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The Implications of Brain Death in Donor Lung Injury:
Investigation and Blockade of the Endothelin Axis

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Abstract

Background: Lungs donated for transplantation are primarily sourced from brain dead organ donors. The process of brain death causes lung injury through haemodynamic instability, catecholamine fluctuations and activation of inflammatory pathways. Recent literature has implicated endothelin-1 in transplantation related lung dysfunction. Therefore, inhibition of endothelin signalling may reduce or reverse endothelin related vasoconstriction and inflammation. Tezosentan, a dual endothelin antagonist, is able to be nebulised to directly target the lungs. This aims to avoid systemic adverse effects, specifically hypotension. Nebulised tezosentan has been previously demonstrated to reduce pulmonary hypertensive responses to systemic inflammatory states.

Objective: This thesis sought to investigate the role of the pulmonary endothelin axis after brain death in a novel, clinically relevant, ovine model of brain death. Furthermore, the effects of nebulised tezosentan on pulmonary haemodynamics and inflammation in donor lungs after brain death were assessed.

Methods: Twenty-four merino cross ewes were randomised into four equal groups ($n=6$ per group). These were control/placebo, control/tezosentan, brain dead/placebo and brain dead/tezosentan. Following induction of general anaesthesia and placement of invasive monitoring, brain death was induced in allocated animals by inflation of an extradural catheter. Animals were then supported in an intensive care unit environment for 24 hours. Management reflected human donor management, including administration of vasopressors, inotropes and hormonal resuscitation therapy. Nebulised tezosentan was administered at 13 and 18 hours. The endothelin axis, and the effects of its antagonism, was assessed by physiological monitoring, blood gas analysis, ELISA, histology and immunohistochemistry. Injury of other organs was assessed using standard biochemical markers.

Results: A total of 25 animals underwent the experimental protocol. One animal died during induction of brain death from ventricular fibrillation and was replaced.

Early evidence of endothelin-1 and big endothelin-1 elevations were seen in brain dead animals that received placebo, reaching maximal levels at one and six hours after brain death, respectively. This was not replicated in the brain dead animals that received tezosentan. Systemic endothelin-1 levels were not increased by tezosentan administration. Immunohistochemistry identified the endothelin axis in pulmonary tissue, but this was not different between groups. Induction of brain death resulted in tachycardia and hypertension, followed by haemodynamic collapse. Mean pulmonary artery pressure rose significantly at induction ($186 \pm 20\%$) and remained elevated throughout the protocol in those that received placebo. Additionally, right ventricular stroke work increased 25.9% above baseline by 24 hours. Mean pulmonary blood pressure in brain dead animals that received tezosentan showed similar elevations with induction of brain death, but was significantly lower at 24 hours compared to those that received placebo. Systemic markers of cardiac and hepatocellular injury were significantly elevated in brain dead animals, with no evidence of renal dysfunction. Tezosentan administration did not adversely affect systemic haemodynamics and there was no evidence of adverse effects on remote organs.

Conclusions: This novel, clinically relevant, ovine model demonstrated that the endothelin axis is able to be modulated after brain death, reducing the observed elevations in pulmonary blood pressure. Early endothelin release possibly contributes to the previously recognised inflammation and cardiopulmonary injury in potential donors. Further investigation is required to determine the exact mechanism of the observed results. In the future, antagonism of the endothelin axis after brain death may lead to novel treatments that improve the function of pulmonary and cardiac allografts for transplantation.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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This paper is presented as Chapter 2 (literature review), with greater expansion of sections specifically relevant to the current thesis.

Contributor	Statement of contribution
Ryan Watts (Candidate)	Wrote the paper (90%)
Ogilvie Thom	Wrote and edited paper (5%)
John Fraser	Overall conception of paper (100%) Wrote and edited paper (5%)

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This paper is presented as Chapter 4, with some additional editing to better contextualise this chapter in the setting of the overall thesis.

Contributor	Statement of contribution
Ryan Watts (Candidate)	Performed animal experiments (35%) Performed laboratory experiments (50%) Design of laboratory experiments (through selection of antibodies, etc - 20%) Statistical analysis of data (30%) Wrote the paper (75%)
Izabela Bilska	Performed laboratory experiments (50%) Statistical analysis of data (20%)

	Wrote and edited paper (5%)
Sara Diab	Performed animal experiments (35%)
Kimble Dunster	Designed experiments (15%) Wrote and edited paper (6%)
Andrew Bulmer	Wrote and edited paper (7%)
Adrian Barnett	Statistical analysis of data (50%) Wrote and edited paper (2%)
John Fraser	Oversaw study and developed concept (100%) Designed animal experiments (100%) Wrote and edited paper (5%)
Margaret Passmore	Designed laboratory experiments (80%)
Other team members for animal clinical work (not contributing directly to writing of paper), acknowledged in paper: Elissa Milford, Scott Temple, Ben Anderson, Michael Manning, Jonathon Taylor and Sue Christie	Performed animal experiments (30%)

Contributions by others to the thesis

Professor Fraser conceptualised the study, developed and validated the 24 hour model of brain death, and received the primary grant for funding of the work. Additionally, he provided technical advice and oversight for the entire project.

Sara Diab assisted with animal experiments, providing clinical skills and interventions in caring for the animals throughout the study. Furthermore, Sara assisted in providing clinical oversight of other team members in caring for the animals.

Kimble Dunster contributed through animal protocol development, device selection, and development and provision of technical skill and support for all of the animal studies. His previous animal work experience was invaluable.

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Andrew Bulmer provided advice and critical analysis of papers submitted for publishing (where named) and presented in this thesis.

Margaret Passmore provided the protocols used for histological and immunohistochemical analysis and taught the laboratory skills necessary to allow processing and analysis of samples. Dan Kilburn assisted with slide preparation.

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Statement of parts of the thesis submitted to qualify for the award of another degree

Biochemical marker data appears through collaboration and was submitted by Izabela Bilska for BSc Honours, Griffith University, 2013, degree awarded December 2013. The current thesis was written separately from Izabela's work, and I (Ryan Watts) independently wrote Chapter 4 (submitted for publishing, with input from colleagues as disclosed). Izabela refers to a subset of pulmonary haemodynamic results in her work. Collaboration with Izabela results in the following specific sections being referred to in her work:

- Sections 4.3.4 and 5.2.5 – Methods of Histological and Tissue Analysis. Independently written from collaborative data (shared laboratory instructions). Note that different antibodies were used
- Section 4.3.6 and 5.2.7 – Method of Biochemical Analysis. Independently written from collaborative data
- Section 4.4 and 5.3 – Statistical Analysis. Analysis of biochemical data (particularly ANOVA) drawn from collaborative plans for data analysis. Statistics replicated independently
- Section 4.5.3 and 5.4.3 – Results of Histological and Tissue Analysis. Independently written from collaborative data. Note that these results reflect the use of different antibodies
- Section 4.5.5 and 5.4.5 – Results of Biochemical Analysis. Independently written from collaborative data
- Figure 12 – Cardiac Markers of Injury. Independently generated from collaborative data
- Section 4.8 – Appendix 1. Adapted from previously submitted work by myself (Ryan Watts) for MPhil confirmation. Izabela Bilska adapted this work
- Section 4.9 – Appendix 2. Adapted in both theses from shared instructions from the Critical Care Research Group. Independently written
- Section 4.10 - Appendix 3. Some aspects of the results of the project have been adapted in both theses. Independently written
- Table 8 - Results of Biochemical Analysis. Data presented in both theses. Independently written for presentation

Dr Scott Temple submitted a related project for his MBBS Honours, University of Queensland, 2012, degree awarded December 2012. Section 4.8 – Appendix 1 was adapted from previous work by me (Ryan Watts) for MPhil confirmation. Scott Temple adapted this work for his thesis.

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Abbreviations Used in the Thesis

ABG - Arterial Blood Gas	ECE - Endothelin Converting Enzyme	NMDA - N-Methyl D-Aspartic Acid
ADM – Aggressive Donor Management	EDTA - Ethylene-Diamine-Tetra-Acetic Acid	NO - Nitric Oxide
ALT - Alanine Aminotransferase	EGFR - Epidermal Growth Factor Receptor	NOS - Nitric Oxide Synthase
AST - Aspartate Aminotransferase	EPO - Erythropoietin	P(A-a)O ₂ - Alveolar-arterial Oxygen Gradient
ALP - Alkaline Phosphatase	ET - Endothelin	PaO ₂ - Arterial Oxygen Partial Pressure
ANOVA - Analysis of Variance	ET _{RA} - Endothelin A Receptor	PAOP - Pulmonary Arterial Occlusion Pressure
ANZOD - Australian and New Zealand Organ Donor (Registry)	ET _{RB} - Endothelin B Receptor	PaCO ₂ - Arterial Carbon Dioxide Partial Pressure
APC - Antigen Presenting Cell	EVLP - Ex-Vivo Lung Perfusion	PGD - Primary Graft Dysfunction
APR - Acute Phase Response	F _i O ₂ - Fraction of Inspired Oxygen	PGI ₂ - Prostacyclin
ARDS - Acute Respiratory Distress Syndrome	G-CSF - Granulocyte Colony Stimulating Factor	PHTN - Pulmonary Hypertension
AT2 - Angiotensin 2	GGT – γ-Glutamyl Transferase	PNS - Parasympathetic Nervous System
BBB - Blood Brain Barrier	GMP - Guanosine Monophosphate	PST - Protocol Start Time
BD - Brain Death	HIF - Hypoxia Inducible Factor	PVRI - Pulmonary Vascular Resistance Index
Big ET-1 - Pro-Endothelin-1	HO - Heme Oxygenase	ROS - Reactive Oxygen Species
BIT - Brain Death Induction Time	ICP - Intra-Cranial Pressure	RV - Right Ventricle

BDP - Brain Dead/Placebo Group	ICU - Intensive Care Unit	RVSWI - Right Ventricular Stroke Work Index
BDT - Brain Dead/Tezosentan Group	IFN - Interferon	SAH - Subarachnoid Haemorrhage
BSA - Body Surface Area	Ig - Immunoglobulin	SIRS - Systemic Inflammatory Response Syndrome
BOS - Bronchiolitis Obliterans Syndrome	IL - Interleukin	SVI - Stroke Volume Index
C - Complement	IRI - Ischaemia-Reperfusion Injury	SvO ₂ - Mixed Central Venous Oxygen Saturation
CD - Cluster of Differentiation	LD - Living Donor	SVRI - Systemic Vascular Resistance Index
CFAS - Calibrator for Automated Systems	LPS - Lipopolysaccharide	TBI - Traumatic Brain Injury
CI - Cardiac Index	LTx - Lung Transplant	TBS(-T) - Tris-Buffered Saline (with Triton)
CK-MB - Creatine Kinase MB Fraction	LV - Left Ventricle	TGF - Transforming Growth Factor
CNS - Central Nervous System	LVSWI - Left Ventricular Stroke Work Index	T _H - Helper T Cell
COX - Cyclo-Oxygenase	MAP - Mean Arterial Pressure	TIMP - Tissue Inhibitor of Metalloproteinases
CP - Control/Placebo Group	MCP - Monocyte Chemoattractant Protein	TLR - Toll Like Receptor
CPP - Cerebral Perfusion Pressure	MMP - Matrix Metalloproteinase	TNF - Tumour Necrosis Factor
CSF - Cerebrospinal Fluid	mPAP - Mean Pulmonary Artery Pressure	TNFR - Tumour Necrosis Factor Receptor
CT - Control/Tezosentan Group	mRNA - Messenger Ribonucleic Acid	T _{reg} - Regulatory T Cell
DCD - Donation after Circulatory Death	NFκB - Nuclear Factor Kappa light chain enhancer of B cells	virIL-10 - Viral Interleukin 10

Chapter 1 - Introduction and Background to Transplantation

Organ transplantation is the gold standard treatment for patients with end stage solid organ failure. Australia has been performing solid organ transplantation for over 50 years.[1] Despite ongoing clinical success, a disparity remains between available organs and potential recipients. This continues to cause substantial morbidity and mortality.[2] According to the Australia and New Zealand Organ Donation (ANZOD) Registry, the number patients on the waiting list exceeds the number of organs transplanted each year (see also Figure 1).[3] As a consequence, patients continue to die waiting for a suitable organ.[4-6]

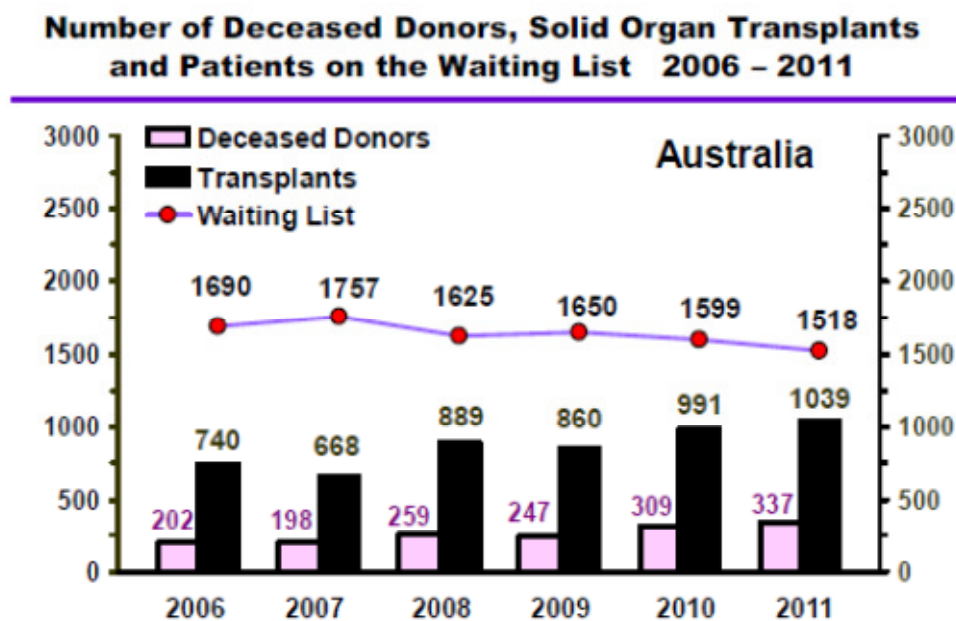


Figure 1: Comparison of Donors and Waiting List in Australia 2006-2011.

From the ANZOD Registry, 2012.[3] This graph demonstrates the number of deceased donors and transplants (bars), and the number of patients on the waiting list (line graph) for solid organ transplants in Australia.

1.1 Lung Transplantation

The first lung transplant (LTx) occurred in 1963, with the recipient surviving 18 days.[7, 8] The first successful heart-lung transplant was done in 1981, quickly followed by single and double LTx over the next five years.[7] Inflammatory and peri-procedural complications affect LTx similarly to other organs. This is further complicated by ongoing exposure to the external environment by the very nature of respiration.[9] Current post-LTx management produces one and three year survival rates of 83.5% and 65.8% overall.[10] Unfortunately, any gains are offset by the relative lack of available donors. Twelve LTx

occurred in Australia for December 2013, yet there were 155 registered patients on the waiting list.[11] The United States also continues to experience a shortage of donor lungs, achieving an average rate of transplants per donor of 0.37.[12]

1.2 Organ Donors

1.2.1 Living Donors

Living donors (LD) provide a viable option for transplantation of a number of solid organs, including kidney and liver.[13, 14] Kidneys from LD have better short and long term outcomes when compared to those from cadaveric donors.[15] This results directly from the pathological and inflammatory changes that occur after death. With rare exceptions, transplanted lungs can only be obtained from deceased donors. Traditionally, these were brain dead (BD)/beating heart donors. Recent work to expand the donor pool has resulted in the inclusion of donation after circulatory death (DCD)/non-beating heart donors.[16, 17]

1.2.2 Donation after Brain Death

Although outcomes from DCD donors have become increasingly successful in recent times,[16, 18] most organs still come from BD donors. The very nature of brain death requires an irreversible central nervous system injury to have occurred, either through direct trauma, hypoxia or other mechanisms. A systemic acute phase reaction occurs with serious brain injury[19] creating a proinflammatory environment. Thus, inflammatory upregulation in potentially transplantable organs has already started prior to BD. Brain death then independently causes inflammatory, haemodynamic and endocrine effects that cause further injury.[20-24] Finally, ischaemia-reperfusion injury (IRI) generates reactive oxygen species (ROS), which are responsible for complement activation and cytokine release, further driving inflammation.[24-26]

1.2.3 Donation after Circulatory Death

Although the first LTx was performed with a DCD donor,[8] BD donors have become the primary source of lung allografts. Animal studies of DCD donors continued until the late 1990's, and lead to the eventual return of this form of donation in humans more recently.[18] This has made some inroads in addressing the donor pool shortfall. Donation after circulatory death donors accounted for 25.5% of all donors in 2012.[11] This number has remained stable, with 24.6% of lung transplants in the first two months of 2015 coming from DCD donors.[27]

Although initial LTx case reports from DCD donors were promising, early observational studies suggested that outcomes were less satisfactory than BD donors.[28, 29] This was refuted by De Vleeschauwer *et al*, who reported their experience with DCD LTx donors.[18] Retrospective analysis of their DCD and BD donors over a four year period demonstrated similar short and medium term outcomes between groups. An Australian collaboration reported the largest cohort of DCD donor outcomes, following 72 recipients for five years.[30] This study observed a greater survival in the DCD group compared to BD at both one and five years.[30] In the United Kingdom, reports of DCD donors showed similar survival up to 7 years when compared to BD donors.[16] Despite this, Kaplan-Meier graphs indicated less Bronchiolitis Obliterans Syndrome (BOS) in recipients of BD donors lungs from three years onward.[16] Of note, the Australian data reported fewer new BOS cases in DCD recipients, (7% Australian data vs 24% United Kingdom data); indeed, the incidence of DCD BOS in the Australian study was less than both DCD and BD groups from the United Kingdom. Machuca *et al* recently reported the experience of a single centre in Canada, similarly demonstrating equal five year survival rates between groups.[31] The incidence of BOS was not reported in this study. These data indicate that donation after circulatory death is associated with good outcomes and remains a critical contributor to addressing current donor shortages.

1.3 Rationale for the Current Study

Despite significant improvements in outcomes after LTx, challenges remain. As long as the majority of donations continue to come from BD patients, each transplanted organ will be exposed to a unique set of insults. Consequently, donor management must consider each step from donor to recipient in order to gain the maximal benefit. Implementation of standardised Aggressive Donor Management (ADM) protocols for BD donors improves quality and quantity of transplantable organs.[32] The objective of the present thesis is to investigate the contribution of the endothelin axis to lung injury after brain death, and apply targeted, local therapy in order to improve the quality of potential donor lungs.

Chapter 2 - Inflammatory Signalling Associated with Brain Dead Organ Donation: From Brain Injury through to Brain Stem Death and Post Transplant Ischaemia Reperfusion Injury

*This Chapter was accepted for publication in the Journal of Transplantation. However, new sections have been added for the thesis. These sections include:

2.3.5 Endothelin and its Receptors

2.3.5.1 Production of Endothelin

2.3.5.2 Endothelin Receptors

2.3.5.3 Influence of Endothelin-1 on Pulmonary Injury and Remodelling

2.7.1 Current Care of the Brain Dead Multi-Organ Donor

2.7.1.1 Respiratory Management

2.7.1.2 Fluid, Haemodynamic and Cardiovascular Management

2.7.1.3 Special Consideration of Cardiac Function after Brain Death

2.7.1.4 Endocrine and Metabolic Management

2.7.2 Duration of Care of Brain Dead Organ Donors

Adjustment has been made to 2.8 Potential Future Directions due to these associated edits

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2.1 Abstract

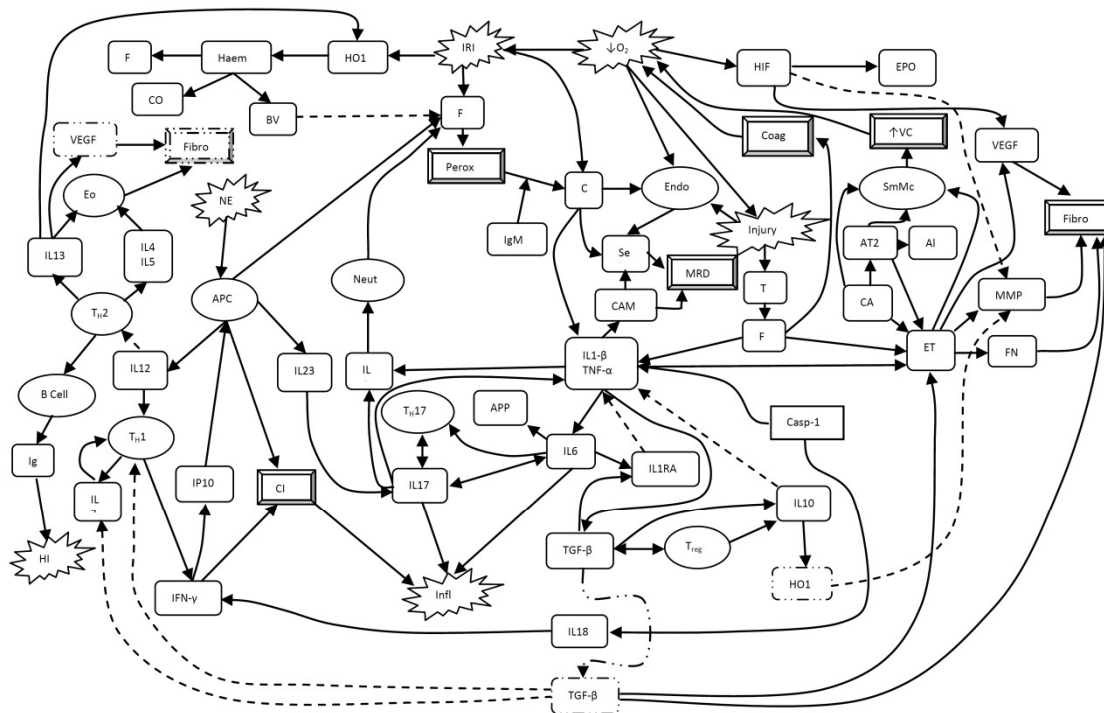
Brain death is associated with dramatic and serious pathophysiologic changes that adversely affect both the quantity and quality of organs available for transplant. To fully optimise the donor pool necessitates a more complete understanding of the underlying pathophysiology of organ dysfunction associated with transplantation. These injurious processes are initially triggered by catastrophic brain injury and are further enhanced during both brain death and graft transplantation. The activated inflammatory systems then contribute to graft dysfunction in the recipient. Inflammatory mediators drive this process in concert with the innate and adaptive immune systems. Activation of deleterious immunological pathways in organ grafts occurs, priming them for further inflammation after engraftment. Finally, post-transplantation ischaemia-reperfusion injury leads to further generation of inflammatory mediators and consequent activation of the recipient's immune system. Ongoing research has identified key mediators that contribute to the inflammatory milieu inherent in brain dead organ donation. This has seen the development of novel therapies that directly target the inflammatory cascade.

2.2 Introduction

Organ transplantation is the gold standard treatment for patients with end stage solid organ failure. An ever increasing disparity between available organs and potential recipients is the cause of avoidable morbidity and mortality.[2, 4-6] Ongoing efforts are being made to increase the quantity and quality of organs available for transplant. Although outcomes from non-heart beating donors have become increasingly successful,[18] the majority of organs are still donated from donors after brain death (BD). Significant brain injury of any aetiology will cause a systemic response,[19] creating a proinflammatory environment prior to the occurrence of brain death itself. BD then also creates a variety of inflammatory, haemodynamic and endocrine effects, which induce adverse sequelae in distant organs.[20-23] Finally, ischaemia-reperfusion injury (IRI), inherent in transplantation, generates reactive oxygen species (ROS), activates complement and independently drives cytokine release and inflammation.[25, 26] Every transplanted organ from a BD donor will face these stages of potential injury. Consequently, donor management must consider each step from donor to recipient in order to maximise recipient outcomes. The purpose of this article is to explore the current understanding of the three main contributors to injury that an organ will travel through from donor to recipient. Additionally, donor management and organ preservation strategies that are currently being investigated will be considered.

2.3 Stage Zero of Potential Organ Injury: Current Concepts in Immunological Signalling

Inflammation secondary to brain injury, BD and IRI is driven by both the innate and adaptive immune systems. The complexity of these systems means that our understanding continues to evolve at a rapid pace (Figure 2). Prior to reviewing the specific inflammatory responses at each major step of the donor organ journey, it is important to discuss current concepts in the normally functioning immune system.



———> - Stimulates, - - - -> - Inhibits, ···> - Mediator Duplicated for Clarity in Diagram
 Ald – Aldosterone, APC - Antigen Presenting Cell, APP - Acute Phase Proteins, AT2 - Angiotensin II, BV – Biliverdin, C – Complement, CA – Catecholamines, CAM - Cellular Adhesion Molecule, Casp-1 - Caspase 1, Cl - Cellular Inflammation, CO - Carbon Monoxide, Coag – Coagulation, Endo - Endothelial Cells, Eo – Eosinophils, EPO – Erythropoietin, ET – Endothelin, F2 - Factor II (Thrombin), Fe – Iron, Fibro – Fibrosis, FN – Fibronectin, FR - Free Radicals, HI – Humoral Immunity, HIF - Hypoxia Inducible Factor, HO1 - Heme Oxygenase 1, IFN – Interferon, Ig – Immunoglobulin, IL – Interleukin, IL1RA - Interleukin 1 Receptor Antagonist, Infi – Inflammation, IP – Interferon-γ Induced Protein, IRI - Ischaemia Reperfusion Injury, MMP - Matrix Metalloproteinases, MRD – Margination/Rolling/Diapedesis, NE - New Antigens/Neopeptides, Neut – Neutrophils, O2 – Oxygen, Perox – Peroxidation, Sel – Selectin, SMC - Smooth Muscle Contraction, TF - Tissue Factor, TGF - Transforming Growth Factor, TH1 - Type 1 Helper T-Cell, TH17 - Type 17 Helper T-Cell, TH2 - Type 2 Helper T-Cell, TNF - Tumour Necrosis Factor, Treg - Regulatory T-Cell, VC – Vasoconstriction, VEGF - Vascular Epithelial Growth Factor.

Figure 2: Primary Mediators of Peri-Transplant Inflammation

Multiple mediators have been identified in peri-transplant inflammation. This diagram represents the complexity and observed interplay of these inflammatory mediators associated with transplantation

Traditionally, T-cell responses are grouped according to the TH1/TH2 paradigm. TH1 lymphocytes (CD4+) are responsible for cell mediated immunity through activation of killer CD8+ T-cells and cytotoxic macrophages.[33, 34] TH2 cells are responsible for control of humoral immunity through antibody producing B cells. Additionally, they regulate eosinophil and basophil function. Recent work has identified TH17 and T-regulatory (Treg) subsets. TH17 cells have been implicated in autoimmunity.[33, 34] Treg cells are related to TH17 cells and function to regulate immunological reactions and prevent uncontrolled inflammation. Each of these T-cells plays a specific role in inflammation and their actions can be identified by certain inflammatory mediators. Although cytokines may interact with multiple T-cell subsets, previous authors have classified the major cytokines into “types” reflecting the major T-cell subtype to which they are related.[9, 35-37] This convention will be used in the current review.

2.3.1 T_H1 Cell Related Cytokines

Communicating via the type 1 cytokines, tumour necrosis factor (TNF)- α , interleukin (IL) - 1, IL-2, IL-12 and IFN- γ , [38-40] T_H1 cells play a fundamental role in acute rejection. Type 1 cytokines are upregulated early in the inflammatory process. After their release, IL-1 β and TNF- α support the inflammatory response via activation of endothelial cells. [41] These cytokines act early in the inflammatory cascade, stimulating generation of cellular adhesion molecules, innate immune defence mechanisms and participating in cross-talk between the various inflammatory pathways. [42, 43] IL-2 plays an essential role in resting T-cell activation and proliferation, contributing to T-cell maturation. [44] After T-cell induction via IL-2, IL-12 directs cellular maturation towards T_H1, leading to a cell-mediated immune response. [45] IFN- γ influences both the innate and adaptive immune systems and is integral in the antigen presenting cell (APC) controlled balance between effector and suppressor T-cells. [46] IFN- γ not only acts as the primary effector cytokine of IL-12 as part of cellular immunity, but also provides negative feedback control of IL-12 and indoleamine dioxygenase mediated T-cell inhibition, under the control of APC's. [46]

2.3.2 T_H2 Cell Related Cytokines

T_H2 cell related cytokines include IL-4, IL-5, IL-10 and IL-13. [34, 47] The type 2 cytokines are generally considered anti-inflammatory when associated with brain injury and BD, and in the early transplant period. [48-50] IL-4 inhibits formation of T_H1 cells and encourages development of T_H2 cells. [48] It also plays an essential role in B-cell generation of IgE. [51] IL-4 may activate macrophages via an alternative pathway that reduces inflammation through sequestration and metabolism of arginine, an essential requirement for nitric oxide generation by inflammatory IFN- γ activated macrophages. [52] IL-4 has been postulated to depress T cell activity through production of indoleamine dioxygenase; Wang et al demonstrated increased indoleamine dioxygenase produced by natural killer cells in IL-4 treated rat livers. [52] IL-13 is best known for its role in allergy. Through interaction with its receptor, IL-13 stimulates inflammatory cells as well as epithelial and smooth muscle cells. [53] This may contribute to smooth muscle hypertrophy and pulmonary hypertension in various lung diseases. [53] IL-13 inhibits cell mediated immunity through downregulation of E-selectin, reduction of neutrophil recruitment and macrophage inhibition. [54] IL-5 is essential for development, recruitment and activation of eosinophils. [55] Once these cells are recruited to inflammatory sites, IL-5 is a potent co-stimulator of eosinophil degranulation and maintains their presence through inhibition of apoptosis. [55, 56] IL-5

also acts as a key mediator for generation of antigen specific IgE. Furthermore, it is important for terminal B cell differentiation, including the switch to mature IgM and IgG1 secreting plasma cells.[57]

IL-10 acts to inhibit production of inflammatory cytokines and upregulate inhibitors of IL-1 and TNF.[35] It may also specifically block the production of IL-1 and TNF.[58] Direct activity on inflammatory cells impairs or reverses the effects of pro-inflammatory mediators.[58] While IL-10 is classified as a type 2 cytokine, it is also able to be produced by T_H1 cells under the influence of transforming growth factor (TGF)- β .[38]

2.3.3 T_H17 Cell Related Cytokines

The T_H17 cells are identified by their association with IL-6, IL-17, IL-21, IL-22 and IL-23.[34, 45, 59] IL-17 and IL-23 direct T_H17 cell differentiation, proliferation and maturation.[45] Apart from directing T_H17 development, IL-17 functions to stimulate production of chemokines, IL-1 β , TNF- α , IL-6 and IL-8.[38, 60] Its production is reinforced by IL-6, IL-23 and TGF- β .[38, 60] IL-8 related neutrophil attraction and activation may contribute partly to the inflammatory action of IL-17.[61] IL-23 is an important upstream regulator of IL-17 expression.[45, 62] Generation of IL-17 by $\gamma\delta$ -T-cells is directly activated by IL-23, and these cells are an important source of IL-17.[62] Furthermore, IL-23 induces IL-17 production from natural killer T-cells.[60] IL-21 stimulates natural killer T cells, CD40 dependent B-cell proliferation and T-cell expansion.[63] Hagn et al recently demonstrated that incompletely activated CD4⁺ T-cells, through expression of IL-21 and CD40 ligand, stimulate B cells to differentiate into Granzyme B generating cytotoxic B cells.[64]

IL-6 has been extensively investigated in many conditions. Its pro- and anti-inflammatory effects have recently been comprehensively reviewed.[65] Briefly, it is a pro-inflammatory agent which has been classified as a type 17 cytokine,[66] although some authors may include it as a type 1.[67] IL-6, the prototypical member of its family, acts through receptor complex formation with glycoprotein gp130 on the cell surface.[65] The IL-6 receptor molecule is present on the surface of hepatocytes, neutrophils, monocytes and macrophages.[65, 68] Direct activation of these receptors is associated with an inflammatory response.[68] Other cells may also respond to IL-6 through a process termed *trans*-signalling.[68] Free soluble IL-6 receptor binds circulating IL-6 and then interacts with the ubiquitous cell surface protein, gp130, to affect cell signalling.[65] The

dual roles of IL-6 may be partly explained by the differing signalling mechanisms. Soluble IL-6 receptor generated from apoptotic neutrophils in areas of inflammation activates signalling pathways after interaction with epithelial gp130, attracting regulatory monocytes and macrophages and contributing to resolution of inflammation.[65]

2.3.4 T_{reg} Cells and Related Cytokines

Named due to their ability to downregulate inflammatory processes, T_{reg} cells are another important source of the anti-inflammatory IL-10. T_{reg} are closely related to T_H17 cells; both lineages are derived from the same naïve T-cell precursor in a similar fashion to T_H1/T_H2 cells.[34, 40] Deknuydt et al recently highlighted the fluidity of the T_H17/T_{reg} balance by demonstrating that T_{reg} cells can be stimulated to become T_H17 cells under the influence of IL-1 β and IL-2.[34] TGF- β also directs differentiation of T cell populations in inflammatory conditions and is important in the T_H17/T_{reg} balance. TGF- β modulates the effects of IL-2, reducing expansion of inflammatory T-cell populations.[38] When acting synergistically with IL-2, TGF- β is able to direct naïve T-cells to become T_{reg} cells.[69] Selective inhibition of T_H1 producing mediators by TGF- β further contributes to the diversion from inflammatory T-cells to T_{reg} cells, mediating the inflammatory response.[38] However costimulation of TGF- β by IL-6 directs T-cell differentiation towards T_H17 cells and production of type 17 cytokines.[40] T_{reg} cells are immunosuppressive through production of IL-10 and TGF- β , cellular anergy and direct contact with inflammatory cells.[70]

2.3.5 Endothelin and its Receptors

Endothelin-1 (ET-1) is the most active member of a family of small polypeptides which are potent vasoconstrictors, mitogens of smooth muscle cells and stimulators of fibroblasts (Table 1).[71-75] Endothelin-1 upregulation has been observed in both brain injury and BD, and increased expression has been associated with adverse outcomes in lung transplantation. Early investigations into endothelin-1 were focussed on its vasoconstrictive properties, though recent research has implicated the endothelin axis in a wide variety of pathophysiological conditions.[76-86] The ubiquitous nature of the endothelin axis, its multiple roles in endothelial function, vascular reactivity and inflammation, and previous research indicating its potential role in BD related pulmonary pathophysiology, highlight ET-1 as a potential therapeutic target in transplant related medicine.[87]

Table 1: Properties of Endothelin*Summary table of the properties of endothelin signalling*

Endothelin	
Endothelin Subtypes[72, 74, 88]	ET-1 ET-2 ET-3
Sites of Production[72]	Smooth Muscle Cells Cardiomyocytes Leukocytes Macrophages Mesangial Cells Airway Epithelium Alveolar Epithelial Cells
Receptor Subtypes[71-73, 89, 90]	Endothelin Receptor A Endothelin Receptor B
Action	Endothelin Receptor A[71-73, 89, 90] Smooth Muscle Contraction Fibrogenesis Endothelin Receptor B[71-73, 89, 90] Smooth Muscle Contraction Smooth Muscle Relaxation ET-1 Clearance
Localisation of Receptors[71, 72, 88]	Heart Endocardium Conducting System Coronary Vessels Lung Kidneys CNS Liver Neutrophils
Stimulators of Release[72]	Endothelial Shear Stress Thrombin AT2 Cytokines Free Radicals Catecholamines
Inhibitors of Release[72]	NO ANP Prostacyclin
ET – Endothelin, AT2 – Angiotensin 2, NO – Nitric Oxide, ANP – Atrial Natriuretic Peptide, CNS – Central Nervous System	

2.3.5.1 Production of Endothelin

The endothelins are produced via step-wise proteolytic cleavage. Initially, prepro-endothelin is cleaved to form pro-endothelin-1 (or “Big ET-1”).[91-93] Big ET-1 is then processed by endothelin converting enzyme (ECE) to produce ET-1.[93, 94] Three isoforms of ECE exist; non-selective ECE-1 localised to the smooth muscle cell membrane, intracellular ECE-2 (which is preferentially selective for Big-ET-1 and operates within the Golgi apparatus of endothelial cells) and ECE-3, which is specific for production of ET-3.[95, 96] Studies measuring systemic levels of ET-1 indicate that it primarily acts in a paracrine/autocrine fashion; more than 80% of ET-1 is released abluminally into the space between endothelium and smooth muscle cells (Figure 3).[95, 97] Clearance of ET-1 occurs through its interactions with its receptors, although some degree of metabolism occurs through endopeptidases associated with vascular smooth muscle cell membranes.[84, 98]

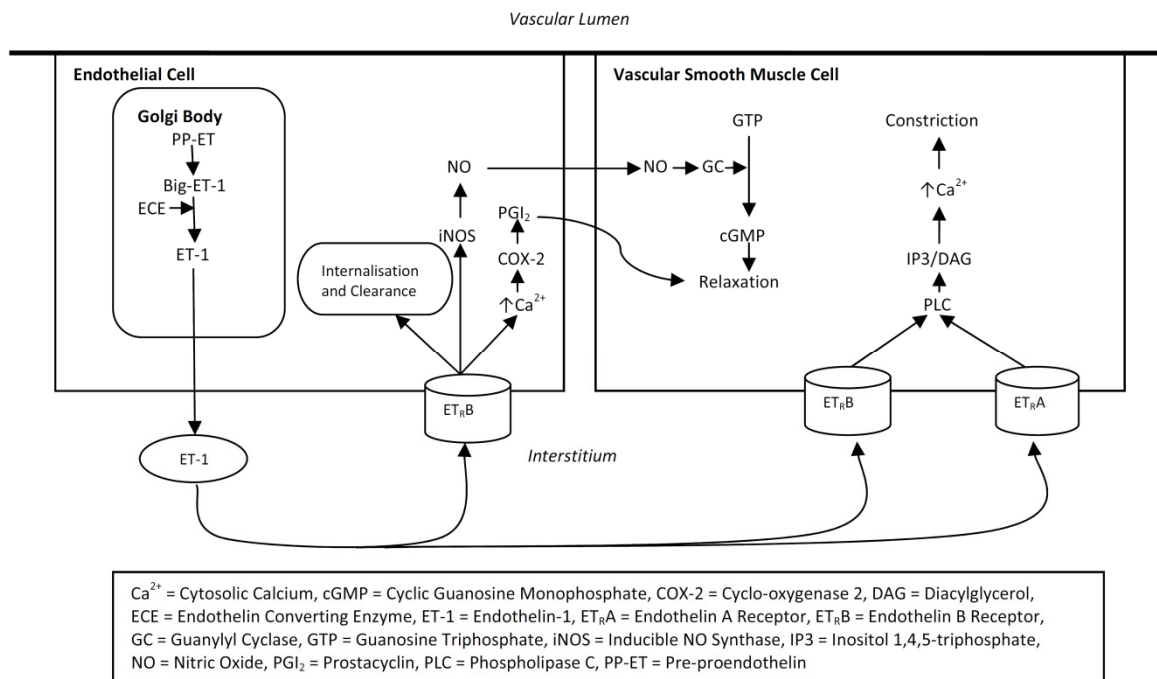


Figure 3: Schematic Diagram of Endothelin Cell Signalling

Endothelin-1 is produced by endothelial cells and primarily released abluminally. Once released, it acts in an autocrine/paracrine fashion to stimulate its receptors. Activation of ET_B on endothelial cells leads to vasorelaxation through production and release of nitric oxide and prostacyclin, while stimulation of vascular smooth muscle cells through ET_A or ET_B leads to vasoconstriction

2.3.5.2 Endothelin Receptors

Endothelin-1 acts on two receptor subtypes, ET_A and ET_B, each with different functions.[99] The ET_A receptor exerts its effects via activation of vascular smooth

muscle cells.[100] Endothelin A receptor stimulation elevates cytosolic calcium through $G_{q/11}$ signalling, with resultant phospholipase C activation.[99] This, in turn, increases inositol triphosphate, stimulating calcium release and leading to calcium-calmodulin dependent activation of myosin light chain kinase.[99] These effects are reinforced via $ET_{RA}-G_{12/13}$ signalling, which activates the Rho-kinase pathway to phosphorylate, and thus inactivate, myosin-phosphatase.[99] Myosin-phosphatase is also inhibited by CPI-17 (protein kinase C potentiated inhibitor protein of 17 kDa), which is itself activated by calcium dependent and independent mechanisms (including phospholipase C mediated production of diacylglycerol) to reinforce smooth muscle contraction and increase calcium sensitivity in vascular smooth muscle cells.[99] The final result is prolonged smooth muscle contraction, increasing systemic and pulmonary vascular resistance.[99] ET_{RA} antagonism has been observed to decrease hepatic inflammatory cytokine release in BD animal models.[101] This may occur indirectly, through attenuation of ischaemic vasospasm, or by direct interruption of cytokine generation secondary to ET-1 signalling.[101] Although the mitogenic effect of ET-1 occurs through ET_{RA} activation, it is modified and enhanced by ET_{RB} cosignalling.[102] After signalling through ET_{RA} , ET-1 is internalised for lysosomal degradation.[98]

The ET_{RB} receptors also utilise G-protein coupled signalling.[103] Their activation produces differing responses dependent on their location. Some authors have sub-classified ET_{RB} receptors into ET_{RB1} (endothelial) and ET_{RB2} (vascular smooth muscle) in accordance with their localisation.[95] Vascular smooth muscle ET_{RB} causes vasoconstriction via G_q related signalling,[104] whereas endothelial ET_{RB} leads to vasodilatation due to activation of inducible nitric oxide synthase (NOS) and production of NO.[99, 105] Stimulation of this subtype also increases cyclo-oxygenase (COX) dependent production of prostacyclin (PGI_2), which acts to reinforce vasodilatation and inhibit platelet aggregation.[99] Nitric oxide and PGI_2 are released from endothelial cells to act on nearby smooth muscle.[99] In vascular smooth muscle cells, NO activates guanylate cyclase, increasing cyclic GMP and protein kinase G to decrease intracellular calcium.[99] Vasorelaxation secondary to prostacyclin is achieved through adenylate cyclase signalling, which also reduces intracellular calcium.[99] In addition to its vasomotor role, ET_{RB} sequesters and clears circulating ET-1, particularly that traversing the pulmonary circulation.[106, 107]

2.3.5.3 Influence of Endothelin-1 on Pulmonary Injury and Remodelling

Endothelin-1 is highly expressed in pulmonary tissue, with ET-1 mRNA production being up to five times that of other organs.[108, 109] Through local signalling, ET-1 is primarily responsible for elevating vascular tone to modulate pulmonary flow.[108] Furthermore, ET_RA stimulation causes proliferation of vascular smooth muscle cells and fibroblasts via phosphatidylinositol 3-kinase/protein kinase B, protein kinase C and mitogen associated protein kinase signalling.[108] These intracellular signalling systems also result in enhancement of extracellular matrix remodelling.[108] Endothelin-1 induced MMP-2 and -9 actively degrade type IV and V collagen, which are important components of the cell basement membrane.[110]

Insights from cancer literature have suggested that these effects of ET-1 are important contributors to extra-cellular matrix remodelling.[85, 110, 111] Signalling by ET_RA may activate the epidermal growth factor receptor (EGFR) through multiple mechanisms. The scaffolding protein, β -arrestin, anchors the ET_RA receptor to the cell membrane and acts as a focal point for facilitation and interaction of complex cell signalling networks.[84] When ET-1 activates ET_RA, β -arrestin may activate EGFR, leading to phosphorylation and activation of β -catenin.[84] Additionally, β -arrestin may itself bind to β -catenin, increasing nuclear uptake and facilitating transcription of molecular targets.[84] These targets include gelatinase transcription and translation.[84, 111] Furthermore, ET-1 has been observed to decrease production of TIMP-1 and -2, significantly tipping the balance towards extracellular matrix degradation.[111, 112] Contrary to this, Felix *et al* reported that both TIMP-1 and -2 expression increased in osteosarcoma cells when stimulated by ET-1, and that this increased expression could be inhibited by the administration of an inhibitor of NF κ B.[110] These contradictory results may still explain increased collagen catabolism secondary to ET-1 stimulation, as TIMP-1 and -2 may activate the precursor forms of MMP-9 and -2, respectively.[110] Such imbalances have been observed in the lung pathology as well; the matrix metalloproteinases are important mediators of acute lung injury and the acute respiratory distress syndrome (ARDS).[113, 114]

2.4 Stage One of Potential Organ Injury: Brain Injury

Most brain dead donors suffer from three main causes of BD: cerebrovascular injury, anoxia or traumatic brain injury (TBI).[115, 116] Donor cause of death can significantly influence recipient survival rates, though this varies according to the organ. Renal transplant outcomes are adversely affected by cerebrovascular causes of BD.[117, 118]

Lung transplant is unaffected by donor cause of death,[119] while heart transplant outcomes remain controversial.[120, 121] For this reason, it is important to consider the pathophysiologic responses to severe central nervous system injury, and their systemic sequelae, prior to brain death.

2.4.1 Systemic Inflammatory Response Secondary to Brain Injury

Central nervous system (CNS) injury is associated with the systemic inflammatory response syndrome (SIRS). This can occur with an intact blood brain barrier (BBB), indicating an additional mechanism distinct from CNS derived cytokine release.[122, 123] The link between the brain and SIRS has been comprehensively reviewed by Lu et al.[124] Briefly, SIRS is associated with leukocyte mobilisation and recruitment to major organs, activation and release of inflammatory mediators, generation of ROS, increased vascular permeability and organ dysfunction.[125, 126] Brain intraparenchymal injection of TNF- α recruits and activates systemic monocytes while IL-1 β activates and recruits neutrophils via release of chemokines from the liver.[122, 127]

TNF- α is released from the spleen in the early stages of brain injury to augment the peripheral inflammatory response.[19, 128] Lee et al demonstrated upregulation of TNF- α , IL-1 β , IL-4 and IL-6 in the spleens of rats with subarachnoid haemorrhage (SAH).[128] Intravenous administration of neural stem cells attenuated the inflammatory response via a chaperone mechanism which was localised to the spleen and reversed on splenectomy.[128] Splenic inflammation may also be directly downregulated via vagal messages from the brain.[19] The SIRS response activates gut derived inflammatory mediators, resulting in leaky gut wall.[129] This contributes to global inflammation through cytokine generation and systemic endotoxin exposure, worsening pulmonary inflammation and impairing oxygenation.[129-131] Similar to the spleen, gut generation of cytokines is also modulated by the CNS through vagal input.[130]

2.4.2 Localised Response to Brain Injury and Loss of Blood Brain Barrier Function

Local responses to severe brain injury can be classified into two phases.[132] The primary phase is due to the insult itself and includes cellular death, direct BBB disruption and cerebral oedema.[132] The secondary phase of injury is caused by elevated intracranial pressure (ICP), global brain ischaemia, excitotoxicity, metabolic derangements and

haemodynamic instability.[132-134] Whatever the cause of brain death, a cytokinaemia secondary to brain injury occurs prior to brain death itself.[135-138]

Local inflammation, and the direct effect of the insult itself, causes the highly selective BBB to become disrupted.[137, 139] Matrix metalloproteinases (MMP), especially MMP-9, act to break down extracellular proteins, including basal lamina and endothelial tight junctions.[140] In a rat model of closed head trauma, Higashida et al investigated the role of MMP-9 and Hypoxia Inducible Factor (HIF) in cerebral oedema resulting from lost BBB integrity.[140] Inhibition of MMP-9 in this model significantly reduced the amount of brain oedema observed after 24 hours. Additionally, inhibition of HIF (an upstream regulator of protein expression associated with hypoxia) also significantly reduced the expression of MMP-9 and brain oedema.[140] This observation was confirmed in an intracranial haemorrhage model in rats; Wu et al showed that MMP-9 is upregulated early post injury and is associated with brain oedema.[141] A post-mortem study of intracranial haemorrhage confirmed these findings in humans.[142]

The effect of the loss of BBB integrity is to allow bidirectional access of inflammatory cells and mediators.[139, 143-147] CNS derived cytokines are then free to interact at receptors within the systemic tissues, inducing local inflammation and 'priming' organs for further injury.[144, 148] The importance of brain injury derived cytokinaemia was recently demonstrated by Graetz et al, who reviewed compartmental levels of IL-6 in SAH and found elevated plasma IL-6 is associated with increased mortality.[135] This provides further evidence that isolated brain injury causes a systemic inflammatory response and upregulates the peripheral immune system.[130, 146]

2.4.3 Type 1 Cytokines

Type 1 cytokines are upregulated in the brain after injury and contribute to BBB breakdown, vasospasm and secondary injury.[133, 149, 150] The general roles of these and other inflammatory mediators have been previously reviewed.[151] Briefly, IL-1 β is a pleiotropic proinflammatory mediator that stimulates multiple pathways of inflammation after brain injury.[151] TNF- α acts as a proinflammatory agent early in the inflammatory process in the CNS.[152] Microdialysis techniques have confirmed the presence of IL-1 β and TNF- α in extracellular fluid after TBI and SAH.[135, 150, 153] Both of these cytokines are also released peripherally as part of a systemic acute phase response (APR).[15, 19, 122, 154] IL-1 β and TNF- α can be detected in blood within as little as one hour after brain

ischaemia, even before significant neuronal cell death can be demonstrated.[146, 155] Quantitative systemic levels of type 1 cytokines may be affected by the type of brain insult; these were decreased in a middle cerebral artery occlusion model in mice, partially explaining the mechanism of the observed shift from T_H1 to T_H2 driven immunity post-stroke.[36]

The soluble TNF Receptors (TNFR), p55 and p75, also contribute to the inflammatory process in traumatic brain injury, though the specifics of their involvement are not currently clear.[147] They act as anti-inflammatory agents through free TNF scavenging, although TNFR levels are more closely correlated to mortality in potential donors than TNF itself.[147, 152] This observation may actually reflect an imbalance in pro- and anti-inflammatory mechanisms or simply be due to the very short half life of TNF.[147]

2.4.4 Type 2 Cytokines

A recent study of stroke in IL-4 knockout mice showed that IL-4 reduces the T_H1:T_H2 cell ratio and infarct volume, and improves neurological outcome.[48] Studies in humans have shown that brain derived IL-4 can be detected in the jugular vein in patients with serious head injury.[156] A post-mortem study of TBI patients confirmed elevated IL-4 in brain tissue.[133] IL-13 has been less studied in brain injury. One in vitro study of IL-13 and IL-4 did show that these mediators induced apoptosis of activated microglia, which may account for part of the observed anti-inflammatory effect.[157] IL-13 is not significantly elevated in the plasma after TBI.[50]

IL-10, a type 2 cytokine with anti-inflammatory properties,[48, 49] plays a protective role in the CNS, reducing infarct size in stroke patients.[58, 158] Analysis of post-mortem TBI brains confirmed the presence of IL-10, though levels were more modest than similarly identified pro-inflammatory cytokines.[133] This was consistent with intraparenchymal levels measured by microdialysis in TBI and SAH patients.[153] Overflow of IL-10 into the cerebrospinal fluid (CSF) after TBI has also been demonstrated.[159] Systemic IL-10 levels peak early in TBI patients, declining to baseline within 48 hours.[147] Although IL-10 decreases inflammation through its immunomodulatory action, it also increases susceptibility to infection through immune system downregulation.[158]

2.4.5 Type 17 Cytokines

In the CNS, IL-6 plays a dichotomous role through modulation of glial responses and neuronal survival, contributing to the early inflammatory response, but modulating later inflammatory pathways to assist with brain recovery.[152, 160-162] While its proinflammatory role is well known, it has also been shown to protect against excitotoxicity *in vitro* and brain ischaemic or excitotoxic states *in vivo*. [160] The specifics of how this balance is achieved is less clear.[160] One suggestion is that the role of IL-6 depends on the amount of neuronal cell damage and is concentration dependent, but it is also probably subject to negative feedback inhibition via crosstalk between NMDA and IL-6 receptors.[160] It may also downregulate inflammation through stimulating IL-1 receptor antagonist.[153] Microdialysis techniques have confirmed that IL-6 is acutely increased after brain injury.[135, 136] Furthermore, Graetz et al demonstrated that IL-6 is released from the brain parenchyma into the systemic circulation after brain injury, particularly in the presence of high ICP.[135] Previous studies have also shown that IL-6 interferes with BBB integrity.[135, 143] Similar to IL-4, IL-6 is detectable in jugular blood, and the transcranial gradient correlates with poor outcome in TBI.[135, 143] The APR is stimulated by circulating IL-6[163] and this may provide a link between central injury and the peripheral immune response seen with intracranial injury.[146]

The roles of IL-17 and IL-23 in acute brain injury remain to be fully elucidated. While a role has been established in central autoimmune disorders including experimental models of multiple sclerosis,[33] less has been published on acute CNS injury. Murine models demonstrate that both of these interleukins are locally upregulated after stroke.[164-166] Currently, there are no published data on their peripheral release after acute brain injury.

2.4.6 The Endothelin Axis in Brain Injury

Endothelin-1 is an important mediator in TBI, stroke and SAH.[94, 167-169] In acute brain injury, ET-1 leads to constriction of large vessels, altering the normal balance between vascular relaxation and constriction, resulting in impaired cerebral blood flow.[169] This alteration of blood flow has been targeted in studies of SAH.[170] Clazosentan, an ET receptor A antagonist, reduces large cerebral artery vasospasm in murine models, but this did not reduce other mechanisms of secondary brain injury.[170] Salonia et al analysed CSF levels of ET-1 in paediatric head trauma.[169] They found that ET-1 is significantly elevated after injury and remains so for up to 5 days. Central production of ET-1 in adult

TBI was confirmed by Chatfield et al.[167] Their analysis of the jugulo-arterial gradient showed that ET-1 is produced intracranially and spills over into the systemic circulation.

2.5 Stage Two of Potential Organ Injury: Brain Death

Serious brain injury, augmented by local inflammation, may eventually lead to an irretrievable state of impaired brain function and brain death. BD then further causes a massive autonomic storm and cytokinaemia which increases the inflammatory state of the individual.[171-173] A complex interplay of immunologic,[174] coagulopathic,[175] autonomic, haemodynamic and endocrine[176, 177] dysregulation drives inflammation through local and global cytokine release, cellular activation, organ priming, IRI and secondary ischaemic insult (Figure 4).

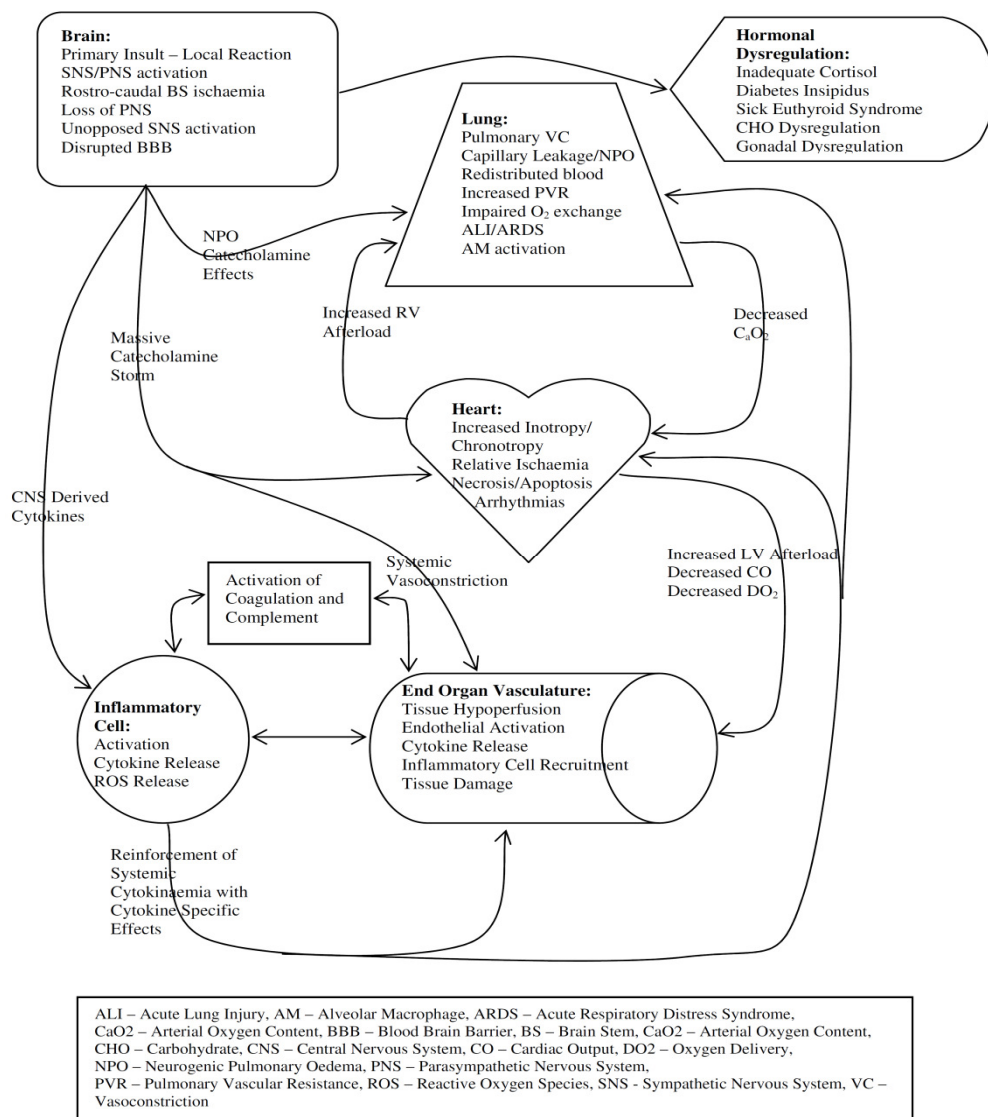


Figure 4: Interaction of Homeostatic Mechanisms Post Brain Death

Multiple organ systems are activated after brain death. In order to maintain homeostasis, these organ systems interact with each other, while reinforcing some of the inflammatory pathways.

2.5.1 The Autonomic Nervous System during Brain Death

Brain stem dysfunction is associated with extreme physiological perturbations due to its 'master control' function.[178-180] Brain stem failure secondary to high ICP occurs in a rostral-caudal direction, with initial hypertension and bradycardia (classically known as Cushing's reflex[181, 182]), followed by an intense 'sympathetic storm' which remains unopposed due to ischaemia of the parasympathetic vagal nucleus.[183, 184] This storm results from an overwhelming release of catecholamines in an attempt to perfuse the brain by increasing the mean arterial pressure (MAP) to overcome the elevated ICP.[185, 186] Such changes in autonomic outflow can be detected prior to the occurrence of brain death.[187] The initial massive upsurge in sympathetic tone results in widespread vasoconstriction and microthrombus formation, impairing organ and tissue perfusion.[178]

As the ICP outpaces the MAP, ischaemia progresses down the brain stem, sympathetic centres become necrotic, vascular and myocardial sympathetic stimulation drops and a second phase of hypotension ensues.[178, 188, 189] The resulting uncontrolled hypotension further impairs the already tenuous end organ perfusion that resulted during the sympathetic storm.[188]

While the effects of the sympathetic nervous system are the most obvious clinically during and after BD, inflammatory and haemodynamic responses are also influenced by the parasympathetic nervous system (PNS). The effect of BD is to inhibit PNS mediated anti-inflammatory responses by direct destruction of vagal centres in the brain stem.[130] Under normal conditions, vagal stimulation directly decreases inflammation via cholinergic receptors on inflammatory cells.[190, 191] Central activation of vagal efferent pathways downregulates inflammation in the brain, gut and spleen.[19, 130] Balance is normally achieved through negative feedback by the innate immune system interacting with the PNS via IL-1 receptors in the parasympathetic paraganglia.[130]

2.5.2 Cytokine Upregulation after Brain Death

2.5.2.1 Type 1 Associated Cytokines

Cytokine upregulation after BD has been recognised for many years.[129, 192] Animal models have shown that serum levels of IL-1 β and TNF- α may be influenced by the rate of induction of brain death.[41, 193] Avlonitis et al reported that explosive brain death

induced a rapid increase in IL-1 β , with significantly elevated levels detectable within one hour and remaining so throughout the duration of the study.[193] TNF- α levels initially rose and then decreased by five hours, though it remained above baseline.[193] Zhu et al showed that gradual induction of brain death lead to steady elevation of IL-1 β over 24 hours in a pig model.[194] Conversely, Damman and colleagues, utilising gradual BD induction in a rat model, showed that IL-1 β and TNF- α did not change significantly over the four hours of their study.[163] Interestingly, this group also analysed serum cytokine levels in human BD donors and showed that they were not significantly elevated.[163] Cypel and colleagues recently reported that TNF- α and IL-1 β mRNA are significantly elevated in lungs rejected for transplant, highlighting the clinical importance of these proinflammatory cytokines.[195]

2.5.2.2 Type 2 Associated Cytokines Including IL-10

Early studies of cytokine upregulation after BD suggested that Type 2 cytokines are not significant contributors to BD induced inflammation.[192] Takada et al did not show upregulation of IL-4 in rat kidneys, hearts, livers or lungs after BD.[192] Weiss et al studied cytokine expression at various timepoints during the liver transplantation process.[15] This group reported that IL-4 expression is increased after brain death.[15] IL-10 is elevated in the plasma of human BD donors.[129, 172, 196] Additionally, IL-10 has been shown to be upregulated in human livers[15] and kidneys.[197] Work undertaken by Li et al suggested that IL-10 expression after BD may be important in stimulating apoptosis of graft infiltrating lymphocytes through activation of the Fas/Fas Ligand pathway.[198] There is little literature investigating the role of IL-5 and IL-13 during brain death. This may be an area for future research.

2.5.2.3 Type 17 Associated Cytokines

IL-6 is heavily implicated in BD related inflammation,[199, 200] where it is an important instigator of the generalised APR.[163] Levels increase in human brain dead donors up until the time of organ retrieval.[163] Systemic venous and CNS derived IL-6 is significantly higher at brain death than at admission to the intensive care unit (ICU) in TBI patients that progress to BD.[143] Brain death induces the production of IL-6 in multiple organs, including the kidney,[201] heart,[202] liver[203] and lung.[173] IL-6 signalling induces nitric oxide synthase in cardiac myocytes[202] and contributes to early

haemodynamic compromise in the donor via direct negative inotropy.[199, 204] IL-6 mRNA and protein are elevated in non-structural donor heart dysfunction.[202]

Damman et al recently investigated IL-6 related renal acute phase protein synthesis in rats.[163] As expected, IL-6 was upregulated after brain death. This correlated with an increase in renal acute phase proteins, notably complement 3 (C3), fibrinogen, α 2-macroglobulin and haptoglobin.[163] Furthermore, in vitro analysis indicated that renal production of C3 is directly related to IL-6 exposure.[163]

Overall, elevated plasma levels of IL-6 are associated with poorer transplantation outcomes.[15, 203] Murugan and colleagues demonstrated an inverse relationship between donor plasma IL-6 levels and recipient six-month hospital free survival.[172] Kaneda et al also showed that higher donor IL-6 levels increased the risk of recipient death within 30 days of lung transplant.[205]

T_H17 cells, through production of IL-17, stimulate inflammation in donor organs.[206] Pretransplant renal biopsies from deceased donors showed little elevation of IL-17 positive cells, though few graft infiltrating cells were demonstrated in the biopsy samples.[206] Although a number of authors have studied IL-17 in the context of chronic rejection, the role of BD donor IL-17 currently remains unexplored.

2.5.2.4 T_{reg} Associated Cytokines

TGF- β is upregulated in heart and lung tissue in animal models.[144] Elevated TGF- β mRNA has been identified in renal and liver biopsies from brain dead donors.[15, 197] Weiss et al showed that the greatest stimulus for TGF- β expression in liver grafts is BD itself.[15] A slight decrease in expression occurred prior to cold storage and to reperfusion. TGF- β mRNA expression increased by one hour after implantation and reperfusion but did not exceed levels measured before surgical manipulation (ie after BD alone).[15] Skrabal et al also demonstrated that TGF- β mRNA transcription is increased in donor heart and lungs in a porcine model of brain death.[144] The role of TGF- β in acute organ injury may relate to its role in the T_H17/T_{reg} balance,[40, 69] however increased expression prior to transplantation may start fibrotic processes through activation of MMP's and tissue inhibitor of metalloproteinases (TIMP's). MMP-2, -9, TIMP-1 and -2 expression is increased after BD in pulmonary tissue.[88]

2.5.2.5 Interleukin 8

IL-8 is a chemokine which attracts and activates neutrophils.[207, 208] Similar to other cytokines, IL-8 is produced peripherally after BD where it stimulates neutrophil driven angiogenesis and fibroproliferation.[207, 209] IL-8 induced neovascularisation, alveolar-capillary disruption and extracellular matrix deposition contribute to the development of acute lung injury after brain death.[207] In lung donors, broncho-alveolar lavage fluid IL-8 levels are positively correlated with neutrophil infiltration in pre-transplant lung tissue, contributing to early graft dysfunction.[207]

2.5.2.6 The Endothelin Axis

ET-1 release from endothelium is stimulated by noradrenaline, thrombin and TGF- β . [210] Animal experiments have shown that ET-1 is upregulated in serum and in donor lung after BD and that this is related to MMP activation.[88, 211] Salama et al demonstrated a correlation between donor ET-1 and primary graft dysfunction (PGD).[212] In this study, ET-1 upregulation (as measured by donor lung mRNA and donor serum levels) adversely affected recipients after transplantation, contributing to the development of PGD.

2.6 Stage Three of Potential Organ Injury: Ischaemia Reperfusion Injury

Ischaemia reperfusion injury is implicated in early and late stage transplant complications.[213] IRI leads to organ dysfunction through induction of cytokines, generation of free radicals and activation of immunocompetent cells.[213, 214] Endothelial cell dysfunction secondary to IRI is key in chronic allograft dysfunction in hearts,[215] lungs,[25] livers,[216] and kidneys.[217] Early injury to cells occurs as a direct result of ischaemia, with impaired oxygen delivery, altered energy metabolism and accumulation of waste products. Cell death occurs through necrosis and apoptosis, the latter through caspase signalling.[129, 218] Further injury occurs upon reperfusion, with recruitment of inflammatory cells, interaction between local and systemic cytokine signalling systems and generation of ROS.[199, 219, 220]

APC's of the innate immune system play a key role providing antigens and co-stimulatory molecules to activate the adaptive immune system, contributing to IRI and early graft dysfunction. Activation of cellular immunity can be classified as direct or indirect.[219]

Direct activation occurs due to the transfer of donor APC's in the allograft, which activate recipient T_H1 cells.[219] Atkinson et al recently demonstrated that passenger leukocytes are recruited to donor hearts after BD in a murine model.[41] This finding was also confirmed in lung[221] and renal allografts.[13] Gelman et al also demonstrated that recipient T-cells interact with donor APC's and that this is sufficient to activate an inflammatory response.[221] Alternatively, the indirect pathway results from the interaction of recipient APC's with native T-cells to stimulate inflammation.

2.6.1 Contribution of Preservation Strategies to Cytokine Expression

Hypothermic preservation strategies are widely used to decrease inflammation, depress the metabolic rate of cells and reduce the effects of ischaemia.[222] However, cold storage does cause cell death via both apoptosis and necrosis.[35] BD donor organs predominantly display the latter mechanism.[216] The duration and type (warm or cold) of ischaemic time may also directly influence cytokine production. A correlation was recently identified between cold ischaemia time and levels of IL-1 and IL-8 in human liver transplants.[223] Warm ischaemia time correlated with IL-6 and IL-10 in the same study. Significantly, the authors found that the excess cytokines generated by hepatic graft warm ischaemia time resulted in systemic adverse effects, most notably increased intraoperative pulmonary shunt.[223] Another study found that, while cold ischaemic time per se did not adversely affect liver function, the associated graft generated IL-8 did correlate with PGD.[224] Weiss et al, in a study of transplanted human livers, showed that IL-4 was increased in BD donors prior to explantation, but cold ischaemia and reperfusion did not result in further increases in the cytokine.[15] Indeed, while it was elevated compared to living donors, it failed to reach statistical significance at time points other than immediately after laparotomy. IL-10 was highly expressed prior to organ preservation, but cold ischaemia and reperfusion did not result in further elevation of this cytokine.[15] Livers from living donors showed a relatively greater increase in expression of IL-10 one hour after reperfusion than BD organs, which may partially contribute to better outcomes with organs from these donors.[15]

Delayed graft function in transplanted kidneys has been shown to be dependent on cold ischaemic time.[206] Kaminska et al showed that while cytokine upregulation occurred associated with brain death, mRNA expression did not increase further after cold ischaemia and prior to reperfusion.[197] In keeping with this, de Vries et al were unable to detect an arteriovenous difference across human BD donor kidneys for multiple cytokines,

including IL-4, IL-5, IL-10 and IL-13.[13] Cold ischaemia and reperfusion does not induce excess TGF- β mRNA production, indicating that the primary stimulus for this mediator is brain death itself.[197]

2.6.2 Other Mediators of Ischaemia Reperfusion Injury

The combination of BD and IRI activates allografts greater than either insult alone. Kusaka et al studied rat renal isografts to analyse gene activation after BD, IRI or combined BD/IRI.[213] They found that BD primarily upregulated cytokines, chemokines and adhesion molecules while IRI tended to upregulate transcription factors. BD/IRI combined was synergistic in enhancing upregulation of these genes. More recent work has maintained these findings. Inhibition of JNK, a phosphorylator of the transcription factor c-Jun, decreases IRI induced renal damage in rats.[217] In humans, de Vries et al demonstrated that reperfusion of BD kidneys generates higher cytokine levels than living donor allografts (ie those that only underwent IRI).[13]

Complement interacts with, and reinforces, the inflammatory process of IRI by increasing TNF- α and IL-1.[41] C3a and C5a, potent anaphylatoxins generated by the complement cascade, activate mast cells and neutrophils.[220] While the specific mechanism of complement activation in BD is unknown, it is postulated that ischaemia leads to defects in cell membranes, uncovering neoepitopes via exposure of internal cellular components to the humoral immune system, which leads to interaction with natural IgM and activation of the classical complement pathway.[220] ROS generated during infarction and IRI may lead to lipid peroxidation and alteration of cellular cytoskeletal structure providing further neo-epitopes for IgM.[220]

The importance of toll like receptors (TLR) in IRI is currently being investigated. It was previously noted that low levels of lipopolysaccharide (LPS) may precondition and therefore protect the lung from IRI.[225] Merry et al demonstrated that low dose preconditioning with LPS in rat lung ischaemia reduced injury.[225] The authors postulated that this may be due to LPS activating TLR-4 via an alternative pathway that results in protective interferon and IL-10 generation. Unfortunately, they did not measure IL-10 protein or mRNA to confirm this hypothesis. The role of TLR's in renal IRI has recently been reviewed elsewhere.[26] TLR's may also contribute to inflammation through interaction with T-cells via cytokine signalling. APC TLR activation leads to generation of cytokines, including IL-6, which may decrease the sensitivity of T_H1 cells to the

immunosuppressive effects of T_{reg} cells.[226] Additionally, TLR on T_{reg} cells may directly inhibit their immunosuppressive effects.[226]

ET-1 contributes to IRI through activated neutrophils, leading to endothelial injury, neutrophil superoxide production and generation of ROS.[88] Both ET-1 and its receptors are upregulated in the lungs after brain death.[88] Alveolar macrophages have been demonstrated to increase expression of endothelin receptors in the donor lung in animal models.[88] This may then prime passenger macrophages for further activation by recipient ET-1, which is generated in the pro-inflammatory environment of chronic lung disease, surgery and the post-transplant course.[88, 227]

Heme-oxygenase-1 (HO-1) is essential for the metabolism of heme to carbon monoxide, free iron and biliverdin.[44] Its ability to reduce injury secondary to IRI, with resulting better recipient outcomes after transplantation has been the subject of much research. HO-1 exerts its beneficial effects through anti-oxidant, anti-apoptotic and anti-inflammatory mechanisms.[44, 228-230] Carbon monoxide contributes to these beneficial effects through inhibiting T-cell proliferation and IL-2 secretion.[44] Zhou et al, in studying a rat model of BD, demonstrated improved lung function and decreased lung injury when carbon monoxide was administered at 250 ppm.[231] Carbon monoxide decreased myeloperoxidase activity, TNF- α and IL-6.[231] More recently, the same group demonstrated that both carbon monoxide and biliverdin reduce myeloperoxidase activity and cytokine signalling while improving respiratory mechanics in rat lung after BD.[232] HO-1 has also been linked to anti-inflammatory cytokine generation. IL-10 production secondary to HO-1 is increased in both BD[229] and non-BD models.[233] HO-1 may also be an important mediator of IL-13's anti-inflammatory effect.[228, 234]

2.7 Management Implications

Recipients of organs from brain dead donors continue to have poorer outcomes than those that receive living donor organs. Aggressive donor management (ADM) improves both quality and quantity of organs available for transplant.[32] Current ADM recommendations include early identification of potential donors, ICU admission, pulmonary artery catheterisation, aggressive fluid management, vasopressors, hormonal resuscitation therapy, pulmonary toilet and bronchoscopy.[180, 235-238] Even with ADM, up to 25% of potential donors are lost due to haemodynamic instability.[180]

2.7.1 Current Care of the Brain Dead Multi-Organ Donor

Management protocols have been developed in order to support potentially transplantable organs, prevent further injury and optimise organ function.[239] Interventions can be broadly classified into systems including respiratory, cardiovascular/renal and endocrine/metabolic.[240]

2.7.1.1 Respiratory Management

Systemic inflammatory responses may injure the lungs after brain death, resulting in pulmonary oedema and impairment of gas exchange.[241] Mascia *et al* first noted, in an observational study, that most donors were ventilated with similar strategies to those with brain injuries.[242] This included no or low positive end expiratory pressure (PEEP) and larger tidal volumes.[242] Subsequently, the same author performed a randomised clinical trial in donors, comparing a conventional ventilation strategy to a low tidal volume strategy.[243] This study reported an increase in lung retrievals with the low tidal volume strategy, despite being stopped early due to funding issues.[243]

Excessive fluid administration may increase pulmonary oedema, compromising potential lung allografts.[244, 245] Therefore, fluid administration must be carefully controlled in order to maintain an appropriate fluid balance without “overloading” the potential donor.

2.7.1.2 Fluid, Haemodynamic and Cardiovascular Management

After an initial, transient, catecholamine storm, loss of sympathetic outflow results in hypotension and cardiovascular collapse.[238, 240] Impaired perfusion pressure leads to tissue ischaemia, local inflammation, necrosis and microthrombus formation.[24] Interventions are therefore necessary to support blood pressure and augment tissue perfusion. Central to this is recognition that prior management efforts (for example mannitol to manage intracranial hypertension) and development of diabetes insipidus may result in intravascular fluid depletion.[238] Optimisation with fluid therapy is therefore essential. Current literature indicates that crystalloid solutions are preferential to colloid; a systematic review found that hydroxyethyl starch may worsen post-transplant renal function.[246] Management of diabetes insipidus promotes haemodynamic stability and reduces fluid administration.[238]

Current recommendations are to support blood pressure with a target mean arterial pressure of at least 60 mmHg and urine output of >0.5 mL/kg/hr.[240] No high level evidence is available for choice of vasoactive agent.[246] Studies comparing use of noradrenaline and vasopressin as pressor agents demonstrate differing effects on transplantable organs. Animal models suggest that both agents decrease lung inflammation and serum cytokine release.[131] While a similar effect is seen in the kidney, hepatic inflammation is increased by both agents.[247] Dopamine decreased monocyte kidney graft infiltration and markers of inflammation in a rat model of BD.[230] In humans, a retrospective cohort study did not find any difference in survival amongst recipients of cardiac allografts from donors who were administered either noradrenaline or dopamine.[248] Schnuelle *et al* observed that donor dopamine infusion was associated with a decreased risk of dialysis after transplant in renal recipients, but this did not translate into a mortality benefit.[249] Based on the available data, and the increased risk of death associated with dopamine use in septic shock patients,[250] it has been recommended that noradrenaline is the first vasopressor of choice in potential organ donors.[246] Vasopressin may be considered for donors with diabetes insipidus.[238, 246]

2.7.1.3 Special Consideration of Cardiac Function after Brain Death

Right ventricular dysfunction contributes to a significant number of early deaths after heart transplant.[251-255] Studies in animal models suggest that preload recruitable stroke work of the right ventricle is impaired to a greater degree than the left,[256] and that the right ventricle has reduced ability to cope with an increased afterload.[257] Despite reduced contractility, right ventricular pump performance may be able to be maintained under conditions of increased afterload, albeit with a greater end diastolic volume.[258] Bittner *et al* observed that the right ventricle had diminished ability to increase total power (compensatory hydraulic power) after brain death.[257] Any observed right ventricular reserve was considered to result from changes in the ratio of oscillatory power (determined by the properties of the pulmonary arterial tree and does not contribute to forward blood flow) to mean power (power generated to overcome vascular resistance).[257]

The preload recruitable stroke work relation deteriorates even further after implantation into the recipient.[251] In a canine model, an attempt was made to transplant BD donor hearts into dogs with chronic pulmonary hypertension, however this was aborted after a series of failures.[251] This included two animals unable to be weaned from

cardiopulmonary bypass secondary to acute right ventricular failure and sustained ventricular dysrhythmias.[251] More recently, Stoica *et al* found similar impairment of right ventricular function in humans.[254] Compared to controls undergoing coronary artery bypass grafting, brain dead donor hearts showed a reduction in contractility, as reflected by the end systolic pressure volume relationship.[254] End diastolic volume index was also increased in this study.[254] Administration of dopamine improved preload recruitable stroke work, without any alteration in the end systolic pressure volume relationship.[254] The increase in stroke volume occurred through an increase in end diastolic volume, reflecting impairment of cardiac output.[254] Right ventricular dysfunction may therefore complicate cardiac transplant, especially when subjected to an increase in recipient pulmonary pressures.[255] Currently, no strategies are specifically recommended to manage these alterations in right ventricular function; this remains a challenge for future studies.[255]

2.7.1.4 Endocrine and Metabolic Management

Loss of hypothalamic thermal regulation often results in temperature abnormalities in brain dead donors. This must be actively managed to maintain a target temperature of greater than 35 °C.[240] Techniques include warming blankets and appropriate warming and humidification of inspired gases.[259]

Destruction of the hypothalamus and pituitary gland also causes hormonal dysregulation in the potential donor. Pituitary dysfunction is not a universal phenomenon, however, as hypophyseal blood flow may be maintained by the lower hypophyseal artery, a branch of the external carotid artery.[188] Loss of anti-diuretic hormone secretion occurs commonly, resulting in diabetes insipidus in up to 80% of brain dead donors.[131] Replacement with vasopressin or its analogues prevents dehydration and electrolyte disturbances in the potential donor.[188]

Normally, afferent vagal nerve signalling activates the hypothalamic-pituitary-adrenal axis, stimulating cortisol release.[190, 260] IL-1, -6 and TNF reinforce this via increasing corticotrophic releasing hormone and adrenocorticotrophic hormone secretion.[145] Hypothalamic or pituitary failure after brain death interferes with this usual process, leading to adrenal insufficiency.[261] In a study of BD versus healthy volunteers, levels of adrenocorticotrophic hormone and cortisol were similar, representing an inadequate stress

response in the BD group.[145] Overall, hypothalamic-pituitary-adrenal axis dysregulation results in an impaired stress response, haemodynamic instability and increased inflammation.[143, 172, 261] Previous observational studies have indicated that methylprednisolone administration improves pulmonary oedema and increases lung retrieval rates.[245] However, a recent systematic review found no clear clinical benefit for routine administration of corticosteroids in potential organ donors.[262]

Thyroid axis dysregulation after brain death is often identifiable as reduced thyroxine and free tri-iodothyronine (fT₃).[177] Some patients may demonstrate the sick euthyroid syndrome (low fT₃, normal thyroxine, elevated inactive rT₃ and low thyroid stimulating hormone).[177] IL-6 may play a role in this latter phenomenon.[145] Thyroid hormone may regulate anaerobic metabolism and cardiac/haemodynamic stability after brain death, however the benefits of routine thyroid hormone administration remains unclear.[177, 186, 188] A recent meta-analysis of routine thyroid hormone administration (as thyroxine and tri-iodothyronine) did not find any benefit on donor haemodynamics, acid-base balance, vasopressor use or cardiac index.[263] In contrast, a retrospective analysis of 106703 patients in the United Network for Organ Sharing (United States) found that administration of thyroid hormones was associated with increased organ procurement/transplantation for lung, heart, kidney, pancreas and intestine but not liver.[264]

Hormone resuscitation is most commonly delivered as a “package”, with the aim of improving donor haemodynamics and increasing the number of organs available for transplant.[236, 265] This package frequently includes methylprednisolone, thyroid hormone, vasopressin and insulin.[259, 266] Routine administration of hormone resuscitation remains controversial, however.[177, 188] Studies in pigs have demonstrated that hormone therapy was associated with improved left ventricular contractility and systemic vascular resistance.[267] This resulted in a reduction in noradrenaline requirements to maintain haemodynamic stability in the hormone resuscitation group.[267] Despite these findings, determining the role and mechanism of each agent in the hormone ‘cocktail’ has been difficult. Management of vasoplegia after brain death with vasopressin is well accepted,[177] though blood flow to the gut may be compromised.[268] As mentioned previously, recent studies of methylprednisolone and thyroid hormone individually have not clearly confirmed a benefit. Furthermore, Venkateswaran *et al* reported no improvement in donor circulatory function after administration of methylprednisolone, liothyronine or their combination compared to

placebo.[177] However, a reduction in noradrenaline administration was noted after institution of vasopressin, and this was associated with an improvement in cardiac index on *post hoc* analysis.[177]

2.7.2 Duration of Care of Brain Dead Organ Donors

The Australian and New Zealand Organ Donation Registry reported that the median time from brain death to aortic cross clamp in Australian donors in 2012 was 18.5 hours.[11] Therefore, a significant amount of time may pass between brain death and organ explantation. It was initially believed that longer intervals prior to allograft retrieval may increase organ injury due to prolonged exposure to inflammatory cytokines.[269] However, more recent data have challenged the notion that organs must be retrieved as early as possible after brain death. Avlonitis *et al* noted in a study of rats that lungs that were transplanted after five hours had improved pulmonary vascular resistance and oxygenation compared to lungs transplanted after 15 minutes.[269] In human lung transplant recipients, Wauters *et al* noted that a brain death to cold preservation (BD-CP) time of greater than 10 hours was associated with increased survival at both five and 10 years.[119] Indeed, on multivariate analysis, this group reported the hazard of dying in the years after transplant was decreased by 5% per hour increase in BD-CP time.[119] This study was a single centre, retrospective analysis, which limits the generalisability of this outcome, and further studies are needed. Renal grafts may similarly benefit from extended duration of brain death prior to transplantation. Nijboer *et al* analysed data from the Organ Procurement and Transplantation Network and found that, in donors younger than 56 years, each hour increase in duration of brain death reduced delayed graft function by 0.4%.[270]

Many confounding factors may account for these findings. This includes selection bias, as unstable potential donors may receive longer duration of care but not ultimately provide organs for transplant. As such, they are not included in databases such as those analysed.[270] Another possible explanation is the ability to apply aggressive donor management strategies, with time to optimise organ function.[119]

2.8 Potential Future Directions

The inflammatory cascade may be downregulated by ADM. Currently, there are no standard interventions specifically directed at individual cytokines, though many are being investigated (Table 2). Steroid administration, as part of hormonal resuscitation, is now

commonplace in the management of organ donors and, in addition to addressing a relatively inadequate adrenal response, reduces inflammatory cytokines to levels similar to living donors.[173, 183, 203]

Other methods directly addressing anti-inflammatory mechanisms are currently being investigated. Gene transfer of IL-10 holds great promise. Manning and colleagues investigated viral IL-10 (virIL-10) transfer into a rat model of lung IRI using mesenchymal stem cells.[25] This study showed that virIL-10 was detectable in the lungs and that presence of this cytokine was related to improved lung function, less microscopic pathology and decreased lung oedema at four hours post injury. Gene transfer pretreatment of rat liver grafts to generate recombinant human IL-10 significantly decreases IRI and markers of apoptosis, with upregulation of HO-1 and the antiapoptotic agent, Bcl-2.[35] HO-1 may then act as a downstream regulator of protective mechanisms in IRI.[35]

HO-1 or its metabolites (carbon monoxide and biliverdin) may offer potential therapeutic benefits.[44, 229, 231, 232] Overexpression of HO-1, through adeno-associated virus gene transfer, was associated with downregulation of IL-2 and TNF- α , decreased infiltration of cytotoxic and helper T-cells and an increase in IL-10, TGF- β and T_{reg} infiltration in transplanted rat livers.[229] IL-13 gene transfer in rat livers increased HO-1 expression with reduced evidence of IRI.[234] Inhibition of HO-1 activity reversed this effect, suggesting that part of IL-13's anti-inflammatory properties in IRI is mediated by HO-1.[234]

Table 2: Major Cytokines Associated with Brain Injury and Brain Death

Cytokine/ Chemokine	Organs/Cells upregulated in BD/CNS injury	Stimulation in BD/TBI	Action	Potential Therapeutic Agents in Brain Injury
TNF- α	CNS – astrocytes, microglia, neurons[133, 271] Endothelial cells[271] Lungs[272] Splenocytes, macrophages[19]	Infection, TBI, SAH[271]	Endothelial cell detachment/apoptosis, activation caspase-3, disruption of BBB[271] Induction of CAM's and other inflammatory cytokines[273] Impairment of cardiac function[204]	IFN- β [272], NNZ-2566[274], etenercept and IFN inhibitors[123] Haemoadsorption[196]
IL-1 β	CNS – neurons, microglia, infiltrating macrophages[275] Endothelial cells[271]	Neuroexcitation, infection, trauma[275] SAH[271]	Synaptic modulation, central regulation of systemic inflammatory response[275] Pro-Inflammatory, activation of NF κ B and SAPK with upregulation of E-selectin/ICAM/VCAM[271]	IL-1RA[275] and NNZ-2566[274] Haemoadsorption[196]
IL-6	CNS - Microglia[276] Kidney, Liver, Spleen, Heart[143, 172, 199, 200, 204] Macrophages[204]	IL-1 β [271] TNF- α [204] Sepsis, major surgery, heart failure, multi-trauma and burns[143, 147, 199, 277, 278]	Regulator of inflammation – Inhibition of TNF and upregulation of Control of glial responses and neuronal survival.[160-162] IL-1RA in CNS, induction of NGF[274] Disruption of BBB[271] Inducer of acute phase reaction[133, 279] Cardiac dysfunction, fibroblast activation[199]	Haemoadsorption[196]
IL-8/CXCL-8/MIP-2	Microglia[276] Lung - alveolar macrophages, endothelial cells[207, 280]	Trauma, ischaemia, SAH, ET-1[271] TNF- α , IL-1 β [273]	Disruption of BBB[271, 273] CXC chemokine – neutrophil migration and activation[273] Induces ROS by neutrophils[279]	Haemoadsorption[196]
IL-10	Macrophages, microglia[276] Splenocytes[146]	TBI[159] Burns, MT, surgery, infection[279]	Anti-inflammatory – downregulates TNF- α , IL-1 β and IFN- γ , upregulates antagonists[58, 281] Reverses effect of proinflammatory cytokines directly on cells[58]	Haemoadsorption[196]
E-Selectin	Endothelial cells in multiple organs[272]	IL-1 β [271] TNF- α [204] TBI[274] SAH[271]	Essential for neutrophil rolling, margination and diapedesis[271]	
ICAM	Endothelial cells in multiple organs[272]	IL-1 β [271] TNF- α [204] SAH[271]	Essential for neutrophil rolling, margination and diapedesis[271]	Monoclonal antibodies[271] and IFN- β [272]
VCAM	Endothelial cells in multiple organs[272]	IL-1 β [271] TNF- α [204] SAH[271]	Essential for neutrophil rolling, margination and diapedesis[271]	Monoclonal antibodies[271] and IFN- β [272]
TGF- β	Macrophages, microglia, astrocytes, neurons[282, 283] Platelets, choroid epithelium[284]	Constitutively expressed by microglia[281] SAH[284]	Anti-inflammatory, may block activation by IL-1 β [276] Regulates T-cell survival and function[283] Suppresses IFN- γ induced macrophage upregulation, cytokine and chemokine generation[283] Downregulation of adhesion molecules[283] Reduces COX-2 production in microglia[276] ECM component generation[285] Angiogenesis[284] ET-1 generation[210]	Haemoadsorption[196]
IFN- γ	Microglia[276] Macrophages[274]	TBI, SAH[274, 276]	Upregulation of CAM's, chemokines and innate immune system cells[274]	IFN inhibitors[123]
COX-2	CNS – Microglia, endothelial cells[276]	Inflammatory mediators including IL-1 β , TNF- α , IL-6[276]	Production of prostaglandins, reinforcement of inflammation[276]	COX inhibitors[276]

BD – Brain Stem Death, TBI – Traumatic Brain Injury, CNS – Central Nervous System, SAH – Subarachnoid Haemorrhage, BBB – Blood Brain Barrier, MT – Multi-Trauma, ECM – Extra-Cellular Matrix, COX – Cyclo-Oxygenase, IL – Interleukin, TNF – Tumour Necrosis Factor, CAM – Cellular Adhesion Molecule (ICAM – Inter Cellular Adhesion Molecule / VCAM – Vascular Cellular Adhesion Molecule), NF κ B – Nuclear Factor κ B, SAPK – Stress Activated Protein Kinases, MIP-2 – Macrophage Inflammatory Protein 2, TGF – Transforming Growth Factor, IFN – Interferon

Hypothermic ischaemic storage prior to transplantation does not allow sufficient metabolic activity for gene transfer to be beneficial.[286] Cypel et al therefore trialled an ex-vivo lung perfusion (EVLP) model to transfer recombinant human IL-10 genes into porcine lungs.[286] Perfusate IL-10 was increased while IL-6 decreased. This effect was maintained after transplantation and four hours of reperfusion. Lung function, as assessed by $P_aO_2:F_iO_2$ ratio, was significantly improved in the transfected lungs. When transfection was trialled in human lungs rejected for transplantation, Cypel and colleagues found similar results including improved gas exchange and pulmonary vascular resistance.[286]

Lung conditioning using EVLP is able to improve function of lungs initially rejected for transplant.[287] Sadaria et al has established a baseline cytokine profile of human lungs undergoing EVLP.[287] Cytokine analysis during 12 hours of EVLP showed an upregulation in IL-6, IL-8, G-CSF and MCP-1.[287] IL-1 β , IL-4, IL-7, IL-12 and TNF- α were detectable but remained unchanged.[287] IL-17 was undetectable, as were IL-10 and IL-13.[287] Kakishita et al also investigated the cytokine profile of EVLP in pigs.[288] Inflammatory cytokines were similarly elevated. Interestingly, based on a previously published concept of haemoadsorption of cytokines,[196] Kakishita investigated the benefit removing perfusate cytokines within the circuit. Cytokine levels were significantly reduced with haemoadsorption, but oxygenation, pulmonary vascular resistance, peak airway pressure and myeloperoxidase activity (as a marker of neutrophil accumulation) were not statistically different.[288]

Numerous other agents have been investigated as part of organ protection and preservation strategies. Donor simvastatin may reduce IRI in cardiac allografts.[215] This agent appears to work through multiple mechanisms and provides a lasting effect after a single dose to the donor prior to graft removal.[215] Organ donors in this animal model were not brain dead, therefore simvastatin's effects seem to be related to downregulation of ischaemia-reperfusion injury. A study of N-acetylcysteine after pig non-BD lung transplantation demonstrated increased glutathione and downregulation of the inflammatory transcription factor NF κ B in tissue samples.[208] IL-6 and IL-8 levels were also reduced. Lung function was improved despite extended cold ischaemia and reperfusion.[208] Intraoperative administration of N-acetylcysteine to human liver transplant recipients significantly increased the transhepatic gradient of IL-4 and IL-10 around the time of reperfusion, but not at other measured time points.[289] The authors

theorised that the presence of these anti-inflammatory cytokines at reperfusion may benefit recipients through downregulation of inflammation. Unfortunately, although the agent was administered as a continuous infusion for 24 hours, no further information is given about levels of cytokines later than the first hour of transplant, nor any information about hepatic biochemistry and patient outcomes.

In renal transplantation, carbamylated erythropoietin (EPO) downregulated renal IL-1 β and IL-6 in a rat model of brain death.[201] This agent retains the protective effects of EPO without stimulating haematopoiesis.[201] Utilising an isolated perfused kidney circuit, Nijboer and colleagues demonstrated that carbamylated EPO downregulated IL-1 β and IL-6, reduced neutrophil infiltration and reversed brain death induced renal impairment. Of note, other authors are also investigating EPO in preventing brain IRI.[290] Such use in pre-BD conditions may eventually spill over to benefit the recipients of organs from these patients in the case of non-survival.

Further research is required into the impact of pre-BD management of organ donors. There are substantial data examining the management of TBI or SAH patients which specifically addresses inflammatory/anti-inflammatory interventions and long term recovery. The impact of such management on the transplanted organs of those that fail treatment and become BD organ donors may reveal interesting results.

2.9 Conclusion

Engrafted organs undergo significant pathophysiological challenges as they are transplanted from the donor to the recipient. Brain injury, brain death, ischaemia and reperfusion all contribute to inflammation and injury. As has been discussed, a vast amount of research is ongoing at each of these steps of transplant. Understanding the molecular inflammatory responses and utilising interventions that can reduce haemodynamic instability, inflammation and IRI is the key to further advancing donor management. With time and more successful interventions, it may be possible to further address the ongoing shortage of donor organs and decrease the number of patients who die whilst waiting for a transplant.

Chapter 3 - Development of an Ovine Model for Investigation of the Implications of the Endothelin Axis after Brain Death

3.1 Development of an Ovine Model of Brain Death

3.1.1 Early Identification of the Endothelin Axis in Brain Death

In searching for models to study brain death, only intact animals are suitable due to the complex pathophysiological changes that occur across and within multiple systems. A rodent model of brain death was initially employed by our group to study the endothelin axis.[88] After brain death was induced by inflation of an extradural catheter, the animals were ventilated for four hours and then sacrificed. Analysis of pulmonary tissue demonstrated increased expression of ET-1, ET_{RA}, ET_{RB}, MMP-2 and MMP-9. Alveolar macrophages were increased in the lungs of BD animals and demonstrated increased expression of each of the investigated markers. Staining indicated upregulation of the endothelin axis in epithelial and endothelial cells, vascular smooth muscle and alveolar macrophages. The importance of these findings in the latter cellular population, and the role of macrophages as a significant source of inflammatory cytokines and gelatinases, also indicates that these cells may be 'primed' prior to transplantation.[24, 144, 227]

Other authors have observed endothelin related organ injury after brain death. In the original paper investigating inflammatory cytokines after BD, Takada *et al* noted that endothelin was upregulated in renal interstitial macrophages and focal capillary endothelial cells.[192] Endothelin expression has been ascribed to detrimental effects in brain dead hearts,[210] lungs,[291] pancreata,[292] and livers.[101] In humans, Salama *et al* observed an association between donor lung tissue endothelin mRNA, recipient serum ET-1 and primary graft dysfunction.[212] However, a recent porcine model presented by Valenza *et al* included pulmonary ET-1 mRNA assessment, and was unable to find any evidence of upregulation.[293] Overall, there remains much more to be learnt about endothelin expression in the pulmonary tissue of potential donors. Inhibition of the endothelin axis in donors may yet provide an opportunity for therapeutic intervention to prevent ET-1 driven production of gelatinases, decreasing extracellular matrix destruction and protecting potentially transplantable lungs.

3.1.2 Limitations of Rodent Animal Models

Although the rat model provided new insights into the role of the endothelin axis after BD, small animal models suffer from many limitations.[294] Challenges that rodent models suffer from include small animal size (limiting blood sampling, interventions and therapeutic manoeuvres), vastly altered physiologic variables compared to humans, significantly different structural anatomy and altered inflammatory responses to injury.[294, 295]

Recent work has also questioned the validity of mouse models for investigating human disease, demonstrating that inflammatory signalling after a variety of insults were greatly dissimilar to humans.[295] Seok *et al* compared genome-wide expression of inflammatory mediators in a variety of human diseases to their murine model counterparts and found that the expression of murine orthologs was essentially random.[295] This paper has been challenged, however, through a re-analysis of the data. Takao *et al* specifically analysed genes that were known to be significantly expressed in both humans and mice in the models under study and demonstrated a close correlation.[296] This is unsurprising considering they selected genes that were already known to be expressed by both humans and mice in validated models. Nonetheless, they considered this a more valid approach to analysing the data as animal models are designed to mimic aspects of the related human disease and cannot be expected to respond entirely the same across the whole genome.[296]

3.1.3 Selection of Sheep as a Large Animal Model of Brain Death

As no single animal model can absolutely replicate human pathology,[297] the process of selecting and developing such models requires careful consideration of known anatomical, physiological and immunological similarities between the animal chosen and the particular human disease of interest.[294] In terms of respiratory pathology, appropriately sized sheep, pigs, dogs and primates have similar body size and respiratory capacity to humans.[294] Compliance, resistance, airflow, tidal volumes and respiratory rates are of great similarity between sheep and humans, and this allows direct comparison to human values.[294] Furthermore, measures of lung function are able to be obtained without anaesthesia or sedation,[294] and targeting a lower P_aCO_2 in sheep facilitates awake ventilatory tolerance.[298, 299] Blood and tissue sampling can be increased due to animal size, and other sampling methods, such as bronchoscopy, can be undertaken.[300] Medications may be delivered effectively via aerosolisation in sheep, allowing

characterisation of both drug deposition and response.[300] Sheep immunology is closely similar to humans and has provided significant insight into innate inflammation.[297] Such properties have resulted in sheep being widely used to model human lung diseases including asthma, chronic obstructive lung disease and cystic fibrosis.[297, 300] Widespread genetic diversity due to out-breeding of sheep may more accurately reflect human disease responses compared to genetically modified rodents.[297] Although such genetic control has led to greater understanding of specific pathophysiological processes, these results may not be completely applicable to clinical human medicine.[294, 297] Endothelial and epithelial production of endothelin has been observed[301] and successfully targeted in sheep.[75, 302, 303] Finally, the placid nature of sheep allows easy housing and reduced risk due to animal handling.[304] One disadvantage of sheep models, however, is the limited range of specific antibodies currently available.[297]

Previously validated large animal models of brain death have included primates,[305] canines[306] and pigs.[307] Each of these animal models are valid and useful; there is no specific literature proving that one type should be utilised to the exclusion of others. No other group has published an ovine model of brain death, and sheep models of varying pathology do not indicate that the development of such a model would be inherently disadvantageous. Until recently, animal models of BD have remained time limited due to the associated extreme haemodynamic instability.[24] Exploratory development of a sheep model by our research group indicated that the duration could be extended to 24 hours.[308] A longer model may better replicate the clinical management of organ donors, as significant delays frequently occur between the time of BD and surgical organ explantation.[309] Sheep allow application of current human donor management strategies by their size; invasive arterial and central venous access can be utilised, as can pulmonary artery monitoring, intermittent bronchoscopy and echocardiography. Clinically employed donor protocols, including vasoactive medications and hormone therapy, may be used in a manner similar to humans, with the same doses and similar physiologic responses. Other large animal models of BD may be considered, however, due to the suitability of sheep, particularly with regard to lung injury models, and our group's significant experience with these animals, we chose to develop a clinically relevant, extended duration, ovine model to investigate the endothelin axis after brain death.

3.1.4 Anaesthesia Selection

Previous experience with the development and implementation of sheep models highlighted the need to find an appropriate anaesthetic agent for the present thesis. Haemodynamic instability associated with BD limits the use of hypotensive anaesthetic agents, such as volatile anaesthetics. This would be most marked at induction of brain death due to the significant and rapid changes in physiology that occur. Although the maintenance phase would require vastly less agent (as the animal was brain dead), hypotensive agents increase the risk of loss of animals early in the protocol. Propofol, which is also cardiodepressant, was not a suitable option, as our group's previous work identified that prolonged infusions lead to inadequate metabolism of the solubilising lipid. Consequently, this impairs sample analysis. Furthermore, prolonged infusions of large doses may be required,[310] placing control animals at risk of propofol related infusion syndrome[311] although there are currently no literature reports of this occurring in sheep. Ketamine and midazolam can be used, however in our group's experience, this combination does not provide smooth anaesthetic conditions over prolonged periods and requires increasing up-titration of doses.

Alfaxalone is a neurosteroid that was previously available in combination with alfadalone in human anaesthesia (Althesin).[312] This agent was removed from the market in 1984 due to hypersensitivity reactions that occurred with the solubilising excipient that was used (Cremophor EL).[313]. More recently, alfaxalone has been reformulated for use in veterinary medicine. This contemporary formulation uses 2-hydropropyl- β -cyclodextran to solubilise the active anaesthetic agent, and is completely devoid of the histamine releasing effects seen with Cremophor EL.[314] Furthermore, it provides rapid anaesthesia with stable haemodynamics in sheep,[314] and would not be expected to cause the previously identified issues with lipid metabolism that occur with propofol. Because of these benefits, alfaxalone was chosen as the primary anaesthetic agent for this thesis. Single agent anaesthesia with alfaxalone was not feasible due to cost[315]; hence a combination of alfaxalone, ketamine and midazolam was used.

3.1.5 Selection of the Endothelin Antagonist

Sutherland *et al* noted that both ET_RA and ET_RB receptors were upregulated after brain death, particularly on alveolar macrophages.[88] These cells play an integral role in inflammatory responses to various stimuli; through multiple cell surface receptors and a significant armamentarium of inflammatory mediators, macrophages can contribute directly

to pulmonary inflammation, or act to recruit neutrophils and further drive the inflammatory process.[316, 317] Gibbs *et al* identified a link between alveolar macrophages and gelatinase expression in lung injury in rats, which was similar to that seen in the human lung.[317] This observation was extended to the brain death model by the Sutherland study.[88] Other authors have identified the relationship between alveolar macrophages, pulmonary IRI and free oxygen radical generation.[318, 319] These findings have led to the conclusion that resident tissue inflammatory cells, including pulmonary macrophages, may be primed prior to transplantation, and that these cell populations may be the initiators of early lung injury after transplant.[24, 318] The selection of a dual endothelin receptor antagonist was therefore based on the observations that alveolar macrophages express both endothelin receptors after brain death[88] and that they contribute significantly to post-transplant pulmonary injury.[318]

Endothelin antagonists have been investigated in a wide variety of conditions, including pulmonary hypertension,[108] sepsis,[74] pain,[80] cancer,[84, 111] meconium aspiration,[320] heart failure,[321, 322] and cerebral vasospasm amongst others.[170] An important recognised class effect of endothelin antagonists is vasodilatation.[76, 96, 323] In the setting of brain death, the haemodynamic deterioration that occurs after the early catecholamine “storm” often requires vasoactive support to maintain organ and tissue perfusion.[24, 238] Thus, nebulisation would allow drug delivery directly to the lung, whilst minimising systemic absorption and possible hypotensive effects. In selecting an endothelin antagonist for the current study, tezosentan fulfils these major criteria (dual receptor antagonism[324] and ability to be nebulised).

3.1.5.1 Tezosentan

Using bosentan as a template, tezosentan was developed to increase water solubility and clearance for use in urgent and emergent indications.[324] Furthermore, the high degree of water solubility allows its administration both intravenously and via nebulisation.[74, 324, 325] Persson *et al* compared intravenous and nebulised tezosentan in endotoxaemic pigs, and demonstrated that a dose of 0.5 mg/kg nebulised effectively reversed pulmonary hypertension.[74] This dose was also used by Mommerot *et al*, who observed improved haemodynamics and oxygenation parameters in a porcine model of cardiopulmonary bypass.[325] Tezosentan has also demonstrated therapeutic efficacy in sheep. Kuklin *et al* demonstrated that tezosentan reduced pulmonary microvascular pressure and evidence

of lung injury in endotoxaemic sheep.[303] Cox *et al* observed that tezosentan decreased pulmonary vascular resistance and bronchiolar obstruction in sheep after smoke inhalation and burn injury, however it did not prevent lung injury overall.[302] Finally, Fitzgerald *et al* observed that tezosentan was able to reduce acute and chronic pulmonary hypertension in a lamb model.[326]

The above studies indicated that tezosentan was able to be administered via nebulisation and that an observable effect may be expected in sheep, further supporting its selection. Bosentan was initially considered, however it is administered orally, and wasn't available for this study in intravenous/nebulisable form. Macitentan was not available. Finally, other preclinical agents were not as easily available as tezosentan; these were not required as tezosentan fulfilled the requirements of this study.

The early clinical application of tezosentan in human pathology focussed on its use in acute heart failure. This included the Randomised Intravenous TeZosentan-4 (RITZ), trial which found no benefit for tezosentan administration in terms of dyspnoea or a composite endpoint including death, worsening heart failure, ischaemia or new or recurrent myocardial infarction.[327] Following up from this, RITZ-5 did not find benefit for intravenous tezosentan for pulmonary oedema.[328] The largest trials investigating tezosentan in acute heart failure (VERITAS)[321] confirmed these results. Consequently, tezosentan is no longer used in acute heart failure. It is yet to find a place in routine clinical medicine.

Chapter 4 - Novel 24-h ovine model of brain death to study the profile of the endothelin axis during cardiopulmonary injury

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4.1 Abstract

Background: Upregulation of the endothelin axis has been observed in pulmonary tissue after brain death, contributing to primary graft dysfunction and ischaemia reperfusion injury. The current study aimed to describe a novel, 24 hour, clinically relevant, ovine model of brain death, developed to investigate the pulmonary endothelin axis and related physiology. We hypothesised that brain death in sheep would also result in demonstrable injury to other transplantable organs.

Methods: Twelve merino cross ewes were randomised into two groups. Following induction of general anaesthesia and placement of invasive monitoring, brain death was induced in six animals by inflation of an extradural catheter. All animals were supported in an intensive care unit environment for 24 hours. Animal management reflected current human donor management, including administration of vasopressors, inotropes and hormonal resuscitation therapy. Activation of the endothelin axis and transplantable organ injury were assessed using ELISA, immunohistochemistry and standard biochemical markers.

Results: All animals were successfully supported for 24 hours. ELISA suggested early Endothelin-1 and Big Endothelin-1 release, peaking one and six hours after BD, respectively, but there was no difference at 24 hours. Immunohistochemistry confirmed activation of the endothelin axis in pulmonary tissue. Brain dead animals demonstrated tachycardia and hypertension, followed by haemodynamic collapse, typified by a reduction in systemic vascular resistance to $46 \pm 1\%$ of baseline. Mean pulmonary artery pressure rose to $186 \pm 20\%$ of baseline at induction and remained elevated throughout the protocol, reaching 25 ± 2.2 mmHg at 24 hours. Right ventricular stroke work increased 25.9% above baseline by 24 hours. Systemic markers of cardiac and hepatocellular injury were significantly elevated, with no evidence of renal dysfunction.

Conclusions: This novel, clinically relevant, ovine model of brain death demonstrated an increase in pulmonary blood pressures in brain dead animals over 24 hours. Confirmation of the presence of the endothelin axis after brain death may implicate its role in generating the observed pulmonary pressures, contributing to inflammation and cardiopulmonary injury. The development of this model will allow for further investigation of therapeutic strategies to minimise the deleterious effects of brain death on potentially transplantable organs.

4.1.1 Medical subject headings key words:

Brain Death; Organ Transplantation; Haemodynamics; Pulmonary Circulation; Ventricular Function, Right; Endothelin-1; Receptors, Endothelin; Sheep.

4.2 Background

After brain death (BD), the lungs are particularly susceptible to injury in the peri-transplant period secondary to direct trauma, soiling with blood or gastric contents, iatrogenic injury, infection and inflammation.[24, 241, 269] Details of the specific mechanisms of catecholamine- and cytokine-induced donor organ injury after BD are yet to be fully elucidated.[24, 119, 329] Peri-transplant injury contributes to the ongoing shortage of transplantable lungs; this is highlighted by United States data showing an average rate of lungs transplanted per donor of 0.37.[12]

Endothelins, their precursors, receptors and associated signalling pathways are collectively referred to as the endothelin axis.[85, 88] Endothelin-1 (ET-1) is a potent vasoconstrictor, smooth muscle cell and fibroblast mitogen and a stimulator of inflammatory cell infiltration.[72, 75, 330] Once released, ET-1 stimulates matrix metalloproteinase (MMP) expression in pulmonary tissue, resulting in protein hydrolysis and interstitial oedema.[24, 88] Our group first demonstrated that the endothelin axis was upregulated after BD in rats, and that this correlated with pulmonary injury.[88] Upregulation of endothelin receptors “primes” the lungs for post-transplant injury,[24] and may partly explain the relationship between endothelin expression and primary graft dysfunction that has been observed in human lung allograft recipients.[212]

Haemodynamic instability has limited the duration of previous BD animal studies and supportive measures used to extend these models to clinically relevant timeframes are difficult to apply to small animals.[186, 210, 306] Interventions utilised in human BD donors, such as fluid or vasoactive agent administration, may have significant effects on genomic expression of inflammatory mediators,[295, 331] further limiting the ability of small animal models to replicate comprehensive, modern, intensive care monitoring and management. To begin to address these issues, porcine models have been extended to 24 hours.[332, 333] Zhai *et al* investigated hepatic injury after BD in BaMa miniature pigs.[332] Although this is a valid extended model, the animals were small compared to humans (average of 25 kg), and the use of other clinically relevant interventions, such as vasopressors, inotropes and hormone resuscitation, were not reported. The model published by Sereinigg *et al* was developed to more closely reflect clinical experience with BD donors, including the use of vasoactive agents, however this publication did not specifically include a control group for comparison.[333]

No animal model can absolutely replicate all aspects of human pathophysiology.[297] For example, controversy exists regarding rodent modelling of human disease, with evidence both supporting and refuting similarities of inflammatory genomic responses to injury between the two species.[295, 296] Both pigs and sheep have been effectively utilised as large animal models of human pathology, with each offering notable benefits.[304, 334] Ovine models have been highlighted as particularly suitable for investigating human lung disease.[304, 331, 335, 336] Furthermore, sheep models have provided detailed insight into the endothelin axis and its contribution to pulmonary haemodynamics, as well as the role of ET-1 in lung inflammation.[294, 301, 304, 337] Therefore, based on these considerations, we have developed a 24 hour ovine model to investigate the role of the endothelin axis in BD related pulmonary inflammation. Additionally, the results of comprehensive investigation of the effects of BD on ovine haemodynamics and systemic markers of transplantable organ injury are presented.

4.3 Materials and Methods

4.3.1 Ethics Approval

This study was conducted with the approval of the Queensland University of Technology Animal Ethics Committee, approval number 0900000319. All experiments were performed in accordance to NHMRC *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* and the *Animal Care and Protection Act 2001 (QLD)*.

4.3.2 Animal Management

Twelve merino-cross ewes were randomly allocated to groups of six animals each (BD vs control) using Statmate (GraphPad Software, La Jolla, California). Initial surgical preparation was the same in all animals. A comprehensive description of the animal management protocol can be found in 4.8 Appendix 1: Animal Management, while Table 3 lists the details of the medications used in this study. After fasting, the right external jugular veins were cannulated, general anaesthesia was induced with midazolam and alfaxalone, and all animals were intubated. Pulmonary arterial and peripheral arterial catheters were placed. Intracranial access was obtained through a burr-hole midway between the midline and lateral edge of the cranium, rostral to the animal's horn base, and an intracranial pressure monitor was introduced. This was designated as the Protocol Start Time (PST) in non-BD animals.

Another burr hole was created on the contralateral side in animals allocated to BD, followed by the extradural placement of a 16 Fr Foley catheter. Brain death was induced by normal saline inflation of the catheter to increase intracranial pressure (ICP) above the mean arterial pressure (MAP) for 30 minutes.[305] Commencement of inflation served as the BD induction time (BIT). Confirmation of brain death was achieved by continuously negative cerebral perfusion pressure (defined as $MAP - ICP$) for greater than 30 minutes, loss of pupillary and corneal reflexes and lack of respiratory efforts. Protocol start time was deemed once BD was confirmed in animals allocated to this group. Due to variability in duration required for induction and confirmation of BD, haemodynamic results are reported as time from BIT. Haemodynamic deterioration was managed with intravenous fluid, and vasopressors or inotropes as appropriate.

At 12 hours after PST, hormone resuscitation therapy was commenced with vasopressin, methylprednisolone and liothyronine. This time point was chosen to reflect the clinical realities of delays in diagnosis and confirmation of brain death, family consent for organ transplantation, and the change from lifesaving to organ preserving treatment.[309] After completion of the 24 hour protocol, the animals were sacrificed using sodium pentobarbitone.

Table 3: Medications Used as Part of the Protocol.

Drug	Bolus	Initial Infusion Rate	Notes
Anaesthetic Induction			
Lignocaine 1%	3-5 mL sub-cutaneously		Over central venous access insertion sites
Buprenorphine	300 mcg		Administered six hourly during protocol
Midazolam	0.5 mg/kg		
Alfaxalone	3 mg/kg		If further boluses needed, dosed at 0.5 mg/kg
Anaesthetic Maintenance			
Alfaxalone		6 mg/kg/hr	Adjusted to surgical plane
Ketamine		3 mg/kg/hr	Adjusted to surgical plane
Midazolam		0.25 mg/kg/hr	Used only if required (if alfaxalone exceeded 250 mg/hr)
Antimicrobial Prophylaxis			
Cefalotin	1000 mg		
Gentamicin	40 mg		
Fluid Management			
Hartmann's Solution	10-20 mL/kg	2 mL/kg/hr	Titrated to CVP 8–12 mmHg. Boluses if needed for low urine output (< 0.5 mL/kg/hr) or hypotension (MAP < 60 mmHg). Initial fluid of choice
Normal Saline 0.9%	10-20 mL/kg	1-2 mL/kg/hr	Boluses if needed for low urine output (UO < 0.5 mL/kg/hr) or hypotension (MAP < 60 mmHg).
Dextrose 5% or Dextrose 4% in Saline 0.18%	10-20 mL/kg	1-2 mL/kg/hr	Utilised for hypoglycaemia (BSL < 6 mmol/L)
Vasopressors, Inotropes and Cardiovascular Support			
Metaraminol	0.5-1 mg		Utilised in emergency situations for hypotension only
Atropine	600 mcg		Utilised in emergency situations for bradycardia (HR < 60 bpm) only
Noradrenaline		0.05 mcg/kg/min	Adjusted to MAP > 60 mmHg
Dopamine		5 mcg/kg/min	Adjusted to MAP > 60 mmHg
Isoprenaline		0.5 mcg/min	Adjusted to MAP > 60 and HR > 60 bpm. Utilised only if considered bradycardia as cause of hypotension
Glyceryl Trinitrate		0.1 mg/hr	For hypertension (SBP > 180 mmHg) if necessary
Amiodarone		5 mg/kg over 2 hours	Infusion for appropriate dysrhythmias (eg atrial fibrillation) if necessary. Could be repeated
Hormonal Management			
Insulin	10 – 20 U	0.5 U/hr	Bolus for BSL > 16 mmol/L. Infusion adjusted to BSL 6 – 10 mmol/L, tested hourly once infusion commenced
Dextrose 50%	25 mL		For management of hypoglycaemia (BSL < 3.5 mmol/L). Please also note Dextrose 5% could be used for ongoing maintenance per above
Desmopressin	4 mcg		If urine output > 300 mL/hr for two consecutive hours

Table 3 (cont): Medications and Ventilation Settings Used as Part of the Protocol.

Hormone Resuscitation at 12 hours			
Vasopressin	1 U	0.5 – 4.0 U/hr (initial dose 2.0 U/hr)	Adjusted to SVR 800 – 1200 dyn.s.cm ⁻⁵
Liothyronine	4 mcg	3 mcg/hr	
Methylprednisolone	15 mg/kg		
Electrolyte Management			
Potassium Chloride		10 – 40 mmol/hr	Adjusted to potassium 3.5 – 5.0 mmol/L
Calcium Chloride 10%	6.8 mmol		Administered to keep ionised calcium > 1.05 mmol/L
Magnesium Sulphate	10 – 20 mmol		Allowed for management of dysrhythmias (eg atrial fibrillation)
Euthanasia			
Sodium Pentobarbitone	100 mg/kg		

Not all agents were used. Agents listed include medications that were able to be used in the case of predetermined outcomes or complications.

4.3.3 Sample Retrieval and Storage

Blood was collected from the peripheral arterial line at baseline (prior to BIT), 1, 6, 12, 18 and 24 hours after confirmation of BD. Blood samples were then centrifuged, supernatant transferred into vials (Eppendorf, North Ryde, Australia) and stored at –80 °C until analysis. After animals were euthanised, the lungs were removed *en bloc* and samples taken from both lower lobes. These were fixed in 10% phosphate buffered formalin, embedded in paraffin and mounted on slides for histological analysis.

4.3.4 Histological and Tissue Analysis

Samples were taken from the right lower lobe to assess for wet:dry weight ratio, as an indicator of inflammatory oedema. These were dehydrated in an oven at 45 °C for 48 hours, at which time they were reweighed and the ratio calculated.

Haematoxylin and eosin staining of lung specimens was performed to allow morphologic assessment of tissue samples. Inflammation was graded semi-quantitatively as previously reported.[88]

Immunohistochemical staining was employed to assess the patency of the endothelin axis using standard methods (see also 4.9 Appendix 2: Immunohistochemistry Staining Procedure).[114] Monoclonal anti-ET-1 (Sigma Aldrich, St Louis, Mo), polyclonal anti-ET_RA, anti-ET_RB and anti-MMP-2 (Merck Millipore, Billerica, MA), polyclonal anti-MMP-9 (Biorbyt, San Francisco, CA) and polyclonal anti-TIMP-1 (Tissue Inhibitor of Metalloproteinase-1) and anti-TIMP-2 (Bioss, Woburn, MA) were selected as primary antibodies. Immunohistochemistry and histological scoring was performed independently

by two investigators (RW and IB) and results compared. Disagreements in scoring were resolved by using the lowest score (ie indicating less injury). Slides were assessed in random order and the assessors were blinded to group of allocation.

4.3.5 ELISA

Systemic concentrations of ET-1 and proendothelin-1 (Big ET-1) were assessed in EDTA plasma using commercially available sandwich ELISA kits (BiomedicaGruppe, Austria). Absorbance was read at 450 nm with reference 630 nm on a 96 well plate spectrophotometer (FluoStar Omega, BMG LabTech, Germany). The results from one animal in the BD group were excluded due to technical reasons preventing accurate spectrophotometric analysis.

4.3.6 Biochemical Analysis

Biomarkers of organ injury were assayed using the COBAS Integra 400 chemical analyser (Roche Diagnostics, Dee Why, Australia), following manufacturer's instructions. Reagent cassettes were calibrated using the Calibrator for Automated Systems (CFAS, Roche Diagnostics). Precision and accuracy of assays were confirmed using standard quality controls (Precinorm Clin Chem Multi 1 and 2, Roche Diagnostics). All tests were performed in duplicate, averaged and compared to CFAS calibrators in order to interpolate sample concentrations. Cardiac markers included myoglobin and CK-MB. Hepatic markers included total and conjugated bilirubin, alkaline phosphatase and γ -glutamyl transferase (cholestatic function) and alanine aminotransferase, aspartate aminotransferase (hepatocellular injury). Albumin and total protein were assessed as markers of synthetic function, while lactate dehydrogenase was a general marker for cellular injury. Urea, creatinine and phosphate were included to assess renal function.

4.4 Statistical Analysis

This study was designed with six subjects for each group. This is able to detect a true difference in the standardised mean change between experimental and control subjects of ± 1.794 with probability (power) 0.8. The Type I error probability associated with this test of the null hypothesis that the population means of the experimental and control groups are equal is 0.05. We assumed the response within each subject group was Normally distributed, standardised to a standard deviation of 1.

Analysis of biochemical data was performed using Prism 6 (GraphPad Software Inc., United States). All regression analyses of physiological data were conducted using R software (www.r-project.org). A two-sided statistical significance level of <0.05 was adopted. Results are reported as mean \pm standard deviation. Two-way repeated-measures Analysis of Variance (ANOVA) was used to test for significant differences in dependent variables. Student's t-test was used to compare changes in physiological variables at specified time points. Fisher's exact test was used to compare semi-quantitative assessment of tissue samples. For continuous physiological variables, a regression model was used to examine the changes in variables over time. A mixed model with a random intercept for each sheep to account for repeated responses from the same animal was used.[338]

4.5 Results

All 12 animals survived the 24 hour protocol. Induction of BD was successful in all animals allocated to this group. Summary tables detailing ventilation, haemodynamics, fluid balance, biochemistry and histology can be found in 4.10 Appendix 3: Summary of Animal Data.

4.5.1 Animal Management and Point of Care Testing

There were no differences between the animal groups with regard to mechanical ventilation. Markers of oxygenation, $P_{(A-a)}O_2$ and $P_aO_2:FiO_2$, deteriorated in BD animals over the first two hours. Mean $P_{(A-a)}O_2$ in BD animals was 66.8 ± 40 mmHg (8.9 ± 5.3 kPa, $p<0.001$) greater at one hour and 45.7 ± 40 mmHg (6.09 ± 5.3 kPa, $p=0.016$) greater at two hours. $P_aO_2:FiO_2$ was 221 ± 81 less in BD animals ($p<0.001$) at one hour and 110 ± 80 ($p=0.003$) less at two hours. These variables were thereafter similar to controls and no difference was found at 24 hours ($p=0.56$ $P_{(A-a)}O_2$ and $p=0.87$ $P_aO_2:FiO_2$, Figure 5). Minute ventilation was similar between the groups, with a trend towards lower P_aCO_2 in the control group by 24 hours (P_aCO_2 27 ± 4 mmHg (3.6 ± 0.5 kPa) control vs 32 ± 5 mmHg (4.3 ± 0.6 kPa) BD, $p=0.051$). Lactate, a surrogate marker of hypoperfusion, was significantly elevated in the BD group ($p=0.03$ at 24 hours), reaching a peak value of 2.75 ± 3.3 mmol/L at 18 hours (Figure 6). There was no difference in blood pH between groups ($p=0.85$). No vasoactive agents were required in the control group, whereas all BD animals required vasoactive support (Figure 7). Each of the six BD animals met predefined criteria for diabetes insipidus and required desmopressin. Cumulative fluid

balance at 24 hours was not different between groups (2.1 ± 0.8 L control vs 2.4 ± 1.7 L BD, $p > 0.9$).

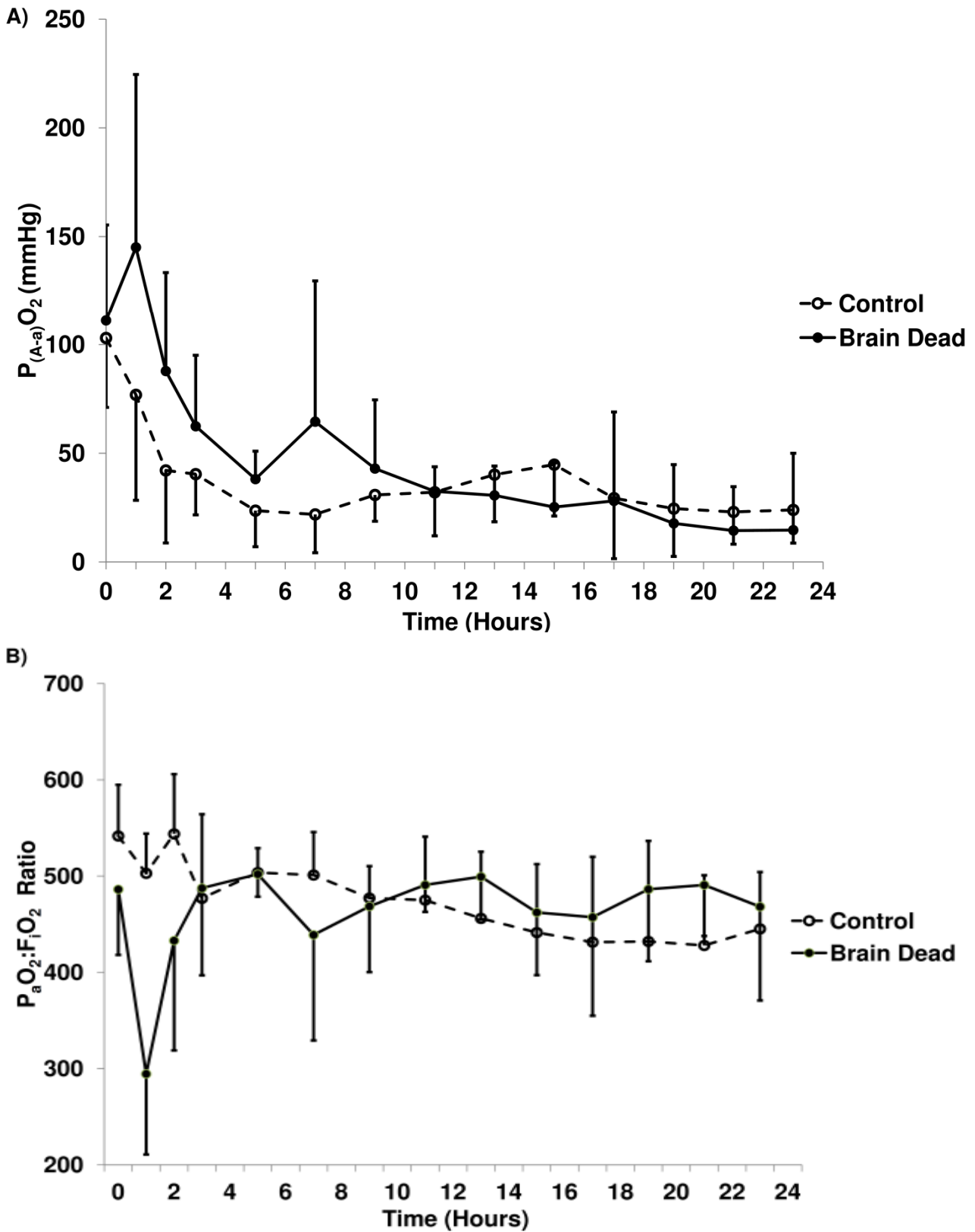


Figure 5: Oxygenation Parameters from Arterial Blood Gases

A) Alveolar-arterial oxygen difference, $P_{(A-a)}O_2$, B) $P_aO_2:F_iO_2$ Ratio. Oxygenation deteriorated early after induction of brain death and was no different to control animals at 24 hours. Brain death was induced immediately after the baseline value at time 0.

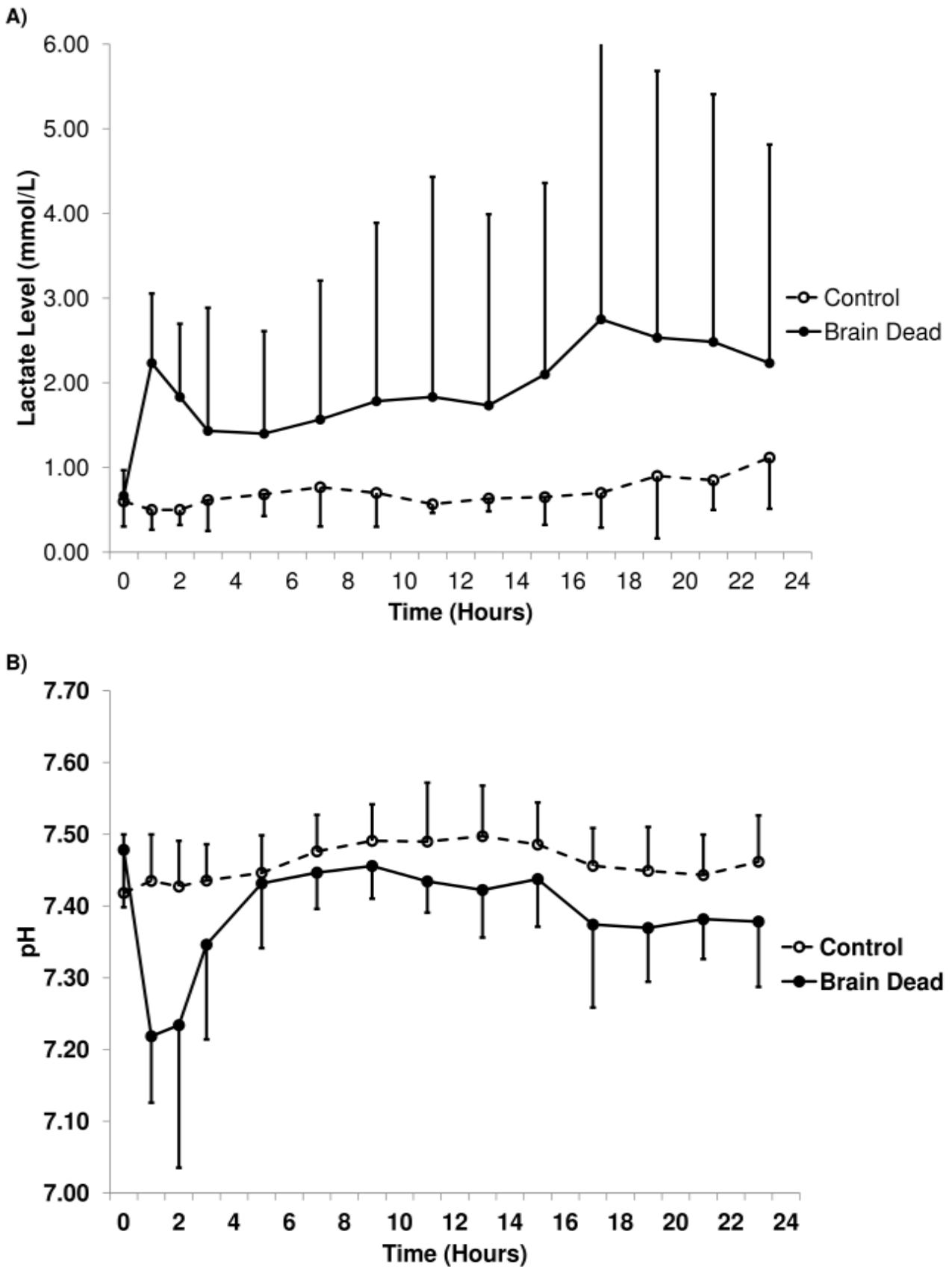


Figure 6: Lactate and pH Values from Arterial Blood Gases

A) Lactate levels and B) pH as measured by arterial blood gases. Lactate levels were elevated in brain dead animals, remaining higher than controls at 24 hours. An early acidosis was noted, but normalised by 24 hours. Brain death was induced immediately after the baseline value at time 0

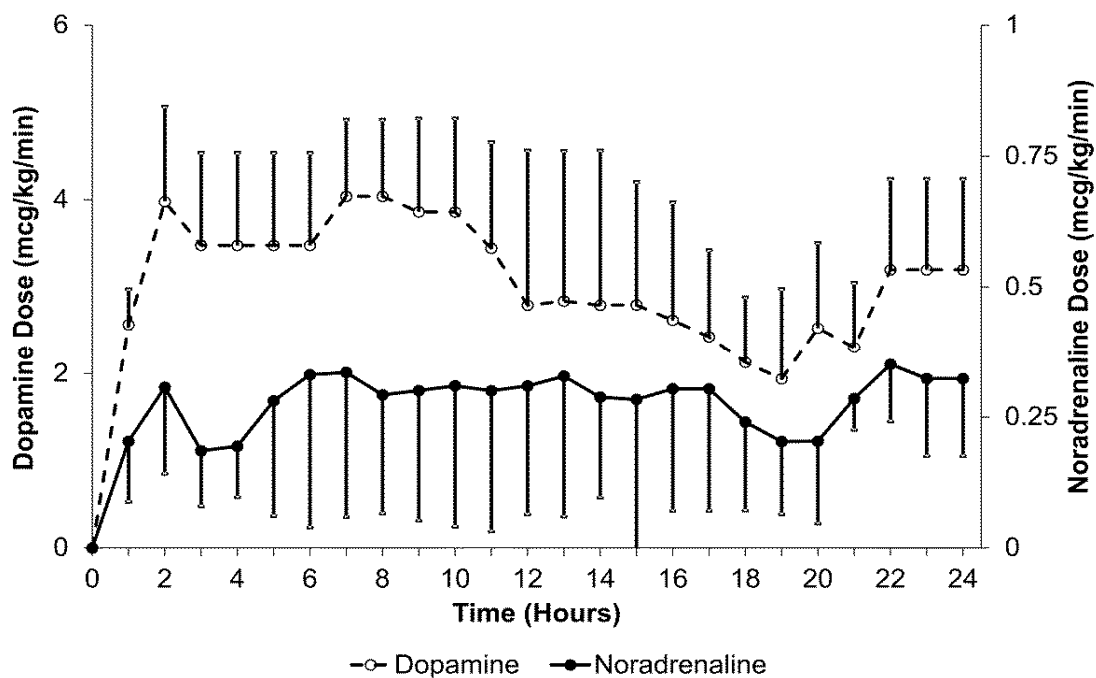


Figure 7: Doses of Vasoactive Agents Administered to Brain Dead Sheep

Mean doses of dopamine and noradrenaline administered to the brain dead animals over the duration of the study.

4.5.2 Physiologic Variables

Time to confirmation of brain death after inflation of the extradural catheter was 50 ± 22 mins. During this time, the highest ICP achieved was 237 ± 79 mmHg, with a resultant CPP of -117 ± 34 mmHg. At completion of the study, ICP was 87 ± 12 mmHg with a CPP of -6.5 ± 12 mmHg.

Brain death caused tachycardia, hypertension and elevated cardiac output (Figure 8A-C). Cardiac index (CI) increased primarily as a consequence of tachycardia with the stroke volume index (SVI) acutely decreasing from 45 ± 1 mL/m² to 30 ± 6 mL/m² ($p < 0.001$). Mean arterial pressure increased from 99 ± 3 mmHg to peak at 193 ± 40 mmHg during induction of BD, decreasing to $58 \pm 2\%$ of baseline at 90 minutes after BIT ($p < 0.001$) and remaining lower than the control group at 24 hours ($p < 0.001$). Systemic vascular resistance index (SVRI - Figure 8D) increased from 1741 dyn.s.cm⁻⁵ to 3718 dyn.s.cm⁻⁵ within five minutes of foley catheter inflation, falling to $46 \pm 1\%$ of baseline by one hour and remaining depressed throughout the remainder of the study. After initiation of hormone resuscitation, SVRI increased to $81 \pm 7\%$ of baseline. Cardiac index increased to a peak

of 7.48 ± 2.1 L/min/m² from a baseline of 4.55 ± 0.18 L/min/m² 30 minutes after BIT and remained $14 \pm 5\%$ above baseline until one hour after hormonal therapy was commenced, whereby it returned to baseline levels. At 24 hours there was no statistical difference ($p=0.79$ compared to baseline, $p=0.91$ compared with controls). Left ventricular stroke work index (LVSWI) was significantly reduced in BD animals compared to controls ($p<0.001$). After decreasing to 19.8 ± 0.69 g.m/m²/beat at 75 minutes post BIT, LVSWI returned to 35.1 ± 1.3 g.m/m²/beat over the following four hours. Hormonal resuscitation therapy increased LVSWI to 44.8 ± 2.8 g.m/m²/beat at 24 hours ($p<0.001$ compared to baseline).

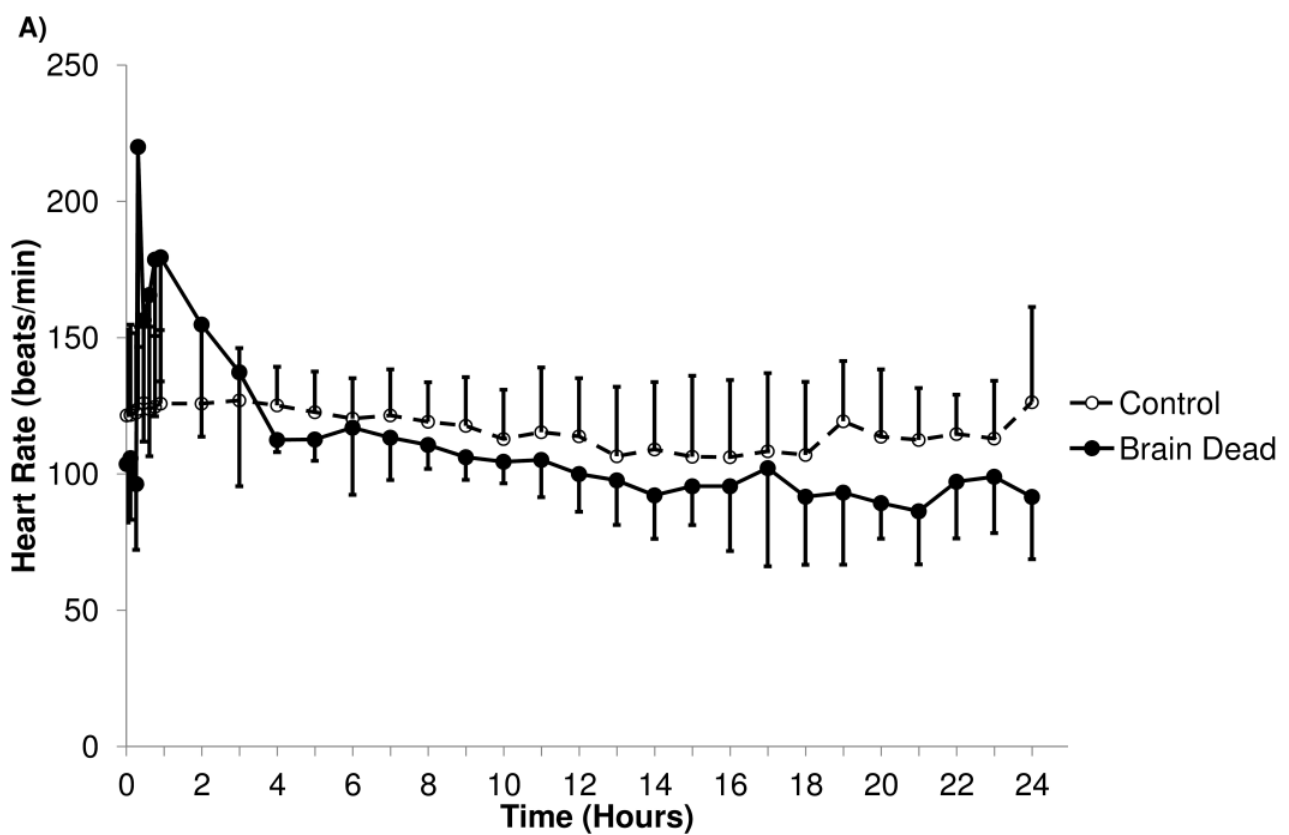


Figure 8: Systemic Cardiovascular Responses Observed in Brain Dead and Control Animals over 24 Hours.

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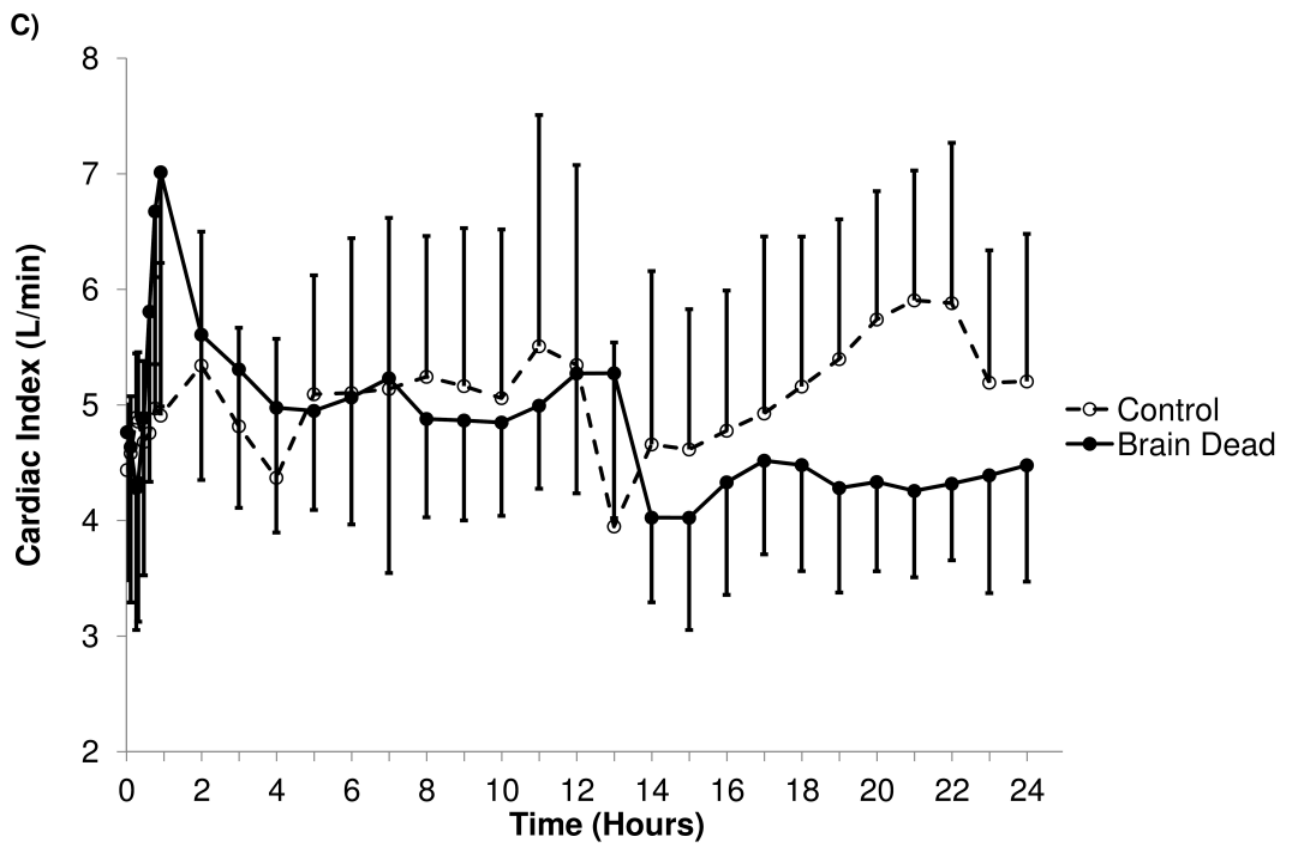
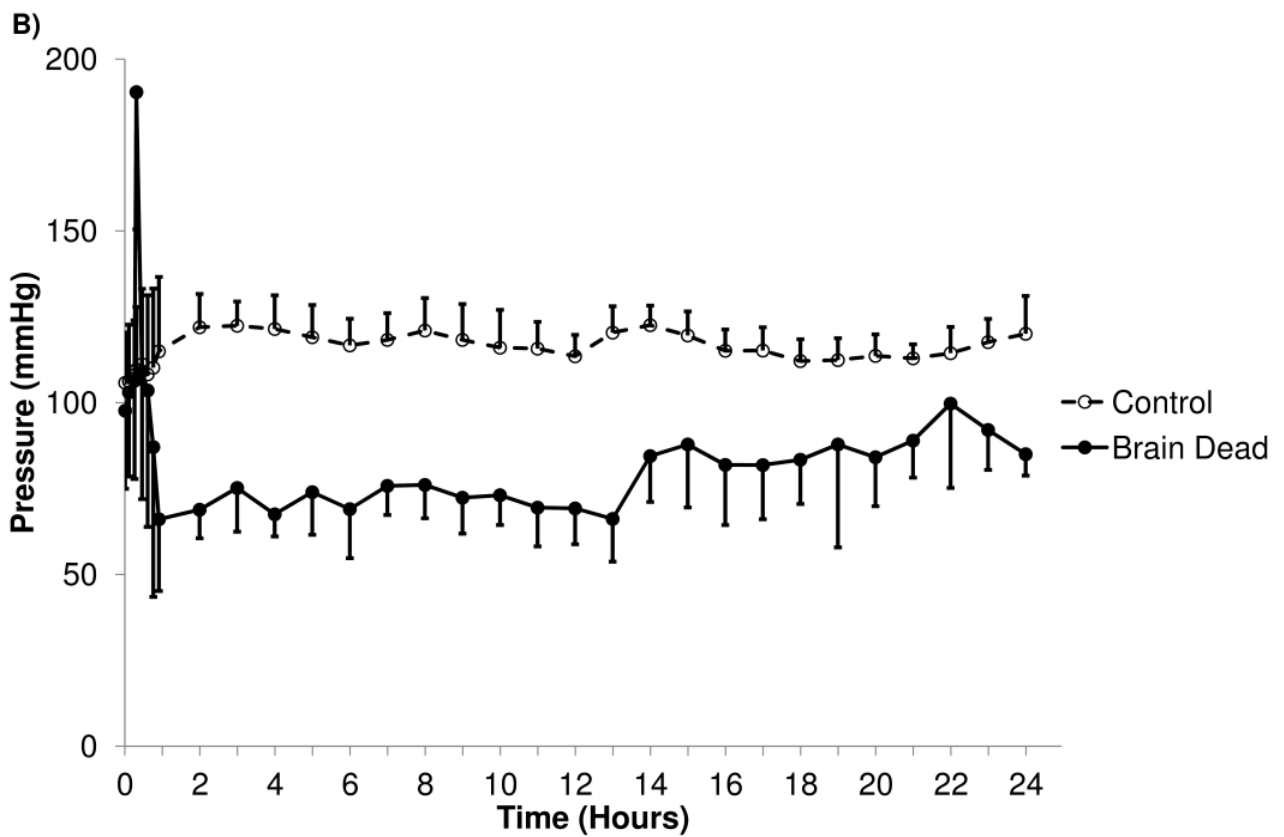


Figure 8 (cont): Systemic Cardiovascular Responses Observed in Brain Dead and Control Animals over 24 Hours.

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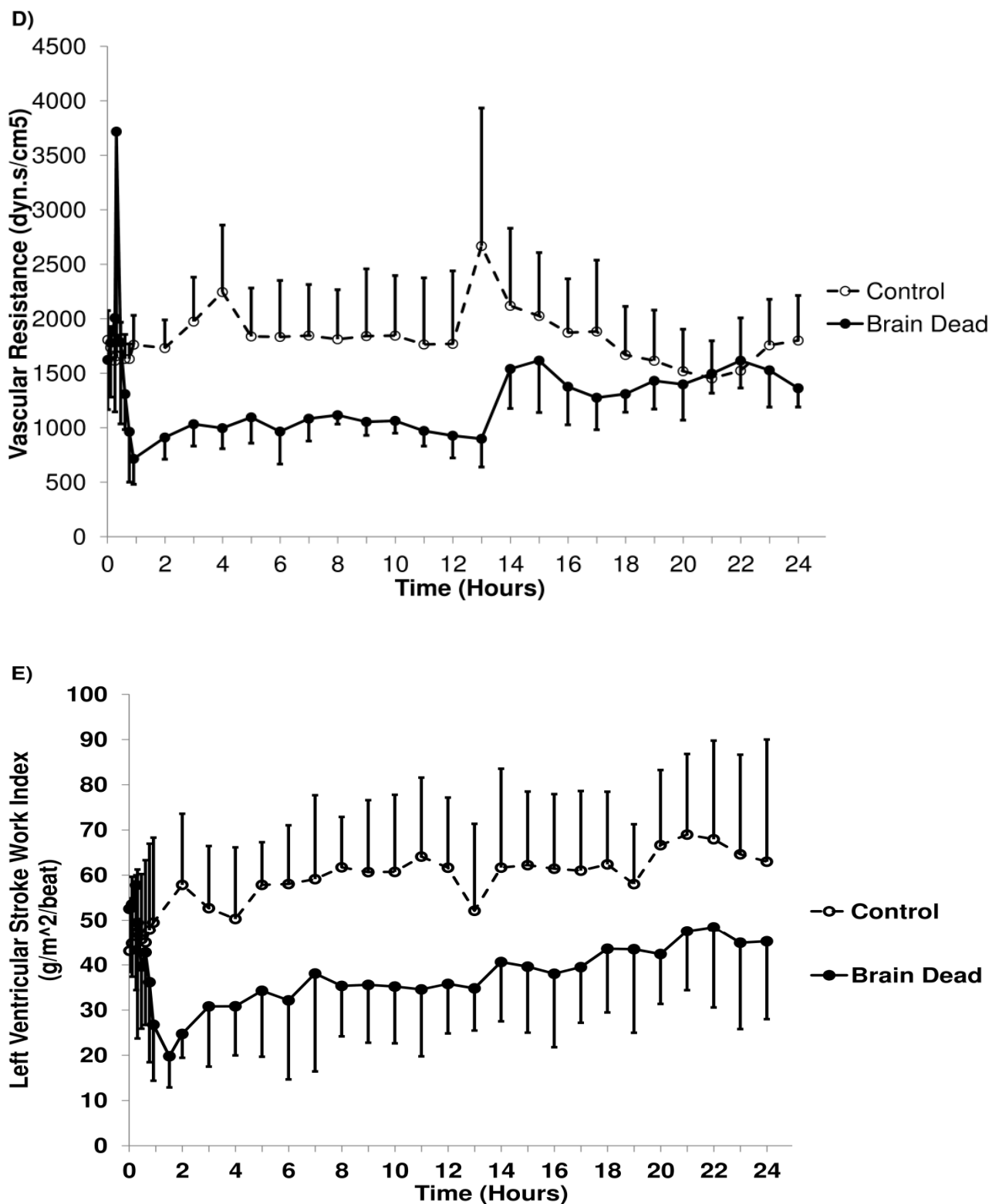
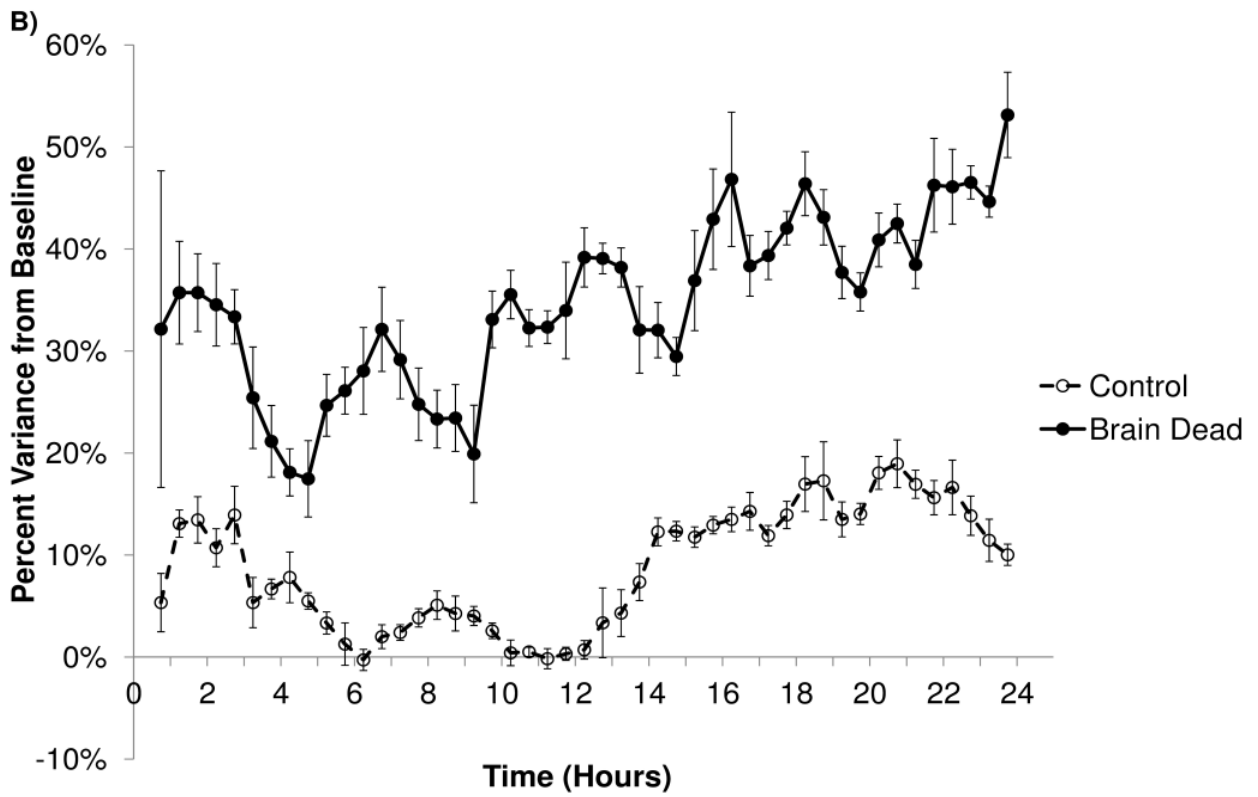
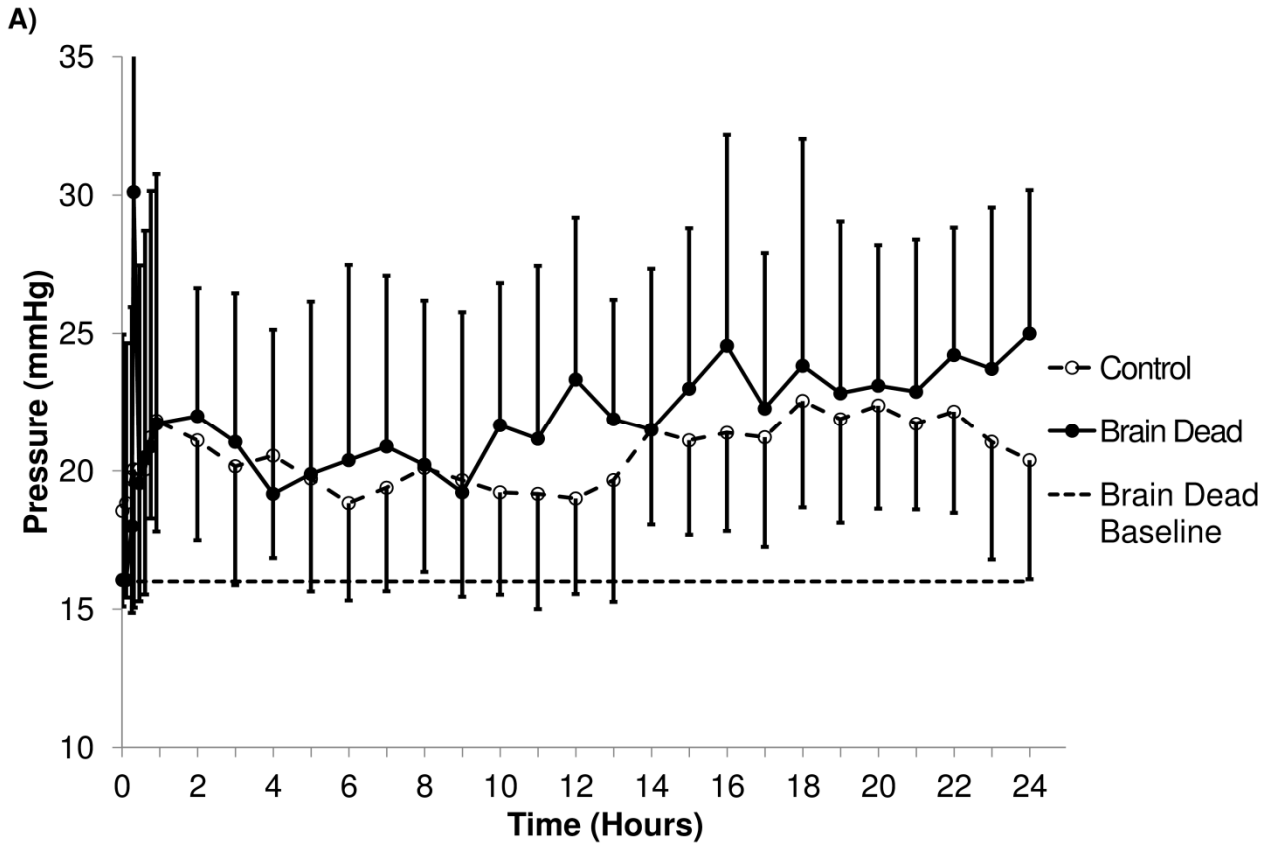


Figure 8 (cont): Systemic Cardiovascular Responses Observed in Brain Dead and Control Animals over 24 Hours.

A) Heart rate. After an early peak, heart rate was similar to controls at 24 hours. B) Mean arterial pressure increased with brain death and then fell below baseline. C) Cardiac index was elevated after brain death, returning to control levels by three hours. It fell after administration of hormone therapy. D) Systemic vascular resistance index was similar to MAP. E) Left ventricular stroke work index fell after brain death. It rose over time but remained less than control animals at 24 hours. Brain death was induced immediately after the baseline value at time 0.

Mean pulmonary artery pressure (mPAP) peaked at $186 \pm 20\%$ of baseline with induction of BD ($p < 0.001$), rising from 16 ± 0.2 mmHg to 30 ± 13 mmHg. After the initial peak, mPAP remained $31 \pm 2\%$ greater than baseline 90 minutes after BIT ($p < 0.001$) and continued to increase for the remainder of the experiment (Figure 9A-B). Pulmonary vascular resistance index (PVRI) increased from 50 ± 3 dyn.s.cm⁻⁵ to 123 ± 77 dyn.s.cm⁻⁵ within five minutes of foley catheter inflation (Figure 9C), decreasing to $55 \pm 4\%$ of baseline (28 ± 12 dyn.s.cm⁻⁵) at four hours after BD. The PVRI returned to baseline after initiation of hormone resuscitation and was not different from the control group at 24 hours ($p = 0.5$). Right ventricular stroke work index (RVSWI) had decreased 15 minutes after BIT (by 1.4 ± 0.7 g.m/m²/beat, $p < 0.001$). However, by 30 minutes, this had increased to be 6.8 ± 0.34 g.m/m²/beat ($5.4 \pm 0.01\%$) above the baseline of 6.5 ± 0.24 g.m/m²/beat ($p = 0.01$) and continued to increase to 25.9% above baseline (8.4 ± 1 g.m/m²/beat) at the end of the study in the BD animals ($p < 0.001$).



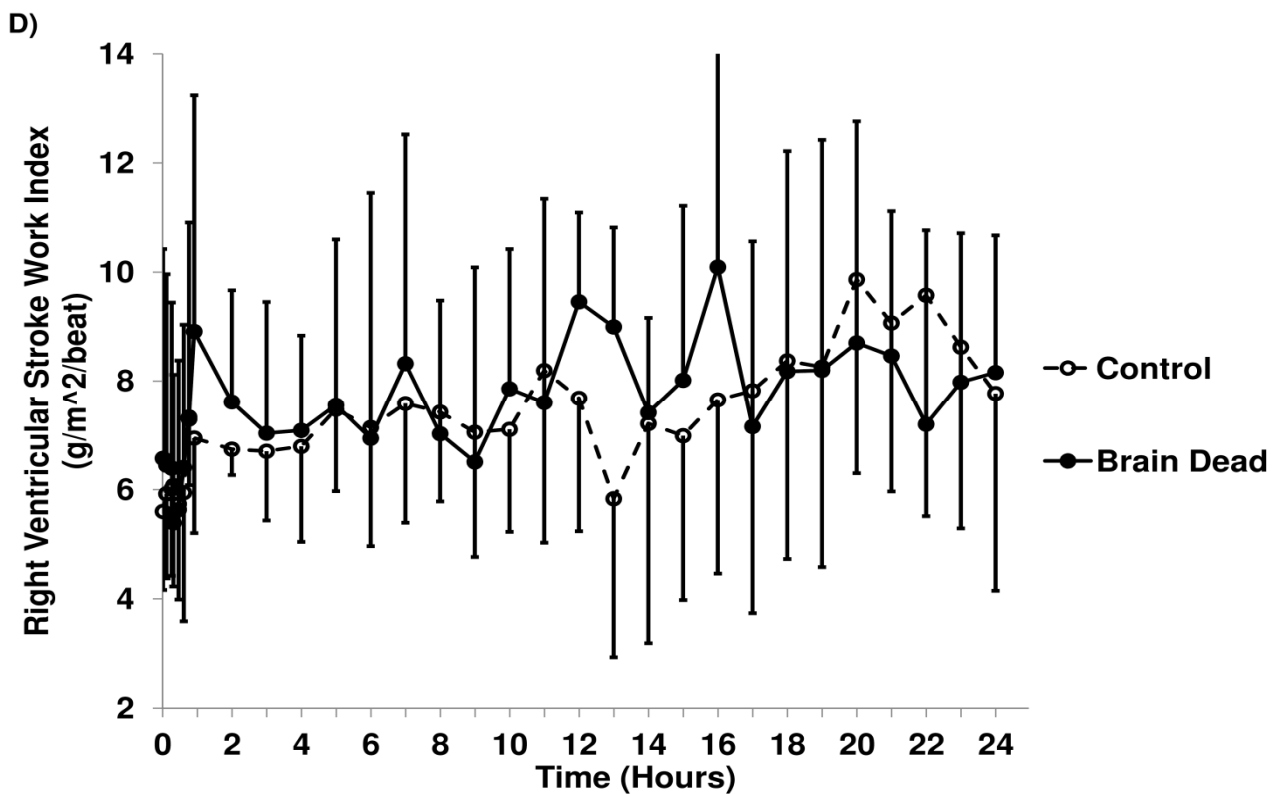
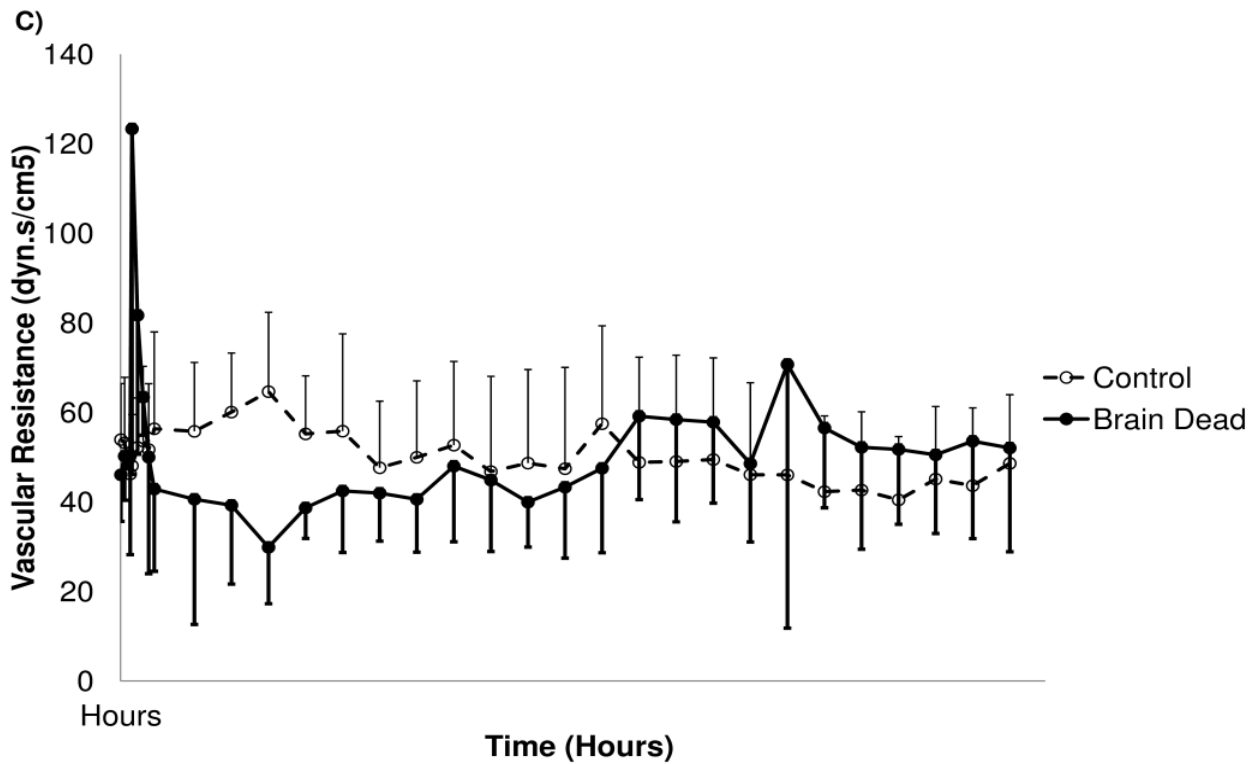


Figure 9: Pulmonary Haemodynamic Responses Observed in Brain Dead and Control Animals during Induction of BD and over 24 Hours.

A) Mean pulmonary arterial pressure peaked early after brain death, remaining above baseline and controls to 24 hours. B) Percent variance from baseline of mPAP demonstrates that this deviated in brain dead animals to a greater degree over time than controls. C) Pulmonary vascular resistance index returned to levels consistent with controls by 24 hours. D) Right ventricular stroke work index increased over time compared to baseline in brain dead animals, but was not different to controls at 24 hours. Brain death was induced immediately after the baseline value at time 0

One animal in the control group met the definition of postcapillary pulmonary hypertension at commencement of the experiment (MPAP 27 mmHg, PAOP 19 mmHg, transpulmonary gradient 8 mmHg, PVRI 101 dyn.s.cm⁻⁵). Mean pulmonary artery pressure, PVRI and RVSWI were not different between control and brain dead animals at 24 hours ($p=0.98$ for mPAP, $p=0.57$ for PVRI and $p=0.55$ for RVSWI). Exclusion of the sheep with baseline pulmonary hypertension did not reach statistical significance, but indicated that this single animal had a large effect on the analysis ($p=0.58$ for mPAP, $p=0.21$ for PVRI and $p=0.24$ for RVSWI).

4.5.3 Histological and Tissue Analysis

There was no statistically significant difference in lung wet:dry ratio between groups, with an average ratio of 3.48 versus 3.39 (non-BD vs BD, $p=0.68$).

Semi-quantitative assessment of lower lobe pulmonary histology demonstrated increased inflammation in BD animals (non-BD animals: none-mild inflammation (0 or +); BD animals: moderate-severe inflammation changes (++ or +++), including increased interstitial oedema and inflammatory cell infiltration, $p=0.014$).

The endothelin axis was detectable by immunohistochemical staining (4.10.5 Summary of Haematoxylin and Eosin and Immunohistochemical Staining). Staining of ET-1 was localised to bronchiolar epithelium and perivascular smooth muscle in both BD and non-BD animals, with no appreciable difference in expression noted. With regard to the endothelin receptors, ET_{RA} stained minimally in both groups within bronchiolar epithelium and smooth muscle, and ET_{RB} was well localised to airway columnar epithelium. There was no difference in expression of either receptor between groups. Overall, there were no differences in intensity of staining for MMP-2 or MMP-9 between groups, with MMP-2 slightly expressed within vascular endothelial and bronchiolar epithelial cells and MMP-9 able to be identified within bronchiolar epithelium and perivascular smooth muscle.

Low intensity of staining for TIMP-1 and -2 was observed for both groups, with no detectable difference. Staining of TIMP-1 was primarily localised within the columnar epithelia in the bronchioles with some staining within the alveolar parenchyma. Similarly to TIMP-1, TIMP-2 expression was mainly observed in bronchiolar epithelia, though some staining in the alveolar parenchyma and pulmonary blood vessels was noted.

4.5.4 ELISA

One animal in the brain dead group was excluded from analysis as blood samples had suffered from haemolysis and were unable to be assessed. Big ET-1 increased in BD sheep six hours after PST compared to baseline ($p=0.002$, Figure 10A). Big ET-1 concentrations also tended to be increased compared to control animals at the same time-point ($p=0.064$). After this early peak, concentrations of Big ET-1 returned to baseline at 12 hours and remained similar to the non-BD group during the remainder of the protocol ($p=0.99$ at 24 hours). Similarly, ET-1 levels rose by 26.9% from baseline at one hour after induction of brain death, approaching statistical significance ($p=0.09$, Figure 10B), and then declined to be equivalent to control animals at 12 hours. Scatter plots indicated no correlation between hourly average doses of administered vasoactive agents and the observed levels of Big ET-1 or ET-1 (Figure 11).

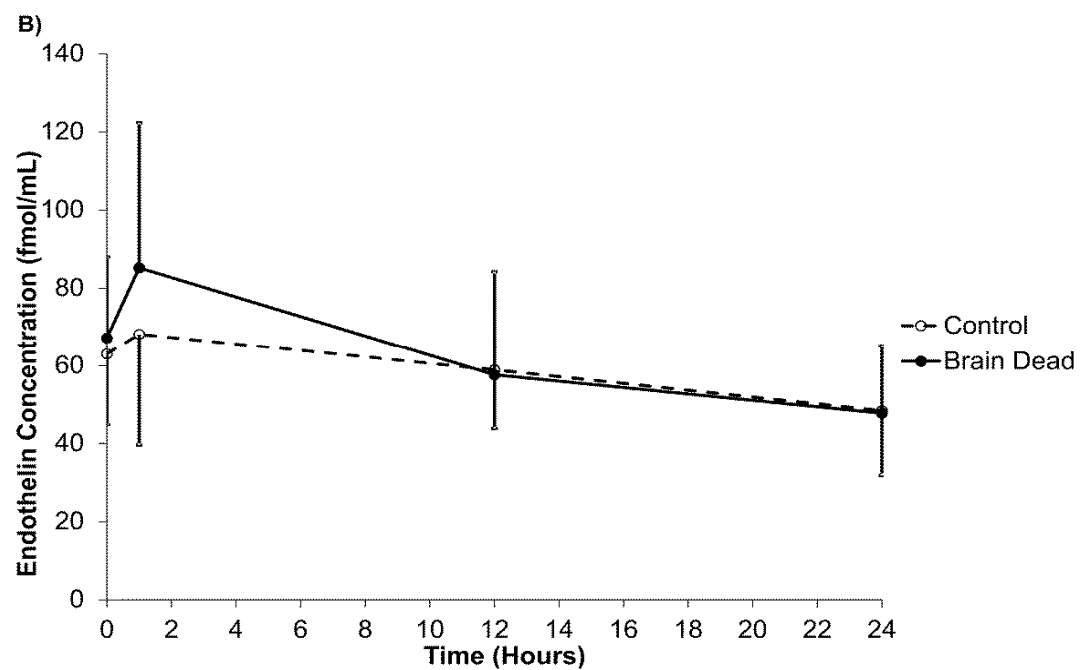
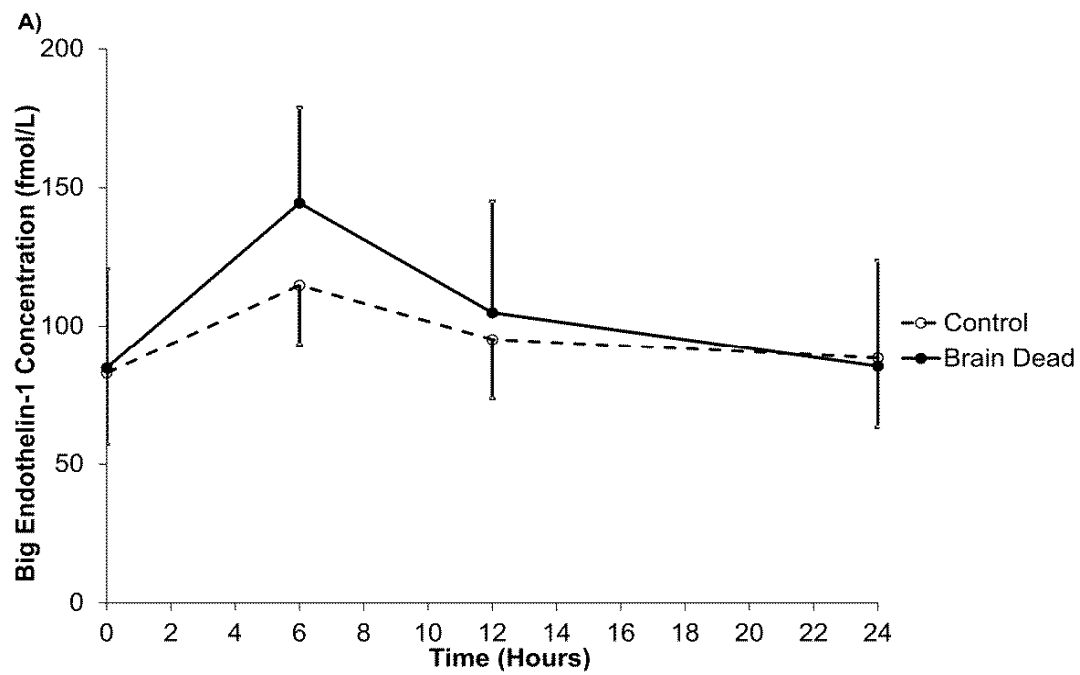


Figure 10: ELISA Analysis of Big Endothelin-1 and Endothelin-1.

A) Big Endothelin concentrations. B) Endothelin-1 concentrations. Samples measured in EDTA plasma. Sheep 6 has been excluded from this analysis (Brain Dead group) due to technical errors in measurement.

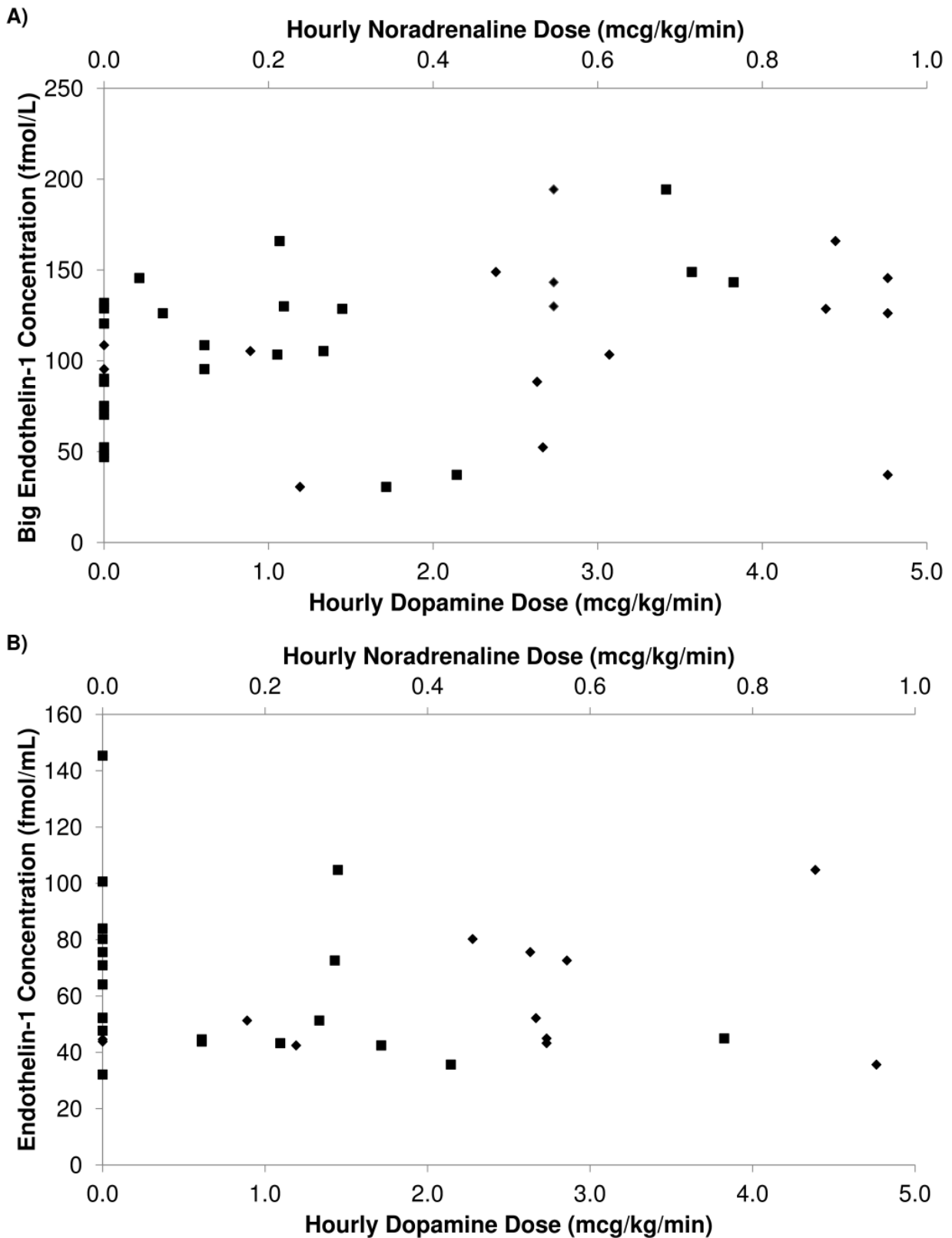


Figure 11: Scatter plot of ELISA Concentrations versus Average Hourly Vasoactive Infusion Doses.

A) Big Endothelin-1. B) Endothelin-1. ■ = noradrenaline, ◆ = dopamine. Although these agents may stimulate endothelin release, scatterplots do not indicate a correlation between dose and levels measured.

4.5.5 Biochemical Analysis

Circulating myoglobin and CK-MB increased over time in BD animals (Figure 12), indicating myocardial necrosis; no change was observed in control animals. In BD animals, myoglobin levels increased earlier than CK-MB, however this did not reach statistical significance compared to control animals ($p=0.13$ at 24 hours). CK-MB was significantly elevated in BD compared to control animals at 24 hours ($p=0.04$). Hepatic injury was also evident at 24 hours with elevation of both alanine aminotransferase and aspartate aminotransferase in BD animals ($p<0.001$ for both). Cholestatic enzymes were not elevated, indicating preferential hepatocellular injury. No evidence of renal dysfunction was indicated by elevated creatinine or urea levels ($p=0.5$ creatinine, $p=0.8$ urea, BD vs control animals at 24 hours).

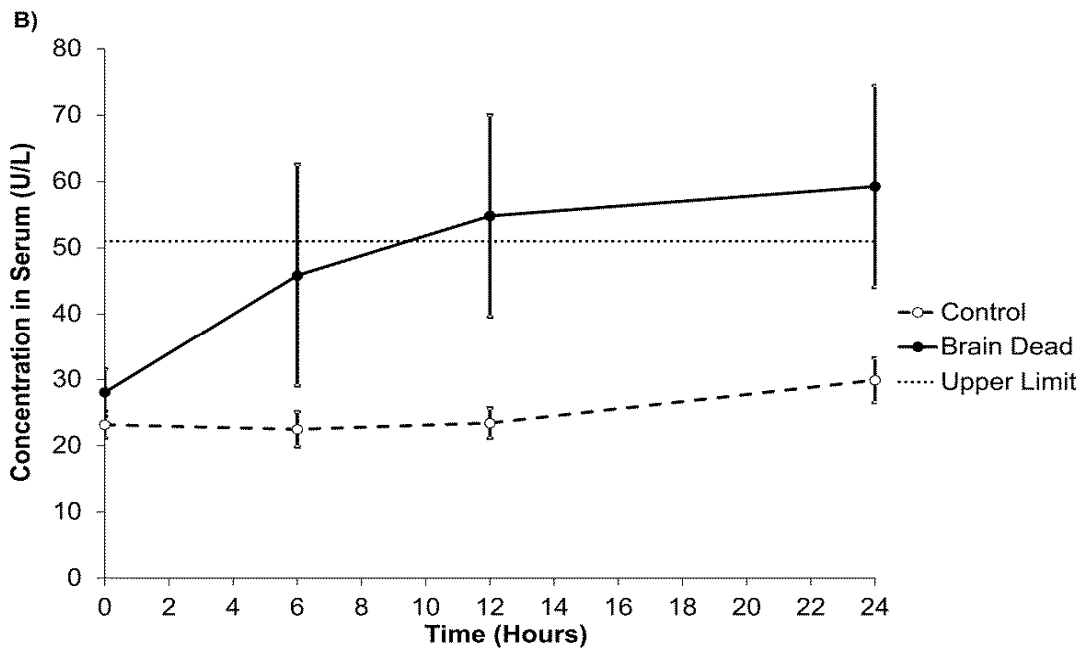
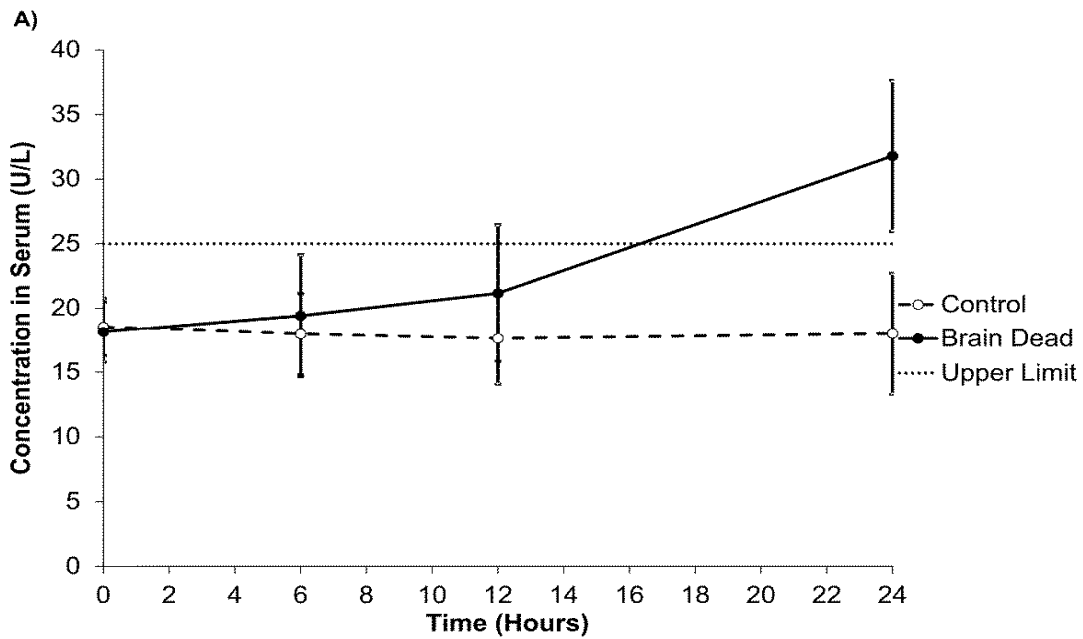


Figure 12: Biochemical Results of Markers of Cardiac Injury in Brain Dead and Control Animals over 24 Hours.

Upper Limit refers to the upper limit of the COBAS reference range. A) Creatine Kinase MB Isoenzyme. B) Myoglobin. Both cardiac markers indicated myocardial injury and necrosis. As expected from their biological properties, myoglobin increased faster, but it did not reach statistical significance. CK-MB increased later in brain dead animals and was statistically significant at 24 hours.

4.6 Discussion

This is the first report to document a 24 hour, clinically relevant, ovine model of brain death and assess systemic and pulmonary endothelin expression. Histological analysis indicated increased inflammation in the BD lung tissue, consistent with previous literature.[88, 241] The components of the endothelin axis were identifiable by immunohistochemical staining, with no demonstrable difference found between groups. This is in contradistinction to previous observations in rodents.[88] ELISA did suggest an early elevation and then resolution of both ET-1 and Big ET-1 in plasma after brain death; this may reflect an early release with rapid clearance,[339] however, no ongoing systemic expression was detected. Observed peak concentrations obtained at one hour and six hours for ET-1 and Big ET-1, respectively, may reflect significant peaks that occurred earlier, but were not captured by the sampling time in this study. Oishi *et al* previously demonstrated that ET-1 peaks as early as 30 minutes in BD canines, however this elevation was still detectable at 60 minutes.[210] Another complicating factor in comparing these results to the current study is the nature of sampling; while Oishi's group sampled coronary venous sinus blood (to detect cardiac generated ET-1), our study analysed peripheral arterial blood. Systemic levels of ET-1 indicate a spillover effect,[97] and therefore may only be detectable in very low concentrations when assessed in this manner due to mixing of blood returning to the pulmonary circulation. Secondly, pulmonary ET_RB may continue to serve its usual function of clearing circulating ET-1, concealing any detectable elevations in mixed central venous blood.[106] Both of these factors may have contributed to our observed results and provide opportunities for future study.

Data is accumulating of the role of ET-1 in brain death and organ donation; activation of the endothelin axis has been demonstrated early in BD related pulmonary inflammation,[88] it contributes to complications associated with human lung donation,[212, 340, 341] and it may contribute to the altered cardiopulmonary haemodynamics observed in the current study. As a potent mitogen, ET-1 stimulates smooth muscle hyperplasia and leads to airway remodelling and oedema.[330] Furthermore, ET-1 initiated cell signalling leads to short- and long-term injury, fibrosis and, ultimately, allograft rejection.[340] Thus, endothelin-1 may induce an inflammatory response that continues to manifest itself long after any detectable elevations in plasma concentrations have resolved; inflammatory cells recruited to the lung by endothelin signalling and increased ET-1 receptors in allograft tissue[88] may be further activated

after transplant by ischemia reperfusion injury[330] and the inflammatory state of the recipient.[24]

Activation of the sympathetic nervous system during brain death results in dramatic increases in vascular resistance and arterial pressure, and contributes to systemic inflammation.[178] Novitzky *et al* observed that the resulting increase in SVRI and left atrial pressure leads to transfer of up to 72% of total blood volume to the lower resistance pulmonary vasculature; mPAP and PVRI were also noted to increase with induction of BD.[305] This has been hypothesised to contribute to pulmonary capillary injury seen after BD. Bittner and colleagues demonstrated that, when observed for six hours, the PVRI decreased below baseline after the initial sympathetic storm of BD, resulting in increased pulmonary flow and vascular congestion, contributing to increased extravascular lung water content.[329] These authors reported that the decrease in PVRI was secondary to sympathetic failure and increased vascular distensibility.[329] The data over the first six hours in the present study supports these observations, replicating the early increase in SVRI and MAP, with reduction in PVRI after an early peak. Cardiac index increased from baseline, reaching a maximal value after the SVRI had dropped, resulting in a hyperdynamic circulation. This may contribute to the observed reduction in PVRI via distension of pulmonary vessels and pulmonary capillary reserve recruitment,[305] in addition to the loss of sympathetic vasoconstriction.

Pulmonary interstitial oedema did not differ between groups. The lack of oedema between the groups may be due to resolution of excess lung water by the end of the protocol. Skilled management likely influenced this outcome; fluid balance was similar between groups, thus preventing iatrogenic pulmonary oedema. The influence of duration of care was posited by Avlonitis *et al*, who observed that, in a prolonged rat model of brain death (15 minutes vs five hours), longer duration of care was associated with better oxygenation and reduced post-transplant PVR despite greater exposure to inflammatory cytokines.[269] This group postulated that the improvement in donor oxygenation at four hours reflected clearance of neurogenic pulmonary oedema.[269] This finding was replicated in our study, demonstrating impaired oxygenation for the first two hours, with subsequent recovery to levels similar to controls. Duration of BD donor care also influences recipient survival in humans; time from BD to cold preservation greater than 10 hours is associated with a survival advantage at both five and 10 years.[119] Hormone resuscitation may have also contributed to the observed effect; methylprednisolone

administration reduces extravascular lung water in BD donors.[245] Furthermore, dopamine stimulates alveolar fluid clearance and is another possible mechanism to explain our findings.[342]

While the absolute values of mPAP did not greatly exceed the defined cut-off for pulmonary hypertension (>25 mmHg)[343] in the present study, the increased pressure was significantly greater than baseline and does reflect greater resistance that needs to be overcome by a damaged myocardium. The observation that mPAP was elevated at 24 hours suggests that the effect of BD on pulmonary pressures may be greater than previously identified. Extended elevations in right ventricular afterload may contribute to the previously identified right ventricular ischaemia and fibrosis, further priming the right ventricle for acute failure in the recipient. Optimisation of ventilation, oxygenation and pH prevented contributions of these factors to the observed increase in pulmonary pressures in the current study.

Administration of catecholamines to the BD animals did represent a difference in care between the two groups. However, this is unlikely to explain the observed findings because elevated pulmonary pressures remained unchanged when doses of vasopressors were decreased after commencement of hormone therapy. The use of vasoactive agents is common in BD donors,[238] and has been demonstrated to reduce inflammation associated with hypotension and resultant poor tissue perfusion.[178] Previous studies of noradrenaline and dopamine infusions in sheep do not support that these agents were causative of the observed alterations in pulmonary pressures.[344, 345] In a study of noradrenaline infusion in healthy and endotoxaemic sheep, Lange *et al* observed an increase in PVRI in endotoxaemic sheep only.[344] Dopamine infusion in sheep has been associated with an increase in mPAP at rates significantly higher than the doses used in the current study.[345, 346] Although ET-1 can both stimulate[347] and be stimulated by catecholamines,[93, 348] comparison of ET-1 and Big ET-1 concentrations over time with the average hourly dose of vasoactive agents did not reveal any correlation in the current study and, therefore do not account for the observed results.

The current study also confirms that BD induces injury in other transplantable organs in sheep. Elevation of hepatic transaminases indicates hepatocellular injury consistent with other animal models.[194, 349] Serum cholestatic enzyme activities did not increase over time and suggest biliary obstruction was not associated with hepatic injury.

All BD animals required management of haemodynamic collapse with vasopressors. Haemodynamic support requirement was reduced after hormone therapy was initiated. This is in part due to the inclusion of vasopressin, but may also reflect improved haemodynamics directly due to hormonal administration. Thyroid hormone may play a role in regulating anaerobic metabolism and cardiovascular stability post-BD, however the benefits of its routine administration remains controversial.[186, 263] The current trial included hormonal resuscitation consistent with local protocols.[239]

Limitations of the Study

Several important limitations have been noted in this study. As previously identified, plasma sampling times may have missed very early peaks in ET-1 or Big ET-1. Big ET-1 and ET-1 were measured at different timepoints early in the study. Assessment of Big ET-1 at six hours, rather than one hour, was aimed to assess changes in protein over the entire duration of the study. However, ET-1 was measured earlier in order to better quantify early elevations of the functional peptide that may have occurred more closely to induction of BD. More frequent sampling around the induction of BD in future studies will better characterise the time-course of ET-1. Small numbers of animals in each group raises the possibility of type 2 error, although pre-clinical animal models have used similar numbers.[332, 333] Myoglobin and CK-MB were chosen to assess for cardiac injury in the current study. Although troponin may reflect cardiac function in the donor, the correlation between troponin levels and recipient outcome remains controversial.[350] Recently published guidelines continue to include CK as a biomarker in assessment of potential heart transplantation donors.[351, 352] Although NT-proBNP has been noted as a potential marker for assessing cardiac function in potential donors,[353] it is yet to be included among standard tests for donors.[354] Inflammatory cytokines have been well characterised in other animal models of BD. An ongoing challenge in developing new models is a relative paucity of validated, species specific analytical methods. Our group continues to develop and validate ovine specific tests,[331, 355] and the presented model will provide a platform to further investigate cytokine expression after BD in future studies.

4.7 Conclusion

The present model replicated the clinical realities leading to delays in organ retrieval upon BD. Haemodynamic disturbances which occur in BD animals has limited the duration of previous studies. By utilising complete haemodynamic monitoring and support in the

same fashion as is applied to human donors, it is possible to maintain a BD sheep for 24 hours. While sheep undergoing BD demonstrate complex haemodynamic changes similar to those seen in humans, our data also suggests that early haemodynamic and inflammatory derangements may improve over time with aggressive donor management. This reduces the urgency for organ retrieval and supports such timeframes as are frequently encountered in daily clinical practice. However, significant increases in pulmonary blood pressure may be noted up to 24 hours after brain death. Big ET-1 and ET-1 are detectable early after BD, and may contribute to the inflammatory cascade that primes allografts for post-transplant dysfunction. Endothelin-1 may also be a factor in the induction of right ventricular dysfunction observed in cardiac transplantation. Further investigation, targeting the endothelin axis, may provide a novel management option in order to improve the condition of transplantable lungs, increasing the number and quality of allografts available.

4.7.1 Competing Interests

This study was funded by the Cellcept Australia Research Grant and The Prince Charles Hospital Foundation New Investigator Grant. Prof Fraser is funded by the Office of Health and Medical Research Fellowship. The authors declare they have no competing interests.

4.7.2 Author's Contributions

RPW participated in all aspects of the study and wrote the manuscript. IB contributed to laboratory analysis of biochemical and histological data. SD and KRD assisted with design and implementation of animal work. AGB undertook statistical analysis of physiological data and provided advice on other statistical work. ACB participated in the design of biochemical analysis and manuscript development. JFF conceived the study and provided oversight and direction. All authors read and approved the final manuscript.

4.7.3 Acknowledgements

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4.8 Appendix 1: Animal Management

4.8.1 Anaesthesia Induction, Surgical Preparation and Monitoring Setup

Animals were divided into two groups (brain dead vs control) of six sheep each, for a total of 12 animals. Initial surgical preparation was identical in both groups of animals. After overnight fasting, animals were administered local anaesthesia (lignocaine 1%) and the right external jugular vein was cannulated using a 7 Fr triple lumen central venous catheter (Arrow-Howes, Research Triangle Park, North Carolina, USA). The left external jugular vein was accessed via an 8 Fr sheath for later placement of a pulmonary artery catheter (Edwards Lifesciences, Irvine, California, USA). Buprenorphine, cefalotin and gentamicin were then administered. Table 3 in the main manuscript lists details of medications used in this study. General anaesthesia was induced with midazolam and alfaxalone, and animals were intubated under direct laryngoscopy. Mechanical ventilation was commenced using a Galileo ventilator (Hamilton Medical, Reno, Nevada, USA) with continuous quantitative capnography. Initial ventilator settings were Synchronised Controlled Mandatory Ventilation, F_iO_2 0.5, tidal volume 10 mL/kg, 20 breaths per minute and positive end-expiratory pressure (PEEP) of 5 cmH₂O. Ventilation was adjusted to keep ETCO₂ at 35-40 mmHg, with mild hyperventilation to reduce animal discomfort and allow invasive ventilation of sheep in the awake state.[298, 299]

Anaesthesia was maintained during surgical preparation with continuous infusions of alfaxalone and ketamine and adjusted to maintain a surgical plane (monitored by observing heart rate, blood pressure, respiratory efforts and rate, eyelash reflexes, chewing and jaw movements). Midazolam infusion was commenced if alfaxalone exceeded 250 mg/hr.

Continuous electrocardiography and oxygen saturation monitoring was commenced directly after intubation. Invasive arterial blood pressure monitoring was performed after

cannulation of the right facial artery under direct surgical visualisation. Physiologic variables were monitored using a Marquette Solar 8000 monitor (GE Healthcare, Little Chalfont, UK) and recorded every five seconds with custom software. A 7.5 Fr pulmonary artery catheter was inserted via the previously placed sheath and continuous monitoring of the mixed venous oxygen saturation (SvO₂), cardiac output and body temperature were commenced using a Vigilance II Monitor (Edwards Lifesciences, Irvine, California, USA). Data were recorded every two seconds using manufacturer provided software. Cardiac index, systemic vascular resistance index (SVRI), pulmonary vascular resistance index (PVRI), stroke volume index and right ventricular and left ventricular stroke work index were calculated according to standard equations.[356]

Pulmonary artery diastolic pressure was used to calculate PVRI in lieu of repeated measurements of pulmonary artery occlusion pressure (PAOP) to minimise the risk of iatrogenic injury. The PAOP was measured every six hours to ensure consistency with the pulmonary diastolic pressure. Calculation of the body surface area (BSA) was performed using the equation, $BSA=0.094 \times (\text{weight})^{0.67}$. [357] A size 10 Portex tracheostomy tube (Smiths Medical, London, UK) was surgically placed and the endotracheal tube removed. A transurethral urinary catheter was inserted to monitor urine output.

Finally, intracranial access was obtained in all animals through a surgical incision midway between the midline and lateral edge of the cranium, rostral to the animal's horn base. A burr hole was created to reveal the dura and an intracranial pressure monitor was placed. This point was designated the protocol start time in control animals.

4.8.2 Induction of Brain Death

In animals allocated to brain death, a second burr hole was created on the opposite side of the head. A 16 Fr Foley catheter was then inserted extradurally. Brain death was

induced via inflation of the catheter balloon with 20 mL saline in 5 mL aliquots. One millilitre aliquots were then used to ensure that the cerebral perfusion pressure (CPP, defined as the mean arterial pressure less the intracranial pressure) remained negative for 30 minutes,[305] preventing cerebral blood flow, and that no additional sympathetic response or seizure activity occurred in response to the boluses. Confirmation of brain death was achieved by continuously negative CPP for greater than 30 minutes, loss of pupillary and corneal reflexes and lack of respiratory efforts. This was designated as the protocol start time for BD animals.

4.8.3 Protocol for Anaesthesia, Sedation and Analgesia in Control Animals after Surgical Preparation

After the protocol start time in control animals, six hourly buprenorphine was continued for analgesia, while anaesthesia remained according to the surgical plane. Anaesthesia was ceased at twelve hours after the protocol start time. Ketamine, alfaxalone and midazolam were able to be administered to non-BD animals after this time to prevent distress and ensure animal well-being. If required, these were titrated to clinical sedation, whereby the animals were calm but easily rousable. Muscle relaxants were not used at any time to allow monitoring for signs of animal distress.

4.8.4 Protocol for Anaesthesia, Sedation and Analgesia in Brain Dead Animals after Surgical Preparation

Anaesthetic and analgesic agents were continued after brain death to maintain consistency of care between groups. Ketamine and alfaxalone were reduced to 2 mg/hr and 1 mg/hr respectively after diagnosis of brain death, reflecting adjustment to maintain a 'surgical plane' in these animals. Midazolam was ceased if it had been used. Six hourly buprenorphine was also continued. Ketamine and alfaxalone were ceased at 12 hours in BD animals.

4.8.5 Protocol for Haemodynamic, Ventilatory and Electrolyte Management in all Animals

Hartmann's solution was initially administered at 2 mL/kg/hr and titrated to maintain a right atrial pressure of 8–12 mmHg. Further fluid boluses of crystalloid could be administered to maintain haemodynamics and urine output greater than 0.5 mL/kg/hr. Hypotension, defined as systolic blood pressure of <90 mmHg or MAP <60 mmHg, was treated with intravascular fluid optimisation and then dopamine or noradrenaline could be commenced. Arterial blood gases (ABG) were measured with a Radiometer ABL-825 analyser (Copenhagen, Denmark) every two hours or as required. Ventilatory adjustments were made to achieve P_aO_2 of greater than 100 mmHg (13.3 kPa) with a minimum F_iO_2 of 0.3, and P_aCO_2 of 35 mmHg (4.6 kPa) and normal pH. Potassium and calcium were replaced as directed by ABG results.

4.8.6 Protocol for Metabolic Management, Hormone Resuscitation and Animal Sacrifice in all Animals

Blood glucose levels were maintained between 6 and 10 mmol/L using insulin or dextrose infusions as necessary. Body temperature was manipulated with warmed fluids, warming blankets, heat lamps and manipulation of the operating theatre environmental temperature to maintain normothermia. Diabetes insipidus was defined as urine output greater than 300 mL/hr for two consecutive hours.[358] This was treated with desmopressin and adequate fluid replacement.

Hormone resuscitation therapy with vasopressin, methylprednisolone and liothyronine was commenced in all animals at 12 hours after the designated protocol start time.[239] Animals were euthanised at 24 hours with sodium pentobarbitone.

4.9 Appendix 2: Immunohistochemistry Staining Procedure

Primary antibodies used for immunohistochemistry included monoclonal anti-ET-1 (Sigma Aldrich, St Louis, Mo), polyclonal anti-ET_{RA}, anti-ET_{RB} and anti-MMP-2 (Merck Millipore, Billerica, MA), polyclonal anti-MMP-9 (Biorbyt, San Francisco, CA) and polyclonal anti-TIMP-1 and anti-TIMP-2 (Bioss, Woburn, MA). After trials for optimisation of concentration, the antibodies were diluted in TBS 0.5% Triton-100 (TBS-T) in the following ratios; ET-1 1:100, ET_{RA} 1:20, ET_{RB} 1:100, MMP-2 1:100, MMP-9 1:50, TIMP-1 1:50 and TIMP-2 1:50.

After sections were rehydrated, they were washed in Tris buffered saline (TBS) and epitope heat retrieval was performed using citrate buffer solution (10mM Sodium Citrate, 0.05% Tween-20, pH 6.0) for 20 minutes. After cooling, the slides were rinsed in distilled water and endogenous peroxidase activity was quenched with 3% hydrogen peroxide solution for 5 minutes. Slides were washed and then placed in TBS prior to application of 1:10 normal horse serum in TBS-T for one hour in a humidified chamber at room temperature. Diluted primary antibodies were applied and slides were then incubated at 4°C (1 hour for ET-1, ET_{RA}, ET_{RB} and MMP-2 and 24 hours for MMP-9, TIMP-1 and TIMP-2).

Following incubation, slides were rinsed with TBS-T and a biotinylated secondary antibody was applied (Vectastain ABC system, Vector Labs, Burlingame, CA) and incubated in a humidified chamber at room temperature for 60 minutes. After rinsing with TBS-T, an avidin/biotin complex was applied and the slides were again incubated in a humidified chamber at room temperature for 60 minutes. Slides were rinsed in TBS-T and then developed with 3,3'-diaminobenzidine to reveal brown staining peroxidase activity. After staining with Mayer's haematoxylin, samples were dehydrated and mounted with DePx (BDH Laboratories, Poole, England). Negative controls were created by completing the same steps with exclusion of the primary antibodies.

4.10 Appendix 3: Summary of Animal Data

4.10.1 Ventilatory, Arterial Blood Gas and Fluid Balance Data

		<u>Control</u>	<u>Brain Dead</u>
Ventilation at 24 Hours			
<i>Minute Ventilation</i>	<i>(L/Min)</i>	8.2 ± 1.2	9.8 ± 1.3
<i>Respiratory Rate</i>	<i>(Breaths/Min)</i>	19.6 ± 2.6	22.1 ± 2.7
<i>Tidal Volume</i>	<i>(Litres)</i>	428.1 ± 42.5	456.9 ± 44.7
<i>Positive End Expiratory Pressure</i>	<i>(cmH₂O)</i>	6.8 ± 1.2	7.5 ± 1.3
<i>Plateau Pressure</i>	<i>(cmH₂O)</i>	18.9 ± 1.8	19.3 ± 2.0
<i>Static Compliance</i>	<i>(L/cmH₂O)</i>	35.9 ± 5.5	39.2 ± 5.8
Blood Gas Results at 24 hours			
<i>P_{(A-a)O₂}</i>	<i>(mmHg)</i>	23.4 ± 25.5	14.6 ± 26.7
<i>P_aO₂:F_iO₂</i>		442.9 ± 44.7	447.6 ± 46.9
<i>PaCO₂</i>	<i>(mmHg)</i>	27 ± 4.5	31.9 ± 4.7
<i>pH</i>		7.46 ± 0.06	7.38 ± 0.09
<i>Lactate</i>	<i>(Mol/L)</i>	1.1 ± 0.5†	1.9 ± 0.6
Fluid Parameters at 24 hours			
<i>Cumulative Fluid Administered</i>	<i>L</i>	4.7 ± 1.7†	15.2 ± 12.5
<i>Cumulative Urine Output</i>	<i>L</i>	2.6 ± 0.9†	12.8 ± 11.6
<i>Fluid Balance</i>	<i>L</i>	2.1 ± 0.8	2.4 ± 1.7

† - p < 0.05 CP vs BDP ~ - p < 0.05 vs baseline

Maximum/Minimum values given occurred within 15 minutes

4.10.2 Summary of Systemic Haemodynamic Variables

		<u>Control</u>	<u>Brain Dead</u>
Systemic Haemodynamics			
<i>Heart Rate (beats/min)</i>	<i>Baseline</i>	122 ± 1	101 ± 5
	<i>Maximum</i>	125 ± 30†	220 ± 73~
	<i>Average First Hour</i>	124 ± 1†	174 ± 15~
	<i>Average at 24 Hours</i>	105 ± 8	100 ± 9
<i>Cardiac Index (L/min/m²)</i>	<i>Baseline</i>	4.0 ± 0.15	4.5 ± 0.18
	<i>Maximum</i>	4.7 ± 1.2†	7.5 ± 2.19~
	<i>Average at 24 Hours</i>	5.2 ± 1.3	4.6 ± 0.6
<i>Mean Arterial Pressure (mmHg)</i>	<i>Baseline</i>	107 ± 2	99 ± 3
	<i>Maximum</i>	110 ± 18†	190 ± 40~
	<i>Average at 24 Hours</i>	122 ± 5†	83 ± 5~
<i>Central Venous Pressure (mmHg)</i>	<i>Baseline</i>	7.5 ± 3.2	6.2 ± 5.0
	<i>Maximum</i>	8.5 ± 4.3	11.3 ± 5.3
	<i>Average at 24 Hours</i>	9.7 ± 1.8	10.9 ± 1.7
<i>SvO₂ (%)</i>	<i>Baseline</i>	78.7 ± 9.5†	67 ± 11.8
	<i>Minimum</i>	77.2 ± 13.1	67.5 ± 6.3
	<i>Average at 24 Hours</i>	78.2 ± 4.2	75.5 ± 5.0
<i>Systemic Vascular Resistance Index (dyn.s.cm⁻⁵)</i>	<i>Baseline</i>	1808 ± 49	1741 ± 60
	<i>Maximum</i>	1885 ± 401†	3718 ± 77~
	<i>Average at 24 Hours</i>	1973 ± 202†	1317 ± 212
<i>Left Ventricular Stroke Work Index (g.m/m²/beat)</i>	<i>Baseline</i>	47 ± 1.7	53 ± 1.7
	<i>Maximum</i>	49 ± 15.9	57 ± 23.4~
	<i>Average at 24 Hours</i>	65 ± 5.8†~	45 ± 6.1~

† - p < 0.05 CP vs BDP ~ - p < 0.05 vs baseline

Maximum/Minimum values given occurred within 15 minutes

4.10.3 Summary of Pulmonary Haemodynamic Variables

		<u>Control</u>	<u>Brain Dead</u>
Pulmonary Haemodynamics			
<i>Mean Pulmonary Artery Pressure (mmHg)</i>	<i>Baseline</i>	19 ± 0.8	16 ± 0.2
	<i>Maximum</i>	20 ± 4.9†	30 ± 13~
	<i>Average at 24 Hours</i>	21 ± 2.2	25 ± 2.2~
<i>Pulmonary Vascular Resistance Index (dyn.s.cm⁻⁵)</i>	<i>Baseline</i>	54 ± 3.9	50 ± 3.1
	<i>Maximum</i>	57 ± 16.9†	123 ± 77~
	<i>Average at 24 Hours</i>	70 ± 19	61 ± 19
<i>Right Ventricular Stroke Work Index (g.m/m²/beat)</i>	<i>Baseline</i>	6 ± 0.4	7 ± 0.2
	<i>Maximum</i>	6 ± 0.4	7 ± 2.6
	<i>Average at 24 Hours</i>	8 ± 1.0	9 ± 1.0~

† - p < 0.05 CP vs BDP ~ - p < 0.05 vs baseline
Maximum/Minimum values given occurred within 15 minutes

4.10.4 Summary of Biochemical Data

			<u>Control</u>	<u>Brain Dead</u>
Cardiac Markers				
<i>Myoglobin</i>	<i>mcg/L</i>	<i>Baseline</i>	23.2 ± 2.0	28.7 ± 3.6
		<i>24 Hours</i>	29.9 ± 3.4	59.3 ± 15
<i>Creatine Kinase MB</i>	<i>U/L</i>	<i>Baseline</i>	18.5 ± 2.2	18.1 ± 2.3
		<i>24 Hours</i>	18 ± 4.7†	31.8 ± 5.8
Hepatic Markers				
<i>Total Bilirubin</i>	<i>µmol/L</i>	<i>Baseline</i>	1.27 ± 0.4	0.9 ± 0.5
		<i>24 Hours</i>	4.0 ± 3.8†~	0.8 ± 0.6
<i>Conjugated Bilirubin</i>	<i>µmol/L</i>	<i>Baseline</i>	1.3 ± 0.6	1.0 ± 0.6
		<i>24 Hours</i>	3.2 ± 1.8†~	1.0 ± 0.7
<i>Alkaline</i>	<i>U/L</i>	<i>Baseline</i>	105.2 ± 29.3	84.1 ± 23.7
<i>Phosphatase</i>		<i>24 Hours</i>	76.2 ± 19.4	84.6 ± 25.5
<i>γ-Glutamyl</i>	<i>U/L</i>	<i>Baseline</i>	58.7 ± 6.8	56.8 ± 2.6
<i>Transferase</i>		<i>24 Hours</i>	55.0 ± 6.4	50.3 ± 4.0
<i>Alanine</i>	<i>U/L</i>	<i>Baseline</i>	9.8 ± 1.2	8.0 ± 1.1
<i>Aminotransferase</i>		<i>24 Hours</i>	16.4 ± 1.1†	75.5 ± 39.5
<i>Aspartate</i>	<i>U/L</i>	<i>Baseline</i>	78.6 ± 4.7	79.9 ± 7.2
<i>Aminotransferase</i>		<i>24 Hours</i>	139.3 ± 9.8†	596.0 ± 291.1
<i>Albumin</i>	<i>g/L</i>	<i>Baseline</i>	30 ± 2.4	31 ± 4.6
		<i>24 Hours</i>	29 ± 0.8	24 ± 4.4~
<i>Total Protein</i>	<i>g/L</i>	<i>Baseline</i>	61 ± 3.9	66 ± 8.6
		<i>24 Hours</i>	60 ± 5.6	51 ± 7.3~
<i>Lactate</i>	<i>U/L</i>	<i>Baseline</i>	470 ± 90	402 ± 83
<i>Dehydrogenase</i>		<i>24 Hours</i>	791 ± 254†	2569 ± 2977~
Renal Markers				
<i>Urea</i>	<i>mmol/L</i>	<i>Baseline</i>	7.7 ± 0.8	6.5 ± 0.7
		<i>24 Hours</i>	4.3 ± 0.2	2.5 ± 0.5
<i>Creatinine</i>	<i>µmol/L</i>	<i>Baseline</i>	68.4 ± 2.6	71.3 ± 7.9
		<i>24 Hours</i>	67.2 ± 3.5	57.1 ± 6.4
<i>Phosphate</i>	<i>mmol/L</i>	<i>Baseline</i>	2.3 ± 0.6	1.9 ± 0.4
		<i>24 Hours</i>	1.2 ± 0.3~	1.5 ± 0.7

† - p < 0.05 CP vs BDP ~ - p < 0.05 vs baseline

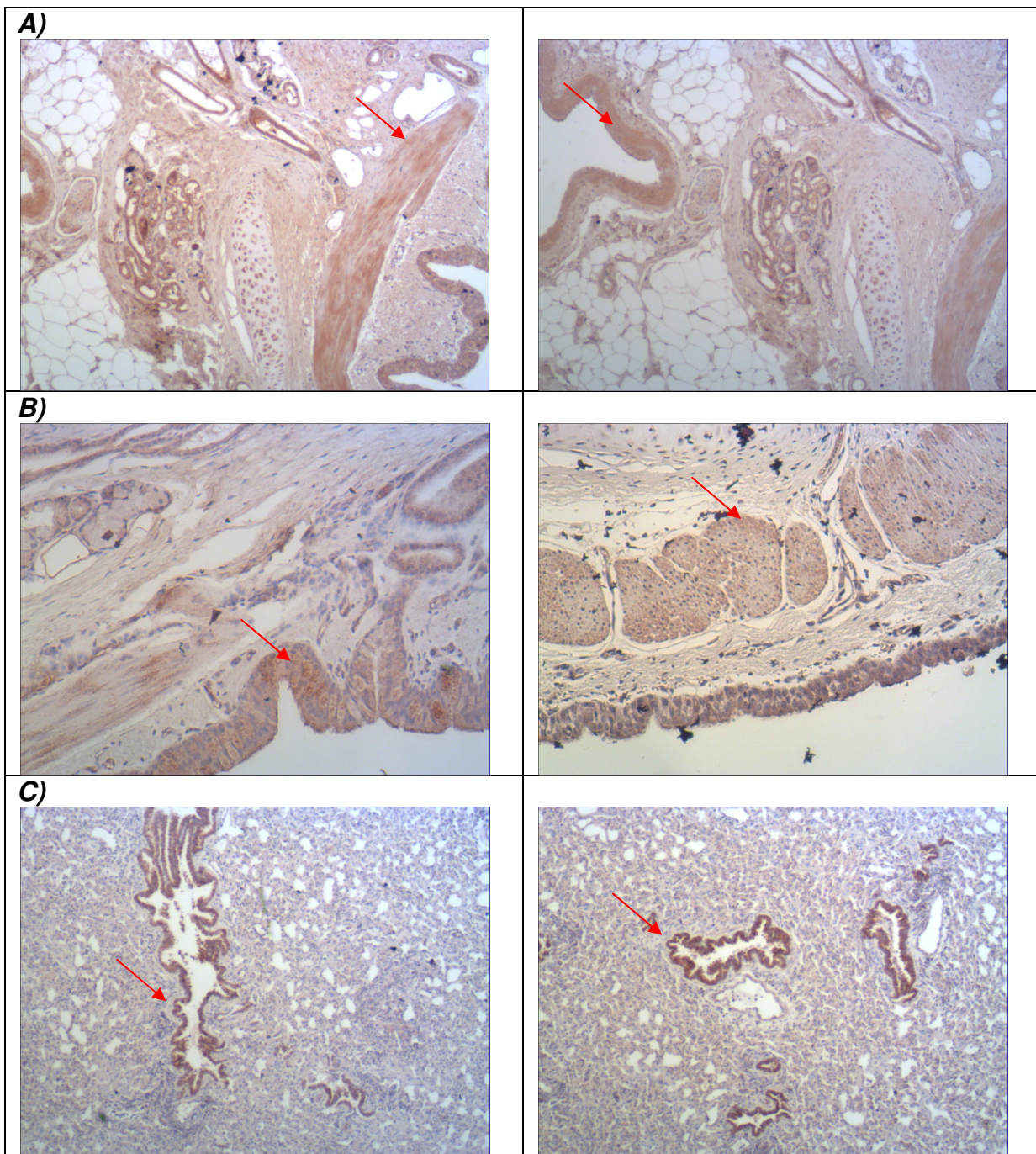
4.10.5 Summary of Haematoxylin and Eosin and Immunohistochemical Staining

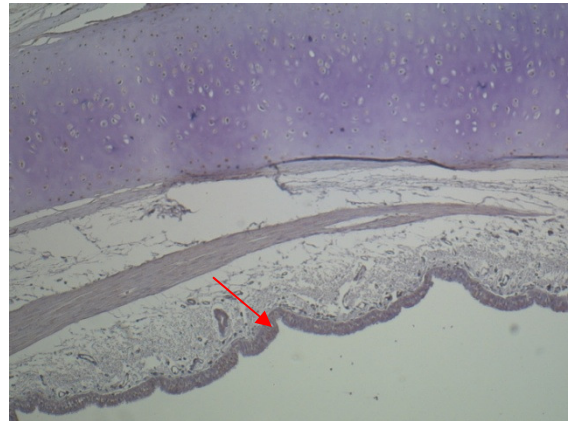
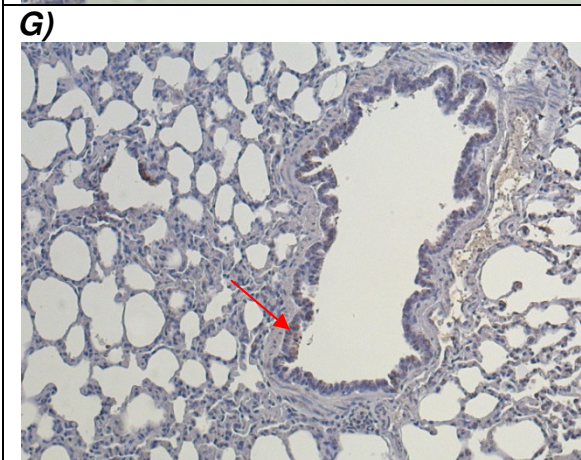
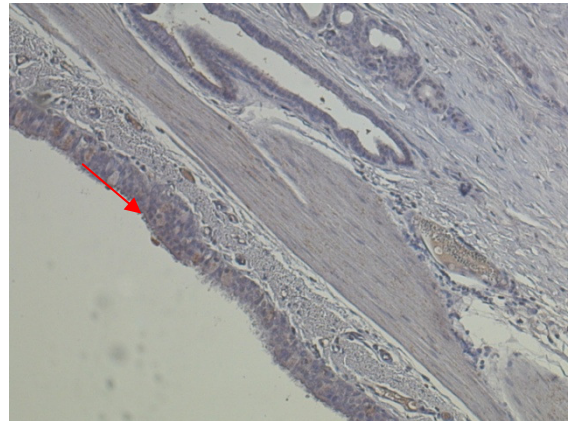
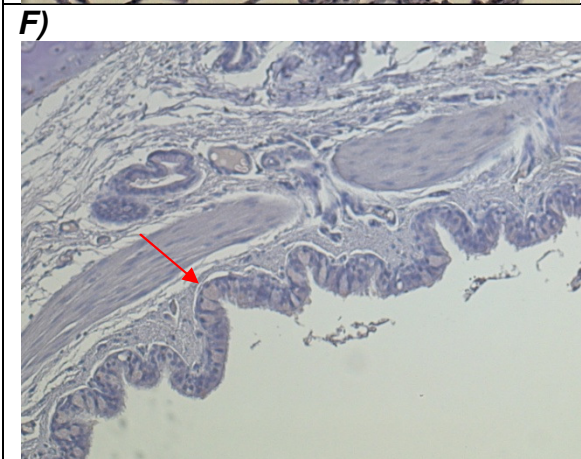
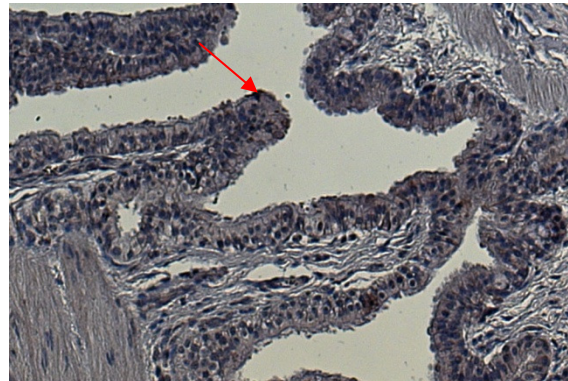
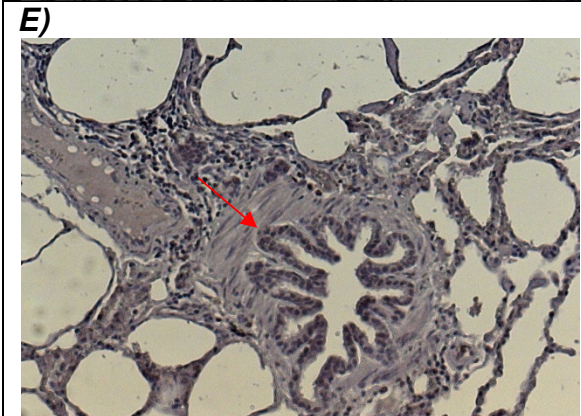
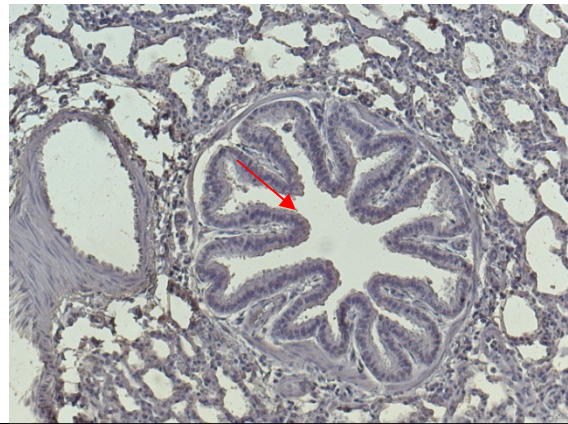
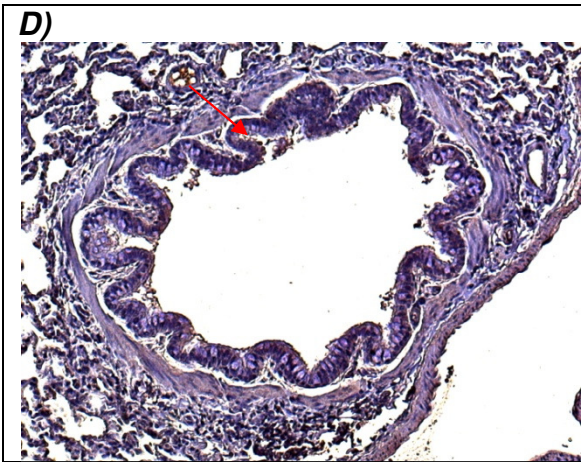
Sheep	HE Score	Neutrophils (/Field)*	ET-1	ET _R A	ET _R B	MMP-2	MMP-9	TIMP-1	TIMP-2
Brain Dead									
1	++	19	+	0	+	0	0	+	++
2	++	10	+	0	+	+	+	+	+
4	+	3	+	0	+	0	0	0	+
6	++	11	0	0	+	0	0	+	+
8	++	6	0	0	+	+	0	0	0
10	+	4	0	0	+	+	0	0	+
Control									
3	0	4	0	0	+	+	+	0	0
5	0	5	+	0	+	0	0	0	+
7	+	9	+	+	+	+	0	0	0
9	++	3	0	0	+	0	0	0	0
11	++	6	+	+	+	0	+	0	+
12	0	4	0	0	+	0	0	+	0
<i>P-Value</i>	0.014	0.018	1.0	1.0	1.0	1.0	1.0	1.0	1.0

* Neutrophils counted by microscopy in 5 fields under 63x power and then averaged.

4.11 Representative Images of Immunohistochemical Staining of the Endothelin Axis

Staining of lung tissue samples and magnification. Brown staining indicates uptake of antibody (arrows to highlight representative staining). The left column represents Control animals. The right column represents Brain Dead animals. A) Endothelin-1 (10x). B) Endothelin A receptor (10x). C) Endothelin B receptor (5x). D) Matrix metalloproteinase 2 (10x). E) Matrix metalloproteinase 9 (10x left panel, 20x right panel). F) Tissue inhibitor of metalloproteinases 1 (10x). G) Tissue inhibitor of metalloproteinases 2 (10x).





Chapter 5 - The Effects of Nebulised Tezosentan Administered After Brain Death in a Clinically Relevant 24-h Ovine Model

5.1 Introduction

Endothelin activity after brain death may contribute to early and delayed lung allograft function via both inflammatory and vasoactive mechanisms.[88, 210, 341] Previous authors have identified that alterations in pulmonary haemodynamics results from transfer of blood from the systemic to pulmonary vasculature,[305] contributing to pulmonary oedema.[178] Ultimately, significant injury may occur to the lungs after brain death, causing pulmonary inflammation, tissue oedema, neutrophil infiltration and impaired gas exchange.[178, 269] Chapter 4 described an ovine model of brain death which was developed to further investigate the role of endothelin in pulmonary injury in potential donors. This model identified an increase in pulmonary inflammation after brain death, and that the endothelin axis was detectable with immunohistochemistry and ELISA. In brain dead animals, pulmonary blood pressures were noted to increase over 24 hours, however this was not statistically significant when compared to control animals. Administration of an endothelin antagonist may provide further insight into the role of endothelin after brain death.

Brain death may adversely affect the right ventricle (RV) more than the left ventricle (LV) after heart transplantation, contributing to early deaths secondary to RV failure.[254, 256, 329] Right ventricular contractility is altered after brain death, an effect that primarily becomes apparent with increased ventricular afterload.[257] Such changes may go undetected by conventional donor assessment techniques.[254] Modulation of pulmonary pressures in the potential donor may therefore represent a target for preserving cardiac function.[359] Endothelin antagonists are able to reverse pulmonary pressures that are elevated in a variety of disease states, including endotoxaemia,[74] meconium aspiration[320] and cardiopulmonary bypass,[325] and therefore may also offer an interventional benefit after BD. Furthermore, inhibition of the endothelin axis may also counteract the 'priming' that occurs after BD[88] and decrease ischaemia-reperfusion injury after transplantation.[101]

Tezosentan is a dual endothelin receptor antagonist that maintains partial selectivity for ET_{RA} in a 30:1 ratio.[74, 325] As BD is associated with significant hypotension resulting

from autonomic failure, intravenous tezosentan may worsen haemodynamic instability and tissue ischaemia. Therefore, nebulisation allows for organ specific therapy while avoiding systemic complications.[322] Nebulised tezosentan is effective in reducing pulmonary hypertension in previous animal models while avoiding systemic hypotension.[74, 325] This chapter describes the effects of administered nebulised tezosentan on pulmonary haemodynamics, myocardial work and histologic evidence of lung injury associated with BD in a 24 hour, clinically relevant, ovine model of brain death.

5.2 Methods

5.2.1 Ethics Approval

The work reported in this chapter was performed under The Queensland University of Technology Animal Ethics Committee approval number 0900000319. All activities were performed under the guidance of the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC)* and the *Animal Care and Protection Act 2001 (QLD)*.

5.2.2 Animal Management

Animal experiments followed the methods outlined in 4.8 Appendix 1: Animal Management. A further 12 merino-cross ewes were included in this analysis, in addition to those animals reported in Chapter 4. Experiments for all 24 sheep were undertaken concurrently to allow randomisation into the four groups. All sheep were between 12 and 24 months of age and weighed 38 ± 4.5 kg. Randomisation into the four groups was via StatMate (GraphPad Software, La Jolla, California), and these groups were termed control/placebo (CP), control/tezosentan (CT), brain dead/placebo (BDP) and brain dead/tezosentan (BDT).

5.2.3 Tezosentan Administration

A standard tezosentan dose of 20 mg was chosen based upon the average expected sheep weight of 40 kg (approximately 0.5 mg/kg/dose).[74, 325] Tezosentan was reconstituted to 4 mL with normal saline. An equivalent volume of normal saline served as a placebo. Nebulised tezosentan or placebo was administered at 13 and 18 hours after PST due to the half-life of the drug. This was delivered by a pneumatically driven nebulizer (Cirrus, Intersurgical, UK), synchronized to the inspiratory phase of the ventilator cycle, and modified for refilling without disconnection from the circuit or loss of PEEP. The

nebuliser was set at 8 L/min to deliver 76% of the volume as particles less than 5 micrometres in diameter.

5.2.4 Sample Retrieval and Storage

Blood was sampled from the peripheral arterial line at baseline, 1, 6, 12, 18 and 24 hours. After centrifugation, supernatant was transferred into vials and stored at -80 °C until analysis. Lungs were removed *en bloc* after animal sacrifice via a lateral thoracotomy and tissue samples collected from both lower lobes. After immediate fixation in 10% phosphate buffered formalin, samples were embedded in paraffin and mounted on slides.

5.2.5 Histological and Tissue Analysis

Wet:dry weight ratio served as a marker of inflammatory oedema using right lower lobe samples. These were weighed immediately after sampling and again after complete dehydration at 48 hours. Morphological changes were randomly assessed by semi-quantitative grading of haematoxylin and eosin stained slides by blinded assessors, grading inflammation and none/mild vs moderate/severe.[88]

Expression of endothelin-1, its receptors, matrix metalloproteinase-2 and -9 and tissue inhibitor of metalloproteinase- and -2 were assessed with immunohistochemistry, as previously described (4.9 Appendix 2: Immunohistochemistry Staining Procedure).

5.2.6 ELISA

Big ET-1 and ET-1 were measured in plasma using sandwich ELISA kits (BiomedicaGruppe, Austria). Absorbance was assessed at 450 nm, with a reference wavelength of 630 nm, on a 96 well plate spectrophotometer (FluoStar Omega, BMG LabTech, Germany).

5.2.7 Biochemical Analysis

The COBAS Integra 400 chemical analyser (Roche Diagnostics, Dee Why, Australia), following manufacturer's directions, was employed to assess markers of cardiac, hepatic and renal injury. Each sample was tested in duplicate, averaged and interpolated via comparison to commercially supplied calibrators. Cardiac markers included myoglobin and CK-MB. Total and conjugated bilirubin, alkaline phosphatase and γ -glutamyl transferase served as markers of hepatic cholestatic function, while alanine aminotransferase and aspartate aminotransferase were measured for evidence of

hepatocellular injury. Synthetic function of the liver was assessed by albumin and total protein, while lactate dehydrogenase was a general marker for cellular injury. Renal function was measured by urea, creatinine and phosphate concentrations.

5.3 Statistical Analysis

Study design and power calculation was performed for the animals presented in Chapter 4 and 5 together. As such, the study included four groups of six animals in total. This is able to detect a true difference in the standardised mean change between experimental and control subjects of ± 1.794 with probability (power) 0.8. The Type I error probability associated with this test of the null hypothesis that the population means of the experimental and control groups are equal is 0.05. We assumed the response within each subject group would be Normally distributed, standardised to a standard deviation of 1.

Statistical analysis of biochemical data was performed with Prism 6. Regression analyses of physiological data were performed with R software (www.r-project.org). A two-sided statistical significance level of <0.05 was set and results reported as mean \pm standard deviation. Two-way repeated-measures Analysis of Variance (ANOVA) was used to compare dependent variables over time and between groups. Tukey's correction was used for multiple comparisons. Student's *t* test was used to compare changes in physiological variables at specified time points. Fisher's exact test was used to compare semi-quantitative assessment of tissue samples. Statistical regression using a mixed model with a random intercept for each individual was used to examine changes in continuous variables over time.[338]

5.4 Results

There was no statistical difference in weight between groups (CP 36.7 ± 3.3 kg, CT 38.6 ± 4.7 kg, BDP 37.2 ± 2.2 kg, BDT 39.0 ± 7.2 , $p=0.8$). Induction of brain death was successful in all animals allocated to these groups. One animal in the BDT group suffered from sustained ventricular fibrillation during brain death induction and was removed from analysis. In order to maintain six animals per group, this sheep was replaced. All other animals completed the protocol to 24 hours as allocated.

5.4.1 Animal Management and Point of Care Testing

Mechanical ventilation and arterial blood gas data are reported in Table 4. Administration of tezosentan did not affect oxygenation as reflected by the PaO₂:FiO₂ ratio. Lactate,

which was measured as a surrogate marker of hypoperfusion, was increased in BD animals. Administration of nebulised tezosentan to BD animals reversed this by -0.78 ± 0.65 mmol/L ($p=0.019$) by the end of the study. All BD animals required haemodynamic support, however there was no difference between groups for the amount of dopamine or noradrenaline provided at any time-point.

Table 4: Ventilation and Blood Gas Results between all Four Groups at 24 Hours

		<u>Control / Placebo</u>	<u>Control / Tezosentan</u>	<u>Brain Dead / Placebo</u>	<u>Brain Dead / Tezosentan</u>
Ventilation Parameters					
Minute Ventilation	(L/Min)	8.2 ± 1.2	8.6 ± 1.3	9.8 ± 1.3	10.0 ± 1.3
Respiratory Rate	(Breaths/Min)	19.6 ± 2.6	20.2 ± 2.7	22.1 ± 2.7	22.2 ± 2.8
Tidal Volume	(Litres)	428.1 ± 42.5	425.2 ± 43.7	456.9 ± 44.7	453.7 ± 46.0
PEEP	(cmH ₂ O)	6.8 ± 1.2	6.6 ± 1.2	7.5 ± 1.3	7.1 ± 1.3
Plateau Pressure	(cmH ₂ O)	18.9 ± 1.8	17.9 ± 1.9	19.3 ± 2.0	18.9 ± 2.0
Static Compliance	(L/cmH ₂ O)	35.9 ± 5.5	39.1 ± 5.8	39.2 ± 5.8	40.1 ± 6.0^e
Blood Gas Results					
$P_{(A-a)}O_2$	(mmHg)	23.4 ± 25.5	22.8 ± 29.5	$14.6 \pm 26.7^\circ$	34.5 ± 30.8
$P_aO_2:F_iO_2$		442.9 ± 44.7	446.2 ± 52.5	447.6 ± 46.9	464.8 ± 54.7
$PaCO_2$	(mmHg)	$27 \pm 4.5\ddagger$	$30 \pm 6.0^\sim$	$31.9 \pm 4.7^\circ$	37.7 ± 6.1
Lactate	(Mol/L)	$1.1 \pm 0.5\ddagger$	$0.8 \pm 0.6^\sim$	$1.9 \pm 0.6^\circ$	1.1 ± 0.6

* - $p < 0.05$ CP vs CT, † - $p < 0.05$ CP vs BDP, ‡ - $p < 0.05$ CP vs BDT, ^ - $p < 0.05$ CT vs BDP, ~ - $p < 0.05$ CT vs BDT, ° - $p < 0.05$ BDP vs BDT, ~ - $p < 0.05$ vs baseline, ° - $p < 0.05$ change with tezosentan

5.4.2 Physiologic Variables

Haemodynamic results are included in

Table 5 and Table 6. Inflation of the extradural catheter rapidly resulted in hypertension and tachycardia. Brain dead animals reached a maximal heart rate (HR) within five minutes of catheter inflation. Tezosentan decreased the HR in BD animals at each administration time-point ($p < 0.001$ both timepoints).

Table 5: Comparison of Mean Systemic Haemodynamic Values between all Four Groups

Systemic Haemodynamics					
		<u>Control / Placebo</u>	<u>Control / Tezosentan</u>	<u>Brain Dead / Placebo</u>	<u>Brain Dead / Tezosentan</u>
<i>Heart Rate (beats/min)</i>	<i>Baseline</i>	122 ± 1	116 ± 2	101 ± 5	118 ± 3
	<i>Maximum</i>	125 ± 30†‡	118 ± 23 [~]	220 ± 73 [~]	206 ± 52 [~]
	<i>24 Hours</i>	105 ± 8	110 ± 9	100 ± 9 [°]	96 ± 9
<i>Cardiac Index (L/min/m²)</i>	<i>Baseline</i>	4.0 ± 0.15	4.0 ± 0.14	4.5 ± 0.18	4.0 ± 0.06
	<i>Maximum</i>	4.7 ± 1.2†	4.3 ± 0.59 [~]	7.5 ± 2.19 [~]	6.3 ± 2.3 [~]
	<i>24 Hours</i>	5.2 ± 1.3‡	5.1 ± 0.6 [^]	4.6 ± 0.6 [°]	3.8 ± 0.6
<i>Stroke Volume Index (ml/m²/beat)</i>	<i>Baseline</i>	39 ± 5.5	34 ± 9.1	47 ± 16.5	41 ± 19
	<i>Minimum</i>	36 ± 5.2†‡	33 ± 6.0 [~]	25 ± 20.8 [~]	23 ± 9.0 [~]
	<i>24 Hours</i>	50 ± 4.3†	50 ± 4.2	53 ± 4.6 [°]	45 ± 4.6
<i>Central Venous Pressure (mmHg)</i>	<i>Baseline</i>	8 ± 4.5	7 ± 5.4	8 ± 5.0	6 ± 4.6
	<i>Maximum</i>	9 ± 3.7	7 ± 4.6	11 ± 5.4	9 ± 6.3
	<i>24 Hours</i>	10 ± 1.6	9 ± 1.7	11 ± 1.7 [°]	9 ± 1.7
<i>Mean Arterial Pressure (mmHg)</i>	<i>Baseline</i>	107 ± 2	107 ± 1	99 ± 3	118 ± 4
	<i>Maximum</i>	110 ± 18†‡	106 ± 18 [~]	190 ± 40 [~]	182 ± 42 [~]
	<i>24 Hours</i>	122 ± 5†‡	117 ± 6 [~]	83 ± 5 [~]	82 ± 5 [~]
<i>Systemic Vascular Resistance Index (dyn.sec/cm⁵/ m²)</i>	<i>Baseline</i>	1808 ± 49	2123 ± 105	1741 ± 60	2230 ± 30
	<i>Maximum</i>	1885 ± 401†‡	2183 ± 547 [~]	3718 ± 77 [~]	3292 ± 795 [~]
	<i>24 Hours</i>	1973 ± 202†	1750 ± 227 [~]	1317 ± 212 [°]	1526 ± 235
<i>Left Ventricular Stroke Work Index (gm.m/m²/ beat)</i>	<i>Baseline</i>	47 ± 1.7	43 ± 1.5	53 ± 1.7	49 ± 2.0
	<i>Maximum</i>	49 ± 15.9‡	42 ± 8 [~]	57 ± 23.4 [~]	70 ± 22 [~]
	<i>24 Hours</i>	65 ± 5.8*†‡ [~]	64 ± 4.8 [~]	45 ± 6.1 [°]	41 ± 6.1 [~]

Maximum values given occurred within 15 minutes of BIT/PST.

* - $p < 0.05$ CP vs CT, † - $p < 0.05$ CP vs BDP, ‡ - $p < 0.05$ CP vs BDT, ^ - $p < 0.05$ CT vs BDP,

[~] $p < 0.05$ CT vs BDT, [°] $p < 0.05$ BDP vs BDT, [~] $p < 0.05$ vs baseline, [°] $p < 0.05$ change with tezosentan

Table 6: Comparison of Pulmonary Haemodynamics between all Four Groups

		<u>Control / Placebo</u>	<u>Control / Tezosentan</u>	<u>Brain Dead / Placebo</u>	<u>Brain Dead / Tezosentan</u>
Pulmonary Haemodynamics					
Mean Pulmonary Artery Pressure (mmHg)	Baseline	19 ± 0.8	17 ± 0.9	16 ± 0.2	15 ± 0.4
	Maximum	20 ± 4.9†	17 ± 8.6 [^]	30 ± 13 [°]	25 ± 8.5 [~]
	Average at 24 Hours	21 ± 2.2	21 ± 2.2	25 ± 2.2 [~]	20 ± 2.2 [~]
Pulmonary Vascular Resistance Index (dyn.sec/cm ⁵ / m ²)	Baseline	54 ± 3.9	44 ± 3.1	50 ± 3.1	47 ± 2.2
	Maximum	57 ± 16.9†	48 ± 12.7 [~]	123 ± 77 [~]	81 ± 34 [~]
	Average at 24 Hours	70 ± 19*	43 ± 20	61 ± 19	67 ± 20
Right Ventricular Stroke Work Index (gm.m/m ² / beat)	Baseline	6 ± 0.4	5 ± 0.5	7 ± 0.2	5 ± 0.3
	Maximum	6 ± 0.4	5 ± 0.1	7 ± 2.6	8 ± 4.7
	Average at 24 Hours	8 ± 1.0‡	8 ± 1.0	9 ± 1.0 [°]	7 ± 1.0

Maximum values given occurred within 15 minutes of BIT/PST

* - $p < 0.05$ CP vs CT, † - $p < 0.05$ CP vs BDP, ‡ - $p < 0.05$ CP vs BDT, ^ - $p < 0.05$ CT vs BDP,

° - $p < 0.05$ CT vs BDT, ~ - $p < 0.05$ BDP vs BDT, ~ - $p < 0.05$ vs baseline, ° - $p < 0.05$ change with tezosentan

Mean arterial pressure similarly reached maximal values in BD animals within five minutes of BIT and then declined to remain significantly below both baseline and the control groups at 24 hrs (all comparisons $p < 0.001$, Figure 13A). However, MAP in BDT animals was not decreased in comparison to BDP animals ($p = 0.88$). Mean pulmonary artery pressure increased from baseline in BD animals within five minutes of BIT ($p < 0.001$), with no difference in peak mPAP between BD groups ($p = 0.86$, Figure 13B). Pulmonary pressures in the BDP animals remained $31 \pm 2\%$ greater than baseline 90 minutes after BIT ($p < 0.001$) and then increased over the remainder of the experiment to reach 25 ± 2.2 mmHg. Although the mPAP in BDP animals was elevated at 24 hours, the mPAP in BDT animals had returned to a level consistent with control animals (CP vs BDT $p = 0.989$). The difference in mPAP between BDP and BDT animals at 24 hours was statistically significant ($p = 0.026$).

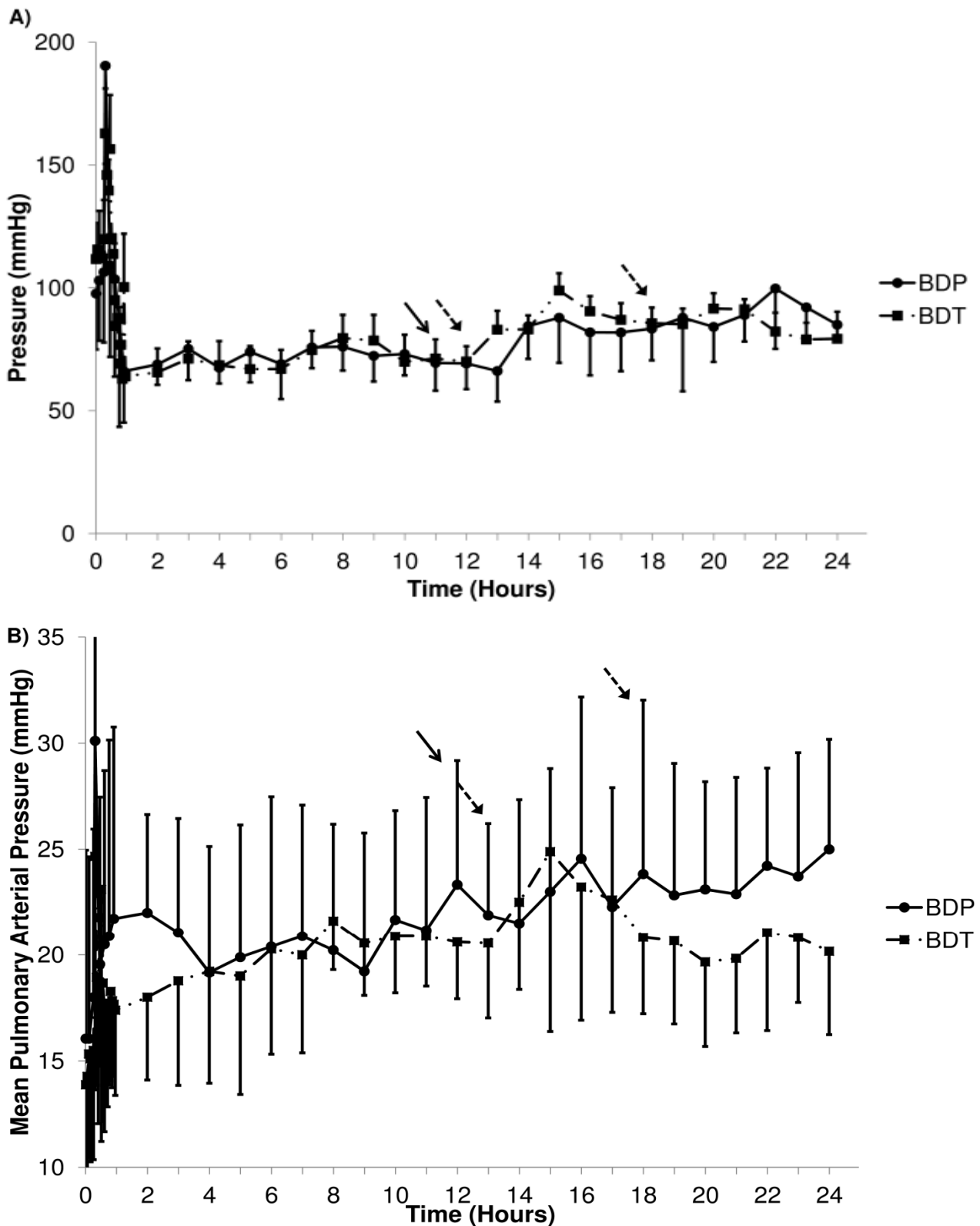


Figure 13: Arterial Pressures Recorded in Brain Dead Animals.

Solid arrows indicate hormone therapy. Dashed lines indicate administration of tezosentan or saline. BDP = Brain dead placebo, BDT = Brain dead tezosentan A) Mean Arterial Pressure was elevated early in both brain dead groups. Some improvement was noted after administration of hormone therapy. B) Mean Pulmonary Arterial Pressure were similar until tezosentan was administered. This was most pronounced after 18 hours. Brain death was induced immediately after the baseline value at time 0

Systemic vascular resistance in BD animals followed a similar time course to MAP and HR, reaching maximal values within five minutes, declining below baseline and control by one hour and remaining lower to the end of the study (Figure 14A). Hormone therapy increased SVRI, reflecting titration of vasopressin according to the protocol. Animals in the BDT group had a higher SVRI at 24 hours compared to BDP animals ($p=0.001$). Pulmonary vascular resistance also peaked within five minutes of BIT (Figure 14B). Both BD groups then fell below baseline, however this gradually improved after initiation of hormone therapy. Administration of tezosentan decreased the PVRI of control animals ($70 \pm 19 \text{ dyn}\cdot\text{sec}/\text{cm}^5/\text{m}^2$ vs $43 \pm 20 \text{ dyn}\cdot\text{sec}/\text{cm}^5/\text{m}^2$, CP vs CT $p=0.011$), however, no difference between BD groups (BDP vs BDT $p=0.46$) existed at 24 hours.

Cardiac index increased in BD animals by 30 minutes, returning to baseline by 3 hours and gradually decreased thereafter. Overall, the administration of tezosentan in BDT decreased the CI by $0.65 \pm 0.46 \text{ L}/\text{min}/\text{m}^2$ ($p=0.006$ at 24 hours). Left ventricular stroke work index followed a similar course to CI, remaining below baseline at 24 hours in the BD animals ($p<0.001$). Tezosentan reduced LVSWI in BDT animals at 24 hours ($p < 0.001$). RVSWI displayed a brief decrease after BD, recovering to baseline by 30 minutes. While the RVSWI continued to increase in BDP animals, nebulised tezosentan reversed this (BDP vs BDT $p<0.001$).

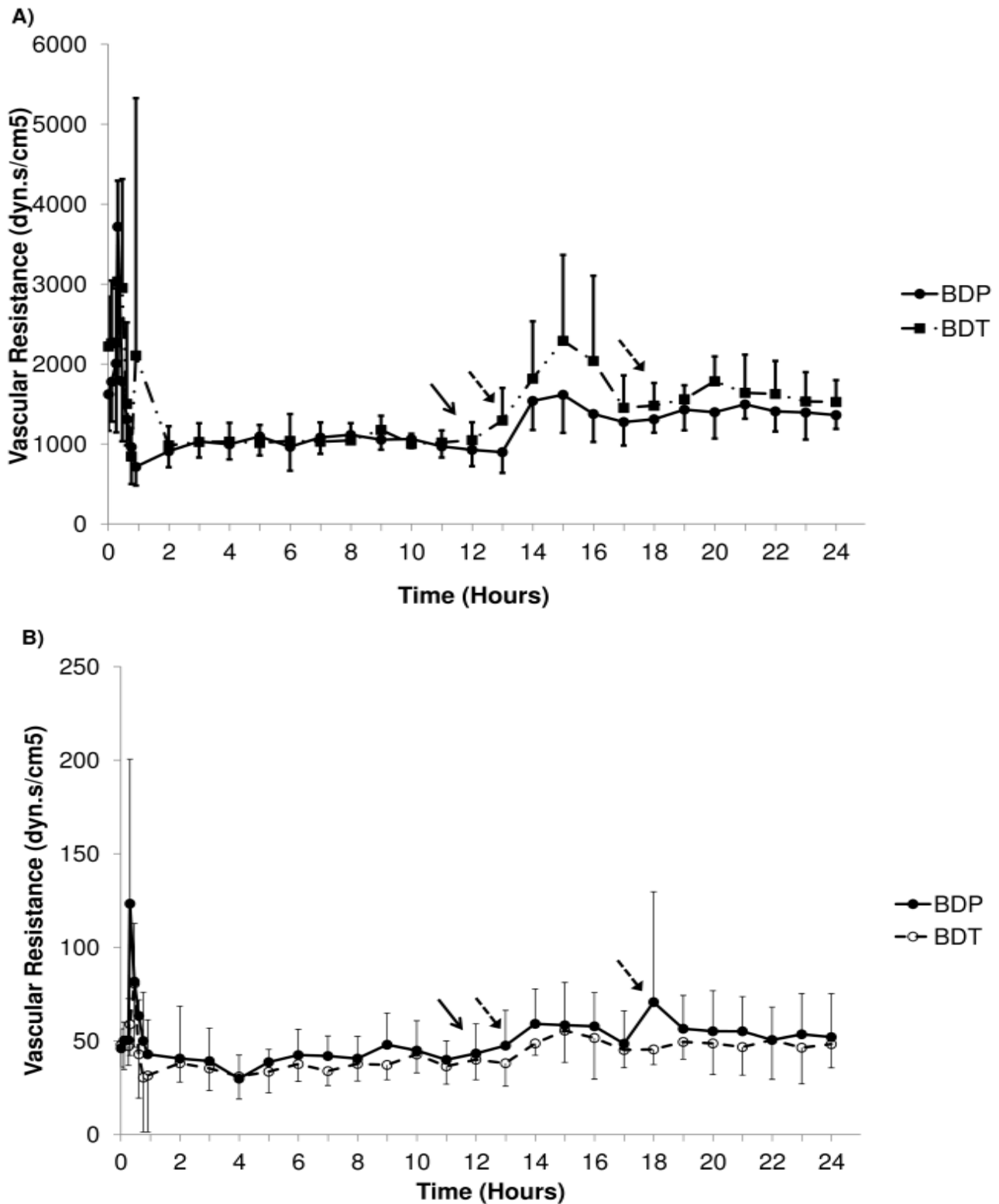


Figure 14: Vascular Resistances of Brain Dead Animals.

Solid arrows indicate hormone therapy. Dashed arrows indicate tezosentan or saline. BDP = Brain dead placebo, BDT = Brain dead tezosentan A) Systemic vascular resistance index increased after brain death and then fell below baseline. Improvement was noted after hormone therapy. In animals receiving tezosentan, a small increase in systemic vascular resistance was noted. B) Pulmonary vascular resistance index was similar to the systemic resistance, with no differences at 24 hours. Brain death was induced immediately after the baseline value at time 0

5.4.3 Histological and Tissue Analysis

Wet:dry ratios indicated no significant difference in inflammatory oedema between all four groups.

Semi-quantitative assessment of lower lobe pulmonary histology did not demonstrate any difference between CP and CT animals ($p=0.07$). As noted previously, BDP animals had increased inflammation compared to CP animals ($p=0.014$); this effect was maintained compared to CT animals ($p=0.1$). However, BDT animals had less inflammation than BDP animals ($p=0.02$). There was no difference in inflammatory scoring between BDT vs CP ($p=1.0$) or BDT vs CT ($p=0.1$)

Staining of the endothelin axis was similar to that previously reported. No difference in intensity of staining was noted between groups for either endothelin receptor, or MMP-2, -9, TIMP-1 or TIMP-2.

5.4.4 ELISA

Analysis of plasma Big ET-1 suggested that CT and BDT had higher levels at baseline; Big ET-1 remained elevated throughout the study in these groups when compared to those receiving placebo (Table 7). When comparing CP vs BDP, there were no significant differences over time. Similarly, analysis of CT vs BDT groups did not reveal any significant changes. The difference in concentrations of Big ET-1 between the BDP and BDT groups did reach statistical significance at 24 hours ($p<0.01$). ET-1 levels rose by 26.9% from baseline at one hour after induction of brain death, approaching statistical significance ($p=0.09$) in BDP animals, however a significant elevation was not observed in BDT animals ($p=0.99$, Table 7). Despite these differing responses, no significant differences between the groups were noted. Furthermore, tezosentan did not affect ET-1 concentrations.

Table 7: Results of ELISA of Big Endothelin-1 and Endothelin-1

	<u>Control / Placebo</u>	<u>Control / Tezosentan</u>	<u>Brain Dead / Placebo</u>	<u>Brain Dead / Tezosentan</u>
Big ET-1 (fmol/L)				
<i>Baseline</i>	83 ± 30*	195 ± 120 [^]	85 ± 40	151 ± 14
<i>6 Hours</i>	115 ± 20*	275 ± 0.16 ^{^^}	145 ± 30	177 ± 50
<i>12 Hours</i>	95 ± 20*	195 ± 110 [^]	105 ± 40	145 ± 60
<i>24 Hours</i>	88 ± 30*‡	205 ± 120 [^]	85 ± 40 [°]	208 ± 16
ET-1 (fmol/mL)				
<i>Baseline</i>	63 ± 18	62 ± 45	67 ± 21	58 ± 16
<i>1 Hour</i>	68 ± 29	60 ± 29	85 ± 37	47 ± 17
<i>12 Hours</i>	59 ± 15	55 ± 37	58 ± 27	45 ± 17
<i>24 Hours</i>	48 ± 16	47 ± 30	48 ± 17	35 ± 10

* - $p < 0.05$ CP vs CT, † - $p < 0.05$ CP vs BDP, ‡ - $p < 0.05$ CP vs BDT, [^] - $p < 0.05$ CT vs BDP, ^{^^} - $p < 0.05$ CT vs BDT, [°] - $p < 0.05$ BDP vs BDT, [~] - $p < 0.05$ vs baseline, [°] - $p < 0.05$ change with tezosentan

Table 8: Results of Biochemical Analysis

		<u>Control / Placebo</u>	<u>Control / Tezosentan</u>	<u>Brain Dead / Placebo</u>	<u>Brain Dead / Tezosentan</u>
Cardiac Markers					
<i>Myoglobin (µg/L)</i>	<i>Baseline</i>	23.2 ± 2.0	21.9 ± 1.5	28.7 ± 3.6	24.5 ± 4.3
	<i>24 Hours</i>	29.9 ± 3.4‡	28.9 ± 0.5 [~]	59.3 ± 15	71.0 ± 9.8
<i>Creatine Kinase MB (U/L)</i>	<i>Baseline</i>	18.5 ± 2.2	19.3 ± 3.5	18.1 ± 2.3	22.6 ± 3.1
	<i>24 Hours</i>	18 ± 4.7†	16.8 ± 2.7 [^]	31.8 ± 5.8	29.7 ± 3.0
Hepatic Markers					
<i>Alkaline Phosphatase (U/L)</i>	<i>Baseline</i>	105.2 ± 29.3	91.1 ± 29.4	84.1 ± 23.7	109.4 ± 35.2
	<i>24 Hours</i>	76.2 ± 19.4	64.5 ± 21.1	84.6 ± 25.5	100.4 ± 42.1
<i>γ-Glutamyl Transferase (U/L)</i>	<i>Baseline</i>	58.7 ± 6.8	71.6 ± 11.0	56.8 ± 2.6	65.8 ± 4.8
	<i>24 Hours</i>	55.0 ± 6.4	62.0 ± 7.7	50.3 ± 4.0	58.7 ± 4.6
<i>Alanine Aminotransferase (U/L)</i>	<i>Baseline</i>	9.8 ± 1.2	9.8 ± 1.9	8.0 ± 1.1	9.5 ± 0.6
	<i>24 Hours</i>	16.4 ± 1.1†‡	16.5 ± 2.2 ^{^^}	75.5 ± 39.5	61.8 ± 16.3
<i>Aspartate Aminotransferase (U/L)</i>	<i>Baseline</i>	78.6 ± 4.7	105.6 ± 40.9	79.9 ± 7.2	73.6 ± 4.8
	<i>24 Hours</i>	139.3 ± 9.8†‡	160.6 ± 18.3 ^{^^}	596.0 ± 291.1	534.0 ± 142.8
Renal Markers					
<i>Urea (mmol/L)</i>	<i>Baseline</i>	7.7 ± 0.8	8.3 ± 1.1	6.5 ± 0.7	7.0 ± 0.5
	<i>24 Hours</i>	4.3 ± 0.2	4.4 ± 0.4	2.5 ± 0.5	3.5 ± 0.5
<i>Creatinine (µmol/L)</i>	<i>Baseline</i>	68.4 ± 2.6	75.4 ± 4.4	71.3 ± 7.9	73.2 ± 7.0
	<i>24 Hours</i>	67.2 ± 3.5	77.8 ± 6.4 ^{^^}	57.1 ± 6.4	57.3 ± 4.9

* - $p < 0.05$ CP vs CT, † - $p < 0.05$ CP vs BDP, ‡ - $p < 0.05$ CP vs BDT, [^] - $p < 0.05$ CT vs BDP, ^{^^} - $p < 0.05$ CT vs BDT, [°] - $p < 0.05$ BDP vs BDT, [~] - $p < 0.05$ vs baseline, [°] - $p < 0.05$ change with tezosentan

5.4.5 Biochemical Analysis

Myoglobin and CK-MB increased over time in both BD groups. However, tezosentan did not significantly reduce markers of myocardial injury (Table 8). Similarly, the elevations in hepatocellular enzymes were not affected by tezosentan administration. No deterioration of renal markers of injury was observed in the brain dead groups (Table 8).

5.5 Discussion

Tezosentan was successfully administered via nebulisation to both brain dead and control animals without causing serious deterioration in haemodynamics. Detectable reductions in mean pulmonary artery pressure and right ventricular stroke work were noted when compared amongst brain dead groups, however, the lack of a statistical difference in mPAP between BDP and CP animals makes the interpretation of these findings difficult. Additionally, there were no detectable differences in immunohistochemical expression of the endothelin axis. As such, while a physiological effect of tezosentan was detectable, the overall significance of these changes, and how they may affect transplantation medicine, remains unclear.

The precise mechanisms of endothelin's involvement in BD related inflammation remain to be fully elucidated, though several authors have observed that it plays an important role. Contrary to the previous findings by our group,[88] the current thesis did not demonstrate any detectable upregulation of the endothelin axis after 24 hours. This may be due to the extended duration of the present study; if early detectable expression did occur, it may have resolved prior to tissue sample retrieval. Alternatively, it may be that no significant tissue upregulation occurred, or did not occur in this model. Previous authors have not attempted to quantify the endothelin axis in sheep models, and more recent studies have not consistently found that ET-1 is upregulated after BD. Salama *et al* demonstrated that an increase in donor lung tissue ET-1 mRNA was associated with PGD in humans.[212] However, it would appear that elevated ET-1 was only found in those patients that developed PGD, meaning that the majority of transplanted lungs (61%) did not have any detectable increase in ET-1 mRNA. This may indicate that, while upregulation of the endothelin axis, when it does occur, adversely affects lung transplant outcomes, it may not be an inherent part of the inflammatory response of BD per se. Consistent with this is the recent report of Valenza *et al*, who did not find any increase in donor ET-1 mRNA in a porcine lung transplantation model.[293] Rather than consistent contributions to BD related inflammation in the donor, detrimental effects of endothelin may be related to IRI

after transplantation, as observed by Shaw *et al* and Shennib *et al*. [87, 291] This may also be consistent with Salama *et al*'s findings that combined elevations in donor ET-1 mRNA and recipient serum ET-1 was associated with the greatest degree of PGD. [212] Such variability between study outcomes implies that greater understanding of the endothelin axis in BD related inflammation is required to allow proper extrapolation of animal data to human transplant medicine.

Further research is required to elucidate the exact mechanisms behind the observed haemodynamic responses to tezosentan in the present study. When comparing the non-brain dead groups, tezosentan's only physiological effect of note was a reduction in PVRI, consistent with its expected mechanism of action. More complex changes were noted among BD animals, however. Pulmonary artery pressure was decreased in BDT compared to BDP animals at 24 hours, without a reduction in PVRI. Cardiac index was also reduced in BDT animals compared to BDP animals, with an associated reduction in both heart rate and stroke volume index. As vascular pressure relies on both vascular resistance and cardiac output, the reduced forward blood flow (through reduction in CI) may contribute to our findings. However, similar reductions in left ventricular output would result in increased left atrial pressure, counteracting any effect of reduced pulmonary flow on mPAP. Also noted was that MAP was maintained in BDT animals, with a small increase in SVRI. It is difficult to explain this increase. There was no difference in administration of pressor, including vasopressin. Brain death precludes a brain-stem reflex response to the reduction in cardiac output, however it has been noted that some degree of reflex sympathetic activity has been maintained in BD donors undergoing surgical organ retrieval. [360] Pennefather *et al* noted increases in systemic vascular resistance after commencement of surgery for organ retrieval. This was associated with increased catecholamine levels via an unknown mechanism. They theorised that a spino-adrenal reflex may remain intact, however further study was recommended.

Pulmonary vasodilators may act as negative inotropes by three main mechanisms. These include afterload reduction, decreases in coronary perfusion (due to a reduction in systemic pressure) or by direct effects on the myocardium. [361] Tezosentan did not decrease the MAP, and hence, did not decrease either the afterload or the driving pressure for coronary perfusion. Rather, tezosentan may have reduced the CI via direct antagonism of myocardial endothelin receptors. [71] Nebulised tezosentan is absorbed via the pulmonary circulation, though levels are significantly lower than those reached by

systemic administration.[74] Konrad *et al*, observed that intravenous tezosentan decreased cardiac inotropy while concurrently improving lusitropy in endotoxaemic pigs.[71] This resulted in an overall increase in cardiac output due to improved diastolic function and reduced afterload. However, Persson *et al*, did not demonstrate any change in CI in endotoxaemic pigs with nebulised tezosentan.[74] These differing findings may be because the effects of endothelin antagonism on myocardial performance is influenced by the pathophysiological state of the heart.[71] The current study was not designed to specifically assess the direct effects of tezosentan on the heart. However, Konrad *et al*'s findings provide a possible contributor for the observed reduction in mPAP in BDT animals; improved lusitropy may shift the end diastolic pressure-volume relationship downwards and rightwards, reducing left ventricular and left atrial end diastolic pressure.[362] Future studies, designed specifically to evaluate tezosentan's effects on the heart, may be able to answer these questions.

Although a reduction in CI may be deemed undesirable, the current findings suggest that overall tissue perfusion was not impaired with administration of tezosentan. Circulating levels of lactate were within the analyser's normal range at the end of the study. Therefore, the statistically significant reduction of lactate in BDT animals is of unlikely clinical significance. More importantly though, a reduction in cardiac output secondary to tezosentan may have worsened haemodynamic stability, impairing tissue perfusion and increasing lactate; this was not observed.

Systemic levels of ET-1 were similar across all groups. Tezosentan did not increase ET-1, although ET_RB antagonism decreases clearance of circulating endothelin.[106] Previous studies have confirmed that intravenous tezosentan increases systemic ET-1 levels,[71, 74] an effect that could adversely affect other transplantable organs. In the current study, the localised effect of nebulisation,[74] or incomplete ET_RB antagonism, may account for the lack of ET-1 elevation after tezosentan administration. Big ET-1 levels tended to be highest in the CT group; this was statistically significant compared to the CP and BDP groups at all points, including at baseline. It is unclear as to why this occurred. No protocol violations were noted across the group, and no one single animal accounted for these results. Big ET-1 did increase in the BDT animals from 12 to 24 hours, an observation that would be unexpected from tezosentan's mechanism of action. Interference with either self-regulation (negative feedback) of endothelin production or NO-mediated inhibition via ET_RB antagonism could be speculated, however ET-1 is not

reported in the literature as an inhibitor of its own production. A clinically relevant effect of this observation remains questionable, as Big ET-1 is required to be converted to ET-1 for maximal signalling; this conversion was not detected by the ET-1 ELISA.

Lung histology indicated increased inflammation in BDP animals compared to all other groups. Overall, the modest inflammatory changes in the BDP group is not unexpected, as previous studies have suggested that ongoing care of brain dead donors may actually lead to improvement in lung function.[119, 269, 363] It is possible that tezosentan prevented or reversed the inflammatory changes observed after BD, accounting for the lower score in BDT animals. However, an explanation for this was not provided by immunohistochemistry. Further study, including techniques such as mRNA analysis, may provide further insight in the future. Brain death resulted in hepatic and cardiac injury, as previously reported. Tezosentan did not decrease levels of cardiac markers. This is most likely because cardiac injury primarily occurred during induction of brain death,[255] well before tezosentan administration. The finding of no differences in hepatic or renal markers of injury between the BDP and BDT groups further supports tezosentan's localised effects.

Interpretation of the results in the current study is potentially confounded by variations in management between groups. This study was designed to be pragmatic, reflecting multiple interventions commonly used in the care of the potential brain dead organ donor. Attempts were made throughout the protocol to maintain consistency, such as sedation adjustment to the surgical plane, or vasoactive support to pre-defined targets. Despite this, inclusion of prolonged sedation, hormone resuscitation therapy and vasoactive agents may have influenced the outcomes of physiological variables. Achieving a protocol of 24 hours is a strength of the current study, but further investigation of endothelin antagonism after BD may require alterations to the protocol. Such variations include removal of non-BD animals (thus removing sedation), or exclusion of hormone resuscitation therapy and vasoactive agents (both of which have measurable cardiovascular effects of their own). Although this may result in shorter studies, greater consistency between groups would allow a more detailed understanding of the effects of tezosentan alone.

5.6 Conclusion

The current study is the first to demonstrate that nebulised tezosentan may be safely administered to potential organ donors. Observed haemodynamic changes after brain death were complex, demonstrating a reduction in cardiac output, with maintained blood

pressure and no evidence of worsening systemic perfusion. It is possible that the reduction in mPAP is due to direct cardiac effects rather than pulmonary activity in brain dead animals. Further studies are required to fully quantify the effects of tezosentan on potential cardiac and pulmonary grafts in the brain dead donor.

Chapter 6 - Conclusions and Opportunities for Future Research

6.1 Summary of Findings in Thesis

The present thesis demonstrates the following major points; 1) the feasibility and validity of an extended, clinically relevant ovine model of brain death, 2) pulmonary and systemic haemodynamics are significantly altered after brain death, 3) nebulised tezosentan can be administered safely to brain dead sheep despite their deranged haemodynamics, and 4) nebulised tezosentan results in complex physiological responses in brain dead animals. As reviewed in Chapter 2, brain death causes widespread inflammation which contributes to multi-organ injury. Fundamental to this process are numerous cytokines, neurotransmitters and other cell signalling molecules, including the endothelin axis. Recent pulmonary transplant literature has identified that ET-1 may play a role in both early and late graft related complications.

In order to further investigate the endothelin axis after BD, the selection of a clinically relevant large animal model was required. Chapter 3 discusses the considerations behind selecting and developing an ovine model for this project. Similar to other large animal models, such as swine, sheep are an appropriate species to investigate human pathology. Extending the model to 24 hours was considered important to replicate delays that occur in human BD organ donor management. Although shorter duration models provide insight into early inflammatory and physiologic changes after brain death, models of greater duration may more accurately simulate the donor after aggressive management. The complexity of such a model may introduce confounders, however. Management between groups in the current thesis did have notable differences. Administration of vasoactive agents to brain dead animals was a source of heterogeneity, as was differing amounts of sedation/anaesthesia. It is unclear the degree to which these confounders may have influenced the final outcomes, but a pragmatic approach was chosen to simulate clinical experience.

The present thesis attempted to quantify the effects of a dual endothelin receptor antagonist on pulmonary inflammation after brain death. Tezosentan was chosen for its non-selectivity and ability to be nebulised. ET_RB antagonism may have increased systemic levels of ET-1, but there was no evidence of this by ELISA. Additionally, nebulised tezosentan did not worsen haemodynamics of BD animals and there was no biochemical evidence of worsened transplantable organ injury. Tezosentan had minimal

effect on cardiac markers of injury; although CK-MB did not reach statistical significance in the BDT animals compared to CP, the level was not much less than the BDP animals, suggesting that clinically relevant injury had already occurred.

6.2 Clinical Relevance of the Presented Findings

In order to completely ascertain the importance of these study findings, one must consider not only their statistical significance, but also their clinical implications. An increase in the pulmonary blood pressure of BDP animals was noted to occur at 24 hours. This was from a baseline of 16 ± 0.2 mmHg to 25 ± 2.2 mmHg, representing a 56% increase. Highest values (outside of the immediate period of BD induction) were observed during the final hour of the study. When nebulised tezosentan was administered to BD animals, mPAP was decreased to 20 ± 2.2 mmHg, a result that was statistically significant. Represented graphically, this appeared to mostly occur after the 18 hour dose. Despite this, the absolute change in mPAP was small, being approximately 5 mmHg. As noted in Chapter 2, the right ventricle is more adversely impacted after brain death than the left. Therefore, the increase in pulmonary artery pressure, despite being small in absolute terms, may contribute to right ventricular dysfunction. The study by Szabo *et al* demonstrated a reduction in the peak positive dP/dT in brain dead animals compared to controls when the increase in pulmonary vascular resistance was increased by 50% from baseline.[258] Although this represented a pulmonary arterial systolic pressure of 30 mmHg, a level greater than that observed in the current thesis, this was from a baseline of 22 ± 2 mmHg. Therefore, further investigation is required, including pressure-volume and pulmonary flow analysis, to fully understand the clinical impact of the observed changes in mPAP in the current study.

Right ventricular stroke work also appeared to decrease in BDT animals compared to BDP. Although this was statistically significant, it was within the normal range of 5-10 $g/m^2/beat$. As RVSWI is a derived variable, it is dependent on cardiac index, heart rate, mPAP and central venous pressure. Comparing these results at 24 hours suggests that the observed RVSWI may be due to either a reduction in mPAP or CI. As already noted, the reduction in mPAP in BDT animals was not due to a decrease PVRI at 24 hours. Therefore, it is possible that the observed changes in RVSWI were due to the reduction in cardiac output induced by tezosentan. Further study is required to better delineate which mechanism predominates.

Acute severe pulmonary hypertension is frequently treated with agents other than endothelin antagonists. Nitric oxide (NO) is a commonly applied inhaled pulmonary vasodilator. Small studies have assessed its potential role in the preservation of pulmonary allografts. Avlonitis *et al* investigated inhaled NO administered to BD rodent donors prior to lung transplantation.[193] This study observed that, contrary to previous living donor experiments, NO worsened oxygenation without improving systemic markers of inflammation. The authors concluded that NO administration to BD organ donors was of no benefit, and could actually lead to harm.[193] Similar to NO, research into inhaled prostacyclin therapy for lung preservation has focussed on preventing IRI. Consequently, animal models have used non-BD donors. Studies investigating BD organ donors are yet to be published. While administration of inhaled NO or PGI₂ to recipients of heart or lung transplants with evidence of pulmonary hypertension in the immediate post-transplant period is associated with improved haemodynamics,[364] further research is required to determine if there is a role for these agents in organ donor management.

Measured systemic haemodynamic changes were similar to those observed in previous studies. Such significant alterations during and after brain death, and the requirement for vasoactive agent administration, indicates that these observed changes were clinically relevant. Lactate was measured as a surrogate marker of global perfusion. Peak levels obtained did not greatly exceed normal values; at 24 hours, all groups were below 2 mmol/L. Animals in the BDT group had a statistically significant reduction in lactate levels at 24 hours, but in absolute terms this was small. Of greater clinical importance is that no increase in lactate levels was observed. This is despite the reduction in cardiac index, and the potential for endothelin antagonists to cause hypotension and worsen perfusion.

6.3 Limitations of the Current Thesis

Limitations occur in all research projects and it is identified that such threats remain in this study. Blinding of groups to BD or control cannot be achieved by nature of the intervention. The investigators caring for the animals must induce brain death and need to be available to manage any complications that may arise from this process. Further, it is impossible to hide the physiological changes that occur. Management of BD animals necessarily differed to controls through use of haemodynamic support and greater intervention for complications. These actions could potentially confound the results. Further, anaesthesia and sedation were adjusted to the surgical plane/comfort, meaning that brain dead animals required less medication. These effects have been attempted to

be controlled for by including four experimental groups. Additionally, administration of the active drug or placebo was blinded from the experimental team caring for the animals.

Each group was allocated six animals for a total of 24 assessed in the current thesis. A power analysis was performed prior to commencement which indicated that these numbers were adequate to allow detection of deviation by one standard error. Animal studies frequently have small numbers per group, for example, Cox *et al* studied four animals per group when assessing smoke and burn injury,[302] while Kuklin used a total of 21 sheep in assessing endothelin in endotoxaemia.[75] In terms of animal BD model reports, Seirenigg *et al* reported 15 pigs,[333] while Valenza *et al* reported a total of 10 pigs, with five donors and five recipients.[293] Although the current study is consistent with recent published literature reporting animal models, such small numbers of animals per group increases the risk of Type II errors. Of course, subjects that are potential outliers could also disproportionately influence study findings. One possible advantage of small studies is that they can successfully identify significant differences between groups, but this still requires careful interpretation and further investigation.[365] Increasing animal numbers may have improved our ability to detect more changes between groups, although logistic and financial limitations prevented this. Each animal study was undertaken over 30 hours, including setup time, study protocol and post-mortem and tissue sampling.

It was identified early that team experience may affect the observed outcomes. As further experiments were performed, the team was able to identify complications faster and intervene at an earlier stage with improved intervention selection. Experience gained with instrumentation and equipment can also affect intra-protocol animal management. Of particular note was the ventilator, which can directly injure the lung (ventilator associated lung injury). In order to control for this, management protocols were put in place to ensure consistent management (for example, ventilator rate and volumes, or criteria for identification and treatment of diabetes insipidus) prior to commencement of the study.

Nebulisation of tezosentan directly delivers the agent to the target site and was selected in order to reduce systemic effects of endothelin antagonism. While this is potentially beneficial in models that result in severe haemodynamic disturbances, heterogeneity in particle size generated by nebulisation may lead to altered deposition of the drug in the small airways and, hence, variability in the exact amount of drug delivered. The nebulisation system used is a standard system that is able to be attached to the inspiratory

limb of a ventilator, with generation of particles consistent with previous studies.[74, 325] Despite this, and the observation of a measurable effect in the current thesis, further investigation is necessary to determine the exact degree of delivery of tezosentan via nebulisation.

The severe physiological insult of BD itself may risk validity through loss of experimental animals. One sheep was lost at induction of BD due to ventricular fibrillation, despite advanced life support protocols being administered. In order to control for this, the animal was replaced to maintain group balance. Although this was seen as a setback in terms of resources and attempts to minimise animal harm, it further demonstrated the validity of the model. Ventricular dysfunction that is unresponsive to intervention and leads to death is reported to occur in 10-40% of cases of brain death.[121]

Although the application of ovine models is gaining increased acceptance,[294, 315, 331] industry development of sheep specific investigational modalities is slow. Antibodies and ELISA kits aimed at identifying a wide range of targets in rodent and human tissue/blood samples have been developed, however not all of these are appropriate for use in ovine models. The antibodies and ELISA kits used in the current thesis were based on previous laboratory experience, sequence homology of molecular targets (and expected cross reactivity) and published works that successfully applied these investigational tools in sheep. Trials were required to identify appropriate dilutions to minimise non-specific staining, yet identify the targets of interest. Initial trials of some of the antibodies were unsuccessful and required new antibodies to be sourced. As the wider research community continues to adopt sheep models for human disease, it is expected that such limitations would disappear.

The duration of the study, and use of a sheep model, provided ample sampling time points and methods. This ability was tempered by sample processing time and financial restraints. Unfortunately, limitations in financial resources prevented ELISA testing for endothelin-1 and big endothelin-1 at all timepoints that blood was collected. As noted previously, elevations in endothelin-1 concentrations have been detected early after brain death, and it is possible that the selective testing of samples to encompass the entire 24 hour period may have resulted in missing peak levels that occurred very early in the protocol. Trial testing of adrenaline and noradrenaline was successfully performed, but complete analysis of all samples was prohibitively expensive. As noted in the animal

protocols, tracheostomy was performed on all animals. This was to facilitate bronchoscopy for broncho-alveolar lavage and bronchial brushings. Unfortunately, a number of bronchial brushing pellets were lost after sample processing, making any results from testing the remaining samples unreliable. Similar to blood and serum testing, broncho-alveolar lavage fluid analysis was also limited by financial restraints. Tissue, serum, urine and broncho-alveolar lavage fluid samples remain appropriately stored to enable possible assessment in the future.

6.4 Opportunities for Future Research

Transplantation remains the gold standard treatment for end stage organ disease, however the complexity of brain death and the peri-transplant period is yet to be fully understood. The extreme physiologic alterations that occur adversely affect transplantable organs, impairing their quality, and increasing the likelihood of post-transplant complications. Endothelin has been observed to play a role in this inflammation and, as has been demonstrated in the current thesis, targeting this axis is possible. However, further research in this area is required to fully elucidate the potential clinical application of endothelin antagonists after brain death. This may include extending the current model to include transplantation of tezosentan treated lungs. Successful lung transplantation has been performed in sheep,[366] although this is not a common model in the literature.

Intermittent administration of tezosentan may reduce its therapeutic benefit due to its short half life. Continuous intravenous infusion may overcome this and provide antagonism of endothelin across multiple organs. It is currently unknown if widespread antagonism would improve the function of these other organs, however. Unfortunately, intravenous administration would reasonably be expected to worsen haemodynamic instability. Although other models of critical illness have successfully used intravenous tezosentan safely,[74, 320] recent developments in lung transplantation provide another modality to administer continuous infusions of tezosentan (including potentially greater doses) to donor lungs. The introduction of *ex-vivo* perfusion techniques now allows reconditioning and salvage of marginal lungs.[367, 368] Cypel *et al* demonstrated that *ex-vivo* lung perfusion (EVLV) is able to improve the quality of lungs prior to transplantation by optimising conditions and allowing lungs to recover from BD associated injury.[367] It has been envisaged that this may significantly contribute to addressing the ongoing disparity between lungs available for transplant and the number of patients on the waiting list.[367]

Ex-vivo lung perfusion may be employed to increase the degree of endothelin antagonism achievable while completely avoiding further haemodynamic compromise in the BD donor. Increasing the dose in this manner may also increase the observed clinical effects. Addition of tezosentan to the perfusate will enhance delivery of the agent directly to pulmonary vasculature, while combination administration with nebulisation would directly target bronchiolar and alveolar epithelium and alveolar macrophages. This would also allow direct comparison of drug delivery between the nebulisation and intravenous routes of administration. Future studies by our group will assess the effects of pulmonary tezosentan administration through EVLP techniques. We have successfully won a competitive grant to develop an ovine pulmonary EVLP model and investigate the effects of tezosentan administration by both nebulisation and perfusate delivery. Comparison of these administration methods will enable quantification of drug delivery by nebulisation, giving greater insight into the actual amount of drug delivered to the alveolar epithelium and pulmonary vascular endothelium. This study will utilise a Vivoline EVLP machine, which has already been acquired. Ethics clearance has been approved and this study has now commenced. Finally, post-transplant outcomes of these interventions, and the possible effects of tezosentan in ischaemia-reperfusion injury, could be assessed by developing and implementing an ovine model of lung transplantation.

Cardiac function after brain death may also be further investigated using the current model. Opportunities include the use of echocardiography to assess the changes in cardiac function that occur during and after brain death. Our group has previously reported on the application of intra-cardiac echocardiography in sheep undergoing extra corporeal membrane oxygenation.[369, 370] The feasibility of intra-cardiac and transthoracic echocardiography was tested in our BD model. Similar to EVLP techniques described above, it is also possible to assess the effects of endothelin antagonism on cardiac function using an *ex-vivo* heart perfusion technique.[371] Our group has recently acquired such a device to use in the ovine brain death model.

6.5 Closing Statements

Brain death may occur due to multiple pathologies and remains a multi-system disease. The very nature of brain death requires evolutionary survival mechanisms to maintain the body in an entirely unphysiological state, that is, in the absence of brain function. Overarching control of physiologic and inflammatory processes is lost and mechanisms for survival become maladaptive, injuring organs and impairing their function. In the natural

order, death results, yet modern technology is able to delay and even reverse these processes. Organs can now be routinely transplanted to preserve and prolong life.

Progressive understanding of complex molecular signalling continues to identify new targets for intervention both before and after transplantation. This includes the identification of the endothelin axis as a potential target. The current thesis has demonstrated that nebulised tezosentan in BD donors is feasible as part of an organ preserving strategy. Pharmacodynamic responses to nebulised tezosentan in brain dead sheep were complex and further study is required to evaluate their underlying mechanisms. Beneficial effects of endothelin antagonism may be realised in the future through utilisation of other techniques, such as EVLP, or studying other complications of transplantation, including ischaemia-reperfusion injury. Ultimately, targeting the endothelin axis may become part of aggressive donor management protocols in the future.

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