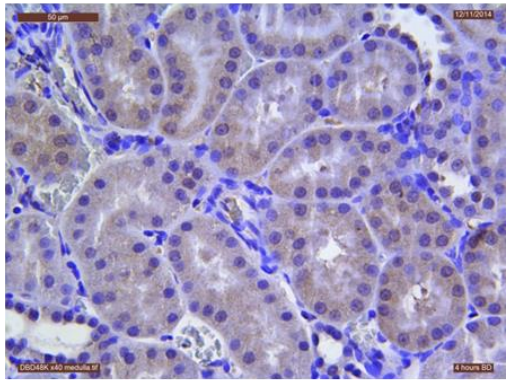
2 hours post BD



Short sham



1 hour post BD



4 hours post BD

Figure 6. High powered (x100) examination of the effects of brain death on HIF1α expression in the medulla. Cytoplasmic and nuclear staining noted in the nephrons.

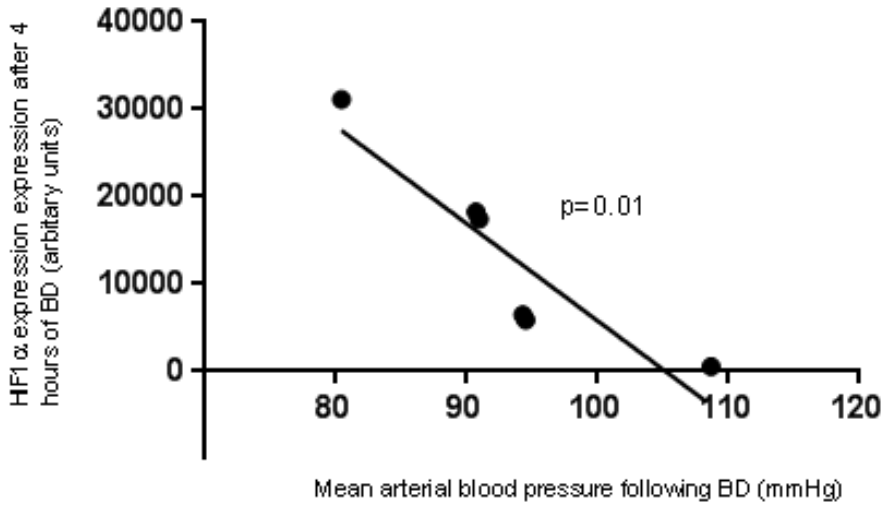


Figure 7. Correlation of HIF1α expression with post BD induction MAPs.

Modulation of mitochondrial function by manipulating HIF

To examine the effect of modulation of HIF on mitochondrial function DMOG was administered to mice in a pilot experiment, the kidney and liver was explanted and RCR's calculated to determine the effect across organ types. Figure 8 illustrates significant increases in state 3 and 4 respiration in both the kidney and liver following 1 hour of DMOG treatment. This resulted in a trend towards a reduction in the RCR in both the kidney and the liver.

To further explore the relationship between the effect of DMOG on the HIF system and mitochondrial function a conditional knock-out experiment was performed (Figure 9). Compared to wild-types, the control conditional knockouts demonstrated a similar response with a similar order of magnitude change to those observed with the previously described mouse experiment. Reproduction of the statistical significance achieved with the liver was not observed, due to the experiment being underpowered. Conditional knock-out of HIF1α did not demonstrate a discernible effect or reliable trend with different patterns observed between the kidney and the liver. A trend towards reduction in the RCR was observed with the kidney, similar to the previous results, this was not recapitulated in the liver however.

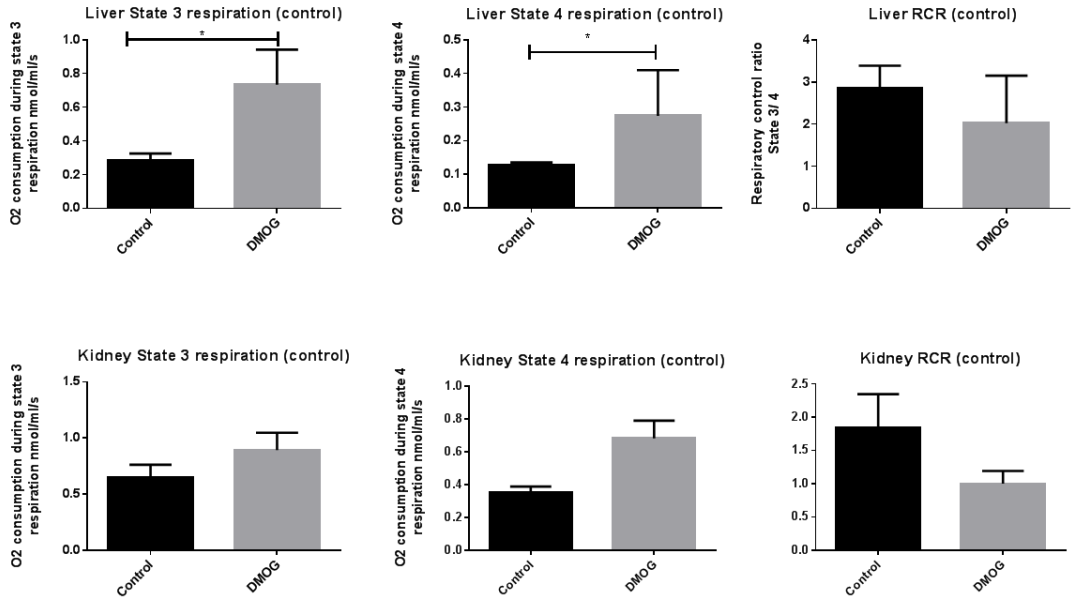


Figure 8. Effect of DMOG on oxygen consumption. A comparison of the effects of DMOG administration on liver and kidney mitochondrial function assessed using the Clarke electrode. An increase in State 3 and State 4 respiration was noted to occur, this was significant (at $P<0.05$ level) in the liver but with a trend towards an increase in the kidney also. The RCR trended downwards following 1 hour of treatment. State respiration was also calculated for mitochondria isolated from rodents after 4 hours of DMOG treatment. Maintained increase in oxygen consumption was noted to occur.

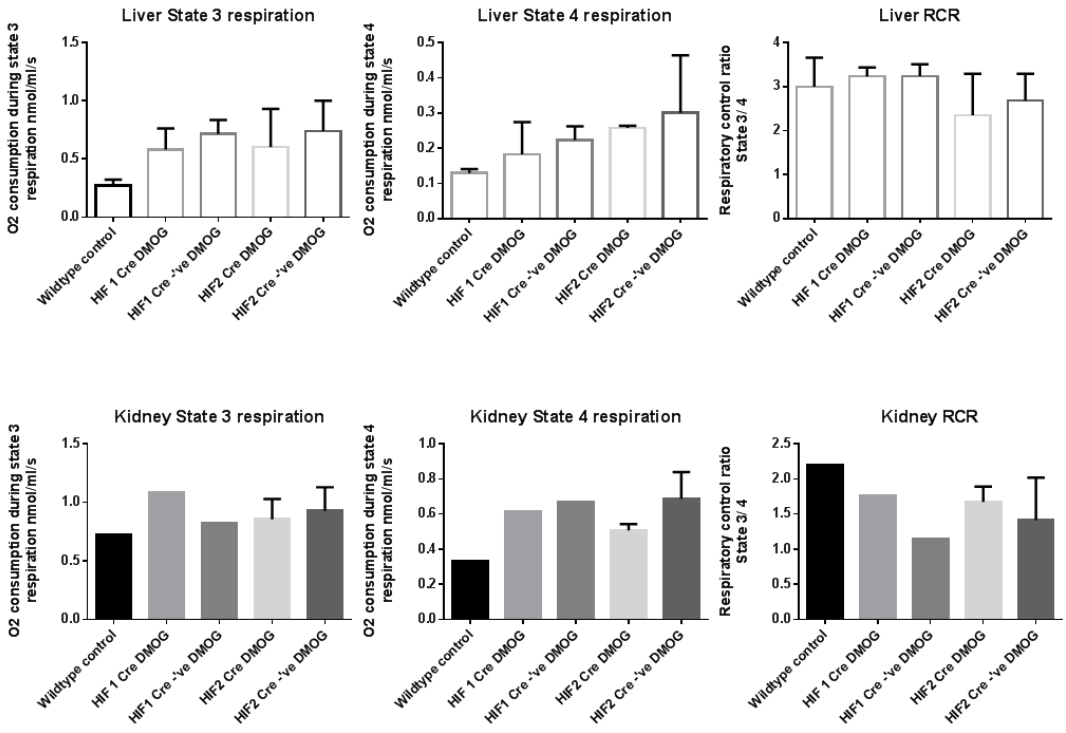


Figure 9. Effect of DMOG on HIF inducible knock-downs. No clear demonstration of the HIF responsible component of the effect of DMOG on the HIF system could be determined. The low n numbers was reflective of the slow breeding rate of the mice.

Due to the unclear effects, the slow breeding rate and expense of the conditional knockdown mice we further explored the relationship between the HIF system and mitochondria using a HIF1α inducible knockdown system in a cell line for which the metabolic response to hypoxia had been previously well characterised (333). Using the mito-express probe we established how oxygen consumption varies over 1 hour with the administration of two different compounds to HIF1α-/- vs HIF1α+/+ cells. The initial observation demonstrated a clear difference between the wildtype and HIF1α-/- in the oxygen consumption rates in normoxia (Figure 10). When challenged with DMOG this resulted in a trend towards reduced oxygen consumption in the wildtype cells, with the most effective dose being 100μM. However, in the HIF1α-/- cells an increase in oxygen consumption was noted with DMOG administration.

When challenged with an alternative compound (Molidustat) the effects on reducing O_2 consumption were more pronounced than with DMOG with the optimal dose being 50μM. However no discernible effect is observed in the HIF1α-/- context.

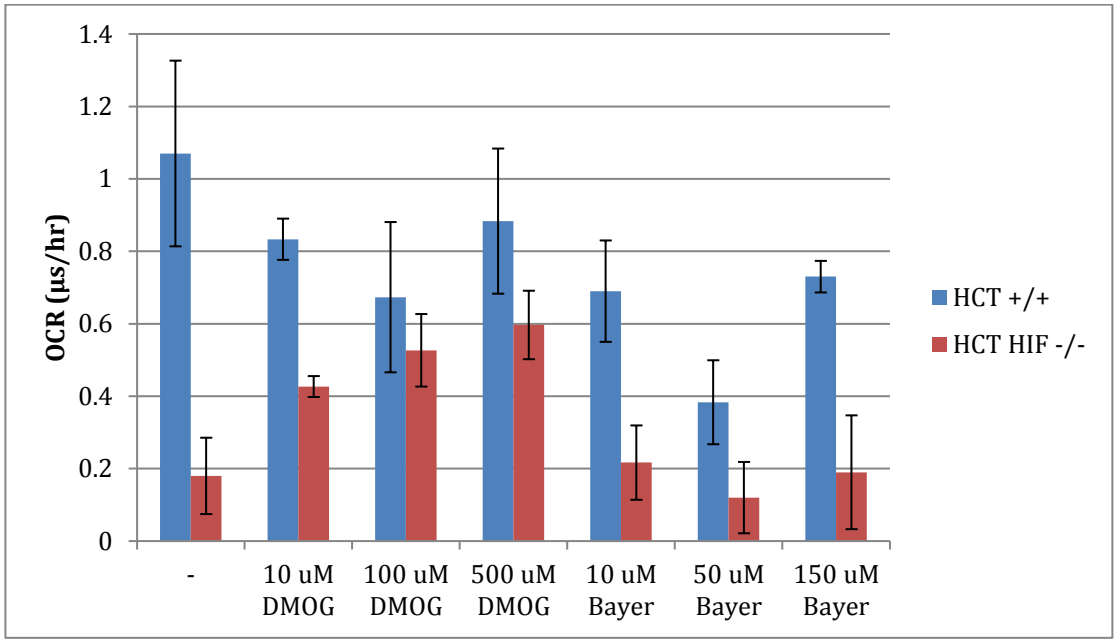


Figure 10. Effect of the Bayer compound and DMOG on oxygen consumption in a cell. Effects of DMOG and Molidustat (Bayer compound) were evaluated using HCT116 cell line, measuring the oxygen consumption ratio using mito-express. Attenuation of the oxygen consumption was most pronounced when administered with the Molidustat at 50µM. In the KO DMOG appeared to cause an up-regulation of oxygen consumption, an effect not observed with the Molidustat.

Discussion

To investigate the role of the HIF machinery in brain death and the potential role for manipulation of the HIF system, I first needed to develop robust methods for HIF1α extraction and quantification at the protein level. As illustrated by the results I could successfully isolate HIF1α following treatment with DMOG. Due to the polyclonal nature of the antibodies other non-specific bands were detected, but the major band was identified at the predicted molecular weight of HIF1α. Concordance of this data across antibody types improved the security of this interpretation.

Kidney tissue samples from DMOG treated animals were examined for HIF1α using immunohistochemistry. This revealed widespread HIF expression, consistent with the western blotting. The absence of any staining in the sham material suggests that the cross-reacting bands seen in the some immunoblots were not contributing to the immunostaining. The predominant location of the staining was in the medullary segment of the kidney, which would also be consistent with data from others demonstrating predominant expression of HIF in the medulla (334, 335). This is thought to be due to the natural oxygen gradient that exists in the kidney, meaning at basal levels higher amounts of HIF1α are expressed in the medulla. This could also be demonstrated for the cortex where relative sparing of the glomeruli was shown, but increased staining in the proximal tubules, where the rich vascular network that surrounds this structure may mean under basal conditions little or no HIF is expressed. Challenging the cells with a compound in this component of the kidney may not result in substantial increases in comparison to other compartments of the kidney.

The immunohistochemistry staining was noted to be both cytoplasmic and nuclear. As HIF1α is predominantly a nuclear protein, cytoplasmic staining could be due to non-specificity of the antibody used or alternatively true detection. If the latter is true this would be reflecting translocatory movement of HIF1α from the cytoplasm to the nucleus. The concordance between the western blotting and immunohistochemistry data however was reassuring.

I then attempted to characterise the effects of brain death on HIF1α expression. Immunoblotting demonstrated a non-significant increase in HIF1α expression in the brain death samples compared to the short sham control. A trend towards increased HIF1α expression was also observed in the long sham. Mean arterial pressures, oxygen saturations and blood oxygen partial pressures were noted to be comparable to between the groups and therefore inadequate ventilation was unlikely to be responsible for causing tissue hypoxia. Unfortunately, isoflurane and injectables, such as propofol, have been shown to be HIF1α induction agents, as are most other inhalational volatile anaesthetics. Due to the length of the surgical procedure however sensitive control of the depth of anaesthesia is pre-requisite; maintaining anaesthesia with IP injection is not possible and thus confounding of HIF levels in the control samples was unavoidable.

In contrast, the brain death animals have had the anaesthetic administration terminated after confirmation of brain death, at the same time point as tissue was harvested from the short sham animals, but with time for the anaesthetic to be flushed out and its effects to be reversed during the period of brain death. Isoflurane therefore

would not be responsible for the increased levels detected. However, the variability of expression made interpretation of a true brain death effect on HIF1α expression difficult to demonstrate with statistical certainty. Concordance between the liver and kidney demonstrated this was not a single organ effect.

Re-powering the experiments based on the variance observed would mean >15 animals per group would be required to demonstrate a statistical significance if one existed. To ensure that we were not missing a transient HIF1α expression I also looked at tissues from animals after 1 and 2 hours of brain death. Increased expression was observed more so after 2 hours in the kidney and 1 hour in the liver, although again this was not statistically significant, but the different time courses suggest that it is not purely due to a residual effect of the initial isoflurane exposure.

A strong correlation existed between HIF1α and the MAPs, suggesting even maintaining MAP of 80mmHg may result in cellular hypoxia responses, although systemic levels of lactate were not different between the groups, this is a non organ specific phenomenon as it was also observed in the liver. The correlation strength was a surprise and the data was interrogated to see whether inotropes actually resulted in the HIF1α expression. However, there was no correlation between the inotrope administration and HIF1α detection levels.

Examining the effects of prolyl hydroxylases (PHD1-3), HIF2a and target genes no discernible impact of brain death was observed on these markers of injury. This may be partly because the signal for induction of target genes may have been too low, or

alternatively due to the duration of the experiment (4 hours) being too short to observe transcriptional and translational changes. HO-1 reported in a previous Chapter to be up-regulated following brain death, is known to also be a target gene of HIF also. However multiple pathways could potentially up-regulate HO1, so this could be mediated by a HIF independent effect.

Examination of the immunohistochemistry data from the brain death animals demonstrated a similar pattern observed with the western blotting. HIF1α staining demonstrated increased expression in the medulla and proximal tubular components of the kidney, consistent with the regions where we observed increased HIF1α expression with the DMOG treatment.

Overall, from this data I was able to demonstrate that HIF1α is variably expressed in the brain death model. No major alterations were observed in target genes at the time points I was able to examine with my experimental model. Therefore, the opportunity to up-regulate HIF target genes by induction of HIF early in the brain death process, may be an opportunity to therapeutically activate cellular pathways and protect organs from injury. To establish whether there was indeed therapeutic potential for this, I evaluated the effects of modulation of the HIF system on mitochondrial function and cellular oxygen consumption.

In order to demonstrate a therapeutic effect of modulation of the HIF system in the brain death model, the up-regulation of HIF would need to occur quickly and instigate protective pathway activation rapidly. Others had previously shown that DMOG can up-

regulate HIF1α protein within 1 hour of administration. In a pilot experiment I first characterised the effect of DMOG on mitochondrial function. After 1 hour of administering the compound I already observed differences in the state 3 and state 4 respirations of the isolated mitochondria, this persisted over a 4 hour time window. No clear difference was observed in the overall RCR of the mitochondria, but the pilot study was underpowered to detect this. There was a trend towards a reduced RCR in both the kidney and liver however.

The effect on the compound on increasing state 3 oxygen consumption markedly within a short time frame was unexpected. State 3 is induced in the presence of substrate (succinate) with the introduction of ADP. State 4 is then induced after the depletion of the ADP but in the presence of excess substrate. The ETC is driven by the presence of ADP allowing oxygen consumption to occur with cytochrome oxidase (complex 4). Higher oxygen consumption after ADP depletion is commonly seen due to an uncoupling effect (336). The RCR reflects how well coupled oxidative phosphorylation is to ATP production. The trend towards reduction of the RCR could be due to increased mitochondrial uncoupling.

The unexpected result led to the examination of how much of the increase in state 3 oxygen consumption was due to HIF and to determine whether this would be counter productive in brain death, where we are trying to reduce oxygen dependency and ETC activity. To evaluate this I initially attempted to use a mouse knock-out system. The slow breeding rate of the mice meant large numbers were not possible to achieve in competition with other experiments being performed in the laboratory. However, the

DMOG in the Cre –'ve controls, yielded a similar result to that of the Cre+'ve, with DMOG causing an increase in state 3 and 4 oxygen consumption. There was no discernible effect of knocking out HIF on the respiratory states, although drawing definitive conclusions from the data was impossible.

For this reason I decided to examine this effect further in a HIF1α KO cell line and test whether this was an effect specific to DMOG or whether other PHD inhibitors also had a similar effect. There was a marked result when examining the difference in the basal oxygen consumption rates between the wild-type and knock-out. The knock out had markedly reduced oxygen consumption rates. This was also an unexpected result and could be explained by reduced mitochondrial density within the cells, as a consequence of the knock out or reduced activity due to an effect on the ETC, Kreb's cycle or other component of energy production.

The response to hypoxia of this cell line had been well characterised by Vialli and colleagues (333, 337). Determining how the differences are related to the intrinsic relation of metabolism with mitochondrial function will be integral to determine how this cell line and others respond to hypoxia. This is an important area in cancer metabolism related research. Given the clear dissociation of pursuing the research question with the initial aims, led me to not pursue this difference in basal oxygen consumption rates in the cell line.

The effects of DMOG on the oxygen consumption rate did not demonstrate an obvious effect in the wild-types. With the alternative compound, effects on reducing oxygen

consumption were more evident, especially at the 50μM dose. Indeed if in the KO, the effect of DMOG was to markedly increase oxygen consumption, an effect not observed with Molidustat. Overall the effect of DMOG to possibly increase oxygen consumption and therefore potentially worsen the metabolic status of a brain death cell could not be ruled out. This appeared to be a HIF independent phenomenon, due to the lack of effect on the KO on attenuating the increased O2 consumption. Indeed, HIF could be counterbalancing the increased oxygen consumption effect seen with DMOG, but this was not fully explored in this series of experiments. The explanation for why this HIF independent effect may occur could in part be driven by DMOG structurally resembling α-ketogluterate (338). Indeed, α-ketoglutarate has been shown to be able to stablise HIF. The presumption has been that these both DMOG and α-ketoglutarate work by stabalisation of the prolyl hydroxylases, by acting as 2-oxyloglyterate analogues. However, to induce marked increases in oxygen consumption within an hour of administration of the compound would unlikely be due to a transcriptional/translational effect which would take longer. Therefore, this would be consistent with DMOG having an off target effect.

If DMOG was to have an off-target effect, inadvertently increasing oxygen consumption, this may have an impact on cellular homeostasis of oxygen, reducing intracellular oxygen concentrations and therefore possibly further driving HIF induction. This however remains speculation and hasn't been proven in these series of experiments but could form part of on-going work. What was clear was that DMOG may not be the best compound to try and reduce the altered mitochondrial function and metabolism observed in brain death animals.

Molidustat offered an alternative compound, which I attempted to test in the brain death setting, however getting sufficient quantities of the compound from the company was challenging. Furthermore, dissolving the compound to a non toxic level for animal i.v. injection, with an appropriate solvent also proved difficult. Overall the compound, which is currently in clinical trials, in tablet form will need chemical manipulation to make it suitable for i.v. injection if it is considered for use in the brain death model.

Conclusion

Overall we have observed that following brain death, HIF1α is increased in donors with lower MAPs. However, this did not translate into detectable effects on HIF target gene expression. Scope for up-regulation of the HIF pathway early in the management of donors is therefore possible. Testing DMOG as a potential compound demonstrated off target effects, that could increase oxygen consumption and drive the electron transport chain, events that we would be trying to prevent based on the data from Chapter 4. Other compounds such as Molidustat offer better prospects but are yet to be tested in clinically relevant models such as ischaemia reperfusion injury or brain death. The brain death model may be limited in being able to detect a discernible change due to the limited 4 hour follow up period. Overall several of the experiments were done with small groups, thus I am cautious about drawing secure conclusions. However, the observations appear to have unearthed complexity in some facets of the control of hypoxia regulation and mitochondrial function that warrants further investigation.

Chapter 7: Conclusion and future avenues for research

When I started my research I can honestly say I was not aware of how difficult it is to scientifically prove something. Much of research is about developing collaborations, planning meticulously and understanding the results of data. I started this research project in 2009 and applied for funding from 12 research bodies before obtaining funding from the Medical Research Council and the Royal College of Surgeons of England to complete the DPhil. I put in over 10 other major grant applications, developed a series of rodent models, helped in the design, structure and execution of a national biobank and 3 clinical trials. I learnt how to perform a number of basic laboratory assays ranging from cell culture, through to immunohistochemistry and mitochondrial functional assays.

In reality, research has been one of the hardest but also most enjoyable things I have ever done so when I summarise the work described in this thesis I ask myself "*what have I really proven and added to our understanding of organ donation?"* I recognise that there remains aspects of the research that requires clarification and further proof. The purpose of this conclusive Chapter is to draw together the Chapters and highlight further and future avenues for research.

Models of organ donation; imperfect science needing access to large scale biobanks

The development of animal models was not novel research. As described in *Chapter 3*, many other research groups have developed and use these models previously. The major challenge for me was to develop these models in a new place, with no previous experience acquiring the required licenses and developing the required skills. I learnt

that the models all have their limitations but provide us with the most controlled environment to investigate the multifaceted injury donor organs are exposed to. In developing the models I gained experience in understanding how animal experiments should be designed and executed. If mistakes and pitfalls are present in the use of animal models I have either found them or unfortunately fell into them.

Creating a control for a model such as the brain death (BD) model is extremely difficult if not impossible. At best, isolating the individual effects of BD and controlling for these, offer the best environment for controlled experiments. However, when these approaches are used they do not take into consideration the impact of interactions of multiple systems or disturbances of multiple homeostatic mechanisms.

One of the other limitations with using samples from these rodent experiments is that the duration of BD was limited to four hours. Ideally I would have repeated the experiments looking at longer periods. It could be that the predomination of mitochondrial and metabolic cellular dysfunction causes changes to other cellular pathways. One of the limitations of the model was to get reproducibility at durations longer than four hours.

Rodent work, for exploring the effect of organ donation on organ quality and pathways of injury and repair, should be backed up with where possible correlations in human samples. Part of the problem with this previously was that there were no large scale biobanks available to support this type of research. Alongside conducting the animal and basic science work described in this thesis during my DPhil I also contributed to the

establishment of the Quality in Organ Donation (QUOD) initiative; a national biobank of now over 800 donors available for research. My exact role in establishment of the QUOD biobank included establishing the business case to support funding, development of the protocols for sample collection, logistics and navigating the course of achieving ethical approval. I also designed the access pathway for researchers to gain access to the samples. This work was carried out alongside others including Maria Kaisar, Sandrine Rendel and others under the guidance of Prof R Ploeg.

Although establishment of a biobank does not constitute basic research, there were a number of research related skills I developed during establishing the biobank including understanding how sample quality affects pre-analytical variability and how this can be minimised in the biobank setting. This was a hugely complex to reconcile in the realms of organ donation whereby organ donors are in different hospitals variable distances from where samples will be processed and housed. In addition, the ethical considerations concerning sample procurement in the deceased donor setting was important and I worked with Karen Melham (Ethicist) to understand this process and achieve ethical approval for the project.

Although I did not have an opportunity to work with any of the samples procured into the QUOD biobank, the biobank remains an important tool for other researchers to use for research into organ quality and to understand how organs become injured during the donation process. Many of the findings from the animal work conducted in this thesis will go on to be validated in human samples. Access to large-scale biobanks will

allow us to circumvent the heterogeneity of the donor population and thus the usual issues concerning using small sample sizes.

Brain death leads to alterations in mitochondrial function and metabolism

Using the BD model and proteomics I surveyed the changes in the protein makeup of cells following BD. The advantage of using this approach was that it was performed in a non biased manner and revealed widespread alterations in the proteome suggesting alterations in mitochondrial function in addition to a number of other pathways following BD. Proteomics itself carries a number of limitations, firstly, we looked at a conglomeration of mixed cells taken from the renal cortex. Although certain proteins are specific to certain cell types we can only develop hypothesise to test based on this technology and need further confirmatory tests. A number of groups are now developing techniques to address this issue by using a number of histological techniques along-side proteomics to either isolate cellular subsets of interest or even look at the proteome of a single cell (339, 340).

Systems biology is emerging as a new area of research that essentially uses techniques such as multi-platofrm –omics together with mathematical modeling . It is a sub-specialty of science bought about by advancements in mass spectrometry and computational science. The integration of –omic techniques described in this thesis forms a rudimentary form of systems biology. Developing systems biology based approach in discoveries of pathways and their interactions will open new frontiers in transplantation.

In Chapter *4* the results of the proteomic analysis were highly suggestive of alterations in mitochondrial function and metabolic changes, in addition to a number of other different pathways. Some of these additional pathways were not fully explored including the NRF2 mediated pathway or fatty acid metabolism, both of which would be of high interest to investigate in tissues from animals or from human samples from the QUOD biobank. Altered fatty acid oxidation (FAO) has been linked to the development of subsequent renal fibrosis (341). In proximal tubular epithelial cells for example, during ischemia leading to acute kidney injury, authors have noted changes in cellular phenotype where proximal tubular epithelial cells transition into mesenchymal phenotype and progressive renal interstitial fibrosis ensues (341, 342). How the process of alterations in BD may lead to alterations in FAO and subsequent effects on renal fibrosis and long term kidney survival need to be investigated.

Indeed, further work into mechanisms of interaction between mitochondria and cellular autophagy networks would be of great interest in the areas of DBD donation. Much work in this area has been described with regards to IRI and parallels could be drawn with investigations into BD (343, 344). Decuypere and colleagues are currently exploring this question with collaborators from the University of Groningen.

Chapter *5* looked into how metabolism is altered following BD. The 1H-NMR spectroscopic evaluation was limited to under 30 metabolites. Our hope is to develop more accurate and in-depth techniques to be able to sample across a wider range of metabolites. Huang and colleagues in the Kessler laboratory in Oxford are developing LC and GC-MS techniques for identifying a range of metabolites. As part of a smaller

project samples from BD animals were prepared for GC-MS analysis, however, reliably and reproducibly identifying the spectral peaks was not possible at the stage of submitting this thesis. Lipidomics would be of interest in the context of determining the effects on FAO as mentioned previously. We recently reviewed the field of lipidomics with regards to clinical research highlighting future avenues for research (345).

Although investigating metabolite alterations in tissues give clues to how metabolic disturbances are interplaying with a stressor, metabolism is often dynamic. Further detailed insights into *in-vivo* metabolism could be gained from evaluating metabolic changes using real time in-vivo assessments through measuring flux or through using imaging. Tyler and colleagues at the department of Anatomy, Physiology and Human Genetics at the University of Oxford have developed techniques for real time in-vivo metabolic imaging of ^{13}C-labelled metabolites using dynamic nuclear polarisation MRI based techniques (346). I plan to try to use this technologies for investigating real time effects of BD on metabolism as part of ongoing work.

The mitochondrial based research looked incompletely at the alterations in complex activity highlighted in Chapter *5*. Further work in this area could be completed by separating the complex II and III analysis allowing us to understand how these complexes work independently and are affected by BD. I speculate that part of the response could be mediated through sub-unit switch in complex IV, through a HIF mediated response, but this was not proven. Measuring RCR and oxygen consumption using a Clarke electrode also does not fully allow us to determine how the organelles function within a cell in-vivo. They are, at best, *ex-vivo* assessments in relatively

artificial conditions often providing substrates at supra-physiological levels. Using more advanced techniques for looking at metabolite handling and oxygen consumption *in vivo* would advance this area of research. The ATP level and levels of phosphocreatine in tissues following BD should be further detailed.

Modulating HIF in the donor could still be of clinical value

Chapter 6 demonstrated that HIF1α was up-regulated heterogenously following BD, but that the level corresponded with the degree of hemodynamic instability. The data was limited by information about other aspects of the control mechanisms. Developing a mouse BD model, although technically challenging would allow the further dissection of this mechanism by increasing the number of usable anti-bodies but also by allowing the use of PHD KO mice. Issues surrounding breeding would need to be considered however, including any associated developmental issues arising as a consequence of the KO system used. Yamamoto and colleagues are currently developing an HIF inducible KO system in the Ratcliffe-Pugh laboratory and this would be of great interest in investigating this area but also within the area of renal IRI. In order for the mouse model to be of particular use in the field of BD, we would need to ensure longer periods of post BD induction were possible i.e. up to 6-8 hours.

I was unable to demonstrate in this thesis any convincing discernable benefit of the PHD inhibitors tested following BD in Chapter 6. Although disappointing, the results were not conclusive regarding a lack of benefit of other compounds, or indeed other preconditioning agents that up-regulate HIF1α or its downstream genes. There were

unclear short-term affects of the compounds on mitochondria and I speculated that the DMOG response was due to an off target effect. This area requires further understanding of the influence of PHD inhibitors on mitochondrial biology.

In retrospect, using isoflurane, the anaesthetic responsible for causing an artificially raised HIF1α level in the long sham controls, could have been tested in the BD setting. This has been shown to have some benefit in models of IRI and could easily be translatable to the clinical setting.

Other avenues to attempt to rescue organs from brain dead donors could have also been tested as alternative hypotheses as suggested in Chapter 4 and 5. For example, a number of groups have recently demonstrated that high ketone diets or short periods of nutrient deprivation can attenuate ischemia reperfusion injury. These strategies would be interesting to test in the BD setting and I have now gained animal licence to do this.

Influence of age and obesity on organ donation

The introduction to the thesis outlined the changing demographics of organ donors. The thesis concentrated on cellular alterations following BD using a haemorrhagic model. If time allowed it would have been interesting to test the influence of age and obesity on the magnitude of the cellular changes following BD. This is an area of significant future potential. Models of age and obesity both carry significant limitations,

but their use combined with the QUOD database of donors of different ages and BMIs could be of interest.

Rescuing organs using machine preservation

Although there are a number of alterations to donor organs during the donation process when is the best time to intervene? Clearly in DCD donors there remains legislative issues concerning the diagnosis of death and the role of pre or ante-mortem inventions. It could be that "conditioning" of an organ could occur once the organ is explanted. The advent of hypothermic machine perfusion (HMP) and now normothermic machine perfusion (NMP) make this period a real opportunity to intervene to rescue an organ. NMP of the kidney, liver and other organs are one of the most exciting and dynamic areas of research currently in the transplant field. The Consortium on Organ Preservation in Europe (COPE) was established to bring together centres across Europe interested in preserving organs for the purposes of transplantation.

During my DPhil I wrote, together with others, an European FP7 grant application for COPE that was awarded in 2012 to support clinical trials in machine preservation. My exact role in this Consortium was to draft the preliminary and main application. To design the outline of the clinical trials and estimate associated costs. To put together a technical annex describing the components of Work Packages responsible for delivering the trials and putting together funding packages as part of the a negotiated agreement between European centres. I also had a more detailed role in designing sample

collection strategies for two of the work packages and also drafted ethical approvals for the clinical trials in the UK.

The COPE trials also aim to deliver biomarkers to predict the outcomes of transplantation. Interventions during NMP could particularly result in an improved metabolic status of an organ and potentially have benefits for mitochondrial function. This is currently being investigated by David Nasralla, Professor Peter Friend and others.

Predicting outcomes following transplantation

Although understanding how organs become injured and developing novel ways to protect and preserve organs is of importance, the other area for major breakthroughs in transplant related research would be in identifying biomarkers to predict the outcomes of transplant. Donor and organ associated risk stratification tools have previously been developed but lack the sensitivity and specificity required to accurately predict the outcomes of transplantation.

This is an area of work the QUOD and COPE biobanks could contribute significant. Maria Kaisar and others are currently investigating novel avenues to predict the eGFR of transplanted kidneys using proteomics of samples taken from the donor. This research could revolutionise the risk stratification of kidneys prior to transplantation.

In addition to identifying biomarkers the increasing role of “big data” in understanding interactions between risk factors could play a significant future role in allowing us to risk stratify organs or donors with better accuracy. As part of ongoing work I am interrogating the UNOS database using data mining tools to develop an algortyhm which will allow us to predict the outcomes of kidney and liver transplantation with a greater degree of accuracy.

Conclusion

As with most research I have ended up probably asking more questions than I have provided answers for. I have shown that metabolic disturbance and alterations in mitochondrial function occur in the kidney following BD, although the exact mechanism for this remains undetermined and whether this is an organ specific event. There are a number of important future avenues for research in the field of organ donation and preservation, some of the most exciting being related to machine preservation.

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Publications arising from DPhil

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Appendix

Chapter 4. IRI proteomic data. A comparison of significantly altered proteins comparing the ischemic kidney against the control groups (P<0.05).

Accession	Anova (p)*	Fold	Description
tr|F1LNM3|F1LNM3_RAT	4.16E-06	587.51	Protein Ank3 OS=Rattus norvegicus GN=Ank3 PE=2 SV=2
tr|G3V7L4|G3V7L4_RAT	0.03	2.18	Protein Cdh16 OS=Rattus norvegicus GN=Cdh16 PE=4 SV=1
sp|Q71LX6|XIRP2_RAT	3.40E-03	4.61	Xin actin-binding repeat-containing protein 2 OS=Rattus norvegicus GN=Xirp2 PE=1 SV=1
tr|D3ZEV8|D3ZEV8_RAT	1.77E-03	2.26	Protein Susd2 OS=Rattus norvegicus GN=Susd2 PE=4 SV=1
sp|P20673|ARLY_RAT	0.01	2.06	Argininosuccinate lyase OS=Rattus norvegicus GN=Asl PE=2 SV=1
tr|F1M1J2|F1M1J2_RAT	0.05	2.58	Protein Neb (Fragment) OS=Rattus norvegicus GN=Neb PE=2 SV=2
tr|D3ZZM8|D3ZZM8_RAT	8.23E-03	2.89	Uncharacterized protein OS=Rattus norvegicus PE=3 SV=1
tr|G3V709|G3V709_RAT	0.02	2.48	Nicotinate phosphoribosyltransferase OS=Rattus norvegicus GN=Naprt1 PE=4 SV=1
sp|Q07439|HSP71_RAT	8.09E-04	4.71	Heat shock 70 kDa protein 1A/1B OS=Rattus norvegicus GN=Hspa1a PE=2 SV=2
sp|D3ZZL9|GCC2_RAT	2.52E-03	2.23	GRIP and coiled-coil domain-containing protein 2 OS=Rattus norvegicus GN=Gcc2 PE=1 SV=1
sp|Q5I0D7|PEPD_RAT	6.20E-03	2.4	Xaa-Pro dipeptidase OS=Rattus norvegicus GN=Pepd PE=2 SV=1
tr|D3ZNI6|D3ZNI6_RAT	0.02	2.47	Protein Dnajc13 OS=Rattus norvegicus GN=Dnajc13 PE=2 SV=2
tr|D4ADC2|D4ADC2_RAT	7.64E-05	4.25	Protein Pric285 OS=Rattus norvegicus GN=Pric285 PE=2 SV=1
tr|D4A0U3|D4A0U3_RAT	8.89E-04	12.85	Protein Zfp638 OS=Rattus norvegicus GN=Zfp638 PE=4 SV=1
tr|D3ZQ89|D3ZQ89_RAT	4.34E-04	63.49	E1A binding protein p400, isoform CRA_a OS=Rattus norvegicus GN=Ep400 PE=4 SV=1
sp|P31232|TAGL_RAT	1.19E-03	3.84	Transgelin OS=Rattus norvegicus GN=Tagln PE=1 SV=2
sp|P14141|CAH3_RAT	0.03	7.5	Carbonic anhydrase 3 OS=Rattus norvegicus GN=Ca3 PE=1 SV=3
sp|O35550|RABE1_RAT	3.43E-03	11.83	Rab GTPase-binding effector protein 1 OS=Rattus norvegicus GN=Rabep1 PE=1 SV=1
sp|P14480-2|FIBB_RAT	0.01	5.28	Isoform 2 of Fibrinogen beta chain OS=Rattus norvegicus GN=Fgb
tr|F1M6P0|F1M6P0_RAT	8.27E-03	3.25	Enolase (Fragment) OS=Rattus norvegicus PE=3 SV=2
tr|F1M4N6|F1M4N6_RAT	1.71E-03	2.48	Protein Dock3 (Fragment) OS=Rattus norvegicus GN=Dock3 PE=2 SV=2
tr|M0R7B0|M0R7B0_RAT	1.23E-03	2.48	Uncharacterized protein (Fragment) OS=Rattus norvegicus PE=4 SV=1
tr|D3ZKI5|D3ZKI5_RAT	0.02	2.23	PHD finger protein 3 (Predicted) OS=Rattus norvegicus GN=Phf3 PE=4 SV=1

tr|D3Z837|D3Z837_RAT	0.01	7.36	CDC42 binding protein kinase gamma (DMPK-like) (Predicted) OS=Rattus norvegicus GN=Cdc42bpg PE=4 SV=2
tr|Q5XFV4|Q5XFV4_RAT	0.03	23.16	Fabp4 protein OS=Rattus norvegicus GN=Fabp4 PE=2 SV=1
tr|D3ZB48|D3ZB48_RAT	1.01E-03	2.53	Hook homolog 1 (Drosophila) (Predicted) OS=Rattus norvegicus GN=Hook1 PE=4 SV=1
tr|D4A9D6|D4A9D6_RAT	0.02	2.85	DEAH (Asp-Glu-Ala-His) box polypeptide 9 (Predicted) OS=Rattus norvegicus GN=Dhx9 PE=4 SV=1
tr|D4AE81|D4AE81_RAT	0.04	2.13	Protein Plekhg1 OS=Rattus norvegicus GN=Plekhg1 PE=4 SV=1
tr|D3ZPR0|D3ZPR0_RAT	0.02	2.68	Chromosome segregation 1-like (S. cerevisiae) (Predicted) OS=Rattus norvegicus GN=Cse1l PE=4 SV=1
sp|Q63151|ACSL3_RAT	0.04	2.05	Long-chain-fatty-acid--CoA ligase 3 OS=Rattus norvegicus GN=Acsl3 PE=1 SV=1
tr|G3V679|G3V679_RAT	0.01	4.68	Transferrin receptor protein 1 OS=Rattus norvegicus GN=Tfrc PE=4 SV=1
sp|P97521|MCAT_RAT	1.01E-04	7.19	Mitochondrial carnitine/acylcarnitine carrier protein OS=Rattus norvegicus GN=Slc25a20 PE=1 SV=1
tr|R9PXZ0|R9PXZ0_RAT	0.01	5.25	Sodium channel protein type 10 subunit alpha OS=Rattus norvegicus GN=Scn10a PE=4 SV=1
sp|Q9EQP5|PRELP_RAT	0.04	3.15	Prolargin OS=Rattus norvegicus GN=Prelp PE=2 SV=1
sp|B1H267|SNX5_RAT	5.12E-05	2.72	Sorting nexin-5 OS=Rattus norvegicus GN=Snx5 PE=1 SV=1
sp|P28826|MEP1B_RAT	0.04	2.55	Meprin A subunit beta OS=Rattus norvegicus GN=Mep1b PE=1 SV=3
sp|P05370|G6PD_RAT	1.86E-04	12.78	Glucose-6-phosphate 1-dehydrogenase OS=Rattus norvegicus GN=G6pdx PE=1 SV=3
sp|Q6AXX6|F213A_RAT	3.87E-04	3.04	Redox-regulatory protein FAM213A OS=Rattus norvegicus GN=Fam213a PE=1 SV=1
sp|P51556|DGKA_RAT	0.01	2.85	Diacylglycerol kinase alpha OS=Rattus norvegicus GN=Dgka PE=2 SV=1
tr|F1M7B8|F1M7B8_RAT	0.04	6.84	Protein Ube3a OS=Rattus norvegicus GN=Ube3a PE=4 SV=2
sp|P0C5H9|MANF_RAT	9.85E-03	4.42	Mesencephalic astrocyte-derived neurotrophic factor OS=Rattus norvegicus GN=Manf PE=1 SV=1
tr|F1LQ70|F1LQ70_RAT	0.03	2.45	Arachidonate 12-lipoxygenase, leukocyte-type OS=Rattus norvegicus GN=Alox12 PE=2 SV=1
tr|Q5M8C3|Q5M8C3_RAT	6.94E-03	3.35	Protein Serpina4 OS=Rattus norvegicus GN=Serpina4 PE=2 SV=1
sp|O55004|RNAS4_RAT	0.02	2.75	Ribonuclease 4 OS=Rattus norvegicus GN=Rnase4 PE=1 SV=1
tr|D3ZCQ8|D3ZCQ8_RAT	0.03	2.62	Protein Rfx8 OS=Rattus norvegicus GN=Rfx8 PE=4 SV=2
sp|Q9ESS6|BCAM_RAT	0.02	6.55	Basal cell adhesion molecule OS=Rattus norvegicus GN=Bcam PE=2 SV=1
sp|Q63802|WEE1_RAT	0.01	5.04	Wee1-like protein kinase OS=Rattus norvegicus GN=Wee1 PE=1 SV=1
sp|O08962|KCNH2_RAT	0.02	4.42	Potassium voltage-gated channel subfamily H member 2 OS=Rattus norvegicus GN=Kcnh2 PE=1 SV=1
tr|F1M9M2|F1M9M2_RAT	0.02	2.15	Uncharacterized protein (Fragment) OS=Rattus norvegicus PE=4 SV=2
sp|P12369|KAP3_RAT	2.19E-03	5.64	cAMP-dependent protein kinase type II-beta regulatory subunit OS=Rattus norvegicus GN=Prkar2b PE=1 SV=3
sp|P14669|ANXA3_RAT	0.01	3.45	Annexin A3 OS=Rattus norvegicus GN=Anxa3 PE=1 SV=4

sp|Q3KRC4|GPC5C_RAT	8.97E-03	3.38	G-protein coupled receptor family C group 5 member C OS=Rattus norvegicus GN=Gprc5c PE=2 SV=2
tr|P70521|P70521_RAT	0.02	5.15	Macrophage stimulating 1 (Hepatocyte growth factor-like) (Precursor) OS=Rattus norvegicus GN=Mst1 PE=2 SV=1
tr|D4A8X0|D4A8X0_RAT	0.02	2.13	Protein Zbtb4 OS=Rattus norvegicus GN=Zbtb4 PE=2 SV=1
tr|D3ZJ70|D3ZJ70_RAT	0.01	5.32	Protein Klk13 OS=Rattus norvegicus GN=Klk13 PE=3 SV=1
tr|M0R430|M0R430_RAT	0.01	5.58	Protein Mpnd OS=Rattus norvegicus GN=Mpnd PE=4 SV=1
tr|Q9EP88|Q9EP88_RAT	0.01	2.44	Brain mitochondrial carrier protein BMCP1 OS=Rattus norvegicus GN=Slc25a14 PE=2 SV=1
sp|Q99NI4|IPMK_RAT	0.03	3.68	Inositol polyphosphate multikinase OS=Rattus norvegicus GN=Ipmk PE=2 SV=1
tr|D3ZU55|D3ZU55_RAT	0.04	2.73	Protein Foxk1 OS=Rattus norvegicus GN=Foxk1 PE=4 SV=1
tr|D3ZHV3|D3ZHV3_RAT	0.02	4.44	Metallothionein OS=Rattus norvegicus GN=LOC100362623 PE=3 SV=1
tr|G3V6S7|G3V6S7_RAT	0.01	12.35	Interleukin 5 receptor, alpha OS=Rattus norvegicus GN=Il5ra PE=4 SV=1
tr|M0RCJ9|M0RCJ9_RAT	5.07E-03	2.47	Protein LOC100911027 OS=Rattus norvegicus GN=LOC100911027 PE=4 SV=1
tr|F1M365|F1M365_RAT	0.02	34.13	Protein Ints9 (Fragment) OS=Rattus norvegicus GN=Ints9 PE=4 SV=2

Western blotting protocol for HIF1α

Running samples on gel:

1. Warm samples to 30oC for 5 min, mix samples
2. Centrifuge samples at 4000 RCT for 1 min
3. Place gel into gasket, wash the wells using running buffer
4. Add samples (6ul marker, 6ul sample)
5. Run gels at 100v (mA is usually 60-80mA for 2 gels) (ensure black to black and red to red)
6. Run the gel until the blue marker is 0.5cm from the bottom

Preparing for transfer:

7. 4x pieces of cut paper and a membrane per gel being run
8. Place membrane into MeOH
9. Wash twice using dH2O
10. Add transfer buffer
11. Rock for 2min
12. Prepare black gasket adding transfer buffer to 1.5 finger breaths from bottom.
13. Soak the sponges in the transfer buffer- placing them on the black gasket to dry
14. Collect tray and fill with the transfer buffer
15. Collect green gasket- empty out the running buffer
16. Place the gel into small plastic tray containing transfer buffer- the gel will begin to peal away
17. Open the cassette and place the sponge with a single piece of white paper
18. Place gel carefully onto paper. Use the glassware to support the gel whilst making the transfer. Ensure the orientation of the gel is correct.
19. Place the membrane on-top of the gel
20. Place 2xtransfer papers on-top of the gel (sometimes more are necessary)
21. Close the sandwich
22. Place into gasket
23. Run for 1.5 hour at 70v

Application of antibodies:

24. Thaw antibodies- placing in water/room temperature
25. Prepare milk for blocking
26. Remove cassette from gasket once complete, open carefully pealing away the sponge and the gel
27. Cut paper accordingly
28. Place in milk for 2 min (to block antigens)
29. Poor off milk
30. Place cut membrane into smaller containers
31. Add primary antibody solution and mix for 1 hour on rocker
32. Poor of antibody and wash with PBS/T (twice)
33. Add secondary antibody if required and mix for 1 hour on rocker
34. Remove antibody and wash with PBS/T for 5 min on rocker, repeat 3 times
35. Leave membrane in PBS/T until ready for making films

36. Samples can be kept with PBS/T, antibodies overnight rocking in the cold room

Preparing to making films:

37. Turn on processor- requires at least 20min to warm up, ensure fluids are accurately filled
38. Prepare SSWD/WS solutions
39. Collect: Acetate sheet/clear plastic cover/exposure cassette
40. Cut the plastic cover in half and open
41. Cut acetate sheet in half
42. Place one half of the acetate sheet into cassette
43. Solution concentration and volumes will depend on the size of membranes
44. Strong solutions: 1:1 ratio (used for weak signals)
45. Weaker solutions: 1:10 (used for stronger signals e.g. B-actin)
46. Add the SSWD first followed by the SSWS
47. Mix using pipette and place into open plastic cover
48. Place membranes onto solutions mixing at least 4 times
49. Aggregate membrane samples you would like to compare
50. Ensure they are lined up but not touching
51. Close the plastic cover and leave for 5 minutes
52. After 5 minutes open plastic cover and place membranes onto acetate sheet ensuring orientation and membranes are aggregated appropriately
53. Place other half of acetate sheet on-top and tape edges

Processing films:

54. Will need: Scissors, tissue paper
55. In dark room ensure red lights are on before taking out films
56. Take out films carefully, ensure that excess films are used is possible
57. Cut corner and fold edge to ensure that the orientation is known and so the film can be lifted off the acetate quickly
58. Place the film on the acetate for the appropriate length of time (1s-5min)- ensure technique
59. Take off and place into processor- evaluate film to see if repeat required