

**The Use of Pharmacological Conditioning Strategies to Improve
Donor Heart Preservation in Cardiac Transplantation**

Gayathri Kumarasinghe

A thesis in fulfilment of the requirements for the degree

Doctor of Philosophy



St. Vincent's Clinical School, Sydney, Australia

Faculty of Medicine, The University of New South Wales, Sydney, Australia

September 2015

PLEASE TYPE

THE UNIVERSITY OF NEW SOUTH WALES
Thesis/Dissertation Sheet

Surname or Family name: KUMARASINGHE

First name: GAYATHRI

Other name/s: HARIPRIYA

Abbreviation for degree as given in the University calendar: PhD

School: ST VINCENT'S CLINICAL SCHOOL

Faculty: MEDICINE

Title: THE USE OF PHARMACOLOGICAL CONDITIONING
STRATEGIES TO IMPROVE DONOR HEART PRESERVATION
IN CARDIAC TRANSPLANTATION

Abstract 350 words maximum: (PLEASE TYPE)

Cardiac transplantation is the gold standard treatment for end-stage heart failure. Over the last 48 years, the profile of cardiac transplantation has evolved to include more higher-risk transplants, that is, longer ischaemic times, more 'marginal' organs and higher-risk recipients. However this is associated with an increased risk of primary graft dysfunction (PGD), for which the main mechanism is ischaemia-reperfusion injury (IRI). This thesis addresses strategies of minimising IRI, using myocardial preservation strategies based on the concept of pharmacological conditioning, and with a focus on improving preservation of marginal donor hearts.

Initially, small animal models were developed for incorporation into myocardial preservation studies:

- i) A rodent model of brain death
- ii) A rodent abdominal heterotopic working heart transplant model and
- iii) Cardiac magnetic resonance (CMR) imaging of rat hearts to allow longitudinal non-invasive functional cardiac imaging.

Using a Langendorff model, hearts from brain dead rats were treated with pharmacological conditioning using three pro-survival kinase agents erythropoietin, zonisporide and glyceryl trinitrate added to Celsior preservation solution used in cardioplegia and 6 hours of hypothermic storage. Compared to controls, conditioning significantly improved the recovery of aortic flow (AF) and cardiac output (CO), while reducing markers of myocardial injury such as lactate dehydrogenase (LDH). Mechanisms of cardioprotection were indicated by increased phosphorylation of pro-survival kinases Akt and ERK1/2 of the reperfusion injury salvage kinase (RISK) pathway.

Older or 'marginal' hearts (from 18-month old rats) were then assessed, and the recovery of AF and CO were again increased; myocardial oedema and LDH were reduced; and phosphorylation of Akt and ERK1/2 were increased.

Sildenafil was tested for its conditioning properties. It was found to be a weak conditioning agent, with activity augmented by the addition of erythropoietin with increased recovery of AF and reduced LDH.

A translational clinical study was performed to assess conditioning by erythropoietin and glyceryl trinitrate, compared to historic controls using Celsior alone or modified St. Thomas' Solution (STS). The conditioned group had older donors and higher-risk recipients. Results demonstrated comparable use of post-transplant mechanical circulatory support and survival up to 12 months compared with the lower risk 'standard criteria' historic controls.

Declaration relating to disposition of project thesis/dissertation

I hereby grant to the University of New South Wales or its agents the right to archive and to make available my thesis or dissertation in whole or in part in the University libraries in all forms of media, now or hereafter known, subject to the provisions of the Copyright Act 1968. I retain all property rights, such as patent rights. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

I also authorise University Microfilms to use the 350 word abstract of my thesis in Dissertation Abstracts International (this is applicable to doctoral theses only).

/s/ /s/

The University recognises that there may be exceptional circumstances requiring restrictions on copying or conditions on use. Requests for restriction for a period of up to 2 years must be made in writing. Requests for a longer period of restriction may be considered in exceptional circumstances and require the approval of the Dean of Graduate Research.

FOR OFFICE USE ONLY

Date of completion of requirements for Award:

ORIGINALITY STATEMENT

'I hereby declare that this submission is my own work and to the best of my knowledge it contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at UNSW or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by others, with whom I have worked at UNSW or elsewhere, is explicitly acknowledged in the thesis. I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.'

COPYRIGHT STATEMENT

'I hereby grant the University of New South Wales or its agents the right to archive and to make available my thesis or dissertation in whole or part in the University libraries in all forms of media, now or here after known, subject to the provisions of the Copyright Act 1968. I retain all proprietary rights, such as patent rights. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

I also authorise University Microfilms to use the 350 word abstract of my thesis in Dissertation Abstract International (this is applicable to doctoral theses only).

I have either used no substantial portions of copyright material in my thesis or I have obtained permission to use copyright material; where permission has not been granted I have applied/will apply for a partial restriction of the digital copy of my thesis or dissertation.'

Signed

Date

05/05/2016

AUTHENTICITY STATEMENT

'I certify that the Library deposit digital copy is a direct equivalent of the final officially approved version of my thesis. No emendation of content has occurred and if there are any minor variations in formatting, they are the result of the conversion to digital format.'

Signed

Date

05/05/2016

ACKNOWLEDGEMENTS

I would like to thank my supervisor Professor Peter Macdonald for his guidance and mentorship throughout my PhD and clinical training years. It has been a privilege working with him, and by leading by example he has been crucial in my professional development.

I would like to thank my co-supervisor Associate Professor Christopher Hayward for his support and guidance during my research and clinical training years. I would also like to thank Dr. Mark Hicks for tirelessly co-editing my thesis and publications, and for his encouragement and enthusiasm.

I would like to thank Dr. Ling Gao for reintroducing me to laboratory work and for sharing with me her expertise in the rat Langendorff system and small animal surgery; Associate Professor Andrew Jabbour for his help in developing the rodent model of cardiac magnetic resonance imaging; Dr. Alexandra Sharland for introducing me to small animal models in her laboratory; and Dr. Chuanmin Wang for demonstrating the rodent heart transplant model. I would also like to thank Dr. Jeanette Villanueva and Ms. Aoife Doyle for their help with laboratory work.

I would like to thank the Victor Chang Cardiac Research Institute, the National Heart Foundation of Australia, the Cardiac Society of Australia and New Zealand, the Transplantation Society of Australia and New Zealand and St. Vincent's Clinic Foundation for scholarship and funding support.

Finally I would like to thank my father Sudath and my mother Padma for being wonderful role models, for gently encouraging me to build on my strengths and for all their sacrifices, and my sister Viraji for her cheerful encouragement and support of all my endeavours.

ABSTRACT

Cardiac transplantation is the gold standard treatment for end-stage heart failure. Over the last 48 years, the profile of cardiac transplantation has evolved to include more higher-risk transplants, that is, longer ischaemic times, more 'marginal' organs and higher-risk recipients. However this is associated with an increased risk of primary graft dysfunction (PGD), for which the main mechanism is ischaemia-reperfusion injury (IRI). This thesis addresses strategies of minimising IRI, using myocardial preservation strategies based on the concept of pharmacological conditioning, and with a focus on improving preservation of marginal donor hearts.

Initially, small animal models were developed for incorporation into myocardial preservation studies:

- i) A rodent model of brain death
- ii) A rodent abdominal heterotopic working heart transplant model and
- iii) Cardiac magnetic resonance (CMR) imaging of rat hearts to allow longitudinal non-invasive functional cardiac imaging.

Using a Langendorff model, hearts from brain dead rats were treated with pharmacological conditioning using three pro-survival kinase agents erythropoietin, zoniporide and glyceryl trinitrate added to Celsior preservation solution used in cardioplegia and 6 hours of hypothermic storage. Compared to controls, conditioning significantly improved the recovery of aortic flow (AF) and cardiac output (CO), while reducing markers of myocardial injury such as lactate dehydrogenase (LDH). Mechanisms of cardioprotection were indicated by increased phosphorylation of pro-survival kinases Akt and ERK1/2 of the reperfusion injury salvage kinase (RISK) pathway.

Older or 'marginal' hearts (from 18-month old rats) were then assessed, and the recovery of AF and CO were again increased; myocardial oedema and LDH were reduced; and phosphorylation of Akt and ERK1/2 were increased.

Sildenafil was tested for its conditioning properties. It was found to be a weak conditioning agent, with activity augmented by the addition of erythropoietin with increased recovery of AF and reduced LDH.

A translational clinical study was performed to assess conditioning by erythropoietin and glyceryl trinitrate, compared to historic controls using Celsior alone or modified St. Thomas' Solution (STS). The conditioned group had older donors and higher-risk recipients. Results demonstrated comparable use of post-transplant mechanical circulatory support and survival up to 12 months compared with the lower risk 'standard criteria' historic controls.

AWARDS AND PUBLICATIONS DURING PERIOD OF CANDIDATURE

Awards

- Transplantation Society of Australia and New Zealand
 - President's Prize for best scientific abstract 2014
 - Young Investigator Award 2014
- Transplantation Society of Australia and New Zealand
 - Young Investigator Award 2013
- Transplantation Society of Australia and New Zealand
 - Young Investigator Award 2012
- St Vincent's Clinic Foundation Travelling Research Fellowship
 - John Radcliffe Hospital, Oxford University Hospitals NHS, UK - 2015/16
- Transplantation Society of Australia and New Zealand
 - Travel Awards to present abstracts at international scientific meetings
2013, 2014 and 2015
- Cardiac Society of Australia and New Zealand
 - Travel Award to present an abstract at an international meeting 2015
 - Travel Award to present an abstract at a national meeting 2012

Published manuscripts

Kumarasinghe G, Gao L, Hicks M, Villanueva J, Doyle A, Rao P, Qiu M, Jabbour A, Iyer A, Chew H, Hayward CS, Macdonald P. Improved heart function from older donors using pharmacological conditioning strategies. *J Heart Lung Transplant*. 2016 May; 35 (5) 636-46

Kumarasinghe G, Yu CY, Chang M, Moffat K, Botnar R, Otton J, Holloway C, McCrohon J, Gao L, Hicks M, Hayward CS, Macdonald P, Jabbour A. Cardiac magnetic resonance imaging produces highly reproducible rodent cardiac volumetric and functional data using a 1.5Tesla clinical scanner. (Submitted to *J Cardiovascular Magnetic Resonance Imaging*, August 2015)

Kumarasinghe G, Iyer A, Hicks M, Watson A, Chew H, Gao L, Villanueva J, Doyle A, Jabbour A, Kotlyar E, Hayward CS, Keogh AM, Granger E, Jansz P, Dhital KK, Macdonald P, Spratt P. Pharmacological conditioning of brain dead donor hearts with erythropoietin and glyceryl trinitrate: initial clinical experience. *Int J Transplantation Res and Medicine* 2016; 2: 018 Vol 2, Issue 1

Iyer A*, Kumarasinghe G*, Hicks M, Watson A, Gao L, Doyle A, Keogh A, Kotlyar E, Hayward CS, Dhital KK, Granger E, Jansz P, Pye R, Spratt P, Macdonald P. Primary graft failure after heart transplantation. *J Transplantation*. 2011;2011:175768. Doi: 10.1155/2011/175768. Epub 2011 Aug 1. * Equal first authors

Published abstracts

Kumarasinghe G, Gao L, Hicks M, Doyle A, Chew H, Iyer A, Hayward CS, Macdonald P. 'Pharmacological conditioning' can allow the use of aged donor hearts in cardiac transplantation. J Heart Lung Transplant 2015;34(4):S278

Kumarasinghe G, Gao L, Hicks M, Jabbour A, Doyle A, Iyer A, Macdonald P. Static cold storage of brain dead donor hearts: can 'pharmacological conditioning' overcome primary graft failure? J Heart and Lung Transplant 2013;32(4):S158-159

Kumarasinghe G, Yu C, Moffat K, Otton J, Holloway C, McCrohon J, Jabbour A. Cardiovascular magnetic resonance (CMR) imaging produces highly reproducible rodent cardiac volumetric and functional data using a 1.5Tesla clinical scanner. Heart Lung Circulation. 2013;22, Supp1:S168-S169

TABLE OF CONTENTS

Thesis dissertation sheet	ii
Originality statement	iii
Copyright statement	iv
Acknowledgements	v
Abstract	vi
Awards and publications during the candidature	viii
Table of contents	xi
List of tables	xviii
List of figures	xix
List of abbreviations	xxii
Appendix A. Other publications during candidature	256

CHAPTER 1. INTRODUCTION	1
1.1 Cardiac transplantation in the 21 st Century	2
1.1.1 Changing recipient and donor profiles	2
1.1.2 Use of marginal donor organs	3
1.1.3 Use of mechanical circulatory support	4
1.1.4 Advances in procedural aspects	4
1.2 Current challenges in cardiac transplantation	5
1.2.1 Primary graft dysfunction	5
1.2.2 Allograft vasculopathy	8
1.3 Ischaemia-reperfusion injury	9
1.3.1 Ischaemia-reperfusion injury of the donor heart	10
1.3.1.1 Donor brain death	10
1.3.2 The pathophysiology of ischaemic injury	11
1.3.2.1 Metabolic alterations	11
1.3.2.2 Disruption of ionic homeostasis	12
1.3.3 Reperfusion injury	14
1.3.3.1 Free radical formation and oxidative stress	14
1.3.3.2 Calcium overload	16

1.3.3.3 Opening of mitochondrial permeability transition pores	17
1.3.3.4 Endothelial injury	18
1.4 Mechanisms of cardioprotection	19
1.4.1 Donor interventions.....	19
1.4.1.1 Donor hormonal resuscitation	19
1.4.2 Organ preservation.....	20
1.4.2.1 Hypothermic preservation solutions.....	20
1.4.2.2 Continuous perfusion techniques	24
1.4.2.2.1 Hypothermic perfusion preservation.....	25
1.4.2.2.2 Normothermic perfusion preservation.....	25
1.4.2.3 Myocardial preservation using ‘conditioning’ strategies	26
1.4.2.3.1 Ischaemic preconditioning.....	26
1.4.2.3.2 Ischaemic postconditioning	27
1.4.2.3.4 Mechanisms of conditioning.....	28
1.4.2.3.5 Pharmacological conditioning.....	30
1.4.2.3.6 Clinical application of pharmacological conditioning	35
1.4.2.4 Myocardial conditioning in the context of donor heart preservation	35
1.4.2.4.1 Sodium-hydrogen exchange inhibitors	35
1.4.2.4.2 Glyceryl trinitrate	37
1.4.2.4.3 Erythropoietin	38
1.4.2.5 Alternative cardioplegic and preservation strategies	39
1.5 Thesis aims	40
CHAPTER 2. METHODS AND MATERIALS	41
2.1 Materials	42
2.3 Ethical conduct.....	44
2.4 Animals.....	45
2.5 The isolated rat heart perfusion circuit.....	46
2.5.1 Description of the perfusion circuit.....	46
2.5.1.1 The Langendorff circuit	48
2.5.1.2 The heart in ‘working’ mode	50
2.5.1.3 Delivery of cardioplegia.....	52
2.6 The surgical procedure.....	53
2.6.1 Preparation for the ex-vivo perfusion experiments	53
2.6.2 Anaesthesia and premedication	54

2.6.3 Explantation of the heart.....	54
2.6.4 Cannulation of the heart onto the perfusion circuit	55
2.7 Assessment of baseline cardiac function	57
2.8 Cardioplegia and hypothermic storage.....	59
2.9 Assessment of post-storage cardiac function	60
2.10 Quantification of lactate dehydrogenase	60
2.11 Tissue collection	60
2.12 Western blot analyses.....	61
2.13 Histopathology	62
2.13.1 Tissue fixation	62
2.13.2 Dehydration, clearing, infiltration and embedding	62
2.13.3 Sectioning and staining.....	62
2.13.4 Histological analysis	63
2.14 Statistical analyses	63

CHAPTER 3. DEVELOPMENT OF A RODENT MODEL OF BRAIN DEATH AND ASSESSING THE ABILITY TO PHARMACOLOGICALLY CONDITION HEARTS FROM BRAIN DEAD DONORS 64

3.1 Introduction	65
3.2 Methods	69
3.2.1 Developing a rodent model of donor brain death.....	69
3.2.1.1 Criteria used to determine brain death	69
3.2.1.2 Materials.....	69
3.2.1.3 Animals	69
3.2.1.4 Anaesthesia	71
3.2.1.5 Intubation and ventilation.....	71
3.2.1.6 Monitoring pulse oximetry and core body temperature	73
3.2.1.7 Invasive haemodynamic monitoring	73
3.2.1.8 Creating the burr hole and placing the intracranial embolectomy catheter .	75
3.2.1.9 Development of a protocol for induction of brain death.....	79
3.2.1.10 Haemodynamic monitoring.....	82
3.2.2 Development of the sham procedure	83
3.2.3 Assessment of cardiac function.....	83
3.2.4 Assessment of the inflammatory response to brain death and sham procedure.	84
3.2.4.1 Serum inflammatory cytokines measured	85
3.2.4.2 Assay for serum inflammatory cytokines	85

3.2.5 Assessing Cardiac Function After Brain Death and Hypothermic Storage, and Assessing the Ability to Condition Hearts from Brain Dead Donors.....	87
3.2.5.1 Assessment of cardiac function on an ex-vivo perfusion circuit.....	89
3.2.5.2 Cardioplegia and hypothermic storage	89
3.2.5.3 Assessment of post-storage cardiac function	90
3.2.5.4 Measurement of lactate dehydrogenase levels.....	90
3.2.5.5 Western blots and histopathology analyses	90
3.2.5.5.1 Western blots.....	90
3.2.5.5.2 Histological analysis	91
3.2.5.6 Statistical analysis.....	91
3.3 Results	92
3.3.1 Neurological changes observed during brain death.....	92
3.3.2 Haemodynamic changes associated with brain death	93
3.3.3 Inflammatory changes associated with brain death	96
3.3.4 Cardiac function after brain death or sham procedure.....	97
3.3.5 Recovery of cardiac function after hypothermic storage in Celsior®	98
3.3.6 Recovery of hearts treated with pharmacological conditioning	99
3.3.7 Lactate dehydrogenase levels	100
3.3.8 Histopathology.....	101
3.3.9 Western blots	102
3.4 Discussion.....	105

CHAPTER 4. ASSESSING THE ABILITY TO PROTECT AGED HEARTS USING

PHARMACOLOGICAL CONDITIONING STRATEGIES.....	110
4.1 Introduction	111
4.2 Methods	112
4.2.1 Anaesthesia.....	112
4.2.2 The surgical procedure	112
4.2.3 Induction of brain death.....	113
4.2.4 Assessment of cardiac function after brain death.....	114
4.2.5 Cardioplegia and hypothermic storage	114
4.2.6 Assessment of post-storage cardiac function.....	114
4.2.7 Measurement of lactate dehydrogenase levels	115
4.2.8 Western blot and histopathology analyses	115
4.3 Results	116

4.3.1 Haemodynamic changes during brain death.....	116
4.3.2 Cardiac function after brain death or sham procedure.....	116
4.3.3 Recovery of cardiac function after hypothermic storage.....	116
4.3.4 Lactate dehydrogenase release.....	120
4.3.5 Histopathology.....	121
4.3.6 Histopathology.....	122
4.4 Discussion.....	123
 CHAPTER 5. PHARMACOLOGICAL CONDITIONING OF BRAIN DEAD DONOR HEARTS WITH ERYTHROPOIETIN AND GLYCERYL TRINITRATE: INITIAL CLINICAL EXPERIENCE.....	126
5.1 Introduction	127
5.2 Methods	128
5.2.1 Definition of extended criteria (marginal) donor hearts.....	129
5.2.2 Cardioplegia and hypothermic preservation.....	129
5.2.3 Composition of preservation solutions	129
5.2.4 Determining post-transplant outcomes and survival.....	130
5.2.5 Definition of primary graft dysfunction.....	130
5.2.6 Statistical analysis.....	131
5.3 Results	132
5.3.1 Donor, recipient and procedural features.....	132
5.3.2 Post-transplant outcomes	135
5.4 Discussion.....	137
 CHAPTER 6. ASSESSING THE CONDITIONING PROPERTIES OF SILDENAFIL IN DONOR HEART PRESERVATION	140
6.1 Introduction	141
6.2 Methods	144
6.2.1 The experimental protocol	144
6.2.2 Animals	144
6.2.3 Anaesthesia.....	144
6.2.4 Study groups	145
6.2.5 Assessment of baseline cardiac function.....	145
6.2.6 Cardioplegia and hypothermic storage	146
6.2.7 Assessment of cardiac function after hypothermic storage.....	146
6.2.8 Analysis of western blots and lactate dehydrogenase levels.....	147

6.2.9 Statistical analysis	147
6.3 Results	147
6.3.1 Baseline cardiac function	148
6.3.2 Recovery of cardiac function after hypothermic storage	148
6.3.2.1 Recovery of hearts after 6 hours of hypothermic storage	148
6.3.2.2 Recovery of hearts after 10 hours of hypothermic storage	150
6.3.2.3 Lactate dehydrogenase levels	151
6.3.2.4 Western blots	152
6.4 Discussion	153
 CHAPTER 7. DEVELOPMENT OF A RODENT CARDIAC MAGNETIC RESONANCE IMAGING	
MODEL	156
7.1 Background	157
7.1.1 Assessments of cardiovascular physiology and disease in rodent models	157
7.1.2 Non-invasive functional imaging	157
7.1.2.1 Echocardiography	158
7.1.2.2 Nuclear imaging	158
7.1.2.3 Computed tomographic imaging	159
7.1.2.4 Cardiac magnetic resonance imaging	159
7.2 Study objectives	160
7.3 Methods	161
7.3.1 Animals	161
7.3.2 Image acquisition	163
7.3.2.1 Image sequences	163
7.3.2.2 Image analysis	163
7.3.3 Statistical Analysis	165
7.3 Results	166
7.3.1 Quantification of left ventricular volumes, mass and function	167
7.3.2 Reproducibility	167
7.3.3 Sample size reduction	171
7.4 Discussion	172
 CHAPTER 8. DEVELOPMENT OF A RODENT ABDOMINAL HETEROTOPIC WORKING HEART	
TRANSPLANT MODEL FOR USE IN DONOR HEART PRESERVATION STUDIES	176
8.1 Introduction	177

8.2 Methods	179
8.2.1 Animals	179
8.2.2 Anaesthesia	179
8.2.3 The donor procedure.....	180
8.2.3.1 Surgical equipment.....	180
8.2.3.2 The surgical procedure	181
8.2.3 The recipient procedure	185
8.2.3.1 Surgical equipment.....	185
8.2.3.2 The surgical procedure.....	186
8.2.4 The transplant procedure	193
8.2.5 Wound closure.....	197
8.2.6 Post-operative analgesia	197
8.2.7 Recovery of the animal	198
8.2.8 Post-operative care and monitoring.....	198
8.2.9 Monitoring the grafted heart	199
8.3 Discussion.....	199
 CHAPTER 9. FINAL DISCUSSION AND CONCLUSIONS	201
9.1 Development of models.....	202
9.1.1 The rodent model of brain death	203
9.1.2 The rodent heterotopic ‘working’ heart transplant model	204
9.1.3 Cardiac magnetic resonance imaging of rat hearts	205
9.2 Myocardial preservation studies.....	206
9.2.1 Myocardial preservation in the context of brain death	206
9.2.2 Preservation of older or ‘marginal’ hearts	208
9.2.3 Sildenafil as a conditioning agent	209
9.2.4 Conditioning human donor hearts with EPO and GTN	210
9.3 Conclusions and future directions	211
 REFERENCES	213

LIST OF TABLES

Table 1.1 The composition of the main cardioplegic/preservation solutions.....	22
Table 2.1 Anaesthesia and pre-medications	42
Table 2.2 Perfusion buffer (modified Krebs-Henseleit solution) components	42
Table 2.3 Composition of modified Krebs-Henseleit solution with $[Ca^{2+}]$ 1.4mM	43
Table 2.4 Composition of Celsior® preservation solution	43
Table 2.5 Pharmacological supplements.....	44
Table 3.1 Equipment for the brain death model.....	70
Table 3.2 Steps involved in developing the rodent model of brain death.....	77
Table 3.3 The concentrations of erythropoietin, glyceryl trinitrate and zoniporide used in pharmacological conditioning studies	89
Table 3.4 Histopathology scoring system.....	91
Table 3.5 Mean weights of animals.....	92
Table 3.6 Mean arterial pressure in brain death and sham groups	95
Table 3.7 Heart rate in brain death and sham groups	95
Table 5.1 The composition of Modified STS and Celsior	130
Table 5.2 Donor, procedural and recipient risk factors for primary graft dysfunction.....	133
Table 5.3 Use of ECMO or IABP post-transplantation.....	135
Table 5.4 Length of stay: all hearts.....	136
Table 6.1 Study groups	145
Table 6.2 Concentrations of sildenafil, erythropoietin and glyceryl trinitrate.....	146
Table 6.3 Mean weights of animals.....	147
Table 6.4 Baseline cardiac function.....	148
Table 7.1 Cardiac volumes, ejection fraction and myocardial mass (Scan 1)	167
Table 7.2 Coefficients of variation.....	170
Table 7.3 Sample size reduction: 1.5T CMR compared with echocardiography.....	171

LIST OF FIGURES

Figure 1.1 Survival by era post-cardiac transplantation.....	5
Figure 1.2 Risk of increased mortality with increasing ischaemic time and donor age	7
Figure 1.3 The additive effects of increasing donor age and ischaemic time on survival.....	8
Figure 1.4 Cellular processes during non-ischaemic and ischaemic conditions.....	13
Figure 1.5 The formation of oxygen free radicals and tissue injury	15
Figure 1.6 Activation of signaling pathways by ischaemic pre- and post-conditioning	28
Figure 1.7 Mechanisms of cardioprotection by IPC and IPost.....	29
Figure 2.1 A schematic overview of the perfusion circuit.....	47
Figure 2.2 Cannulation of the rat heart at the aorta and left atrium.....	48
Figure 2.3 The perfusion circuit in Langendorff mode	49
Figure 2.4 The perfusion circuit in working mode.....	51
Figure 2.5 Delivery of cardioplegia	52
Figure 2.6 Surgical instruments	53
Figure 2.7 Cannulation of the heart onto the perfusion circuit	56
Figure 2.8 Experimental protocol	57
Figure 2.9 Representative image of aortic flow and heart rate in working mode	58
Figure 2.10 Hypothermic storage of the donor heart	59
Figure 3.1 Surgical instruments	70
Figure 3.2 Intubation of the rat.	72
Figure 3.3 Monitoring pulse oximetry and core body temperature	73
Figure 3.4 Placement of a pressure catheter in the carotid artery	74
Figure 3.5 Creating a burr hole and placement of the intracranial balloon catheter	76
Figure 3.6 Examples of haemodynamic changes observed in the trial protocols for induction of brain death.	80
Figure 3.7 Study groups.....	87
Figure 3.8 The experimental protocol	88
Figure 3.9 Mean arterial pressure in brain death and sham groups.....	94
Figure 3.10 Heart rate in brain death and sham groups	94
Figure 3.11 Inflammatory cytokines at baseline, post BD and sham procedures.....	96
Figure 3.12 Cardiac function after sham or brain death	97

Figure 3.13 Recovery of cardiac function after hypothermic storage in Celsior®	98
Figure 3.14 The recovery of cardiac function in hearts treated with pharmacological conditioning compared with untreated hearts	99
Figure 3.15 Lactate dehydrogenase release.....	100
Figure 3.16 Histopathology scores for the severity of ischaemic injury	101
Figure 3.17 Western blots for hearts stored for 1 hour	102
Figure 3.18 Western blots for hearts stored for 3 hours	103
Figure 3.19 Western blots for hearts stored for 6 hours	104
Figure 4.1 Study groups.....	112
Figure 4.2 The experimental timeline	113
Figure 4.3 Haemodynamic changes observed during brain death or sham procedure	117
Figure 4.4 Cardiac function after brain death or sham procedure	118
Figure 4.5 The recovery of cardiac function after 6 hours of hypothermic storage	119
Figure 4.6 Lactate dehydrogenase levels	120
Figure 4.7 Histopathology of 18-month rat hearts	121
Figure 4.8 Western blots of pro-survival kinases in 18-month hearts.	122
Figure 5.1 Survival (all hearts) at 370 days post-transplant.....	136
Figure 6.1 The experimental protocol.....	144
Figure 6.2 Recovery of hearts after 6 hours hypothermic storage	149
Figure 6.3 Recovery of hearts after 10 hours hypothermic storage	150
Figure 6.4 Lactate dehydrogenase levels in hearts stored for 6 hours	151
Figure 6.5 Lactate dehydrogenase levels in hearts stored for 10 hours	151
Figure 6.6 Western blots in hearts stored for 6 hours.....	152
Figure 6.7 Recovery of hearts after 10h storage.....	154
Figure 7.1 The position of the rat within the clinical wrist coil and the 1.5T MR scanner....	162
Figure 7.2 Close-up view of rat within wrist coil with monitoring equipment.....	162
Figure 7.3 Acquisition of three-lead electrocardiograph signals.	164
Figure 7.4 Electrocardiograph and respiratory monitoring during image acquisition.	164
Figure 7.5 Representative images of rat CMR using a 1.5T scanner.	166
Figure 7.6 Intra-observer correlations.	168
Figure 7.7 Inter-observer correlations.	169
Figure 8.1 Rodent models of abdominal heterotopic heart transplantation.....	178
Figure 8.2 Equipment for donor surgery	180
Figure 8.3 Donor animal preparation.....	181

Figure 8.4 Laparotomy and thoracotomy.....	182
Figure 8.5 Donor heart preparation for cardioplegia and explantation	183
Figure 8.6 Cardioplegia of the donor heart.....	184
Figure 8.7 Further dissection of the heart-lung bloc.....	185
Figure 8.8 Equipment for recipient surgery	187
Figure 8.9 Preparation and abdominal dissection of the recipient animal.....	188
Figure 8.10 Preparation of the recipient's abdominal surgical field	189
Figure 8.11 Isolation and cautery of the iliolumbar vessels.....	190
Figure 8.12 Cautery of spinal arteries and veins	191
Figure 8.13 Placing a partial ligature around the recipient inferior vena cava	191
Figure 8.14 Clamping the aorta and inferior vena cava	192
Figure 8.15 Performing an incision in the recipient abdominal aorta.....	193
Figure 8.16 Anastomosis of the donor aorta to the recipient aorta	194
Figure 8.17 Anastomosis of the donor SVC to the recipient IVC.....	195
Figure 8.18 Completion of SVC-IVC anastomosis	195
Figure 8.19 The transplanted heart after reperfusion.	196

LIST OF ABBREVIATIONS

μM	micromolar
ACE	angiotensin-converting enzyme
AF	aortic flow
AIF	apoptosis inducing factor
Akt	protein kinase B
AMPK-α	adenosine monophosphate-activated protein kinase alpha
ANOVA	analysis of variance
ATP	adenosine triphosphate
BD	brain death
BSA	bovine serum albumin
bSSFP	balanced steady-state free precession
°C	degrees Celsius
Ca ²⁺	Calcium ion
CABG	coronary artery bypass graft
CF	coronary flow
cGMP	cyclic guanosine monophosphate
CK-MB	creatinine kinase MB isoform
CMR	cardiac magnetic resonance
CO	cardiac output
CO ₂	carbon dioxide
CoA	coenzyme-A
CXCL-1	chemokine (C-X-C motif) ligand-1
DCD	donation after circulatory death
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
ECG	electrocardiograph
ECMO	extra-corporeal membrane oxygenation
EDV	end-diastolic volume
EF	ejection fraction
ESV	end-systolic volume

eNOS	endothelial nitric oxide synthase
EPO	erythropoietin
Erk	extracellular signal regulated kinase
FOV	field of view
GIK	glucose-insulin-potassium
GMP	guanosine monophosphate
GPCR	g-protein coupled receptor
GRO/KC	synonyms for CXCL-1 [chemokine (C-X-C motif) ligand-1]
GSH	glutathione-SH
GSK-3 β	glycogen+C109 synthase kinase 3 beta
GTN	glyceryl trinitrate
H ⁺	hydrogen ion
H ₂ O ₂	hydrogen peroxide
HLA	human leucocyte antigen
HO•	hydroxyl radical
HR	heart rate
HTK	histidine-tryptophan-ketoglutarate
IABP	intra-aortic balloon pump
IgG	Immunoglobulin G
IL	interleukin
IPC	ischaemic preconditioning
IPost	ischaemic postconditioning
IRI	ischaemia-reperfusion injury
IVC	inferior vena cava
IU	international units
K ⁺	potassium ion
L	litre
LA	left atrium
LAD	left anterior descending artery
LDH	lactate dehydrogenase
LVEF	left ventricular ejection fraction
MAP	mean arterial pressure
MAPK	mitogen activated protein kinase
MCS	mechanical circulatory support

MgSO ₄	magnesium sulphate
MCP	monocyte chemoattractant protein
MEK	mitogen-activated protein kinase
MIP	macrophage inflammatory protein
mKATP	mitochondrial potassium ATP
mm	millimeter
MM	myocardial mass
mOsm	milliosmolar
MPA	main pulmonary artery
mPTP	mitochondrial permeability transition pore
mRNA	messenger ribonucleic acid
mTOR	mechanistic target of rapamycin
MUW	modified University of Wisconsin
Na ⁺	sodium ion
NaHCO ₃	sodium bicarbonate
NHE	sodium-hydrogen exchange
nM	nanometer
NO	nitric oxide
NOS	nitric oxide synthase
NRG-1	neuregulin-1
O ₂	oxygen
O ₂ ⁻	superoxide anion
ONOO ⁻	peroxynitrite
PCI	percutaneous coronary intervention
PGD	primary graft dysfunction
PDE-5	phosphodiesterase type 5
PFA	paraformaldehyde
PI3-k	phosphoinositide-dependent kinase 1
PKC	protein kinase C
PKC-ε	protein kinase C epsilon
PKG	protein kinase G
PVDF	polyvinylidene fluoride
RISK	reperfusion injury salvage kinase
ROS	reactive oxygen species

SAFE	survivor activating factor enhancement
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SOD	superoxide dismutase
STAT3	signal transducer and activator of transcription 3
STS	St Thomas' Solution
STS 2	St Thomas' Solution no. 2
SVC	superior vena cava
T3	triiodothyronine
T4	thyroxine
TE	echo time
TLR	toll-like receptor
TNF- α	tumour necrosis factor alpha
TR	repetition time
UW	University of Wisconsin
VLA	ventricular long axis
ZON	zoniporide

CHAPTER 1. INTRODUCTION

1.1 Cardiac transplantation in the 21st Century

Cardiac transplantation was made possible by pioneering experimental work by Drs Alexis Carrell, Frank Mann, Norman Shumway and Richard Lower^{1,2}, and the first human to human heart transplant was subsequently performed by Dr. Christiaan Barnard in 1967³. Less than 50 years later, significant advances in donor and recipient selection, immunosuppression and the control of infection have made cardiac transplantation the gold standard treatment for end-stage heart failure. Significant improvement in post-transplant survival, in particular over the last two decades, has seen survival exceed 85% at one-year, 70% at five-years and median survival exceeding 12 years⁴.

1.1.1 Changing recipient and donor profiles

The profile of cardiac transplantation has also been changing in this rapidly evolving field. Significant progress in recipient management has led to the acceptance of 'higher risk' candidates for transplantation, such as patients of older age, more comorbidities and those with prior cardiac surgery^{4,5}. Between the eras of 1982-1985 and 2006-2012, the proportion of recipients aged 60-69 years increased from 14% to 24% and recipients over 70 years has increased from 0.2% to 1.3%⁶.

The increasing use of mechanical circulatory support (MCS) has also introduced higher risk transplant candidates, due to an increased incidence of pre-sensitisation and post-transplant morbidity^{4,7-9}. Other important changes in recipient profiles over the last two decades include increasing body mass index, higher numbers of recipients with prior dialysis, increasing proportion of female recipients, increasing numbers of patients with congenital heart disease, a higher prevalence of diabetes and hypertension in recipients, and inclusion of those with prior malignancies⁴.

1.1.2 Use of marginal donor organs

The donor profile has also changed significantly over recent decades. The widening gap between the number of waiting list candidates and the number of donors has led to liberalisation of donor acceptance criteria, and there has been a notable rise in the number of extended criteria or 'marginal' donor hearts used in transplantation¹⁰⁻¹³.

In Australia, extended criteria heart donation is defined by the presence of one or more of the following^a:

- i) Donor age 50-60 years,
- ii) Anticipated ischaemic time > 360 minutes
- iii) Donor requiring high-dose inotropes
 - Noradrenaline > 0.2 µg/kg/min or equivalent
- iv) Donor graft dysfunction on echocardiogram
 - Left ventricular ejection fraction (LVEF) < 50% or major wall motion abnormalities
- v) Donor comorbidities including hepatitis B, C or high-risk behaviour.

Increasing reliance on extended criteria donors and an older donor pool have resulted in a significant rise in the median donor age over recent years, from 24 years in 1982-1985 to 31 years in 2006-2012⁴, with the proportion of transplants from donors aged > 50 years of age rising from 0% to over 12% during the same time period⁶. The increase in donor age has been particularly prominent in Europe and Australia¹⁴. Transplants performed using extended criteria hearts demonstrate inferior 30-day, 1- and 2-year survival compared to those with standard criteria hearts¹².

Although not a widely applied practice, some institutions have allocated extended criteria organs for transplantation of 'alternate listing' recipients such as those with systemic illnesses who would otherwise be deemed ineligible for transplantation¹⁵⁻¹⁷. This practice extends survival in severely ill patients and expands the number of transplants performed, however the high-risk nature of these transplants is associated with increased rates of primary graft dysfunction (PGD) and lower survival at 30-days, 1- and 4-years post transplantation^{16,17}.

^a The definition for extended criteria donation was obtained from the Transplantation Society of Australia and New Zealand's Consensus Statement on Eligibility Criteria and Allocation Protocols for Organ Transplantation from Deceased Donors, Version 1.4, 15 April 2015.

1.1.3 Use of mechanical circulatory support

As discussed above, a significant change in cardiac transplantation is the increasing use of mechanical circulatory support (MCS)¹⁸⁻²⁰. In the era 1992-2000, 18.8% of recipients were on MCS, which grew to 35.1% in 2006-2013⁴. Although allowing survival of severely ill transplant candidates, MCS introduces risks of cerebrovascular accidents, bleeding, infection, thrombosis, pre-sensitisation and device failure as well as increased risk of post-transplant complications such as graft dysfunction, rejection and poorer survival compared with non-MCS patients^{4,19}. Registry data reveal survival of 80% at 1 year and 70% at 5 years post transplantation, a significant improvement compared to expected survival without MCS in these patients with decompensated advance heart failure⁴. Transplant centres are now attempting temporary use of MCS, aiming to unload the left ventricular and allow reverse remodelling of the myocardium, or a strategy of 'bridge to recovery', however these practices involve small numbers of patients at present^{21,22}.

1.1.4 Advances in procedural aspects

The last two decades have seen evolving progress in surgical techniques. The bicaval anastomotic technique, developed in 1991²³, is increasingly used, as it minimises disruption of atrial geometry, reduces tricuspid and mitral regurgitation, and reduces the incidence of sinus node dysfunction²⁴. Heterotopic transplants, associated with increased morbidity, have been gradually reducing over the years, as female donor to male recipient transplants also associated with higher risk of PGD. Other more challenging procedures have been increasing such as transplanting recipients with congenital heart disease, patients with prior sternotomies and combined transplants such as heart-kidney and heart-liver transplants⁴. Procedural times have also been increasing over the years⁴, which may be attributed to more complicated surgical procedures. Increasing numbers of non-regional transplants have increased the mean ischaemic time from 3.0 hours in 1992-2002, to 3.4 hours in 2006-2013⁴. Ischaemic times over 4 hours are seen in 40% of transplants in Europe and approximately 50% of transplants in Australia over the last decade, with ischaemic times over 6 hours seen in almost 5% of transplants in Europe and Australia^b.

^b Data obtained from the EuroTransplant and Australia and New Zealand Cardiothoracic Organ Transplant (ANZCOTR) annual reports.

1.2 Current challenges in cardiac transplantation

The expansion of donor and recipient acceptance criteria and increasing use of MCS reflects the growing experience and confidence of cardiac transplantation surgeons and physicians. It also places emphasis on the need to ensure graft and patient survival are optimised in this higher-risk setting. Of note is that while overall survival has improved over the eras, mortality within the first months after transplantation remains high, as demonstrated in the Kaplan-Meier survival graphs of registry data from the International Society for Heart and Lung Transplantation⁶, shown in Figure 1.1. The need to improve graft preservation in this critical early period is evident in this data.

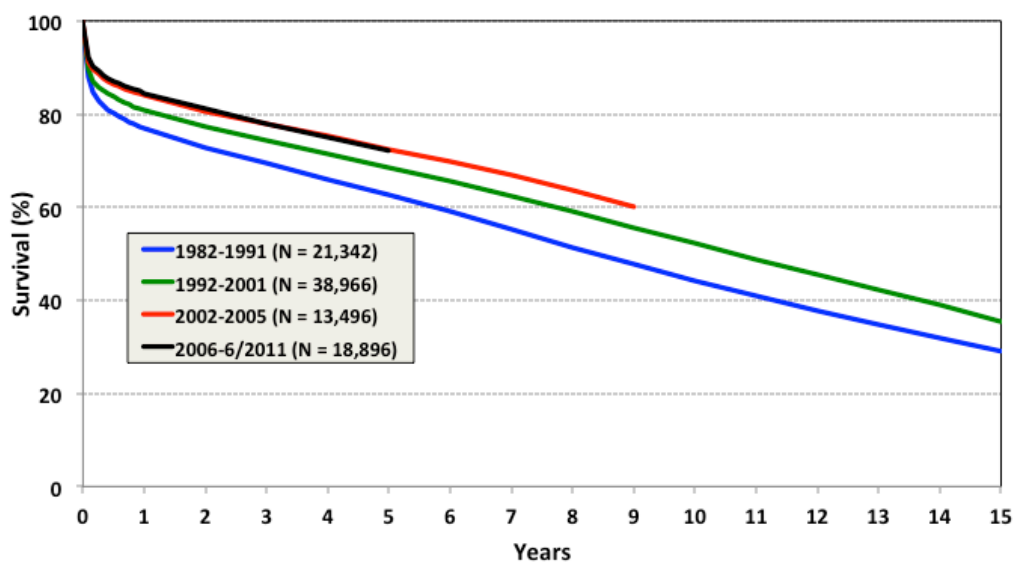


Figure 1.1 Survival by era post-cardiac transplantation

Lund et al. J Heart Lung Transplant. 2013 Oct;32(10):951-964

1.2.1 Primary graft dysfunction

Primary graft dysfunction (PGD) is the leading cause of early morbidity and mortality after cardiac transplantation²⁵⁻²⁷. Registry data reveal that 39% of deaths within the first 30 days are due to PGD, with an additional 18% attributed to secondary effects such as multi-organ failure^{4,25}. Primary graft dysfunction is defined as the occurrence of either left, right or biventricular dysfunction within the first 24 hours after transplant, without any discernible secondary causes such as hyperacute rejection, recipient pulmonary hypertension or known surgical complications²⁵.

The degree of graft dysfunction can range from mild to severe, classified using echocardiography, invasive right heart catheter haemodynamic parameters and the degree of circulatory support required in the recipient²⁵. Several risk factors for PGD have been identified in the donor, transplant procedure and the recipient. The main donor associated risks include increasing age, donor brain death in particular of 'explosive onset', cardiac dysfunction, high dose inotropic support, comorbidities such as diabetes and hypertension, left ventricular hypertrophy and sepsis^{25,28-31}. Procedural risk factors include prolonged ischaemic times, donor-recipient sex mismatch (in particular female donor to male recipient), donor to recipient weight mismatch < 0.8, multi-organ donation, increased blood transfusion requirement and emergency transplants. The main recipient factors include increasing age, multiple reoperations, renal or liver dysfunction, combined organ transplantation, infection, retransplant and the presence of mechanical circulatory support^{25,28-32}.

Literature on the pathology of PGD is limited, however a multi-centre survey of autopsy results revealed ischaemia-reperfusion injury (IRI) as the main histopathological finding in 48% of mortalities attributed to PGD²⁵. Other findings such as myocyte necrosis (28%), multifocal oedema and/or haemorrhage (14%) and freeze injury (7%) may also be attributed to IRI incurred during prolonged hypothermic storage and reperfusion-related injury.

The additive effects of increasing donor age and prolonged ischaemic times

Particular emphasis has been placed on the additive effects of prolonged ischaemic times and increasing donor age on the incidence of graft dysfunction and early mortality^{25,28,29,33,34}. The 2013 registry report from the International Society for Heart and Lung Transplantation (ISHLT) demonstrates the individual effects of ischaemic time and donor age on one-year mortality (Figure 1.2).

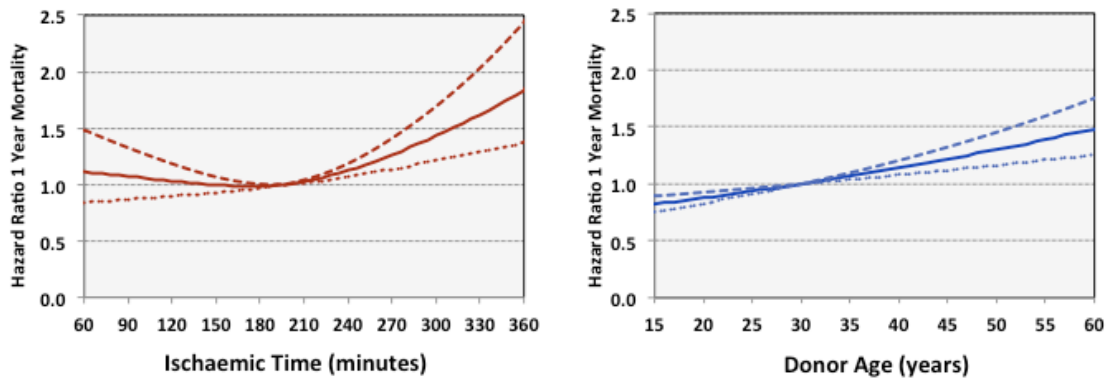


Figure 1.2 Risk of increased mortality with increasing ischaemic time and donor age
 Graphs adapted from Lund et al. J Heart and Lung Transplant 2014; 33(10):996-1008.

An analysis by Russo et al in 2007³⁵ demonstrated that long-term survival is significantly affected by exposing older donor hearts to prolonged ischaemic times. Median survival was almost halved when donor age exceeded 34 years and ischaemic time was greater than 6 hours (Figure 1.3), which highlights the vulnerability of older donor hearts to ischaemic injury and the need for improved preservation of hearts in an era of increased use of extended criteria organs.

Transplantation of higher risk recipients and the use of marginal donor hearts may explain the lack of significant improvements in early survival rates. However, maintenance of current recipient survival rates despite higher risk transplantation is credited to advances in acute peri-operative care of the recipient in intensive care units, early implementation of extra-corporeal membrane oxygenation and treatment of infections and secondary complications of graft failure³⁶.

Graft function and recipient survival may be improved by implementing myocardial preservation strategies that minimise IRI incurred by the donor heart and thereby reduce PGD and early mortality. Primary graft dysfunction remains a challenge, and it needs to be minimised in the contemporary era of higher-risk cardiac transplantation.

Average Post-Transplant Survival in years

Donor Age Ischaemic Time	0-19 yrs (n = 10,814)	20-33 yrs (n = 11,410)	> 34 yrs (n = 11,416)
< 3.5 hrs	11.4	10.6	9.1
3.5 – 6 hrs	11.4	9.9	8.5
> 6 hrs	11.4	7.3	6.3

Figure 1.3 The additive effects of increasing donor age and ischaemic time on survival

Adapted from Russo et al. J Thoracic Cardiovascular Surgery 2007; 133:554-559³⁵

1.2.2 Allograft vasculopathy

Allograft vasculopathy is the most common cause of late graft failure, and together with malignancy, accounts for most deaths beyond the first year⁴. Allograft vasculopathy is characterised by diffuse intimal hyperplasia that affects the epicardial vessels and microcirculation, with luminal stenosis occurring both concentrically and longitudinally³⁷⁻⁴⁰. Approximately 30% to 50% of patients have allograft vasculopathy at 5 years post transplantation^{4,37}.

Causes of allograft vasculopathy have been identified as arising from ischaemia-reperfusion injury (IRI), immune-mediated injury to the endothelium and cytomegalovirus infection. An increased incidence is associated in hearts from older donors and female to male mismatches^{37,41-44}. As with cardiomyocytes, the endothelium is susceptible to injury during donor brain death, haemodynamic fluctuations during donor resuscitation, warm and cold ischaemia during organ procurement and warm blood reperfusion injury after transplantation^{37,42,43}. It has demonstrated that the glycocalyx, which coats the endothelium and is integral to multiple endothelial functions, undergoes severe destruction by IRI⁴⁵. This is an important contributor to both primary graft dysfunction and allograft vasculopathy.

Immune mediated injury contributes to IRI with possible causes identified as human-leucocyte antigen (HLA) mismatching between donor and recipient, the risk increased with preformed antibodies directed against the donor heart (sensitised patients) and cellular rejection^{8,37}. Protocols for detection include regular surveillance coronary angiography and intravascular ultrasound. Treatment includes the use of statin therapy, control of cardiovascular risk factors and substitution of mycophenolate or azathioprine immunosuppression with mTOR inhibitors specifically aimed at reducing further intimal proliferation⁴⁴. Complications of allograft vasculopathy include fatal arrhythmias and late graft failure. It is one of the most common indications for retransplantation, although subsequent survival remains poor^{46,47}. Importantly, allograft vasculopathy and late graft dysfunction may potentially be minimised with improvements to donor heart preservation during organ procurement and transplantation.

1.3 Ischaemia-reperfusion injury

As discussed, ischaemia-reperfusion injury (IRI) is a significant contributor to primary graft dysfunction, and is also recognised as a contributor to allograft vasculopathy and potentially late graft dysfunction^{25,44}. Ischaemia-reperfusion injury will therefore be described initially, with a discussion of mechanisms of myocardial protection against IRI to follow.

The concept of ischaemia, indicating deficient blood supply to tissues, was first described by Virchow in 1858^c. In the 1960s, Jennings discovered the concept of 'reperfusion injury', where restoration of blood to ischaemic tissue could paradoxically exacerbate cellular injury and necrosis⁴⁸. The extent of ischaemic injury and vulnerability to reperfusion injury was dependent on the magnitude and duration of ischaemia⁴⁹. Short periods, such as 15-20 minutes of ischaemia was considered reversible, however a prolonged insult caused irreversible myocyte injury. Both ischaemic and reperfusion injury therefore contributes to overall infarct size. The term 'lethal reperfusion injury' has been used to define death of cardiomyocytes that were potentially viable at the termination of an ischaemic insult⁵⁰⁻⁵². Ischaemia-reperfusion injury has been studied in detail in the context of acute coronary syndromes and in patients undergoing cardiac surgery^{51,53}. After prolonged ischaemia, reperfusion of myocardial territory supplied by a coronary artery by either thrombolysis or deflation of the angioplasty balloon is associated with decreased cardiac contractility due to

^c Virchow R. Die Cellularpathologie in ihrer Begründung auf physiologische und pathologische Gewebelehre. Berlin: Hirschwald. 1858:122

cellular death^{51,52}. Similarly, the release of the aortic cross-clamp during coronary artery bypass grafting can be associated with myocardial injury, cell necrosis and reduced contractility⁵⁴.

1.3.1 Ischaemia-reperfusion injury of the donor heart

In the context of cardiac transplantation, ischaemia-reperfusion injury of the donor heart has added complexity compared to an acute coronary event. Ischaemia-reperfusion of the donor heart includes i) donor brain death, ii) global ischaemia without blood supply from collateral vessels, iii) global ischaemia accompanied by hypothermia during organ storage, iv) warm ischaemia during grafting of the donor heart, v) physiological changes in the recipient during cardiopulmonary bypass and vi) reperfusion injury⁵⁵. The risk of IRI increases with distant procurement of donor organs and the use of marginal donor organs.

1.3.1.1 Donor brain death

The effects of donor brain death on peripheral organs are discussed in detail in Chapter 3, and are discussed in brief below. Donor brain death is associated with significant haemodynamic fluctuations, a pro-inflammatory state and hormonal dysregulation, and is the first point of exposure of donor organs to ischaemic injury⁵⁶⁻⁵⁸. A catecholamine 'storm' is created during brain death resulting in severe hypertension, tachycardia and systemic vasoconstriction^{59,60}. Increased myocardial workload is enforced due to inotropic effects and increased vascular resistance, while cellular hypoxia occurs due to catecholamine-induced coronary vasoconstriction^{56,60-64}. Brain death has been associated with increased myocardial interstitial lactate and adenosine in experimental animal studies⁶⁵ and significant cardiac contractile impairment⁶⁶. Hearts from brain dead donors have demonstrated histological evidence of ischaemic injury such as contraction bands, cellular and mitochondrial oedema, subendocardial petechial haemorrhages and coagulative necrosis⁶⁷.

Brain death also induces a significant systemic inflammatory reaction and causes upregulation of proinflammatory mediators and cell surface molecules in peripheral organs^{68,69}. Organs from brain dead donors demonstrate infiltration of peripheral organs with mononuclear cells that contribute to coagulative necrosis⁷⁰. Studies by Birks et al^{71,72} compared hearts declined for transplantation due to poor left ventricular ejection fraction

(LVEF) with accepted hearts demonstrating normal LVEF, and found increased circulating IL-6 and TNF- α , increased cardiac mRNA for pro-inflammatory mediators TNF- α and IL-6, and increased apoptotic markers cleaved caspase-9, pro-caspase 3 and caspase-3 substrate poly (ADP-ribose) polymerase. This study highlights the correlation between the severity of the physiological response to brain death and its effects on end-organ dysfunction. Furthermore, hormonal derangements are seen in brain death, with depletion of thyroid stimulating hormone⁷³⁻⁷⁵, vasopressin and corticotrophin releasing hormone^{58,76} adding to the unstable pathophysiological milieu of the brain dead organ donor.

Injurious effects induced by brain death at times manifest in the donor heart as temporary mechanical dysfunction, or myocardial 'stunning'^{63,64,77}. It is postulated that this may be a manifestation of reversible myocardial injury as myocardial contractility is regained within hours or days. However, it is also possible that cellular injury may remain although not apparent at a macroscopic level, that may lead to primary graft dysfunction with combined insults of warm and cold ischaemia and reperfusion. Additive effects of ischaemic injury during organ procurement and reperfusion injury are discussed below in sections 1.4.2 and 1.4.3 respectively.

1.3.2 The pathophysiology of ischaemic injury

1.3.2.1 Metabolic alterations

Ischaemia leads to several metabolic alterations that can potentially cause cellular injury. Under normoxic conditions, cardiomyocytes produce adenosine triphosphate (ATP) mainly through fatty acid oxidation (60-90%) during aerobic metabolism^{78,79}. Sixty six percent of ATP produced is used in contractile function of the heart and approximately 34% is used for basal metabolic activity including maintenance of intra-cellular ionic gradients, pumps and cellular biosynthetic function⁷⁹. Hypoxic conditions are induced during donor heart procurement when blood flow and oxygen supply to the heart ceases during aortic clamping. Metabolism therefore shifts to the anaerobic route, where ATP is less efficiently produced via glycolysis. The glycolytic pathway converts glycogen or glucose to pyruvate, which is subsequently converted to lactate, creating an acidic intracellular environment. Fatty acids are unused in anaerobic metabolism, therefore accumulate in the cytosol⁸⁰ and with their acyl metabolites (fatty-acyl-CoA and fatty acylcarnitine) insert into the inner membrane of the lipid bilayer,

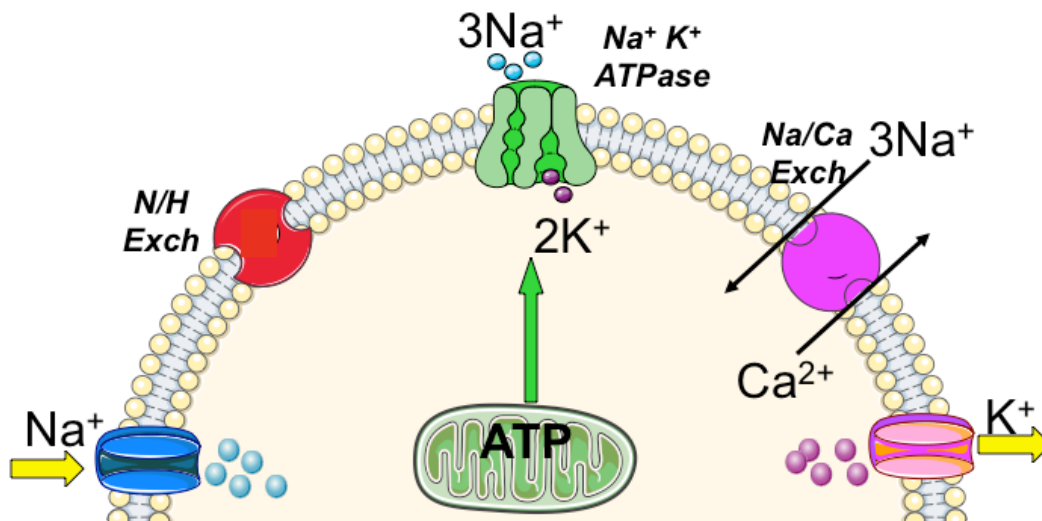
creating alteration of physical properties with potential cellular injury⁸¹. Breakdown products of ATP such as hypoxanthine and xanthine accumulate intracellularly, and may be potential substrates for free radical and oxidant production at reperfusion⁸². Prolonged ischaemia with sustained intracellular acidosis may eventually inhibit glycolysis and residual ATP formation, causing paralysis of ATP-dependent pumps and irreversible cell injury.

1.3.2.2 Disruption of ionic homeostasis

During explantation of the donor heart, the heart is arrested with hyperkalaemic and hypothermic (2°C to 6°C) cardioplegia^{55,83,84}. Hypothermia arrests cardiac contractility, reduces oxygen consumption and metabolic rate, however does not cause complete arrest of metabolic activity⁸⁵. Anaerobic metabolism continues, albeit at a slower rate, with accumulation of lactate and H⁺ ions, which as discussed previously, reduces intracellular pH⁸⁶. The acidic intracellular environment triggers activity of the Na⁺/H⁺ exchanger, which acts as a buffer by exchanging H⁺ for Na⁺ ions (Figure 1.4), creating a large influx of Na⁺ into the cell⁸⁷⁻⁸⁹. In addition, reduced ATP production markedly impairs activity of the Na,K-ATPase, causing further accumulation of intracellular Na⁺⁹⁰. Hyperkalaemic cardioplegia induces rapid depolarisation of the cardiomyocytes membrane, which facilitates influx of negative Cl⁻ ions and efflux of positive K⁺ ions. The Na,K-ATPase is also the driving force for extrusion of Ca²⁺ by the Na⁺/Ca²⁺ exchanger⁹¹. Raised intracellular Na⁺ due to the Na⁺/H⁺ exchanger and reduced Na,K-ATPase function, drives the Na⁺/Ca²⁺ exchanger in 'reverse mode', causing accumulation of intracellular Ca²⁺⁹². The large influx of solutes into the cell induces the diffusion of water into cells, causing cellular oedema^{93,94} (Figure 1.4B).

Thus, ischaemia, hypothermia and hyperkalaemia-induced alterations in metabolism and ion channels cause deleterious effects such as i) intracellular acidosis, ii) intracellular sodium accumulation, iii) abnormal calcium homeostasis and iv) cellular swelling. Furthermore, intracellular Ca²⁺ overload leads to cell contracture, sarcolemmal bleb formation and rupture⁹⁵. Altered electrochemical gradients cause cell-to-cell interactions, and cellular injury is propagated in a 'wave-front' mechanism leading to progressive myocardial necrosis⁴⁹. Disruption of metabolic, ionic and osmotic homeostasis renders the cell more vulnerable to further injury during reperfusion⁵¹.

A: Non-Ischaemic Conditions



B: Ischaemic Conditions

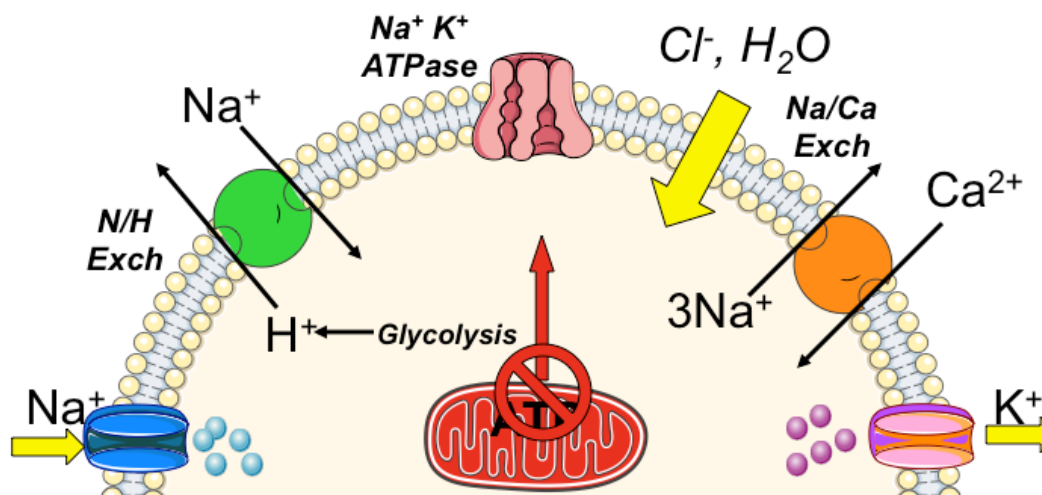


Figure 1.4 Cellular processes during non-ischaemic and ischaemic conditions

During normoxic conditions (A), efficient ATP production ensues mainly through fatty acid metabolism. Activity of ion channels and ATP-dependent pumps maintains ionic homeostasis. During ischaemia (B), ATP production occurs less efficiently through anaerobic metabolism, leading to intracellular acidosis, paralysis of ATP-dependent pumps, and disruption of ionic homeostasis by activation of the N^+/H^+ exchanger, reverse activity of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and diffusion of Cl^- and water down electrochemical and osmotic gradients.

1.3.3 Reperfusion injury

Reperfusion of ischaemic tissue provides a source of oxygen and nutrients, and removes waste products from the extra-cellular space. Jennings et al⁴⁸ discovered that reperfusion of ischaemic tissue with oxygenated blood can paradoxically exacerbate cellular injury and necrosis. It has been postulated that reperfusion injury can contribute up to 50% of myocardial infarct size in studies on acute coronary events⁵³. Reperfusion results in a sudden oxidative load and a rapid correction of pH in a damaged cell with impaired calcium handling⁹⁶.

Four principal phenomena have been described in myocardial reperfusion injury: (i) myocardial 'stunning' or mechanical dysfunction that persists temporarily despite restoration of blood flow^{97,98}; (ii) the 'no reflow' phenomenon, or the impedance of microvascular blood flow due to microvasculature or endothelial injury^{99,100}; (iii) arrhythmias¹⁰¹ and (iv) irreversible cell damage and necrosis, or 'lethal reperfusion injury'^{102,103}. Myocardial stunning refers to hypokinesis or akinesis of either a region of the myocardium or a global myocardial effect, with temporary cardiac contractile dysfunction which improves over days, weeks or months¹⁰⁴. The 'no reflow' phenomenon occurs when cell reperfusion is incomplete as oedema and injury to the microvascular endothelial cells precludes adequate tissue perfusion, and reperfusion arrhythmias refer to either ventricular or atrial arrhythmias which arise upon reperfusion on an ischaemic region, related to oxygen free radicals and altered membrane potentials^{101,105}. The main mediators of reperfusion injury are postulated as calcium overload¹⁰⁶ and free radical damage^{107,108}, both of which induce opening of mitochondrial permeability transition pores, cardiomyocyte hypercontracture and apoptotic cell death⁵¹.

1.3.3.1 Free radical formation and oxidative stress

The restoration of oxygenated blood rapidly generates free radicals that mediate oxidative stress giving rise to injury of essential cellular components such as mitochondria and the sarcoplasmic reticulum and eventually cell death^{107,109}. This phenomenon has therefore been termed the 'oxygen paradox'¹¹⁰.

Free radicals, the mediators of oxidative stress, are species containing one or more unpaired electrons and are capable of independent existence^{105,107,111}. Of relevance to reperfusion

injury are those generated by mitochondria, the vascular endothelium and neutrophils, where molecular oxygen undergoes a series of single electron reductions to form the free radicals superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO\cdot$)^{111,112}. Figure 1.5 outlines the biological routes of formation and decomposition of oxygen free radicals.

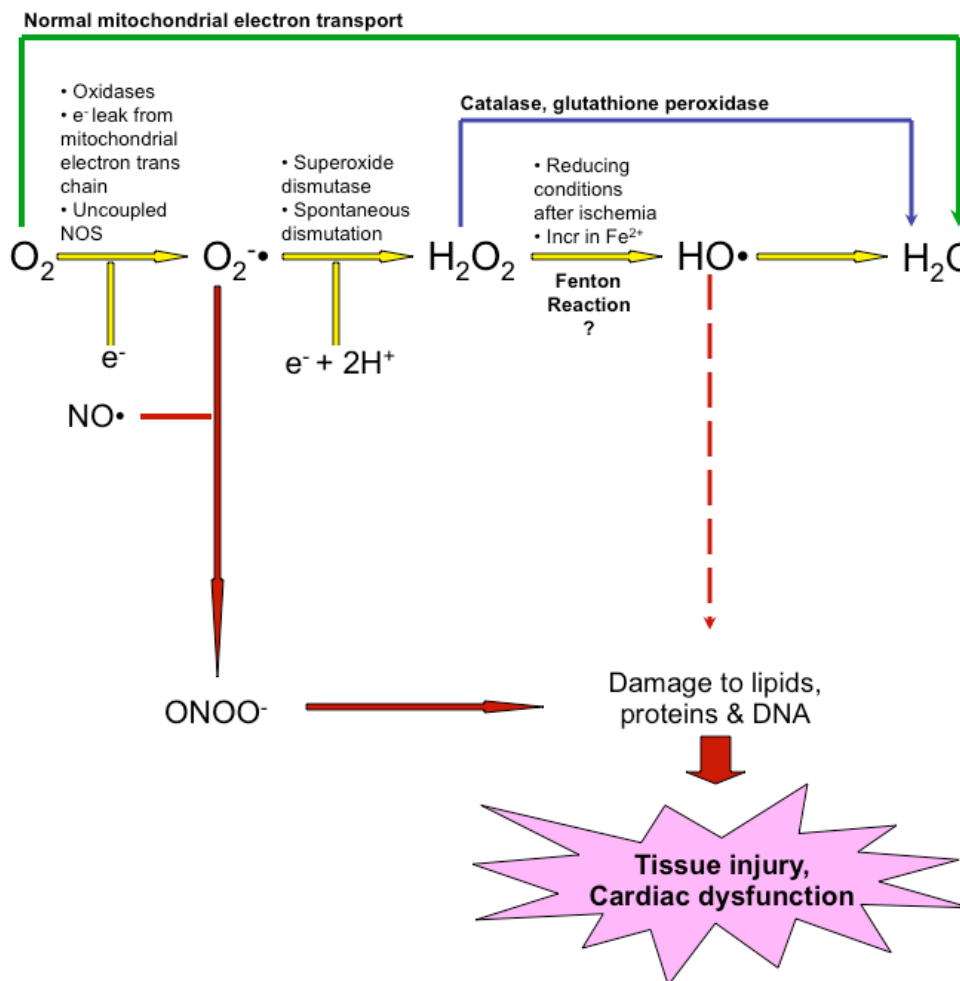


Figure 1.5 The formation of oxygen free radicals and tissue injury

Molecular oxygen undergoes a series of single electron reduction to form the free radicals superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO\cdot$). The unstable oxidant peroxynitrite ($ONOO^-$) is formed by the reaction of superoxide with the nitric oxide radical ($NO\cdot$).

Ischaemia produces a highly reduced intra-mitochondrial environment that favours single electron donation to oxygen in the mitochondrial electron transport chain, thereby favouring the formation of oxygen free radicals^{111,113} (Figure 1.5). Hydrogen peroxide (H_2O_2) is also formed by monoamine oxidase in the outer mitochondrial membrane, and may diffuse into the cytosol or inner mitochondrial space¹¹⁴. Ischaemia also causes changes in substrate specificity of the enzyme xanthine oxidoreductase, favouring electron transfer to oxygen instead of NAD^+ , with consequent formation of hydrogen peroxide¹¹⁵. Nitric oxide synthase (NOS) is another source of superoxide formation. Ischaemia oxidises cofactors, which 'uncouples' NOS to preferentially transfer electrons to oxygen instead of its usual substrate arginine, increasing formation of superoxide¹¹⁶. Superoxide may be converted to hydrogen peroxide by the antioxidant manganese-superoxide dismutase, and then to the hydroxyl radical by the Fenton reaction. Superoxide may also react with nitric oxide (NO) to form highly oxidising peroxynitrite species ONOO^- , which has similar reactivity and toxicity profiles^{117,118}.

Oxidative stress induces cellular apoptosis, DNA damage, oxidation of polyunsaturated fatty acids (lipid peroxidation) and deactivation of enzymes by oxidation of cofactors¹⁰⁷. Oxidative stress also reduces the bioavailability of nitric oxide (NO), thereby removing its cardioprotective effects such as inhibition of neutrophil accumulation, inactivation of superoxide radicals, and improvement of coronary blood flow^{107,118,119}. The influx of neutrophils to ischaemic tissue plays a critical role in the inflammatory process associated with necrosis and production of large quantities of free radicals¹²⁰.

1.3.3.2 Calcium overload

Under physiological conditions, cellular calcium homeostasis is tightly controlled by multiple pumps, transporters and binding proteins^{121,122}. Extracellular Ca^{2+} is maintained in the millimolar range¹²³, cytosolic Ca^{2+} in the nanomolar range (20-50nM)¹²⁴, mitochondrial Ca^{2+} at approximately 100nM¹²⁵, and the sarcoplasmic reticulum has the largest intracellular Ca^{2+} store at 100 μM ¹²⁶. At myocardial reperfusion, there is an abrupt rise in intracellular Ca^{2+} secondary to oxidative-stress induced damage to the sarcoplasmic reticulum¹²⁷. Normal Ca^{2+} handling mechanisms are overwhelmed, with cytosolic Ca^{2+} entering the mitochondrion via the Ca^{2+} uniporter, driven by the repolarised mitochondrial membrane potential. This leads to mitochondrial Ca^{2+} accumulation which, in conjunction with oxidative stress generated at

time of reperfusion, triggers opening of mitochondrial permeability transition pores (mPTP) resulting in cell death¹²⁷⁻¹²⁹.

Increased intracellular Ca^{2+} also activates degradative enzymes such as phospholipases which hydrolyse phospholipids and may have a detergent effect on biological membranes. Calcium-induced activation of calpains induces cell injury by localising to lysosomes and leading to their rupture and release of proteases such as cathepsin and lysosomal hydrolases^{130,131}. Calpains also cause dysfunction of mitochondria¹³², the sarcoplasmic reticulum and the Na,K-ATPase¹³³ as well as alteration of key metabolic pathways in the cytosol and mitochondria leading to cell death^{134,135}. Increased intracellular calcium leads to increased contractile activity. The combination of oxygen restoration and high cytosolic calcium thereby leads to the hypercontractures or 'contraction band necrosis' associated with reperfusion injury.

1.3.3.3 Opening of mitochondrial permeability transition pores

The mitochondrial permeability transition pore (mPTP) is a non-selective channel located in the inner mitochondrial membrane, and is permeable to molecules with a size limit $\leq 1.5\text{kDa}$ ¹³⁶. It remains closed during ischaemia, but may open within minutes of reperfusion in response to an increase in accumulated Ca^{2+} , oxidative stress, with depleted ATP and a resultant decrease in the mitochondrial membrane potential¹³⁷⁻¹³⁹. The return of cytosolic pH to normal range at reperfusion has also been determined as a key requirement for opening of the mPTP¹⁴⁰, and is known as the 'pH paradox'¹⁴¹. In neonatal rat cardiomyocytes, experimental studies have shown that reoxygenation with acidic buffer is cardioprotective¹⁴², an effect that may be mediated by inhibition of mPTP opening¹⁴³.

Opening of mPTP at reperfusion initiates a cascade of deleterious processes such as altering the membrane potential and uncoupling mitochondria, thereby impairing oxidative phosphorylation and depleting ATP¹³⁹. In addition, increased permeability of the inner mitochondrial membrane leads to entry of solutes and water, leading to mitochondrial swelling^{136,138,144}. Rupture of the mitochondrial outer membrane follows, leading to release of cytochrome c and apoptosis inducing factor (AIF), which cause activation of other proapoptotic proteins and caspases leading to apoptotic cell death^{51,137,138}.

1.3.3.4 Endothelial injury

Ischaemia and reperfusion not only cause injury to cardiomyocytes, but also has deleterious effects on the endothelium of the main coronary arteries and the microvasculature. Under physiological conditions, the endothelium synthesises vasodilators prostacyclin¹⁴⁵ and nitric oxide (NO)¹⁴⁶. Nitric oxide binds to and activates guanylate cyclase, producing a rise in cyclic GMP, and with a resultant cascade of reactions, leads to vasodilation. Nitric oxide also minimises platelet aggregation and prevents neutrophil adhesion to the endothelium¹⁴⁷.

The synthesis of NO is reduced during ischaemia, and its availability is further compromised by reperfusion¹⁴⁸. Decreased nitric oxide in the vascular smooth muscle leads to vasoconstriction, and reduced luminal levels of nitric oxide is associated with the upregulation of adhesion molecules the selectins, beta2 integrins and the immunoglobulin superfamily¹⁴⁷. These adhesion molecules act as chemoattractants to inflammatory cells, which migrate through endothelial junctions to cause inflammatory damage to the myocardium, and also physically plug the microvasculature, leading to the 'no reflow' phenomenon^{99,149}. Endothelial injury or 'activation' to produce adhesion molecules and luminal recruitment of inflammatory cells during ischaemia and reperfusion has been linked to rejection and allograft vasculopathy^{150,151}.

The luminal endothelial glycocalyx was first described as a site of injury during ischaemia and reperfusion by Haack et al in 1981¹⁵². The glycocalyx is composed of glycosaminoglycans¹⁵³, enzymes (endothelial NOS, extracellular superoxide dismutase, angiotensin converting enzyme and lipoprotein lipase), chemokines, apolipoproteins and growth factors. It acts as a direct barrier between the endothelial surface and red and white blood cells and plasma proteins, and is crucial in the regulation of vascular permeability, oncotic forces and endothelial signalling¹⁵⁴⁻¹⁵⁶. The glycocalyx is rapidly modified during IRI with stimulation of formation of ROS from the high concentration of xanthine oxidoreductase present in the glycocalyx, activated leucocytes, and vascular NADPH oxidase with further fragmentation of glycocalyceal proteins such as hyaluronan¹⁵⁷. Destruction of the glycocalyx is associated with release of clotting factors such as antithrombin III, apolipoproteins, selectins and chemokines¹⁵⁷.

Histologically, ischaemia-reperfusion injury in mild forms is evident as cellular swelling, contracture of myofibrils, disruption of the sarcolemma, appearance of intramitochondrial

calcium phosphate particles, and in more severe forms as contraction bands, coagulative necrosis and significant inflammatory cell infiltrate^{51,96}. These histological changes have been demonstrated in the context of myocardial infarctions as well as in cardiac transplantation²⁵.

1.4 Mechanisms of cardioprotection

While the heart tolerates periods of ischaemia up to 3 hours during hypothermic storage, actual ischaemic times including warm ischaemia can extend up to or beyond 6 hours. As discussed, although metabolism is slowed during hypothermia, it is not entirely arrested, and the combination of hyperkalaemia-induced membrane depolarisation and ongoing metabolic and ionic alterations lead to accumulated ischaemic injury, directly proportional to the period of hypothermic storage^{158,159}. The ability of the heart to recover therefore gradually deteriorates over this time. Optimising myocardial protection to increase the tolerance of cardiomyocytes to hypothermic storage and minimise injury due to IRI is of utmost importance, however still remains a challenging goal in experimental and clinical transplantation. Potential opportunities for intervention targeted at myocardial protection include donor intervention, intervention during organ procurement and transport, and intervention at the time of reperfusion or recipient interventions.

1.4.1 Donor interventions

The period between brain death and heart retrieval is one in which cardiac function can deteriorate rapidly. Several strategies of early intervention to minimise haemodynamic, hormonal and inflammatory stressors on donor organs have been evaluated. Intervention in the donor must ensure that all potential organs are provided maximal protection, and ensure that practices comply with ethical standards.

1.4.1.1 Donor hormonal resuscitation

The role of hormonal resuscitation of the donor has been investigated with the replacement of pituitary dependent hormones during donor management in intensive care units. Replacement of triiodothyronine (T3), thyroxine (T4) and cortisol has been suggested based on promising results from animal studies¹⁶⁰, however their role in donor management clinically remains controversial. Many retrospective analyses have supported a role for these

interventions, however prospective randomised controlled trials have failed to demonstrate improved cardiac function after transplantation^{161,162}. Vasopressin has also been proposed as an effective alternative to noradrenaline to maintain blood pressure and prevent the use of high dose inotropes^{163,164}, thereby reserving myocardial energy stores and minimising additional stress on the donor heart. Although vasopressin is routinely used in donor management, and many centres are increasing the use of T3, T4 and cortisol, inconclusive results from clinical studies has led to a lack of consensus on hormonal resuscitation protocols¹⁶⁵.

1.4.2 Organ preservation

Hearse described the three main components of cardioprotection as first, energy conservation through the rapid induction of diastolic arrest; secondly slowing metabolic and degenerative processes through the use of hypothermia; third selective prevention or reversal of various unfavourable ischaemic changes¹⁶⁶. Several strategies have been investigated to improve preservation of the donor heart during organ procurement and transport. The development of hypothermic preservation solutions and novel developments of hypothermic and normothermic ex-vivo perfusion of the donor heart will be discussed below.

1.4.2.1 Hypothermic preservation solutions

Hypothermic storage is currently the main method of donor heart preservation during transport to the recipient. The composition of the preservation solution is a critical determinant of the tolerance of the organ to hypothermic storage. Improved donor heart preservation during cold storage involves minimising ischaemic injury and vulnerability to subsequent reperfusion injury. However, targeting the multiple pathophysiological processes of IRI is extremely complex, with a multifaceted approach required to prevent cellular oedema, minimise ionic imbalances, prevent calcium overload and myocyte contracture, minimise oxygen-derived free radical injury and preserve endothelial integrity.

Strategies employed to protect the myocardium include the rapid induction of asystole to minimise electromechanical activity and conserve myocardial energy stores^{167,168}. Hyperkalaemia, calcium channel blockers and sodium channel blockers have been used to induce rapid asystole¹⁶⁹, however hyperkalaemia remains the most commonly used method

of arresting the heart in preservation solutions used clinically^{55,84,158,170}. However, as discussed, hyperkalaemia can induce ionic changes due to alteration of the membrane potential, and can cause endothelial damage, coronary vasoconstriction and contribute to myocardial stunning and hypercontracture^{171,172}. In addition to impermeants and measures to minimise calcium overload, preservation solutions should comprise acid-base buffers, metabolic substrates for optimal recovery of mitochondrial ATP production, contain optimal osmotic and ionic compositions, and ideally contain antioxidant capacity to overcome free radical injury. A variety of formulas and solutions have been developed and tested over the years with sodium and potassium composition based on 'intracellular' or 'extracellular' ionic concentrations. Intracellular-based solutions have high potassium and low sodium, while extracellular-like solutions have high sodium and lower potassium concentrations, although the latter is often raised to 9-16mM since the mode of cardioplegia is through hyperkalaemia¹⁶⁶. Varying impermeants, metabolic substrates, acid-base buffers and free radical scavengers have been added to optimise myocardial protection¹⁷⁰.

Table 1.1 outlines the more widely used cardioplegic and preservation solutions: the intracellular solutions with low sodium and high potassium concentrations, University of Wisconsin (UW) and histidine-tryptophan-ketoglutarate (HTK), and two extra-cellular solutions with high sodium and low potassium, St. Thomas' Solution (STS) and Celsior®.

University of Wisconsin Solution

University of Wisconsin solution was developed by Belzer and Southard in 1988¹⁷³ as a hypothermic renal preservation solution. It contains cell impermeant agents (lactobionic acid, raffinose, hydroxyethyl starch) with reduced glutathione and adenosine to augment antioxidant capacity, maintain ATP-dependent ionic gradients and stimulate high-energy phosphate generation upon reperfusion. Lactobionate and allopurinol provide added free-radical scavenging properties to mitigate IRI. It replaced Euro-Collins solution^{174,175}, the widely used renal preservation solution at the time, and was successfully adopted in liver and pancreas transplantation¹⁷⁶⁻¹⁷⁹. Although UW preserved intra-abdominal organs well, its success in preservation of the donor heart was less consistent, particularly when used during prolonged hypothermic storage^{84,158}. The presence of adenosine allowed superior cardiac protection to existing preservation solutions, however UW failed to prevent ischaemic contractures and did not consistently demonstrate optimal contractile recovery. This led to testing of modifications to UW with the addition of potential cardioprotective agents¹⁸⁰.

Table 1.1 The composition of the main cardioplegic/preservation solutions

	UW	HTK	STS 2	Celsior
<u>Electrolytes (mmol/L)</u>				
Sodium	30	15	120	100
Potassium	125	9	16	15
Calcium		0.015	1.2	0.25
Magnesium	5	4	16	13
Chloride		50	160	41.5
Bicarbonate			10	
SO ₄	5			
HPO ₄				
H ₂ PO ₄	25			
<u>Metabolic Substrates</u>				
Glucose				
Aspartate				
Lactate				
Glutamate				20
<u>Osmotic Agents</u>				
Lactobionate	105			80
Mannitol		30		60
Starch (g/L)	50			
Raffinose	30			
<u>Acid/base Buffers</u>				
Histidine		198		30
<u>Other</u>				
Adenosine	5			
Allopurinol	1			
Glutathione	3			3
Tryptophan		2		
Ketoglutarate		1		
Insulin (units)	40*			
Dexamethasone (mg)	16*			
Penicillin (U/L)	200,000*			
pH	7.4	7.2	7.8	7.3
Osmolality (mOsm/L)	320	320	320	360

Compositions were obtained from original descriptions of UW (University of Wisconsin solution or ViaSpan®)¹⁷³, HTK (histidine-tryptophan-ketoglutarate, Bretschneider or Custodiol®) solution¹⁸¹, STS 2 (St. Thomas No. 2¹⁸² or Plegisol®) and Celsior®¹⁸³. All components are expressed in mmol/L unless otherwise specified.

* Substances added to UW solution to enhance cellular protection, as described in ViaSpan®

HTK (Bretschneider) Solution

Histidine-tryptophan-ketoglutarate (HTK or Custodial®,) was developed by Bretschneider in the 1970s as a cardioplegic solution¹⁸¹. It was widely applied and found to be comparable to UW in kidney, liver and pancreas preservation¹⁸⁴⁻¹⁹². Unlike UW solution, It is a low-viscosity solution using mannitol as an impermeant and antioxidant agent; a potent buffer, histidine, to counteract acidosis caused by anaerobic metabolism and stabilise the content of energy-rich phosphates and two metabolic substrates, tryptophan and ketoglutarate. Its lower potassium concentration was reported to have superiority over other cardioplegic agents, however showed comparable results to UW and Celsior^{159,193-195}. Several of the intra-cellular solutions have been used as cardioplegia, while an extra-cellular solution or Krebs solution has been used as the organ preservation solution used during transport.

St Thomas' Solution

St Thomas' solution (STS) was developed by Hearse et al¹⁹⁶ in 1975 and modified in 1981 to optimise calcium (2.0 to 1.2mM), sodium (147 to 120mM) and potassium (20 to 16mM) concentrations to provide St Thomas' solution No. 2 (STS 2 or Plegisol®)¹⁸². Both were primarily designed as cardioplegic solutions. STS 2 and UW solution, either in original composition or with minor variations are the most widely used cardioplegic and preservation solutions in transplantation. Comparative studies have demonstrated STS 2 to be equivocal to UW^{197,198}, HTK and Celsior¹⁹⁹ in minimising PGD and in long-term patient and graft survival.

Celsior

Celsior®, developed in 1993 by Menasché et al^{183,200}, is specific for donor heart preservation. It is an extracellular solution designed for use as both cardioplegic and hypothermic preservation as either crystalloid or blood-based solutions. It contains high concentrations of mannitol and lactobionate as impermeants; high magnesium and a mildly acidic pH to prevent calcium overload; reduced glutathione, a powerful thiol-based antioxidant and an agent that maintains thiol groups in a reduced state for optimal enzyme function; and glutamate to enhance anaerobic energy production and prevent contracture. Celsior® was designed to provide superior myocardial preservation and endothelial protection^{201,202}, especially in prolonged hypothermic storage^{203,204}. Reduced glutathione was considered essential for myocardial preservation^{200,205}, as replacement with N-acetyl-L-cysteine failed to improve recovery beyond that provided by antioxidant-free solutions.

These four main solutions use different approaches to achieve myocardial preservation. Individual comparative studies have shown benefits of one solution over another, however overall have shown comparable results between the main preservation solutions UW, HTK, STS 2 and Celsior for the incidence of PGD and for long term patient and graft survival^{83,159,193-195,199,206-209}. The number of variations of these solutions, either prepared in-house or commercially, has resulted in to over 160 different solutions being used worldwide^{83,170}. This highlights not only the lack of an ideal preservation solution, or a 'gold standard', but also the complexity of the molecular and cellular mechanisms that underlie ischaemia-reperfusion injury and the lack of a consensus on the optimal cardioplegic and preservation storage strategy to minimise PGD²¹⁰.

1.4.2.2 Continuous perfusion techniques

The use of cold, hyperkalaemic cardioplegia and the immersion of the donor heart in preservation solutions have several limitations, as discussed above. Alternatives to static hypothermic storage have been evaluated, with the most promising of which includes continuous perfusion of the donor heart during organ transport²¹¹. Continuous perfusion has three basic advantages over static storage. These advantages include i) perfusion with oxygen carrying solutions preventing ischaemia, anaerobic metabolism and the 'oxygen paradox' associated with sudden reperfusion; ii) provision of nutritional supplementation and metabolic substrates and iii) enabling the clearance of metabolic waste products from the coronary circulation^{211,212}. It was recognised that the main factors needed for survival of organs *ex vivo* were reliable pumps, non-thrombogenic interactions between perfusion circuits and the perfusate, a method of oxygenation such as membrane oxygenators, and control of temperature²¹¹. Pulsatile perfusion has also been evaluated, however the crucial factor during *ex-vivo* perfusion has been stated as the production of the lowest flow compatible with good perfusion in order to minimise the risk of damage to the endothelium by abnormal shear stresses²¹³. Several perfusion solutions have been evaluated to obtain the optimal oncotic pressure by use of high-molecular anions and colloids to minimise myocardial oedema, the use of blood-based solutions, and addition of anti-oxidants to minimise reperfusion injury. *Ex-vivo* perfusion techniques have added advantages of allowing the evaluation of cardiac function using biochemical parameters and the ability to apply therapeutic interventions during organ transport²¹⁴.

1.4.2.2.1 Hypothermic perfusion preservation

Continuous hypothermic perfusion was designed to have the added advantage of a reduction of overall metabolism and oxygen consumption at sub-physiologic temperatures^{211,215}. Reduced metabolism and oxygen consumption would require lower flow rates to sustain oxidative activity and the removal of metabolic waste compared to normothermic temperatures, and would therefore reduce potential shear stress on the endothelium related to high flow rates and high perfusion pressures. It has also been demonstrated that oxidative energy production via mitochondrial electron transport is sustained at hypothermic temperatures as low as 10°C, hence energy production would be maintained²¹⁶. The use of hypothermic perfusion preservation has demonstrated improved graft function in experimental models compared to static storage²¹⁷⁻²¹⁹, with continuous hypothermic perfusion reducing exposure of the heart to ischaemic injury sustained during the implantation period of heart transplantation²¹⁹. Despite concerns of endothelial damage and tissue oedema, optimising perfusion solutions and improved preservation of the donor heart by continuous perfusion has demonstrated the ability to self-limit tissue oedema considerably²²⁰. Clinical studies on hypothermic perfusion have demonstrated good preservation of graft function²²¹, with this technique holding promise in improving preservation of the donor heart, in particular when extra protection is required when using marginal donor hearts or exposure to long ischaemic times.

1.4.2.2.2 Normothermic perfusion preservation

Normothermic perfusion of the donor heart allows preservation of the heart in a warm beating state during transport. Although energy utilisation is increased in a normothermic beating heart system, it also allows the ability to assess functional as well as biochemical parameters to enable better evaluation of organ viability. A recent randomised controlled multicentre trial on human heart transplantation of hearts preserved using normothermic ex-vivo perfusion (PROCEED II trial), with an initial 67 patients in the normothermic perfusion group, has demonstrated non-inferiority to static cold storage in 30-day graft and patient survival²²². The Organ Care System used in the PROCEED II trial evaluated the heart in 'resting' mode which does not allow true contractile assessment, however modifications to the circuit are being developed at several transplant centres to incorporate a 'working' mode circuit that would allow an afterload for evaluation of cardiac output. At present, biochemical parameters of troponin I and lactate levels are being used as surrogate markers

of myocardial preservation. As with hypothermic perfusion, the ability to offer therapeutic intervention is an advantage of normothermic continuous perfusion. A recent case series demonstrated that hearts from donation after circulatory death (DCD) preserved and transported using the normothermic ex-vivo perfusion Organ Care System allowed assessment of the viability of hearts prior to transplantation, enabling excellent post-transplant graft and recipient survival²²³. Continuous perfusion of the donor heart during organ retrieval, either using normothermic or hypothermic methods, holds great promise in improving the utility of marginal hearts and allowing improve preservation in distant retrievals.

1.4.2.3 Myocardial preservation using 'conditioning' strategies

Discussion of existing preservation solutions has highlighted the need to investigate alternative strategies of optimising myocardial preservation. One such strategy of myocardial 'conditioning' has been studied extensively in the context of myocardial infarctions, and has shown promise in reducing IRI of the donor heart^{55,224}. The term 'conditioning' broadly describes a phenomenon of protection of the myocardium against IRI, thereby reducing the extent of myocardial injury at the point of reperfusion. Development of the concept of 'conditioning' through studies on ischaemic pre- and post-conditioning, and the eventual application of pharmacological conditioning, are discussed below.

1.4.2.3.1 Ischaemic preconditioning

Ischaemic preconditioning (IPC) was originally described by Murry et al²²⁵ in 1986, where a canine study demonstrated that multiple short cycles of ischaemia and reperfusion of the myocardium prior to the onset of prolonged ischaemia, resulted in a reduction in infarct size. The success of animal studies allowed this strategy to be tested in patients undergoing coronary artery bypass grafts, where IPC with one minute of aortic cross-clamping followed by five minutes of reperfusion demonstrated enhanced postoperative cardiac function and a reduced need for inotropic support²²⁶. In brief, IPC acts as a stimulus that induces the release of substances such as adenosine, bradykinin, endogenous opioids and other growth factors which bind to cell surface receptors, which in turn activate cardioprotective pathways. Enzymes that have been shown to be involved in these pathways include phosphatidylinositol-3-phosphate kinase (PI3K), protein kinase B (PKB or Akt), endothelial

nitric oxide synthase (eNOS), extracellular signal-regulated protein kinase 1 and 2 (ERK1/2), protein kinase C (PKC) and Janus kinase and signal transducer and activator of transcription (JAK-STAT), a set of kinases termed by Yellon et al as the reperfusion injury salvage kinase (RISK) pathway^{51,227}. Other pathways suggested to contribute to the conditioning phenomenon include the survivor activating factor enhancement (SAFE) pathway²²⁸ and possibly the 5'-adenosine monophosphate-activated kinase pathway²²⁹.

Preconditioning of the cell causes activation of these pathways at reperfusion, resulting in a cascade of reactions that ultimately prevent the formation of mPTP. The mitochondria continue to function without the release of proapoptotic factors, preventing cell death. Myocardial infarct size is therefore reduced and cardiac contractility improved. Details of the mechanisms of IPC induced cardioprotection will be discussed in Section 1.5.2.3.3, however prior to this, a brief discussion of ischaemic postconditioning will be performed due to activation of common mechanistic pathways.

1.4.2.3.2 Ischaemic postconditioning

In patients with unanticipated onset of myocardial ischaemia, such as patients with an acute myocardial infarction, an IPC strategy would be impossible to implement. A more clinically relevant model where an interventional strategy could be applied at the time of myocardial reperfusion was needed. Zhao et al described the phenomenon of ischaemic postconditioning (IPost) in 2003²³⁰, where transient, repetitive cycles of coronary occlusion during early reperfusion demonstrated a reduction in infarct size, with evidence of reduced oxidative stress. Several studies have independently reported IPost to be an effective cardioprotective strategy in patients undergoing percutaneous coronary intervention (PCI), using an invasive protocol of serial coronary angioplasty balloon inflations and deflations²³¹⁻²³⁴. It has also been demonstrated that remote ischaemic conditioning, where ischaemic cycles are applied at a remote site, such as inflation and deflation of an upper arm blood pressure cuff, had similar protective effects on cardiac muscle that had been subjected to prolonged ischaemia²³⁵. Both IPC and IPost have been demonstrated to offer myocardial protection through common pathways. The combination of applying IPC and IPost has not been demonstrated to confer additional myocardial protection²³⁶.

1.4.2.3.4 Mechanisms of conditioning

Both IPC and IPost have been demonstrated to confer cardioprotection in the first few minutes of myocardial reperfusion following a period of ischaemia²³⁷. The signaling pathways activated by IPC have been demonstrated as common to the signaling pathways in IPost, with eventual convergence of signaling cascades to confer mitochondrial protection by inhibiting the formation of mPTP²³⁸. The two major signalling pathways that have been implicated in the cardioprotection offered by IPC and IPost are the reperfusion injury salvage kinase (RISK) pathway and the survival activating factor enhancement (SAFE) pathway^{239,240}.

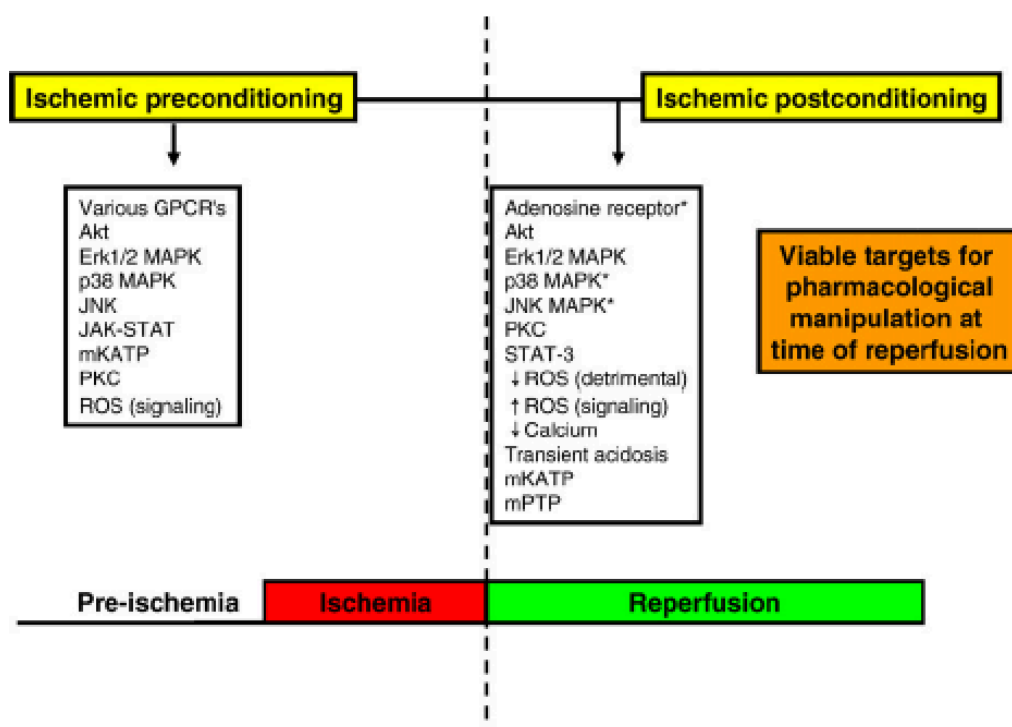


Figure 1.6 Activation of signaling pathways by ischaemic pre- and post-conditioning

Hausenloy DJ, Yellon DM. Pharmacology and Therapeutics. 2007;116(2):173-91²³⁷

The common cardioprotective pathways recruited at reperfusion by ischaemic pre- and post-conditioning are schematically outlined above.

The RISK pathway

The reperfusion injury salvage kinase (RISK) pathway includes a group of survival protein kinases which provide powerful cardioprotection²⁴¹. The protein kinases PI3K-Akt and 42- and 44-kDa extracellular signal regulated (ERK1/2) mitogen activated protein kinase (MAPK) are key signaling proteins of the RISK pathway²⁴², demonstrated to be activated at early reperfusion by extracellular signaling stimuli such as transforming growth factor- β ²⁴³ and insulin²⁴⁴. Both Akt and ERK1/2 mediate cell survival through activation of various antiapoptotic pathways that prevent opening of mPTP^{137,245}.

The SAFE pathway

The SAFE pathway includes activation and recruitment of TNF- α and signal transducer and activator of transcription-3 (STAT-3) during IPC and IPost, and is recognised as a separate pathway that confers cardioprotection in IPC and IPost^{228,239}. Activation of the SAFE pathway has been shown to prevent the formation of mPTP.

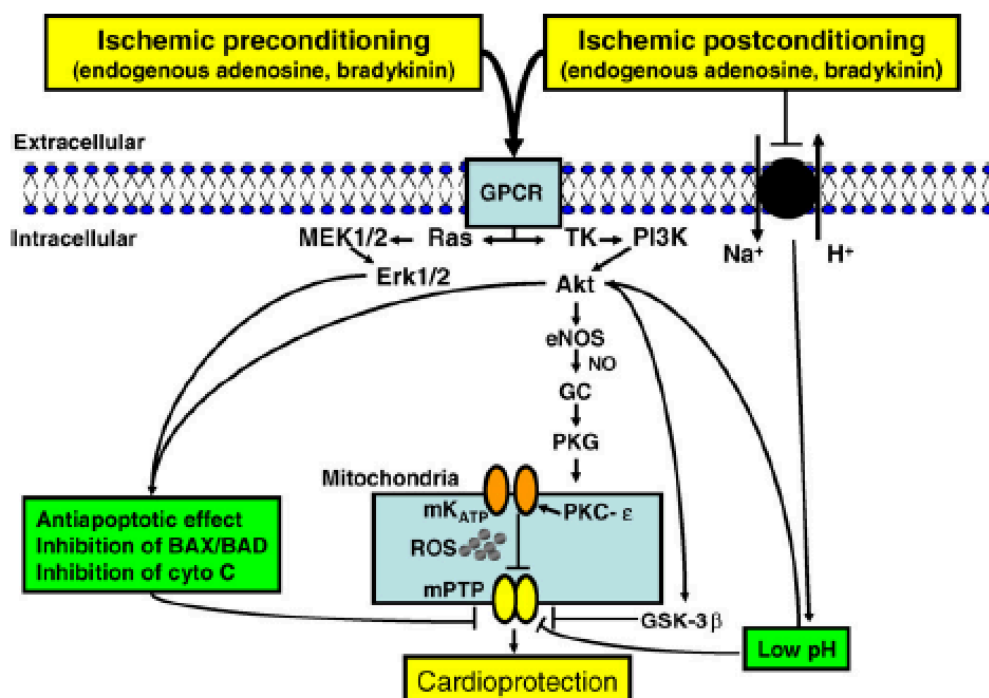


Figure 1.7 Mechanisms of cardioprotection by IPC and IPost

Hausenloy DJ, Yellon DM. Pharmacology and Therapeutics. 2007;116(2):173-91²³⁷

This schematic diagram outlines the mechanisms of cardioprotection by pathways common to IPC and IPost. Many of the signal transduction pathways converge on mPTP which appears to be at the end-effector of cardioprotection in both pathways.

Reduction of reactive oxygen species generation

Both IPC and IPost attenuate the generation of reactive oxygen species (ROS) at reperfusion^{246,247}. Mechanisms implicated are the activation of protein kinase C (PKC) and the mitochondrial ATP-sensitive potassium (mKATP) channel²⁴⁸. Opening of mKATP channels at reperfusion are described to be associated with maintenance of efficient mitochondrial energy production and a reduction in electron flow in the coenzyme Q cycle which is a key component in ROS generation²⁴⁹.

Reduction of calcium overload

The prevention of cytosolic and mitochondrial calcium overload has been demonstrated in studies on ischaemic conditioning²⁵⁰. The role of mKATP have been implicated in reduced mitochondrial calcium overload, however it is unclear if calcium handling is improved due to reduction of ROS activity, or through ROS-independent mechanisms.

Cell-surface receptor binding at reperfusion

Endogenous ligands such as adenosine, opioids and bradykinin have been demonstrated to be stimulated by IPC, with stimulation of cell-surface receptors such as G protein-coupled receptors (GPCR) thought to provide the initial trigger for signal-transduction pathways²⁵¹. Activation of adenosine receptors has been demonstrated to activate PI3K-Akt²⁵² and guanylyl cyclase, and bradykinin has been shown to activate PI3K-Akt. Activation of cell-surface receptors such as GPCR induce phosphorylation of ERK1/2 and PI3K-Akt signalling cascades that either inhibit proapoptotic enzymes by the former, or induce further signal transduction through endothelial nitric oxide synthase (eNOS), guanylate cyclase, protein kinase G and C^{51,53,137}. Protein kinase G (PKG) has been implicated as the terminal mediator of the signal transduction pathway relaying the initial conditioning stimulus received at the cell surface to PKC- ϵ , a secondary mediator at the level of the mitochondria²⁵³. PKG phosphorylates mitochondrial PKC- ϵ , which then inhibits the opening of the mPTP through intermediary steps involving the mKATP channel, matrix alkalinisation and mitochondrial ROS²⁵³.

1.4.2.3.5 Pharmacological conditioning

Determination of the many signaling pathways common to IPC and IPost have led to research on the activation of these pathways through exogenous mechanisms. The concept of pharmacological conditioning, where exogenous agents are used to confer cardioprotection during ischaemia and reperfusion are discussed below.

Adenosine

The conditioning effects of adenosine were discovered in 1991 by Liu et al, where a rabbit study demonstrated that adenosine receptor antagonists blocked the protection offered by IPC²⁵¹. They demonstrated that intracoronary administration of adenosine A₁ receptor antagonist reduced infarct size as seen in preconditioned hearts. A translational clinical study, the Acute Myocardial Infarction Study of Adenosine (AMISTAD)²⁵⁴ demonstrated a 33%

reduction in infarct size in patients treated with an infusion of adenosine after thrombolysis. Animal studies have suggested that activation of the A_{2b} adenosine receptor is crucial in activating downstream cell survival signalling cascades that offer cardioprotection^{255,256}. The mechanism of downstream signalling is linked to PKC and the RISK pathway^{241,257}. The use of adenosine is not standardised treatment in acute myocardial infarctions, perhaps due to no significant improvements demonstrated in survival in a subsequent AMISTAD-II trial, however adenosine has been added to preservation solutions^{173,258}.

Bradykinin

Bradykinin was shown to reduce infarct size equivalent to IPC in rabbit study of intra-atrial infusion by Wall et al²⁵⁹. A bradykinin receptor antagonist was demonstrated to block the protective effect of IPC. It was postulated that IPC may stimulate the release of bradykinin physiologically. Subsequent studies have demonstrated that the bradykinin B2 receptor is crucial in cardioprotection²⁶⁰. The inhibition of angiotensin-converting enzyme (ACE) causes accumulates bradykinin. Since ACE-inhibitors have demonstrated the ability to enable myocardial reverse remodelling after myocardial infarction, several studies were conducted to test its efficacy as a conditioning agent, however with mixed results^{261,262}. Treatment with bradykinin infusion during coronary artery bypass grafting (CABG) also demonstrated no change in troponin I levels²⁶³.

Opioids

The cardioprotective effect of opioids was first described by Schultz et al in 1995, in a study where naloxone, a nonselective opioid receptor antagonist, blocked the protective effects of IPC in Wistar rats²⁶⁴. A clinical study where remifentanyl infusions were administered to patients having CABG demonstrated reduced troponin I and CK-MB release, however did not show any difference in intensive care and hospital length of stay, and the study was not powered to look at survival outcomes²⁶⁵. In the setting of acute myocardial infarctions, patients routinely receive opioids for analgesia and reduction of myocardial preload, which can confound clinical studies. Animal studies demonstrated that kappa and delta opioid receptor activation was the mechanism of action of remifentanyl induced cardioprotection²⁶⁶, however has been shown to be active in opioid μ receptors in humans, which may explain the absence of cardioprotection of the same magnitude in translational studies. To date, opioids have not been used in the context of donor heart preservation.

Erythropoietin

Erythropoietin (EPO), a glycoprotein hormone effecting through a transmembrane erythropoietin receptor²⁶⁷, has been demonstrated to activate cardioprotective signalling cascades involving PI3-K, Akt, JAK-STAT and glycogen synthase kinase (GSK)-3 β that prevent cellular apoptosis and the formation of mPTP²⁶⁸⁻²⁷⁰. Clinical studies using lower doses of EPO administered to patients with acute myocardial infarctions demonstrated reduction in CK-MB, reduced antiapoptotic gene expression and improvement in left ventricular volume and function²⁷¹⁻²⁷⁴. Studies where EPO was provided at higher doses (50,000-60,000 IU) as boluses either before and/or after reperfusion did not demonstrate improvements in myocardial protection, and in some cases showed adverse effects of microvascular obstruction, or increased risk of death, myocardial infarction and stroke in older patients²⁷⁵⁻²⁷⁸. A recent trial of intracoronary infusion of darbopoietin at the time of reperfusion, the 'Epo-Co-EpoMI' trial²⁷⁹, has failed to demonstrate a reduction of infarct size, however results of other ongoing trials are pending.

Atrial natriuretic peptide

Atrial natriuretic peptide (ANP) is produced by atrial cardiomyocytes in response to distension and ischaemia. It has been shown that ANP acts on cyclic guanosine monophosphate (cGMP)-coupled protein kinase G (PKG), components of the RISK pathway, and on mPTP to offer cellular protection^{280,281}. A clinical trial where ANP was administered to patients after PCI, demonstrated reduced creatinine kinase, improved ejection fraction at 6 months and reduced rates of rehospitalisation for heart failure and death²⁸², however other trials have been negative^{283,284}.

Insulin

Insulin binds to a tyrosine kinase transmembrane receptor to exert effects²⁸⁵, and it has been linked to mechanisms of cardioprotection similar to EPO, with the additional effect of closure of mPTP^{244,286}. Insulin has been administered in the combination of glucose-insulin-potassium (GIK) in order to avoid hypoglycaemia. Animal studies showed reduction of myocardial infarct size²⁸⁷⁻²⁸⁹, however clinical trials have not shown any improvements in morbidity or mortality, although reduction of cardiac arrest was noted in treated groups²⁹⁰⁻²⁹².

Volatile anaesthetics

Halothane, enflurane and isoflurane and subsequently sevoflurane and desflurane have been shown to reduce myocardial infarctions in animal studies²⁹³⁻²⁹⁵. The mechanism of action is proposed to work through the adenosine receptor, PI3-K/Akt, MEK1/2, ERK1/2, PKC, calcium-activated potassium (BKCa) channels and mPTP²⁹⁶. Human studies where enflurane or isoflurane were used as the primary anaesthetic agent throughout surgery demonstrated reduced troponin I and improved left ventricular contractility^{297,298}. In contrast, negative findings have been demonstrated in a study on sevoflurane or desflurane compared to a non-volatile anaesthetic propofol, with no differences in troponin I release or 1-year mortality²⁹⁹.

Glyceryl trinitrate

The nitric oxide donor, glyceryl trinitrate (GTN), has effects on the endothelium and myocardium. In the endothelium it inhibits platelet aggregation¹¹⁹, smooth muscle cell migration¹¹⁹ and adhesion of inflammatory cells¹²⁰. In the myocardium, NO prevents reperfusion injury, as it is an important component of the RISK pathway that leads to inhibition of opening of mPTP. Animal studies have shown conditioning properties of NO donors that increase the bioavailability of NO, or increase intracellular NO signalling within cardiomyocytes³⁰⁰⁻³⁰². A meta-analysis of ten studies on patients³⁰³ receiving intravenous nitrates in the setting of acute myocardial infarctions demonstrated a significant reduction in mortality, warranting the assessment of nitric oxide donors in larger randomised studies.

Phosphodiesterase 5 inhibitors

Phosphodiesterase (PDE) 5 inhibitors degrade cGMP, hence PDE-5 inhibitors increase cellular cGMP, which then has downstream effects on signalling cascades including PKG, PKC, ERK1/2 and GSK-3 β ³⁰⁴. Nitric oxide levels are also increased, activating the RISK pathway³⁰⁵. Of the PDE-5 inhibitors, sildenafil has been shown to offer endothelial effects in pulmonary hypertension³⁰⁶. Animal studies have demonstrated that sildenafil reduces myocardial infarction size³⁰⁷⁻³¹⁰, however human studies are lacking.

Atorvastatin and rosuvastatin

Atorvastatin and rosuvastatin, inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme (HMG-CoA) reductase, have been shown to reduce infarct size in animal studies^{311,312}. Conditioning is mediated through the RISK pathway including PKC, ERK1/2, Akt and eNOS³¹³. Clinical studies on chronic atorvastatin use prior to elective PCI demonstrated reduced periprocedural myocardial injury, and reduced death, myocardial infarctions and revascularisation rates³¹⁴. These findings were not reproduced in acute myocardial infarction studies to date³¹⁵.

Cyclosporin

Calcineurin inhibitor cyclosporin binds to cyclophilin D, a component of the mPTP and has conditioning effects by directly inhibiting opening of the pore³¹⁶. It thereby protects mitochondrial integrity and prevents release of apoptotic factors that cause cell death. A pilot clinical study by Ovize et al, where cyclosporine was administered in acute ST elevation myocardial infarctions, demonstrated reduced troponin I, reduced number of abnormally contracting myocardial wall segments, and reduced infarct size as measured by CMR³¹⁷. However, a recent randomised clinical trial by the same group, the Cyclosporine and Prognosis in Acute Myocardial Infarction Patients (CIRCUS) trial, where bolus injections of cyclosporine were administered prior to PCI in anterior ST elevation myocardial infarctions, failed to demonstrate significant differences in early mortality, worsening heart failure or ventricular reverse remodelling at 1 year³¹⁸.

1.4.2.3.6 Clinical application of pharmacological conditioning

The pharmacological agents discussed above have all shown promise in reducing ischaemia reperfusion injury in animal studies, however have demonstrated less consistency and success in clinical trials. Reasons for less success in humans have been postulated as heterogeneity of receptor subtypes, polypharmacy use that may have cardioprotective effects in control groups, and improvements in time to revascularisation and post-infarct care. Some animal studies have also highlighted the need to carefully tailor dosage and timing and route of administration, hence pharmacological conditioning may still provide cardioprotection once the above factors have been optimised. Assessment of pharmacological conditioning in context of donor heart preservation is discussed in section 1.5.2.3. Results from clinical myocardial infarction studies have been noted and attempts made to enhance the efficacy of conditioning in donor heart preservation are also discussed below.

1.4.2.4 Myocardial conditioning in the context of donor heart preservation

Several pharmacological conditioning agents that have showed promising results in experimental myocardial infarction studies have been assessed in the context of donor heart preservation. These studies have been conducted at the Cardiac Transplantation Laboratory at Victor Chang Cardiac Research Institute over the last decade, and will be discussed below along with strategies used to enhance the efficacy of conditioning, aiming to allow translation of findings in clinical studies.

1.4.2.4.1 Sodium-hydrogen exchange inhibitors

Sodium-hydrogen exchange (NHE) inhibitors are proposed to mediate a rise in intracellular Na^+ and subsequent Ca^{2+} overload causing myocardial damage during reperfusion. Evidence exists that NHE-1 is mostly inactive during the ischaemic period, due to factors such as equalisation of intracellular and extracellular pH (making NHE-1 activity chemically unfavourable), acidosis directly inhibiting NHE-1, or metabolites in ischaemia inactivation NHE-1^{87,319}. It has been hypothesized that it is only during reperfusion when the extracellular acidosis and metabolites are washed out that NHE-1 becomes activated and results in overwhelming Na^+ influx⁸⁷. NHE-inhibitors maintain intracellular acidosis during the early stage of reperfusion as well as reducing intracellular Na^+ influx. Although prevention of Na^+

and Ca²⁺ overload are key parts of NHE-1 inhibitor action, maintenance of acidosis may also be important as a low pH will inhibit opening of mPTP and activate the RISK pathway (specifically the ERK1/2 component).

NHE-inhibitor cariporide was initially tested in a rodent model of donor heart preservation. Rodent hearts were arrested with an extra-cellular preservation solution supplemented with cariporide at concentrations of 1, 3.2, 10 or 10µmol/L and stored for 2-3°C for 6 hours and compared to control hearts arrested without cariporide³²⁰. Cardiac function was assessed on an isolated working-heart model. Significant improvement in the recovery of cardiac function was demonstrated in hearts treated with cariporide, compared to poor recovery in untreated hearts. The optimal concentration of cariporide in cardioplegia was determined as 10µmol/L, and the optimal timing of treatment was determined as during cardioplegia. The mechanism of cardioprotection was inhibition of the rapid correction of pH at reperfusion, which has previously been demonstrated as a deleterious mechanism leading to opening of mPTP^{140,141}. By inhibiting NHE channels, cariporide aids in retaining an acidic intracellular pH, thereby preventing the opening of mPTP, preserving mitochondrial integrity, preventing oxygen free radical injury to mitochondrial and preventing the triggering of proapoptotic pathways^{114,136,139}. The cardioprotective properties of cariporide were validated in a porcine orthotopic heart transplant study, where improvements were demonstrated in left ventricular contractility (measured using preload recruitable stroke-work relationship) and left ventricular compliance (end-diastolic pressure-volume relationship) in hearts arrested and stored for 4 hours in crystalloid solution supplemented with cariporide³²¹. A subsequent porcine orthotopic heart transplant study on extended (14 hours) hypothermic storage demonstrated that recipients were able to be weaned off cardiopulmonary bypass only in transplants with hearts treated with cariporide³²².

Cariporide was tested in a phase III clinical study by Mentzer et al (the EXPEDITION trial), where cariporide or placebo were administered to patients prior to high-risk coronary artery bypass graft surgery. Results of the study were published in 2008³²³, and revealed significantly lower incidence of death or myocardial infarction in patients treated with cariporide, however also reported an increased incidence of cerebrovascular events in this group. Although it showed promise in myocardial protection, safety concerns led to the discontinuation of clinical use of cariporide subsequent to this study. However, in context of donor heart preservation, cariporide is not administered in the recipient. It is used in

cardioplegia and hypothermic preservation, with the potential for small amounts to enter the recipient circulation at time of reperfusion. These would be significantly lower concentrations than used as bolus doses in the EXPEDITION trial, and may not lead to adverse cerebrovascular events. The ability to assess cariporide as a supplement in cardioplegia and hypothermic storage in clinical cardiac transplantation has been halted, and it remains to be seen whether approval will eventually be granted.

An alternative NHE-inhibitor zoniporide (ZON), was subsequently tested in an isolated rodent working heart model, to determine whether it had similar cardioprotective properties to cariporide³²⁴. Rat hearts stored for 6 hours in hypothermic conditions (1-4°C) demonstrated significantly improved recovery of cardiac function compared with control hearts. Conditioning mechanisms were demonstrated by increased phosphorylation of pro-survival kinases ERK1/2 and STAT3, and myocardial preservation was further demonstrated by reduced lactate dehydrogenase release in coronary effluent, and a reduced marker of apoptosis, cleaved caspase 3. Since performing this study, the clinical use of zoniporide has also been restricted, mainly due to concerns over the use of NHE-inhibitors having a 'class-effect' of cerebrovascular accidents.

1.4.2.4.2 Glyceryl trinitrate

The nitric oxide donor glyceryl trinitrate (GTN) was assessed for its conditioning properties in donor heart preservation in a rodent isolated working heart study³²⁵. Hearts arrested and stored with an extracellular crystalloid solution supplemented with glyceryl trinitrate at a concentration of 0.1mg/mL had significantly improved myocardial recovery after 6 hours of hypothermic storage. In order to augment the cardioprotective properties of GTN, the solution was supplemented with the NHE-inhibitor Cariporide, in order to target multiple conditioning pathways. Hearts were stored for 10 hours, and the recovery of cardiac function was significantly improved in hearts treated with both cariporide and GTN, but not with each agent used individually. This study was conducted prior to the discontinuation of cariporide from clinical use, and cariporide is currently unavailable for use clinically, this study has importantly highlighted the ability to enhance myocardial preservation when more than one conditioning pathway is targeted.

Findings from rodent studies were validated in a porcine orthotopic heart transplantation model³²⁶. This study used either GTN, cariporide or a combination of both agents in cardioplegia and extended (14 hours) hypothermic storage of porcine hearts, and demonstrated that recipient animals transplanted with hearts stored with a combination of the two agents were able to be weaned off cardiopulmonary bypass, while hearts stored with GTN or cariporide singly were unable to be weaned. Incidentally, donor and recipient animals treated with intravenous cariporide prior to explantation of the heart and at reperfusion were also able to be weaned off cardiopulmonary bypass.

Supplementation of MUW with GTN was also demonstrated to improve myocardial protection in a rodent model by Baxter et al^{327,328}, allowing extension of preservation to 16 hours without compromise of graft function. The mechanism of action proposed as the action of GTN with the thiol group of reduced glutathione of MUW, to release biologically active NO. Glyceryl trinitrate was demonstrated to improve myocardial preservation when added to Celsior or MUW, however not STS, which was attributed to the lack of reduced glutathione (GSH) in STS, which was considered essential for facilitation of vasodilation and cardioprotection. Baxter et al also demonstrated at 0.1ml/ml was the optimal concentration of GTN to be added (to MUW). This study highlighted the point that when adding extra components to preservation solutions, it is critical to examine what other compounds are present in the solution in order to ensure that the desired effect and no unwanted effects are produced.

1.4.2.4.3 Erythropoietin

The conditioning properties of the glycoprotein hormone, erythropoietin (EPO), was also assessed in a rodent isolated working heart study³²⁹. This study investigated the effects of erythropoietin alone, and in combination with GTN and NHE-inhibitor, zoniporide. Hearts arrested and stored at 2-3°C with an extracellular crystalloid solution supplemented with EPO had superior recovery of cardiac function compared to controls, and the optimal concentration of EPO was determined as 5IU/mL. Extended (10 hour) storage revealed that the use of EPO, GTN or ZON alone did not provide adequate cardioprotection, however myocardial recovery improved with the use of a combination of two agents, such that EPO + ZON provided < 10% recovery of cardiac function, GTN + ZON approximately 15% recovery, EPO + GTN approximately 25% of recovery. Maximum recovery of cardiac function was demonstrated when all three agents were used together, allowing 50% recovery of cardiac

output after 10 hours of hypothermic storage. Cardioprotection by EPO was mediated through ERK1/2 and STAT3, as determined by Western blot analyses, demonstrating that both the RISK and SAFE pathways were activated. This study was important in highlighting that maximum cardioprotection was provided by conditioning when pharmacological agents were used to provide synergistic effects by activating multiple cytoprotective pathways. A porcine orthotopic heart transplantation study validated these findings, demonstrating that recipient animals were able to be weaned off cardiopulmonary bypass only when hearts were treated with GTN, EPO and ZON used in combination in cardioplegia and hypothermic storage³³⁰.

The studies described in subsequent Chapters 3 and 4 were performed to test this combination of pharmacological agents in a rodent model of brain death, and in aged hearts representing extended criteria donors. Prior to outlining these studies, a brief discussion will follow on alternative preservation solutions and normothermic perfusion techniques developed to improve myocardial preservation.

1.4.2.5 Alternative cardioplegic and preservation strategies

Standard methods of cardioplegia involve hypothermia and hyperkalaemia-induced depolarisation of the cardiomyocytes membrane. Studies by Dobson et al have demonstrated that an alternative nondepolarising cardioplegic solution using sodium channel blocker lignocaine provides superior myocardial preservation²⁵⁸. Rat hearts were arrested at temperatures between 22 and 35°C, using lignocaine and adenosine added to Krebs-Henseleit solution (AL solution), and compared with modified St. Thomas' Hospital solution no. 2. Hearts arrested for up to 4 hours using AL solution demonstrated superior recovery of aortic flow, heart rate, systolic pressures and rate-pressure products. This solution is currently in clinical use in cardiac surgery, and remains to be tested in the context of donor heart preservation involving brain death and transplantation.

1.5 Thesis aims

The literature reviewed in this chapter reveals that there has been significant progress made in determining mechanisms mediating cardiac ischaemia-reperfusion injury. Primary graft dysfunction still remains high, urging the need to develop and translate mechanisms of myocardial preservation of the donor heart to improve graft and patient survival. Given the complex interplay of elements of ischaemia reperfusion injury, it is likely that a combination of therapeutic approaches may be required to maximize cardiac protection post reperfusion. The development of clinically relevant small animal screening models is likely to improve the translation of preclinical studies.

The overall aims are to improve myocardial preservation by testing the optimal pharmacological conditioning mechanism that can be implemented in the context of hypothermic static storage. By developing such strategies, the aim is to ensure that graft function is improved in standard criteria and extended criteria organs transplanted in higher-risk clinical settings. The translation of these strategies from preclinical studies to the clinic would be improved with the use of more clinically relevant small animal screening models. Another aim was therefore to improve on current small animal models used by incorporation of brain death and transplantation to allow the full extent of ischaemia-reperfusion injury to be present when testing myocardial preservation strategies.

The studies involved:

- Establishing a rodent model of brain death and assessing the efficacy of pharmacological conditioning of hearts from brain dead donors (Chapter 3)
- Assessing the ability to condition older hearts, by using an aging rodent model (Chapter 4)
- Determining the efficacy of two clinically available pharmacological conditioning agents at improving preservation of human hearts, in a pilot clinical trial (Chapter 5)
- Assessing the efficacy of sildenafil as an alternative conditioning agent (Chapter 6)
- Developing a non-invasive method of measurement of cardiac function – using cardiac magnetic resonance imaging to image rodent hearts (Chapter 7)
- Developing a rodent abdominal heterotopic heart transplant model to allow longitudinal assessment of cardiac function (Chapter 8)

CHAPTER 2. METHODS AND MATERIALS

This chapter outlines the isolated working rat heart perfusion system used to assess myocardial preservation strategies, and the general experimental protocol. Methods used to develop specific models such as the rodent model of brain death, the heterotopic heart transplant model and cardiac magnetic resonance imaging of rodent hearts will be described in subsequent chapters.

2.1 Materials

Tables 2.1 to 2.5 list all the drugs, chemicals and experimental compounds used in the study along with their suppliers.

Table 2.1 Anaesthesia and pre-medications

Drug	Dose	Source
Ketamine	80mg/kg	Parnell Laboratories Aust, Alexandria, NSW, AU
Xylazine	10mg/kg	Troy Laboratories Aust, Smithfield, NSW, AU
Isoflurane	1-5ml/min	Cenvet Aust, Kings Park, NSW, AU
Heparin	250IU	Pfizer Pty Ltd, North Ryde, NSW, AU

Medications given to animals to induce anaesthesia and to prepare for surgery are listed above.

Table 2.2 Perfusion buffer (modified Krebs-Henseleit solution) components

Compound	Source
Sodium chloride (NaCl)	Sigma Aldrich (St Louis, MO, USA)
Potassium chloride (KCl)	Sigma Aldrich (St Louis, MO, USA)
Potassium dihydrogen phosphate (KH ₂ PO ₄)	Sigma Aldrich (St Louis, MO, USA)
Magnesium sulphate (MgSO ₄)	Sigma Aldrich (St Louis, MO, USA)
Sodium bicarbonate (NaHCO ₃)	Sigma Aldrich (St Louis, MO, USA)
Glucose (C ₆ H ₁₂ O ₆)	BDH Chemicals (Kilsyth, Vic, AU)
Calcium chloride (CaCl ₂ ·2H ₂ O) in 1M solution	BDH Chemicals (Kilsyth, Vic, AU)

Compounds used to formulate the buffer used to perfuse the isolated heart are outline above.

Table 2.3 Composition of modified Krebs-Henseleit solution with [Ca²⁺] 1.4mM

Electrolytes	Concentration (mM)
Sodium (Na ⁺)	143.0
Potassium (K ⁺)	4.7
Calcium (Ca ²⁺)	1.4
Magnesium (Mg ²⁺)	1.2
Chloride (Cl ⁻)	127.7
Bicarbonate (HCO ₃ ⁻)	25.0
Sulphate (SO ₄ ²⁻)	1.2
Phosphate (PO ₄ ²⁻)	1.2
Glucose	11.0
Osmolarity	300mOsm/l

Krebs-Henseleit solution used in perfusion of the isolated rat heart was modified as per Hearse et al, 1975¹⁹⁶. Water for solution was purified through a MilliQ purification system (Millipore, Australia).

Table 2.4 Composition of Celsior® preservation solution

Component	Concentration (mM)
Sodium	100
Potassium	15
Chloride	41.4
Magnesium	13
Calcium	0.25
Histidine	30
Lactobionate	80
Mannitol	50
Glutamate	20
Reduced glutathione	3
pH	7.3 ± 0.2 at 20°C
Osmolarity	360mOsm/l

Celsior® solution (Genzyme, Naarden, NE) was used as the control cardioplegia and preservation solution. It was supplied as a sterile solution and stored at 2-3°C in the dark in an air-tight bag.

Table 2.5 Pharmacological supplements

Component	Class	Concentration	Source
Erythropoietin alpha	Glycoprotein hormone and activator of PI3K-Akt and JAK-STAT signalling pathways	5000U/l	Janssen-Cilag (North Ryde, AU)
Zoniporide	Sodium-hydrogen exchange inhibitor	1µmol/l	Pfizer, Inc (Groton, CT, USA)
Glyceryl trinitrate	Nitric oxide donor	100mg/l	David Bull Laboratories (AU)
Sildenafil	Phosphodiesterase type 5 inhibitor	50nM	Pfizer, Inc (Groton, CT, USA)

The above pharmacological supplements were added to Celsior® either singly or in combination, and used in cardioplegia and hypothermic storage to enhance cardiac preservation. Zoniporide was obtained under a Materials Transfer Agreement from Pfizer, Inc. The concentrations of erythropoietin, zoniporide and glyceryl trinitrate were obtained from prior dose-response studies performed in the laboratory^{325,329}, and the dose of sildenafil was obtained from published dose-response studies³³¹.

All pharmacological supplements were added to Celsior solution fresh on the day of use. Erythropoietin-alpha (Eprex®) was supplied as 5000IU/0.5ml, glyceryl trinitrate as 50mg/ml solution and sildenafil as 10mg/ml solution (Revatio®, Pfizer, Inc. Groton, CT, USA). The appropriate amounts were drawn up and added directly to Celsior. Zoniporide was weighed on a 6-place microbalance (Sartorius MC 210 S, Goettigen, GE), and dissolved in 0.5ml DMSO. All components were mixed in Celsior for 5 minutes using a magnetic stirrer, immediately prior to use in cardioplegia and hypothermic storage.

2.3 Ethical conduct

All animals received humane care in compliance with the *Guidelines to Promote the Wellbeing of Animals Used for Scientific Purposes* (National Health and Medical Research Council, Australia) and the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, Bethesda, MD). All studies were approved by the Garvan Institute Animal Ethics Committee (Animal Research Authorities #11/12, 12/13, 12/08, 12/25 and 12/28).

2.4 Animals

Male rats from two strains were used in the studies. Six animals were used per study group in all experiments. Wistar rats (Animal Resources Centre, Western Australia) aged 10-12 weeks (320-380g) were used in all studies involving the initial screening of new preservation techniques. This outbred strain was used to allow comparison with previous donor heart preservation studies conducted in the same laboratory.

Lewis rats (Animal Resources Centre, Western Australia), a syngeneic strain, aged 10-14 weeks (300-380g), were used in the development of three new models: a rodent donor heart model incorporating brain death, a rodent abdominal heterotopic working heart transplant model and developing a reproducible protocol for rodent cardiac magnetic resonance imaging that allows non-invasive *in-vivo* functional assessment of donor hearts. A syngeneic strain was used in order to prevent allograft rejection.

Older Wistar rats aged 12 and 18 months (Charles River Laboratories, Wilmington, MA, USA) were used in an aging study which evaluated the viability of aged hearts in transplantation. The outbred Wistar strain was used due to a lower predicted attrition with aging, compared to inbred strains more susceptible to malignancies³³²⁻³³⁴.

Housing and monitoring

Animals were housed two to three per cage in the BioCORE Animal Facility of the Victor Chang Cardiac Research Institute. Light:dark cycles of 12:12 hours were used and room temperature maintained at 22°C. Animals were fed standard pelleted laboratory food (Gordon's Specialty Stock Feeds, Yanderra, AU) and sterile water *ad libitum*. Animals were weighed at arrival and regularly thereafter until they attained the required weight as for each experiment. All animals were allowed an acclimatisation period of at least 1 week prior to use.

2.5 The isolated rat heart perfusion circuit

A custom-built *ex-vivo* rat heart perfusion circuit was used to assess the efficacy of preservation solutions in all experiments. The isolated heart perfusion circuit was first described by Langendorff in 1895^d, as an *ex-vivo* circuit to study the physiology of small mammalian hearts. The aorta is cannulated to allow flow of perfusate (most commonly Krebs-Henseleit buffer) into the coronary arteries in an antegrade fashion, however hearts are in 'resting' mode without contraction against a fixed afterload. In 1967, Neely et al modified the circuit by placing a second perfusion cannula in the left atrium, allowing flow of perfusate from the left atrium into the left ventricle and ejection into the aorta against a fixed afterload, thereby allowing functional assessment of the heart in 'working' mode³³⁵.

Strengths and limitations of the isolated rat heart perfusion circuit

The isolated heart perfusion circuit allows assessment of the whole organ with high throughput of results at a comparatively low cost³³⁶. It provides a stable model with fixed preload and afterload enabling reliable comparisons between control and intervention groups. This model is less resource intensive compared with large animal studies, and is more clinically relevant than methods such as cell culture³³⁷. Disadvantages of this model are that the conditions in the circuit differ from normal physiology with a lack of input from autonomic nerves and hormones, as well as a lack of blood components when using crystalloid perfusates^{336,337}. The study of small mammalian hearts provides an adequate screening model, however due to intrinsic physiological differences to the human heart, application of the results to clinical use may have limitations.

2.5.1 Description of the perfusion circuit

The custom-made circuit schematically outlined in (Figure 2.1) is based on the Langendorff perfusion circuit with modifications incorporated as per Neely et al as previously described³³⁵. This allows stabilisation of the heart in 'resting' (Langendorff) mode, and performing functional measurements by switching to 'working' mode.

^d Langendorff O. Untersuchungen am uberlebenden Säugethierherzen. Pflügers Arch für die Gesamte Physiologie des Menschen und der Tiere 1895;61:291-332

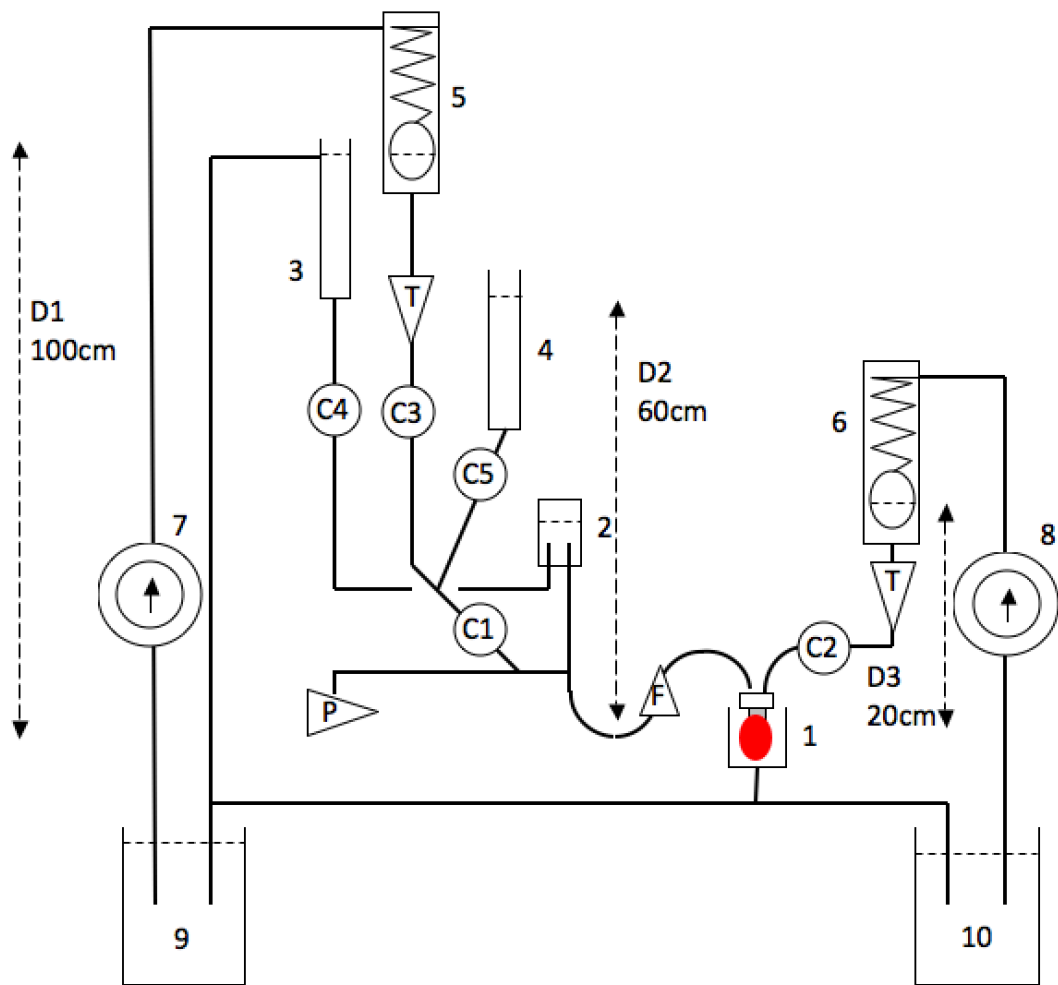


Figure 2.1 A schematic overview of the perfusion circuit

- | | |
|---|---|
| 1) Isolated heart and heart chamber with heart cannulated through the aorta and left atrium | C1-C5: clamps 1,2,3,4,5 |
| 2) Air trap | F: in-line flow probe |
| 3) Afterload chamber | P: pressure probe |
| 4) Cardioplegia chamber | T: temperature probe |
| 5) Langendorff heat exchanger | D1: distance between heart and Langendorff heat exchanger and afterload chamber |
| 6) Working-heart heat exchanger/preload chamber | D2: distance between heart and cardioplegia chamber |
| 7) Langendorff perfusion pump | D3: distance between heart and working-heart heat exchanger/preload chamber |
| 8) Working heart perfusion pump | — Bold dark lines: PVC tubing |
| 9) Langendorff buffer reservoir | - - - Dashed lines: fluid levels |
| 10) Working-heart buffer reservoir | |

2.5.1 1 The Langendorff circuit

The aorta and left atrium (LA) are cannulated onto the ex-vivo circuit as demonstrated in Figure 2.2. The aortic cannula is unclamped and the LA cannula is clamped during Langendorff or 'resting' mode. Figure 2.3 schematically outlines the perfusion of the heart in Langendorff mode. Two litres of modified Krebs-Henseleit solution (Table 2.3) are used as the Langendorff buffer reservoir. The solution is warmed to 37°C using a water bath and bubbled for 1 hour prior to use and during the experiment using carbogen (5% CO₂, 95% O₂) at 1.5L/min. This ensures oxygenation and maintenance of a physiologic pH of 7.3 - 7.4. A peristaltic pump drives the oxygenated buffer via an inline filter (5µm pore size) to the top of the Langendorff heat exchanger, and the perfusate flows through the heat exchanger, then into the aortic cannula via gravity at a pressure of 100cmH₂O. A high flow rate is maintained in the Langendorff circuit, however only 10-20ml enters the coronary circulation per minute to perfuse the heart. Excess buffer is diverted via the afterload circuit which acts as a conduit to return excess buffer back to the Langendorff reservoir. The aortic valve remains closed in Langendorff mode, hence the buffer flows into the coronary arteries, then enters the coronary sinus, right atrium and right ventricle and flows out via an incision made in the pulmonary artery. The coronary effluent drains into a chamber surrounding the heart and is returned to the Langendorff buffer reservoir.

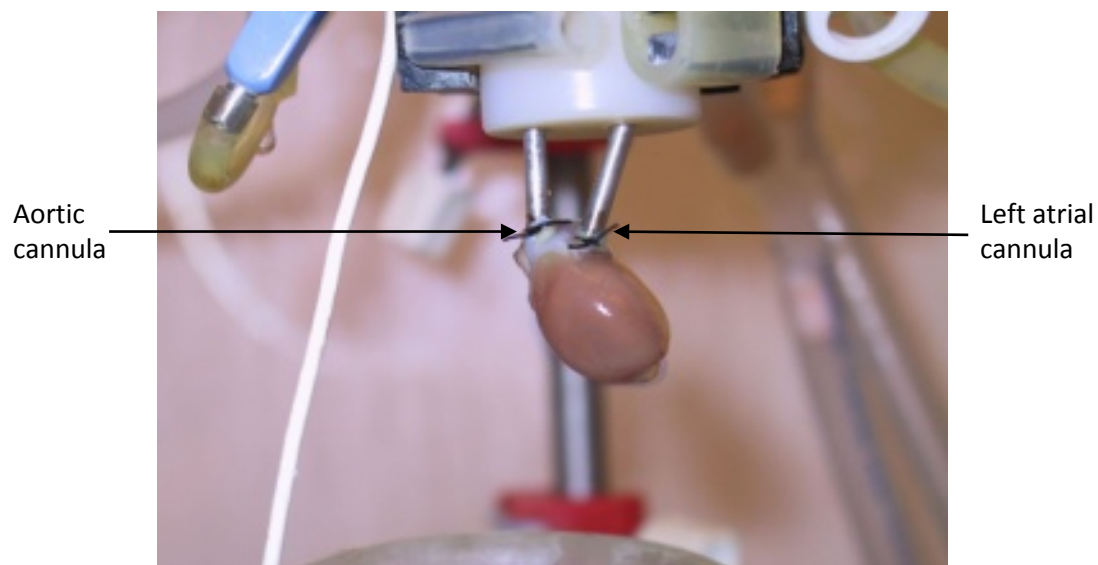


Figure 2.2 Cannulation of the rat heart at the aorta and left atrium

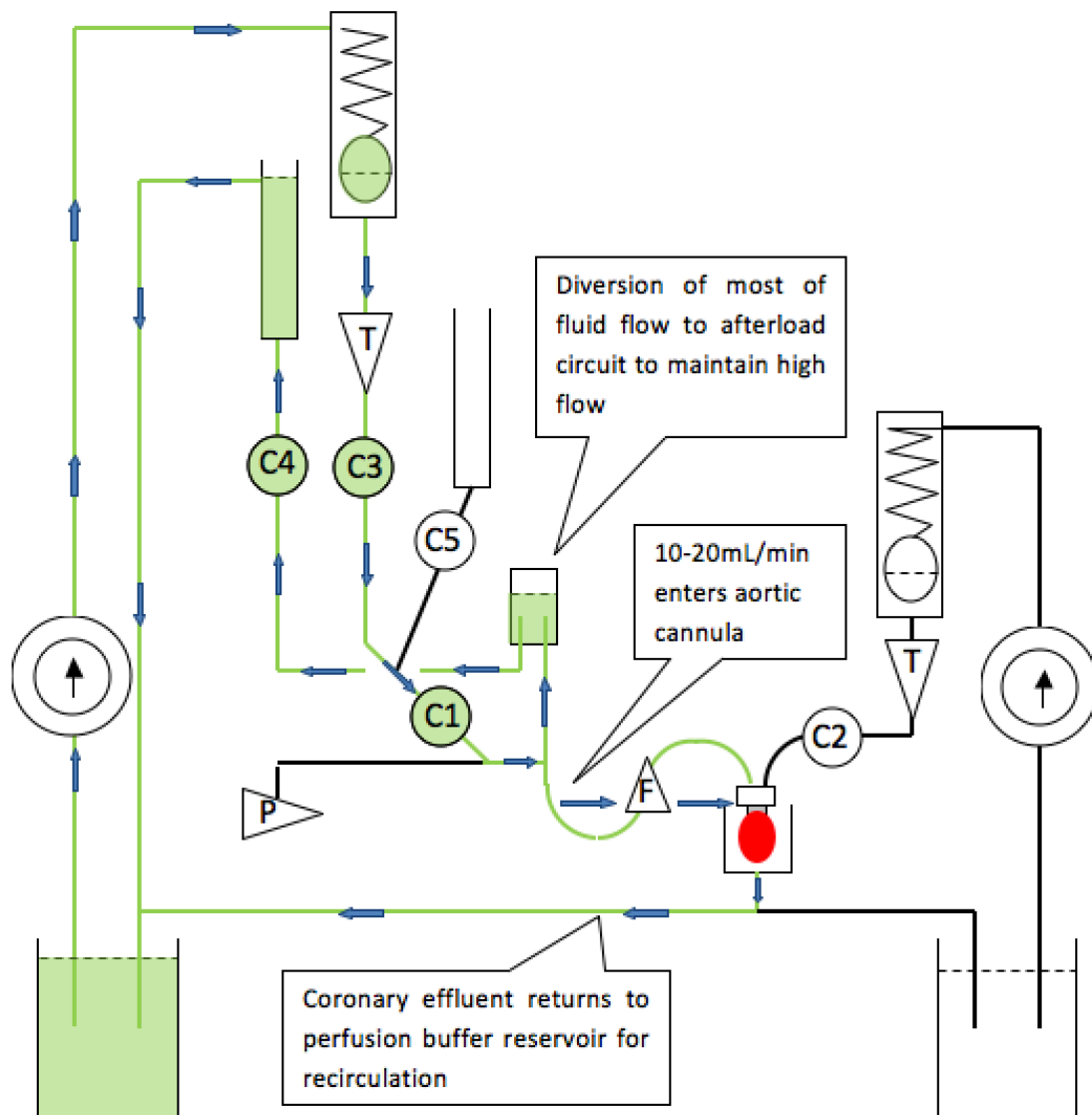
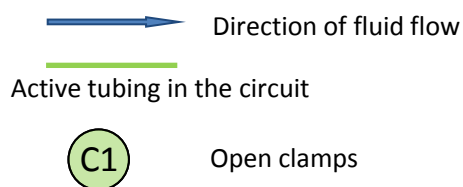


Figure 2.3 The perfusion circuit in Langendorff mode

Above is a schematic overview of flow through the perfusion circuit in Langendorff mode.



2.5.1.2 The heart in 'working' mode

Perfusion of the heart in working mode is outlined in Figure 2.4. Prior to perfusion in working mode, the heart is always stabilized in Langendorff mode for at least 10 minutes. For perfusion in the working mode circuit, two litres of modified Krebs-Henseleit solution is warmed, preoxygenated and pumped via a peristaltic pump through an inline filter, identical to that described for the Langendorff circuit.

The buffer is pumped into the working-heart heat exchanger/preload chamber, which is placed 20cm above the heart. The left atrial cannula is unclamped, allowing entry of buffer into the left atrium via gravity, at a pressure of 20cmH₂O (preload). Excess buffer drains out via side arms in the heat exchanger and is returned to the working-heart buffer reservoir (not indicated in diagram).

Buffer enters the left ventricle via the left atrium, and is ejected into the aorta against a fixed afterload of 100cmH₂O. Buffer ejected into the aorta perfuses the heart by entering the coronary circulation in diastole. The buffer ejected via the afterload chamber is drained back into the working-heart buffer reservoir and the circuit continues.

A flow probe placed in the aortic cannula measures the rate of flow (aortic flow, ml/min) of perfusate ejected from the heart. A pressure probe placed in the same cannula measure the pressure of buffer ejected from the heart into the aortic cannula.

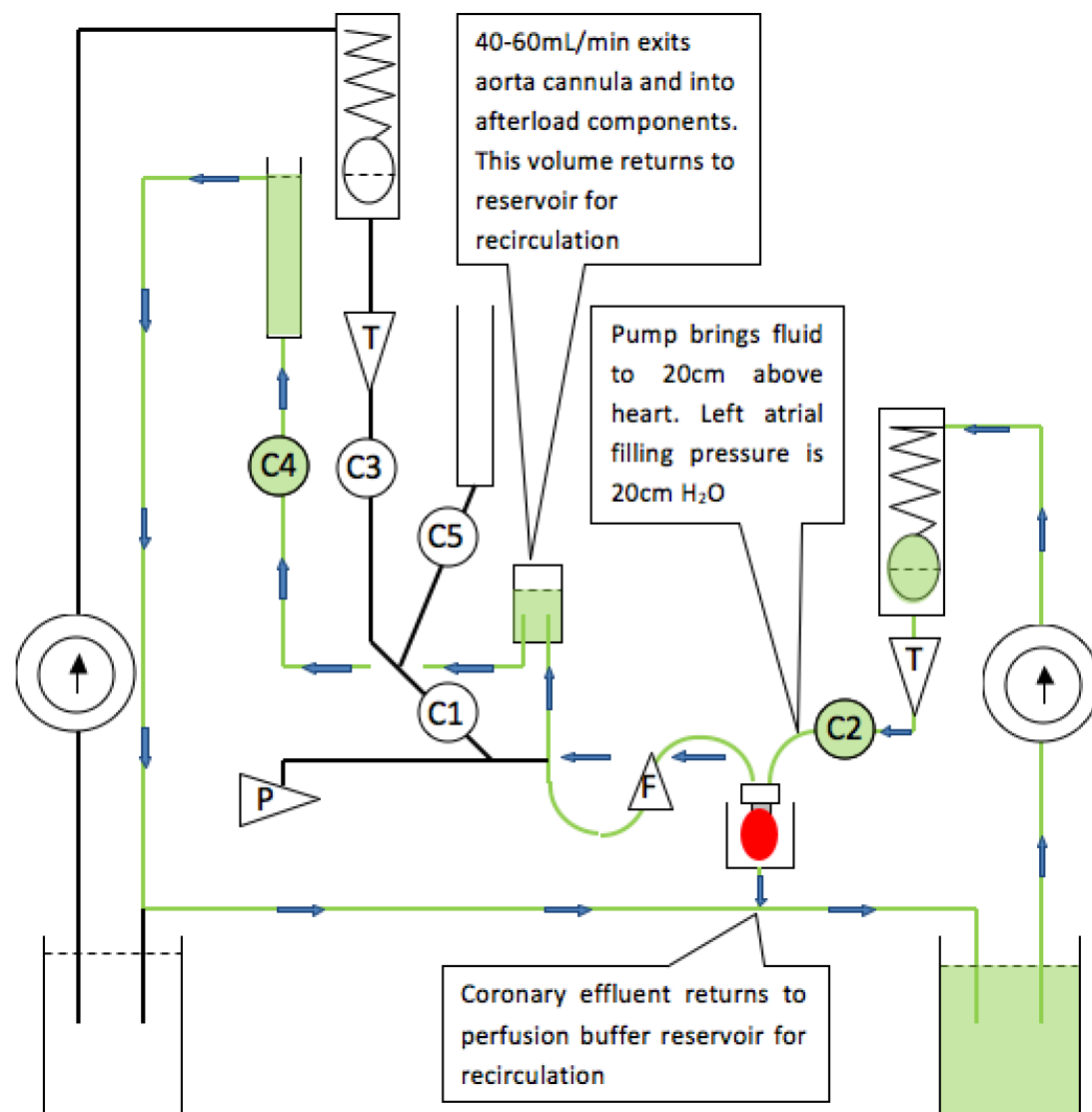
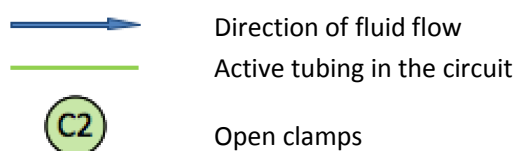


Figure 2.4 The perfusion circuit in working mode

A schematic overview of flow through the perfusion circuit in working mode.



2.5.1.3 Delivery of cardioplegia

Cardioplegia is delivered via a side arm as outline in Figure 2.5. The left atrial cannula is clamped. The cardioplegia chamber is filled with 45ml of hypothermic (2-3°C) cardioplegic solution, which enters the aortic cannula from a height of 60cm to perfuse the coronary arteries over 3 minutes.

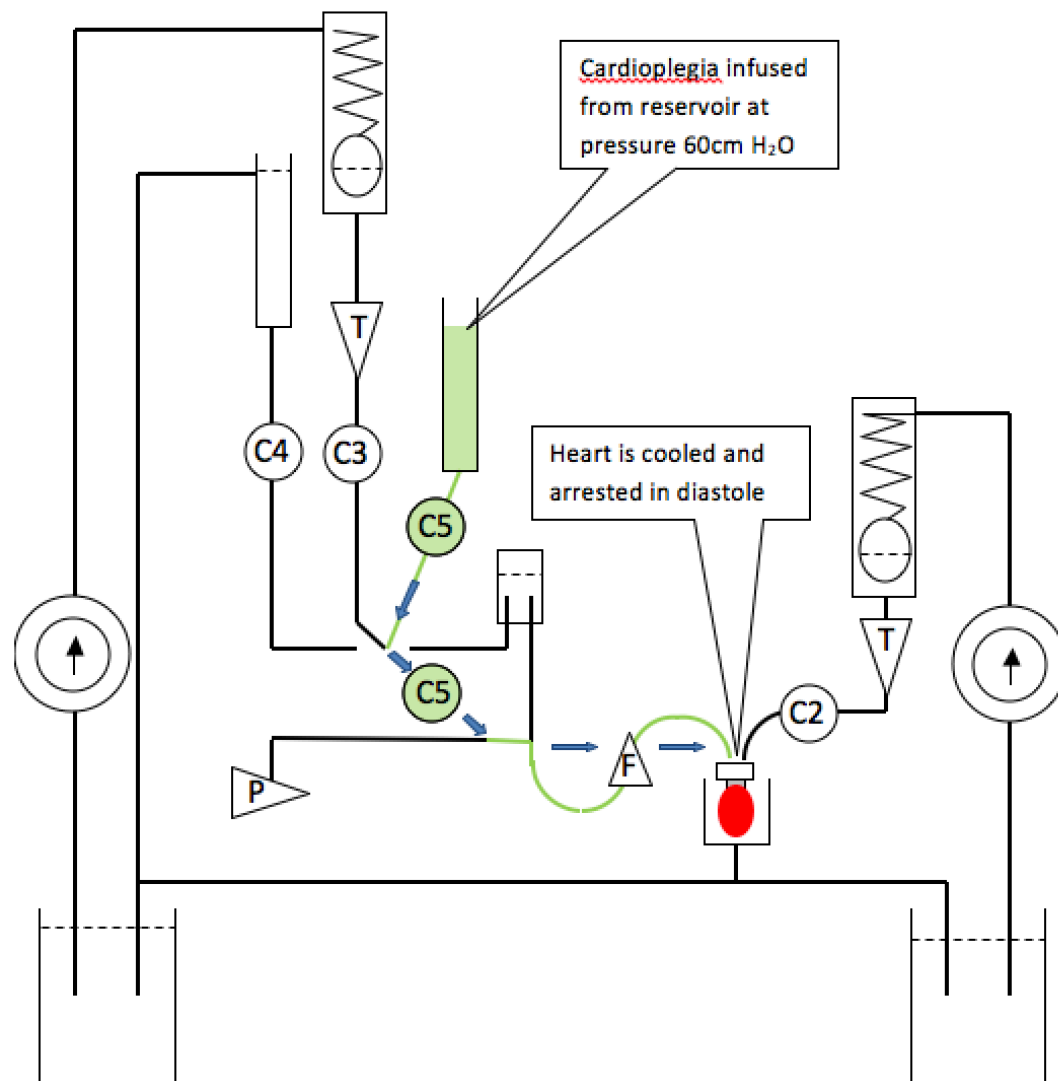


Figure 2.5 Delivery of cardioplegia

A schematic diagram of the flow of cardioplegia through the perfusion circuit.

2.6 The surgical procedure

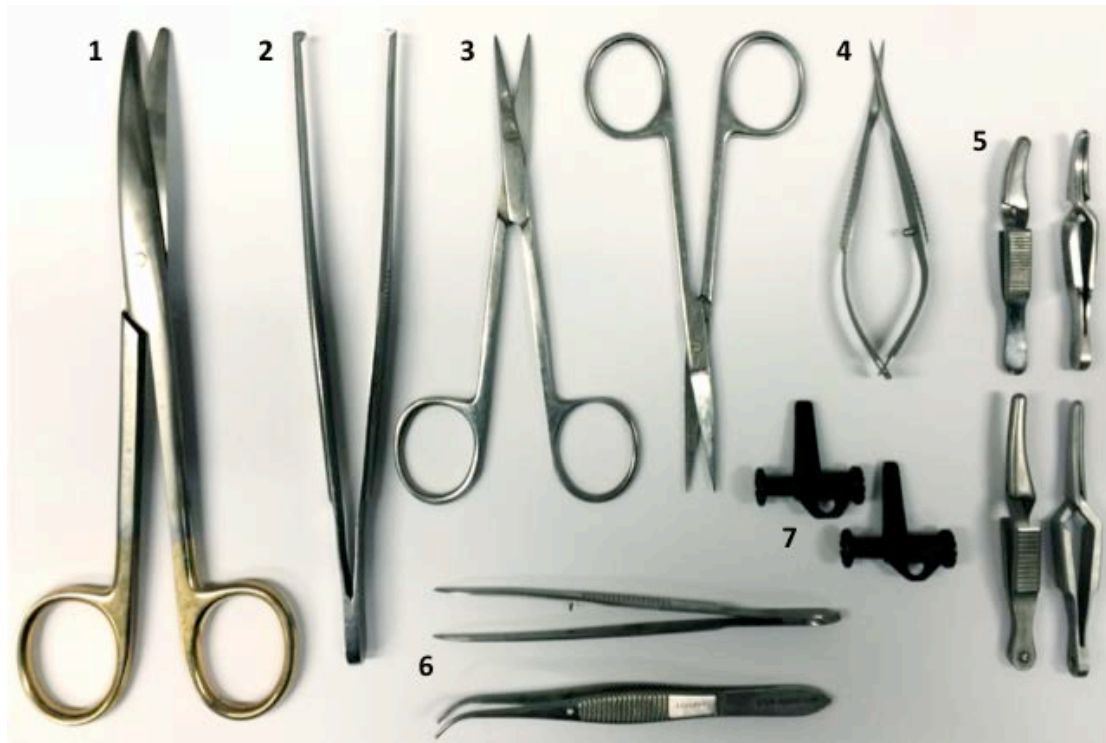


Figure 2.6 Surgical instruments

1. Curved Mayo scissors
2. Medium-toothed forceps
3. Curved sharp point scissors x 2
4. Straight-tipped micro-dissecting scissors
5. Small vascular clamps x 4
6. Curve-tipped fine iris forceps with serrated tips x 2
7. Plastic vascular clamps x 2

2.6.1 Preparation for the ex-vivo perfusion experiments

Four litres of Krebs-Henseleit buffer (Table 2.3) prepared on the day of the experiment and bubbled with carbogen for 1 hour as described above. The buffer is warmed in a water bath for 1 hour to 37.5°C. Filters (5µm pore size) are placed in the Langendorff and working-heart circuit lines. Approximately 100ml of buffer is chilled (but not frozen) in a -20°C freezer, for subsequent use in washing the heart soon after explantation. The buffer is circulated in the perfusion circuit and the aortic and LA cannulae are primed to remove air bubbles.

2.6.2 Anaesthesia and premedication

- i) Animals are anaesthetized with an intraperitoneal injection of ketamine (80mg/kg) and xylazine (10mg/kg). The depth of anaesthesia is determined by a toe-pinch after 5 minutes of injection of anaesthesia and the surgical procedure is commenced when there is absence of pedal reflexes. A supplementary intraperitoneal dose of ketamine (20mg/kg) is provided if pedal reflexes are persistent.
- ii) The animal is placed supine on a surgical mat and a transverse abdominal incision is made using Mayo scissors. Using a 25G needle and a 1.0ml tuberculin syringe, heparin (250 IU) is injected into the left renal vein for anticoagulation, and allowed to circulate for 1 minute.

2.6.3 Explantation of the heart

- i) The diaphragms are excised and two lateral thoracotomies are performed. The anterior thoracic cavity is reflected superiorly to expose the thoracic cavity.
- ii) Using curved sharp-point scissors, the heart, lungs and aortic arch are rapidly dissected *en bloc* and submerged in a petri dish containing the pre-chilled Krebs-Henseleit buffer to gently wash excess blood, and to achieve temporary cardioplegia for approximately 3.5 minutes.
- iii) The heart-lung bloc is placed on a cold cotton swab on a bed of ice during the period of temporary cardioplegia, to allow dissection prior to cannulation on the isolated heart perfusion circuit. The thymus and pericardial fat are dissected and the aorta is trimmed proximal to the subclavian and carotid branch vessels ensuring adequate length remains for cannulation.

2.6.4 Cannulation of the heart onto the perfusion circuit

- i) The aorta is cannulated first. The flow of buffer from the aortic cannula is slowed to approximately one drop per second, and the aorta is gently lifted using two curved forceps and placed on the aortic cannula on the Langendorff circuit (Figure 2.7A).
- ii) Small plastic clamps are used to temporarily secure the aorta on the cannula, and the flow of buffer is returned to the normal flow rate to allow adequate coronary perfusion (Figure 2.7B).
- iii) The aorta is then firmly secured on the cannula using 2-0 silk ties (Figure 2.7B)
- iv) Using micro-dissecting scissors, an incision is made in the pulmonary artery to allow the outflow of coronary effluent (Figure 2.7C).
- v) The lungs are tied at the hila and dissected free from the heart (Figure 2.7D).
- vi) An incision is made in the left atrial appendage (Figure 2.7E) and the left atrium is placed on the primed cannula in the working-heart circuit (Figure 2.7F).

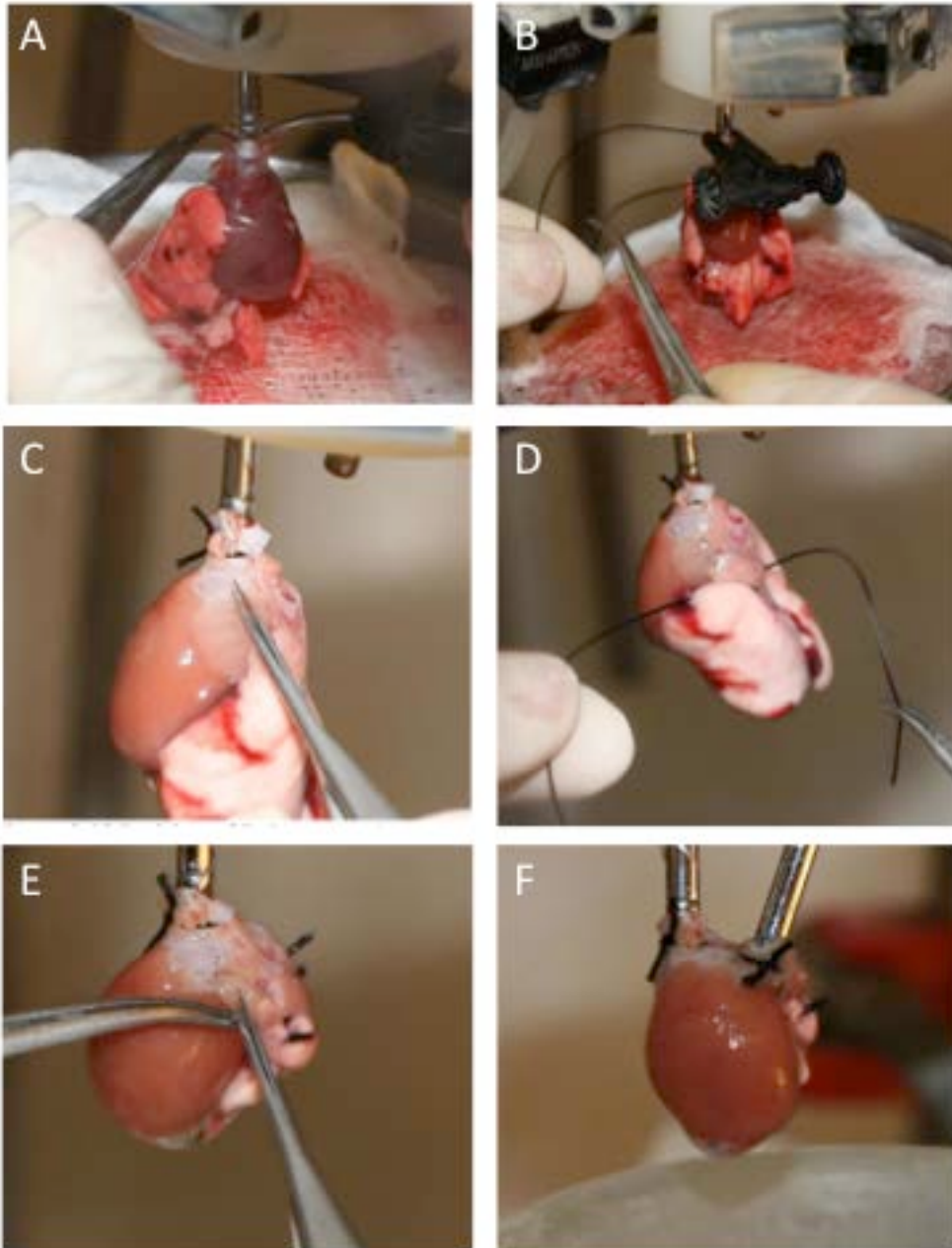


Figure 2.7 Cannulation of the heart onto the perfusion circuit

The aorta is cannulated first (A), then secured with 2-0 silk ties (B). An incision is made in the pulmonary artery using microdissecting scissors for outflow of coronary effluent (C). The lungs are tied at the hila and dissected free (D). An incision is made in the left atrial appendage (E) and the left atrium is cannulated and secured with 2-0 silk ties (F).

2.7 Assessment of baseline cardiac function

The heart is stabilised for 10 minute by perfusion in Langendorff mode. The circuit is then switched to 'working' mode for 15 minutes (Figure 2.8), and baseline cardiac function is evaluated.

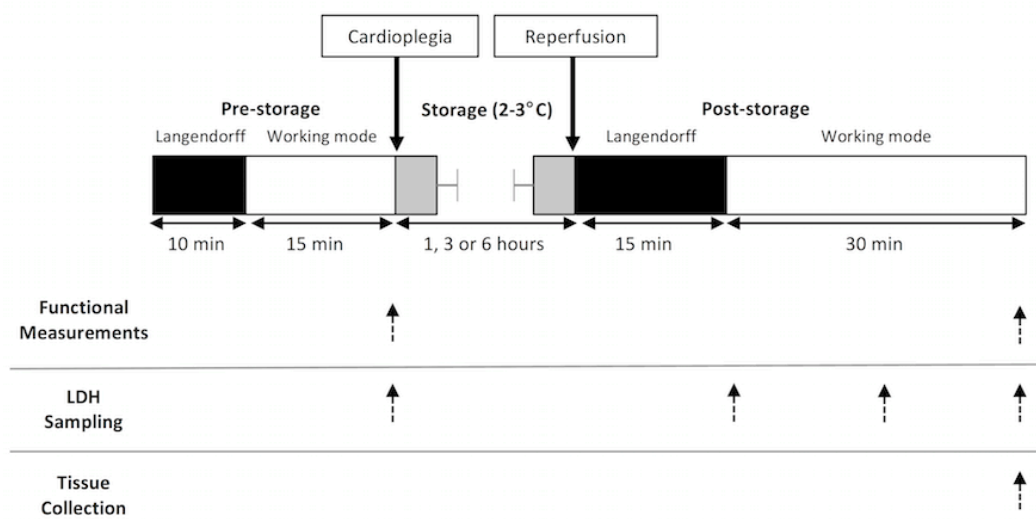


Figure 2.8 Experimental protocol

The above diagram outlines the protocol used in all studies using the ex-vivo functional assessment of the donor heart. Time-points for obtaining functional measurement, biochemical analysis and tissue collection are marked with arrows. LDH indicates lactate dehydrogenase.

The following four parameters are measured in working mode:

- Aortic flow (AF, ml/min)
- Coronary flow (CF, ml/min)
- Heart rate (HR, bpm)
- Cardiac output (CO, ml/min)

Aortic flow (AF, ml/min) is measured via an ultrasound flow probe placed at the outlet of the aortic cannula and a flow meter set (Transsonics Instruments Inc, Ithaca, NY). Data is acquired using a MacLab/ 4E realtime acquisition system and displayed and recorded using Chart 3 software (ADI instruments, Bella Vista, NSW, AU) as shown in Figure 2.9. Heart rate (HR, beats per minute) is derived from the aortic flow wave (number of peaks per minute) using Chart 3 software.

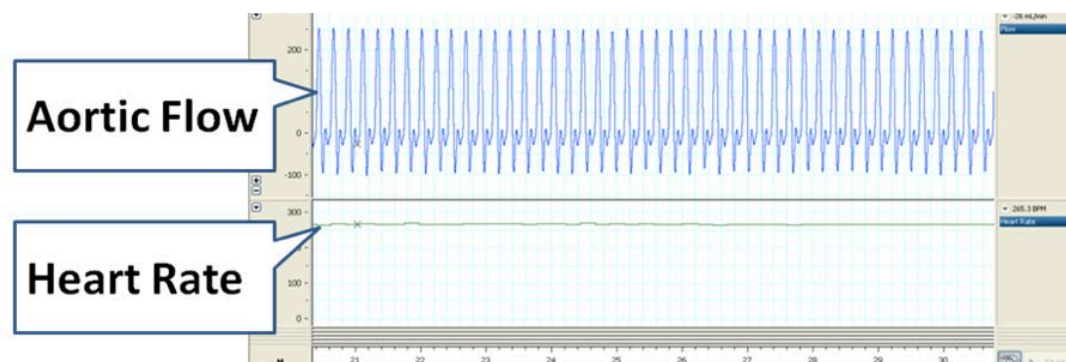


Figure 2.9 Representative image of aortic flow and heart rate in working mode

Aortic flow (AF, ml/min) in working mode is read by the flow meter and recorded as shown above. Heart rate (HR, bpm) is derived from the aortic flow reading (number of peaks in the aortic flow wave per minute).

Coronary flow (CF, ml/min) is determined by measuring the coronary effluent collected over 1 minute. Cardiac output (CO, ml/min) is determined by adding the aortic flow and coronary flow as shown below:

$$\text{Cardiac output (ml/min)} = \text{Aortic flow (ml/min)} + \text{Coronary flow (ml/min)}$$

Measurements of AF, CF, HR and CO at 15 minutes in working mode are recorded as 'baseline' parameters.

2.8 Cardioplegia and hypothermic storage

At the end of 15 minutes of working mode, the left atrial cannula is clamped and cardioplegia (2-3°C) is delivered via the aortic cannula over 3 minutes (Figure 2.5). Celsior preservation solution is used as the control in all experiments, with intervention groups comprised of treatment with pharmacological conditioning agents added to Celsior used in cardioplegia and hypothermic storage, described in subsequent chapters.

The heart is disconnected from the perfusion circuit. The tubing of aortic and left atrial cannulae remain attached to the heart, and are clamped using vascular clamps in order to prevent entry of air into the cannulae. Hearts are placed in a beaker with 100ml of preservation solution (Figure 2.10) and stored for a specified time period according to the experimental protocol. The heart is placed in a container with ice and placed in a 2°C refrigerator during the storage period.



Figure 2.10 Hypothermic storage of the donor heart

2.9 Assessment of post-storage cardiac function

After hypothermic storage, hearts are re-cannulated onto the circuit and perfused with 37.5°C Krebs-Henseleit buffer. The buffer is again bubbled with carbogen and warmed for 1-hour prior to use. Hearts are initially stabilised in Langendorff mode for 15 minutes, then placed in working mode for 30 minutes, during which the recovery of cardiac function is measured. Aortic flow and heart rate are continuously recorded during working mode. Coronary flow is measured every 5 minutes, as described previously, and cardiac output is determined by the addition of aortic and coronary flow.

Measurements of AF, CF, HR and CO at the end of 30 minutes in working mode are used as 'post-storage' values. The percentage recovery of AF, CF, HR and CO are determined by the following calculation:

$$\text{Percent recovery (\%)} = \frac{\text{Post-storage measurement}}{\text{Baseline measurement}} \times 100$$

2.10 Quantification of lactate dehydrogenase

The release of lactate dehydrogenase (LDH) into the coronary effluent was used as a surrogate marker for myocardial cell necrosis. Coronary effluent (1.5ml) was collected at baseline (end of 15 minutes of working mode, prior to cardioplegia) and at 16, 30 and 45 minutes at reperfusion after hypothermic storage (indicated in Figure 2.8). Duplicate aliquots were assayed with a TOX 7 assay kit (Sigma Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. Released LDH into the coronary effluent was measured by the absorbance of a tetrazolium product formed at 490nm in a 96-well plate reader.

2.11 Tissue collection

At the completion of post-reperfusion functional evaluation, hearts were removed from the perfusion circuit and rapidly dissected free from the cannulae. The heart was then bisected in cross section at the mid-ventricular level, and the basal anterolateral wall of the left ventricle was sectioned into 2-3mm cubes and placed in an eppendorff tube and submerged in liquid nitrogen. The samples were stored at -80°C for immunoblotting.

Of the remaining cardiac tissue, a 5mm thick cross-sectional sample between the apex and mid-ventricle was stored in 4% formaldehyde in phosphate-buffered saline PFA at 4°C for processing for histological analysis.

2.12 Western blot analyses

Sixty milligrams of left ventricular tissue from each heart were homogenized in ice-cold lysis buffer [150 mM NaCl, 50 mM Tris HCl, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM glycerophosphate, 5 mM DTT, protease inhibitor cocktail, (Roche complete “mini”; Roche Diagnostics, Australia), pH 7.4]. Samples were then centrifuged at 10,000 rpm (9,400 x g) for 5 minutes at 4°C. The protein concentration of each lysate was measured using a Bradford assay kit (Pierce Biotechnology Inc, Rockford IL), with bovine serum albumin used as standard.

Protein samples were then boiled in sample loading buffer for 5 minutes before loading (15µg per lane) onto 10% pre-cast SDS-polyacrylamide gel (Bio–Rad Laboratories Pty. Ltd., Regents Park, Australia). After electrophoretic separation on a Protean III system (Bio–Rad, Australia), proteins were transferred to a PVDF membrane (Millipore, Australia). Membranes were blocked for 1 hour in Tris buffered saline (pH 7.4) containing 1% BSA and 0.1% Tween–20.

Membranes were probed overnight with rabbit polyclonal antibodies raised against total and phospho-AMPKα (Thr¹⁷²), total and phospho-Akt (Ser⁴⁷³), total and phospho-STAT3 (Tyr⁷⁰⁵, Cell Signaling, St Louis, MO, USA) or mouse polyclonal antibodies against total and phospho-Erk1/2 (Thr²⁰²/Tyr²⁰⁴, Cell Signaling, St Louis, MO, USA) and α-tubulin (Sigma Aldrich, St. Louis, MO, USA). The presence of these antibodies was detected with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (GE Health Care Rydalmere, NSW).

The protein bands were visualised using enhanced chemiluminescence (GE Health Care Rydalmere, NSW). Band intensities were digitised then quantified with Image J freeware (Version 1.41, National Institute of Health, Bethesda, MA). The phosphorylation status of each protein is expressed as the ratio of phosphorylated to total antibody.

2.13 Histopathology

2.13.1 Tissue fixation

Fixation of myocardial samples was performed by storing the samples in 4% formaldehyde in phosphate-buffered saline at 4°C as described in section 2.11

2.13.2 Dehydration, clearing, infiltration and embedding

The samples were transferred through baths of increasing concentrations of ethanol (70%, 90%, 95% and 100% ethanol) at room temperature, each over 1 hour, and further dehydrated by storing in an overnight bath of 100% ethanol at -20°C.

Samples were then transferred to two consecutive baths of pure xylene. Paraffin infiltration was performed by transferring into a solution of 50:50 concentration of xylene and paraffin at 60°C over one hour, followed by transfer into 100% molten paraffin at 60°C over one hour. A further overnight transfer was performed by storing samples in 100% molten paraffin at 60°C. The samples were placed in molds filled with molten paraffin. A plastic cassette was placed over the mold and filled with molten paraffin which was solidified by cooling.

2.13.3 Sectioning and staining

The paraffin embedded samples were sectioned (5µm thick slices) using a steel knife microtome and mounted on a glass microscope slide. The sections were stained with haematoxylin and eosin using the following protocol:

- i) Dewax in xylene – 3 minutes
- ii) Dewax in separate container with xylene – 3 minutes
- iii) Ethanol 100% – 5 minutes
- iv) Ethanol 100% in separate container – 5 minutes
- v) Ethanol 70% – 5 minutes
- vi) Running distilled water – 2 minutes
- vii) Haematoxylin – 5 minutes
- viii) Distilled water – 3 x dips
- ix) Ethanol 70% + HCl 1% – 2 dips
- x) Rinse in distilled water briefly
- xi) Scott's blue (1L distilled water + 3.5g NaHCO₃ + 20g MgSO₄) – 2 minutes

- xii) Rinse in distilled water – 5-10 seconds
- xiii) Ethanol 70% – 30 seconds
- xiv) Ethanol 95% – 30 seconds
- xv) Eosin – 20-30 seconds (test slide required as intensity varies)
- xvi) Ethanol 95% – 30 seconds
- xvii) Ethanol 95% in separate container – 1 minute
- xviii) Ethanol 100% – 2 minutes
- xix) Ethanol 100% in separate container – 2 minutes
- xx) Xylene – 3 minutes
- xxi) Xylene in separate container – 3 minutes.

Slides were allowed to dry, and mounting medium (DPX mounting medium, Sigma Aldrich, St Louis, MO, USA) was placed over the section and sealed with a coverslip.

2.13.4 Histological analysis

An experienced anatomical pathologist, blinded to the experimental treatment assessed the above sections. Histological analysis included evidence of ischaemia-reperfusion injury (IRI), in particular the presence of myocardial oedema, contraction bands and cellular necrosis. A combined semi-quantification system (score of 0-4) was applied based on the degree of morphological changes from normal or near-normal to most severe changes for each marker of IRI. A score of 0 indicated normal myocardium and 4, severe IRI.

2.14 Statistical analyses

All continuous variables are expressed as mean \pm standard error of the mean (SEM) unless otherwise specified. All categorical data are expressed as number and percentage. Differences between groups were compared using one-way ANOVA with Tukey's post-hoc analysis. The unpaired t-test was used to compare differences between two groups. A p value < 0.05 was considered statistically significant. Data were analysed using Prism 6 software (GraphPad Software Inc., CA).

**CHAPTER 3. DEVELOPMENT OF A RODENT MODEL OF BRAIN
DEATH AND ASSESSING THE ABILITY TO
PHARMACOLOGICALLY CONDITION HEARTS FROM BRAIN
DEAD DONORS**

3.1 Introduction

Hearts used in cardiac transplantation are commonly obtained from brain dead donors who have suffered extensive central nervous system damage due to subarachnoid haemorrhage or traumatic brain injury⁴. Brain death is caused by a significant rise in intracranial pressure that leads to ischaemia and herniation of supratentorial structures and the pons and medulla vital to cardiorespiratory control, and it is defined as the irreversible cessation of cerebral and brainstem functions³³⁸.

Brain death is associated with complex haemodynamic, inflammatory, endocrine and metabolic dysfunction that can lead to inferior function of donor organs³³⁹. The haemodynamic instability of brain death is characterised by an initial Cushing response of bradycardia and hypertension caused by mixed vagal and sympathetic responses associated with pontine ischaemia³⁴⁰. This is followed by extreme tachycardia, vasoconstriction and hypertension caused by loss of vagal and cardiomotor nuclei during medullary ischaemia³⁴⁰. Finally, progressive loss of spinal sympathetic pathways leads to total sympathetic denervation with profound vasodilation and hypotension.

A catecholamine 'storm' is described during brain death, with supraphysiological levels of plasma adrenaline and noradrenaline measured in brain dead organ donors⁷⁴ and in experimental animal models of acutely raised intracranial pressure⁵⁹. It is attributed as a main causes of donor organ dysfunction^{60,74,77,341,342}. Of particular relevance to the donor heart is the increased myocardial work induced by direct catecholamine stimulation and a significantly increased afterload due to severe systemic hypertension and vascular resistance⁵⁸. Catecholamine-induced coronary vasoconstriction impairs myocardial oxygen supply, leading to ischaemic injury of the myocardium^{58,343}. Elevated plasma catecholamines have additional cardiotoxic effects such as causing myocardial calcium overload, depleting magnesium and phosphate, increasing intracellular sodium, uncoupling oxidative phosphorylation, increasing ATP hydrolysis and inducing the formation of oxygen free radicals^{77,344}. Myocardial hypoxia is evident by increased levels of lactate and adenosine in the interstitium^{62,65,345}.

The rate of increase of intracranial pressure has been described to range from induction of slower 'gradual' onset of brain death to more rapid or 'explosive' onset of brain death, with the latter associated with more severe end-organ injury^{56,59,68}. Histological analysis of unused hearts from brain dead donors and experimental animal models of brain death demonstrate subendocardial haemorrhage, cellular and mitochondrial oedema, interstitial mononuclear infiltrates, cardiomyocyte contraction bands, focal myocytolysis or widespread ischaemic necrosis^{56,59-61,63,64,342,346}. Similar myocardial damage has been demonstrated in patients dying from acute cerebral lesions^{347,348}.

The inflammatory response to brain death is also well documented. It is associated with elevation of a number of serum pro-inflammatory cytokines including interleukin (IL)-6, IL-1 α , tumour necrosis factor (TNF)- α , monocyte chemotactic protein (MCP)-1, E- and P-selectins, toll-like receptor (TLR) 4, heme-oxygenase-1, markers of injury p21 and Kim-1^{57,349-351}. Furthermore, pro-inflammatory genes are expressed in peripheral organs after brain death, and increased chemoattractants expressed on the endothelium lead to mononuclear cell and neutrophil migration, exacerbating donor organ injury^{68,69,349,352}. Studies by Birks et al^{71,72} demonstrated elevated serum and myocardial TNF- α , IL-6 and the activation of proapoptotic pathways in unused donor hearts with poor contractile function, indicating that donor organ dysfunction may be due to a more severe inflammatory reaction and what has been termed an 'explosive' onset of brain death. The pro-inflammatory state has also been implicated in increased susceptibility to allograft rejection^{353,354}. In addition to inflammatory and hemodynamic changes, hypothalamic and pituitary destruction during brain death causes thermoregulatory impairment and endocrinopathy of the brain dead, with depletion of thyroid stimulating hormone, corticotrophin releasing hormone and vasopressin release, with the latter resulting in diabetes insipidus commonly encountered in patients with brain death^{76,355-357}.

The endothelin axis, comprised of endothelins, their precursors and associated signalling pathways, has been demonstrated to be upregulated in animal studies of brain death (BD)³⁵⁸. Endothelin-1 causes vasoconstriction, stimulates inflammatory cell infiltration and is a smooth muscle cell and fibroblast mitogen. Activation of the endothelin axis by brain death has been studied in more detail in donor lung injury, where it has been implicated as a cause of donor right heart dysfunction secondary to elevated pulmonary pressures³⁵⁹.

Injury incurred due to unstable haemodynamics, the pro-inflammatory state and hormonal and electrolyte disturbances explain the regional or global cardiac contractile dysfunction that can be seen after brain death even in young donors without a prior history of cardiac disease^{75,360}. High dose inotropes and vasopressors are used for circulatory support of the brain dead donor, further exacerbating physiological stress on the heart by increasing myocardial oxygen demand and depleting high energy phosphates³⁶¹. Contractile dysfunction or myocardial 'stunning' is often transient and reversible at a macroscopic level^{58,66,362}, however injury at a cellular level often becomes evident with additional insults incurred during warm and cold ischaemia involved with organ procurement and transport. Brain death therefore sensitises donor organs to further injury during warm and cold ischaemia and warm blood reperfusion⁵⁹. Inferior function of renal and liver transplants from deceased donors over living unrelated donors is largely attributed to inflammatory and ischaemia-reperfusion injury incurred during brain death and warm and cold ischaemia^{354,363}. In context of cardiac transplantation, this combination of insults may manifest in the immediate post-transplant period as mechanical dysfunction and rejection, or manifest as later complications such as allograft vasculopathy and late graft dysfunction^{26,37,70,349,364}.

Previous studies in our laboratory have focused on developing myocardial preservation against ischaemia-reperfusion injury (IRI) by methods of pharmacological conditioning of the donor heart, which have been applied during cardioplegia and hypothermic storage^{55,324,325,329,365,366}. Significant functional improvements have been demonstrated with histological and biochemical evidence of reduced IRI. Several pharmacological agents have been tested for cardioprotective properties, and these include the nitric oxide donor glyceryl trinitrate (GTN)^{325,326,367}, activator of the ERK1/2, AKT-PI3k and JAK-STAT pathways erythropoietin (EPO)^{329,330}, sodium hydrogen exchange inhibitors zoniporide (ZON)³²⁴ or cariporide (CAR)³²⁰⁻³²² and the ligand of the ErbB family of tyrosine kinase receptors neuregulin-1 (NRG-1)³⁶⁶. Optimal cardioprotection was demonstrated when a combination of agents were used to provide synergistic action by targeting multiple pathways of cellular protection, with most cardioprotection provided with the combination of EPO, GTN and ZON^{329,330}.

However, these screening studies were performed on rodent hearts obtained from anaesthetised animals, rather than those that have undergone brain death. Given the significant impact of brain death and its contribution to inflammatory and ischaemic injury of

donor organs, one of the aims of the present study was to develop a small animal model that incorporates a valid model of brain death, in order to evaluate the efficacy of previously successful cardioprotective strategies on hearts subjected to brain death. Another aim was to develop a more clinically relevant small animal screening model that could be used to test future therapeutic strategies of maximising donor heart preservation.

In summary, the aims of the present study are:

- i) To develop a reproducible rodent model of donor brain death for use in screening studies
 - a. To be validated by assessing neurological, haemodynamic and inflammatory changes
- ii) To assess the recovery of hearts after brain death and hypothermic storage
- iii) To determine the efficacy of pharmacological conditioning with EPO, GTN and ZON in providing myocardial preservation in the context of brain death and hypothermic storage.

3.2 Methods

The process of developing and refining a rodent model of donor brain death is initially discussed. Investigation of the effects of brain death and hypothermic storage on cardiac function, and the application of pharmacological conditioning to hearts from brain dead donors are discussed in Section 3.2.5.

3.2.1 Developing a rodent model of donor brain death

3.2.1.1 Criteria used to determine brain death

The criteria for brain death were adopted from the guidelines of the American Academy of Neurology³⁶⁸. These were i) the absence of brainstem reflexes, ii) coma and iii) apnoea.

The following signs were used in the rodent model to meet the above criteria for brain death:

- i) Sustained absence of pedal reflexes
- ii) Sustained absence of corneal reflexes
- iii) Sustained absence of spontaneous respiration

3.2.1.2 Materials

The surgical instruments used are outlined in Figure 3.1 and the equipment used are listed in Table 3.1.

3.2.1.3 Animals

Male Lewis rats weighing 320-380g (aged 11-14 weeks) were used in this study. Twelve animals were used in developing the brain death protocol, which involved refining surgical techniques and determining the optimal method of induction of brain death. The steps involved in development of the brain death model are outlined in Table 3.2

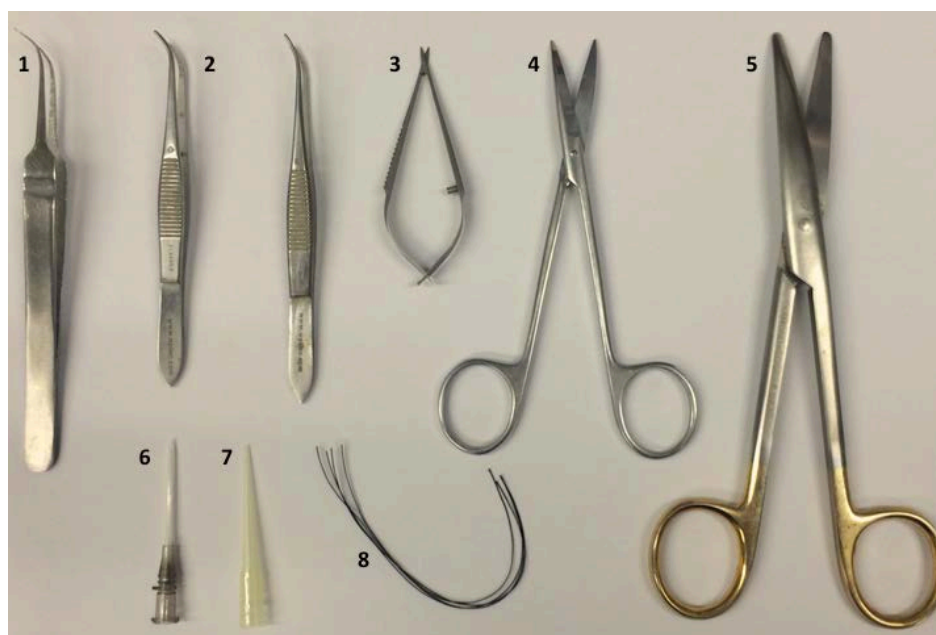


Figure 3.1 Surgical instruments

1) Curve-tipped delicate tissue forceps 2) Two curve-tipped fine iris forceps 3) Straight-tipped microdissecting scissors 4) Curved sharp point scissors 5) Curved Mayo scissors 6) 16-gauge plastic intravenous catheter sleeve 7) Pipette tip Gilson (200µl) 8) 2-0 silk ties

Table 3.1 Equipment for the brain death model

Equipment	Source
Small animal heat pad	Cenvet, Kings Park, AU
Rodent ventilator (Harvard Model 683)	Harvard Apparatus Inc, Houston, MA
Pulse oximeter (Masimo Rad-8)	Masimo, Frenchs Forest, AU
Fibreoptic illuminator	Dolan-Jenner Industries, Boxborough, MA
Micro thermocouple wire (IT-18)	Physitemp, Clifton, NJ
Micro-tip pressure catheter (SPC-320)	Millar Inc, TX
3F Embolectomy catheter (0.20ml)	Le Maitre Vascular Inc, Burlington, MA
Rotary micromotor (2.35mm)	Foredom Electrical Co, Bethel, CT

3.2.1.4 Anaesthesia

Anaesthesia was induced by an intraperitoneal injection of 80mg/kg ketamine and 10mg/kg xylazine as described in Section 2.6.2. The depth of anaesthesia was assessed by a toe pinch and corneal stimulation. If reflexes were present, supplemental anaesthesia was provided with 10mg of ketamine via intraperitoneal injection prior to commencing the surgical procedure.

3.2.1.5 Intubation and ventilation

i) All animals are placed supine on a small-animal heat pad to maintain body temperature between 36.5-37.5°C.

ii) Endotracheal intubation is performed via the oropharynx using a 16-gauge intravenous cannula sleeve (BD Insyte Autoguard, Becton Dickinson & Co, New Jersey) under direct vision. A light source is placed over the neck to aid visualisation by illuminating the larynx and vocal cords (Figure 3.2A and B).

iii) In the event of laryngospasm or difficulty with intubation, a midline neck incision is made and the intravenous catheter sleeve is inserted directly via a tracheotomy (Figure 3.2C and D) and secured with 2-0 silk sutures.

iv) Ventilation is provided using a rodent ventilator set to 2.5ml of tidal volume and 60 breaths/minute. Inflow and outflow lines from the ventilator are connected to a t-tube (Figure 3.2B) and the third end attached to the endotracheal cannula sleeve. All lines are secured onto the surgical bench to ensure the endotracheal tube remains in place.

v) Oxygen is supplied via the ventilator at a flow rate of 0.8 l/min, with wall suction set at 0.4 l/min.

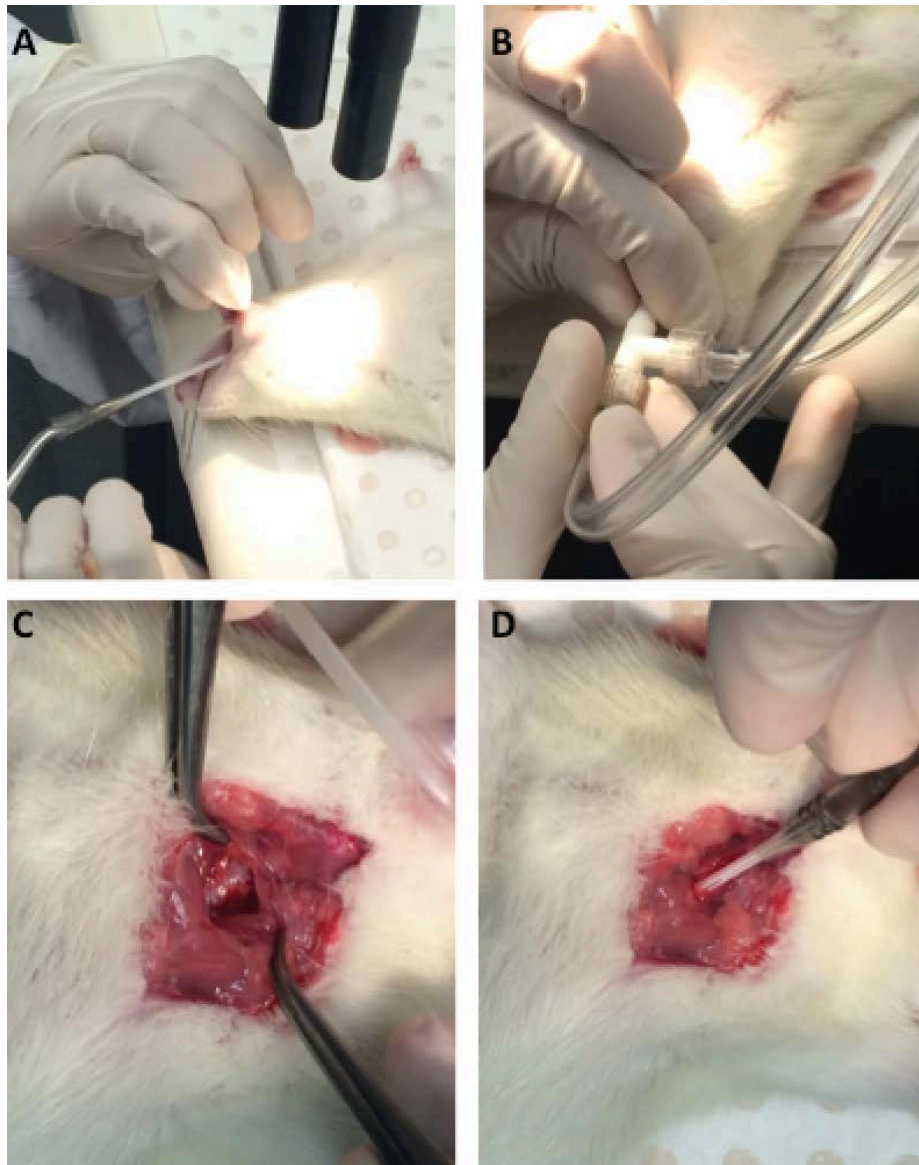


Figure 3.2 Intubation of the rat.

Orotracheal intubation is performed as shown in (A) using a 16-gauge cannula sleeve and a light source to illuminate the vocal cords, and connection of the cannula sleeve to the ventilator lines using a t-tube (B). If orotracheal intubation is hindered by persistent laryngospasm, a midline neck incision is made to expose the trachea (C) and the cannula sleeve inserted via a tracheotomy (D).

3.2.1.6 Monitoring pulse oximetry and core body temperature

- i) A veterinary pulse oximeter is secured onto a hind-paw for non-invasive continuous measurement of oxygen saturations. Pulse oximetry saturations are maintained above 95%.
- ii) The abdomen is covered with aluminium foil for insulation of body heat. Core body temperature is continuously monitored using a rectal micro thermocouple wire with readings displayed and recorded using LabChart Software (ADI Instruments, Bella Vista, AU).

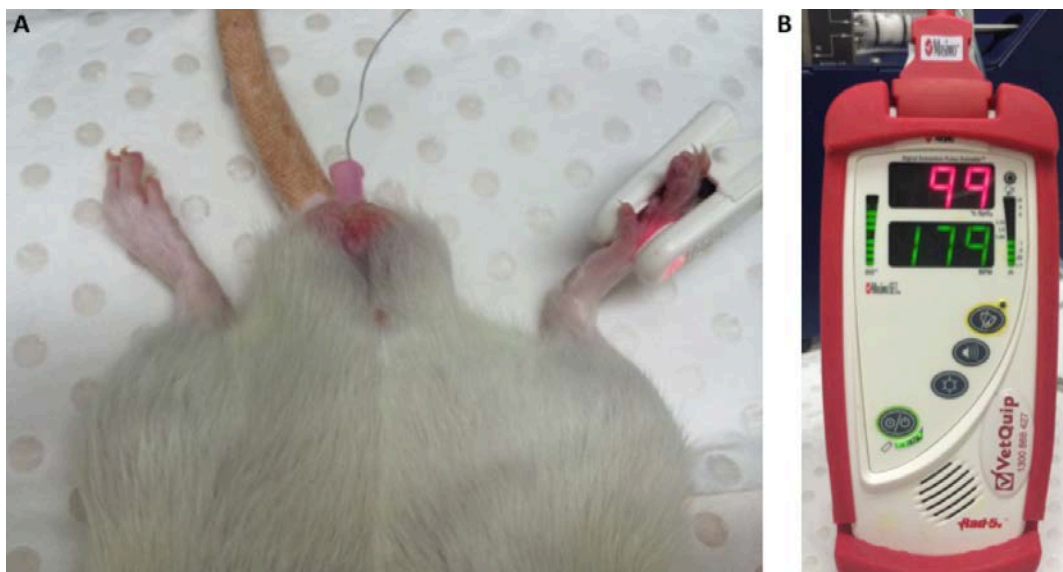


Figure 3.3 Monitoring pulse oximetry and core body temperature

Core body temperature is recorded using a micro-thermocouple wire inserted per-rectally (A) and pulse oximetry monitored from a hind-paw using a veterinary oximeter (A and B).

3.2.1.7 Invasive haemodynamic monitoring

- i) An anterior midline neck incision is made and the right common carotid artery is exposed beneath the sternomastoid and sternohyoid muscles (Figure 3.4A).
- ii) A 200µl pipette tip (Neptune Scientific, San Diego, CA, USA) is used to lift and stabilise the artery (Figure 3.4B) and 2-0 silk ties (Sof silk, Covidien, Ireland) are placed around the artery at the inferior and superior ends for retraction. A third tie is looped twice around the artery, just superior to the inferior tie, and loosely tied for later use to secure the pressure catheter. A partial incision is made on the anterior wall of the carotid artery using microdissecting

scissors (Figure 3.4C). Haemostasis is achieved by retracting the artery with the ties, and cotton tips are used to clear the surgical field of blood.

iii) Curved delicate tissue forceps are used to lift the distal end of the incised lumen of the carotid artery and a micro-tip pressure transducer is inserted under direct vision and secured in place with the double-looped 2-0 silk tie (Figure 3.4D).

iv) Continuous invasive arterial blood pressure readings from the pressure transducer are displayed and recorded using LabChart Software (ADI Instruments, Bella Vista, AU). Heart rate is derived from these readings by calculating the number of systolic peaks per minute.

vi) Mean arterial pressure (MAP) is calculated using systolic and diastolic pressure readings according to the following relationship:

$$\text{Mean arterial pressure (MAP)} = 1/3 [\text{systolic pressure} + 2 \times \text{diastolic pressure}]$$

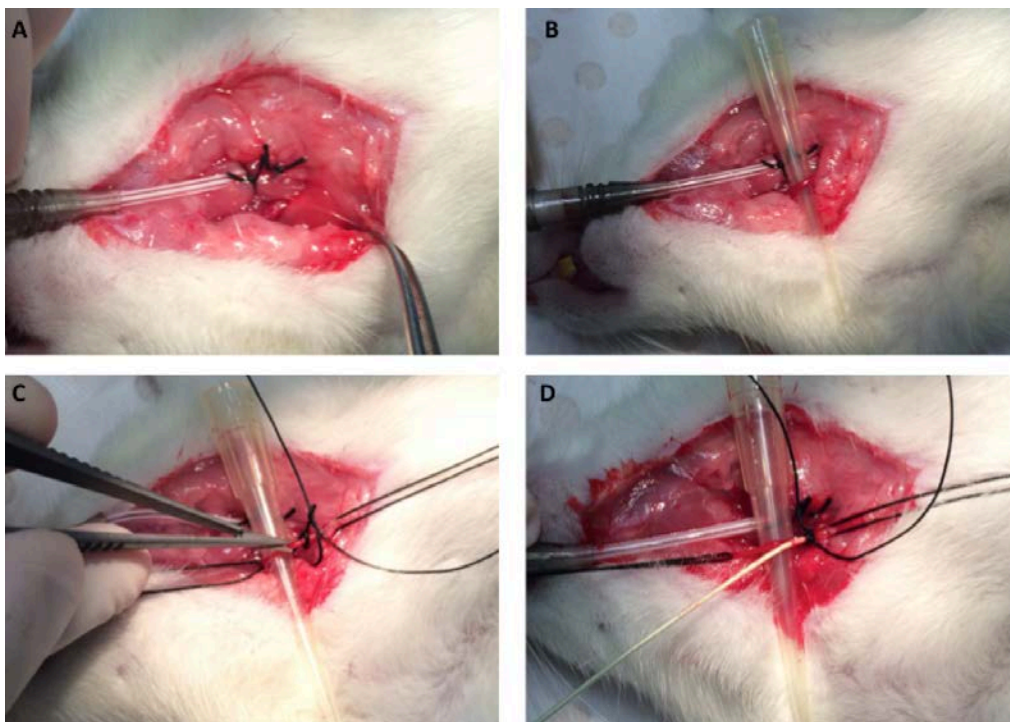


Figure 3.4 Placement of a pressure catheter in the carotid artery

3.2.1.8 Creating the burr hole and placing the intracranial embolectomy catheter

A rodent model of brain death described by Pepe et al³⁶⁹ was used as a guide in developing the model. The anatomical location for creation of the burr hole was initially chosen as the occipital skull to provide maximum pressure to the brain stem during inflation of the intracranial balloon catheter. However, drilling the burr hole at this location was associated with significant bleeding and at times death of the animal (Rat 1 and 3, -). The position of the burr hole was therefore revised to the right temporo-parietal bone, which proved a better position due to ease of drilling with less bleeding thus maximising animal survival (Rat 5, -).

i) Animals are placed prone with care taken to prevent dislodgement of orotracheal tube or the intravascular pressure catheter.

ii) An incision is made in the right temporo-parietal skin at a location where the skull is felt flat on palpation. The scalp is dissected to expose the bone beneath (Figure 3.5A).

iii) A burr hole is created using a 2.35mm rotary micromotor (Figure 3.5B). Care is taken to prevent injury to the underlying cerebrum. Bleeding is controlled with a cotton tip.

iv) A pretested, deflated 3F embolectomy catheter (specified balloon volume 0.20ml) was inserted gently via the burr hole ensuring that the full length of the balloon is placed subdurally (Figure 3.5C). The placement of the catheter was often associated with bleeding (< 0.50ml) and a small rise in heart rate and blood pressure.

v) The catheter is pre-loaded with water to ensure that air was removed from the catheter lumen. It is secured in place by inserting a 1.5 x 1.5cm piece of gauze for compaction of the burr hole and further secured by suturing the scalp incision using 2-0 Vicryl sutures (Ethigard, Ethicon Inc, Sommerville, NJ).

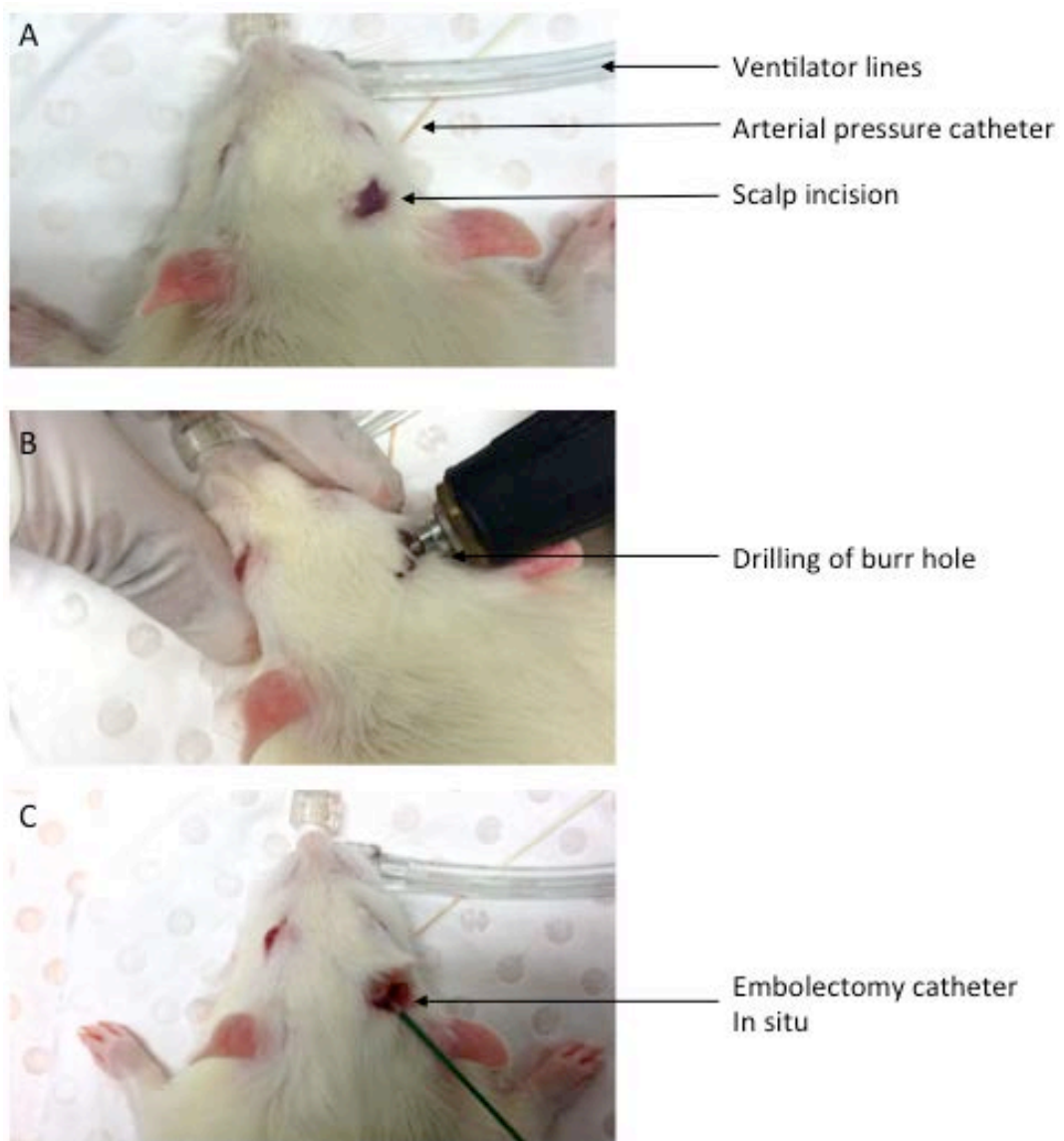


Figure 3.5 Creating a burr hole and placement of the intracranial balloon catheter

An incision is made in the temporoparietal scalp (A), and a burr hole is drilled using a handheld micromotor (B). An embolectomy catheter is gently placed subdurally, minimizing trauma and bleeding.

Table 3.2 Steps involved in developing the rodent model of brain death

Trial animal	Weight	Experimental procedure	Result
Rat 1	328g	Observing a demonstration of orotracheal intubation and dissection of the neck for insertion of the intravascular pressure catheter. Trial of creation of a burr hole at occiput.	Successful intubation, neck dissection and insertion of pressure catheter. Significant bleeding during burr hole with animal death.
Rat 2	335g	Practicing intubation and neck dissection for insertion of the intravascular catheter. Trial of creating a burr hole at the region of the brainstem and insertion of the embolectomy catheter.	Successful intubation, insertion of pressure catheter and creation of burr hole. Twenty min of hemodynamic observation performed.
Rat 3	380g	Practicing intubation and neck dissection for insertion of the intravascular catheter. Burr hole created at the region of the brainstem and insertion of the embolectomy catheter.	Successful intubation and insertion of pressure catheter. Significant intracranial bleeding at time of creation of burr hole with animal death.
Rat 4	373g	Practicing intubation	Animal death due to prolonged hypoxia during intubation (laryngospasm, tracheal injury).
Rat 5	358g	Intubation and intravascular pressure catheter insertion. Practicing creating the burr hole in right temporo-parietal bone. Practicing the protocol for induction of brain death: Hand injection of 0.20ml water into embolectomy catheter over 1min.	Minimal bleeding with burr hole and placement of intracranial embolectomy catheter. Explosive onset of brain death with rapid hemodynamic deterioration and animal death at 30min.
Rat 6	346g	Intubation, insertion of intravascular pressure catheter. Burr hole created in the right temporo-parietal bone. Refining the protocol for induction of brain death: Hand injection of 0.20ml of water into embolectomy catheter over 2min.	Minimal haemodynamic changes observed. Animal observed for 1 hour. Return of reflexes at 30min requiring additional anaesthesia.

Table 3.2 (continued). Steps involved in developing the rodent model of brain death

Trial animal	Weight	Experimental procedure	Result
Rat 7	343g	Refining the protocol for induction of brain death: Hand injection of 0.25ml of water into embolectomy catheter over 2min. Additional 0.05ml injected to obtain haemodynamic changes.	A significant rise seen in BP and HR after injection of a total of 0.30ml of water. The animal survived for 1h of hemodynamic observations.
Rat 8	372g	Refining the protocol for induction of brain death: Hand injection of 0.3ml of water into embolectomy catheter over 2min. Aimed for three hours of hemodynamic observations.	A significant rise seen in BP and HR with injection of 0.30ml of water. The animal died after 2h (hypoxic and hypothermic).
Rat 9	342g	Established the protocol for induction of brain death as injection of 0.30ml of water over 2min into embolectomy catheter. Attempted to maintain animal hydration during observation by injection of 1ml of saline intraperitoneally at time = 0 and 1h.	Haemodynamic changes as with rats 7 and 8. The animal survived for 3 hours.
Rat 10	365g	Assess stability of animal during protocol of induction of brain death as described for Rat 9 and during monitoring. Injection of 1ml of saline intraperitoneally at time=0 and 1h.	Haemodynamic changes as with rats 7-9. The animal died at 2h with deteriorating BP and HR.
Rat 11	338g	Assess stability of animal during protocol of induction of brain death as described for Rat 9. Injection of 1ml of saline intraperitoneally at time=0 and 1hour for hydration.	Haemodynamic changes as with rats 7-10. The animal survived for 3h.
Rat 12	340g	Assess stability of animal during protocol of induction of brain death as described for Rat 9. Intraperitoneal saline not injected.	Haemodynamic changes as with rats 7-11. The animal died at 2h with deteriorating BP and HR.

3.2.1.9 Development of a protocol for induction of brain death

Optimising the speed and volume of water used to inflate the balloon catheter was critical to the success of the final model. The ideal protocol would allow a rise in intracranial pressure adequate to cause ischaemia and herniation of the brainstem with evidence of haemodynamic changes associated with brain death. However, it should not cause such rapid changes in intracranial pressure that would cause 'explosive' onset of brain death with severe hemodynamic instability leading to animal death⁵⁹.

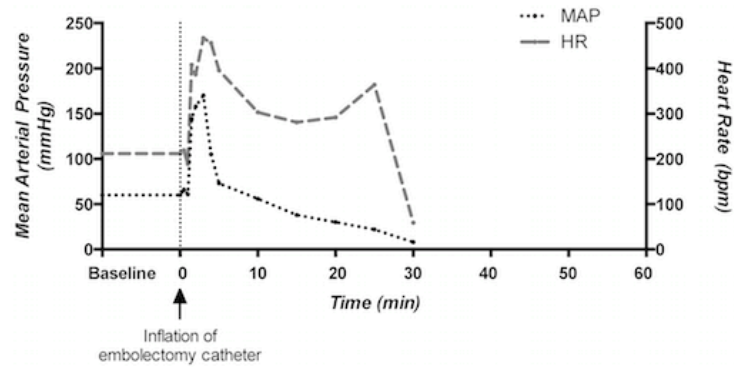
The details of the procedures conducted to refine the protocol are outlined in Table 3.2. Representative graphs of haemodynamic changes observed in each protocol are shown in Figure 3.6.

i) Protocol 1

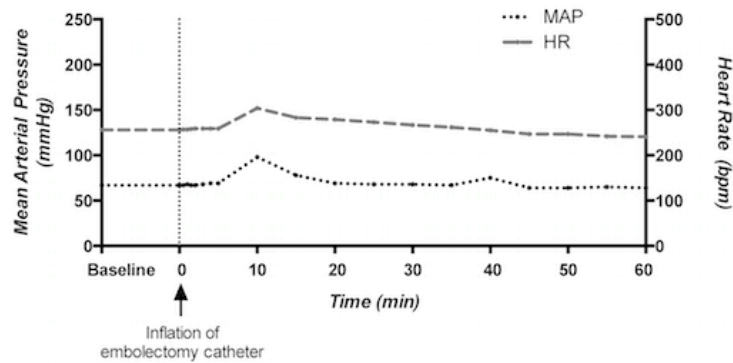
The initial protocol tested involved inflation of the embolectomy catheter with 0.20ml of water over 1 minute (Rat 5, Table 3.2). Injection via a syringe driver was attempted but hand injection was preferred due to technical difficulties with the syringe driver. The speed of hand injection was approximately 0.03ml per 10 seconds to inject the above volume in 1 minute. A timer and a graduated 1.0ml tuberculin syringe (Beckington Dickson Inc, North Ryde, NSW, AU) were used. After inflation over 2 minutes, the balloon was kept inflated by closing the lumen of the catheter with a three-way tap.

After 0.1ml was injected, the animal was noted to undergo twitching of the facial muscles and tremor of the limbs. At completion of inflation of the embolectomy catheter, kicking of the hindlimbs and extensor posturing was noted. The haemodynamic changes are shown in Figure 3.6(A). Baseline heart rate (HR) was 212bpm and mean arterial pressure (MAP) 60mmHg.

(A) Injection of 0.20ml water into embolectomy catheter over 1 minute (Rat 5)



(B) Injection of 0.2ml water into embolectomy catheter over 2 minutes (Rat 6)



(C) Injection of 0.30ml water into embolectomy catheter over 2 minutes (Rat 10)

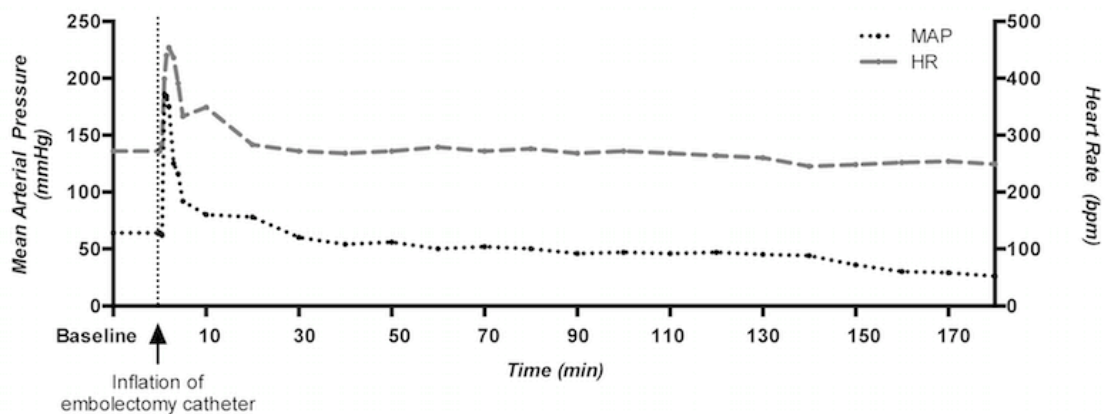


Figure 3.6 Examples of haemodynamic changes observed in the trial protocols for induction of brain death.

Rapid inflation of the embolectomy balloon (A) resulted in death of the animal at 30 minutes. Slower inflation (B) produced mild haemodynamic changes. A moderate speed of inflation produced haemodynamic changes demonstrated in (C) and allowed animal survival up to 3 hours.

A significant haemodynamic rise was noted with a peak MAP 170mmHg and peak HR 467bpm at 3 minutes after commencing inflation of the embolectomy catheter [Figure 3.6(A)]. However, there was subsequent rapid deterioration of the MAP associated with initial tachycardia then bradycardia, and ultimately animal death. This protocol created hemodynamic changes similar to the 'explosive' onset of brain death described in the literature⁵⁹ and was therefore modified as described below.

ii) Protocol 2

A slower injection speed of 2 minutes using the same volume of water was used in this protocol (Rat 6, Table 3.2). Injection was performed at 0.015ml per 10 seconds. The balloon was kept inflated after the 2 minutes of injection. Mild twitching of the facial muscles and tremor of the limbs were noted at the completion of balloon inflation.

Figure 3.6(B) demonstrates the haemodynamic changes observed. The baseline HR was 256bpm and MAP 67mmHg. The MAP and HR rose with less intensity to a peak of 98mmHg and 304bpm respectively at 10 minutes after commencing inflation of the embolectomy balloon as shown in Figure 3.6(B). Haemodynamic observations were performed for 1 hour. At 30 minutes, pedal reflexes returned and the animal required supplemental anaesthesia of 10mg of intraperitoneal ketamine. This indicated that although a rise in MAP and HR were observed, the speed and intensity of the pressure created was inadequate to cause brain death.

iii) Protocol 3

The balloon was inflated over 2 minutes using 0.25ml of water (approximately 0.02ml per 10 seconds). An additional 0.05ml was injected at the end of 2 minutes, since a hemodynamic change was not noted with 0.25ml, therefore a total volume of 0.30ml was used. Although the specified capacity of the embolectomy balloon was 0.20ml, the further 0.10ml volume was accommodated without damage to the balloon. The balloon was kept inflated after the initial injection. The animal was noted to undergo facial twitching, tremor of the limbs during inflation and kicking with extensor posturing at completion of inflation of the embolectomy catheter.

Representative haemodynamic changes using this protocol are shown in Figure 3.6(C), with haemodynamics of Rat 10 was chosen for representation of MAP and HR for 3 hours after induction of brain death. The baseline HR and MAP were 272bpm and 67mmHg respectively. A peak MAP of 186mmHg was noted at 1 minute and peak HR 454 at 2 minutes after commencing inflation of the embolectomy catheter.

Over the 3 hours of haemodynamic monitoring, pedal and corneal reflexes remained absent and respirations did not recur when ventilation was temporarily ceased. The MAP was noted to gradually reduce over the 3 hours, although HR remained relatively stable, which indicated that this protocol created a valid model of brain death with a significant initial haemodynamic rise, but also evidence of gradual haemodynamic deterioration consistent with what is seen clinically. The total of 0.30ml was sufficient to raise the blood pressure (Rat 7) and therefore used in Rats 8-12 (Table 3.2) and established as the brain death protocol.

3.2.1.10 Haemodynamic monitoring

The optimal time period for monitoring hemodynamic changes was then assessed (Rats 7-12, Table 3.2). The initial protocol included 3 hours of hemodynamic monitoring, however most rats underwent a gradual deterioration in blood pressure and did not survive beyond 2 hours. Pharmacological inotropic agents or vasopressors were not used to maintain hemodynamic stability, hence a protocol of 1 hour of hemodynamic observations was used to prevent the loss of animals. The inflation volume and speed were not reduced, as these reliably produced brain death.

During the 1 hour of hemodynamic monitoring, pedal and corneal reflexes were checked every 5 minutes and none of the animals subjected to the brain death protocol regained reflexes. Ventilation was ceased every 15 minutes for 10-15 seconds to assess for the return of spontaneous respirations, and again none of the animals regained spontaneous respirations. Body temperature, heart rate (HR), MAP and oxygen saturations were continually monitored during the 1-hour observation period.

3.2.2 Development of the sham procedure

Sham animals underwent the same anaesthetic protocol, intubation and ventilation protocol, methods of maintenance of body temperature and continuous monitoring of oxygen saturations and temperature. The surgical procedure of insertion of an intravascular pressure catheter using the right common carotid artery was also kept constant as was the location and method of creation of a burr hole.

The sham model initially included placement of the embolectomy catheter subdurally, however since this created bleeding and at times a rise in blood pressure and HR, it was decided to omit this step due to a risk of potential intracranial injury that may confound results. Sham animals therefore did not have any further surgical intervention after creation of the burr hole.

Checks for pedal and corneal reflexes and the return of spontaneous breathing were performed as for animals subjected to brain death. Spontaneous respiration did not recur, however all sham animals regained pedal and corneal reflexes between 30-40 minutes into haemodynamic observations. All sham animals were provided with one supplemental doses of 10mg of ketamine via an intraperitoneal injection, and reflexes checked to ensure they remained absent until explantation of the heart.

3.2.3 Assessment of cardiac function

The cardiac function in brain death and sham animals were assessed on an ex-vivo perfusion circuit as described in Chapter 2, Section 2.7. A study on the assessment of cardiac function after brain death and hypothermic storage will be discussed in detail below, in Section 3.2.5.

3.2.4 Assessment of the inflammatory response to brain death and sham procedure.

Circulating inflammatory cytokines were measured at baseline and after one hour of observation after induction of the sham or BD procedure. Methodological steps were as follows:

- i) Collection of 1ml of blood at the time of carotid artery incision (Figure 3.4C) for measurement of baseline inflammatory cytokines.
- ii) Collection of 1ml of blood from sham and BD animals after 1 hour of haemodynamic observations following sham or BD procedure respectively. The second blood sample was obtained from the left renal vein, which was accessed to inject heparin prior to explanting the heart, labelled 'POST (Sham)' and 'POST (BD)' respectively.
- iii) Blood samples were placed in Eppendorf tubes at room temperature for 30minutes to allow coagulation.
- iv) The samples were centrifuged for 10 minutes at 1000xg. Prior to centrifugation, the edge of the meniscus of blood was gently separated from the tube to ensure adequate separation of the supernatant during centrifugation.
- v) The serum supernatant (100-250ml) was immediately pipetted and centrifuged again for 10 minutes at 1000xg to remove any residual red blood cells. The resulting purified supernatant was stored at $\leq -20^{\circ}\text{C}$ for later assay.

3.2.4.1 Serum inflammatory cytokines measured

Eight inflammatory cytokines listed below were analysed using a Milliplex rat cytokine/chemokine magnetic bead luminex assay (RCYTO-80K, EMD Millipore Corp, Billerica, MA):

- i) Tumour necrosis factor alpha (TNF- α)
- ii) Interleukin-6 (IL-6)
- iii) Interleukin-10 (IL-10)
- iv) Interleukin-1 beta (IL-1 β)
- v) Interleukin-12 p70 (IL-12p70)
- vi) Monocyte chemoattractant protein-1 (MCP-1)
- vii) Macrophage inflammatory protein-2 (MIP-2)
- viii) GRO-alpha or keratinocyte-derived cytokine (GRO/KC), a rat homologue of the human chemokine CXC motif ligand 1 (CXCL1)

These cytokines were chosen from a panel of rat cytokines.

3.2.4.2 Assay for serum inflammatory cytokines

Samples of frozen serum were chosen to represent PRE, POST (Sham) and POST (BD) groups, with ten samples per group. Samples with the least haemolysis (lightest yellow colouring) were chosen to avoid interference with the assay, as per manufacturer's instructions.

- i) At the time of assay, the serum was warmed to room temperature, vortexed and centrifuged for 10 minutes at 1000xg to remove particulates.
- ii) Twenty-five microliters of serum were diluted 1:2 using an assay buffer provided in the Milliplex cytokine kit and 25 μ l of the diluted serum was added in duplicate samples to a 96-well assay plate.
- iii) Two wells were used for background assays using the provided assay buffer.
- iv) Four wells were used for quality control and 14 wells for standard controls. Standards were prepared by reconstituting the provided Rat Cytokine/Chemokine Standard with deionised water for serial dilutions to give the following known concentrations of chemokines:

- TNF- α and IL-1 β : 2.4 to 10,000pg/ml a
- IL-6: 73.2 to 300,000pg/ml
- IL-10: 7.3 to 30,000pg/ml
- IL-12p70: 12.2 to 50,000pg/ml
- MCP-1: 29.3 to 120,000pg/ml
- MIP-2: 24.4 to 100,000pg/ml
- GRO/KC: 14.6 to 60,000pg/ml

v) Twenty-five microliters of provided 'matrix solution' was added to all wells along with 25 μ L of premixed antibody-immobilised magnetic beads. The plate was then incubated with agitation at room temperature.

vi) Washing of the wells was then performed by adding 200 μ L of a provided wash buffer into each well and shaking the plates for 30 seconds. A hand-held magnet was attached to the bottom of the plate and the magnetic beads allowed to settle for 60seconds. The solution in the wells was removed by gently decanting the plate while attached to the magnet. This step was repeated.

vii) Detection antibodies (25 μ L) were added to each well and the plate was incubated on a plate shaker for 1 hour at room temperature.

viii) Streptavidin-Phycoerythrin (25 μ L) was added to each well and the plate was incubated with agitation on a plate shaker for 30 minutes at room temperature.

ix) The well contents were removed by resting on a hand-held magnet then gently decanting. The plate was washed twice with wash buffer as described above.

x) Sheath fluid (125 μ L) was added to each well and the beads were resuspended on a plate shaker for 5 minutes.

xi) The plate was run on Luminex 200™ (Life Technologies Australia Pty Ltd, Victoria AU) with xPONENT® Software (Luminex Corporation, Austin TX).

xii) The concentration of each cytokine was measured per well. The mean and standard deviation of the duplicate samples were also available in the calculations using the xPONENT software. The mean of the two observed concentrations for each cytokine was then graphed to compare the concentration at 'PRE', 'POST (sham)' and 'POST (BD)' groups.

3.2.5 Assessing Cardiac Function After Brain Death and Hypothermic Storage, and Assessing the Ability to Condition Hearts from Brain Dead Donors

The optimised rodent models of brain death and sham procedure described above were used to:

- i) Determine the effects of brain death on baseline cardiac function.
- ii) Assess the recovery of hearts after brain death and hypothermic storage
- iii) Assess the ability to provide cardioprotection by pharmacological conditioning of hearts subjected to brain death and hypothermic storage

Hearts from Lewis rats subjected to brain death or sham procedure, as described above, were used in this study. Study groups are outlined in Figure 3.7. Animals were randomly allocated to brain death or sham procedure. Blood was collected from all animals for the measurement of inflammatory cytokines and all animals underwent one hour of haemodynamic observations as described above.

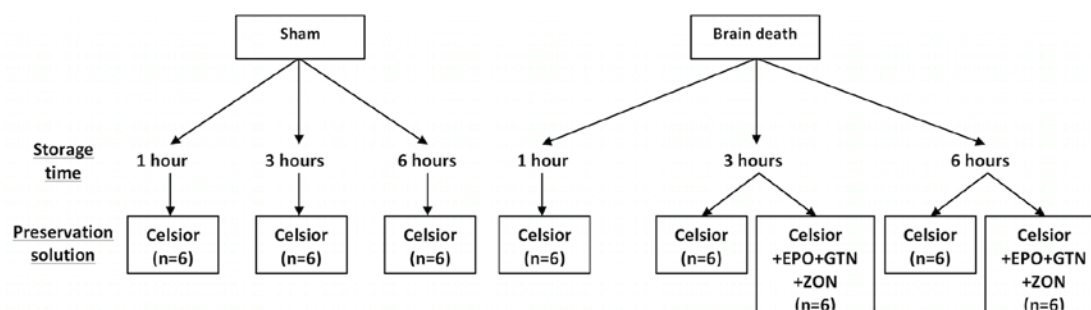


Figure 3.7 Study groups

The above diagram outlines the study groups used to assess the effects of brain death and prolonged hypothermic storage on the donor hearts. BD indicates brain death; EPO, erythropoietin; GTN, glyceryl trinitrate; ZON, zonisipride.

The experimental timeline is outlined in Figure 3.8. Ventilation was ceased after obtaining blood from the renal vein and injection of 250IU of heparin sodium, and hearts were explanted and cannulated onto the ex-vivo cardiac perfusion circuit as described in Chapter 2, Sections 2.6.3 and 2.6.4.

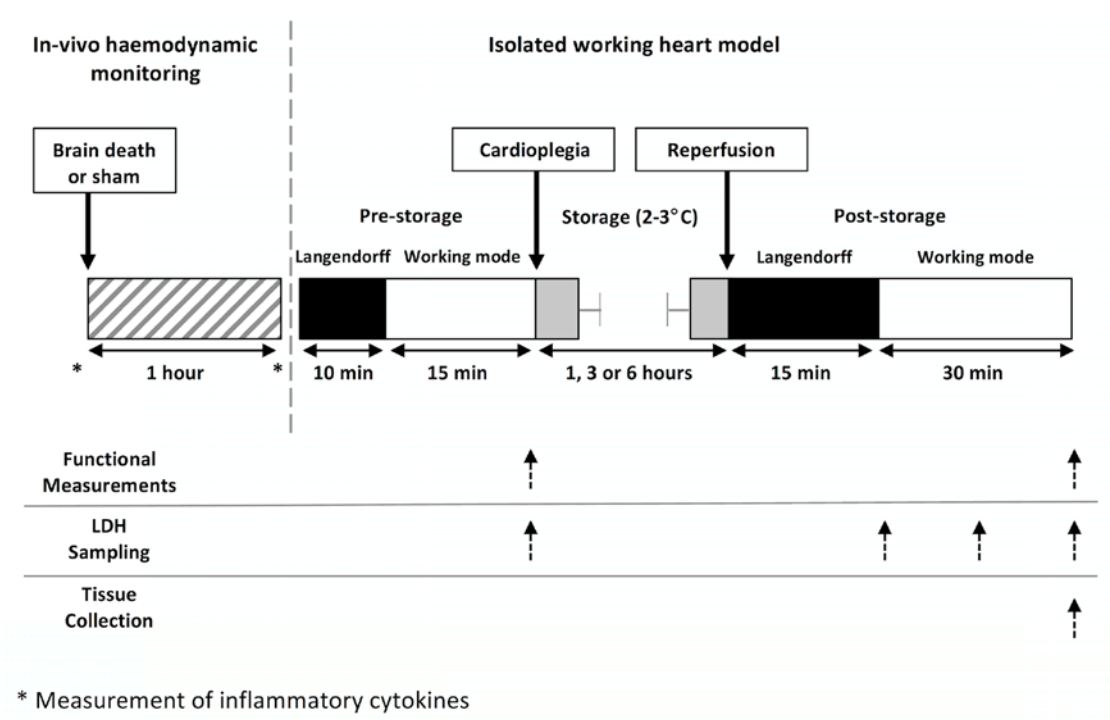


Figure 3.8 The experimental protocol
The experimental protocol involves induction of brain death or performing sham procedure on Wistar rats, with one hour of hemodynamic observations. Times of measurement of serum inflammatory cytokines are shown above. Cardiac function is then assessed on an ex-vivo perfusion circuit with times of Langendorff and ‘working’ mode shown above. Cardioplegia is delivered with hypothermic storage for 1, 3 or 6 hours followed by reperfusion on the ex-vivo perfusion circuit. The times for measurement of lactate dehydrogenase (LDH) and tissue collection are shown above.

3.2.5.1 Assessment of cardiac function on an ex-vivo perfusion circuit

As described in Chapter 2, Section 2.7, the following parameters of cardiac function were measured on the ex-vivo perfusion circuit:

- v) Aortic flow (AF, ml/min)
- vi) Coronary flow (CF, ml/min)
- vii) Heart rate (HR, bpm) *and*
- viii) Cardiac output (CO, ml/min)

3.2.5.2 Cardioplegia and hypothermic storage

Hearts were arrested and stored in Celsior® preservation solution, as described in Chapter 2, Section 2.8. Celsior® was obtained commercially (Genzyme, Naarden, The Netherlands) with the composition as provided in Table 2.3.

Twelve hearts underwent cardioplegia and hypothermic storage with Celsior® supplemented with erythropoietin, glyceryl trinitrate and zoniporide. These pharmacological agents were added to Celsior® on the day of the experiment at concentrations shown in Table 3.3.

Table 3.3 The concentrations of erythropoietin, glyceryl trinitrate and zoniporide used in pharmacological conditioning studies⁵

	Concentration	Source
Erythropoietin alpha	5000U/l	Janssen-Cilag, North Ryde, AU
Glyceryl trinitrate	100mg/l	David Bull Laboratories, AU
Zoniporide	1µmol/l	Pfizer Inc, Groton , CT

⁵ The concentrations were based on previous studies conducted in the laboratory (Watson A et al, Enhanced preservation of the rat heart after prolonged hypothermic storage with erythropoietin-supplemented celsior solution. J Heart and Lung Transplant. 2013;32:633-40).

3.2.5.3 Assessment of post-storage cardiac function

The recovery of cardiac function after hypothermic storage was assessed by reperfusion on the ex-vivo perfusion circuit as described in Section 2.9. Post-storage values for AF, CF, CO and HR were measured continuously during reperfusion, and the recovery of each parameter was calculated with values obtained at the end of 45 minutes of reperfusion (30 minutes in working mode, Figure 3.8) The percentage recovery of AF, CF, HR and CO were calculated using the formula:

$$\text{Percentage recovery (\%)} = \frac{\text{Post-storage measurement}}{\text{Baseline measurement}} \times 100$$

3.2.5.4 Measurement of lactate dehydrogenase levels

Coronary effluent was collected at time points shown in Figure 3.8 for the measurement of lactate dehydrogenase (LDH) levels released from hearts in working mode. Pre-storage samples were obtained prior to cardioplegia (labelled 'baseline'). Post-storage samples were obtained at 16, 30 and 45 minutes on the ex-vivo perfusion circuit and labelled 'R16', 'R30' and 'R45'. The LDH assay was performed as described in Section 2.10.

3.2.5.5 Western blots and histopathology analyses

At the end of the assessment of post-storage cardiac function, myocardial samples were stored for molecular and histological studies, as described in Chapter 2, Section 2.11.

3.2.5.5.1 Western blots

Western blot analyses was performed to assess the degree of phosphorylation of pro-survival kinases ERK1/2, AMPK α , Akt and STAT3, indicating the activation of these kinases. The phosphorylation status of each kinase is expressed as the ratio of phosphorylate to total protein. The level of phosphorylation of pro-survival kinases was compared for groups (with

or without pharmacological conditioning) according to storage times. A 15-well 10% pre-cast SDS-polyacrylamide gel (Bio-Rad Laboratories Pty. Ltd., Regents Park, Australia) was used in order to compare at least four samples per group.

3.2.5.5.2 Histological analysis

Histological analysis was performed on haematoxylin and eosin stained cross-sections of the mid-ventricle of hearts obtained at the end of ex-vivo reperfusion. An experienced clinical anatomical pathologist, blinded to the experimental treatment, assessed each sample as described in section 2.13.4. A semi-quantitative score was given for evidence of ischaemia-reperfusion injury as shown in **Table 3.4**

Table 3.4 Histopathology scoring system

Score	Description
0	No evidence of ischaemia-reperfusion injury
1 +	One isolated area of contraction bands or myocardial necrosis per high power field view
2 -	≥ two areas of contraction bands/myocardial necrosis per high power view <u>without</u> oedema
2 +	≥ two areas of contraction bands/myocardial necrosis per high power view <u>with</u> oedema
3 +	≥ two areas of contraction bands/myocardial necrosis per high power field <u>with</u> oedema and evidence of monocyte infiltrate

3.2.5.6 Statistical analysis

All results are expressed as mean ± SEM. Prism 6 software (GraphPad Software Inc., CA) was used for all statistical analyses. One-way ANOVA was used to compare the difference between three or more groups, and unpaired t-test used to compare the differences between two groups. A p value < 0.05 was considered statistically significant

3.3 Results

Thirty-six animals were used (n=18 sham and n=18 BD) as shown in Figure 3.7. There were no significant differences in the weights of animals between groups (Table 3.5, p=ns).

Table 3.5 Mean weights of animals

Sham or BD groups	Preservation time	Preservation solution	Number of animals	Weight (g, mean \pm SD)
Sham	1h	Celsior	6	345.0 \pm 13.5
Sham	3h	Celsior	6	353.0 \pm 12.8
Sham	6h	Celsior	6	350.7 \pm 5.9
BD	1h	Celsior	6	340.3 \pm 13.7
BD	3h	Celsior	6	346.3 \pm 8.9
BD	6h	Celsior	6	357.5 \pm 11.7
BD	3h	Celsior +EPO+GTN+ZON	6	349.3 \pm 10.7
BD	6h	Celsior +EPO+GTN+ZON	6	352.4 \pm 9.5
All Sham			18	348.1 \pm 11.7
All BD			30	349.5 \pm 12.6

p=ns between groups. BD indicates brain death.

3.3.1 Neurological changes observed during brain death

During inflation of the intracranial embolectomy catheter, animals were noted to initially undergo twitching of the facial muscles and tremor of the lower limbs. As the blood pressure and heart rate peaked, animals underwent increasing tremor particularly of the lower limbs, with kicking and extensor posturing. All animals in the brain death group met neurological criteria for brain death, with the sustained absence of pedal and corneal reflexes and no return of spontaneous respirations when ventilation was ceased. Sham animals did not regain spontaneous respirations when ventilation was temporarily ceased, however regained pedal reflexes requiring supplemental anaesthesia at approximately 30-40min after sham procedure.

3.3.2 Haemodynamic changes associated with brain death

The MAP and HR during BD and sham procedure and for 1 hour of hemodynamic monitoring are outlined in Table 3.6 and

Table 3.7 and graphically demonstrated in Figure 3.9 and Figure 3.10 respectively. There were no significant differences in the baseline MAP and HR between BD and sham groups ($p=0.26$ and 0.82 respectively).

With inflation of the embolectomy catheter, a gradual rise in blood pressure and heart rate were initially noted. In the last 15-30 seconds of inflation of the embolectomy catheter, a sharp rise in blood pressure and heart rate were noted. The classic 'Cushing' response characterised by bradycardia and hypertension was not observed prior to the rise in heart rate and blood pressure. The blood pressure continued to peak then fall gradually to levels below baseline, but there were no significant differences compared with baseline or with sham animals (Figure 3.9). The heart rate rose and remained significantly elevated compared to baseline and sham animals (Figure 3.10). Blood pressure in the sham animals remained stable throughout the observation period, with a small non-significant rise after receiving ketamine supplemental anaesthesia between 30-40 minutes after the sham procedure.

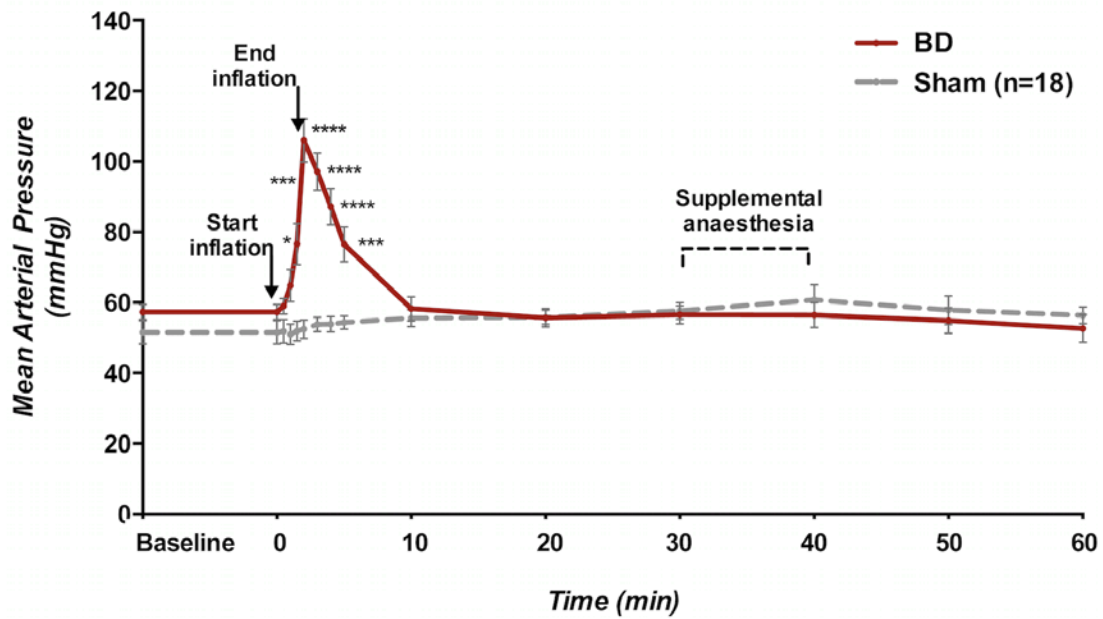


Figure 3.9 Mean arterial pressure in brain death and sham groups.

The mean \pm SD of the MAP for brain death (BD) and sham groups are shown above. The arrows indicate the time of start and end of inflation of the embolectomy catheter in BD animals. The dashed bracket shows the time period during which supplemental anaesthesia was provided for sham animals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

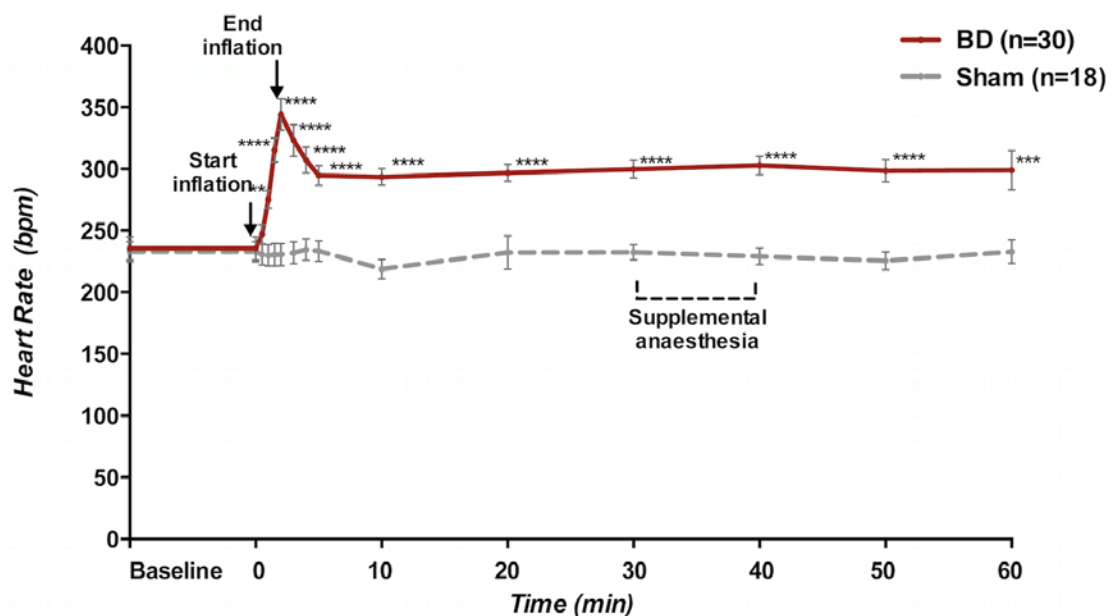


Figure 3.10 Heart rate in brain death and sham groups

The mean \pm SD of HR for brain death (BD) and sham animals represented above. The arrows indicate the time of start and end of inflation of the embolectomy catheter in BD animals. The dashed bracket shows the time period during which supplemental anaesthesia was provided for sham animals. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Table 3.6 Mean arterial pressure in brain death and sham groups

Time (min)	MAP in BD group (mmHg, mean \pm SD) n=30	MAP in sham group (mmHg, mean \pm SD) n=18	p-value
Baseline	57.33 \pm 8.91	52.89 \pm 13.95	ns
0.0 (Start of Inflation)	57.33 \pm 8.91	52.89 \pm 13.95	ns
0.5	58.94 \pm 9.35	51.88 \pm 14.29	ns
1.0	64.83 \pm 19.21	52.33 \pm 11.91	0.03
1.5	76.55 \pm 24.82	52.67 \pm 11.31	0.0008
2.0 (End of inflation)	106.00 \pm 26.00	53.22 \pm 11.11	< 0.0001
3.0	97.11 \pm 22.27	54.33 \pm 8.55	< 0.0001
4.0	87.22 \pm 21.87	54.44 \pm 8.55	< 0.0001
5.0	76.50 \pm 20.88	55.00 \pm 7.96	0.0003
10.0	58.22 \pm 14.35	55.72 \pm 10.03	ns
20.0	55.67 \pm 10.83	56.22 \pm 8.52	ns
30.0	56.50 \pm 10.81	57.83 \pm 9.90	ns
40.0	56.44 \pm 14.90	61.06 \pm 18.09	ns
50.0	54.89 \pm 15.29	57.72 \pm 17.14	ns
60.0	52.67 \pm 16.64	56.41 \pm 9.69	ns

All values are expressed as mean \pm standard deviation (SD). MAP indicates mean arterial pressure

Table 3.7 Heart rate in brain death and sham groups

Time (min)	HR in BD group (bpm, mean \pm SD) n=30	HR in sham group (bpm, mean \pm SD) n=18	p-value
Baseline	236 \pm 41	233 \pm 35	ns
0.0 (Start of Inflation)	236 \pm 41	233 \pm 35	ns
0.5	247 \pm 31	231 \pm 38	ns
1.0	275 \pm 42	230 \pm 40	0.002
1.5	315 \pm 54	230 \pm 39	<0.0001
2.0 (End of inflation)	344 \pm 54	231 \pm 39	<0.0001
3.0	323 \pm 44	232 \pm 38	<0.0001
4.0	307 \pm 34	235 \pm 36	<0.0001
5.0	295 \pm 29	233 \pm 35	<0.0001
10.0	294 \pm 30	219 \pm 59	<0.0001
20.0	297 \pm 30	232 \pm 27	<0.0001
30.0	300 \pm 32	232 \pm 30	<0.0001
40.0	303 \pm 38	229 \pm 32	<0.0001
50.0	298 \pm 67	226 \pm 37	<0.0001
60.0	299 \pm 40	233 \pm 35	0.0003

All values are expressed as mean \pm standard deviation (SD). HR indicates heart rate, BD brain death.

3.3.3 Inflammatory changes associated with brain death

The inflammatory cytokines measured at baseline (pre-intracranial manipulation) and after 1 hour of hemodynamic observations in BD and sham animals are shown in Figure 3.11 (n=10 samples per group).

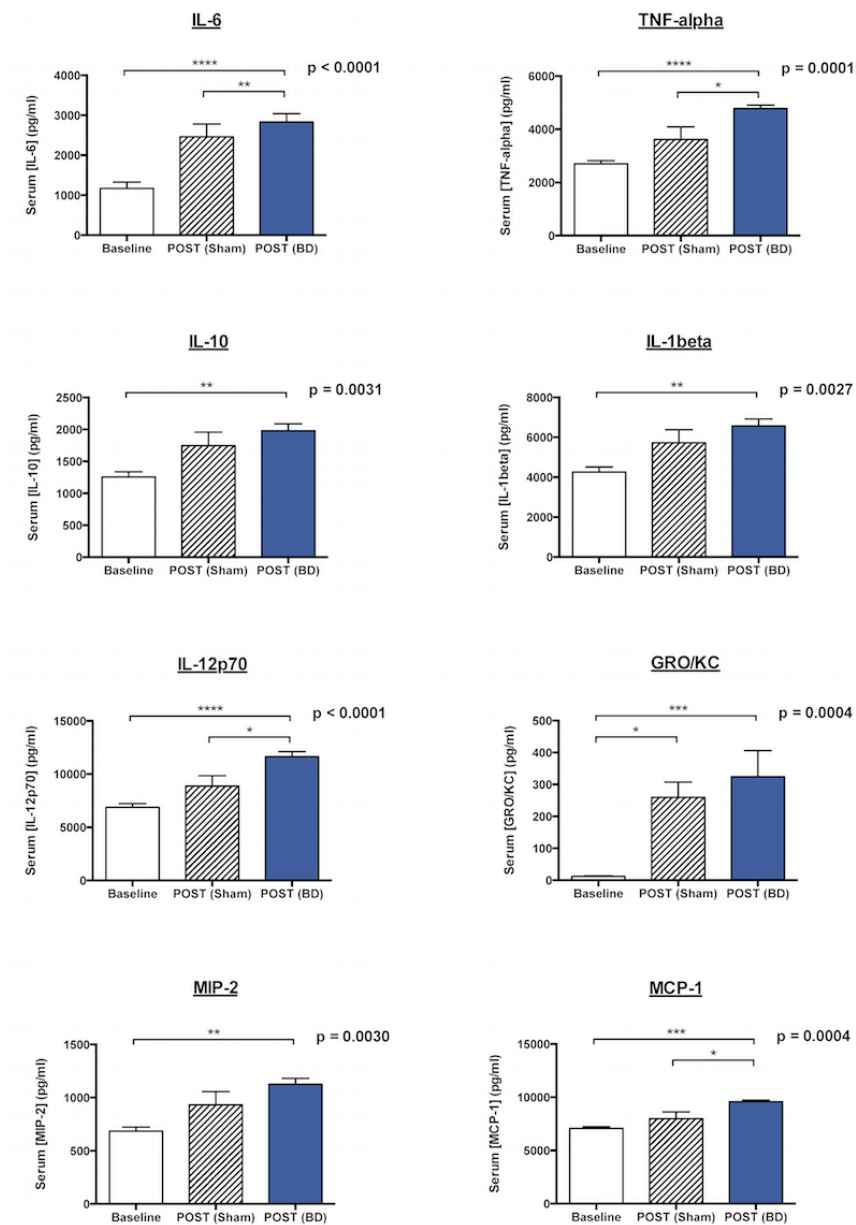


Figure 3.11 Inflammatory cytokines at baseline, post BD and sham procedures

The mean value \pm SEM are shown for each group (n=10 per group). P-values were calculated using one-way ANOVA, with differences between individual groups calculated using Tukey's post-hoc analysis. * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001

There was a significant rise in all inflammatory markers in BD animals compared with baseline values ($p<0.01$ to $p<0.0001$). Compared with the sham animals, the BD group had significantly higher levels of IL-6 ($p<0.01$), TNF- α ($p<0.05$), IL-12p70 ($p<0.05$) and MCP-1 ($p<0.05$) after 1 hour, but no significant changes in IL-10, IL-1 β , GRO/KC or MIP-2. There was a trend towards higher inflammatory markers in the sham groups compared with baseline values, however only significantly elevated in GRO/KC ($p<0.05$).

3.3.4 Cardiac function after brain death or sham procedure

Values for aortic flow, coronary flow, cardiac output and heart rate obtained on the ex-vivo perfusion circuit at working mode prior to cardioplegia are shown in Figure 3.12. There were no significant differences between sham and BD groups.

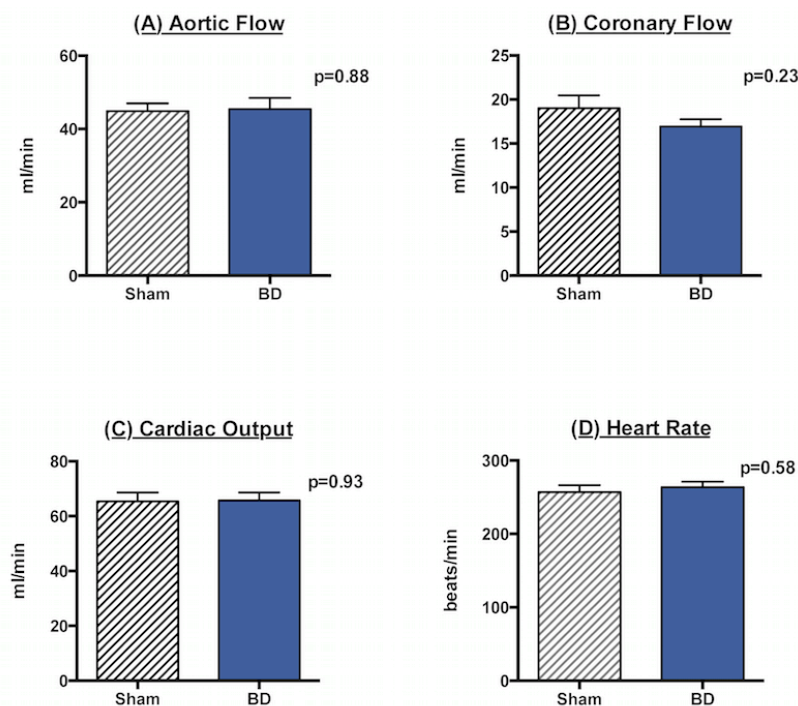


Figure 3.12 Cardiac function after sham or brain death

Values for aortic flow (A), coronary flow (B), cardiac output (C) and heart rate (C) are shown for sham and brain death groups prior to cardioplegia (mean \pm SEM, $n=18$ in sham and $n=30$ in BD). The differences between groups were compared using the unpaired t-test.

3.3.5 Recovery of cardiac function after hypothermic storage in Celsior®

The percent recovery of AF, CF, CO and HR after hypothermic storage in Celsior® alone are shown in Figure 3.13. There was a significant decline in the recovery of AF and CO with prolonged hypothermic storage, which was more pronounced with brain death [Figure 3.13 (A) and (C)]. There were no significant differences in CF or HR recovery due to brain death or varying durations of hypothermic storage.

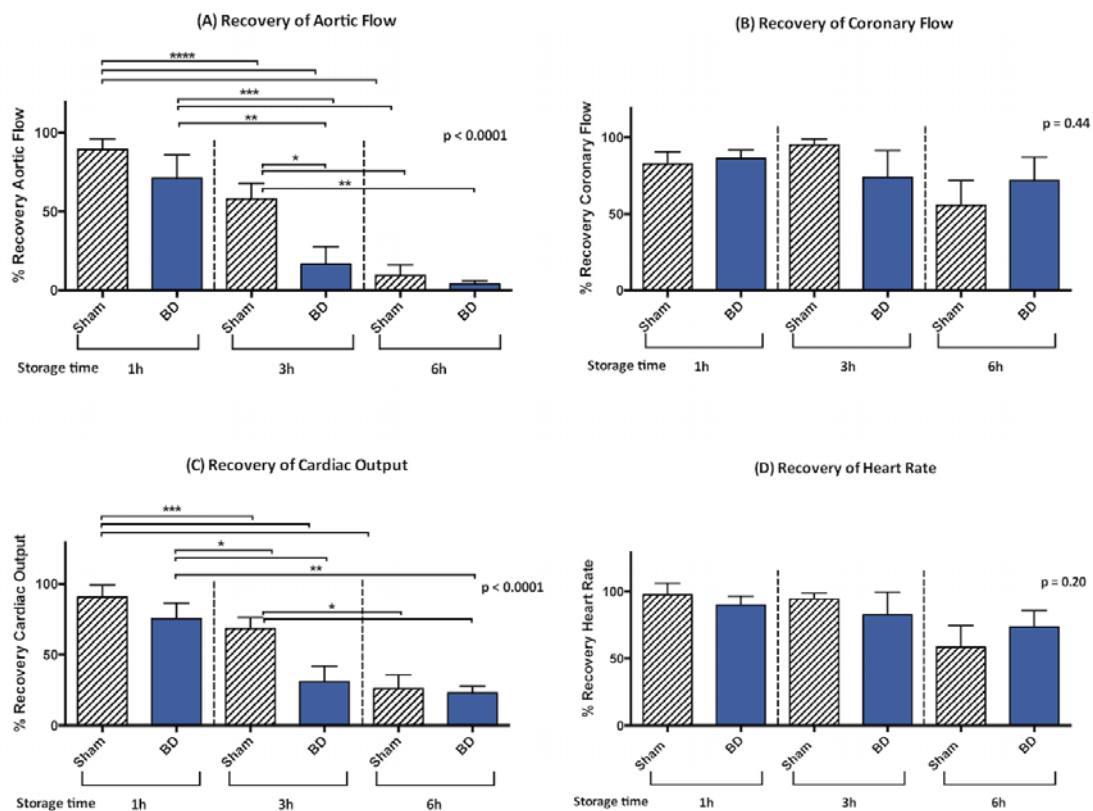


Figure 3.13 Recovery of cardiac function after hypothermic storage in Celsior®

The recovery of aortic flow (A), coronary flow (B), cardiac output (C) and heart rate (D) for all hearts stored with Celsior® alone are shown as a percentage of pre-storage values (mean \pm SEM, n=6 per group). * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001

3.3.6 Recovery of hearts treated with pharmacological conditioning

Figure 3.14 demonstrates the recovery of cardiac function in BD hearts treated with pharmacological conditioning (in red) compared with untreated sham and BD hearts. Compared with BD hearts stored in Celsior alone, significantly improved recovery of AF and CO were seen in hearts treated with pharmacological conditioning and hypothermic storage for 3 hours ($p < 0.0001$ and $p < 0.001$ respectively) and for 6 hours ($p < 0.001$).

In the 3-hour storage group, the recovery of AF and CO in BD hearts treated with conditioning agents was comparable to sham hearts. In the 6-hour storage group, treated hearts had improved recovery of AF compared with sham hearts.

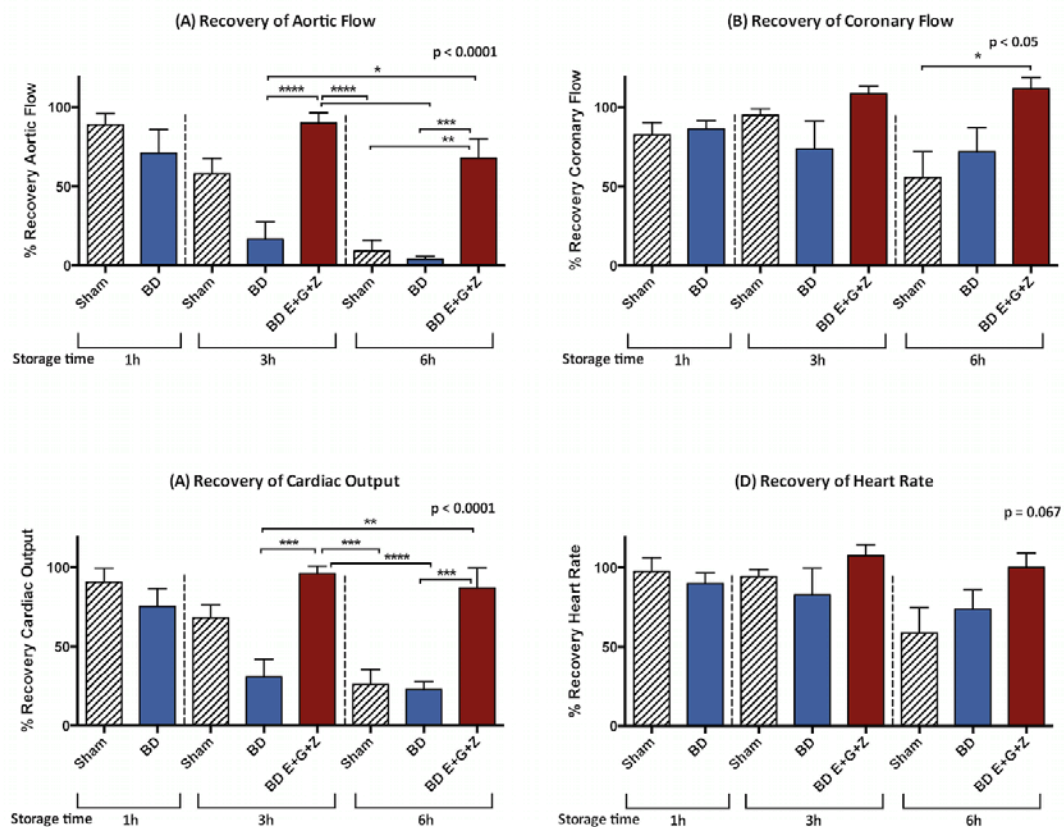


Figure 3.14 The recovery of cardiac function in hearts treated with pharmacological conditioning compared with untreated hearts

The recovery of AF, CF, CO and HR for all hearts are presented above (mean \pm SEM, $n=6$ per group). Statistically significant differences are only shown for hearts treated with EPO, GTN and ZON (E+G+Z).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

3.3.7 Lactate dehydrogenase levels

Lactate dehydrogenase (LDH) levels for all groups are shown in Figure 3.15. There were no significant differences in LDH levels prior to cardioplegia (baseline). During reperfusion, LDH values rose in all groups, with significantly higher levels in BD and hearts cardiopleged and stored in Celsior® alone.

At 30 and 45 minutes into reperfusion, LDH release was significantly higher in BD hearts stored for 6 hours Celsior® compared with hearts stored for 1 or 3 hours ($p < 0.05$ to < 0.001), and BD hearts treated with pharmacological conditioning and stored for 3 or 6 hours ($p < 0.05$ to $p < 0.01$).

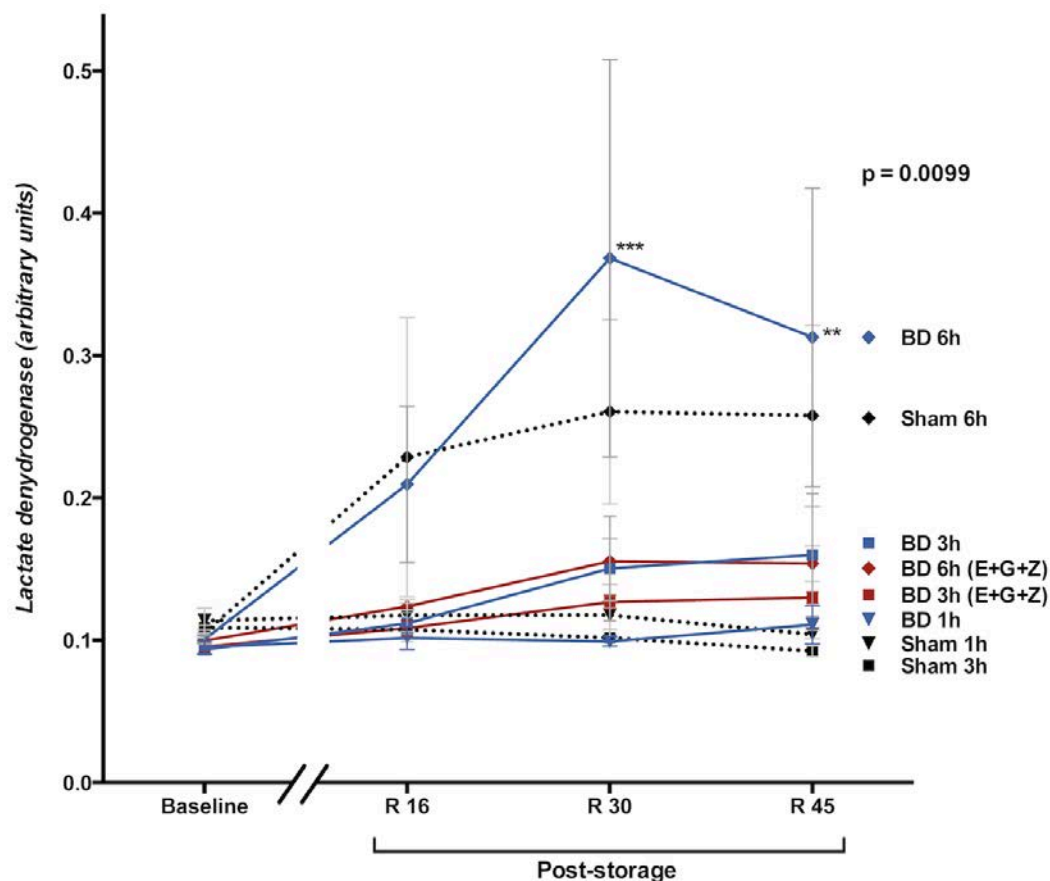


Figure 3.15 Lactate dehydrogenase release

Lactate dehydrogenase (LDH) levels are expressed as arbitrary units of colorimetric absorbance (mean \pm SEM, $n=6$ per group) for sham and brain death (BD) groups stored for 1, 3 or 6 hours.

** $p < 0.01$ and **** $p < 0.0001$ represent significant differences in LDH for BD hearts stored in Celsior for 6 hours compared to all groups, except sham hearts also stored for 6 hours.

3.3.8 Histopathology

Histopathological scores of the degree of IRI are shown for all groups in **Figure 3.16**. Sham hearts stored for 3 hours had more histological evidence of IRI compared to BD hearts stored for 1 or 3 hours. There were no differences in histological grading of IRI across other groups.

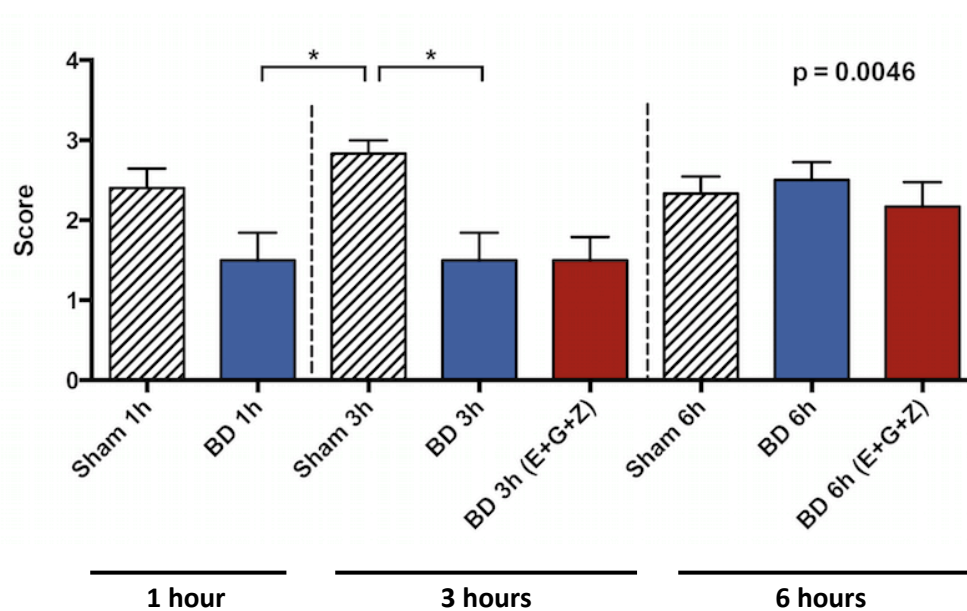


Figure 3.16 Histopathology scores for the severity of ischaemic injury

A score for the severity of IRI including the presence of contraction bands, cellular oedema and monocyte infiltrate are shown for all groups (n=6 per group, mean ± SEM), with 0 indicating no injury and 4, severe injury.

3.3.9 Western blots

One-hour storage group

Western blots for hearts stored for 1, 3 and 6 hours are shown in Figure 3.17 to Figure 3.19. In the 1-hour storage group, hearts sham hearts had increased phospho-Akt compared to BD hearts (B). There were no differences in the phosphorylation ratio of ERK1/2, AMPK α or STAT3 between groups.

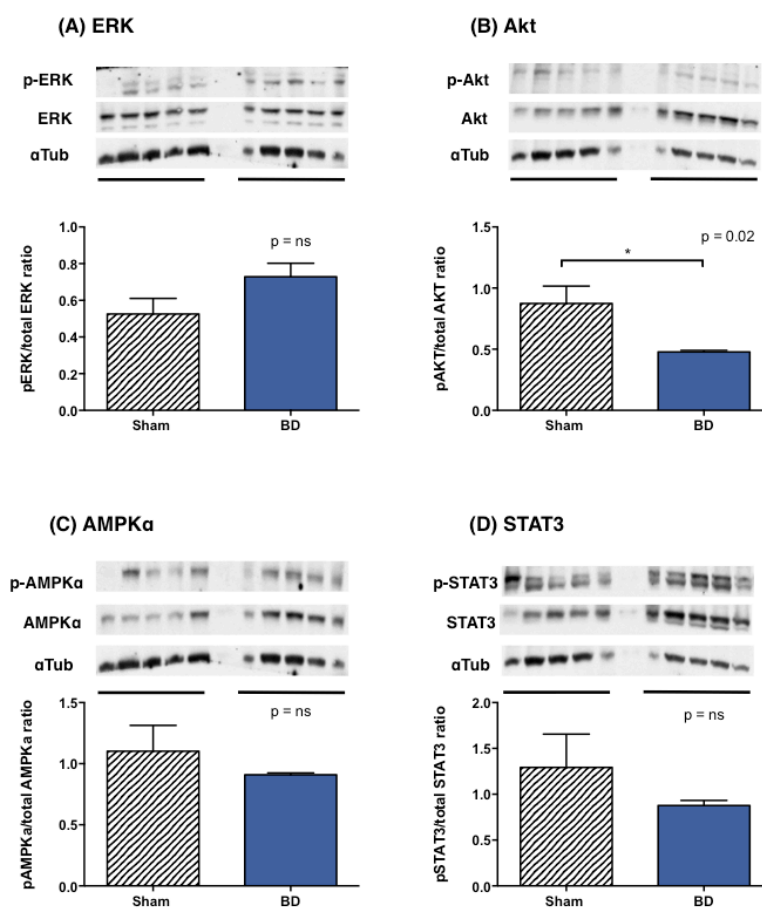


Figure 3.17 Western blots for hearts stored for 1 hour

The graphs display the phospho:total ratio of the respective pro-survival kinases. The western blots with alpha-tubulin (α -tub) are shown for each protein. All values are represented as mean \pm SEM, $n=5$ per group. * $p<0.05$

Three-hour storage group

In the 3 hour storage group, hearts treated with EPO, GTN and ZON had increased phospho-ERK1/2 compared to sham hearts stored in Celsior ($p < 0.05$), and increased phospho-Akt compared to sham and BD hearts stored with Celsior alone ($p = 0.0019$, Figure 3.18A and B).

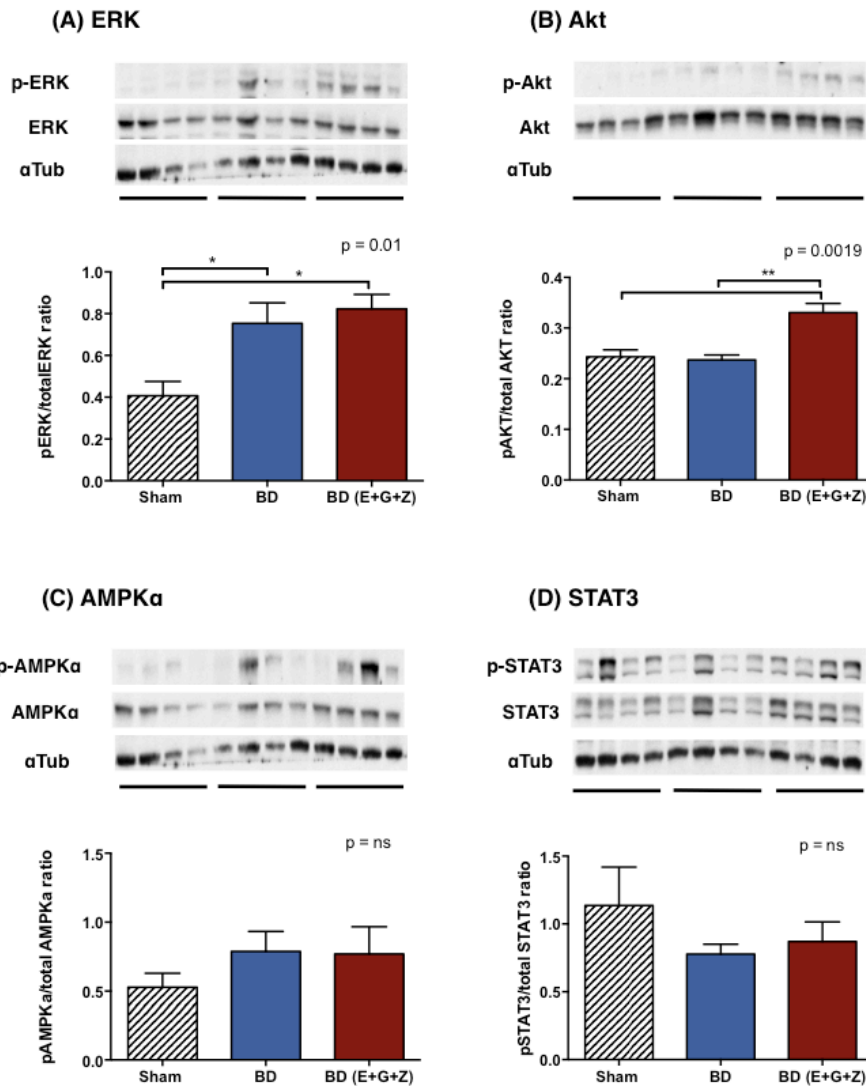


Figure 3.18 Western blots for hearts stored for 3 hours

The graphs display the phospho:total ratio of the respective pro-survival kinases. The western blots with alpha-tubulin bands (α-tub) are shown for each protein. All values are represented as mean ± SEM, $n = 4$ per group. * $p < 0.05$, ** $p < 0.01$.

Six-hour storage group

In the 6-hour group, hearts treated with EPO, GTN and ZON did not demonstrate increased phosphorylation of ERK1/2, Akt, AMPK α and STAT3 (Figure 3.19). Hearts subjected to brain death and stored in Celsior demonstrated elevated phospho-ERK levels compared to sham hearts, and elevated phospho-STAT3 levels compared to hearts treated with EPO, GTN and ZON.

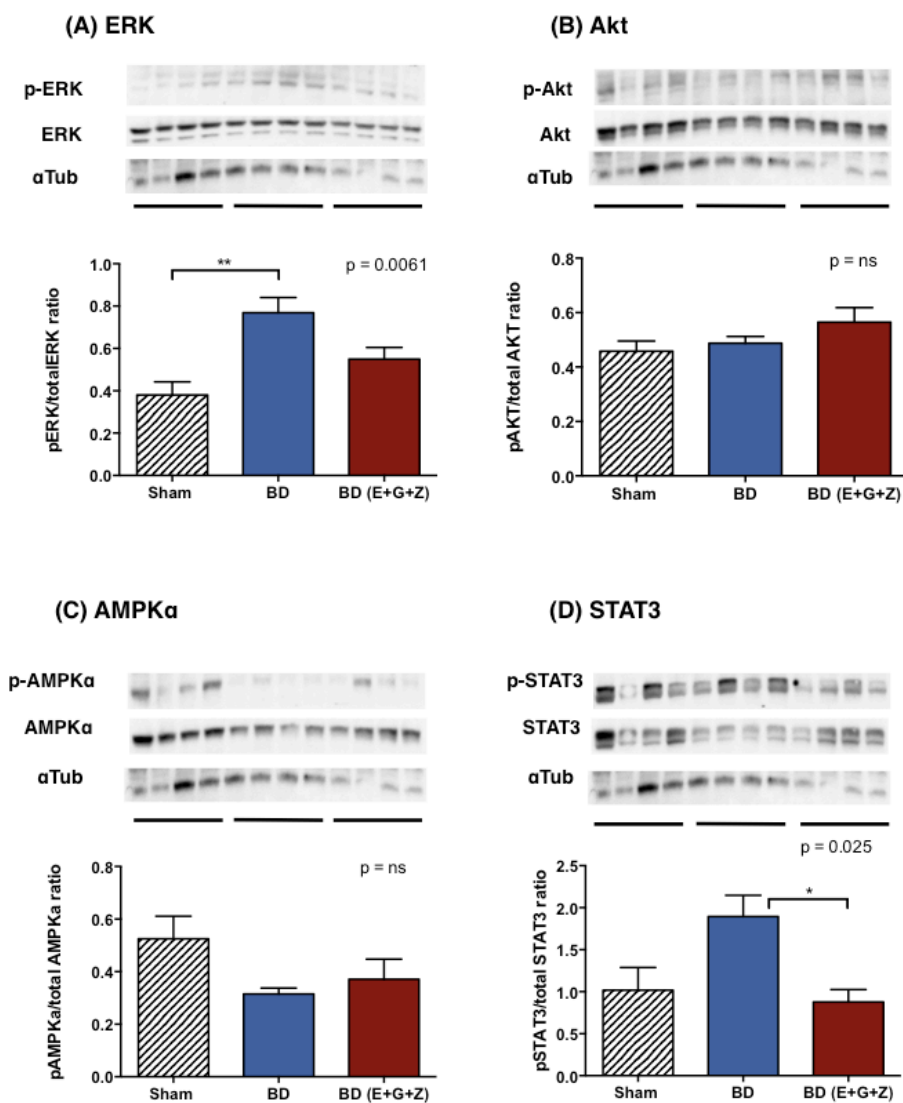


Figure 3.19 Western blots for hearts stored for 6 hours

The graphs display the phospho:total ratio of the respective pro-survival kinases. The western blots with alpha-tubulin (α -tub) are shown for each protein. All values are represented as mean \pm SEM, n=4 per group. * p<0.05

3.4 Discussion

This study has demonstrated that pharmacological conditioning with erythropoietin, glyceryl trinitrate and zoniporide provides myocardial protection against IRI in rat hearts subjected to brain death and hypothermic storage. Evidence supporting this includes significant functional recovery of aortic flow and cardiac output in hearts treated with pharmacological conditioning, and significantly reduced release of lactate dehydrogenase, indicating less cardiomyocytes injury compared to untreated hearts.

The rodent model of brain death

This study developed a reproducible model of brain death, validated by i) the absence of key neurological features, ii) the presence of typical haemodynamic changes associated with brain death, and iii) the presence of a significant inflammatory reaction as described clinically^{58,343,368} and in other experimental animal studies^{56,59,66,68}. The model was refined to ensure neurologic and haemodynamic changes of brain death were induced without causing 'explosive' brain death that would compromise animal survival over the hour of haemodynamic observations. Extension of haemodynamic observations over 3 hours was performed while developing the model, and indeed deteriorating blood pressure and ultimately cardiac arrest was noted in some of these animals, indicating significant hemodynamic instability, as seen in human brain dead organ donors^{58,343,356}. This highlights the need for judicious use of high dose inotropes and vasopressors to manage and maintain adequate perfusion pressure in many patients with traumatic brain injury or subarachnoid haemorrhage. Signs of sympathetic denervation were evident by the sustained elevation in heart rate, which are consistent with hemodynamic changes observed clinically and in other animal models^{56,58-60,64,164,343}.

The model of brain death demonstrated a significant rise in all inflammatory cytokines tested when compared with baseline, and to sham animals for IL-6, TNF- α , IL-12p70 and MCP-1. Sham animals tended to have raised serum inflammatory cytokines, however a significant rise compared to baseline values was only seen in GRO/KC. The rise in inflammatory cytokines may have been induced by cerebral injury caused by creation of the burr hole, or due to right anterior cerebral ischaemia caused by cannulation and ligation of the right carotid artery. An inflammatory reaction may also have been induced by tissue

trauma during the surgical procedure in sham animal³⁷⁰⁻³⁷². However, seven of the eight inflammatory cytokines tested were only significantly elevated in the brain death group. It was also determined that sham animals may suffer further neurological injury during intracranial placement of the embolectomy catheter, hence this step was omitted. These animals may therefore not represent a true 'sham' group due to omission of this step, however all other steps were kept uniform between the two groups.

The effect of brain death on cardiac function

This effect of brain death on cardiac function was not evident at initial functional assessment on the ex-vivo perfusion circuit, with no differences demonstrated in baseline values of aortic flow, coronary flow, cardiac output and heart rate between BD and sham groups. After hypothermic storage, it was evident that hearts had inferior recovery of aortic flow and cardiac output proportional to the length of storage, however with much worse recovery in hearts subjected to brain death. The difference in recovery was significant between sham and BD groups stored for 3 hours. The lack of exposure to significant periods of ischaemia in 1-hour storage groups may explain the lack of significant differences in recovery of cardiac function between sham and BD groups, although the data recovery tended to be lower in BD hearts. In the 6-hour storage groups, exposure to prolonged hypothermic storage may have caused severe ischaemic injury in both BD and sham groups, which again failed to show a difference in the recovery of cardiac output between groups, both with < 20% recovery of aortic flow and < 25% recovery of cardiac output. It is possible that a more prolonged period of haemodynamic observation of the donor heart in the current model may have exposed the heart to longer periods of inflammation and haemodynamic instability leading to poorer baseline cardiac function and worse recovery.

The effects of varying degrees of induction of brain death on cardiac function have been demonstrated in experimental animal studies, where recipient canine animals were able weaned off cardiopulmonary bypass after transplantation of hearts from donors that underwent 'gradual' but not 'explosive' onset of brain death⁵⁹. Histological changes of myocardial injury were demonstrated to be proportional to the speed of induction of brain death, highlighting the injurious effects of brain death on cardiomyocytes and cardiac function. Inflammatory and haemodynamic changes seen in brain death in the current study certainly explains some of the mechanisms of myocardial injury. The effects of brain death

have been considered 'reversible' due to apparent normal cardiac function at organ retrieval, however the current study demonstrates that cumulative insults either unmask irreversible changes occurring at a cellular level, or that it impairs recovery of cells that have undergone potentially reversible ischaemic effects.

Studies by Birks et al also demonstrated that human donor hearts with poor left ventricular ejection fraction (LVEF) and therefore declined for use in transplantation demonstrated elevated inflammatory cytokines and upregulation of myocardial TNF- α and IL-6, compared to hearts with normal LVEF that were used in transplantation^{71,72}, again highlighting that the severity of physiological changes induced by brain death, perhaps related to 'gradual' versus 'explosive' onset of brain death, are directly proportional to the degree of donor organ dysfunction. These studies highlight that donor intervention is a therapeutic opportunity to minimise ongoing injury in a 'hostile' physiologic environment. Strategies such as donor hormonal replacement have not had consistently positive results^{161,162}. Myocardial infarction studies on targeted anti-inflammatory agents against cell adhesion molecules P-selectin³⁷³, CD 11³⁷⁴ and CD 18³⁷⁵ have also failed to demonstrate protection against IRI, however it has been suggested that etanercept, which binds to and inactivates TNF- α ³⁷⁶, may be useful in protecting the donor heart from prolonged exposure to inflammation in-vivo, although it remains to be tested in humans. Corticosteroids have also been used in donor management^{350,355}, and additional anti-inflammatory agents may augment its protection of donor organs against inflammatory injury. Although donor intervention aimed purely at improving organ quality for transplantation may not be allowed due to ethical reasons, these are potential areas that could be addressed in future to further improve the preservation of organs from brain dead donors.

Pharmacological conditioning of brain dead hearts

Hearts from brain dead rats treated with pharmacological conditioning with EPO, GTN and ZON during cardioplegia and hypothermic storage unequivocally demonstrated superior recovery of aortic flow and cardiac output. Hence pharmacological conditioning is a robust mechanism of donor heart preservation despite brain death and prolonged hypothermic storage. Although functional measurements were obtained on the ex-vivo perfusion circuit with hearts in 'working' mode, it was evident within seconds of commencing reperfusion while hearts were 'rested' in Langendorff mode that the recovery of myocardial contractility

was far superior to untreated hearts. There were fewer arrhythmias and less variability in aortic flow during working mode. All hearts retained stable aortic flow throughout the 30 minutes of functional assessment in working mode.

The mechanisms of cardioprotection were suggested by demonstration of increased phosphorylation of Akt and ERK1/2, although the latter was only raised in comparison to sham hearts, in the three-hour storage group. Both Akt and ERK1/2 are key signalling kinases in the reperfusion injury salvage kinase (RISK) pathway. Increased ERK1/2 phosphorylation was also demonstrated in BD hearts stored in Celsior alone, despite the lack of a conditioning stimulus. Unpublished data in the laboratory has demonstrated that cold storage alone activates ERK1/2, although sustained activation to the end of 45 minutes of reperfusion was only present with the addition of pharmacological conditioning agents glyceryl trinitrate and NHE-inhibitor cariporide. It may be that brain death and hypothermic storage may activate ERK1/2, the Extracellular Signal-Related Kinase, which is known to transduce extra-cellular signals or stresses into intracellular activity.

In the 6-hour storage group, BD hearts stored in Celsior alone demonstrated increased phospho:total ERK1/2 compared with sham hearts and increased phospho:total STAT3 compared with BD hearts treated with pharmacological conditioning. These findings may again represent stimulation of cell-survival kinase due to brain death. Hearts treated with pharmacological conditioning did not demonstrate increased phosphorylation of pro-survival kinases after 6 hours of hypothermic storage, which was inconsistent with the higher functional recovery seen in these hearts. However, significant improvements in functional recovery of all hearts treated with pharmacological conditioning show promise in the ability to apply pharmacological conditioning brain dead donor hearts from 3 to 6 hours of hypothermic storage.

Significant reduction of LDH in groups treated with EPO, GTN and ZON support that myocardial injury as prevented by pharmacological conditioning despite brain death and hypothermic storage for 3 or 6 hours. Histopathological analysis of myocardial samples however failed to reveal significant differences in reduction of IRI with pharmacological conditioning. The sham group with 3 hours of hypothermic storage demonstrated worse IRI histologically, however this was not consistent with good functional recovery seen in this group. The lack of significant differences between groups may be due cellular damage

occurring during long fixation times in formalin and during sample processing. The presence of mononuclear cell infiltrate in myocardial samples may be due to the inflammatory reaction induced by brain death, since all hearts were perfused using crystalloid bloodless solutions.

Limitations

While this model incorporates BD, measures hemodynamic and inflammatory changes associated with BD and assesses various storage times for hypothermic preservation of the heart, a limitation of the study is that it is a small animal model. Results may vary significantly in humans, as has recently been demonstrated in genomic studies on immunological responses between rodents and humans³⁷⁷. Findings from rodent studies in will therefore always be reassessed in large animal studies, for validation across species.

Another significant limitation is that animals were observed for only one hour following BD. This was chosen for pragmatic reasons since i) significant haemodynamic compromise was noted towards the end of one hour, and ii) it was difficult to support haemodynamics with cannulation and infusion of catecholamines and to maintain ventilation for longer periods of time in small animal models. It is acknowledged that this varies from the clinical situation where patients with severe brain death are managed for days in the ICU, where donor organs are subjected to a longer periods of inflammatory, haemodynamic and hormonal disturbances. This again points to the need to validate any results obtained in large animal studies where it is more feasible to observe animals for longer periods of time following BD.

In summary, pharmacological conditioning appears to be an attractive strategy of myocardial preservation, as it allows superior functional recovery and reduced LDH release in brain dead hearts stored for 3 or 6 hours of hypothermic storage. Its success across species, as demonstrated in porcine studies conducted in the laboratory^{326,330,378}, shows promise in its potential to confer myocardial preservation of human donor hearts. This rodent brain death model has allowed incorporation of an important pathophysiological process experienced by the donor heart into screening studies. Subsequent chapters will discuss the development of a rodent heterotopic working heart transplantation model that incorporates an additional insult of warm blood reperfusion in a physiological recipient setting.

**CHAPTER 4. ASSESSING THE ABILITY TO PROTECT AGED
HEARTS USING PHARMACOLOGICAL CONDITIONING
STRATEGIES**

4.1 Introduction

The increasing demand for heart transplantation has led to the utilization of 'extended criteria' organs including those from older donors⁴. As discussed in the literature review, the mean age of cardiac donors has increased significantly over the last two decades particularly in Australia and Europe where the median donor age is now over 40 years^{4,11,379}. Registry data from the International Society for Heart & Lung Transplantation demonstrate that the risk of primary graft failure and early mortality after heart transplantation rises steadily above a donor age of 30 years⁶.

In addition, there is a powerful adverse interaction between donor age and ischemic time. Recipients of older-donor hearts subjected to prolonged ischemic times have the highest post-transplant mortality^{35,380}. Pre-clinical studies have demonstrated that older hearts are more susceptible to ischemia-reperfusion injury (IRI) due to age-related dysregulation of mitochondrial oxidative phosphorylation and calcium handling, impaired antioxidant capacity and changes in protein expression resulting in myocardial injury, hypercontracture and myocardial stunning³⁸¹⁻³⁸⁵. Identifying strategies to overcome these aged-related defects in the cellular defence against IRI are essential if hearts from older donors are to become a safe and effective source of organs for transplantation.

In this study, previously evaluated strategies of pharmacological conditioning using erythropoietin (EPO), glyceryl trinitrate (GTN) and zoniopride (ZON) will be used in an aging rodent model. The ability of the triple therapy with EPO, GTN and ZON to condition hearts from older brain dead rats will therefore be evaluated. The activation of pro-survival kinases will also be assessed in order to determine whether the ability to induce activation of cytoprotective signaling cascades is preserved in aging. These include kinases ERK1/2, Akt, STAT3 and AMPK α .

4.2 Methods

Male Wistar rats aged 3, 12 and 18 months (Charles River Laboratories, Wilmington, MA and Animal Resource Centre, Canning Vale, Western Australia) were used to represent adolescent, 30 and 45 year old human cardiac donors respectively³⁸⁶⁻³⁸⁹, as these ages were associated with diminishing post-transplant survival in a large registry analysis by Russo et al³⁵. Animals were allocated to brain death (BD) or sham groups (n=6 per group) as outlined in (Figure 4.1).

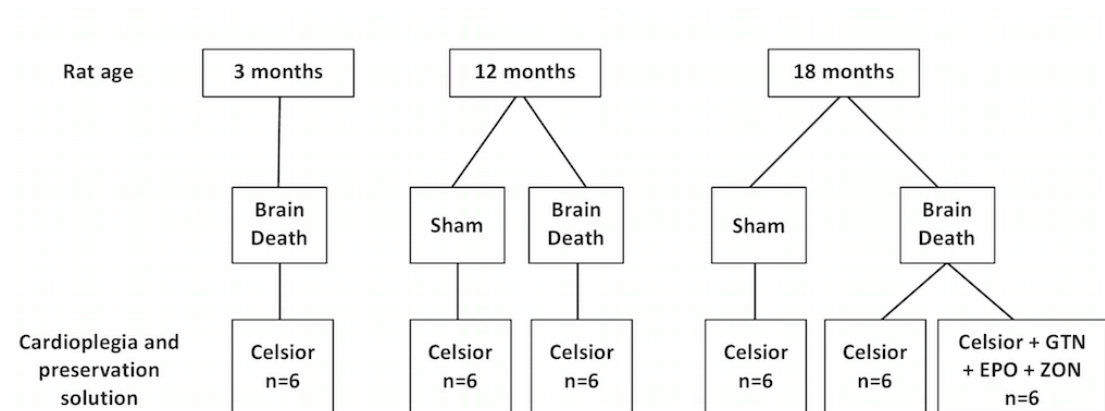


Figure 4.1 Study groups

Study groups comprised of 3, 12 and 18-month old Wistar rats, and were subjected to either brain death or sham procedure and cardioplegized and stored in Celsior or Celsior supplemented with EPO, GTN and ZON as outlined above.

4.2.1 Anaesthesia

Due to large animal sizes of older rats (up to 1kg), induction anaesthesia was provided for all animals with inhaled isoflurane in a closed chamber (5%, provided with oxygen) for ease of handling. Maintenance anaesthesia was provided with intraperitoneal ketamine and xylazine as described in Chapter 2, Section 2.6.2.

4.2.2 The surgical procedure

The timeline of the study protocol is outlined in Figure 4.2. All animals were intubated and ventilated as described in Chapter 3, Section 3.2.1.5, with pulse oximetry and core body temperature monitored as described in Section 3.2.1.6. Invasive haemodynamic monitoring

was performed on all animals by insertion of a micro-tip pressure catheter into the carotid artery as described in Section 3.2.1.7.

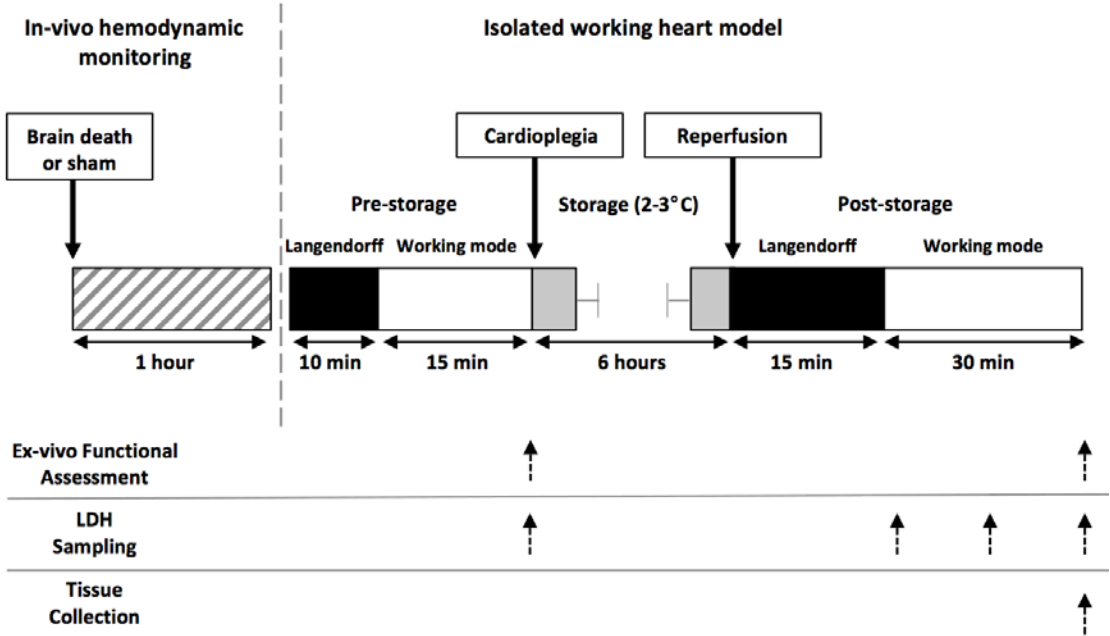


Figure 4.2 The experimental timeline

4.2.3 Induction of brain death

A burr hole was created in all animals, and animals allocated to brain death had an embolectomy catheter placed subdurally as described in Section 3.2.1.8. Brain death was induced by inflation of 0.30ml of water into the embolectomy catheter over 2 minutes with hand inflation as described in Protocol 3 in Section 3.2.1.9 and haemodynamic changes during brain death were recorded. All animals (sham and BD) underwent invasive haemodynamic monitoring for 1 hour. Sham animals required a supplemental dose of ketamine at 30 minutes into haemodynamic observations. Of note, despite larger animal size, a larger embolectomy catheter was not required to induce a large rise intracranial pressure, with all neurologic criteria for brain death met during the 1 hour of observation.

4.2.4 Assessment of cardiac function after brain death

Hearts were explanted after 1 hour and placed on an ex-vivo 'Langendorff' perfusion circuit as outlined in Chapter 2, Section 2.7. All hearts were perfused with Krebs-Henseleit buffer with a $[Ca^{2+}]$ 1.4mM (Table 2.3).

The following parameters of cardiac function were measured on the ex-vivo perfusion circuit:

- i) Aortic flow (AF, ml/min/g heart weight)
- ii) Coronary flow (CF, ml/min/g heart weight)
- iii) Heart rate (HR, bpm)
- iv) Cardiac output (CO, ml/min/g heart weight)

Of note, in this aging study, AF, CF and CO were calculated as a percentage of heart weight. Heart weight was not measured directly due to the need to rapidly cannulated the heart onto the ex-vivo perfusion circuit, and since it is in a heart-lung *bloc* at the time. Heart weight was also unable to be measured after the experiment due to possible confounding effects of myocardial oedema. Heart weight was therefore calculated as 0.24% of total body weight^{381,390,391}.

4.2.5 Cardioplegia and hypothermic storage

Antegrade cardioplegia was provided using Celsior® preservation solution or Celsior supplemented with EPO, GTN and ZON as described in Chapter 3, Section 3.2.5.2, with concentrations of pro-survival kinase agents kept consistent with previous experiments (Table 3.3). All hearts were stored for 6 hours of hypothermic storage using Celsior or Celsior supplemented with EPO, GTN and ZON as per study group.

4.2.6 Assessment of post-storage cardiac function

The recovery of cardiac function after hypothermic storage was assessed by reperfusion on the ex-vivo perfusion circuit as described in Section 2.9. Post-storage values for AF, CF, CO and HR were measured continuously during reperfusion, and the recovery of each

parameter was calculated with values obtained at the end of 45 minutes of reperfusion (30 minutes in working mode, Figure 4.2). The percentage recovery of AF, CF, HR and CO were calculated using the formula:

$$\text{Percentage recovery (\%)} = \frac{\text{Post-storage measurement}}{\text{Baseline measurement}} \times 100$$

4.2.7 Measurement of lactate dehydrogenase levels

Coronary effluent was collected at time points shown in Figure 4.2 for the measurement of lactate dehydrogenase (LDH) levels released from hearts in working mode. Pre-storage samples were obtained prior to cardoplegia (labelled 'baseline'). Post-storage samples were obtained at 16, 30 and 45 minutes on the ex-vivo perfusion circuit and labelled 'R16', 'R30' and 'R45'. The LDH assay was performed as described in Section 2.10.

4.2.8 Western blot and histopathology analyses

At the end of the assessment of post-storage cardiac function, myocardial samples were stored for molecular and histological studies, as described in Chapter 2, Section 2.11. Western blots were performed as previously described, to assess the degree of phosphorylation of pro-survival kinases ERK1/2, AMPK α , AKT and STAT3, which indicates the activation of these kinases.

Histological analysis was performed on haematoxylin and eosin stained cross-sections of the mid-ventricle of 18-month hearts obtained at the end of ex-vivo reperfusion. An experienced clinical anatomical pathologist, blinded to the experimental treatment, assessed each samples for the presence of ischaemia-reperfusion injury, with individual scores given for i) myocardial oedema and ii) contraction bands using a semi-quantification system (scored 0-4) based on the degree of morphological changes from normal or near-normal to most severe changes. Two hearts from 18-month old rats explanted directly after anesthesia (without BD, sham procedure or hypothermic storage) were used as controls for comparison.

4.3 Results

The mean weights of 3, 12 and 18-month old rats were $371 \pm 27\text{g}$, $763 \pm 99\text{g}$ and $826 \pm 110\text{g}$ respectively. The calculated mean heart weights for each group were $0.89 \pm 0.06\text{g}$, $1.83 \pm 0.23\text{g}$ and $1.98 \pm 0.27\text{g}$ respectively.

4.3.1 Haemodynamic changes during brain death

Induction of BD was associated with a significant rise in mean arterial pressure (MAP) and heart rate (HR) (Figure 4.3). The MAP decreased over one hour of hemodynamic observation to values significantly below baseline, while HR remained significantly elevated.

4.3.2 Cardiac function after brain death or sham procedure

Cardiac function after BD or sham procedure are presented in Figure 4.4. The combination of increasing age and BD were associated with a significant decline in AF, CF and CO, with the lowest values seen in 18-month BD hearts ($p < 0.0001$ to $p < 0.001$ compared with 3-month hearts). Heart rate was relatively unaffected by age and BD (Figure 4.4C). Pre-storage cardiac function did not differ between 18-month BD hearts allocated to be stored in Celsior® or Celsior® supplemented with GTN, EPO and ZON ($p = \text{ns}$, Figure 4.4A-D).

4.3.3 Recovery of cardiac function after hypothermic storage

After 6 hours of hypothermic storage, all 18-month hearts stored in Celsior® alone had poor recovery of AF, CF and CO, with the weakest recovery seen in 18-month BD hearts ($p < 0.001$ to < 0.01 compared with 3 and 12-month hearts,). The percentage recovery of AF, CF and CO in both 12-month BD and sham groups were equivalent to the 3-month group ($p = \text{ns}$). Again there were no appreciable differences in the recovery of HR between groups. The recovery of 18-month BD hearts treated with EPO, GTN and ZON was significantly higher than 18-month BD hearts stored in Celsior® alone ($p < 0.001$ to < 0.01 for AF, CF and CO). These hearts had similar percentage recovery of AF, CF and CO to 3 and 12-month hearts ($p = \text{ns}$, figure 5).

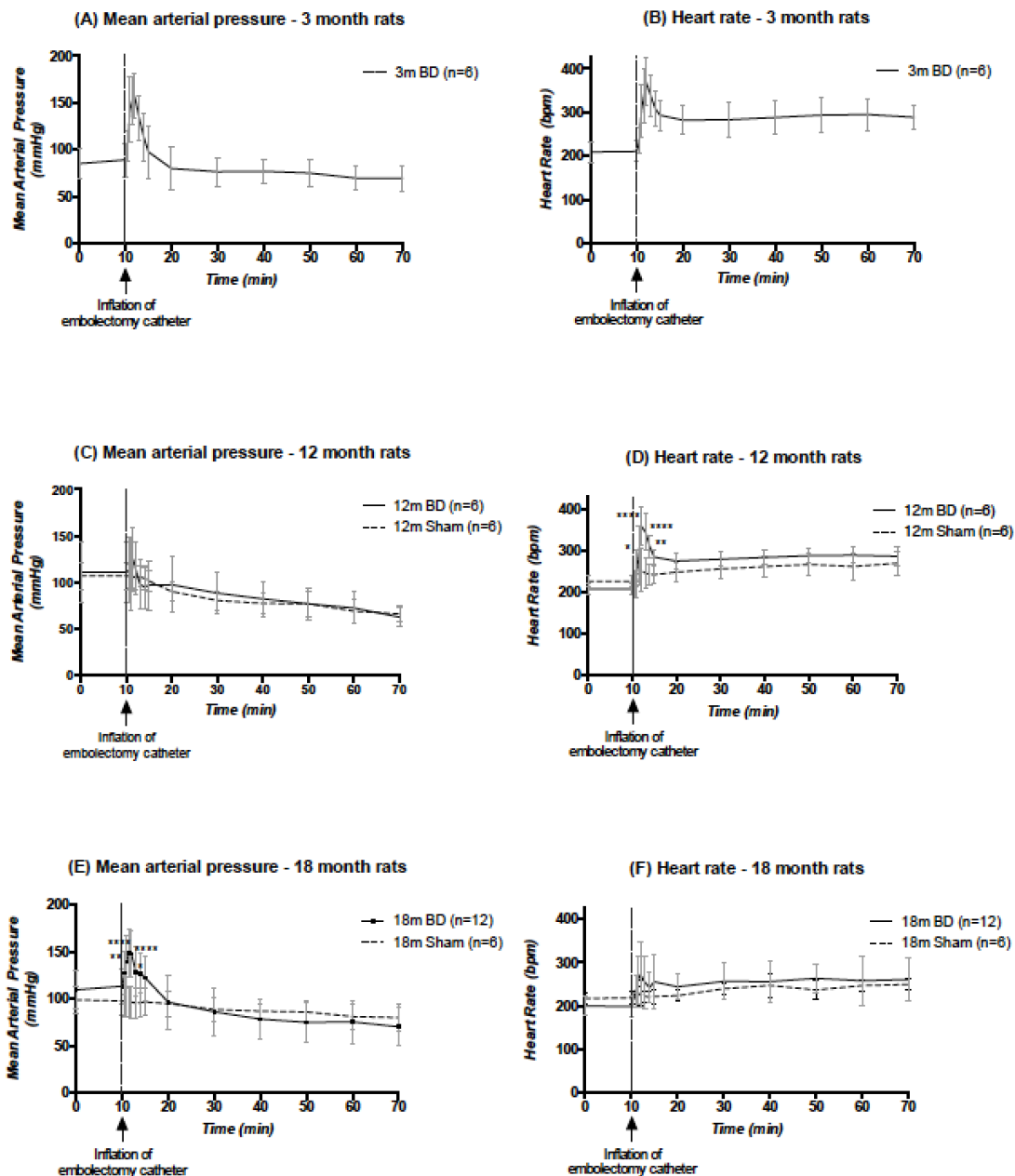


Figure 4.3 Haemodynamic changes observed during brain death or sham procedure

The mean arterial pressure (MAP) and heart rate (HR) during induction of BD versus sham procedure for 3-month animals (A and B), 12-month animals (C and D) and 18-month animals (E and F) are shown above (n=6 per group, n=12 for 18-month BD rats). A sharp rise in MAP and HR were observed during BD in all age groups. Significant differences compared to sham groups are seen in the HR of the 12-month group (D) and MAP of the 18-month group (E). **** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$

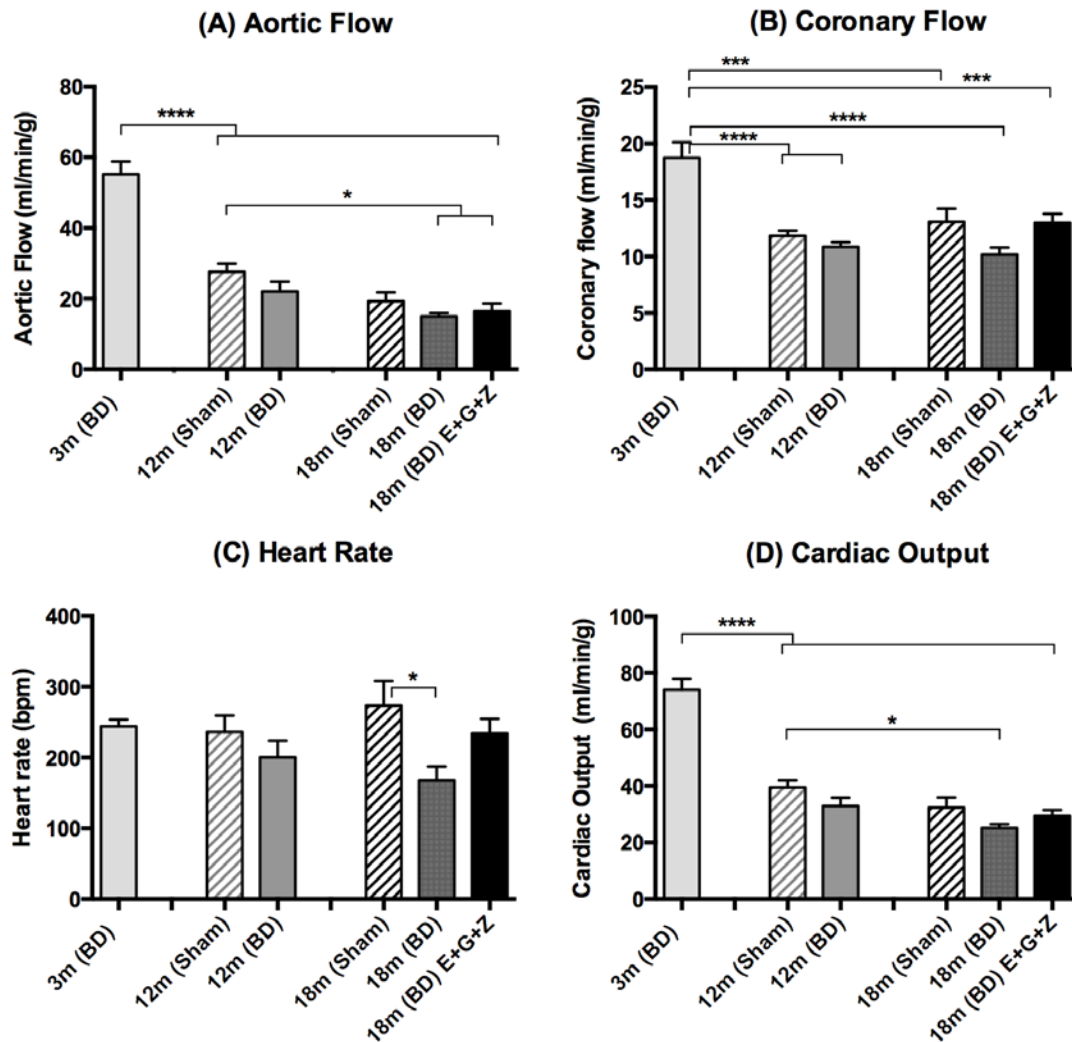


Figure 4.4 Cardiac function after brain death or sham procedure

Cardiac functional parameters measured on an ex-vivo perfusion circuit after BD or sham procedure are presented above for all study groups (mean \pm SEM, n=6 per group). Aortic flow (A), coronary flow (B) and cardiac output (D) are expressed as ml/min/g of calculated heart weight. BD indicates brain death; m, months and E+G+Z, erythropoietin, glyceryl trinitrate and zoniporide. ****p < 0.0001, ***p<0.001 **p < 0.01 and *p<0.05

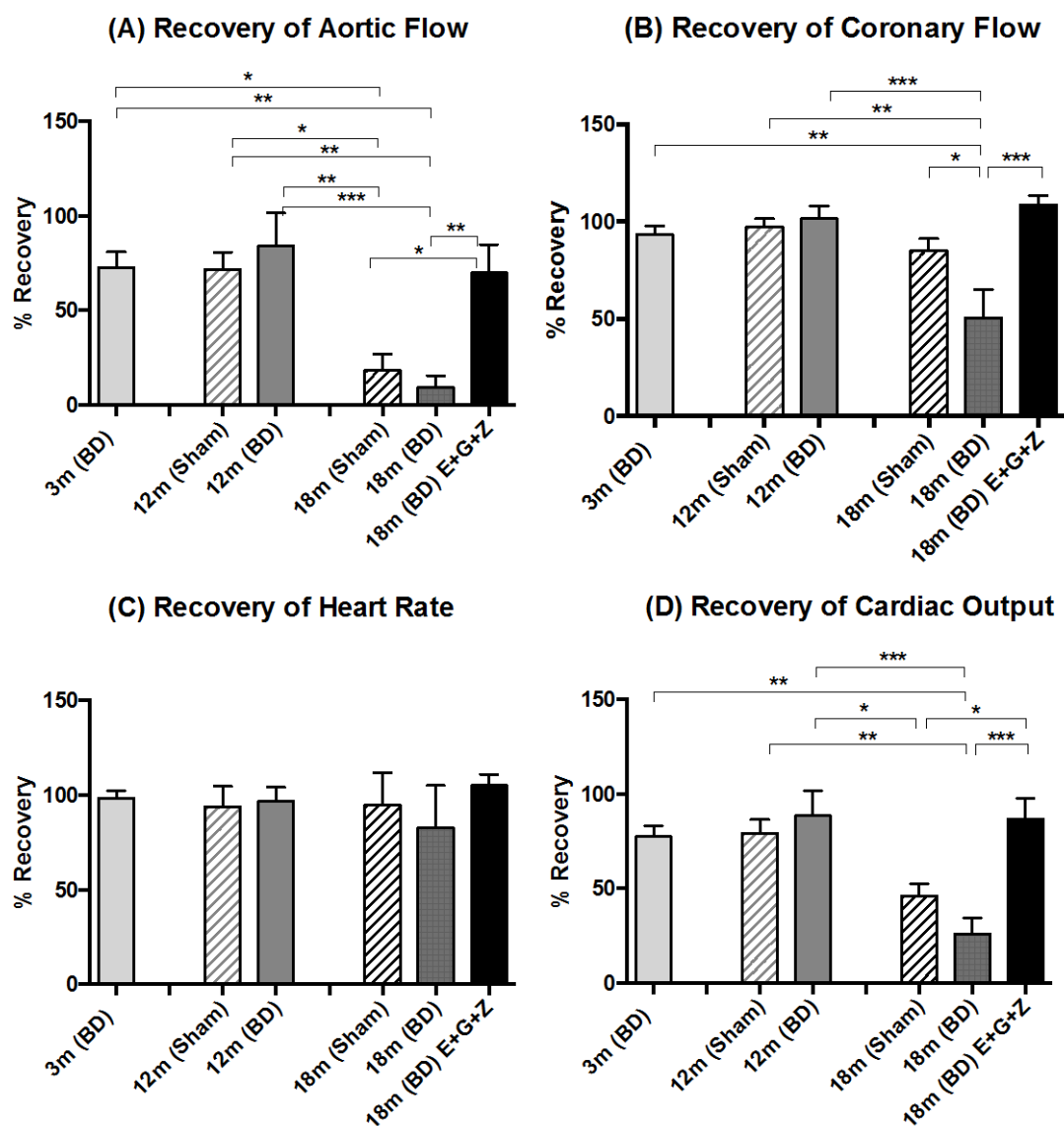


Figure 4.5 The recovery of cardiac function after 6 hours of hypothermic storage

Post-storage myocardial functional recovery in 3, 12 and 18-month hearts BD or sham hearts stored in Celsior and 18-month BD hearts stored in Celsior supplemented with erythropoietin, glyceryl trinitrate and zoniporide (E+G+Z). Data is presented as mean \pm SEM. BD indicates brain death; m, months. *** $p < 0.001$ ** $p < 0.01$ and * $p < 0.05$

4.3.4 Lactate dehydrogenase release

Lactate dehydrogenase (LDH) levels in the coronary effluent at reperfusion were significantly higher in 18-month BD hearts stored in Celsior® ($p<0.05$ at 30 min and $p<0.0001$ at 45 min, Figure 4.6). The LDH levels in 18-month BD hearts treated with EPO, GTN and ZON remained low during reperfusion, comparable to those of all 3 and 12-month hearts and 18-month sham hearts stored in Celsior® ($p=ns$).

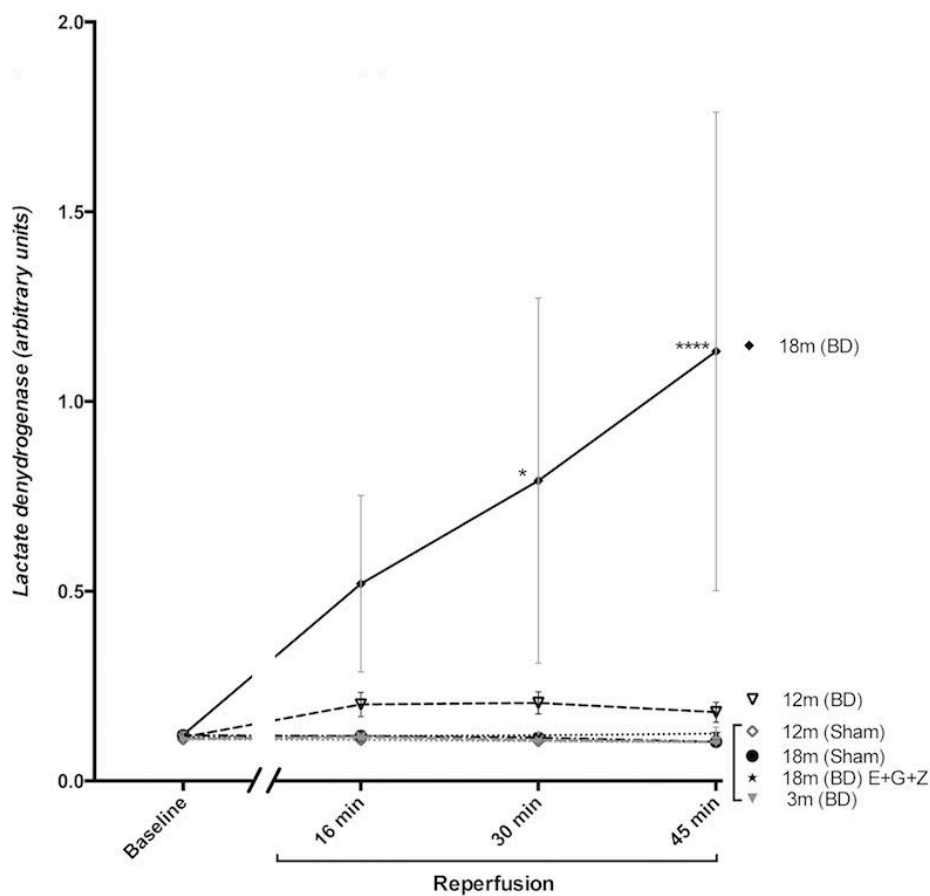


Figure 4.6 Lactate dehydrogenase levels

Lactate dehydrogenase (LDH) levels in the coronary effluent pre-cardioplegia (baseline) and at reperfusion (16, 30 and 45 minutes) are shown above. Values are represented as mean \pm SEM. Hearts from 18-month BD rats had significantly higher LDH levels at 30 and 45 minutes of reperfusion ($p<0.05$ and $p<0.0001$ respectively). Hearts from 12-month rats (both BD and sham) did not have significantly elevated LDH levels compared to hearts from rats aged 3 months. Hearts from rats treated with erythropoietin, glyceryl trinitrate and zoniporide (E+G+Z) did not have elevated LDH levels compared to 3-month hearts. **** $p<0.0001$, * $p<0.05$

4.3.5 Histopathology

Significantly increased myocardial edema ($p < 0.05$) was seen in 18-month BD hearts stored in Celsior® (figure 7A) and a trend towards increased contraction bands was noted in 18-month BD and sham hearts stored in Celsior® (Figure 4.7B). In 18-month hearts stored in Celsior® alone, myocyte injury was so severe that the ability to quantify contraction bands was limited. Representative images from the anterior left ventricle demonstrate severe interstitial oedema and ischemic necrosis in 18-month BD hearts stored in Celsior® (figure 7C). In comparison, 18-month BD hearts treated with EPO, GTN and ZON had significantly less interstitial oedema ($p < 0.05$) and myocyte injury (figure 7D). Control hearts (Figure 4.7E) demonstrated minimal interstitial oedema ($p < 0.001$) and had no evidence of myocyte injury.

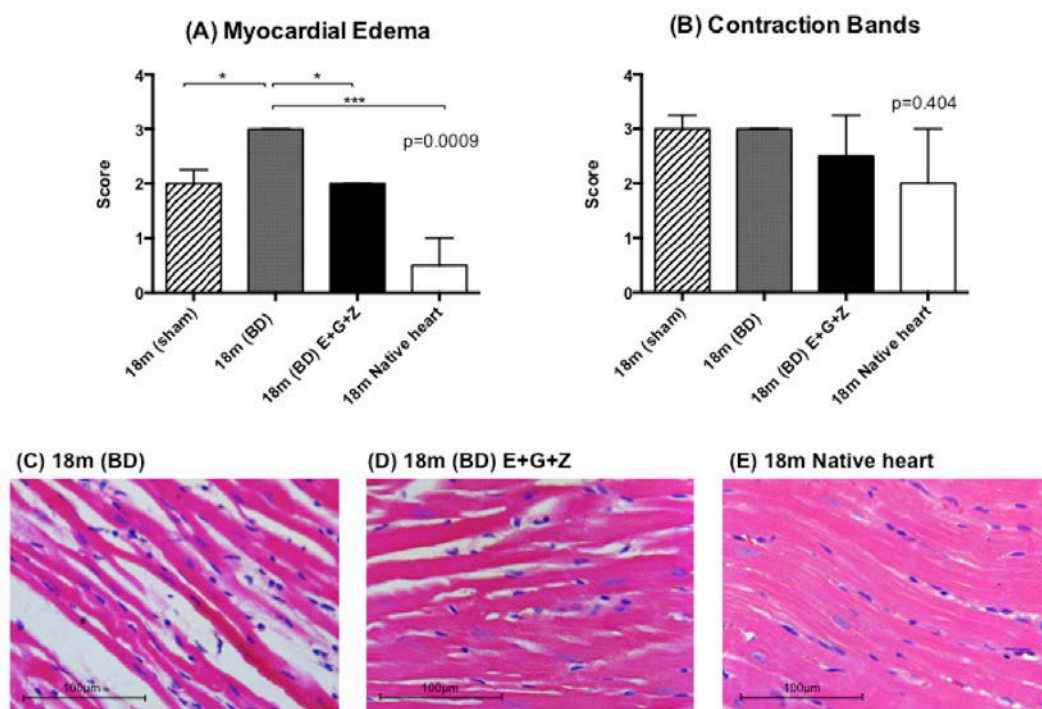


Figure 4.7 Histopathology of 18-month rat hearts

Semi-quantified scores for myocardial oedema and contraction bands are shown in figures A and B. The lack of error bars for certain values is due to presentation of the data as median \pm interquartile range. Representative light microscopy images (x 20 magnification) from the anterior left ventricle demonstrate significant interstitial edema and ischemic necrosis in 18-month BD hearts stored in Celsior® alone (figure C), less oedema and necrosis in 18-month BD hearts treated with EPO+GTN+ZON (figure D) and no oedema or necrosis in 18-month native hearts explanted immediately after anaesthesia (figure E). *** $p < 0.001$, * $p < 0.05$

4.3.6 Histopathology

Compared with 18-month sham and BD hearts stored in Celsior alone, increased phosphorylation of ERK 1/2 and Akt were seen in 18-month BD hearts treated with GTN, EPO and ZON ($p < 0.05$ to < 0.01 , figure 8A and B). A trend was also noted towards increased phosphorylation of AMPK α in the treated hearts (figure 8C). There were no differences in the phosphorylation of STAT3 across groups.

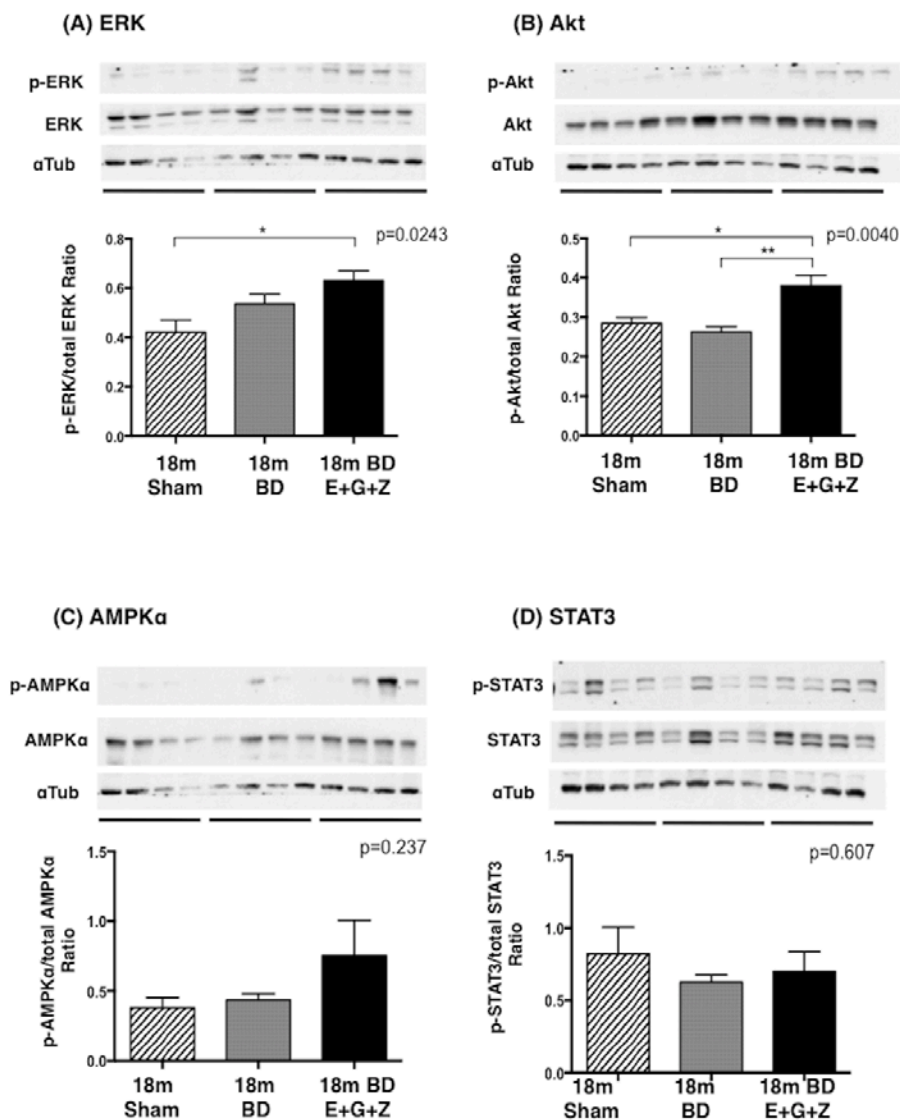


Figure 4.8 Western blots of pro-survival kinases in 18-month hearts.

The ratio of phospho:total ERK 1/2 and Akt were significantly increased in hearts treated with Celsior® supplemented with EPO, GTN and ZON ($n=4$ per group). There was a tendency towards increased phosphorylation of AMPK α , and no difference in the phosphorylation status of STAT3.

* $p < 0.05$, ** $p < 0.01$

4.4 Discussion

This study confirms that hearts from older donors are significantly more vulnerable to injury during donor brain death, prolonged hypothermic storage and reperfusion. It has importantly addressed two essential concepts that have long been debated. That is, that conditioning provides protection against IRI in the context of brain death as well as in aging.

Successful conditioning of brain dead donor hearts

The literature has been divided on the ability to condition hearts from BD donors. Studies using rabbit myocardial infarction models have shown an inability of ischemic preconditioning (IPC) to protect BD hearts, attributed to the extent of ischemic and inflammatory damage caused by BD³⁹²⁻³⁹⁴. Contrarily, a subsequent rabbit myocardial infarction study showed successful conditioning of BD hearts with either ischemic or pharmacological (isofluorane) preconditioning³⁹⁵. Our study supports the latter findings, demonstrating significant functional improvement, reduced LDH release and less histological evidence of myocardial injury with conditioning of BD hearts. Previous work in our laboratory further supports the ability to condition BD hearts, with significant improvement demonstrated in post-transplant cardiac function of porcine BD hearts conditioned with GTN, EPO and ZON³³⁰.

Successful conditioning of older donor hearts

The ability to successfully condition older hearts has also been debated. Studies on conditioning older hearts are limited and have shown either no benefit or only weak responses³⁹⁶. One study showed that significantly increased conditioning stimuli were required to achieve appreciable myocardial protection in older hearts³⁹⁷. The waning power of cardioprotection with aging was attributed to increased oxidative stress, myocardial fibrosis, increased cytosolic calcium accumulation during ischemia, reduced extracellular ligands and impaired signaling responses, mutations in mitochondrial nucleic acid, impaired transcription and expression of downstream signaling proteins and increased activity of phosphatases^{381,382,384,398}. However, again our study showed that despite brain death, older

hearts treated with pharmacological conditioning had significant improvement in functional, histopathological and biochemical parameters. Furthermore, the concentrations of EPO, GTN and ZON used, that is the conditioning stimuli, did not differ from those used in younger hearts^{329,330}.

Mechanisms of pharmacological conditioning in hearts from older donors with brain death

Increased phosphorylation of ERK 1/2 and Akt in our study indicates the ability to activate the RISK pathway despite BD and aging. The RISK pathway is central to EPO-mediated cardioprotection³⁹⁹⁻⁴⁰², and is comprised of MEK-ERK 1/2 and PI3K-Akt signaling cascades that converge to activate GSK-3 β , preventing opening of mitochondrial permeability transition pores (mPTP), a vital element in conditioning against IRI^{137,139}. Furthermore, ERK activation is stimulated by a low intra-cellular pH maintained during initial reperfusion by GTN and sodium-hydrogen exchange (NHE) inhibitors^{403,404}.

The exogenous supply of NO through GTN further abrogates IRI by activating cGMP-mediated vasodilation, inhibition of platelet and leucocyte adhesion and cGMP-independent opening of mitochondrial ATP-sensitive K⁺ channels^{325,405}. In addition, NO causes cysteine S-nitrosylation of xanthine oxidase and NADPH oxidase, reducing oxidative stress, preventing opening of mPTP and uncoupling of oxidative phosphorylation^{406,407}. Erythropoietin further increases NO availability by stimulating endothelial nitric oxide synthase (eNOS)⁴⁰⁸. Due to the bimodal action of NO, where it induces myocardial contractility at lower concentrations, but has negative inotropic effects at higher doses^{409,410}, the optimal concentrations of GTN and EPO had been determined in prior studies in order to prevent oversupply of NO^{329,330}. In addition to activating the RISK pathway and increasing NO availability, EPO reduces BD and ischemia-related uncoupling of endothelial nitric oxide synthase (eNOS), minimizing production of reactive oxygen species and nitrosative stress⁴⁰⁰.

The NHE-inhibitor ZON activates ERK 1/2 and STAT3, augmenting protection against mPTP opening during reperfusion, thereby minimizing ATP loss and decreasing mitochondrial superoxide production^{324,411-413}. It also prevents acidosis-triggered activation of NHE, minimizing intracellular accumulation of Na⁺ and water and subsequent activation of the Na⁺/Ca²⁺ exchanger, preventing intracellular Ca²⁺ overload⁴¹⁴. A trend towards increased phospho-AMPK α may also indicate activation of autophagy, central to overcoming cellular

injury due to IRI⁴¹⁵. The lack of increased phospho-STAT3 in our study may suggest impaired JAK-STAT signaling in aging, or that STAT3-mediated cardioprotection is seen in late conditioning, appreciated at 24 to 72 hours after reperfusion⁴¹⁶. A combination of protective mechanisms with retention of the ability to condition hearts despite aging and BD explains the significant functional recovery, lack of LDH release and histological evidence of minimal myocyte injury seen in hearts treated with EPO, GTN and ZON in our study.

Clinical application

The ability to condition hearts during cardioplegia and hypothermic preservation provides a model that is clinically applicable. As we are faced with an aging donor pool and the increased reliance on marginal donor organs, improved myocardial preservation is essential to ensure continued success in cardiac transplantation. The applicability and success of this method was demonstrated in a pilot clinical trial using EPO and GTN, two clinically available conditioning agents, where successful outcomes were seen after transplantation despite higher-risk donor and recipient profiles⁴¹⁷.

Conclusions

Pharmacological conditioning significantly protects aged hearts against IRI induced by brain death and subsequent hypothermic storage. In this first study assessing the efficacy of pharmacological conditioning of older donor hearts, we conclude that it is a simple yet effective approach to improving the quality of hearts retrieved from older donors, with potential to allow an increase in the acceptable donor age in cardiac transplantation.

**CHAPTER 5. PHARMACOLOGICAL CONDITIONING OF BRAIN
DEAD DONOR HEARTS WITH ERYTHROPOIETIN AND
GLYCERYL TRINITRATE: INITIAL CLINICAL EXPERIENCE**

5.1 Introduction

The ongoing success and progress in heart transplantation has led to acceptance of higher risk candidates for transplantation. These include patients of older age, with comorbidities, prior malignancies, redo sternotomies, and candidates on mechanical circulatory support (MCS)⁴. The rising demand for transplantation has also led to liberalization of donor acceptance criteria, with a significant increase in the use of extended criteria or 'marginal' donor hearts, in particular from older donors⁴.

Transplanting higher risk recipients and using marginal donor organs increases the risk of post-transplant complications such as primary graft dysfunction (PGD)^{25,28,31,33,418}. At present, 39% of deaths in the first 30 days post-transplantation are due to PGD, with a further 18% attributed to secondary causes such as multi-organ failure^{4,25}. Particular emphasis has been placed on the additive effect of combining older donor age and prolonged ischemic time on the risk of PGD^{4,35,419}. Certainly, longer ischaemic times are encountered in the current era of transplantation, due to use of non-local donors and longer procedural times associated with technically complex recipients⁴.

As discussed previously, literature on the pathology of graft dysfunction is limited, however a multi-center survey of autopsy results revealed ischemia-reperfusion injury (IRI) as the main histopathological finding in 48% of deaths attributed to PGD²⁵. Other findings such as myocyte necrosis (28%), multifocal edema and/or hemorrhage (14%) and freeze injury (7%) may also be attributed to IRI incurred following prolonged hypothermic storage. Improving donor heart preservation is therefore of utmost importance, with organ retrieval a potential opportunity for therapeutic intervention.

The addition of the nitric oxide donor, glyceryl trinitrate (GTN), the glycoprotein hormone erythropoietin (EPO), and sodium-hydrogen exchange (NHE)-inhibitors to cardioplegia and hypothermic storage has demonstrated significantly improved post-storage recovery of cardiac output, myocardial contractility and reduced biochemical and histological markers of IRI in several preclinical studies^{320-322,324-326,329,330,365,378} including studies on rodent hearts subjected to brain death (Chapter 3) and in hearts from aging rats subjected to brain death (Chapter 4). The mechanisms of action of pharmacological conditioning are through activation of pro-survival signaling cascades mediated by ERK1/2, Akt of the reperfusion

injury salvage kinase (RISK) pathway^{240,241}, STAT 3 of the survivor activating factor enhancement (SAFE) pathway^{228,239}, and cytoprotective effects of nitric oxide^{103,300}, that prevent oxidative stress, cellular oedema, intracellular calcium overload and preserve mitochondrial integrity to prevent cellular apoptosis and necrosis due to IRI^{51,53,137,138,238,316,420}.

Given the success of these measures in multiple small and large animal studies including models that incorporate donor brain death, we aimed to assess the efficacy of pharmacological conditioning in reducing IRI in clinical cardiac transplantation. Two clinically available pharmacological conditioning agents EPO and GTN were added to cardioplegia and hypothermic preservation of the donor heart, with the aim of reducing primary graft dysfunction and improving patient survival. We report initial findings from this clinical experience.

5.2 Methods

A retrospective analysis was conducted on donor and recipient data for cardiac transplants performed at a quaternary institution between January 2000 and November 2013. Transplants performed between August 2010 and November 2013 used Celsior preservation solution supplemented with GTN and EPO for cardioplegia and hypothermic storage (n=61), while transplant performed during April 2005 to June 2010 (n=104) used Celsior alone; and those between January 2000 to March 2005 (n=100) used modified St. Thomas' solution (STS). The study was conducted in compliance with the Australian Code for the Responsible Conduct of Research, and approved by the institution's ethics committee.

Donor, recipient and procedural data were obtained from a transplant database and medical records. Detailed data were collected to determine donor, recipient and procedural risk factors for PGD as defined by Kobashigawa et al²⁵. Transplants performed with organ retrieval by non-regional centers were excluded due to the use of other cardioplegic and hypothermic storage solutions.

5.2.1 Definition of extended criteria (marginal) donor hearts

Donor hearts were classified as 'marginal' (extended criteria) according to the definition stated in the Transplantation Society of Australia and New Zealand Consensus Statement on Eligibility Criteria and Allocation Protocols for Organ Transplantation from Deceased Donors, 2010. Marginal organs were defined by one of more of the following criteria: donor age > 50 years, ischemic time > 360minutes, donor left ventricular ejection fraction (LVEF) < 50% or regional wall motion abnormalities, the use of high dose inotropes (noradrenaline > 0.2µg/kg/min or equivalent) or donor hepatitis B, C or high risk behaviour.

5.2.2 Cardioplegia and hypothermic preservation

Antegrade cardioplegia was delivered via the aortic root in all transplants. Hearts were stored in hypothermic conditions (2-3°) during transport, using the same preservation solution as used in cardioplegia. All retrievals were performed by the study institution's transplant team.

5.2.3 Composition of preservation solutions

Celsior® was obtained commercially (Genzyme, Naarden, The Netherlands). Modified St. Thomas' Solution (STS) was prepared in-house, with the composition outlined in Table 5.1. Both solutions were 'extracellular'-based preservation solutions with hyperkalaemia and hypothermia the modes of induction of cardiac arrest. In the study group, Celsior® was supplemented with glyceryl trinitrate (Hospira Australia, Pty, Ltd, Mulgrave, AU) at a concentration of 0.1g/L, and erythropoietin-alpha (Eprex; Janssen-Cilag, North Ryde, AU) at 5000U/L. Glyceryl trinitrate and EPO were added to Celsior® at the recipient institution, just prior to cardioplegia and hypothermic storage. The optimal concentrations of pharmacological supplements were determined from preclinical studies^{320,329,330}.

Table 5.1 The composition of Modified STS and Celsior

	Modified STS	Celsior®
Electrolytes		
Na ⁺	149 mmol/L	100 mmol/L
K ⁺	19 mmol/L	15 mmol/L
Ca ²⁺	2.0 mmol/L	0.25 mmol/L
Mg ²⁺	19 mmol/L	13 mmol/L
Cl ⁻	120 mmol/L	41.5 mmol/L
Metabolic substrates		
Glucose	39 mmol/L	0
Aspartate	24 mmol/L	0
Osmotic agents		
Lactobionate	0	80 mmol/L
Mannitol	0	60 mmol/L
Acid-base buffers		
Histidine	0	30 mmol/L
Bicarbonate	28 mmol/L	0
Osmolarity	400mosm/L	360mosm/L

5.2.4 Determining post-transplant outcomes and survival

Immediate post-transplant outcomes were determined by analysis of the use of mechanical circulatory support [extra-corporeal membrane oxygenation (ECMO) or intra-aortic balloon pump (IABP)] within the first 24 hours of transplantation. The length of stay in the intensive care unit (ICU) and length of hospital admission were compared between groups. Data were obtained for survival for 1, 3 and 12-months post-transplantation, with survival outcomes for all recipients obtained for the total 12 month follow up period.

5.2.5 Definition of primary graft dysfunction

Primary graft dysfunction was defined by the use of ECMO or IABP in the first 24 hours after completion of cardiac transplantation surgery. Utilisation of ECMO to treat severe PGD was only instituted in our institution from 2005 onwards. Prior to 2005, all cases of PGD were managed by IABP support. Due to the retrospective nature of data collection, the use of

inotropes and nitric oxide were not included in the analysis to determine mild left ventricular or right ventricular dysfunction. Echocardiography was routinely performed in the first hours post transplantation, however data were not used due to the absence of standardized reporting and recording in medical records. Reports of the first endomyocardial biopsy (week 1 post-transplant) were analyzed to exclude severe rejection as a cause of allograft dysfunction.

5.2.6 Statistical analysis

Continuous variables are represented as mean \pm standard deviation and categorical data as the number of events and percentage. Differences between groups for continuous variables were calculated using one-way ANOVA with Tukey's post-hoc multiple comparisons test. Categorical data were analyzed using the Chi-square test. Survival data were analyzed using the Kaplan-Meier method. A p value < 0.05 was considered significant. All statistical analyses were performed using Prism 6 software (GraphPad Software, Inc., CA).

5.3 Results

5.3.1 Donor, recipient and procedural features

Donor, procedural and recipient risk factors for PGD for each group are outline in Table 5.2. There was a significantly higher proportion of hearts from donors > 50 years of age in the group where Celsior was supplemented with GTN and EPO (25%) and Celsior alone (23%) compared with STS (9%, $p<0.01$). Donors with anoxic brain injury, cerebral edema and meningitis were higher in the supplemented Celsior group (28%) compared with Celsior and STS groups (19% and 8.5% respectively, $p<0.01$).

A higher proportion of recipients were on mechanical circulatory support prior to transplantation in the supplemented Celsior group (36%) compared with Celsior (22%, $p<0.0001$) and STS (6.0%, $p<0.0001$ supplemented Celsior; $p<0.01$ Celsior). Procedural cross-clamp times were higher in the supplemented Celsior group (111 ± 36 minutes, $p<0.0001$) compared with Celsior (83 ± 24 minutes) and STS (82 ± 20 minutes).

Other trends observed were an increase in the mean donor age in supplemented Celsior (37.7 ± 12.8 years) and Celsior groups (36.8 ± 13.8 years), compared with STS (33.4 ± 12.6 years, $p=ns$), and a higher proportion of redo-sternotomies in the supplemented Celsior group (48%) compared with the Celsior (35%) and STS groups (35% and 34% respectively, $p = ns$). The proportion of donor hearts with left ventricular dysfunction at retrieval was similar in the 3 groups: supplemented Celsior (13%), Celsior (11%) and STS (10%, $p=0.85$). There were no differences in mean recipient age or the presence of pulmonary hypertension across groups. Between 33 to 43% of ischemic times were > 240 minutes, with no significant differences across groups.

Table 5.2 Donor, procedural and recipient risk factors for primary graft dysfunction

Preservation Solution	STS (n=100)	Celsior (n=104)	Celsior+EPO+GTN (n=61)	p-value
DONOR FACTORS				
Donor age (years, mean ± SD)	33.4 ± 12.6	36.8 ± 13.8	37.7 ± 12.8	ns
Donor weight (kg, mean ± SD)	79.3 ± 14.3	77.5 ± 15.1	80.9 ± 15.1	ns
Cause of death (n, %)				
Head trauma	36 (36%)	29 (28%)	17 (28%)	ns
CVA or ICH	55 (55%)	53 (51%)	25 (41%)	ns
Cardiac arrest	1 (1.0%)	2 (1.9%)	2 (3.3%)	ns
Other^ø	8 (8.0%)	20 (19%)	17 (28%)	<0.01
Valvular heart disease	3 (3.0%)	3 (2.9%)	3 (4.9%)	ns
Marginal donors (total)	44 (44%)	59 (57%)	34 (56%)	ns
High dose inotropes	30 (30%)	32 (31%)	20 (33%)	ns
Age 50-60 years	9 (9%)*	24 (23%)	15 (25%)	<0.01
LV dysfunction	10 (10%)	11 (11%)	8 (13%)	ns
Ischaemic time > 360min	4 (4.0%)	4 (3.8%)	1 (1.6%)	ns
PROCEDURAL FACTORS				
Ischaemic time (min, mean ± SD)	235 ± 68	232 ± 67	216 ± 63	ns
Ischaemic time > 240min (n, %)	43 (43.0%)	42 (40.4%)	20 (32.8%)	ns
Cross-clamp time (min, mean ± SD)	82 ± 20	83 ± 24	111 ± 36	<0.0001
Female to male transplants (n, %)	13 (13%)	16 (15%)	8 (13%)	ns
Donor : Recipient weight <0.8 (n, %)	3 (3.0%)	10 (9.6%)	2 (3.3%)	ns
Emergency transplant (n,%)	2 (2.0%)	5 (4.8%)	3 (4.9%)	ns

RECIPIENT FACTORS				
Recipient age (years, mean \pm SD)	47.6 \pm 11.8	48.4 \pm 12.8	46.4 \pm 13.2	ns
Recipient weight (kg, mean \pm SD)	76.1 \pm 14.3	73.1 \pm 14.9	74.1 \pm 17.0	ns
MCS (n, %) (LVAD, BiVAD or TAH)	6 (6.0%)	23 (22%)	22 (36%)	<0.0001
ECMO or IABP pre-transplant (n, %)	1 (1.0%)	0	0	ns
Congenital heart disease (n, %)	4 (4.0%)	6 (5.8%)	3 (4.9%)	ns
Redo sternotomy (n, %)	34 (34.0%)	36 (34.8%)	29 (47.5%)	ns
Multiorgan transplant	1 (1.0%)	3 (2.9%)	2 (3.3%)	ns
Heart-Lung	1 (1.0%)	1 (0.9%)	0	ns
Heart-Kidney				
Recipient PHT[#]	23 (23%)	22 (21%)	13 (21%)	ns
Retransplant	0	0	0	ns

∅ Anoxic brain death (asthma, hanging), cerebral edema from overdoses and meningitis.

[#] Recipient pulmonary hypertension was defined by a transpulmonary gradient \geq 12mmHg.

STS indicates St. Thomas' Solution; EPO, erythropoietin; GTN, glyceryl trinitrate; CVA, cerebrovascular accident; ICH, intracranial hemorrhage; MCS, mechanical circulatory support; LVAD, left ventricular assist device; BiVAD, biventricular assist device; TAH, total artificial heart; ECMO, extra-corporeal membrane oxygenation; IABP, intra-aortic balloon pump; and PHT, pulmonary hypertension

5.3.2 Post-transplant outcomes

Primary graft dysfunction as defined by the use of ECMO or IABP within the first 24 hours of transplantation was present in 25% of supplemented Celsior, 32% of Celsior and 32% of STS transplants (p=ns, Table 5.3). Venous-arterial (VA) ECMO was used in all cases except one patient in the Celsior group who received veno-pulmonary arterial (V-PA) ECMO but also had an IABP. In marginal hearts, PGD occurred in 29% of supplemented Celsior, 37% of Celsior and 39% of STS transplants (p=ns). Severe rejection was not identified as the reason for implementation of MCS in any of the transplants. There were no significant differences in the length of stay in ICU or hospital between groups (Table 5.4).

Of note, recipients in the STS group only received IABP support post-transplantation, which reflects practices in that era of transplantation (Table 3). The use of ECMO increased and the use of IABP decreased in Celsior and supplemented Celsior groups, also reflecting practices at the time.

Table 5.3 Use of ECMO or IABP post-transplantation

	STS	Celsior	Celsior+EPO+GTN	p-value
<u>ALL HEARTS</u>				
ECMO or IABP (n, %)	32 (32.0%) (32 IABP)	33 (31.7%) (22 ECMO, 11 IABP)	15 (24.6%) (13 ECMO, 2 IABP)	ns
<u>MARGINAL HEARTS</u>				
ECMO or IABP (n, %)	17/44 (38.6%) (17 IABP)	22/59 (37.2%) (16 ECMO, 6 IABP)	10/34 (29.4%) (9 ECMO, 1 IABP)	ns

The use of extra-corporeal membrane oxygenation (ECMO) or an intra-aortic balloon pump (IABP) within the first 24 hours post-transplant is shown above for all hearts and for marginal hearts.

Table 5.4 Length of stay: all hearts

	STS (n = 48)	Celsior (n = 59)	Celsior+EPO+GTN (n = 34)	p-value
LOS ICU (days, mean \pm SD)	6.1 \pm 6.3	6.2 \pm 6.3	8.9 \pm 10.9	0.08
LOS hospital (days, mean \pm SD)	24 \pm 20	29 \pm 28	27 \pm 18	0.40

LOS indicates length of stay.

Survival

Survival at 30-days post transplant was 92%, 95% and 98% in STS, Celsior and supplemented respectively (p=ns, Figure 5.1). Survival at 3-months was 89%, 91% and 93% (p=ns) and at 12-months was 86%, 89% and 90% respectively (p=ns).

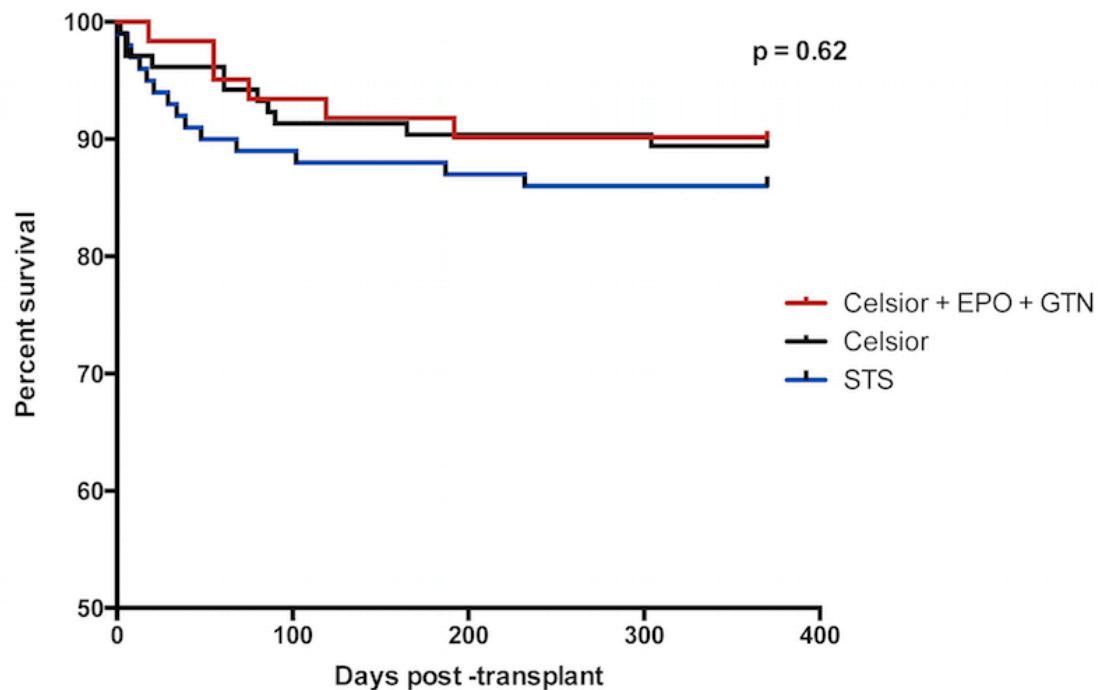


Figure 5.1 Survival (all hearts) at 370 days post-transplant

5.4 Discussion

The introduction of brain dead donor heart conditioning with GTN and EPO at arrest, perfusion and storage at our institution has resulted in high patient survival despite increasing donor, procedural and recipient risk factors for PGD. The survival rate observed in our series compares favourably with ISHLT registry data which reported post-transplant survival of 92% at 1-month, 90% at 3- months and 85% at 1-year in contemporaneous years between 2006 and 2011^{4,25}. To our knowledge, this is the first study investigating the efficacy of pharmacological conditioning of donor hearts with the combination of EPO and GTN in clinical cardiac transplantation. Excellent post-transplant survival and a trend towards reduced requirement for MCS compared with historical controls despite the acceptance of higher risk donors and recipients suggest that pharmacological conditioning is a promising approach to donor heart preservation. Longer-term follow-up will be important to determine if this conditioning strategy protects the vascular endothelium and reduces the incidence of cardiac allograft vasculopathy.

The need for MCS to treat PGD in our series is relatively high compared with other published series^{16,421}. We believe this is explained in part by our relatively high utilization of hearts from marginal donors in an era when more than one third of transplant candidates were supported on MCS at time of transplant. Indeed, in a recent US registry analysis, Fudim et al⁴²² reported that implantation of marginal donors into VAD-supported patients was an independent risk factor for early graft failure and death. In the most recent era, 56% of donors had one or more marginal characteristics. Of particular note, 25% of donors in the most recent cohort were over the age of 50 years and another 13% had evidence of left ventricular systolic dysfunction at the time of retrieval. The advent of peripheral ECMO as a relatively simple and safe option for short-term mechanical support may have introduced two biases in the more recent era, firstly a greater preparedness on the part of surgeons to utilize marginal donors and secondly a move towards earlier utilization of ECMO to allow the heart more time to recover from the transplant process rather than attempt repeated weaning of a heart that is clearly struggling at the first attempt.

Future directions

This first study on pharmacological conditioning of donor hearts in clinical cardiac transplantation has demonstrated safety and non-inferiority of this strategy of myocardial preservation. The translation of the pharmacological conditioning strategy was based on robust pre-clinical data which demonstrated significant improvements in the recovery of donor hearts in small and large animals^{320-322,324-326,329,330,365,367}. These studies also demonstrated reduced biochemical markers of myocardial injury such as lactate dehydrogenase and troponin I, increased phosphorylation of key cell survival signaling enzymes ERK1/2, Akt, GSK-3 β and reduction of markers of apoptosis such as cleaved caspase 3. A limitation in the translation of our pharmacological conditioning protocol from animal studies to man was that only two pharmacological agents, GTN and EPO are approved for clinical use. The third agent, an NHE-inhibitor, either zoniporide or cariporide, was not used due to discontinuation of clinical development of these agents due to safety concerns following the Expedition trial (Ref 33). In that trial, repeated bolus intravenous doses of cariporide for myocardial preservation in high-risk coronary artery bypass grafts (EXPEDITION trial)³²³ demonstrated a higher incidence of fatal cerebrovascular accidents despite significantly improved myocardial protection. In the context of cardiac transplantation, NHE-inhibitors would only be used in cardioplegia and hypothermic storage of the donor heart and not administered systemically in the recipient, hence similar adverse effects are unlikely. Based on our previous finding of a synergistic interaction between zoniporide, EPO and GTN in preclinical studies, we believe that the addition of an NHE inhibitor would result in further improved myocardial preservation and reduced PGD if this 'triple supplementation' strategy is able to be implemented clinically.

We have also added EPO and GTN to STS in clinical heart transplants from donation after circulatory death donors conducted at our institution²²³. These transplants utilized normothermic ex vivo perfusion (NEVP) with for transport of the donor heart. The decision to flush the donor heart with STS rather than Celsior was based on the recommendation of the NEVP manufacturer.

Limitations

There are some limitations to our study. Although all data were prospectively entered into a dedicated database, the analysis was conducted retrospectively. This was a single centre study and historical controls were used as a basis for comparison. The use of historical controls however does highlight the dramatic changes that have occurred in both donor and recipient populations in little more than a decade. This study did not use hemodynamic criteria or echocardiographic data, hence all mild and some moderate cases of primary graft dysfunction were not captured in our data. Short-term use of inotropes and nitric oxide was often part of standard post-transplant treatment, and it was difficult to retrospectively discern true hemodynamics in all groups in the presence of inotrope use.

An era effect was demonstrated in practices of IABP and ECMO use, and may also reflect improved survival in the more contemporary supplemented Celsior group. The use of ECMO, as opposed to ventricular assist device use for PGD has been associated with improved survival and is now considered a safer and more effective method of maintaining circulatory support in severe PGD⁴²³. Of patients treated with ECMO in our study, 80% one-year survival was demonstrated. A prospective multi-centre randomized controlled trial would be required to address these limitations and provide further support for our hypothesis that pharmacological conditioning improves donor heart preservation in the current era.

Conclusion

Early results of pharmacological conditioning demonstrate excellent survival rates in the immediate and early post-transplant period. The rate of primary graft dysfunction remains high and indicates an ongoing need for further improvements in donor heart preservation in an era of increasing donor and recipient risk profiles.

CHAPTER 6. ASSESSING THE CONDITIONING PROPERTIES OF SILDENAFIL IN DONOR HEART PRESERVATION

6.1 Introduction

The aim of this study was to assess the efficacy of sildenafil in minimising ischaemia-reperfusion injury (IRI) when added to cardioplegia and hypothermic preservations used in donor heart preservation. As previously discussed (chapters 3 and 4), pharmacological conditioning by triple supplementation with erythropoietin (EPO), glyceryl trinitrate (GTN) and sodium-hydrogen (NHE) inhibitor, zoniopride (ZON), significantly improves recovery of young and aged rodent hearts subjected to brain death and prolonged hypothermic storage. These protective effects have been also confirmed in prior rodent and porcine studies^{329,330}.

However, NHE-inhibitors, a crucial component of combined pharmacological conditioning, have been recently withdrawn from clinical use. The withdrawal of the NHE-inhibitor cariporide occurred after findings of the 2008 'EXPEDITION' trial³²³, where cariporide injected systemically in patients undergoing cardiopulmonary bypass for coronary artery surgery, increased mortality secondary to cerebrovascular accidents. Although the study involved injection of bolus doses of cariporide at high systemic concentrations, safety concerns led to its general discontinuation from clinical use. This was subsequently followed by clinical inaccessibility to zoniopride, which was of the same therapeutic class.

The clinical translation of pharmacological supplementation with pro-survival kinase agents during cardioplegia and hypothermic storage, was therefore limited to two of the three agents, EPO and GTN. As discussed in chapter 5, the clinical study demonstrated non-significant trends of reduced primary graft dysfunction (PDG) and improved survival when EPO and GTN were used to supplement Celsior in a cohort of higher risk donors and recipients. However the efficacy of pharmacological conditioning at reducing PGD and improving early survival may have been blunted due to the absence of the NHE-inhibitor ZON. This hypothesis was strengthened by a preclinical porcine study³³⁰ that highlighted the requirement of all three agents to allow recovery of cardiac function after transplantation. Hearts were unable to be weaned off cardiopulmonary bypass when one or two of the above-mentioned pharmacological agents were used in cardioplegia and hypothermic storage. However 80% of hearts were weaned with the presence of all three agents. The need for a third agent, preferentially targeting NHE-inhibition, is therefore urgently needed to allow successful clinical translation of pharmacological conditioning strategies.

This study therefore aimed to assess the efficacy of sildenafil, a selective inhibitor of cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type-5 (PDE-5), as an additive pharmacological conditioning agent. Sildenafil is known for its vasodilatory effects that occur due to increased levels of cGMP due to inhibition of PDE-5, and is used clinically to treat erectile dysfunction⁴²⁴⁻⁴²⁶ and pulmonary hypertension⁴²⁷⁻⁴²⁹.

Studies have detected PDE-5 in cardiomyocytes⁴³⁰, with specific conditioning effects of sildenafil against IRI demonstrated in both in situ³¹⁰ and isolated^{331,431-433} rabbit, rat and mouse studies. Mechanisms of cardioprotection have been thought to be mediated via increased cGMP due to direct inhibition of PDE-5 as well as activation of guanylyl cyclases⁴³⁴. Activation of protein-kinase G (PKG) by cGMP, and subsequent opening of sarcolemmal³³¹ and mitochondrial potassium ATP channels^{309,310,434} confers ultimate cardioprotective 'conditioning' effects. Opening of mitochondrial ATP channels (mKATP) enables a H⁺ electrochemical gradient that allows ATP synthesis, calcium transport and protection of mitochondrial function⁴³⁵⁻⁴³⁷. Effects of sildenafil on mKATP channels may occur directly through increased cGMP levels, or through signaling pathways, such as activation of protein kinase C (PKC)⁴³⁸ and mitogen-activated protein kinases (MAPK), in particular ERK 1/2^{308,439}.

A delayed phase of pharmacological conditioning has also been demonstrated with activation of inducible nitric oxide synthase (iNOS)⁴³¹, which generates nitric oxide (NO) with known effects of opening mKATP channels⁴⁴⁰. Sildenafil has also been demonstrated to activate mitochondrial Ca²⁺ activated K⁺ (mitoK_{Ca}) channels⁴⁴¹ in delayed preconditioning and preventing apoptosis and cellular necrosis through an increased Bcl2:BAX ratio⁴⁴². In addition, sildenafil has been demonstrated to prolong repolarization of cardiac muscle and to reduce ventricular arrhythmias during ischaemia⁴⁴³, although conversely has been shown to increase reperfusion arrhythmias at higher doses⁴³². Interestingly, studies have also reported that sildenafil inhibited NHE-1 phosphorylation at reperfusion^{433,444}. Although the inhibition of NHE-1 was weaker than seen with cariporide in one study, reducing NHE-1 phosphorylation raises in the possibility of using sildenafil as an alternate pharmacological conditioning agent to ZON. Furthermore, given the clinical availability of sildenafil, and its low systemic side-effect profile, successful findings in preclinical studies may be more feasible to apply clinically.

A screening rodent study was therefore designed to add sildenafil to Celsior®, either alone, or in combination with EPO and/or GTN, and assess the recovery of cardiac function in an ex-vivo perfusion circuit after 6 or 10 hours of hypothermic storage. Results from a prior study where EPO, GTN and ZON were added to Celsior®³²⁹, were used as a comparative 'gold standard'. The activation of ERK 1/2 signaling, a proposed key mechanisms of sildenafil-mediated cardioprotection, was tested by assessing phosphorylation of ERK 1/2 with western blots of ventricular tissue.

An ex-vivo dose-response study demonstrated maximum cardioprotective effects of sildenafil at concentrations between 20-50nmol/l³³¹. This was attributed to a higher cGMP:cAMP ratio which was reversed at higher concentrations, demonstrated to worsen cardiac function at reperfusion. An in-vivo study further demonstrated a narrow therapeutic window between 0.01 and 0.05mg/kg in rats⁴³², with lower doses being ineffective and higher doses associated with a risk of ventricular fibrillation at reperfusion. Based on these studies, a dose of 50nmol/l was used in this study, and findings are reported below.

6.2 Methods

6.2.1 The experimental protocol

An ex-vivo perfusion circuit (as described in Chapter 2, Section 2.5) was used in cardiac functional assessment. Brain death was not used in this screening study. The experimental protocol is outlined in Figure 6.1.

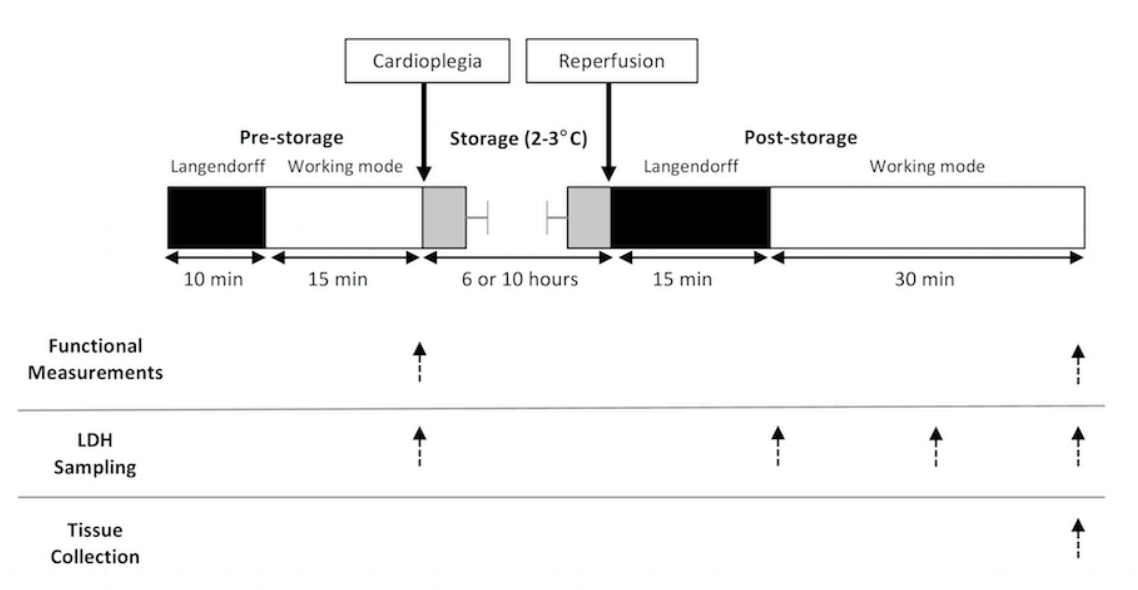


Figure 6.1 The experimental protocol

6.2.2 Animals

Male Wistar rats aged 10-12 weeks (320-380g) were used in this study. The outbred Wistar strain was used for consistency with prior screening studies conducted in the laboratory.

6.2.3 Anaesthesia

Anaesthesia was provided with an intraperitoneal injection of ketamine (80mg/kg) and xylazine (10mg/kg), as described in Section 2.6.2.

6.2.4 Study groups

Study groups are outlined in Table 6.1 below. Six animals were allocated per group. A control group comprised of hearts with cardioplegia and 6 hours of hypothermic storage using Celsior® preservation solution. Test groups comprised of hearts allocated to cardioplegia and 6 hours of hypothermic storage with Celsior® supplemented with sildenafil alone, or in combination with erythropoietin (EPO) or glyceryl trinitrate (GTN).

Preservation solutions that demonstrated significantly improved recovery of cardiac function after six hours were used to store hearts over an extended time period of 10 hours of hypothermic storage (n=6 per group, Table 6.1). An additional group was studied, comprising cardioplegia and hypothermic storage with Celsior® with triple supplementation with sildenafil, EPO and GTN and extended hypothermic storage over 10 hours.

Table 6.1 Study groups

Duration of Hypothermic Storage	Cardioplegic and hypothermic preservation Solution	Number of rats
6 hours	Celsior	6
6 hours	Celsior + Sildenafil	6
6 hours	Celsior + Sildenafil + EPO	6
6 hours	Celsior + Sildenafil + GTN	6
10 hours	Celsior + Sildenafil	6
10 hours	Celsior + Sildenafil + EPO	6
10 hours	Celsior + Sildenafil + EPO + GTN	6

6.2.5 Assessment of baseline cardiac function

Hearts were explanted and cannulated onto the ex-vivo perfusion circuit as described in Chapter 2, Section 2.7.

Baseline functional measurements of aortic flow (AF, ml/min), coronary flow (CF, ml/min), heart rate (HR, beats/min) and cardiac output (CO, ml/min) were obtained at 15 minutes into working mode (Figure 6.1).

6.2.6 Cardioplegia and hypothermic storage

Celsior® was used alone or supplemented with sildenafil, glyceryl trinitrate and/or erythropoietin at concentrations outlined in Table 6.2. Sildenafil was obtained as the citrate salt available as a solution for injection, Revatio 10mg/12.5ml (Pfizer Australia, West Ryde, AU). All pharmacological supplements were added to Celsior® within 5 minutes of use, and the solution was mixed using a magnetic stirrer for 2-3 minutes to ensure uniform distribution. The mode of delivery of cardioplegia was antegrade at a temperature 2-3°C as described in Chapter 2, Section 2.8.

Table 6.2 Concentrations of sildenafil, erythropoietin and glyceryl trinitrate

	Concentration	Source
Sildenafil citrate	50nmol/l	Pfizer Australia Pty Ltd., West Ryde, AU
Erythropoietin alpha	5000U/l	Janssen-Cilag, North Ryde, AU
Glyceryl trinitrate	100mg/l	Hospira Australia Pty Ltd, Mulgrave, AU

The optimal concentration of 50nmol/l of sildenafil was determined from a published ex-vivo rodent study³³¹, and concentrations for erythropoietin and glyceryl trinitrate were based on previous studies conducted in the laboratory³²⁹

6.2.7 Assessment of cardiac function after hypothermic storage

The recovery of cardiac function after hypothermic storage was assessed on the ex-vivo perfusion circuit as described in Chapter 2, Section 2.9. Aortic flow, coronary flow, heart rate and cardiac output at 30 minutes in working mode were expressed as percentages of baseline values using the formula:

$$\text{Percentage recovery (\%)} = \frac{\text{Post-storage measurement}}{\text{Baseline measurement}} \times 100$$

6.2.8 Analysis of western blots and lactate dehydrogenase levels

Samples of the basal anterolateral wall of the left ventricle were stored at -80°C for immunoblotting as described in Chapter 2, Section 2.12. Three samples of each 6 hour storage group were analysed to determine the ratio of phosphorylated to total protein for ERK ½, AMPKα, Akt and STAT 3.

Coronary effluent was collected at baseline and reperfusion, at time points shown in Figure 6.1 and the presence of lactate dehydrogenase was quantified for all 6 and 10 hour storage groups, as described in Chapter 2, Section 2.10.

6.2.9 Statistical analysis

All variables are expressed as mean ± standard deviation unless otherwise specified. Differences between groups were compared using one-way ANOVA with Tukey's post-hoc analysis. A p value <0.05 was considered statistically significant. Data were analysed using Prism 6 software (GraphPad Software Inc., CA).

6.3 Results

The mean weights of animals in each study group are shown in Table 6.3. There were no significant differences between groups.

Table 6.3 Mean weights of animals

Preservation time	Preservation solution	Number of animals	Weight (g, mean ± SD)
6h	Celsior	6	342.2 ± 12.8
6h	Celsior + Sildenafil	6	349.8 ± 11.6
6h	Celsior + Sildenafil + EPO	6	350.9 ± 9.6
6h	Celsior + Sildenafil + GTN	6	348.3 ± 10.2
10h	Celsior + Sildenafil	6	353.5 ± 9.5
10h	Celsior + Sildenafil + EPO	6	342.8 ± 14.2
10h	Celsior + Sildenafil + EPO + GTN	6	349.3 ± 10.7

All values are expressed as mean ± standard deviation. EPO indicates erythropoietin and GTN, glyceryl trinitrate. P = ns between groups.

6.3.1 Baseline cardiac function

Baseline (pre-cold storage) aortic flow, coronary flow, heart rate and cardiac output are demonstrated in Table 6.4. There were no significant differences in baseline parameters between study groups.

Table 6.4 Baseline cardiac function

Study Group	AF (ml/min)	CF (ml/min)	CO (ml/min)	HR (bpm)
6h Celsior	47.2 ± 11.2	15.2 ± 2.6	62.3 ± 12.1	223 ± 47
6h Sildenafil	47.2 ± 10.1	19.0 ± 9.1	63.0 ± 13.9	243 ± 32
6h Sildenafil + EPO	46.0 ± 5.5	14.2 ± 0.8	49.8 ± 5.6	210 ± 18
6h Sildenafil + GTN	42.5 ± 8.6	16.8 ± 3.0	62.0 ± 8.4	245 ± 49
10h Sildenafil	43.8 ± 9.8	15.0 ± 2.1	47.0 ± 7.8	224 ± 28
10h Sildenafil + EPO	42.2 ± 6.3	19.3 ± 6.0	59.6 ± 9.5	261 ± 12
10h Sildenafil + EPO + GTN	43.8 ± 11.1	15.1 ± 1.5	56.4 ± 8.7	240 ± 24
p value	0.49	0.41	0.14	0.21

All values are expressed as mean ± standard deviation, with n=6 per group.

6.3.2 Recovery of cardiac function after hypothermic storage

6.3.2.1 Recovery of hearts after 6 hours of hypothermic storage

The percentage recovery of AF, CF, CO and HR after 6 hours of hypothermic storage, are shown in Figure 6.2(A) to (D) respectively.

The recovery of AF was highest in hearts supplemented with sildenafil + EPO (74 ± 15%), with significant differences compared with hearts stored in Celsior alone (24 ± 25%, $p < 0.01$) and hearts supplemented with sildenafil + GTN (32 ± 30%, $p < 0.05$). Supplementation with sildenafil alone did not have significantly higher recovery of AF (52 ± 26%, $p = \text{ns}$ compared with Celsior).

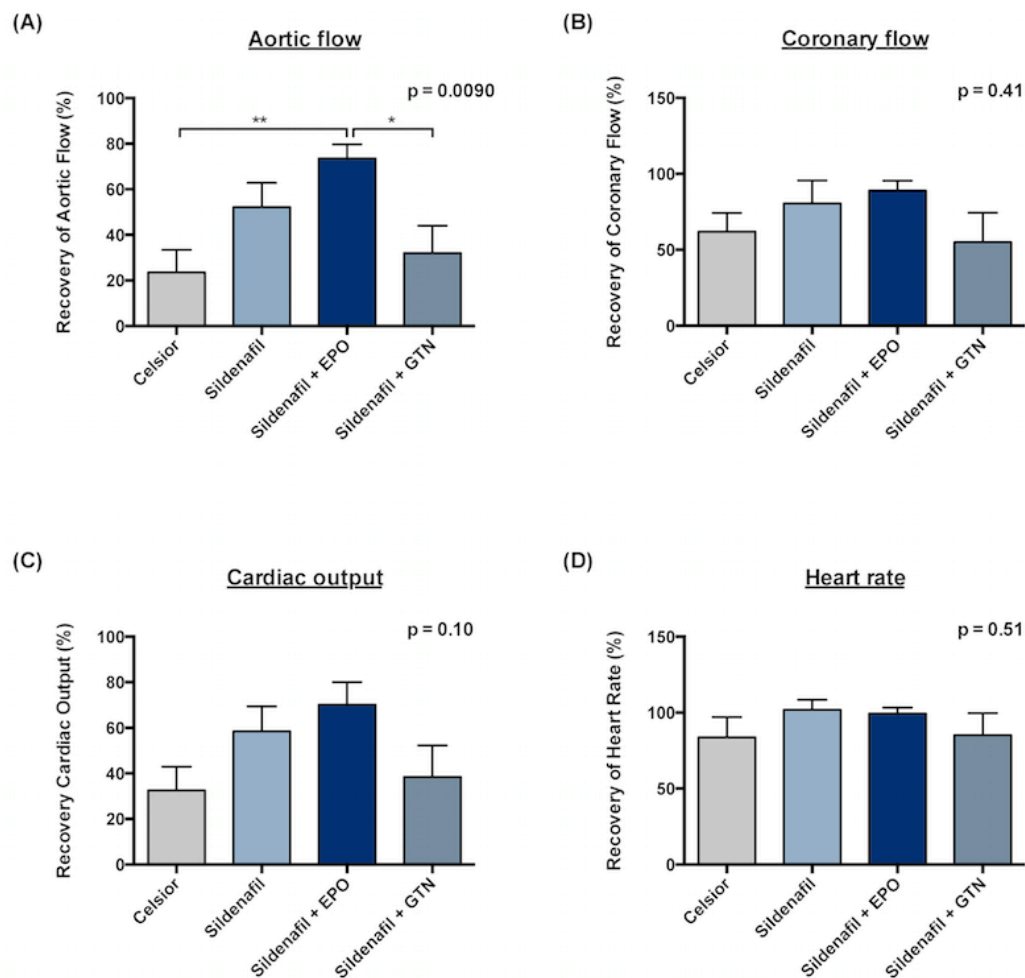


Figure 6.2 Recovery of hearts after 6 hours hypothermic storage

EPO indicates erythropoietin and GTN, glyceryl trinitrate.

There were no significant differences between groups in the recovery of CF or HR. The recovery of CO tended to be higher in hearts supplemented with sildenafil + EPO ($70 \pm 30\%$), however due to intra-group variability of results, it was not significantly higher than Celsior or the other supplemented groups.

6.3.2.2 Recovery of hearts after 10 hours of hypothermic storage

The percentage recovery of AF, CF, CO and HR for hearts stored for 10 hours are outlined in Figure 6.3 (A) to (D) respectively. There were no significant differences in the recovery of AF, CF, HR or CO.

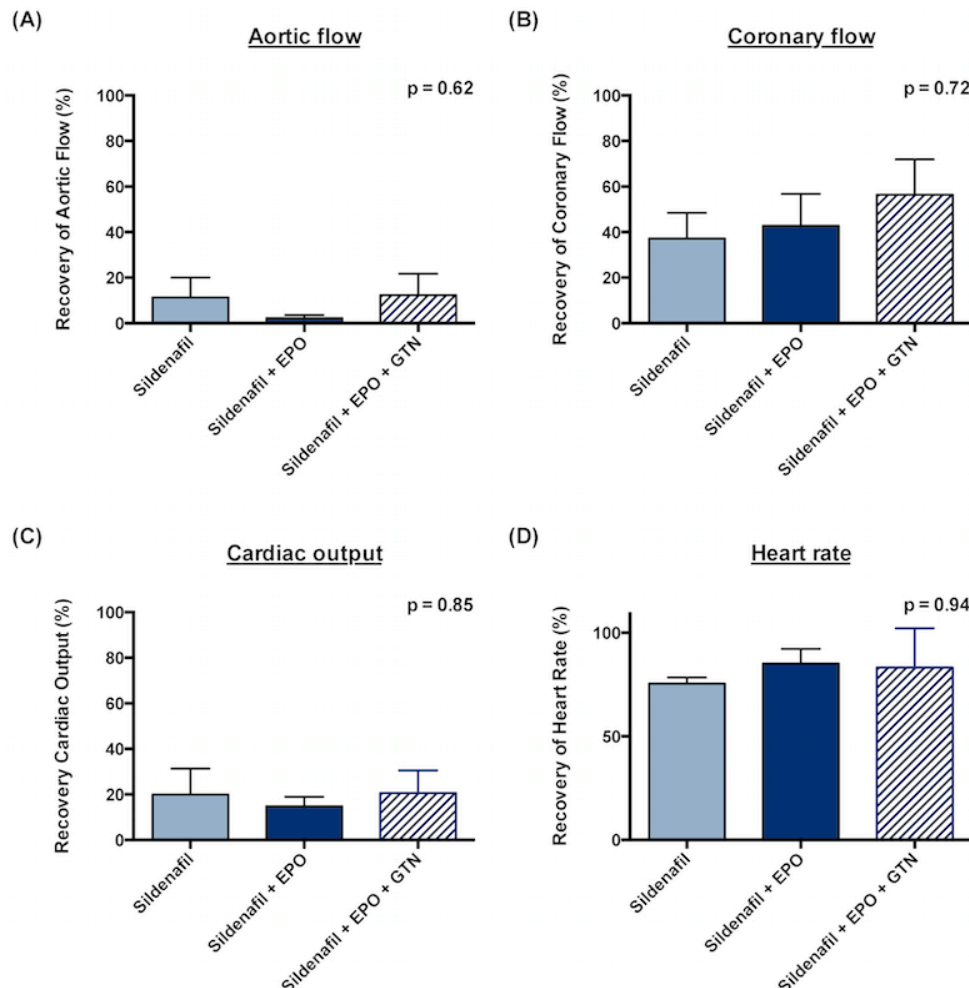


Figure 6.3 Recovery of hearts after 10 hours hypothermic storage

All values are expressed as mean \pm SEM. EPO indicates erythropoietin, GTN, glyceryl trinitrate

In the 10 hour storage group, the recovery of CO in hearts supplemented with sildenafil was 20 \pm 16%, with sildenafil + EPO 15 \pm 10% and with triple supplementation with sildenafil + EPO + GTN 21 \pm 24% ($p=ns$). These results had higher variability and inferior recovery compared to hearts stored for 10 hours in a prior study with EPO + GTN + ZON³²⁹, where 50% recovery of cardiac output was demonstrated. In the same study, 10 hour storage with dual supplementation demonstrated cardiac output recovery as 5% (EPO + ZON), 15% (GTN + ZON) and 22% (EPO + GTN).

6.3.2.3 Lactate dehydrogenase levels

Levels of lactate dehydrogenase (LDH) in hearts stored for 6 hours are shown in Figure 6.4, and for 10 hour storage groups in Figure 6.5. Hearts stored with sildenafil + EPO for 6 hours had the lowest LDH levels, with a significant difference compared to the Celsior group at 30 minutes into reperfusion ($p < 0.05$). There were no significant differences between Celsior and other groups. In hearts stored for 10 hours, LDH levels were lowest in the triple supplemented group sildenafil + EPO + GTN, however not significantly lower than hearts stored with sildenafil + EPO or sildenafil alone.

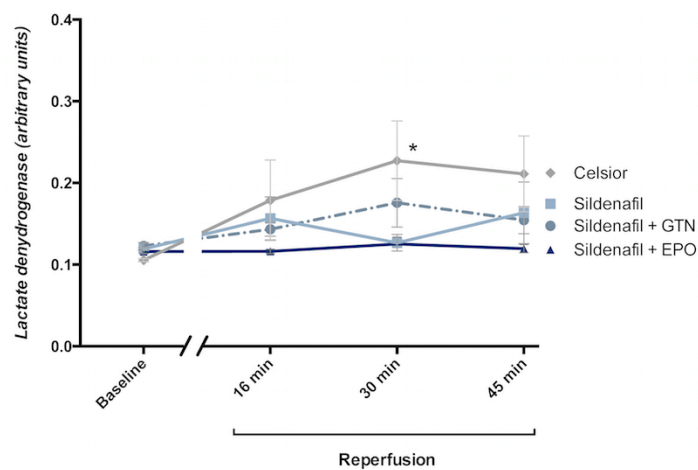


Figure 6.4 Lactate dehydrogenase levels in hearts stored for 6 hours

Lactate dehydrogenase levels at baseline (pre-storage) and at 16, 30 and 45 minutes at reperfusion for all hearts stored for 6 hours are demonstrated above. All values are expressed as mean \pm SEM. * $p = 0.012$ for Celsior compared with Sildenafil + EPO at 30 minutes into reperfusion. $P = ns$ at other time points and between all other groups.

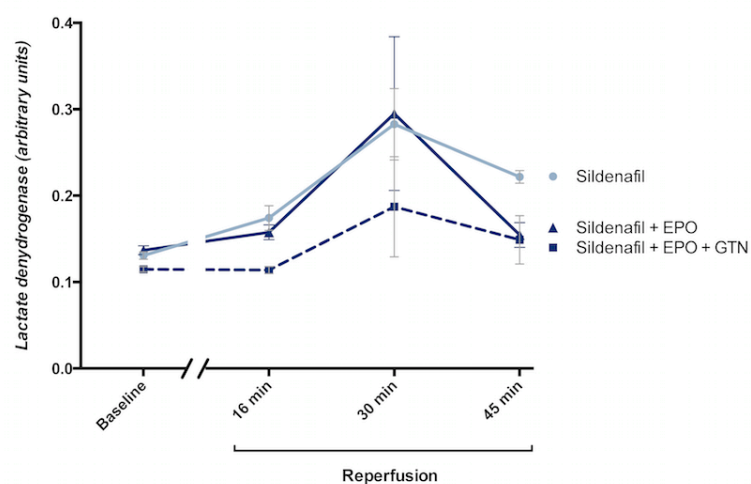


Figure 6.5 Lactate dehydrogenase levels in hearts stored for 10 hours

Lactate dehydrogenase levels at baseline (pre-storage) and at 16, 30 and 45 minutes at reperfusion for all hearts stored for 10 hours are demonstrated above. All values are expressed as mean \pm SEM. $P = ns$ between groups.

6.3.2.4 Western blots

Figure 6.6 demonstrates phospho:total ERK1/2, Akt, AMPK α and STAT 3 for hearts stored for 6 hours. An unanticipated increase in phosphorylation of ERK1/2 was seen in the Celsior group ($p < 0.001$ compared with sildenafil + GTN, and $p < 0.01$ compared with sildenafil + EPO). Phosphorylation of ERK1/2 was increased in the sildenafil group compared with sildenafil + GTN ($p < 0.01$). There were no significant differences in phospho:total Akt, AMPK α or STAT3 between groups.

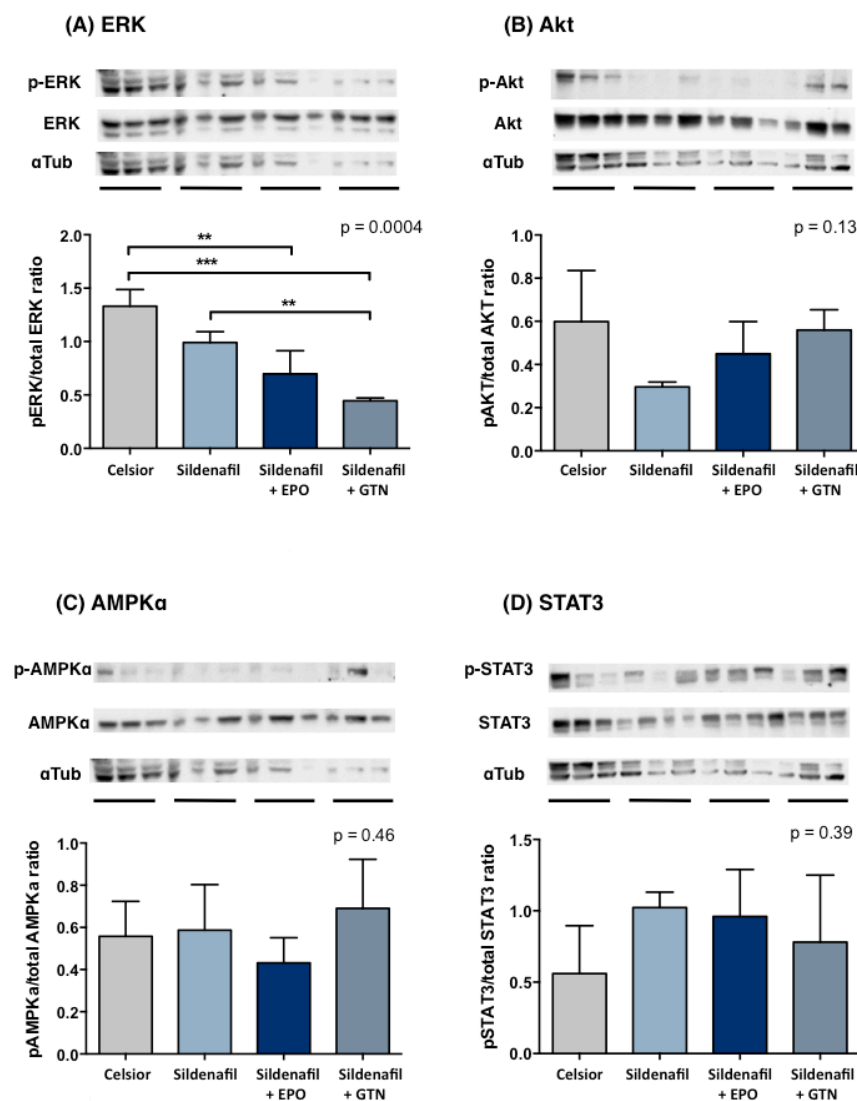


Figure 6.6 Western blots in hearts stored for 6 hours

All values are expressed as mean \pm SD

6.4 Discussion

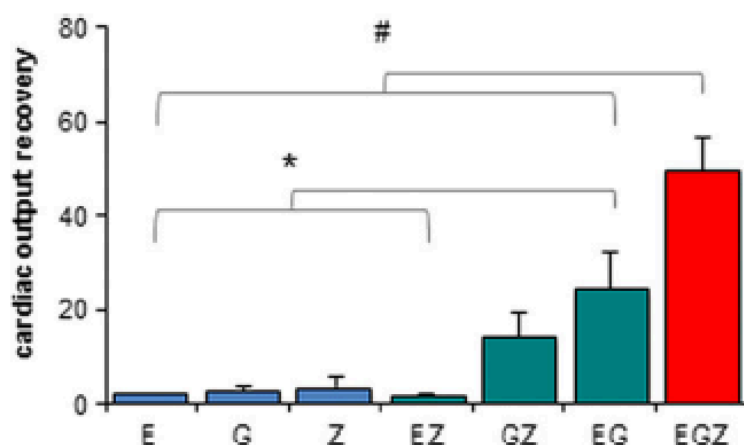
This study has demonstrated that sildenafil has modest cardioprotective effects, which were not of significant benefit to allow appreciable advantages in the recovery of donor hearts after prolonged hypothermic storage.

In hearts stored for 6 hours, supplementation with sildenafil alone recovered cardiac output between 19 to 96% of pre-storage values, and due to large intragroup variability, was not significantly better than Celsior alone (2 to 64% recovery). Combined supplementation with sildenafil + EPO improved aortic flow ($p < 0.001$ compared with Celsior). However, again moderate intra-group variability, resulted in a non-significant rise in the recovery of cardiac output, when adding further variable recoveries of coronary flow. Overall, hearts stored for 6 hours with sildenafil either with or without EPO, had lower recovery of CO compared with prior studies where GTN, EPO and ZON were used where 75 to 130% recovery of cardiac output were demonstrated, even with the presence of brain death (Figure 3.14, Section 3.3.6).

Of particular prominence was the impaired recovery of aortic flow in hearts treated with the combination of sildenafil + GTN. This may have resulted from high levels of nitric oxide from the combined effects of PDE-5 inhibition and NO supply, resulting in relaxation or impaired contractility of cardiomyocytes. Recovery of cardiac output in sildenafil + GTN was 2 to 64%, comparable to Celsior alone (2 to 65%). For this reason, and due to variability of results in sildenafil + EPO hearts, along with non-significant recovery of CO, studies of triple supplementation with sildenafil + GTN + EPO were not performed. Sildenafil + GTN was also not studied at extended periods of hypothermic storage over 10 hours.

The reduced phosphorylation of ERK1/2 in sildenafil groups compared with Celsior controls was unanticipated, ERK1/2 has been an accepted form of sildenafil-mediated cardioprotection. In the absence of significant cardioprotective effects in this study, these findings may support the modest cardioprotective effects of sildenafil.

In groups tested over 10 hour storage times, the recovery of CO was comparable between groups, but inferior to hearts stored with GTN + EPO + ZON in a prior study in the laboratory³²⁹, where triple supplementation allowed 50% recovery of CO, and supplementation (Figure 6.7). These findings further highlight the weaker cardioprotective effects of sildenafil in addition to EPO and GTN, compared to NHE-inhibitors.



Watson A et al, J Heart Lung Transplant 2013;32(6):633-40

Figure 6.7 Recovery of hearts after 10h storage.

E indicates erythropoietin; G, glyceryl trinitrate and Z, zoniporide supplementing Celsior.

* $p < 0.05$ compared with E, G, Z and EZ hearts and # $p < 0.05$ compared with all other hearts.

The weaker cardioprotective effects of sildenafil compared with NHE-inhibitors are highlighted in some studies that fail to show improvement in ventricular contractility despite reduced infarct size, increased phosphorylation of ERK1/2 and activation of PKC. Salloum et al using doses of 0.7mg/kg in mouse studies showed reduced infarct size, however no improvement in ventricular contractility^{431,439}. In another study, Reffelmann et al⁴⁴⁵ showed comparable infarct sizes in sildenafil and control hearts, and in addition demonstrated impaired myocardial contractility, reduced dP/dT(max) and dP/dT(min) in hearts treated with sildenafil. This in-vivo rabbit myocardial infarction study used higher doses of sildenafil (1.45mg/kg), which may account for the negative findings, given the narrow therapeutic window of Sildenafil⁴³². These studies highlight the absence of significant functional recovery of hearts treated with sildenafil, although it is possible that cGMP-mediated inhibition of contractility may have masked protection against IRI.

It is acknowledged that only one concentration of sildenafil was tested. The rationale for the dose chosen has been discussed, however it is possible that in this model, a smaller or larger dose may have provided more effective cardioprotection. Nonetheless, given the biphasic response to sildenafil observed in earlier studies, investigation of other concentrations was not warranted.

In context of donor heart preservation, the conditioning effects of sildenafil have not been consistent or adequate to warrant its use in future preservation studies. Sildenafil may not be able to overcome significant IRI from prolonged hypothermic ischaemia experienced by the donor heart. An agent with strong NHE-inhibiting properties would be an ideal supplement to add to EPO and GTN, however such an agent is clinically unavailable at present. While amiloride, a NHE-inhibitor is only available in tablet form, the need to develop a NHE-inhibitor, or test alternative agents is urgently needed.

CHAPTER 7. DEVELOPMENT OF A RODENT CARDIAC MAGNETIC RESONANCE IMAGING MODEL

7.1 Background

7.1.1 Assessments of cardiovascular physiology and disease in rodent models

Small rodent studies are the most common initial models of assessment of cardiovascular physiology, disease states and the efficacy of surgical and pharmacological interventions. Furthermore, development of transgenic and knockout strains have substantially increased the complexity and the number of small rodent models used today⁴⁴⁶⁻⁴⁴⁸.

Traditionally, small animal studies have relied on either invasive in-vivo measurements of cardiac physiology and disease states, or ex-vivo measurements that necessitate sacrifice of the animal. Examples of in-vivo methods of measurements include the use of intra-ventricular balloon catheters, intravascular or intraventricular conductance catheters, and the use of crystals sewn onto cardiac chambers which require open chest surgical procedures. Ex-vivo measurements involve perfusion circuits and necropsy myocardial analysis^{320,329}.

The need for non-invasive methods of measurements was realised as this would allow the longitudinal study of cardiovascular physiology and disease, and also minimise the unnecessarily large numbers of number of animals sacrificed to study specific stages of diseases or therapeutic interventions.

7.1.2 Non-invasive functional imaging

Non-invasive cardiovascular functional imaging modalities have been developed and are in extensive use clinically. The common modalities used include echocardiography, nuclear imaging, computed tomography (CT) and more recently cardiac magnetic resonance (CMR), which provide valuable information in diagnosis and monitoring of cardiovascular disease. While these modalities provide excellent image quality and valuable diagnostic information clinically, their application in small rodent studies has proved to be challenging due to small heart sizes and rapid heart rates^{449,450}. Protocols have been adapted to allow improved spatial resolution of small animal cardiovascular imaging, however this has often necessitated development of scanners dedicated to small rodent imaging, which is less cost-effective and less easily accessible⁴⁵⁰⁻⁴⁵⁴. A cost-effective, widely accessible modality that is easily adaptable to image hearts of small rodents would be of most value. The available cardiovascular imaging modalities and their utility in small animal studies are discussed in detail below.

7.1.2.1 Echocardiography

Echocardiography was first described in 1954 by Edler and Hertz⁴⁵⁵, assessed in post-mortem human studies⁴⁵⁶⁻⁴⁵⁸, and applied clinically in the 1960s⁴⁵⁹⁻⁴⁶². Initially using m-mode for cardiac geometry and functional assessment, echocardiography has since developed to allow detailed analysis of cardiac anatomy, volumes, haemodynamics and function using 2-dimensional imaging^{463,464} doppler^{465,466} and more recent techniques such as 3-dimensional imaging^{467,468} and speckle tracking^{469,470}. Semi-invasive transoesophageal echocardiography has allowed more detailed imaging and accurate measurement of cardiac valves and atria^{471,472} and invasive intravascular ultrasonography (IVUS) has been used to enhance diagnostic accuracy of detecting functionally significant coronary artery disease^{473,474}.

Echocardiography was soon adopted for use in animal research and it remains the most widely used non-invasive method of assessment of cardiovascular disease in animal studies. While echocardiography is easily accessible and has excellent temporal resolution, it has modest spatial resolution, and can prove challenging when imaging small rodents due to small heart size and rapid heart rates (250-450 bpm in rats and 350-600 bpm in mice). High frequency ultrasound probes have therefore been developed to improve image quality and reproducibility of cardiac measurements^{450,454,475-477}. A limitation of echocardiography is that it relies on geometric assumptions for quantification of ventricular volumes and mass, which can lead to over- or underestimation of measurements, particularly in disease states⁴⁷⁸⁻⁴⁸¹. In particular, the quantification of myocardial mass in rats using two-dimensional echocardiography has shown poor correlation with necropsy weights⁴⁸⁰.

7.1.2.2 Nuclear imaging

Nuclear cardiac imaging modalities are frequently used clinically to determine myocardial ischaemia and viability. The use of thallium-201 based scans measure viability by assessing tracer uptake by intact cell membranes^{482,483}, technetium-99m is used to determine ischaemia by assessing intact mitochondrial function^{484,485} and fluorine-18 deoxyglucose is used in positron-emission tomography (PET) to detect preserved myocardial metabolism^{486,487}.

However, due to limited spatial resolution (5-6mm), nuclear imaging methods are unable to provide detailed imaging of cardiac structures and its use is mostly restricted to assessment of ischaemia, myocardial viability, cardiac volumes and ejection fraction⁴⁸⁸. Its application in small rodent studies has required dedicated small animal research scanners to provide improved spatial resolution for quantification of small cardiac volumes⁴⁸⁹⁻⁴⁹¹ making this a less economically feasible mode of imaging. Long image acquisition times and the inability to provide detailed cardiac anatomy have also limited the use of nuclear cardiac imaging in small rodent research models.

7.1.2.3 Computed tomographic imaging

Computed tomography (CT) has wide clinical application in the non-invasive assessment of coronary artery disease, imaging of cardiac anatomy such as in congenital heart disease or detection of cardiac malignancies and for pre-operative assessment of valvular heart disease⁴⁹². It has been applied in small rodent cardiovascular imaging, however has also met with challenges due to poor image quality as a result of low signal to noise ratio caused by smaller voxels, motion from rapid rodent heart rates and long acquisition times. Micro-CT scanners have therefore been designed with improved spatial resolution (10msec) and the ability to overcome motion-related image degradation with electrocardiograph (ECG) and respiratory gating^{451,453}. Improving contrast between blood and myocardium has also been enhanced with the use of iodine-based contrast agents allowing detailed imaging of the myocardium and accurate calculation of cardiac volumes, cardiac output and ejection fraction^{493,494}. The need for dedicated small animal micro-CT scanners limits its economical viability. Furthermore, compared to magnetic resonance imaging (MRI), CT has limited ability to detect myocardial fibrosis and oedema.

7.1.2.4 Cardiac magnetic resonance imaging

Cardiac magnetic resonance (CMR) imaging is the current gold standard for non-invasive imaging of cardiovascular structure and function in clinical practice. Magnetic resonance imaging provides excellent spatial resolution allowing detailed tissue characterization, with additional advantages of the ability to determine myocardial oedema with high sensitivity using T2 weighted imaging⁴⁹⁵ and detection of myocardial fibrosis using late gadolinium enhancement⁴⁹⁶. The application of CMR in animal studies has provided the ability to study disease models in greater detail and increased accuracy.

Applying CMR to small animal models has again proved to be complex. Poor signal to noise ratio reduces image clarity in small heart sizes, and the moderate temporal resolution of magnetic resonance imaging creates image artifact at rapid heart rates⁴⁹⁷. The use of clinical scanners with low field strengths (1.5T or 3.0T) have therefore historically led to poor image quality and long acquisition times. Studies have reported modified image sequencing protocols to improve image resolution using clinical scanners^{479,498}, however these are under-utilised in current studies. Furthermore, dedicated small animal scanners with high field strengths (4.7T, 7T or higher) and specialised vertical bores have been developed and are increasingly been used in more recent small rodent CMR studies⁴⁹⁹⁻⁵⁰². However, substantial costs limit the accessibility of these scanners to specialized research units. The ability to use clinical MR scanners to image small rodents would increase if protocols were validated and made accessible in the literature.

Additional advantages of CMR are its highly reproducibly imaging, with minimal inter- and intra-observer variability compared with echocardiography. In particular, poor correlation of CMR and echocardiography has been shown in measurement of ventricular mass and ejection fraction after cardiac transplantation^{503,504}. The ability to develop protocols using clinical scanners would provide less expensive, reproducible and accurate images that would allow more widespread use in small rodent cardiovascular studies.

7.2 Study objectives

The objectives of this study were:

- To develop a protocol that would provide high-resolution images of rat hearts using a low field strength (1.5T) clinical MR scanner.
- To measure left ventricular volumes, ejection fraction and mass using these images.
- To assess the reproducibility of measurements obtained by determining intra-observer, inter-observer and inter-study reproducibility of measurements.
- To enable quantitative longitudinal functional measurements of transplanted rat hearts in future studies.

7.3 Methods

7.3.1 Animals

Cardiac magnetic resonance imaging was conducted on eight healthy male Lewis rats weighing 250-350g (Animal Resources Centre, Canning Vale, Western Australia). Animals received humane care in compliance with the *Guidelines to Promote the Wellbeing of Animals Used for Scientific Purposes* (National Health and Medical Research Council, Australia) and the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, Bethesda, MD). The study was approved by the Animal Ethics Committee of the Garvan Institute of Medical Research, Sydney, Australia (Animal Research Authorities #12/08).

Anaesthesia was provided with inhaled isoflurane at a concentration of 5% delivered into a closed chamber via a rodent vapouriser (Vetquip Inc, Livermore, CA) for induction, and maintenance anaesthesia provided with 1.5% isoflurane at a flow rate of 1-1.5 ml/min delivered via a nose cone.

Animals were placed supine in a clinical wrist coil (Philips Achieva SENSE 8 elements, Philips Healthcare, The Netherlands). An MR-compatible rodent monitoring device (SA instruments, NY, USA) recorded three-lead electrocardiograph (ECG) signals using subcutaneous electrodes. Body temperature was recorded using a rectal probe and respiration monitored with a pneumatic monitor placed over the abdomen. This data was redirected into a 1.5T clinical MR scanner (Achieva, Philips Healthcare, Best, The Netherlands), which was used for image acquisition. The Lewis rats were scanned then removed from the table briefly, remaining anaesthetised. They were then immediately returned and repositioned in the coil for rescanning to determine inter-study reproducibility.



Figure 7.1 The position of the rat within the clinical wrist coil and the 1.5T MR scanner.

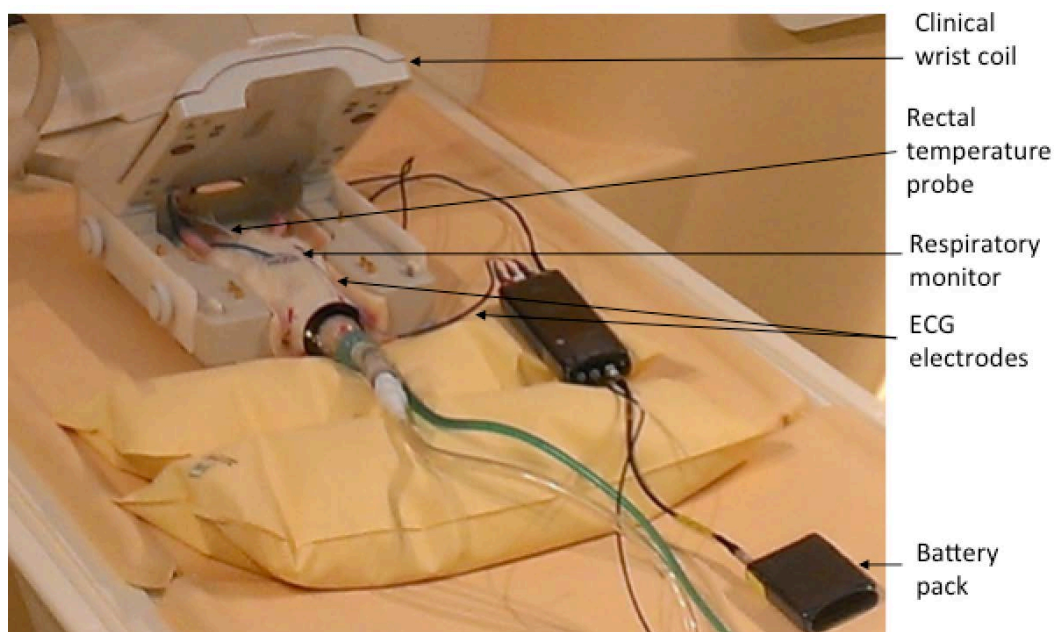


Figure 7.2 Close-up view of rat within wrist coil with monitoring equipment

The rat is placed within the clinical wrist coil with electrocardiograph leads and respiratory monitor. A nose cone delivers anaesthesia to the animal, with the isoflurane vapouriser placed outside the MR scanner room.

7.3.2 Image acquisition

7.3.2.1 Image sequences

Three-dimensional plane localisation was determined using a sagittal image plane to identify the ventricular long-axis (VLA) and ventricular four-chamber (4CH) views. Balanced steady state free precession (bSSFP)-based, ECG-triggered, free-breathing cine images were acquired with 8 signal averages in multiple planes including VLA and 4CH views as well as a contiguous ventricular short-axis stack from base to apex, which were used to calculate left ventricular end-diastolic volume (EDV), left ventricular end-systolic volume (ESV), ejection fraction (EF) and myocardial mass (MM).

The bSSFP cine imaging sequence details were as follows: 28-35 frames per cycle depending on heart rate, repetition time (TR) 9.8ms, echo time (TE) 3.2ms, band width 166.9Hz, field of view (FOV) 70 x 50 mm, mean pixel size 0.3 x 0.5mm, flip angle 30 degrees, 12 slices, slice thickness 1.5 mm, 8 signal averages and 50 cardiac phases. The temporal resolution was 10 msec.

7.3.2.2 Image analysis

Volumetric analyses were performed using clinically validated software (cmr42, Circle Cardiovascular Imaging Inc, Calgary, Alberta, Canada). Measurement of EDV, ESV, MM and EF were assessed according to standard definitions by manual tracing of contiguous short-axis cine images. Measurements were performed by two experienced investigators on two separate occasions a minimum of two weeks apart, with blinding to the original results to determine inter- and intra-observer variability and inter-study reproducibility.

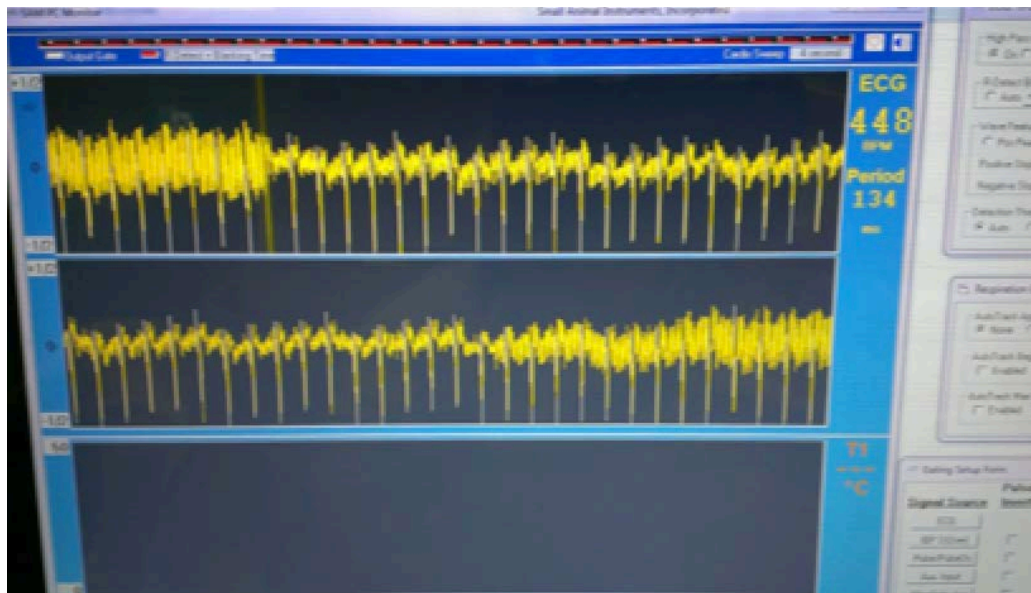


Figure 7.3 Acquisition of three-lead electrocardiograph signals.

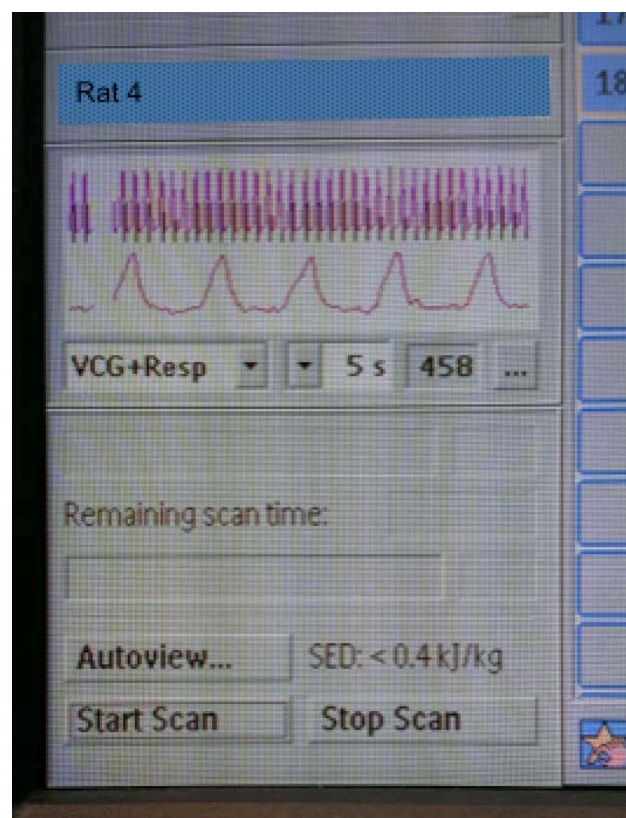


Figure 7.4 Electrocardiograph and respiratory monitoring during image acquisition.

7.3.3 Statistical Analysis

Measurements are shown as the mean \pm standard deviation (SD). Intra-observer and inter-observer measurement agreements were determined as described by Bland and Altman⁵⁰⁵. Coefficients of variation (COV), defined as the standard deviation of the difference between measurements divided by the mean difference of measurements, were calculated to determine intra- and inter-observer variability and inter-study reproducibility.

The sample sizes required to detect a 10% change in a parameter measured by 1.5T CMR were compared to sample sizes required for studies using two-dimensional echocardiography. Calculations were performed to show a 10% change with a power of 90% and a α error of 0.05 using the following formula:

$$N = \frac{2 \times C(\alpha, \beta)}{(ES)^2} \quad \text{and} \quad ES = \frac{\delta}{SD}$$

N indicates the sample size, $\alpha = 0.05$, $\beta = (1 - \text{power})$ or 0.10, and $C(\alpha, \beta)$ a prespecified constant of 10.5. ES indicates effect size, δ the required difference, and SD standard deviation. All statistical analyses were conducted using Prism 6 software (GraphPad Software Inc., CA).

7.3 Results

High quality images were obtained despite rapid heart rates of 280-480 bpm and the acquisition of images during free breathing. Figure 7.5 demonstrates representative images of rat hearts in end-diastole and end-systole in ventricular four-chamber, long axis and mid-ventricle short axis views. Good contrast was observed between myocardium and blood pool with little image degradation or artefact. Spatial resolution was comparable to images obtained using higher field-strength $3T^{498,506}$, $4.7T^{507}$ and $7T^{499}$ scanners.

The time required for three-dimensional plane localisation was 10 minutes, and acquisition time for cine-images of a short-axis stack with 8NSA was 25-30 minutes, which were within the acquisition times described for higher field-strength scanners in the literature^{498,508,509}.

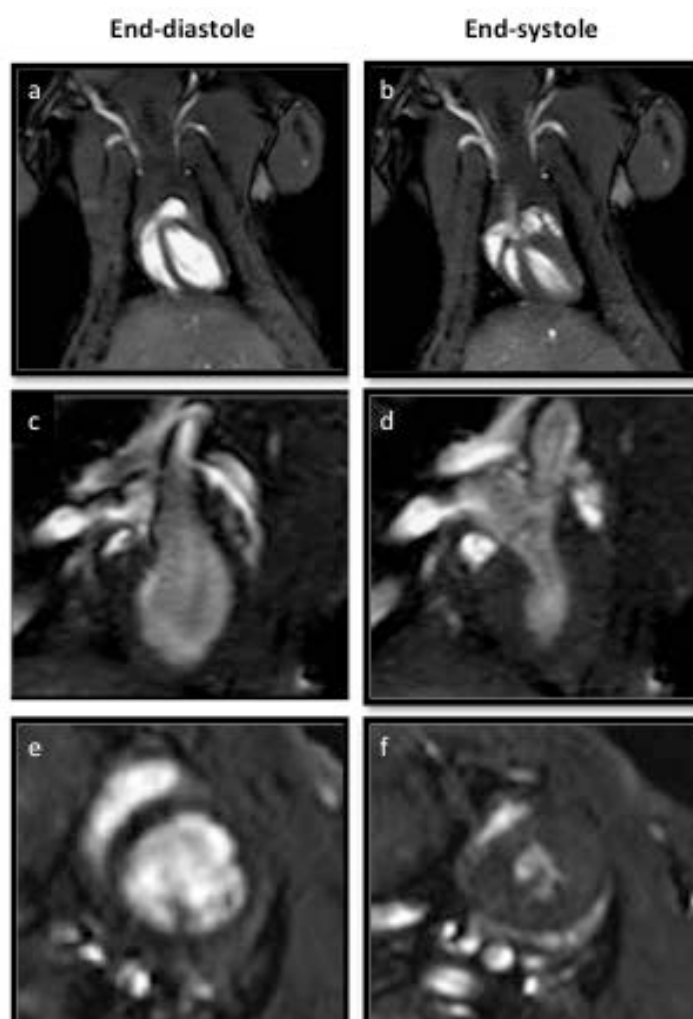


Figure 7.5 Representative images of rat CMR using a 1.5T scanner. Ventricular four-chamber (a and b), ventricular long axis (c and d) and mid-ventricle short axis (e and f) views are shown at end-diastole and end-systole respectively.

7.3.1 Quantification of left ventricular volumes, mass and function

Measurements performed by the first investigator for EDV, ESV, EF and MM from Scan 1 for each animal are outlined in Table 7.1. Cardiac volumes and myocardial mass varied according to animal size. Of note, Rat 1 was smaller (250g) than the other animals, with reduced EDV and a less pronounced reduction in ESV and MM.

Table 7.1 Cardiac volumes, ejection fraction and myocardial mass (Scan 1)

	Animal weight	EDV (ml)	ESV (ml)	EF (%)	MM (g)
Rat 1	250g	0.26	0.04	84.6	0.42
Rat 2	350g	0.42	0.08	81.0	0.52
Rat 3	345g	0.41	0.09	78.0	0.54
Rat 4	365g	0.46	0.05	89.1	0.58
Rat 5	340g	0.40	0.08	80.0	0.49
Rat 6	365g	0.48	0.07	83.3	0.52
Rat 7	345g	0.39	0.06	84.6	0.38
Rat 8	360g	0.42	0.09	78.6	0.48
Mean \pm SD	340 \pm 38g	0.41 \pm 0.07	0.07 \pm 0.02	82.4 \pm 3.7	0.49 \pm 0.06

Individual measurements made by investigator 1 from Scan 1 images are shown above for each animal for end-diastolic volume (EDV) and end-systolic volume (ESV), ejection fraction (EF) and myocardial mass (MM).

7.3.2 Reproducibility

Intra-and inter-observer measurement variability is presented as Bland-Altman plots (Figure 7.6 and Figure 7.7 respectively). The COV for intra- and inter-observer and inter-study measurements are shown in Table 7.2.

Intra-observer COV for EDV, ESV, EF and MM were 3.17%, 7.04%, 1.61% and 5.39% which were favourable compared with published data on clinical CMR⁵¹⁰⁻⁵¹⁴, rodent CMR with higher field strength scanners^{479,515,516} and superior to 2- and 3-dimensional clinical^{513,517} and rodent^{515,516,518-520} echocardiography. Inter-observer COV for EDV, EF and MM were 7.88%, 5.88% and 10.91% respectively, which were again favourable compared with clinical CMR^{511-513,521}, rodent CMR using high-field strength scanners^{479,515}, and superior to 2- and 3-dimensional clinical^{513,517} and rodent^{479,515,518,520} echocardiography. Inter-study COV were 6.93%, 4.56% and 3.66% respectively, which were also favourable compared with variability

in clinical CMR^{511,521-523}, rodent CMR using higher field strength scanners⁵¹⁵ and superior to 2- and 3-dimensional clinical^{513,517,522,523} and rodent⁵¹⁸ echocardiography.

Inter-observer and inter-study COV were higher for end-systolic volumes (18.92% and 14.80% respectively) due to small ventricular cavity size resulting in larger variability. However, these results were comparable to clinical CMR variability of 4-17%^{510,514,521} and 4.4-10%^{514,522} respectively and rodent CMR with higher field strength scanners⁵¹⁶. Results were also either comparable or superior to variability of ESV measurements in clinical echocardiography (6.4%⁵¹³ and 13-20%⁵²² respectively) and rodent echocardiography (up to 20%)^{515,516,518}

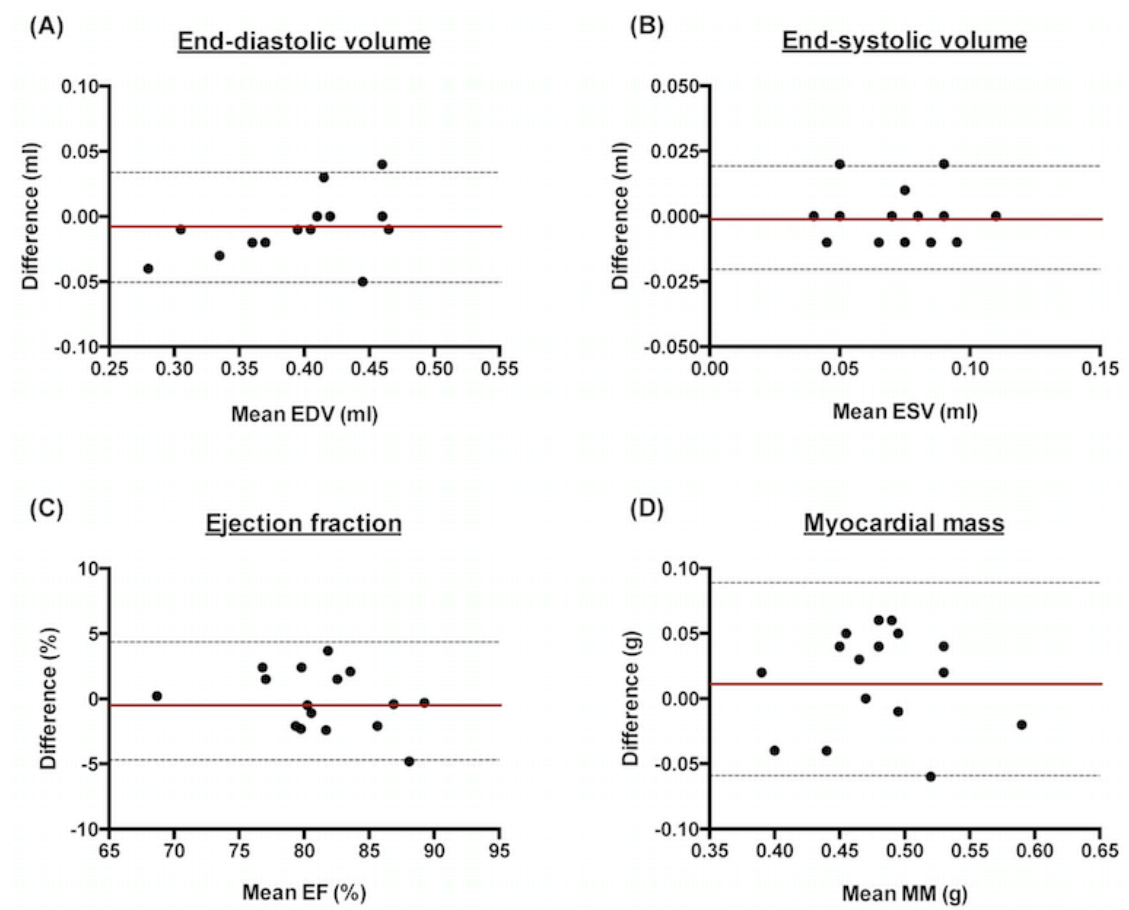


Figure 7.6 Intra-observer correlations.

Bland and Altman representations of intra-observer correlations are demonstrate for EDV, ESV, EF and MM (n=16 scans per analysis). The mean difference is indicated with a red line and 95% limits of agreement in dashed lines.

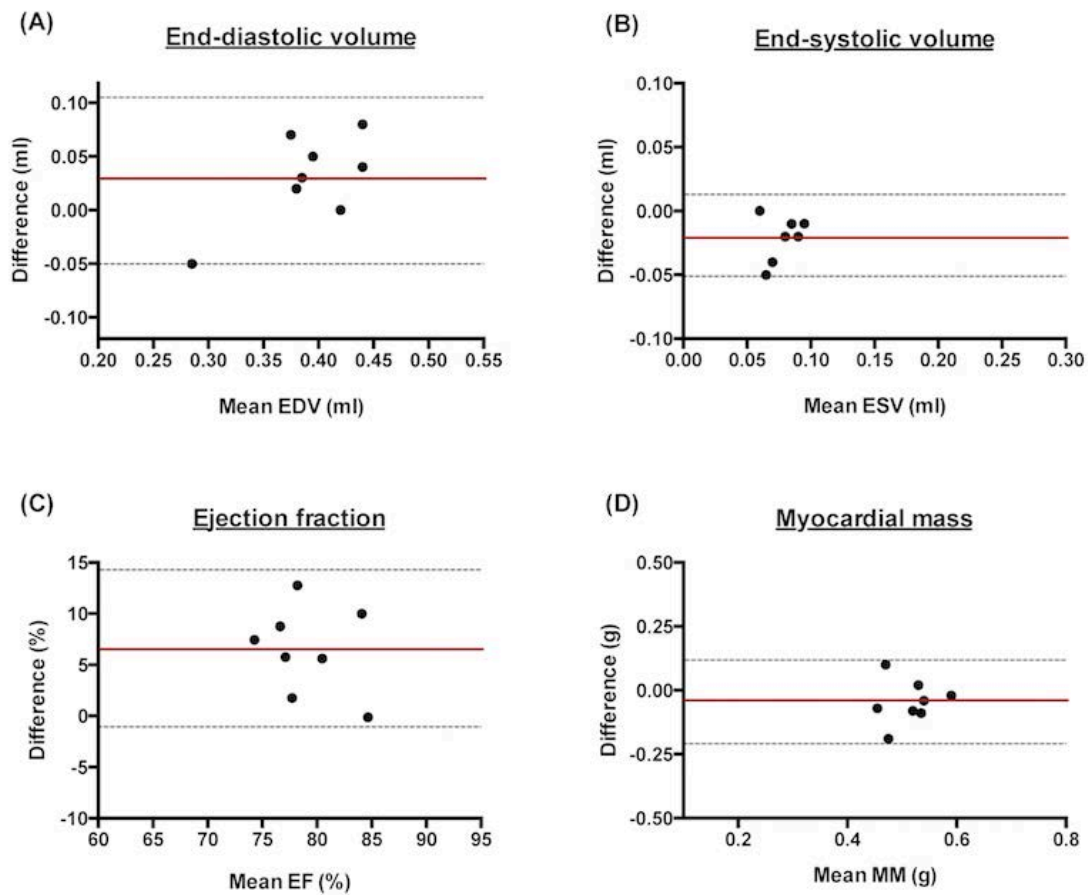


Figure 7.7 Inter-observer correlations.

Bland and Altman representations of inter-observer correlations are demonstrated for EDV, ESV, EF and MM (n=8 scans). The mean difference is indicated with a red line and 95% limits of agreement with dashed lines.

(A) Intra-observer measurements

	Measure 1 (Mean \pm SD)	Measure 2 (Mean \pm SD)	Bias	95% limits of agreement	Coefficient of variation
EDV (ml)	0.39 \pm 0.06	0.40 \pm 0.05	0.0081	-0.052 to 0.036	3.17%
ESV (ml)	0.07 \pm 0.02	0.08 \pm 0.02	0.0006	-0.020 to 0.019	7.04%
EF (%)	81.3 \pm 4.8	81.4 \pm 5.5	0.138	-4.62 to 4.35	1.61%
MM (g)	0.48 \pm 0.05	0.47 \pm 0.05	0.015	0.061 to 0.091	5.39%

(B) Inter-observer measurements

	Investigator 1 (Mean \pm SD)	Investigator 2 (Mean \pm SD)	Bias	95% limits of agreement	Coefficient of variation
EDV (ml)	0.40 \pm 0.07	0.38 \pm 0.04	0.030	-0.051 to 0.11	7.88%
ESV (ml)	0.07 \pm 0.02	0.09 \pm 0.01	0.0088	-0.049 to 0.032	18.92%
EF (%)	82.4 \pm 3.7	80.1 \pm 5.7	6.50	-1.79 to 14.78	5.88%
MM (g)	0.49 \pm 0.06	0.54 \pm 0.06	0.046	-0.021 to 0.121	10.91%

(C) Inter-study measurements

	Scan 1 (Mean \pm SD)	Scan 2 (Mean \pm SD)	Bias	95% limits of agreement	Coefficient of variation
EDV (ml)	0.41 \pm 0.07	0.38 \pm 0.06	0.024	-0.062 to 0.11	6.93%
ESV (ml)	0.07 \pm 0.02	0.08 \pm 0.02	0.0088	-0.050 to 0.032	14.80%
EF (%)	82.4 \pm 3.7	80.2 \pm 5.7	2.2	-9.5 to 14.0	4.56%
MM (g)	0.49 \pm 0.06	0.48 \pm 0.04	0.0075	-0.052 to 0.067	3.66%

Table 7.2 Coefficients of variation

The mean measurements, bias, 95% limits of agreement and coefficients of variation are shown above for intra-observer (A), inter-observer (B) and inter-study (C) correlations.

7.3.3 Sample size reduction

Calculated sample size reduction for estimation of a 10% change in EDV, ESV, EF and MM using CMR in our study compared with published echocardiography data⁵¹⁹ were 65%, 55%, 78% and 96% respectively (Table 7.3).

Table 7.3 Sample size reduction: 1.5T CMR compared with echocardiography

	Echocardiography		CMR in our study		Reduction in sample size by 1.5T CMR
	SD	Sample Size	SD	Sample Size	
10% change in EDV in CMR compared with 10% change in end-diastolic area in echocardiography	4.56 ⁵¹⁹ (mm ²)	40	0.032 (ml)	14	65%
10% change in ESV in CMR compared with 10% change in end-systolic area in echocardiography	3.14 ⁵¹⁹ (mm ²)	193	0.015 (ml)	86	55%
10% change in EF	6.39 (%) ⁵¹⁹	18	3.21 (%)	4	78%
10% change in MM	0.208 (g) ⁵¹⁹	77	0.016 (g)	3	96%

Sample sizes required to detect a significant change in left ventricular end-diastolic volume (EDV), end-systolic volume (EDV), ejection fraction (EF) and left ventricular mass (MM) with 90% power and an α error of 0.05 are demonstrated above.

7.4 Discussion

This study has demonstrated that rodent CMR imaging on a 1.5T clinical scanner provides highly reproducible cardiac volumetric and functional data which is comparable to data afforded by high-field-strength MR scanners and allows significant sample size reduction when compared to dedicated 2D small-animal echocardiography.

The methods used for image acquisition were simple and cost effective. A relatively light anaesthetic protocol of 1-1.5% isoflurane, the preferred anaesthetic for rodent cardiovascular studies, was employed as it was expected to have least cardiodepressant effects⁵²⁴. The overall speed of image acquisition was within the timeframes described in other rodent imaging studies^{498,508,509}.

Image resolution

Although a low field strength 1.5T scanner was used, image clarity from cine sequences of short-axis stacks using 8NSA were comparable to images produced using higher field-strength 3T^{498,506}, 4.7T⁵⁰⁷ and 7T⁴⁹⁹ scanners published in the literature.

The temporal resolution of 10msec used in this study balanced well with the acquisition of eight signal averages to provide optimal image resolution. This is comparable to the temporal resolution from 3T-scanner studies. Historical protocols using lower temporal resolution such as 39 msec, have required multiple acquisitions to obtain images for quantification of small rodent cardiac volumes, mass and function⁵⁰⁹; our protocol avoids this whilst maintaining high reproducibility.

Access to a respiratory monitor and gating system was available, however respiratory gating was not used in image acquisition due to the inability to successfully register respiratory signals by the available software. Despite the lack of respiratory gating, image resolution was excellent with minimal motion artefact.

Reproducibility

To the best of our knowledge, this is the first study to describe inter-study reproducibility of rodent volumetric and functional data on a clinical 1.5T scanner.

Intra- and inter-observer variability were favourable when compared to published data on rodent CMR using high field-strength scanners. In our study, intra-observer variance was low for EDV (3.17%), ESV (7.04%), EF (1.61%) and MM (5.39%), as were most parameters for inter-observer variance: 7.88%, 18.92%, 5.88% and 10.91% respectively. Rodent CMR using 11.7T scanners have also demonstrated intra-observer and inter-observer variance of 3-7% and 3-11% respectively for EDV, ESV, EF, MM in healthy and infarcted hearts⁵⁰¹, also comparable to our results. A study by Stuckey et al⁵¹⁶, using an 11.7T scanner to image hearts from healthy rats demonstrated percentage error of $7 \pm 2\%$ for end-diastolic area, $8 \pm 3\%$ for end-systolic area, and $2 \pm 1\%$ for ejection fraction with repeated measurements. Two-dimensional echocardiography was shown to be inferior in measurement accuracy of the same animals in this study, with percentage error for intra-observer end-diastolic area $8 \pm 2\%$, end-systolic area $20 \pm 8\%$ and ejection fraction $7 \pm 2\%$.

Improved measurement accuracy of CMR compared with echocardiography was also demonstrated in a study by Richardson et al⁴⁷⁹ in a rodent myocardial infarction model, where intra- and inter-observer variance was lowest in CMR (1.5T scanner), with doubling of variance of measurements when using transthoracic and transoesophageal echocardiography. Other rodent studies have demonstrated intra-and inter-observer variance of 9.4% and 14.5% respectively when measuring cardiac output using transesophageal echocardiography⁵²⁰ or wider variability between 4% and 22% using echocardiographic parameters for ventricular dimensions and ejection fraction⁴⁵⁰.

Cardiac magnetic resonance has invariably demonstrated significantly more reliable measurements for ventricular mass when compared with echocardiography in human and animal studies. Poor accuracy and agreement of measurements using echocardiography is attributed to assumptions made on cardiac geometry⁵²⁵⁻⁵²⁷. In rodent studies, echocardiographic intra-observer variance in mass estimates have been as high as $37 \pm 19\%$ ⁵²⁸, with poor accuracy during validation at necropsy^{475,480} compared with CMR⁵²⁹. The use of three-dimensional echocardiography has improved accuracy and reproducibility of volumes, cardiac function and mass, with variance ranging from 3-7% for intra- and inter-

observer and inter-study measurements⁵¹⁸, however access is again limited even within specialised institutions.

Improved accuracy of CMR has therefore allowed significant reductions in sample size, compared with echocardiography^{479,519}. This was confirmed in our study, with sample size reductions by 65%, 55%, 78% and 96% achievable for a 10% change in ESV, EDV, EF and MM respectively using 1.5T CMR. This would significantly reduce the number of animals used in rodent cardiovascular studies compared with echocardiography, allowing more ethical practices and reduction of cost.

Finally we compared our data to clinical CMR studies, and found that variance in our study were again comparable^{510,530}. Variance between intra- and inter-observer and inter-study measurements have been reported between 3.5%-5.6% for cardiac volumes^{511,531-533} and 3.5-10% for ventricular mass^{511,512,531-533}. Our study had higher variance for ESV measurements, which attributed to the small cavity size, which causes large variations in differences of measurements of extremely small cavity sizes. Wide intra-observer variability between 16% and 30% were reported by Pattynama et al⁵¹⁴, however most studies have shown comparable volumetric and functional data variance between 2.9% to 6.5% (Grothues et al⁵²²), 3.6% to 10.0% (Baur et al)⁵³² or 3.6% to 7.2%^{512,531,533}.

We also compared our data with clinical 2-dimensional and 3-dimensional echocardiography, and our study using a low-field strength scanner has shown comparable or lower variance of measurements. Echocardiographic variability for EF has ranged from 2.6% to 24%^{513,522,523}, EDV from 5.5% to 12%^{513,522,523}, ESV from 3% to 13.7%^{513,522,523} and as previously mentioned, higher variance for MM. Increased accuracy of CMR measurements using a low field strength scanner even in small rodent hearts, is again attributed to superior image quality due to improved spatial resolution, and the use of true short-axis planes of the left ventricle for measurements.

A limitation of our study is that only healthy Lewis rats were scanned. Although slightly higher variability would be expected in cardiomyopathy states^{501,524}, ethical considerations prevented us from performing reproducibility studies in cardiomyopathic rodents, given the comparability of our results for healthy rodents to published high-field-strength CMR data⁵⁰¹.

Conclusion

Cardiovascular magnetic resonance is the gold standard in assessment of left ventricular volumes and function in humans and large animals. The utilization of CMR in small animal models of cardiac disease has been limited due to inaccessibility to specialized high-field strength scanners. This study has demonstrated that rodent CMR imaging on a 1.5T clinical scanner provides highly reproducible cardiac volumetric and functional data which is comparable to data afforded by high-field-strength MR scanners and allows significant sample size reduction when compared to dedicated small-animal echocardiography. The ability to use low field strength scanners will improve the utilization of CMR in small rodent models of cardiovascular disease. Using the protocol developed in this study, with modifications made to electrocardiograph gating, functional image acquisition of small animal heterotopic abdominal transplant models will be performed in future studies. In doing so, longitudinal functional measurements of the heart will be possible in-vivo.

CHAPTER 8. DEVELOPMENT OF A RODENT ABDOMINAL HETEROTOPIC WORKING HEART TRANSPLANT MODEL FOR USE IN DONOR HEART PRESERVATION STUDIES

8.1 Introduction

Small rodent studies are the initial modes of assessment of donor heart preservation strategies. These studies most often assess donor heart function using established ex-vivo perfusion circuits such as the Langendorff circuit, which provide excellent functional measurements. However, ex-vivo studies omit an important aspect of injury of the donor, which is warm blood reperfusion injury⁵¹.

In order to develop effective donor heart preservation techniques, rodent models should incorporate all aspects of ischaemia-reperfusion injury (IRI) relevant to transplantation. This includes donor brain death, warm ischaemia during explantation of the heart, representative periods of hypothermic storage, warm ischaemia during transplantation of the heart and finally injury during reperfusion of warm blood. If any aspect is omitted, positive findings from small animal trials may not show similar results in subsequent large animal transplant models, and may be unable to be translated into clinical practice. For this reason, donor brain death has been incorporated into future donor heart preservation studies, as described in chapter 3.

A review of the literature found several techniques. Among these, the most widely used was the abdominal heterotopic heart transplant technique. This was first described by Abbott et al⁵³⁴ in 1964 and involved anastomosing the donor ascending aorta and main pulmonary artery (MPA) end-to-end with the recipient's abdominal aorta and inferior vena cava (IVC) respectively (Figure 8.1A). However, this procedure was poorly tolerated by animals, as it required transection of the recipient aorta and IVC leading to lower extremity ischaemia and animal death. The technique was later modified by Ono and Lindsey⁵³⁵ to anastomose the same vessels end-to-side (Figure 8.1B), which improved recipient survival.

Alternate sites of transplantation were also described, such as a sutureless 'cuff technique' described by Heron⁵³⁶, where the donor heart was grafted to the recipient's carotid and jugular vessels, and a technique of transplantation to the femoral vessels described by Rao and Lisitza⁵³⁷. However, these latter techniques were not associated with improved survival, hence abdominal heterotopic heart transplantation was applied more widely⁵³⁸⁻⁵⁴⁴.

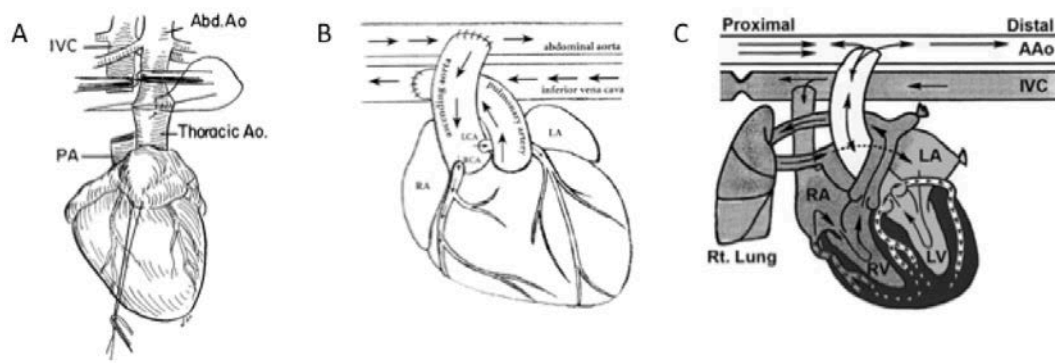


Figure 8.1 Rodent models of abdominal heterotopic heart transplantation

An Image of first model described in the literature (A) was obtained from Abbott CP et al, Arch surg 1964;89:645-52; a 'resting' heart model (B) obtained from Wang D et al, Microsurgery 2006; 26:470-472; and of the 'working' heart model (C) from Wu YL et al, JACC Cardiovascular Imaging 2009; 2:731-741.

The abdominal heart transplant method has since been refined, with wide application in transplant immunology research. Many of these studies use a 'resting' heart model⁵⁴⁵ (Figure 8.1B) where coronary perfusion is provided through gurgitation of blood from the aorta, with minimal ventricular volume loading. While appropriate for studying immunological aspects of rejection, it is not suitable for functional assessment of the isograft. Application in donor heart preservation studies has required the use of invasive methods functional measurement such as intraventricular pressure balloons.

More recently, a 'working' rodent abdominal heart transplant model has been described by Wu et al⁵⁴⁶. This technique transplants the donor heart and right lung with end-to-side anastomosis of the donor aorta to recipient aorta, and donor right superior vena cava (SVC) to the recipient's IVC (Figure 8.1C). This 'biventricular' model allows loading of the heart with blood flow from the SVC to the right atrium (RA) with pressure created from a partial ligature placed in the recipient IVC proximal to the grafted heart. Blood from the RA is then circulated via the intact pulmonary circulation to the left atrium (LA) and left ventricle (LV) and blood is ejected against physiological pressure into the recipient's systemic circulation. With the use of cardiac magnetic resonance (CMR) imaging, isografts were demonstrated to exhibit patterns of early left ventricular strain similar to native hearts. This 'working' heart model would be ideal to assess graft function in organ preservation studies, as it allows incorporation of warm blood reperfusion as well as physiological loading and pressure conditions.

Therefore, the aim of this study was to develop this 'working' heart transplant model in the laboratory for use in future organ preservation studies. With adaptation of steps described in the literature, and with observation of an experienced surgeon in small animal heart transplants, this method was developed as described below.

This study involved developing the surgical model, hence results of graft function have not been assessed with CMR. With the incorporation of this surgical model with rodent CMR imaging described in Chapter 7, it is aimed to allow its use in future organ preservation studies.

8.2 Methods

The abdominal heterotopic heart transplant model was developed into the protocol described below.

8.2.1 Animals

Male Lewis rats were used since a syngeneic strain was required to prevent allograft rejection. Donor and recipient animals were aged 10-11 weeks were used as the ideal body weight was 300g, where the recipient animal had minimal abdominal fat and therefore provided a better surgical field with minimal bleeding. A donor weight of 300g was chosen as it provided a close approximation of the large thoracic vessels of the donor to the abdominal vessels of the recipient. Females were not used due to significantly smaller body size of 180g at 10-11 weeks of age. Animals were obtained from Animal Resources Centre (Western Australia, AU) and housed as described in Chapter 2, Section 2.4.

8.2.2 Anaesthesia

All animals received induction anaesthesia with isoflurane (Abbott Laboratories, Abbott Park, IL) delivered at 5ml/min with oxygen into a closed chamber. Maintenance anaesthesia was provided with inhaled isoflurane delivered at a rate of 1.5-2.0ml/min with oxygen via a nose cone. The depth of anaesthesia was assessed with a toe pinch and the surgical procedure was commenced when pedal reflexes were absent.

8.2.3 The donor procedure

8.2.3.1 Surgical equipment

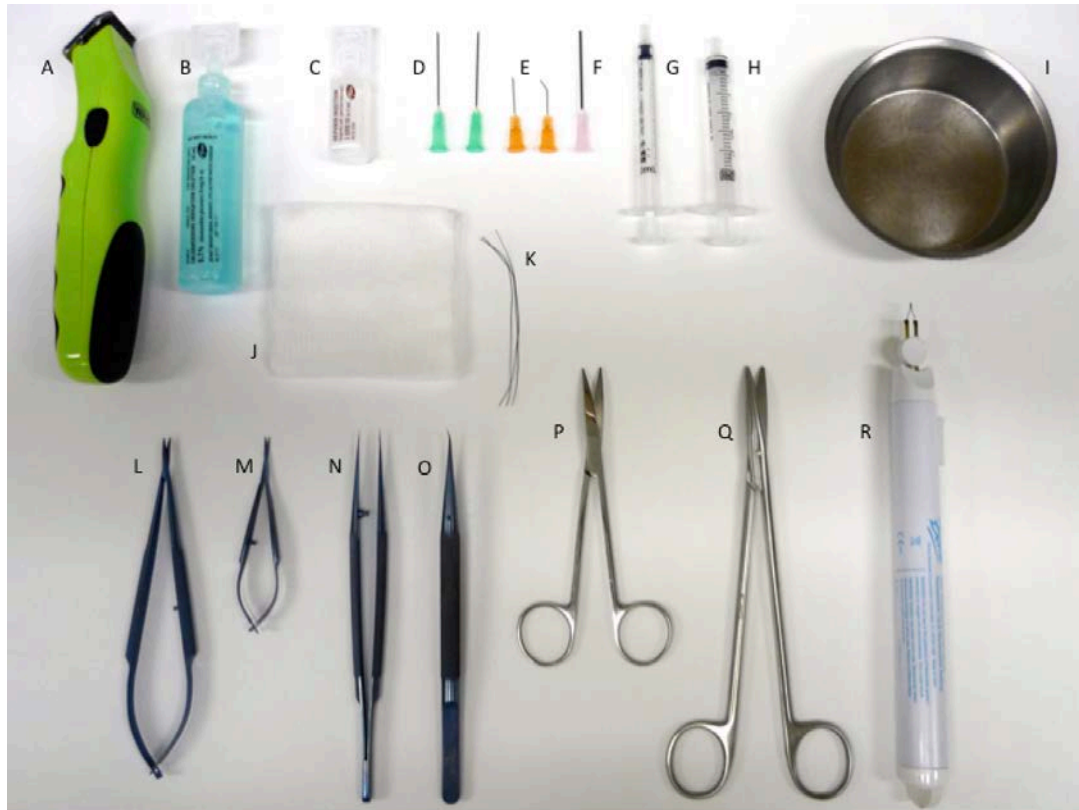


Figure 8.2 Equipment for donor surgery

- A. Animal Hair Clipper (Wahl, Sterling IL, USA)
- B. Chlorhexidine 0.1% irrigation solution (Pfizer Inc., Melrose Park, NSW, AU)
- C. Heparin sodium 5000units/5ml (Pfizer Inc., Melrose Park, NSW, AU)
- D. 21G needles x 2 (Becton, Dickinson & Co., North Ryde, NSW, AU)
- E. 25G needles x 2 (Becton, Dickinson & Co., North Ryde, NSW, AU)
(one needle angled at 45 degrees)
- F. 18G drawing up needle (Becton, Dickinson & Co., North Ryde, NSW, AU)
- G. 1.0ml tuberculin syringe (Becton, Dickinson & Co., North Ryde, NSW, AU)
- H. 3.0ml syringe (Becton, Dickinson & Co., North Ryde, NSW, AU)
- I. Stainless steel bowl, 10cm diameter (Key Surgical, Minneapolis, MN, USA)
- J. Gauze swabs (Multigate Medical Products, Villawood, NSW, AU)
- K. 4-0 'Sof silk' wax-coated braided silk (Covidien, Lane Cove, NSW, AU)

Titanium microsurgical instruments (Microsurgery Instruments Inc., Bellaire, USA)

- L. Micro-scissors (14cm, curved)
- M. Micro-scissors (9cm, straight)
- N. Micro-forceps (15cm, straight tip)
- O. Micro-forceps (15cm, curved tip)
- P. Curved sharp point scissors (Microsurgery Instruments Inc., Bellaire, USA)
- Q. Metzenbaum scissors - curve tip (SSS Healthcare Supplies, Murarrie, QLD, AU)
- R. Fine tip micro cauterization set (Bovie Med Corporation, Clearwater, FL, USA)

All surgical instruments were sterilized between uses. All disposable items such as needles, syringes, gauze and silk ties were obtained in sterile packaging, which were opened on the day of surgery.

8.2.3.2 The surgical procedure

The surgical procedure was developed as outlined below:

- i) The donor animal was placed supine on a small animal surgical mat (Figure 8.3A) with continuous inhaled isoflurane provided via a nose cone. The limbs were secured onto the surgical mat with tape and the upper abdomen and anterior thorax were shaved as shown in Figure 8.3B.
- ii) Chlorhexidine 0.1% irrigation solution (Pfizer Inc., Melrose Park, NSW, AU) was used to disinfect the shaved region of the anterior thorax and abdomen.

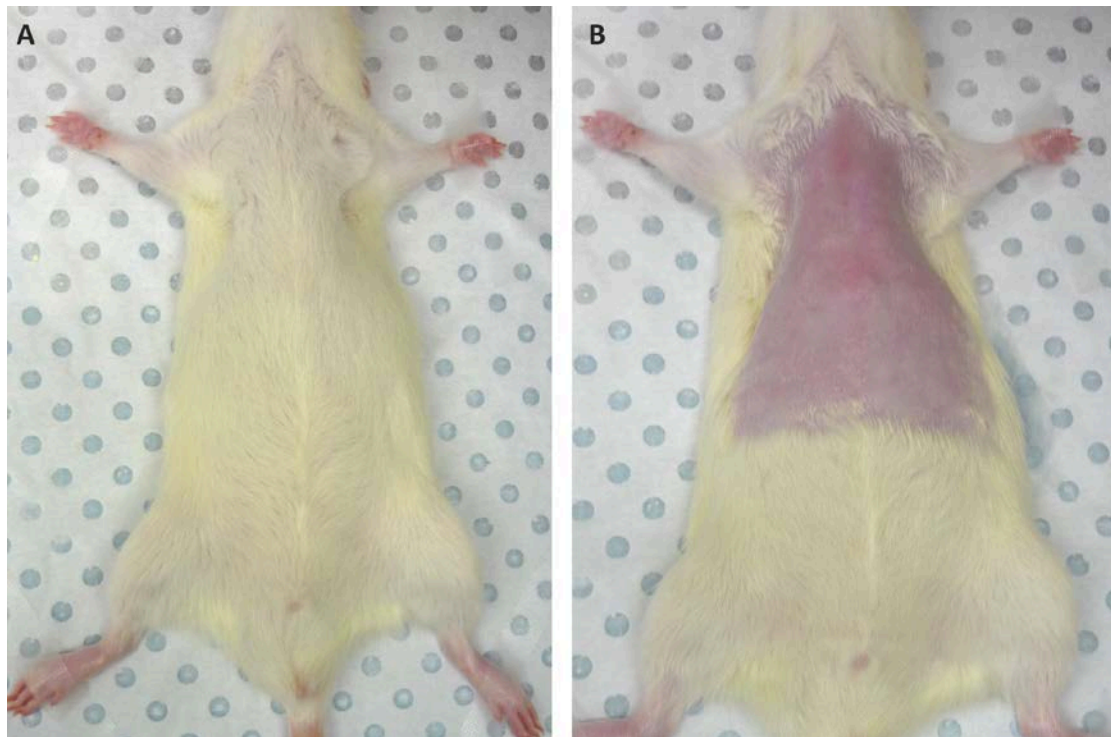


Figure 8.3 Donor animal preparation

- iii) A transverse upper abdominal incision was made using Metzenbaum scissors. Heparin (250units) was injected into a renal vein and allowed to circulate for 1 minute (Figure 8.4A).

- iv) The diaphragm was separated from the anterior thoracic cage. Two incisions made (supero-inferior) along the lateral thoracic walls. The time of diaphragm incision was marked as the start of warm ischaemia.
- v) The anterior thoracic wall was reflected superiorly and secured with two 21G needles. This provided maximum exposure with a bloodless field (Figure 8.4B).

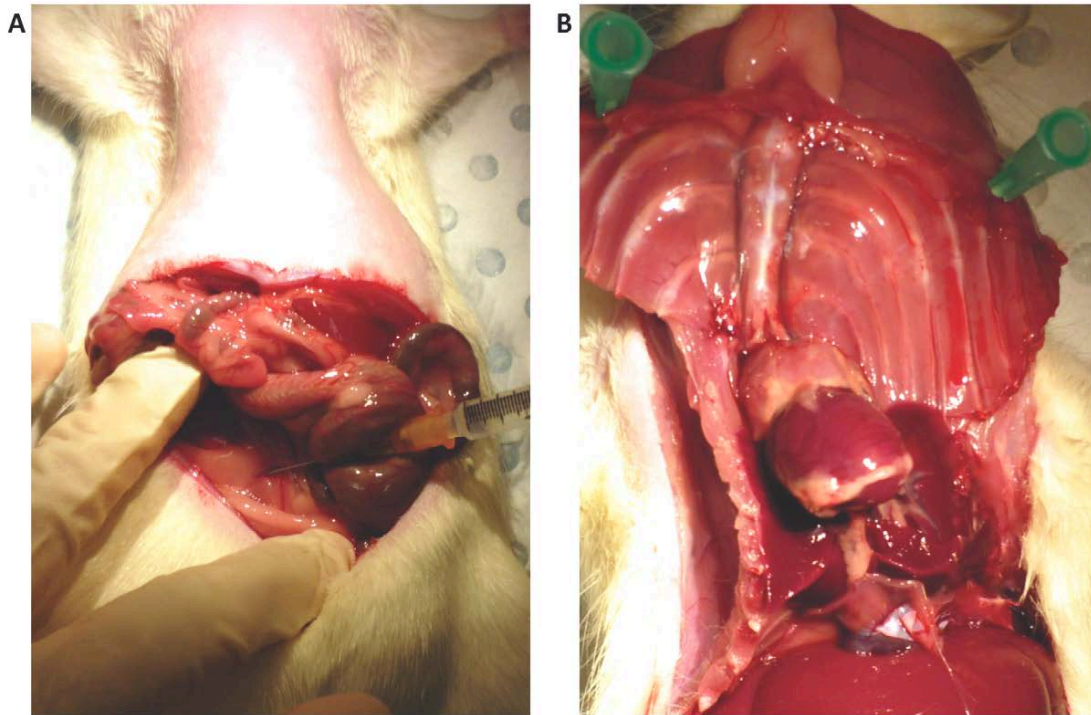


Figure 8.4 Laparotomy and thoracotomy

- vi) The thymus was dissected to expose the ascending aorta and a loose ligature was placed around the distal ascending aorta (just proximal to the branch vessels) using 4-0 silk (Figure 8.5).
- vii) Loose ligatures were placed around the inferior vena cava (IVC) and the left superior vena cava (SVC) using 4-0 silk ties (Figure 8.5). The right SVC did not require a ligature.

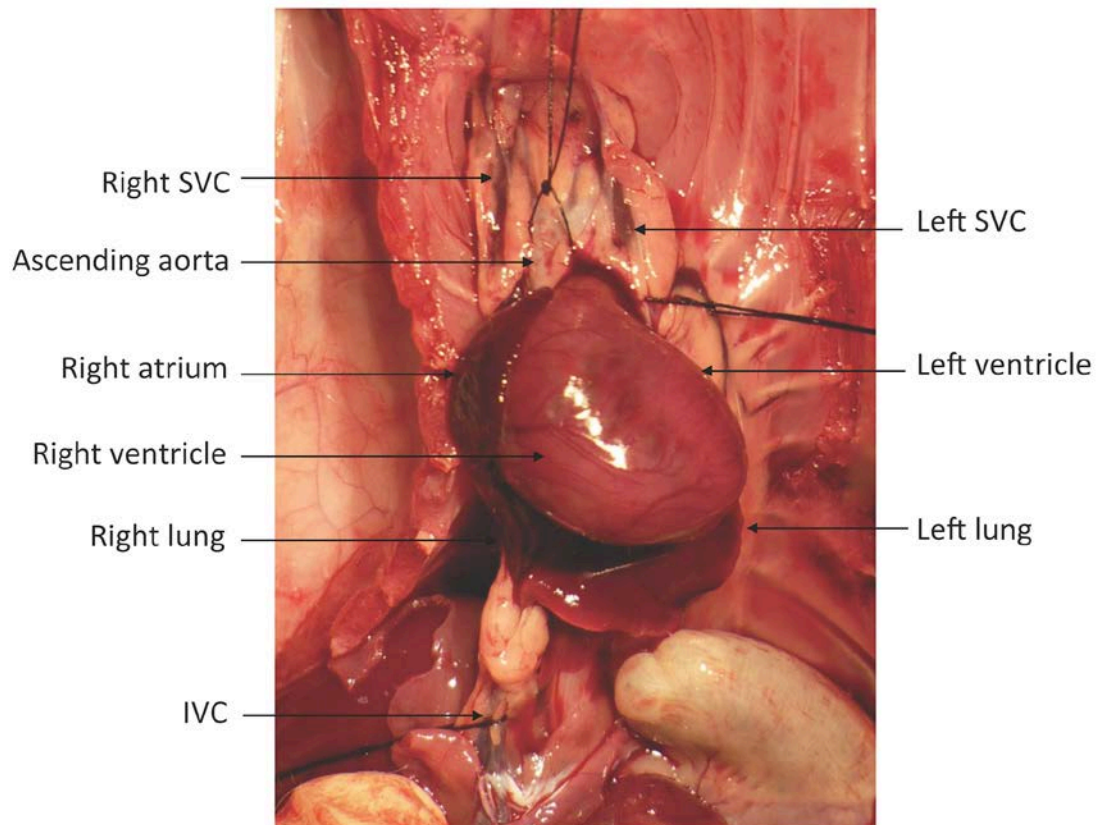


Figure 8.5 Donor heart preparation for cardioplegia and explantation

The thymus has been dissected and loose ligatures have been placed around the ascending aorta, left superior vena cava (left SVC) and inferior vena cava (IVC).

- viii) The IVC and both right and left SVC were partially transected to allow venting of blood. Partial, rather than complete transection of these three veins maintained the heart in position within the thoracic cavity for cardioplegia.

- ix) The aortic ligature was tightened and antegrade cardioplegia was delivered proximal to the ligature using an angled 25G needle and a 3.0ml syringe (Figure 8.6). Celsior® (Genzyme, Naarden Denmark) at 2-3°C was used as cardioplegia, delivered at a rate of 3ml over 20 seconds. This rate ensured a low pressure that would minimise coronary endothelial injury. The angled needle prevented puncture of the posterior aortic wall. Extra cardioplegia (3ml over 20 seconds) was often required for complete cardiac arrest.

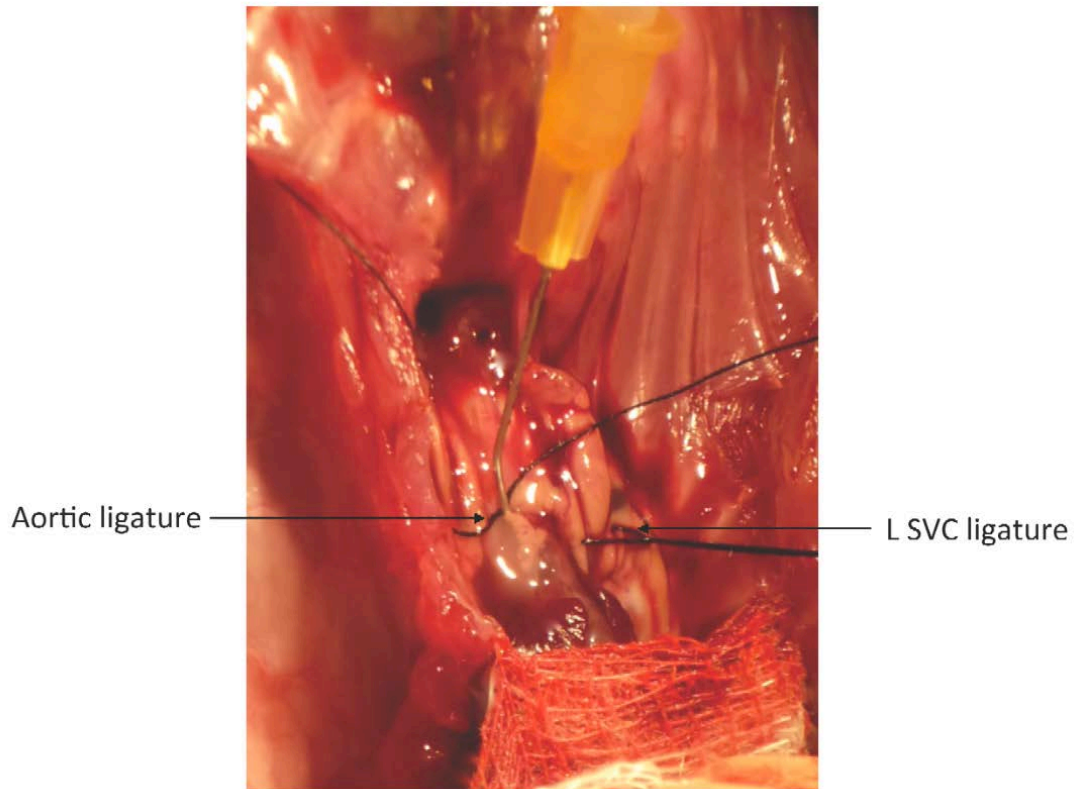


Figure 8.6 Cardioplegia of the donor heart

- x) After arrest of cardiac contractility, the aorta was transected with one straight cut, proximal to the entry point of the cardioplegia needle, ensuring adequate length was present for grafting to the recipient abdominal aorta.
- xi) The IVC and left SVC ligatures were secured and the vessels dissected distal to the ligatures. The right SVC was dissected 1cm distal to the right atrium allowing adequate length for grafting onto the recipient IVC.
- xii) The heart-lung bloc was placed in a small stainless steel bowl with gauze and 2-3°C cardioplegia for further dissection and preparation of the donor heart. Light microscopy (x 6 or x 10 magnification) was used to dissect structures. An 18G drawing up needle was used to flush blood from the aorta and right SVC to ensure minimal residual blood remained in the coronary arteries (Figure 8.7). An artery leading from the aortic root distally along the aorta was cauterised at this point, as it was often a source of bleeding after transplantation.

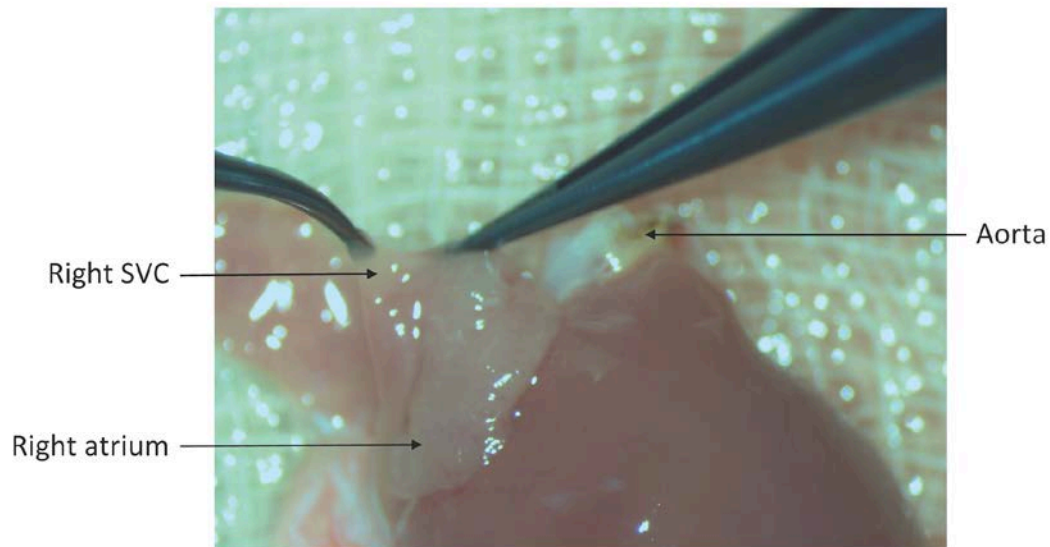


Figure 8.7 Further dissection of the heart-lung bloc

- xiii) The left lung was ligated with 4-0 silk ties (upper and lower lobes ligated separately), and dissected from the heart. The oesophagus was carefully dissected from the heart-lung bloc using microscissors. Cautery was used to seal any vasculature leading to the oesophagus.
- xiv) The heart and right lung bloc was placed in 2-3°C Celsior preservation solution in a 100ml beaker in an esky with ice. Gauze was placed at the bottom of the beaker to prevent freeze injury of the donor heart.

8.2.3 The recipient procedure

8.2.3.1 Surgical equipment

Equipment used in the recipient procedure are shown in Figure 8.8. Surgical instruments were not sterilised between donor and recipient surgical procedures. All disposable items such as needles, syringes, gauze, silk ties and cotton tips were obtained in sterile packaging, which were opened on the day of surgery.

9.2.3.2 The surgical procedure

The surgical procedure was developed as outlined below:

- i) The recipient animal was placed supine on a small animal surgical heatmat which was pre-warmed. Animals were supplied continuous isoflurane via a nose cone as described in Section 9.2.2. The limbs were secured onto the surgical mat with tape and the abdomen was shaved in the midline and cleaned with chlorhexidine solution.
- ii) Ampicillin 0.15mg/kg (Aspen Pharmacare Australia, St. Leonards, NSW AU) was administered intramuscularly into a thigh muscle for antibiotic cover during the surgery. The antibiotic powder was obtained in powder form as 1g/vial and pre-mixed with sterile water. A 1.0ml tuberculin syringe was used with a 25G needle for administration of the antibiotic.
- iii) Analgesic premedication was provided at this point with subcutaneous buprenorphine (Abbott Australia, Macquarie Park, NSW, AU) at a dose of 0.05mg/kg. Premedication is associated with reduce post-operative pain, and was therefore adopted as part of the recipient surgical protocol.
- iv) A midline skin incision was then made using sharp point scissors from the xiphisternum to the pubis, as shown in Figure 8.9A. Bleeding from superficial vessels was controlled with cautery.



Figure 8.8 Equipment for recipient surgery

- A. Animal Hair Clipper (Wahl, Sterling IL)
- B. Fine tip micro cautery set (Bovie Med Corporation, Clearwater, FL, USA)
- C. 21G needles x 4 (Becton, Dickinson & Co., North Ryde, NSW, AU)
- D. Large paper clips (angled as demonstrated) x 4
- E. 3.0ml and 1.0ml syringes (Becton, Dickinson & Co., North Ryde, NSW, AU)
- F. 25G needles x 2 (Becton, Dickinson & Co., North Ryde, NSW, AU)
(one needle point cut to create a straight end)
- G. Microvascular clamps (1.8cm length) x 2 (Microsurgical Instruments Inc., Bellaire, USA)
- Double clamps shown above may be used, but single clamps were preferred.
- H. 0.9% sodium chloride 10ml ampoule x 10 (Pfizer Inc., Melrose Park, NSW, AU)
- I. Chlorhexidine 0.1% irrigation solution (Pfizer Inc., Melrose Park, NSW, AU)
- J. OPSITE spray on dressing (Smith & Nephew Pty, Ltd., North Ryde, NSW, AU)
- K. Gauze swabs (Multigate Medical Products, Villawood, NSW, AU)
- L. Cotton tips
- M. Curved sharp point scissors (Microsurgery Instruments Inc., Bellaire, USA)

Titanium microsurgical instruments (Microsurgery Instruments Inc., Bellaire, USA)

- N. Micro-scissors (9cm, straight)
- O. Micro-forceps (15cm, straight tip)
- P. Micro-forceps (15cm, curved tip)
- Q. Micro-scissors (14cm, straight)
- R. Micro-scissors (14cm, curved)
- S. Micro needle holder (14cm, straight)
- T. Micro-needle holder (14cm, curved)
- U. Gelfoam dressings 80 x 125mm (SSS Healthcare Supplies, Murarrie, QLD, AU)
- V. 8-0 Prolene sutures (Ethicon, Johnson & Johnson, North Ryde, NSW, AU)
- W. 4-0 Vicryl sutures (Ethicon, Johnson & Johnson, North Ryde, NSW, AU)

- v) The abdominal muscles were dissected in the midline. Dissection along the *linea alba* minimized bleeding (Figure 8.9B). Care was taken to prevent injury to the bladder. Cautery was used to control bleeding.

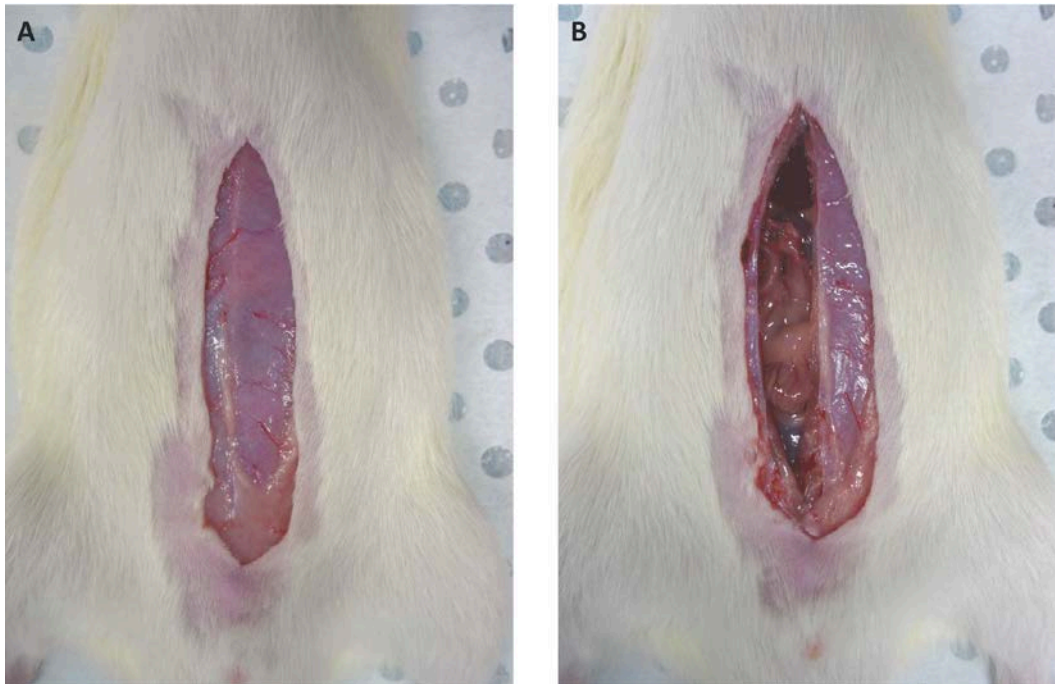


Figure 8.9 Preparation and abdominal dissection of the recipient animal.

A midline skin incision was made (A) followed by a midline dissection of the abdominal wall muscles along the linea alba (B).

- vi) The abdominal skin and muscles were retracted to expose abdominal contents. Angled paperclips were used for retraction with 21G needles to secure them on the surgical mat (Figure 8.10A).
- vii) Surgery was performed under microscopy (x10 or x25 magnification) in the subsequent steps.

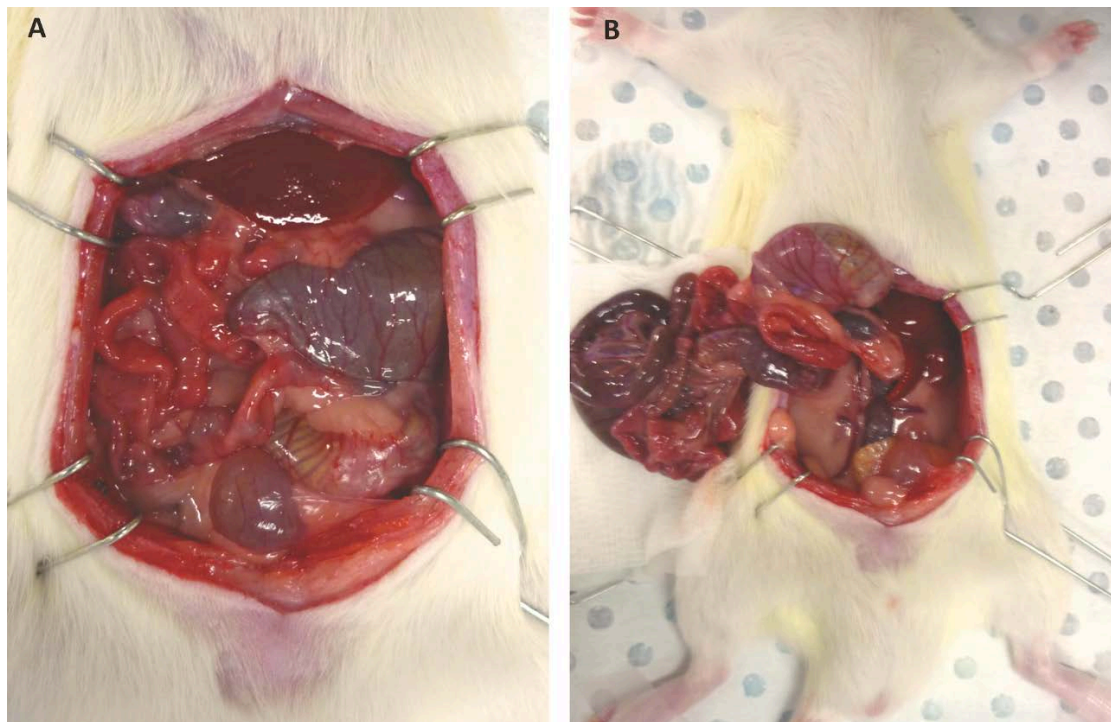


Figure 8.10 Preparation of the recipient's abdominal surgical field

- viii) The stomach and mobile large and small intestines were displaced and the avascular mesentery of the sigmoid colon was divided. The stomach and intestines were then reflected to lie on the right side of the recipient's abdomen (Figure 8.10B). Care was taken to prevent twisting of the intestines, and temperature and hydration was maintained by placing the abdominal contents on warm saline-soaked gauze with addition of saline every 30 minutes. Care was taken to prevent twisting of the intestines. Cotton tips (blunt dissection) or micro-forceps with fine tips were used to divide the peritoneum and mesentery.

- ix) The right and left iliolumbar vessels (artery and vein) were identified as shown in arrows in Figure 8.11A and cauterised. This was performed by gently dissecting the retroperitoneum with microforceps to allow the vessels to surface (Figure 8.11B), then compression of the vessels to arrest blood flow by using cotton tips (Figure 8.11C), followed by cautery to separate the two ends shown in arrows in Figure 8.11D. This was to prevent blood flow to the IVC and abdominal aorta during anastomosis of the donor heart vessels. Compression of the iliolumbar vessels was required to prevent bleeding during cautery.

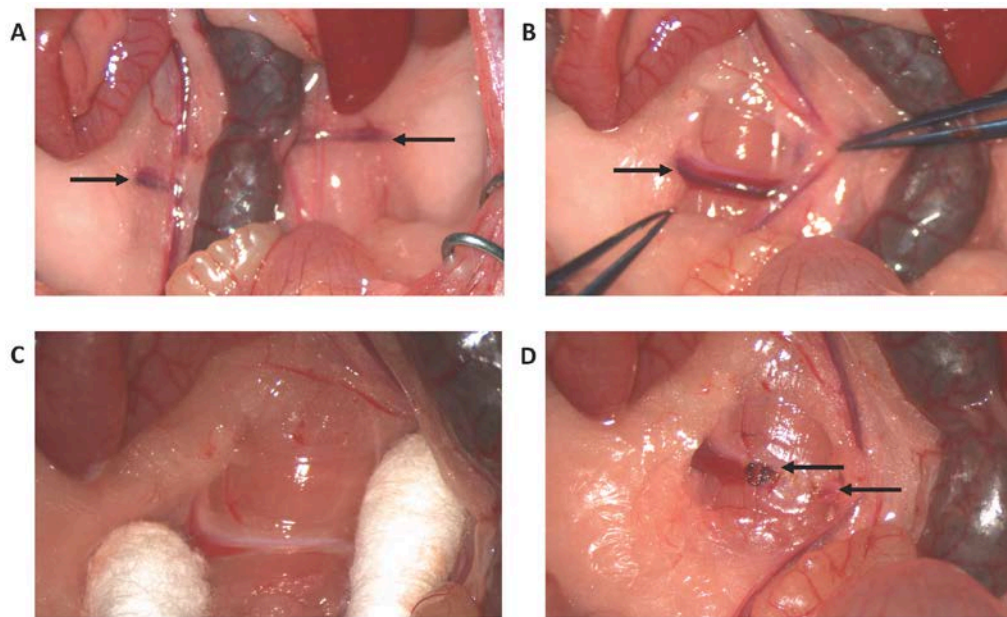


Figure 8.11 Isolation and cautery of the ilio-lumbar vessels

- x) The abdominal aorta and IVC were exposed by fine dissection of the retroperitoneum and cautery was used to free the two vessels from where they were freed of spinal arteries and veins from the level of the renal to iliac vessels. Gonadal vessels were spared (Figure 8.12). Freeing the aorta and IVC of collateral blood flow prevented bleeding during the transplant procedure. Care was taken to prevent dissection of the ureters.

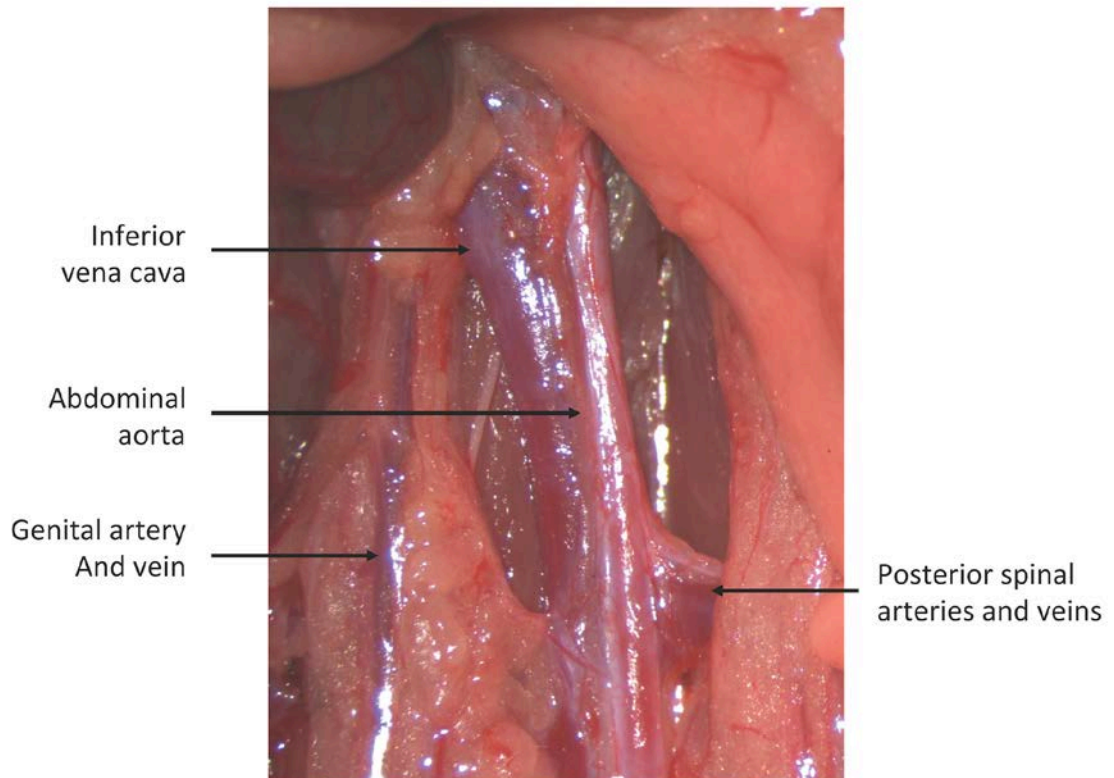


Figure 8.12 Cautery of spinal arteries and veins

- xi) Once branch arteries and veins were cauterised, the heat mat was rotated to 90 degrees so that the animal was positioned horizontal to the operator, with the head towards the left and tail to the right.
- xii) A partial ligature was placed around the IVC using a 4-0 silk tie (Figure 8.13), in order to create adequate perfusion of blood to the donor heart after transplantation.



Figure 8.13 Placing a partial ligature around the recipient inferior vena cava

- xiii) Using two small vascular clamps, the abdominal aorta and IVC were clamped superiorly (inferior to the genital vessels), and inferiorly (inferior to the cauterised iliolumbar vessels) (Figure 8.14).

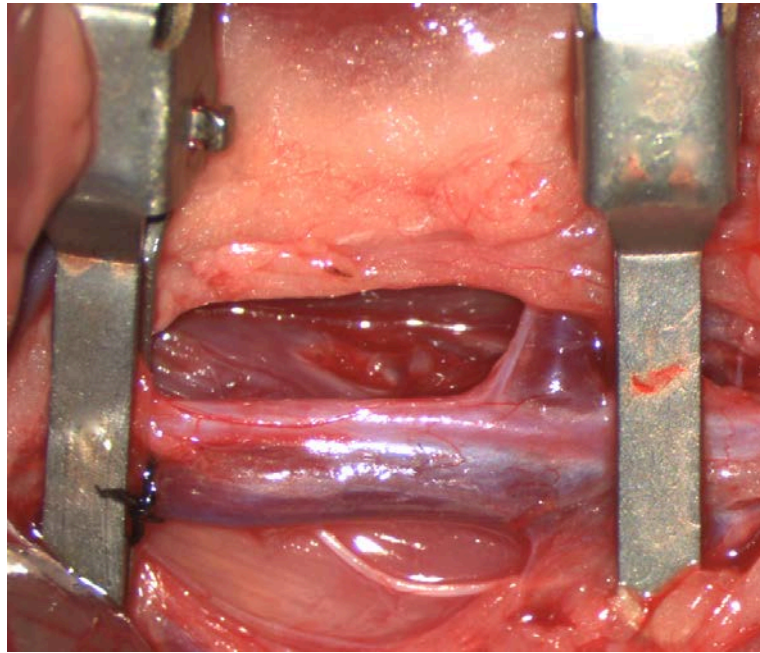


Figure 8.14 Clamping the aorta and inferior vena cava

- xiv) The ideal timing for the clamps to remain on should be less than 30minutes, as significant hindlimb ischaemia can occur with longer durations of clamping of the aorta. A timer was used to time the period of clamping.
- xv) A 25G needle was used to puncture the abdominal aorta at the inferior end (just proximal to the inferior clamp), and at the superior end of the IVC (approximately 1/3 of the distance from the proximal clamp). The 9cm microscissors were used to cut along the aorta and IVC to the length of the donor vessels (aorta and SVC respectively), as shown in Figure 8.15.

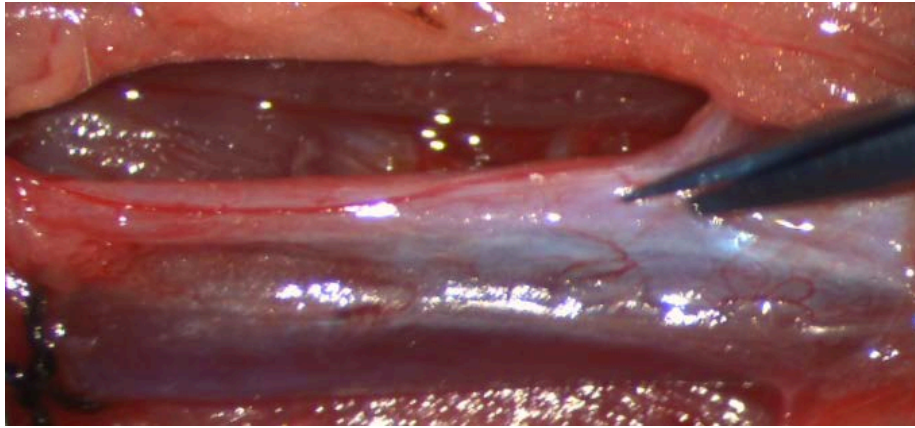


Figure 8.15 Performing an incision in the recipient abdominal aorta

- xvi) The lumen of the aorta and IVC were flushed with saline using a 3.0ml syringe and a 21G needle which was cut to have a relatively blunt, straight end. This was done to prevent puncture of the aorta or IVC during flushing. It was imperative to flush the vessel since it was common for residual blood to form clots and occlude the lumen, which would impair perfusion of the grafted heart as well as the recipient hindlimbs.

8.2.4 The transplant procedure

- i) The donor heart and right lung bloc were placed on cold saline gauze, with the apex towards the right side of the recipient animal and the anterior surface facing anteriorly. The donor aorta was aligned with the incision made in the recipient aorta, and the donor right SVC aligned with the incision made in the recipient IVC.
- ii) Using 8-0 prolene sutures, the donor aorta was anastomosed onto the recipient aorta. The anterior wall of the donor aorta was initially anastomosed onto the left wall of the recipient aorta using running sutures. Stay sutures were placed in the initial transplants performed, however these were not necessary and were replaced with good aligning of the vessels and continuous running sutures. Approximately 10-14 sutures were required, varying according to the vessel diameter. The sutures were placed close to prevent bleeding after reperfusion (Figure 8.16). The heart was reflected to the left of the recipient to anastomose the posterior wall of the donor

aorta to the right side of the incision on the recipient aorta. One reef knot was used to secure the sutures at the completion of aortic anastomosis.

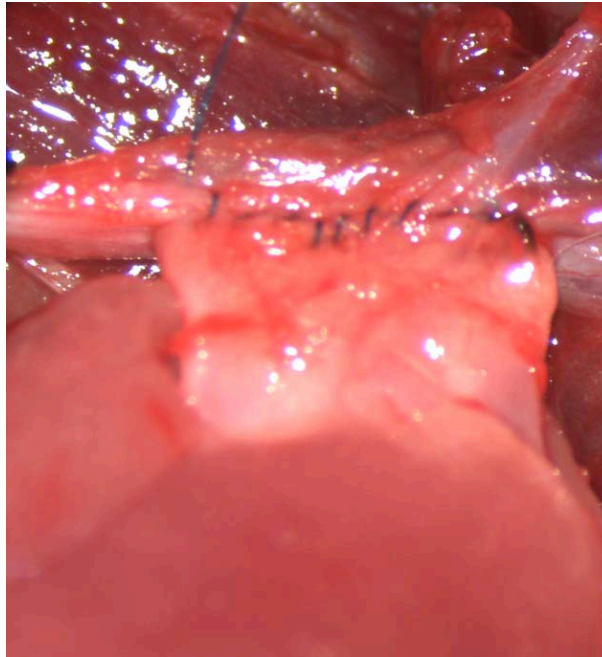


Figure 8.16 Anastomosis of the donor aorta to the recipient aorta

- iii) The donor SVC was anastomosed to the recipient IVC using the same technique as the aortic anastomosis. Approximately 8-14 running sutures were used for each wall. The heart was reflected to the left of the recipient to anastomose the posterior wall of the donor SVC, and a double knot was used to secure the two ends of the sutures after completion of anastomosis of anterior and posterior walls. The completed SVC-IVC anastomosis is shown in Figure 8.18.

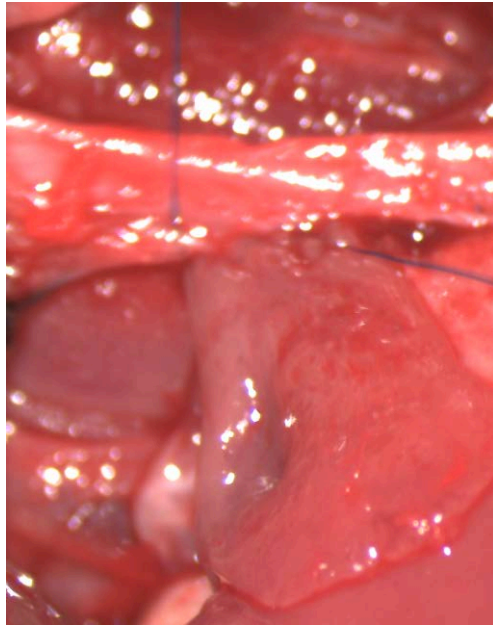


Figure 8.17 Anastomosis of the donor SVC to the recipient IVC

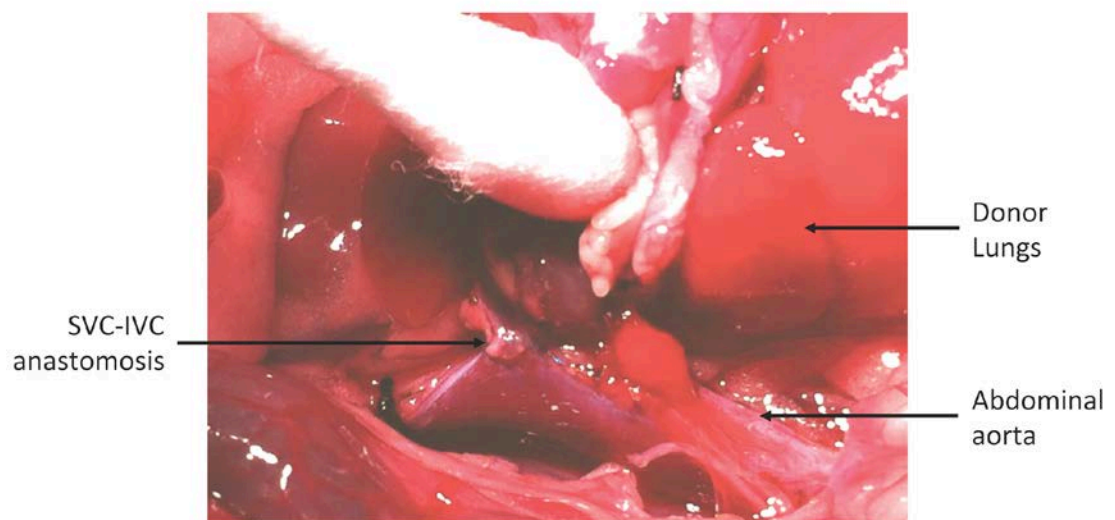


Figure 8.18 Completion of SVC-IVC anastomosis

- iv) Strips of Gelfoam (10mm x 3mm) were placed on the anterior and posterior walls of the anastomosed vessels to minimize bleeding during reperfusion.
- v) The inferior clamp was momentarily released, allowing blood to enter the recipient IVC from the hindlimbs. This clamp was released first in order to initially provide low-pressure flow into the SVC-IVC anastomosis. Intermittent reclamping for not more than

45 seconds and application of pressure effectively sealed minor defects in the anastomotic site. Moderate bleeding required reclamping and reinforcement with simple sutures.

- vi) The superior clamp was then released to allow blood flow into the aorta. As with the IVC, intermittent reclamping was performed to seal minor defects. Moderate bleeding was controlled by reclamping and reinforcing the defect with a simple suture
- vii) After release of both clamps, the heart rapidly distends (Figure 8.19) and multifocal ventricular contractions are initially noted. Effective regular contractions of the atria and ventricles followed. The heart rate after reperfusion was approximately 70-120bpm in the model thus far.

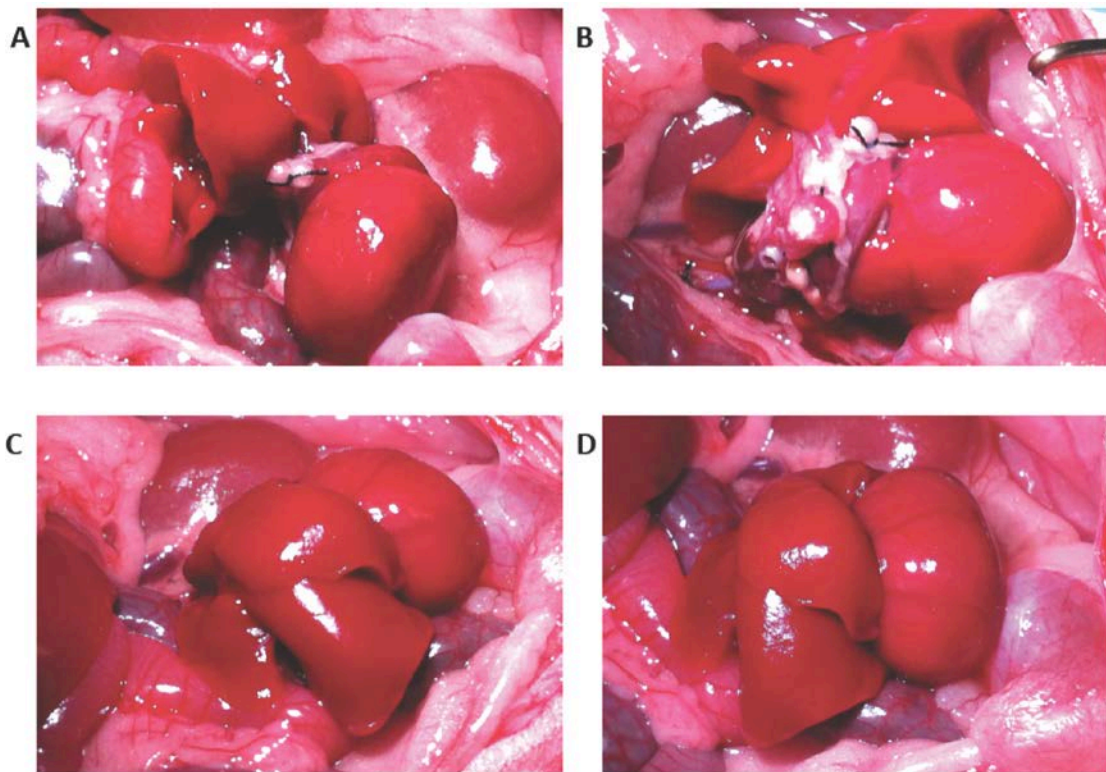


Figure 8.19 The transplanted heart after reperfusion.

Views of the transplanted heart and lung are shown anteriorly (A) and turned posteriorly (B). Good perfusion of the heart and both lobes of the right lung can be seen in (C) and (D).

8.2.5 Wound closure

- i) The stomach, small and large intestines were placed back in the abdominal cavity with care taken to prevent twisting of the bowel. Saline (2.0-2.0ml) was used to hydrate the intestines in the abdominal cavity.
- ii) The abdominal muscle was closed with running sutures using 4-0 Vicryl. An Aberdeen knot was performed to bury the ends of the sutures beneath the muscle to prevent the rats from gnawing at subcutaneous masses that could be felt. Care was taken to appose the muscle layers well.
- iii) The skin was closed with 4-0 Vicryl using subcuticular sutures and an Aberdeen knot to bury the ends subcutaneously. Care taken to ensure the edges of the skin were well apposed, without any uneven or protruding ends. This was again done to prevent the animal from gnawing at foreign material or protruding skin. Spray-on opsite was used to seal the wound.

8.2.6 Post-operative analgesia

Multimodal analgesia was provided, with opioids and non-steroidal anti-inflammatories for optimal pain control.

- i) After closure of the abdominal skin with a subcuticular suture, drops of 0.25% bupivacaine (2.5mg/ml, Astra Zeneca, Eight Mile Plains, QLD, AU) were placed along the incision site prior to application of the spray-on Opsite dressing.
- ii) A bolus dose of non-steroidal anti-inflammatory agent ketoprofen (5mg/kg, Ilium Veterinary Laboratories, Smithfield, NSW, AU) was administered once intramuscularly soon after closure of all wounds, expected to last 24 hours.
- iii) Buprenorphine (0.05mg/kg) was provided subcutaneously every 8-24 hours from the time of wound closure until at least 48 hours post-operatively. The frequency of dosing depended on signs of pain behaviour and on how alert the animal was. Signs of pain were arching of the back and decreased movement.

- iv) Pain score (1 point each for reduced movement, arching of the back and reduced appetite) was recorded over the seven days of animal monitoring. The score was recorded hourly in the first 6 hours, then 8 hourly in the first 24 hours and daily for the 7 post-operative days.

8.2.7 Recovery of the animal

- i) After provision of post-operative analgesia, isofluorane was ceased and the animal was placed individually in a ventilated cage (Tecniplast Aust., Pty. Ltd, Rydalmere, NSW, AU), that was placed over a heat mat to ensure half the floor of the cage was heated. The animal was placed prone over the heated area with soft bedding provided. Oxygen was provided via the nose cone until the animal regained consciousness.
- ii) During the recovery period, the animal was closely monitored for signs of pain and for signs of complications of the surgical procedure, such as decreased movement or paralysis of the hindlimbs due to intra-operative clamping of the aorta and IVC .

8.2.8 Post-operative care and monitoring

- i) Soft bedding was placed in the cage to minimise irritation of the surgical wound and the cage was placed over a heat mat to ensure half the cage was heated for at least 24 hours of the recovery period (Post-operative 'Day 0').
- ii) Food and water were provided in nectar form (Able Nectar, Able Scientific, West Ryde, NSW, AU), placed at the bottom of the cage for easier access. After 24 hours, if the animal's pain was well-controlled (as per absence of signs of pain behaviour) nectar food and hydration was supplemented with pelleted food and water.
- iii) Animal movement was observed closely to detect for signs of decreased movement of the hindlimbs, and recorded in a chart along with the pain scale.

8.2.9 Monitoring the grafted heart

- i) The grafted heart was palpated daily to feel the strength of contraction and heart rate. Heart rate was recorded daily.
- ii) Functional assessment of the heart was not performed, however the model was developed to be able to assess with non-invasive functional imaging on post-operative days 1 and 7.

8.3 Discussion

An abdominal heterotopic 'working' heart transplantation model was developed as described above. The aim of developing this model was for application in future organ preservation studies, with non-invasive functional assessment to be performed with CMR.

The surgical procedure required significant practice and optimisation. Steps optimized in the donor include i) minimising warm ischaemic time to < 8 minutes during dissection of the thorax and thymus and applying loose ligatures around the large veins and arteries; ii) optimizing the mode of delivery of cardioplegia to the donor heart by evaluating delivery through the inferior vena cava (IVC) and the aorta; iii) ensuring complete flushing of blood from coronary arteries, the microcirculation and atrial and ventricular cavities; iv) tying of large vessels under microscopy; and v) ensuring the donor heart was placed in hypothermic storage within 20 minutes of dissection of the donor diaphragms (ie. total warm and cold ischaemic time during organ procurement).

Steps optimised in the recipient include i) the optimal method of providing an adequate surgical field by dissecting peritoneal attachments of abdominal viscera, while moving abdominal contents from the donor in a manner that prevents twisting of the bowel, and ensuring regular hydration while performing the surgical procedure; ii) optimizing the method of terminating blood supply to the aorta and IVC from abdominal and spinal branch vessels, with light cautery proved to be the optimal method as it avoids significant blood loss and death of the animal; ii) determining appropriate clamps and the sites of clamping of the abdominal aorta and IVC; iii) separating the delicate fascia between the IVC and aorta

without causing blood loss, which allowed placement of a loose ligature at the IVC to direct blood flow to the donor heart; iv) the optimal method of suturing of the donor vessels (ie. use of stay sutures or one continuous running suture and optimizing the direction of suturing); iv) ensuring warm ischaemia of the donor heart is minimized by applying intermittent cold saline while suturing; v) minimizing hindlimb ischaemia during transplantation of the donor heart by reducing suture time to < 30 minutes, with realistic times of < 60 minutes were achieved; v) preventing blood from needle entry sites at reperfusion by using gelfoam and applying intermittent clamping; and vi) practicing subcuticular sutures to close the skin.

This model has not been used in studies described in this thesis, however has been established for use in future screening experiments on preservation strategies, with skills passed on to laboratory staff.

CHAPTER 9. FINAL DISCUSSION AND CONCLUSIONS

Considerable progress and success has been made in cardiac transplantation over the last few decades. The growth of knowledge and experience is allowing increasing numbers of higher risk transplants to be performed. As discussed, these include the use of hearts from extended criteria donors, transplantation into higher risk recipients, and more challenging surgical procedures. The donor heart is more vulnerable to injury in this setting, hence it is of utmost importance to improve on current myocardial preservation techniques to ensure graft and patient survival are optimised.

This thesis describes the development of clinically relevant small animal models for use in screening studies on donor heart preservation. Using these models, the results of pharmacological conditioning of hearts brain dead and older or 'marginal' hearts are discussed. The development of a non-invasive method of functional assessment of small animal hearts using cardiac magnetic resonance (CMR) imaging is also discussed. Lastly results of a translational clinical study on preservation of the donor heart using pharmacological conditioning are discussed.

9.1 Development of models

Animal models used to evaluate the efficacy of newly developed or modified cardiac preservation solutions should closely resemble the clinical situation. Large animal models are ideal, however are expensive and require a large team and hence are not practical as screening models. A small animal orthotopic heart transplant model would be an ideal initial screening model, however it would require cardiopulmonary bypass, therefore not be feasible for routine screening of new myocardial preservation strategies. Orthotopic heart transplant models would also result in significant animal morbidity in the event of graft failure, which would be unethical due to animal discomfort, and it would prevent longitudinal functional assessment of the donor heart.

A routine screening model should offer quantitative functional evaluation of the donor heart as well as allow longitudinal assessment. Furthermore, donor heart function should be evaluated in the setting of a circulatory load, and animal models should incorporate all reversible and irreversible changes that a human donor heart would undergo. These include

brain death, warm ischaemia during organ procurement, cold ischaemia during organ transport, warm ischaemia during transplantation and finally reperfusion with warm blood.

The rodent isolated working heart model that had been used in the laboratory provides some of these features, such as quantitative functional evaluation of the heart in the setting of a fixed workload^{320,325,329,366,367}. This model incorporates warm and cold ischaemia, however does not include donor brain death. Reperfusion with oxygenated crystalloid solution provides oxygen to induce free radical damage, however does not provide the full extent of oxidative injury and calcium overload that would be seen with warm blood reperfusion in a recipient animal.

9.1.1 The rodent model of brain death

A rodent model of brain death was developed to incorporate the initial physiological insults incurred by human donor hearts. This was based on the model described by Oto et al³⁶⁹. Brain death in the small animal model was validated by loss of neurologic reflexes and spontaneous respirations³³⁸. Animals subjected to brain death demonstrated haemodynamic changes of a 'Cushing response', extreme tachycardia and hypertension, followed by hypotension that are typical of brain death described clinically^{58,340} and in other experimental animal studies^{59,60,342,369}. An inflammatory response was also demonstrated with a significant rise in systemic inflammatory cytokines IL-6, TNF- α , IL-10, IL-1 β , IL-12p70, GRO/KC, MIP-2 and MCP-1, as seen clinically^{58,71,350} and in experimental animal studies^{352,353}.

The effects of brain death (BD) on cardiac function were evaluated on an isolated working heart circuit and compared with sham hearts. There were no initial functional differences noted between sham and BD groups (Section 3.3.4), however they became apparent after subjecting hearts to additional insults of hypothermic storage and reperfusion with oxygenated crystalloid buffer (Section 3.3.5). Significant differences in the recovery of aortic flow (AF) in sham and BD hearts were noted after storage for 3 hours, and not in 1- or 6-hour groups. This is likely to be due to minimal injury incurred during 1 hour of storage, and severe injury at 6 hours of storage that did not allow appreciable differences to be noted between groups. The model was an accurate and reproducible model of brain death, hence used to evaluate the efficacy of pharmacological conditioning in subsequent studies.

One of the criticisms of the model is that hearts were only subjected to 1 hour of brain death. If the hearts were kept for a longer duration of time in-vivo, more injury may have been incurred due to ongoing inflammatory activity and haemodynamic and hormonal dysregulation. However, haemodynamic deterioration in the absence of inotropic or vasopressor support led to animal loss within 1 to 3 hours of brain death, hence hearts were explanted after 1 hour.

9.1.2 The rodent heterotopic ‘working’ heart transplant model

An abdominal heterotopic working heart transplant model was subsequently developed, as discussed in Chapter 9. This model was designed to allow quantitative and longitudinal data of donor heart function, with inclusion of reperfusion of warm blood, which the current ex-vivo perfusion model does not provide. Longitudinal assessment of cardiac function would be possible by using cardiac magnetic resonance imaging of the donor heart, also described in the thesis. The transplant model developed allows the donor heart to work against a systemic circulatory load, therefore negates the need for further intervention to provide loading for evaluation of work-related functional changes, such as use of a left ventricular balloon catheter described in ‘resting’ heart transplantation models^{328,547}.

This model was refined considerably, with time taken to optimize several crucial steps by evaluating several procedural techniques as discussed in Chapter 8.

This model required considerable time to develop, hence it was not used in studies described in this thesis. It has however been set-up for future studies. For functional assessment of the transplanted heart, non-invasive imaging methods were developed, also to be applied to future studies. Functional assessment of the heart is required rather than palpation for heart rate. Heart rate is an unreliable marker of myocardial preservation, with no differences appreciated between well-preserved or poorly preserved hearts as demonstrated in Section 3.3.5 and in other studies^{327,328}. It will also be possible to incorporate donor brain death in future studies using this abdominal heterotopic working heart transplantation model.

9.1.3 Cardiac magnetic resonance imaging of rat hearts

The rodent working heart transplant model allows the ability to perform longitudinal non-invasive functional assessment of the transplanted heart. Such measurements could be performed using traditional echocardiography, however better tissue characterization and more accurate measures of cardiac volumes and function can be achieved with cardiac magnetic resonance (CMR) imaging^{451,489,500,501}. A model was therefore developed to provide high-resolution images of rat hearts to allow accurate, highly reproducible volumetric and functional data. This model was developed using native hearts in rats without prior surgical intervention. Ease of access to CMR was paramount in developing this model for application in other studies, hence image were acquired using a low field strength (1.5 Tesla) clinical scanner and a clinical wrist coil. Challenges of image acquisition due to rapid heart rates and small heart sizes were overcome by refining acquisition settings and using rodent electrocardiograph (ECG) gating, and images of high-resolution were obtained, comparable to images from dedicated high field strength small animal scanners^{449,498,506,507}.

The assessment of intra- and inter-observer variability and inter-study reproducibility demonstrated highly reproducible data with measurement variability lower than published clinical^{513,517} and rodent^{515,516,518-520} echocardiography data, and comparable to clinical^{503,504,510,512,513,548,549} and rodent CMR using higher field strength scanners^{479,515,516}. Increased reproducibility of measurements allowed significant sample size reduction compared to echocardiography. The application of CMR in imaging abdominal heterotopic heart transplants would require repositioning of ECG leads to detect the signal of the transplanted heart. Image localization and planning of sequences allows heart to be imaged in its 'true' long and short axes, regardless of its position in the animal. True short axis stack images will be able to be obtained in abdominally transplanted hearts in order to accurately evaluate ejection fraction, cardiac dimensions, mass and volumes. The extension of CMR to determine myocardial oedema and inflammation using T1 and T2 weighted images would allow more detailed analysis of injury due to IRI, and the use of gadolinium contrast would allow assessment of rejection, however these techniques remain to be developed in future rodent CMR studies. At present, the model has been firmly established to image cardiac volumes, mass and ejection fraction of rodent hearts.

9.2 Myocardial preservation studies

Existing myocardial preservation solutions have been developed for either short-term open heart surgery^{182,550,551} or for preservation of abdominal organs for transplantation^{176,552,553}. The most recently developed preservation solution, Celsior, was developed specifically for cardiac transplantation^{183,200}, however has demonstrated weak myocardial preservation capacity during prolonged hypothermic storage, and has not been tested for preservation of extended criteria organs. Indeed hyperkalaemia and hypothermia, the main methods of cardioplegia in all currently used preservation solutions in transplantation have been shown to have detrimental effects by inducing ionic imbalances and myocardial oedema.

9.2.1 Myocardial preservation in the context of brain death

Previous studies in the laboratory had demonstrated significant improvement of donor heart preservation made possible by implementing pharmacological conditioning strategies^{320,322,324,325,329,365-367}. These rodent studies were performed on hearts that had not been subjected to brain death, hence initial studies outlined in this thesis focused on ensuring that pharmacological conditioning provided adequate preservation of the donor heart with additive insults incurred during brain death (section 3.2.3). The pharmacological conditioning strategy tested was ‘triple therapy’ with erythropoietin (EPO), glyceryl trinitrate (GTN) and zoniporide (ZON), a combination that had been demonstrated as providing synergistic effects in donor heart preservation³²⁹.

The literature has been divided on the ability to condition hearts that have suffered injurious insults during brain death³⁹²⁻³⁹⁵. However, in the study described in this thesis, using a validated reproducible model of brain death, the addition of EPO, GTN and ZON to Celsior preservation solution used in cardioplegia and hypothermic storage demonstrated significantly improved recovery of cardiac function in hearts subjected to brain death and additional insults of 3 or 6 hours of hypothermic storage (section 3.3.6). Differences in other studies may be due to the mechanism of conditioning implemented, such as potassium-channel openers used by Kirsch et al^{393,394}, and ischaemic preconditioning used by Farhat et al³⁹². This highlights the synergistic effects of combining all three pharmacological agents to target multiple cytoprotective pathways, that may explain successful outcomes described in the current study. Certainly, in the 6-hour storage group, the recovery of cardiac output

(CO) was 90% in treated hearts, compared to 25% in untreated hearts, and over 95% in BD hearts stored for 3 hours, compared to 30% in untreated hearts.

Supporting evidence for improved myocardial protection by pharmacological conditioning of brain dead hearts were reduced levels of lactate dehydrogenase levels (section 3.3.7). The mechanism of cardioprotection was demonstrated through increased phosphorylation of pro-survival kinases Akt and ERK in 3- hours storage groups, but not in hearts stored for 6 hours. It was noted however, that increased phosphorylation of the ERK was also demonstrated in untreated BD hearts compared to sham hearts. A similar study, however excluding brain death, demonstrated that EPO, GTN and ZON used in conditioning of rat hearts increased phosphorylation of PI3K/Akt and STAT3 kinases³²⁹. The lack of significance in STAT3 phosphorylation at 3 hours may be due to brain death mediated injury inhibiting activation of the SAFE pathway. The lack of increased phosphorylation of pro-survival kinases in the 6 hours storage group was inconsistent with significant functional improvement and with biochemical data, however it may explain cardioprotection that may be mediated through other signaling proteins involved in pharmacological conditioning.

This study further highlighted the need to optimise donor management strategies in order to minimise injurious effects of brain death at an earlier stage. Due to the donation of multiple organs, such strategies should ensure protection and avoid adverse effects to all potential donor organs. As discussed in the literature review, donor hormonal management strategies have been implemented including replacement of thyroid hormone, the use of vasopressin over inotropes, and treatment with methylprednisone. However, prospective clinical studies have not demonstrated improved graft function from these donors^{161,162}. Methylprednisone in particular would have additional anti-inflammatory properties that may minimise endothelial injury and the migration of inflammatory cells into the myocardium, thereby potentially reducing rejection and allograft vasculopathy. Combining such treatment with additional anti-inflammatory therapy such as the TNF- α antagonist etanercept has been proposed by some authors⁷¹. If such donor management strategies were ethically allowed to be implemented early after brain death, organ preservation against inflammation may potentially be improved. The use of anti-inflammatory treatment to prevent ischaemia-reperfusion injury in the context of myocardial infarctions has not shown significance in translational human studies^{373-375,554}, however the application of such strategies in donor organ preservation remains to be evaluated.

9.2.2 Preservation of older or 'marginal' hearts

The success of pharmacological conditioning of hearts from brain dead rats led to studies on myocardial preservation of older or 'marginal' donor hearts. Rats aged 3, 12 and 18 months were used to represent adolescent, 30 and 45 year old human donors. Brain death and increasing age were independently associated with declining aortic flow and cardiac output per gram of body weight. After 6 hours of hypothermic storage, the recovery of function of 18-month hearts treated with EPO, GTN and ZON added to Celsior preservation solution was significantly improved compared to untreated BD or sham hearts of the same age group. These findings were in contrast to studies described by Boengler et al³⁸³ where ischaemic preconditioning of aged mouse hearts failed to provide myocardial protection, Ebrahim et al³⁹⁶, where ischaemic preconditioning only provided weak conditioning of older, hypertensive rat hearts, and Schulman et al³⁹⁷, who demonstrated that stronger conditioning stimuli were required to produce myocardial preservation of aging hearts. The increased vulnerability of older hearts to IRI has been described as an increased tendency to cytosolic calcium accumulation during ischaemia, reduced expression of extracellular ligands, impaired signaling responses and increased activity of phosphatases³⁸¹⁻³⁸⁴. The synergistic effect of triple therapy with EPO, GTN and ZON, through activation of multiple signaling cascades, has been demonstrated in the study described in this thesis to provide an adequately strong stimulus to conditioning hearts from brain dead, older animals from IRI.

Supporting data for improved myocardial preservation of older hearts in this study are reduced levels of LDH and myocardial oedema. Mechanisms of cardioprotection were indicated by increased phosphorylation of ERK1/2 and Akt signaling proteins which are key components of the RISK pathway. It has therefore been demonstrated that the RISK pathway can be activated in aged hearts by pharmacological conditioning. Extending the animal age to 24 or 30 months (equivalent to 60 and 75 human years) was also considered. Due to large costs associated with aging small animal colonies, the study was not extended to cover ages beyond 18 months. The proof of concept of the ability to condition older hearts from brain dead hearts has been demonstrated by the use of GTN, EPO and ZON added to Celsior during cardioplegia and hypothermic storage.

9.2.3 Sildenafil as a conditioning agent

This study evaluated the ability of sildenafil to condition rat hearts, either used as single therapy, double therapy with EPO or GTN, or triple therapy during prolonged hypothermic storage for 10 hours with EPO and GTN. Animals were not subjected to brain death in order to compare directly to historical controls used as a gold standard (hearts stored with EPO, GTN and a NHE-I zonisipride)³²⁹. The optimal dose of sildenafil was obtained from published data on an isolated working rat heart study on myocardial preservation³³¹. Sildenafil used singly did not improve the recovery of aortic flow or cardiac output, however double therapy of sildenafil + EPO significantly improved the recovery of aortic flow, but not cardiac output. Synergism of pharmacological agents was demonstrated with these results, with supporting data of reduced LDH release at 30 minutes into reperfusion in this group.

Of note, double therapy with sildenafil + GTN trended to have worse recovery of aortic flow and cardiac output (although not statistically significant), compared to the use of sildenafil alone, but had significantly lower recovery of aortic flow compared with sildenafil + EPO combination. The reason for poorer cardiac function is most likely due to the oversupply of nitric oxide from i) the exogenous supply of NO by GTN and ii) by increasing endogenous availability of NO due to the inhibition of its metabolism sildenafil. Hence the optimal concentration of NO was exceeded and may have led to myocardial relaxation or impaired contractility. The additive effects of increasing NO bioavailability thereby leading to sustained vasodilation and systemic hypotension is one of the reasons why sildenafil and glyceryl trinitrate are rarely administered together systemically.

The narrow therapeutic windows of protective effects of pharmacological agents have also been demonstrated in previous studies, with doses over the optimal level being ineffective or causing adverse events. For example, a single centre study by Ludman et al demonstrated that high bolus doses (50,000 IU) of EPO administered intravenously prior to and after percutaneous coronary intervention failed to reduce myocardial infarction size, but caused microvascular obstruction and increased left ventricular dilatation²⁷⁵. Similarly, the REVEAL study demonstrated that a single dose of 60,000 IU of EPO administered within 4 hours of the onset of acute myocardial infarction failed to reduce infarct size, and was associated with more adverse events²⁷⁶. In contrast, lower doses of EPO (12,000 or 33,000 IU) were shown to reduce myocardial infarct size and increase left ventricular ejection fraction^{271,272}.

After 10 hours of hypothermic storage, the recovery of hearts stored with sildenafil, double therapy with sildenafil + EPO or triple therapy with sildenafil + EPO + GTN had mean recovery of cardiac output close to 20%, with no significant differences between groups. Overall sildenafil was demonstrated to have some cardioprotective properties, with synergistic effects when used in combination with EPO. Combination therapy has been shown to provide superior myocardial protection, hence the efficacy of sildenafil and EPO may be enhanced with the addition of an appropriate third agent such as a mitochondrial permeability transition pore blocker (eg. cyclosporine), or a strong activator of the SAFE pathway, however these remain to be evaluated in future studies.

9.2.4 Conditioning human donor hearts with EPO and GTN

The experience of prior translational studies has led to an understanding that preclinical studies should demonstrate consistent results across several animal species, and ideally with results obtained from more than one laboratory, prior to application in human studies⁵¹. The lack of significance of translational studies has been attributed in part to the heterogeneity of receptor subtypes across species. . Due to consistent findings of superior recovery of the donor heart when applied in rodent^{320,325,329,366,367} and porcine^{321,326,330} studies, including brain death and recently in aging (Chapter 4), this strategy was applied in human cardiac transplantation.

Despite a higher numbers of older donors (> 50 years), and an increased number of higher risk recipients, pharmacological conditioning with EPO and GTN allowed comparable, if not reduced use of mechanical circulatory support post-transplantation, and excellent operative and one-year survival. These findings highlight the safety of pharmacological conditioning, and show promise in the ability to condition human hearts in context of cardiac transplantation. The translational study excluded an NHE-inhibitor due to clinical inaccessibility, and it may be possible that more significant reduction of MCS use and improved patient survival may have been demonstrated with triple therapy. Survival advantages may have occurred due to an 'era effect' when comparing pharmacological conditioning with historic controls, however the use of MCS post-transplantation has not reduced over the years, hence it is likely that pharmacological conditioning would have led to the improved recovery of transplanted human hearts.

The translation of preclinical studies in this human study has shown encouraging results. Based on these results, further studies on pharmacological conditioning of the donor heart are warranted, and should ideally occur in large, randomized multicenter trials in order to ensure adequate patient numbers allow matching of donor, procedural and recipient risk factors for primary graft dysfunction. This would be a formidable undertaking. For example, a study of approximately 400 recipients (200 in each group) would have 80% power to demonstrate a statistically significant reduction (at a significance level of 5%) in the incidence of severe PGD (and the need for mechanical circulatory support post transplant) from 20% to 10%.

9.3 Conclusions and future directions

Results of studies described in this thesis support the following:

- Pharmacological conditioning with erythropoietin, glyceryl trinitrate and zoniporide triple therapy applied during cardioplegia and hypothermic storage provides superior myocardial preservation of rat hearts subjected to brain death and hypothermic storage.
- Pharmacological conditioning with erythropoietin, glyceryl trinitrate and zoniporide provides superior myocardial preservation of older hearts subjected to brain death and hypothermic storage.
- Sildenafil provides modest conditioning used singly, with synergistic effects when used as double therapy in combination with erythropoietin in myocardial preservation of rat hearts subjected to hypothermic storage.
- Sildenafil used in combination with glyceryl trinitrate for conditioning of the donor heart, is associated with poor recovery of cardiac contractility.
- Pharmacological conditioning of human donor hearts with erythropoietin and glyceryl trinitrate used in cardioplegia and hypothermic storage allows high patient survival

post-transplantation despite the use of older donor hearts and higher risk transplant recipients, however the requirement for mechanical circulatory support remains high.

- Cardiac magnetic resonance imaging allows superior quantitative assessment of rat heart volumes, mass and ejection fraction compared to echocardiography.

The recent emergence of normothermic ex vivo machine perfusion (NEVP) machine ie OCS as an alternative to cold storage Normothermic ex vivo perfusion has found to produce equivalent outcomes to cold storage in low risk brain dead donors (Proceed II trial Lancet 2015). Uncontrolled clinical studies suggest favourable outcomes when OCS is used in higher risk donors and recipients but as with our pharmacological conditioning, further randomized controlled trials are needed. Finally pharmacological conditioning and EVP are not mutually exclusive. A complementary strategy combining pharmacological conditioning with NEVP has already been applied in human DCD heart transplantation.

Further animal studies are required to evaluate the conditioning properties of other pharmacological agents accessible for clinical use. These agents should be tested in single, double and triple therapy combinations, given clear demonstration of synergism or additive effects of myocardial preservation by targeting multiple potential signalling and protective pathways. The use of screening models can be advanced to allow closer resemblance to the clinical situation by using models of brain death and heterotopic heart transplantation, with longitudinal assessment using non-invasive imaging such as cardiac magnetic resonance imaging. The ability to attenuate ischaemia-reperfusion injury in older donor hearts by using pharmacological conditioning shows promise in allowing improved utility of the available donor pool. Large, multicentre, prospective, randomised controlled trials are required to evaluate pharmacological conditioning strategies in human heart transplantation.

REFERENCES

- 1 Shumway NE, Stinson EB. Two decades of experimental and clinical orthotopic homotransplantation of the heart. *Perspect Biol Med* 1979; 22:S81-S88
- 2 Cooper DK. Experimental development of cardiac transplantation. *Br Med J* 1968; 4:174-181
- 3 Barnard CN. The operation. A human cardiac transplant: an interim report of a successful operation performed at Groote Schuur Hospital, Cape Town. *S Afr Med J* 1967; 41:1271-1274
- 4 Lund LH, Edwards LB, Kucheryavaya AY, et al. The Registry of the International Society for Heart and Lung Transplantation: Thirty-first Official Adult Heart Transplant Report-2014; Focus Theme: Retransplantation. *J Heart Lung Transplant* 2014; 33:996-1008
- 5 Hunt SA, Haddad F. The changing face of heart transplantation. *J Am Coll Cardiol* 2008; 52:587-598
- 6 Lund LH, Edwards LB, Kucheryavaya AY, et al. The Registry of the International Society for Heart and Lung Transplantation: Thirtieth Official Adult Heart Transplant Report--2013; focus theme: age. *J Heart Lung Transplant* 2013; 32:951-964
- 7 John R, Lietz K, Schuster M, et al. Immunologic sensitization in recipients of left ventricular assist devices. *J Thorac Cardiovasc Surg* 2003; 125:578-591
- 8 Kerman RH. Understanding the sensitized patient. *Heart Fail Clin* 2007; 3:1-9
- 9 Khan MS, Mery CM, Zafar F, et al. Is mechanically bridging patients with a failing cardiac graft to retransplantation an effective therapy? Analysis of the United Network of Organ Sharing database. *J Heart Lung Transplant* 2012; 31:1192-1198
- 10 Zaroff JG, Rosengard BR, Armstrong WF, et al. Consensus conference report: maximizing use of organs recovered from the cadaver donor: cardiac recommendations, March 28-29, 2001, Crystal City, Va. *Circulation* 2002; 106:836-841
- 11 Wolfe RA, Merion RM, Roys EC, et al. Trends in organ donation and transplantation in the United States, 1998-2007. *Am J Transplant* 2009; 9:869-878
- 12 Schumer EM, Ising MS, Trivedi JR, et al. Early Outcomes With Marginal Donor Hearts Compared With Left Ventricular Assist Device Support in Patients With Advanced Heart Failure. *Ann Thorac Surg* 2015; 100:522-527

- 13 Maurer MS, Raina A, Hesdorffer C, et al. Cardiac transplantation using extended-donor criteria organs for systemic amyloidosis complicated by heart failure. *Transplantation* 2007; 83:539-545
- 14 Stehlik J, Edwards LB, Kucheryavaya AY, et al. The Registry of the International Society for Heart and Lung Transplantation: twenty-seventh official adult heart transplant report--2010. *J Heart Lung Transplant* 2010; 29:1089-1103
- 15 Chen JM, Russo MJ, Hammond KM, et al. Alternate waiting list strategies for heart transplantation maximize donor organ utilization. *Ann Thorac Surg* 2005; 80:224-228
- 16 Lima B, Rajagopal K, Petersen RP, et al. Marginal cardiac allografts do not have increased primary graft dysfunction in alternate list transplantation. *Circulation* 2006; 114:127-32
- 17 Schulze PC, Jiang J, Yang J, et al. Preoperative assessment of high-risk candidates to predict survival after heart transplantation. *Circ Heart Fail* 2013; 6:527-534
- 18 Rose EA, Gelijns AC, Moskowitz AJ, et al. Long-term use of a left ventricular assist device for end-stage heart failure. *N Engl J Med* 2001; 345:1435-1443
- 19 Hunt SA. Mechanical circulatory support: new data, old problems. *Circulation* 2007; 116:461-462
- 20 Patel CB, Cowger JA, Zuckermann A. A contemporary review of mechanical circulatory support. *J Heart Lung Transplant* 2014; 33:667-674
- 21 Miyagawa S, Toda K, Nakamura T, et al. Building a bridge to recovery: the pathophysiology of LVAD-induced reverse modeling in heart failure. *Surg Today* 2015
- 22 Halbreiner MS, Cruz V, Starling R, et al. Myocardial recovery: a focus on the impact of left ventricular assist devices. *Expert Rev Cardiovasc Ther* 2014; 12:589-600
- 23 Sievers HH, Weyand M, Kraatz EG, et al. An alternative technique for orthotopic cardiac transplantation, with preservation of the normal anatomy of the right atrium. *Thorac Cardiovasc Surg* 1991; 39:70-72
- 24 Schnoor M, Schafer T, Luhmann D, et al. Bicaval versus standard technique in orthotopic heart transplantation: a systematic review and meta-analysis. *J Thorac Cardiovasc Surg* 2007; 134:1322-1331
- 25 Kobashigawa J, Zuckermann A, Macdonald P, et al. Report from a consensus conference on primary graft dysfunction after cardiac transplantation. *J Heart Lung Transplant* 2014; 33:327-340
- 26 Jahania MS, Mullett TW, Sanchez JA, et al. Acute allograft failure in thoracic organ transplantation. *J Card Surg* 2000; 15:122-128

- 27 Beiras-Fernandez A, Kaczmarek I, Weis F. Primary graft failure after heart transplantation: urgent need for a consensus guideline. *Transplantation* 2011; 91:e31; author reply e31-32
- 28 Segovia J, Cosio MD, Barcelo JM, et al. RADIAL: a novel primary graft failure risk score in heart transplantation. *J Heart Lung Transplant* 2011; 30:644-651
- 29 Fujino T, Kinugawa K, Nitta D, et al. Donor age is a predictor of early low output after heart transplantation. *J Cardiol* 2015
- 30 D'Alessandro C, Golmard JL, Barreda E, et al. Predictive risk factors for primary graft failure requiring temporary extra-corporeal membrane oxygenation support after cardiac transplantation in adults. *Eur J Cardiothorac Surg* 2011; 40:962-969
- 31 Chen JW, Chen YS, Chi NH, et al. Risk factors and prognosis of patients with primary graft failure after heart transplantation: an Asian center experience. *Transplant Proc* 2014; 46:914-919
- 32 Hong KN, Iribarne A, Worku B, et al. Who is the high-risk recipient? Predicting mortality after heart transplant using pretransplant donor and recipient risk factors. *Ann Thorac Surg* 2011; 92:520-527; discussion 527
- 33 Russo MJ, Iribarne A, Hong KN, et al. Factors associated with primary graft failure after heart transplantation. *Transplantation* 2010; 90:444-450
- 34 Cosio Carmena MD, Gomez Bueno M, Almenar L, et al. Primary graft failure after heart transplantation: characteristics in a contemporary cohort and performance of the RADIAL risk score. *J Heart Lung Transplant* 2013; 32:1187-1195
- 35 Russo MJ, Chen JM, Sorabella RA, et al. The effect of ischemic time on survival after heart transplantation varies by donor age: an analysis of the United Network for Organ Sharing database. *J Thorac Cardiovasc Surg* 2007; 133:554-559
- 36 Santise G, Panarello G, Ruperto C, et al. Extracorporeal membrane oxygenation for graft failure after heart transplantation: a multidisciplinary approach to maximize weaning rate. *Int J Artif Organs* 2014; 37:706-714
- 37 Valantine H. Cardiac allograft vasculopathy after heart transplantation: risk factors and management. *J Heart Lung Transplant* 2004; 23:S187-193
- 38 Rahmani M, Cruz RP, Granville DJ, et al. Allograft vasculopathy versus atherosclerosis. *Circ Res* 2006; 99:801-815
- 39 Mitchell RN, Libby P. Vascular remodeling in transplant vasculopathy. *Circ Res* 2007; 100:967-978

- 40 Gao SZ, Alderman EL, Schroeder JS, et al. Progressive coronary luminal narrowing after cardiac transplantation. *Circulation* 1990; 82:IV269-275
- 41 Valantine HA. The role of viruses in cardiac allograft vasculopathy. *Am J Transplant* 2004; 4:169-177
- 42 Sipahi I, Starling RC. Cardiac allograft vasculopathy: an update. *Heart Fail Clin* 2007; 3:87-95
- 43 Costanzo MR, Naftel DC, Pritzker MR, et al. Heart transplant coronary artery disease detected by coronary angiography: a multiinstitutional study of preoperative donor and recipient risk factors. *Cardiac Transplant Research Database. J Heart Lung Transplant* 1998; 17:744-753
- 44 Schmauss D, Weis M. Cardiac allograft vasculopathy: recent developments. *Circulation* 2008; 117:2131-2141
- 45 Mulivor AW, Lipowsky HH. Inflammation- and ischemia-induced shedding of venular glycocalyx. *Am J Physiol Heart Circ Physiol* 2004; 286:H1672-1680
- 46 Magee JC, Barr ML, Basadonna GP, et al. Repeat organ transplantation in the United States, 1996-2005. *Am J Transplant* 2007; 7:1424-1433
- 47 Johnson MR, Aaronson KD, Canter CE, et al. Heart retransplantation. *Am J Transplant* 2007; 7:2075-2081
- 48 Jennings RB, Sommers HM, Smyth GA, et al. Myocardial Necrosis Induced by Temporary Occlusion of a Coronary Artery in the Dog. *Archives of Pathology* 1960; 70:68-78
- 49 Reimer KA, Lowe JE, Rasmussen MM, et al. The wavefront phenomenon of ischemic cell death. 1. Myocardial infarct size vs duration of coronary occlusion in dogs. *Circulation* 1977; 56:786-794
- 50 Braunwald E, Kloner RA. Myocardial reperfusion: a double-edged sword? *J Clin Invest* 1985; 76:1713-1719
- 51 Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. *N Engl J Med* 2007; 357:1121-1135
- 52 Yellon DM. Myocardial reperfusion and reperfusion injury: current views. *Bratisl Lek Listy* 1991; 92:66-76
- 53 Hausenloy DJ, Yellon DM. Myocardial ischemia-reperfusion injury: a neglected therapeutic target. *J Clin Invest* 2013; 123:92-100
- 54 Bulkely BH, Hutchins GM. Myocardial consequences of coronary artery bypass graft surgery. The paradox of necrosis in areas of revascularization. *Circulation* 1977; 56:906-913

- 55 Hicks M, Hing A, Gao L, et al. Organ preservation. *Methods Mol Biol* 2006; 333:331-374
- 56 Wilhelm MJ, Pratschke J, Laskowski IA, et al. Brain death and its impact on the donor heart-lessons from animal models. *J Heart Lung Transplant* 2000; 19:414-418
- 57 Westendorp WH, Leuvenink HG, Ploeg RJ. Brain death induced renal injury. *Curr Opin Organ Transplant* 2011; 16:151-156
- 58 Szabo G. Physiologic changes after brain death. *J Heart Lung Transplant* 2004; 23:S223-226
- 59 Shivalkar B, Van Loon J, Wieland W, et al. Variable effects of explosive or gradual increase of intracranial pressure on myocardial structure and function. *Circulation* 1993; 87:230-239
- 60 Rosner MJ, Newsome HH, Becker DP. Mechanical brain injury: the sympathoadrenal response. *J Neurosurg* 1984; 61:76-86
- 61 Beckman DL, Iams SG. Circulating catecholamines in cats before and after lethal head injury. *Proc Soc Exp Biol Med* 1979; 160:200-202
- 62 Cooper DK, Novitzky D, Wicomb WN. The pathophysiological effects of brain death on potential donor organs, with particular reference to the heart. *Ann R Coll Surg Engl* 1989; 71:261-266
- 63 Baroldi G, Di Pasquale G, Silver MD, et al. Type and extent of myocardial injury related to brain damage and its significance in heart transplantation: a morphometric study. *J Heart Lung Transplant* 1997; 16:994-1000
- 64 Rona G. Catecholamine cardiotoxicity. *J Mol Cell Cardiol* 1985; 17:291-306
- 65 Halejcio-Delophont P, Siaghy EM, Devaux Y, et al. Increase in myocardial interstitial adenosine and net lactate production in brain-dead pigs: an in vivo microdialysis study. *Transplantation* 1998; 66:1278-1284
- 66 Ryan JB, Hicks M, Cropper JR, et al. Functional evidence of reversible ischemic injury immediately after the sympathetic storm associated with experimental brain death. *J Heart Lung Transplant* 2003; 22:922-928
- 67 DePasquale NP, Burch GE. How normal is the donor heart? *Am Heart J* 1969; 77:719-720
- 68 Takada M, Nadeau KC, Hancock WW, et al. Effects of explosive brain death on cytokine activation of peripheral organs in the rat. *Transplantation* 1998; 65:1533-1542

- 69 Venkateswaran RV, Dronavalli V, Lambert PA, et al. The proinflammatory environment in potential heart and lung donors: prevalence and impact of donor management and hormonal therapy. *Transplantation* 2009; 88:582-588
- 70 Atkinson C, Floerchinger B, Qiao F, et al. Donor brain death exacerbates complement-dependent ischemia/reperfusion injury in transplanted hearts. *Circulation* 2013; 127:1290-1299
- 71 Birks EJ, Burton PB, Owen V, et al. Elevated tumor necrosis factor-alpha and interleukin-6 in myocardium and serum of malfunctioning donor hearts. *Circulation* 2000; 102:III352-358
- 72 Birks EJ, Yacoub MH, Burton PS, et al. Activation of apoptotic and inflammatory pathways in dysfunctional donor hearts. *Transplantation* 2000; 70:1498-1506
- 73 Macoviak JA, McDougall IR, Bayer MF, et al. Significance of thyroid dysfunction in human cardiac allograft procurement. *Transplantation* 1987; 43:824-826
- 74 Lopau K, Mark J, Schramm L, et al. Hormonal changes in brain death and immune activation in the donor. *Transpl Int* 2000; 13 Suppl 1:S282-285
- 75 Novitzky D, Cooper DKC. Thyroid hormone and the stunned myocardium. *Journal of Endocrinology* 2014; 223:R1-R8
- 76 Dimopoulou I, Tsagarakis S, Anthi A, et al. High prevalence of decreased cortisol reserve in brain-dead potential organ donors. *Crit Care Med* 2003; 31:1113-1117
- 77 Apostolakis E, Parissis H, Dougenis D. Brain death and donor heart dysfunction: implications in cardiac transplantation. *J Card Surg* 2010; 25:98-106
- 78 Naveri L, Naveri H, Harkonen M. Myocardial energy metabolism. *Ann Chir Gynaecol* 1987; 76:3-11
- 79 Suga H. Ventricular energetics. *Physiol Rev* 1990; 70:247-277
- 80 Liedtke AJ. Alterations of carbohydrate and lipid metabolism in the acutely ischemic heart. *Prog Cardiovasc Dis* 1981; 23:321-336
- 81 Katz AM, Messineo FC. Lipid-membrane interactions and the pathogenesis of ischemic damage in the myocardium. *Circ Res* 1981; 48:1-16
- 82 McCord JM. Oxygen-derived free radicals in postischemic tissue injury. *N Engl J Med* 1985; 312:159-163
- 83 Wildhirt SM, Weis M, Schulze C, et al. Myocardial preservation in clinical cardiac transplantation. *Transplant Proc* 1999; 31:147-148
- 84 Southard JH, Belzer FO. Organ preservation. *Annu Rev Med* 1995; 46:235-247

- 85 Buckberg GD. Myocardial temperature management during aortic clamping for cardiac surgery. Protection, preoccupation, and perspective. *J Thorac Cardiovasc Surg* 1991; 102:895-903
- 86 King LM, Opie LH. Glucose and glycogen utilisation in myocardial ischemia--changes in metabolism and consequences for the myocyte. *Mol Cell Biochem* 1998; 180:3-26
- 87 Lazdunski M, Frelin C, Vigne P. The sodium/hydrogen exchange system in cardiac cells: its biochemical and pharmacological properties and its role in regulating internal concentrations of sodium and internal pH. *J Mol Cell Cardiol* 1985; 17:1029-1042
- 88 Murphy E, Cross H, Steenbergen C. Sodium regulation during ischemia versus reperfusion and its role in injury. *Circ Res* 1999; 84:1469-1470
- 89 Frolich O, Karmazyn M. The Na-H exchanger revisited: an update on Na-H exchange regulation and the role of the exchanger in hypertension and cardiac function in health and disease. *Cardiovasc Res* 1997; 36:138-148
- 90 Cross HR, Radda GK, Clarke K. The role of Na⁺/K⁺ ATPase activity during low flow ischemia in preventing myocardial injury: a ³¹P, ²³Na and ⁸⁷Rb NMR spectroscopic study. *Magn Reson Med* 1995; 34:673-685
- 91 Schmidt TA, Kjeldsen K. Human myocardial Na,K-ATPase--quantification, regulation and relation to Ca. *Cardiovasc Res* 1998; 37:335-345
- 92 Barcenas-Ruiz L, Beuckelmann DJ, Wier WG. Sodium-calcium exchange in heart: membrane currents and changes in [Ca²⁺]_i. *Science* 1987; 238:1720-1722
- 93 Leaf A. Cell swelling. A factor in ischemic tissue injury. *Circulation* 1973; 48:455-458
- 94 Leaf A, Chatillon JY, Wrong O, et al. The mechanism of the osmotic adjustment of body cells as determined in vivo by the volume of distribution of a large water load. *J Clin Invest* 1954; 33:1261-1268
- 95 Dong Z, Saikumar P, Weinberg JM, et al. Calcium in cell injury and death. *Annu Rev Pathol* 2006; 1:405-434
- 96 Kalogeris T, Baines CP, Krenz M, et al. Cell biology of ischemia/reperfusion injury. *Int Rev Cell Mol Biol* 2012; 298:229-317
- 97 Braunwald E, Kloner RA. The stunned myocardium: prolonged, postischemic ventricular dysfunction. *Circulation* 1982; 66:1146-1149
- 98 Bolli R, Marban E. Molecular and cellular mechanisms of myocardial stunning. *Physiol Rev* 1999; 79:609-634

- 99 Ito H. No-reflow phenomenon and prognosis in patients with acute myocardial infarction. *Nat Clin Pract Cardiovasc Med* 2006; 3:499-506
- 100 Krug A, Du Mesnil de R, Korb G. Blood supply of the myocardium after temporary coronary occlusion. *Circ Res* 1966; 19:57-62
- 101 Manning AS, Hearse DJ. Reperfusion-induced arrhythmias: mechanisms and prevention. *J Mol Cell Cardiol* 1984; 16:497-518
- 102 Park JL, Lucchesi BR. Mechanisms of myocardial reperfusion injury. *Ann Thorac Surg* 1999; 68:1905-1912
- 103 Granger DN. Ischemia-reperfusion: mechanisms of microvascular dysfunction and the influence of risk factors for cardiovascular disease. *Microcirculation* 1999; 6:167-178
- 104 Pomblum VJ, Korbmacher B, Cleveland S, et al. State-of-the-art - Cardiac general Cardiac stunning in the clinic: the full picture. *Interactive Cardiovascular and Thoracic Surgery* 2010; 10:86-91
- 105 Manning AS. Reperfusion-induced arrhythmias: do free radicals play a critical role? *Free Radic Biol Med* 1988; 4:305-316
- 106 Shen AC, Jennings RB. Myocardial calcium and magnesium in acute ischemic injury. *Am J Pathol* 1972; 67:417-440
- 107 Zweier JL, Talukder MA. The role of oxidants and free radicals in reperfusion injury. *Cardiovasc Res* 2006; 70:181-190
- 108 Zweier JL, Flaherty JT, Weisfeldt ML. Direct measurement of free radical generation following reperfusion of ischemic myocardium. *Proc Natl Acad Sci U S A* 1987; 84:1404-1407
- 109 Zweier JL. Measurement of superoxide-derived free radicals in the reperfused heart. Evidence for a free radical mechanism of reperfusion injury. *J Biol Chem* 1988; 263:1353-1357
- 110 Hearse DJ, Humphrey SM, Chain EB. Abrupt reoxygenation of the anoxic potassium-arrested perfused rat heart: a study of myocardial enzyme release. *J Mol Cell Cardiol* 1973; 5:395-407
- 111 Halliwell B. Free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis. *Br J Exp Pathol* 1989; 70:737-757
- 112 Halliwell B. Superoxide, iron, vascular endothelium and reperfusion injury. *Free Radic Res Commun* 1989; 5:315-318
- 113 Turrens JF. Mitochondrial formation of reactive oxygen species. *J Physiol* 2003; 552:335-344

- 114 Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic Biol Med* 2000; 29:222-230
- 115 Kelley EE, Khoo NK, Hundley NJ, et al. Hydrogen peroxide is the major oxidant product of xanthine oxidase. *Free Radic Biol Med* 2010; 48:493-498
- 116 Stuehr D, Pou S, Rosen GM. Oxygen reduction by nitric-oxide synthases. *J Biol Chem* 2001; 276:14533-14536
- 117 Koppenol WH. The basic chemistry of nitrogen monoxide and peroxynitrite. *Free Radic Biol Med* 1998; 25:385-391
- 118 Huie RE, Padmaja S. The reaction of no with superoxide. *Free Radic Res Commun* 1993; 18:195-199
- 119 Radomski MW, Palmer RM, Moncada S. Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet* 1987; 2:1057-1058
- 120 Lefer AM. Nitric oxide: nature's naturally occurring leukocyte inhibitor. *Circulation* 1997; 95:553-554
- 121 Carafoli E, Santella L, Branca D, et al. Generation, control, and processing of cellular calcium signals. *Crit Rev Biochem Mol Biol* 2001; 36:107-260
- 122 Berridge MJ, Bootman MD, Roderick HL. Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol* 2003; 4:517-529
- 123 Schanne FA, Kane AB, Young EE, et al. Calcium dependence of toxic cell death: a final common pathway. *Science* 1979; 206:700-702
- 124 Dipolo R, Requena J, Brinley FJ, Jr., et al. Ionized calcium concentrations in squid axons. *J Gen Physiol* 1976; 67:433-467
- 125 Silverman HS. Mitochondrial free calcium regulation in hypoxia and reoxygenation: relation to cellular injury. *Basic Res Cardiol* 1993; 88:483-494
- 126 Bers DM. Calcium fluxes involved in control of cardiac myocyte contraction. *Circ Res* 2000; 87:275-281
- 127 Piper HM, Garcia-Dorado D, Ovize M. A fresh look at reperfusion injury. *Cardiovascular Research* 1998; 38:291-300
- 128 Shen AC, Jennings RB. Kinetics of calcium accumulation in acute myocardial ischemic injury. *Am J Pathol* 1972; 67:441-452
- 129 Crompton M, Costi A. Kinetic evidence for a heart mitochondrial pore activated by Ca^{2+} , inorganic phosphate and oxidative stress. A potential mechanism for mitochondrial dysfunction during cellular Ca^{2+} overload. *Eur J Biochem* 1988; 178:489-501

- 130 Yamashima T. Implication of cysteine proteases calpain, cathepsin and caspase in ischemic neuronal death of primates. *Prog Neurobiol* 2000; 62:273-295
- 131 Syntichaki P, Tavernarakis N. Death by necrosis. Uncontrollable catastrophe, or is there order behind the chaos? *EMBO Rep* 2002; 3:604-609
- 132 Inserre J, Garcia-Dorado D, Hernando V, et al. Calpain-mediated impairment of Na⁺/K⁺-ATPase activity during early reperfusion contributes to cell death after myocardial ischemia. *Circ Res* 2005; 97:465-473
- 133 Chen M, Won DJ, Krajewski S, et al. Calpain and mitochondria in ischemia/reperfusion injury. *J Biol Chem* 2002; 277:29181-29186
- 134 Norberg E, Gogvadze V, Ott M, et al. An increase in intracellular Ca²⁺ is required for the activation of mitochondrial calpain to release AIF during cell death. *Cell Death Differ* 2008; 15:1857-1864
- 135 Ferri KF, Kroemer G. Organelle-specific initiation of cell death pathways. *Nat Cell Biol* 2001; 3:E255-263
- 136 Crompton M. The mitochondrial permeability transition pore and its role in cell death. *Biochem J* 1999; 341 (Pt 2):233-249
- 137 Ong SB, Samangouei P, Kalkhoran SB, et al. The mitochondrial permeability transition pore and its role in myocardial ischemia reperfusion injury. *J Mol Cell Cardiol* 2015; 78C:23-34
- 138 Hausenloy DJ, Yellon DM. The mitochondrial permeability transition pore: its fundamental role in mediating cell death during ischaemia and reperfusion. *J Mol Cell Cardiol* 2003; 35:339-341
- 139 Halestrap AP. The mitochondrial permeability transition: its molecular mechanism and role in reperfusion injury. *Biochem Soc Symp* 1999; 66:181-203
- 140 Di Lisa F, Canton M, Menabo R, et al. Mitochondria and reperfusion injury. The role of permeability transition. *Basic Res Cardiol* 2003; 98:235-241
- 141 Lemasters JJ, Bond JM, Chacon E, et al. The pH paradox in ischemia-reperfusion injury to cardiac myocytes. *EXS* 1996; 76:99-114
- 142 Bond JM, Herman B, Lemasters JJ. Protection by acidotic pH against anoxia/reoxygenation injury to rat neonatal cardiac myocytes. *Biochem Biophys Res Commun* 1991; 179:798-803
- 143 Kim JS, Jin Y, Lemasters JJ. Reactive oxygen species, but not Ca²⁺ overloading, trigger pH- and mitochondrial permeability transition-dependent death of adult rat myocytes after ischemia-reperfusion. *Am J Physiol Heart Circ Physiol* 2006; 290:H2024-2034

- 144 Crompton M. Mitochondrial intermembrane junctional complexes and their role in cell death. *J Physiol* 2000; 529 Pt 1:11-21
- 145 Armstrong JM, Dusting GJ, Moncada S, et al. Cardiovascular Actions of Prostacyclin (Pgi₂), a Metabolite of Arachidonic-Acid Which Is Synthesized by Blood-Vessels. *Circulation Research* 1978; 43:I112-I119
- 146 Loscalzo J, Welch G. Nitric oxide and its role in the cardiovascular system. *Prog Cardiovasc Dis* 1995; 38:87-104
- 147 Lefer AM, Lefer DJ. The role of nitric oxide and cell adhesion molecules on the microcirculation in ischaemia-reperfusion. *Cardiovasc Res* 1996; 32:743-751
- 148 Tsao PS, Aoki N, Lefer DJ, et al. Time course of endothelial dysfunction and myocardial injury during myocardial ischemia and reperfusion in the cat. *Circulation* 1990; 82:1402-1412
- 149 Rezkalla SH, Kloner RA. No-reflow phenomenon. *Circulation* 2002; 105:656-662
- 150 Belperio JA, Ardehali A. Chemokines and transplant vasculopathy. *Circ Res* 2008; 103:454-466
- 151 Al-Lamki RS, Bradley JR, Pober JS. Endothelial cells in allograft rejection. *Transplantation* 2008; 86:1340-1348
- 152 Haack DW, Bush LR, Schlafer M, et al. Lanthanum staining of coronary microvascular endothelium: effects of ischemia reperfusion, propranolol, and atenolol. *Microvasc Res* 1981; 21:362-376
- 153 Rix DA, Douglas MS, Talbot D, et al. Role of glycosaminoglycans (GAGs) in regulation of the immunogenicity of human vascular endothelial cells. *Clin Exp Immunol* 1996; 104:60-65
- 154 Henry CB, Duling BR. Permeation of the luminal capillary glycocalyx is determined by hyaluronan. *Am J Physiol* 1999; 277:H508-514
- 155 Hu X, Weinbaum S. A new view of Starling's hypothesis at the microstructural level. *Microvasc Res* 1999; 58:281-304
- 156 Rubio R, Ceballos G. Role of the endothelial glycocalyx in dromotropic, inotropic, and arrhythmogenic effects of coronary flow. *Am J Physiol Heart Circ Physiol* 2000; 278:H106-116
- 157 Rubio-Gayosso I, Platts SH, Duling BR. Reactive oxygen species mediate modification of glycocalyx during ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* 2006; 290:H2247-2256
- 158 Stringham JC, Southard JH, Hegge J, et al. Limitations of heart preservation by cold storage. *Transplantation* 1992; 53:287-294

- 159 Garlicki M, Kolcz J, Rudzinski P, et al. Myocardial protection for transplantation. *Transplant Proc* 1999; 31:2079-2083
- 160 Novitzky D, Cooper DK, Rosendale JD, et al. Hormonal therapy of the brain-dead organ donor: experimental and clinical studies. *Transplantation* 2006; 82:1396-1401
- 161 Mariot J, Jacob F, Voltz C, et al. [Value of hormonal treatment with triiodothyronine and cortisone in brain dead patients]. *Ann Fr Anesth Reanim* 1991; 10:321-328
- 162 Venkateswaran RV, Steeds RP, Quinn DW, et al. The haemodynamic effects of adjunctive hormone therapy in potential heart donors: a prospective randomized double-blind factorially designed controlled trial. *Eur Heart J* 2009; 30:1771-1780
- 163 Pennefather SH, Bullock RE, Mantle D, et al. Use of low dose arginine vasopressin to support brain-dead organ donors. *Transplantation* 1995; 59:58-62
- 164 Yoshioka T, Sugimoto H, Uenishi M, et al. Prolonged hemodynamic maintenance by the combined administration of vasopressin and epinephrine in brain death: a clinical study. *Neurosurgery* 1986; 18:565-567
- 165 Macdonald PS, Aneman A, Bhonagiri D, et al. A systematic review and meta-analysis of clinical trials of thyroid hormone administration to brain dead potential organ donors. *Crit Care Med* 2012; 40:1635-1644
- 166 Hearse DJ. Cardioplegia: the protection of the myocardium during open heart surgery: a review. *J Physiol (Paris)* 1980; 76:751-768
- 167 Nelson RL, Fey KH, Follette DM, et al. Intermittent infusion of cardioplegic solution during aortic cross-clamping. *Surg Forum* 1976; 27:241-243
- 168 Hearse DJ, Stewart DA, Braimbridge MV. Cellular protection during myocardial ischemia: the development and characterization of a procedure for the induction of reversible ischemic arrest. *Circulation* 1976; 54:193-202
- 169 Lowe JE, Kleinman LH, Reimer KA, et al. Effects of cardioplegia produced by calcium flux inhibition. *Surg Forum* 1977; 28:279-280
- 170 Wheeldon D, Sharples L, Wallwork J, et al. Donor heart preservation survey. *J Heart Lung Transplant* 1992; 11:986-993
- 171 Chambers DJ. Mechanisms and alternative methods of achieving cardiac arrest. *Ann Thorac Surg* 2003; 75:S661-666
- 172 Fallouh HB, Kentish JC, Chambers DJ. Targeting for cardioplegia: arresting agents and their safety. *Curr Opin Pharmacol* 2009; 9:220-226

- 173 Ploeg RJ, Goossens D, Vreugdenhil P, et al. Successful 72-hour cold storage kidney preservation with UW solution. *Transplant Proc* 1988; 20:935-938
- 174 Squifflet JP, Pirson Y, Gianello P, et al. Safe Preservation of Human Renal Cadaver Transplants by Euro-Collins Solution up to 50 Hours. *Transplantation Proceedings* 1981; 13:693-696
- 175 Dreikorn K, Horsch R, Rohl L. 48-Hour to 96-Hour Preservation of Canine Kidneys by Initial Perfusion and Hypothermic Storage Using the Euro-Collins Solution. *European Urology* 1980; 6:221-224
- 176 Todo S, Tzakis A, Starzl TE. Preservation of livers with UW or Euro-Collins' solution. *Transplantation* 1988; 46:925-926
- 177 Kalayoglu M, Sollinger HW, Stratta RJ, et al. Extended preservation of the liver for clinical transplantation. *Lancet* 1988; 1:617-619
- 178 Jamieson NV, Sundberg R, Lindell S, et al. Preservation of the canine liver for 24-48 hours using simple cold storage with UW solution. *Transplantation* 1988; 46:517-522
- 179 Wahlberg JA, Love R, Landegaard L, et al. Successful 72 hours' preservation of the canine pancreas. *Transplant Proc* 1987; 19:1337-1338
- 180 Stringham JC, Paulsen KL, Southard JH, et al. Prolonging myocardial preservation with a modified University of Wisconsin solution containing 2,3-butanedione monoxime and calcium. *J Thorac Cardiovasc Surg* 1994; 107:764-775
- 181 Bretschneider HJ. Myocardial protection. *Thorac Cardiovasc Surg* 1980; 28:295-302
- 182 Jynge P, Hearse DJ, Feuvray D, et al. The St. Thomas' hospital cardioplegic solution: a characterization in two species. *Scand J Thorac Cardiovasc Surg Suppl* 1981; 30:1-28
- 183 Menasche P, Termignon JL, Pradier F, et al. Experimental evaluation of Celsior, a new heart preservation solution. *Eur J Cardiothorac Surg* 1994; 8:207-213
- 184 Hatano E, Kiuchi T, Tanaka A, et al. Hepatic preservation with histidine-tryptophan-ketoglutarate solution in living-related and cadaveric liver transplantation. *Clin Sci (Lond)* 1997; 93:81-88
- 185 Mangus RS, Tector AJ, Agarwal A, et al. Comparison of histidine-tryptophan-ketoglutarate solution (HTK) and University of Wisconsin solution (UW) in adult liver transplantation. *Liver Transpl* 2006; 12:226-230
- 186 Isemer FE, Ludwig A, Schunck O, et al. Kidney procurement with the HTK solution of Bretschneider. *Transplant Proc* 1988; 20:885-886

- 187 Agarwal A, Goggins WC, Pescovitz MD, et al. Comparison of histidine-tryptophan ketoglutarate and University of Wisconsin solutions as primary preservation in renal allografts undergoing pulsatile perfusion. *Transplant Proc* 2005; 37:2016-2019
- 188 de Boer J, De Meester J, Smits JM, et al. Eurotransplant randomized multicenter kidney graft preservation study comparing HTK with UW and Euro-Collins. *Transpl Int* 1999; 12:447-453
- 189 Gubernatis G, Pichlmayr R, Lamesch P, et al. HTK-solution (Bretschneider) for human liver transplantation. First clinical experiences. *Langenbecks Arch Chir* 1990; 375:66-70
- 190 Potdar S, Malek S, Eghtesad B, et al. Initial experience using histidine-tryptophan-ketoglutarate solution in clinical pancreas transplantation. *Clin Transplant* 2004; 18:661-665
- 191 Erhard J, Lange R, Scherer R, et al. Comparison of histidine-tryptophan-ketoglutarate (HTK) solution versus University of Wisconsin (UW) solution for organ preservation in human liver transplantation. A prospective, randomized study. *Transpl Int* 1994; 7:177-181
- 192 Fridell JA, Agarwal A, Milgrom ML, et al. Comparison of histidine-tryptophan-ketoglutarate solution and University of Wisconsin solution for organ preservation in clinical pancreas transplantation. *Transplantation* 2004; 77:1304-1306
- 193 Reichenspurner H, Russ C, Uberfuhr P, et al. Myocardial Preservation Using Htk Solution for Heart-Transplantation - a Multicenter Study. *European Journal of Cardio-Thoracic Surgery* 1993; 7:414-419
- 194 Reichenspurner H, Russ C, Wagner F, et al. Comparison of UW versus HTK solution for myocardial protection in heart transplantation. *Transpl Int* 1994; 7 Suppl 1:S481-484
- 195 Wieselthaler GM, Chevtchik O, Konetschny R, et al. Improved graft function using a new myocardial preservation solution: Celsior. Preliminary data from a randomized prospective study. *Transplant Proc* 1999; 31:2067-2068
- 196 Hearse DJ, Stewart DA, Braimbridge MV. Hypothermic arrest and potassium arrest: metabolic and myocardial protection during elective cardiac arrest. *Circ Res* 1975; 36:481-489
- 197 Demertzis S, Wippermann J, Schaper J, et al. University of Wisconsin versus St. Thomas' Hospital solution for human donor heart preservation. *Ann Thorac Surg* 1993; 55:1131-1137

- 198 Weyand M, Konertz W, Deiwick M, et al. Donor heart preservation with three different solutions: donor heart function at more than 1 year after transplantation. *Transplant Proc* 1992; 24:2665-2666
- 199 Cannata A, Botta L, Colombo T, et al. Does the cardioplegic solution have an effect on early outcomes following heart transplantation? *Eur J Cardiothorac Surg* 2012; 41:e48-52; discussion e52-43
- 200 Menasche P, Pradier F, Grousset C, et al. Improved recovery of heart transplants with a specific kit of preservation solutions. *J Thorac Cardiovasc Surg* 1993; 105:353-363
- 201 Perrault LP, El-Hamamsy I, Dumont E, et al. Effects of crystalloid, blood and Celsior solutions on porcine coronary endothelial function after heart transplantation. *J Heart Lung Transplant* 2005; 24:912-920
- 202 Perrault LP, Nickner C, Desjardins N, et al. Improved preservation of coronary endothelial function with Celsior compared with blood and crystalloid solutions in heart transplantation. *J Heart Lung Transplant* 2001; 20:549-558
- 203 Mohara J, Morishita Y, Takahashi T, et al. A comparative study of Celsior and University of Wisconsin solutions based on 12-hr preservation followed by transplantation in canine models. *J Heart Lung Transplant* 1999; 18:1202-1210
- 204 Mohara J, Takahashi T, Oshima K, et al. The effect of Celsior solution on 12-hour cardiac preservation in comparison with University of Wisconsin solution. *J Cardiovasc Surg (Torino)* 2001; 42:187-192
- 205 Kevelaitis E, Nyborg NC, Menasche P. Protective effect of reduced glutathione on endothelial function of coronary arteries subjected to prolonged cold storage. *Transplantation* 1997; 64:660-663
- 206 George TJ, Arnaoutakis GJ, Baumgartner WA, et al. Organ storage with University of Wisconsin solution is associated with improved outcomes after orthotopic heart transplantation. *J Heart Lung Transplant* 2011; 30:1033-1043
- 207 George TJ, Arnaoutakis GJ, Beaty CA, et al. A novel method of measuring cardiac preservation injury demonstrates University of Wisconsin solution is associated with less ischemic necrosis than Celsior in early cardiac allograft biopsy specimens. *J Heart Lung Transplant* 2012; 31:410-418
- 208 Vega JD, Ochsner JL, Jeevanandam V, et al. A multicenter, randomized, controlled trial of Celsior for flush and hypothermic storage of cardiac allografts. *Ann Thorac Surg* 2001; 71:1442-1447
- 209 Llosa JC, Rodriguez Lambert JL, Naya JL, et al. Celsior, a novel cardioplegic solution for arrest and storage in heart transplantation. *Transplant Proc* 2000; 32:2589-2590

- 210 Demmy TL, Biddle JS, Bennett LE, et al. Organ preservation solutions in heart transplantation--patterns of usage and related survival. *Transplantation* 1997; 63:262-269
- 211 Fuller BJ, Lee CY. Hypothermic perfusion preservation: the future of organ preservation revisited? *Cryobiology* 2007; 54:129-145
- 212 Alijani MR, Cutler JA, DelValle CJ, et al. Single-donor cold storage versus machine perfusion in cadaver kidney preservation. *Transplantation* 1985; 40:659-661
- 213 Cerra FB, Raza S, Andres GA, et al. The endothelial damage of pulsatile renal preservation and its relationship to perfusion pressure and colloid osmotic pressure. *Surgery* 1977; 81:534-541
- 214 Light J. Viability testing in the non-heart-beating donor. *Transplant Proc* 2000; 32:179-181
- 215 Proctor E, Parker R. Preservation of isolated heart for 72 hours. *Br Med J* 1968; 4:296-298
- 216 Tago M, Seno S, Bando K, et al. [High energy phosphates and mitochondrial respiratory function after 24 hours cardiac preservation--comparison between simple hypothermic immersion and continuous hypothermic perfusion]. *Kokyu To Junkan* 1989; 37:61-63
- 217 Jones GR, Matthews G, Jones T, et al. The metabolism and viability of the canine heart during hypothermic (4 degrees C) asanguinous perfusion for 72-96 hr. *Cryobiology* 1973; 10:177-180
- 218 Lockett CJ, Busza AL, Toffa SK, et al. Resuscitation of cardiac energy metabolism in the rabbit heart by brief hypothermic reperfusion after preservation studied by ³¹P NMR spectroscopy. *Transpl Int* 1995; 8:8-12
- 219 Nickless DK, Rabinov M, Richards SM, et al. Continuous perfusion improves preservation of donor rat hearts: importance of the implantation phase. *Ann Thorac Surg* 1998; 65:1265-1272
- 220 Claes G, Aurell M, Blohme I, et al. Experimental and clinical results of continuous hypothermic albumin perfusion. *Proc Eur Dial Transplant Assoc* 1972; 9:484-490
- 221 Wicomb WN, Cooper DK, Novitzky D, et al. Cardiac transplantation following storage of the donor heart by a portable hypothermic perfusion system. *Ann Thorac Surg* 1984; 37:243-248
- 222 Ardehali A, Esmailian F, Deng M, et al. Ex-vivo perfusion of donor hearts for human heart transplantation (PROCEED II): a prospective, open-label, multicentre, randomised non-inferiority trial. *Lancet* 2015; 385:2577-2584

- 223 Dhital KK, Iyer A, Connellan M, et al. Adult heart transplantation with distant procurement and ex-vivo preservation of donor hearts after circulatory death: a case series. *Lancet* 2015
- 224 Hausenloy DJ, Yellon DM. The evolving story of "conditioning" to protect against acute myocardial ischaemia-reperfusion injury. *Heart* 2007; 93:649-651
- 225 Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986; 74:1124-1136
- 226 Illes RW, Swoyer KD. Prospective, randomized clinical study of ischemic preconditioning as an adjunct to intermittent cold blood cardioplegia. *Ann Thorac Surg* 1998; 65:748-752; discussion 752-743
- 227 Yellon DM, Downey JM. Preconditioning the myocardium: from cellular physiology to clinical cardiology. *Physiol Rev* 2003; 83:1113-1151
- 228 Lecour S. Activation of the protective Survivor Activating Factor Enhancement (SAFE) pathway against reperfusion injury: Does it go beyond the RISK pathway? *J Mol Cell Cardiol* 2009; 47:32-40
- 229 Paiva MA, Goncalves LM, Providencia LA, et al. Transitory activation of AMPK at reperfusion protects the ischaemic-reperfused rat myocardium against infarction. *Cardiovasc Drugs Ther* 2010; 24:25-32
- 230 Zhao ZQ, Corvera JS, Halkos ME, et al. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. *Am J Physiol Heart Circ Physiol* 2003; 285:H579-588
- 231 Yang XC, Liu Y, Wang LF, et al. Permanent reduction in myocardial infarct size by postconditioning in patients after primary coronary angioplasty. *Circulation* 2006; 114:812-812
- 232 Staat P, Rioufol G, Piot C, et al. Postconditioning the human heart. *Circulation* 2005; 112:2143-2148
- 233 Ma X, Zhang X, Li C, et al. Effect of postconditioning on coronary blood flow velocity and endothelial function and LV recovery after myocardial infarction. *J Interv Cardiol* 2006; 19:367-375
- 234 Laskey WK. Brief repetitive balloon occlusions enhance reperfusion during percutaneous coronary intervention for acute myocardial infarction: a pilot study. *Catheter Cardiovasc Interv* 2005; 65:361-367
- 235 Hausenloy DJ, Yellon DM. Remote ischaemic preconditioning: underlying mechanisms and clinical application. *Cardiovasc Res* 2008; 79:377-386
- 236 Halkos ME, Kerendi F, Corvera JS, et al. Myocardial protection with postconditioning is not enhanced by ischemic preconditioning. *Ann Thorac Surg* 2004; 78:961-969; discussion 969

- 237 Hausenloy DJ, Yellon DM. Preconditioning and postconditioning: united at reperfusion. *Pharmacol Ther* 2007; 116:173-191
- 238 Lim SY, Davidson SM, Hausenloy DJ, et al. Preconditioning and postconditioning: the essential role of the mitochondrial permeability transition pore. *Cardiovasc Res* 2007; 75:530-535
- 239 Lacerda L, Somers S, Opie LH, et al. Ischaemic postconditioning protects against reperfusion injury via the SAFE pathway. *Cardiovasc Res* 2009; 84:201-208
- 240 Hausenloy DJ, Yellon DM. New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. *Cardiovasc Res* 2004; 61:448-460
- 241 Hausenloy DJ, Yellon DM. Reperfusion injury salvage kinase signalling: taking a RISK for cardioprotection. *Heart Fail Rev* 2007; 12:217-234
- 242 Bopassa JC, Ferrera R, Gateau-Roesch O, et al. PI 3-kinase regulates the mitochondrial transition pore in controlled reperfusion and postconditioning. *Cardiovasc Res* 2006; 69:178-185
- 243 Baxter GF, Mocanu MM, Brar BK, et al. Cardioprotective effects of transforming growth factor-beta1 during early reoxygenation or reperfusion are mediated by p42/p44 MAPK. *J Cardiovasc Pharmacol* 2001; 38:930-939
- 244 Jonassen AK, Sack MN, Mjos OD, et al. Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling. *Circ Res* 2001; 89:1191-1198
- 245 Davidson SM, Hausenloy D, Duchon MR, et al. Signalling via the reperfusion injury signalling kinase (RISK) pathway links closure of the mitochondrial permeability transition pore to cardioprotection. *Int J Biochem Cell Biol* 2006; 38:414-419
- 246 Tosaki A, Cordis GA, Szerdahelyi P, et al. Effects of preconditioning on reperfusion arrhythmias, myocardial functions, formation of free radicals, and ion shifts in isolated ischemic/reperfused rat hearts. *J Cardiovasc Pharmacol* 1994; 23:365-373
- 247 Narayan P, Mentzer RM, Jr., Lasley RD. Adenosine A1 receptor activation reduces reactive oxygen species and attenuates stunning in ventricular myocytes. *J Mol Cell Cardiol* 2001; 33:121-129
- 248 Vanden Hoek T, Becker LB, Shao ZH, et al. Preconditioning in cardiomyocytes protects by attenuating oxidant stress at reperfusion. *Circ Res* 2000; 86:541-548

- 249 Ozcan C, Bienengraeber M, Dzeja PP, et al. Potassium channel openers protect cardiac mitochondria by attenuating oxidant stress at reoxygenation. *Am J Physiol Heart Circ Physiol* 2002; 282:H531-539
- 250 Wang L, Cherednichenko G, Hernandez L, et al. Preconditioning limits mitochondrial Ca^{2+} during ischemia in rat hearts: role of K(ATP) channels. *Am J Physiol Heart Circ Physiol* 2001; 280:H2321-2328
- 251 Liu GS, Thornton J, Van Winkle DM, et al. Protection against infarction afforded by preconditioning is mediated by A_1 adenosine receptors in rabbit heart. *Circulation* 1991; 84:350-356
- 252 Solenkova NV, Solodushko V, Cohen MV, et al. Endogenous adenosine protects preconditioned heart during early minutes of reperfusion by activating Akt. *Am J Physiol Heart Circ Physiol* 2006; 290:H441-449
- 253 Costa AD, Garlid KD, West IC, et al. Protein kinase G transmits the cardioprotective signal from cytosol to mitochondria. *Circ Res* 2005; 97:329-336
- 254 Mahaffey KW, Puma JA, Barbagelata NA, et al. Adenosine as an adjunct to thrombolytic therapy for acute myocardial infarction - Results of a multicenter, randomized, placebo-controlled trial: The Acute Myocardial Infarction Study of Adenosine (AMISTAD) Trial. *Journal of the American College of Cardiology* 1999; 34:1711-1720
- 255 Eckle T, Krahn T, Grenz A, et al. Cardioprotection by ecto-5'-nucleotidase (CD73) and A_2B adenosine receptors. *Circulation* 2007; 115:1581-1590
- 256 Philipp S, Yang XM, Cui L, et al. Postconditioning protects rabbit hearts through a protein kinase C-adenosine A_2b receptor cascade. *Cardiovasc Res* 2006; 70:308-314
- 257 Liu GS, Cohen MV, Mochly-Rosen D, et al. Protein kinase c-e is responsible for the protection of preconditioning in rabbit cardiomyocytes. *Journal of Molecular and Cellular Cardiology* 1999; 31:1937-1948
- 258 Dobson GP, Jones MW. Adenosine and lidocaine: a new concept in nondepolarizing surgical myocardial arrest, protection, and preservation. *J Thorac Cardiovasc Surg* 2004; 127:794-805
- 259 Wall TM, Sheehy R, Hartman JC. Role of bradykinin in myocardial preconditioning. *J Pharmacol Exp Ther* 1994; 270:681-689
- 260 Manolis AJ, Marketou ME, Gavras I, et al. Cardioprotective properties of bradykinin: role of the $\text{B}(2)$ receptor. *Hypertens Res* 2010; 33:772-777

- 261 Morris SD, Yellon DM. Angiotensin-converting enzyme inhibitors potentiate preconditioning through bradykinin B2 receptor activation in human heart. *J Am Coll Cardiol* 1997; 29:1599-1606
- 262 Jaberansari MT, Baxter GF, Muller CA, et al. Angiotensin-converting enzyme inhibition enhances a subthreshold stimulus to elicit delayed preconditioning in pig myocardium. *J Am Coll Cardiol* 2001; 37:1996-2001
- 263 Pedersen CM, Schmidt MR, Barnes G, et al. Bradykinin does not mediate remote ischaemic preconditioning or ischaemia-reperfusion injury in vivo in man. *Heart* 2011; 97:1857-1861
- 264 Schultz JE, Rose E, Yao Z, et al. Evidence for involvement of opioid receptors in ischemic preconditioning in rat hearts. *Am J Physiol* 1995; 268:H2157-2161
- 265 Wong GT, Huang Z, Ji S, et al. Remifentanyl reduces the release of biochemical markers of myocardial damage after coronary artery bypass surgery: a randomized trial. *J Cardiothorac Vasc Anesth* 2010; 24:790-796
- 266 Wong GT, Li R, Jiang LL, et al. Remifentanyl post-conditioning attenuates cardiac ischemia-reperfusion injury via kappa or delta opioid receptor activation. *Acta Anaesthesiol Scand* 2010; 54:510-518
- 267 Chong ZZ, Kang JQ, Maiese K. Erythropoietin is a novel vascular protectant through activation of Akt1 and mitochondrial modulation of cysteine proteases. *Circulation* 2002; 106:2973-2979
- 268 Chong ZZ, Li FQ, Maiese K. Erythropoietin requires NF-kappa B and its nuclear translocation to prevent early and late apoptotic neuronal injury during beta-amyloid toxicity. *Current Neurovascular Research* 2005; 2:387-399
- 269 Ong SG, Hausenloy DJ. Hypoxia-inducible factor as a therapeutic target for cardioprotection. *Pharmacology & Therapeutics* 2012; 136:69-81
- 270 Nishihara M, Miura T, Miki T, et al. Modulation of the mitochondrial permeability transition pore complex in GSK-3beta-mediated myocardial protection. *J Mol Cell Cardiol* 2007; 43:564-570
- 271 Ferrario M, Arbustini E, Massa M, et al. High-dose erythropoietin in patients with acute myocardial infarction: a pilot, randomised, placebo-controlled study. *Int J Cardiol* 2011; 147:124-131
- 272 Ozawa T, Toba K, Suzuki H, et al. Single-dose intravenous administration of recombinant human erythropoietin is a promising treatment for patients with acute myocardial infarction - randomized controlled pilot trial of EPO/AMI-1 study. *Circ J* 2010; 74:1415-1423
- 273 Prunier F, Biere L, Gilard M, et al. Single high-dose erythropoietin administration immediately after reperfusion in patients with ST-segment elevation

myocardial infarction: results of the erythropoietin in myocardial infarction trial. *Am Heart J* 2012; 163:200-207 e201

- 274 Taniguchi N, Nakamura T, Sawada T, et al. Erythropoietin prevention trial of coronary restenosis and cardiac remodeling after ST-elevated acute myocardial infarction (EPOC-AMI): a pilot, randomized, placebo-controlled study. *Circ J* 2010; 74:2365-2371
- 275 Ludman AJ, Yellon DM, Hasleton J, et al. Effect of erythropoietin as an adjunct to primary percutaneous coronary intervention: a randomised controlled clinical trial. *Heart* 2011; 97:1560-1565
- 276 Najjar SS, Rao SV, Melloni C, et al. Intravenous erythropoietin in patients with ST-segment elevation myocardial infarction: REVEAL: a randomized controlled trial. *JAMA* 2011; 305:1863-1872
- 277 Ott I, Schulz S, Mehilli J, et al. Erythropoietin in patients with acute ST-segment elevation myocardial infarction undergoing primary percutaneous coronary intervention: a randomized, double-blind trial. *Circ Cardiovasc Interv* 2010; 3:408-413
- 278 Voors AA, Belonje AM, Zijlstra F, et al. A single dose of erythropoietin in ST-elevation myocardial infarction. *Eur Heart J* 2010; 31:2593-2600
- 279 Roubille F, Micheau A, Combes S, et al. Intracoronary administration of darbepoetin-alpha at onset of reperfusion in acute myocardial infarction: results of the randomized Intra-Co-EpoMI trial. *Arch Cardiovasc Dis* 2013; 106:135-145
- 280 Hong L, Xi J, Zhang Y, et al. Atrial natriuretic peptide prevents the mitochondrial permeability transition pore opening by inactivating glycogen synthase kinase 3beta via PKG and PI3K in cardiac H9c2 cells. *Eur J Pharmacol* 2012; 695:13-19
- 281 Yang XM, Philipp S, Downey JM, et al. Atrial natriuretic peptide administered just prior to reperfusion limits infarction in rabbit hearts. *Basic Res Cardiol* 2006; 101:311-318
- 282 Kitakaze M, Asakura M, Kim J, et al. Human atrial natriuretic peptide and nicorandil as adjuncts to reperfusion treatment for acute myocardial infarction (J-WIND): two randomised trials. *Lancet* 2007; 370:1483-1493
- 283 Kasama S, Furuya M, Toyama T, et al. Effect of atrial natriuretic peptide on left ventricular remodelling in patients with acute myocardial infarction. *Eur Heart J* 2008; 29:1485-1494
- 284 Kuga H, Ogawa K, Oida A, et al. Administration of atrial natriuretic peptide attenuates reperfusion phenomena and preserves left ventricular regional wall motion after direct coronary angioplasty for acute myocardial infarction. *Circ J* 2003; 67:443-448

- 285 Lou M, Garrett TP, McKern NM, et al. The first three domains of the insulin receptor differ structurally from the insulin-like growth factor 1 receptor in the regions governing ligand specificity. *Proc Natl Acad Sci U S A* 2006; 103:12429-12434
- 286 Baines CP, Wang L, Cohen MV, et al. Myocardial protection by insulin is dependent on phosphatidylinositol 3-kinase but not protein kinase C or KATP channels in the isolated rabbit heart. *Basic Res Cardiol* 1999; 94:188-198
- 287 Zhang HF, Fan Q, Qian XX, et al. Role of insulin in the anti-apoptotic effect of glucose-insulin-potassium in rabbits with acute myocardial ischemia and reperfusion. *Apoptosis* 2004; 9:777-783
- 288 Zhang HX, Zang YM, Huo JH, et al. Physiologically tolerable insulin reduces myocardial injury and improves cardiac functional recovery in myocardial ischemic/reperfused dogs. *J Cardiovasc Pharmacol* 2006; 48:306-313
- 289 Zhu P, Lu L, Xu Y, et al. Glucose-insulin-potassium preserves systolic and diastolic function in ischemia and reperfusion in pigs. *Am J Physiol Heart Circ Physiol* 2000; 278:H595-603
- 290 Diaz R, Goyal A, Mehta SR, et al. Glucose-insulin-potassium therapy in patients with ST-segment elevation myocardial infarction. *JAMA* 2007; 298:2399-2405
- 291 Mehta SR, Yusuf S, Diaz R, et al. Effect of glucose-insulin-potassium infusion on mortality in patients with acute ST-segment elevation myocardial infarction: the CREATE-ECLA randomized controlled trial. *JAMA* 2005; 293:437-446
- 292 Selker HP, Beshansky JR, Sheehan PR, et al. Out-of-hospital administration of intravenous glucose-insulin-potassium in patients with suspected acute coronary syndromes: the IMMEDIATE randomized controlled trial. *JAMA* 2012; 307:1925-1933
- 293 Cope DK, Impastato WK, Cohen MV, et al. Volatile anesthetics protect the ischemic rabbit myocardium from infarction. *Anesthesiology* 1997; 86:699-709
- 294 Davis RF, DeBoer LW, Rude RE, et al. The effect of halothane anesthesia on myocardial necrosis, hemodynamic performance, and regional myocardial blood flow in dogs following coronary artery occlusion. *Anesthesiology* 1983; 59:402-411
- 295 Preckel B, Schlack W, Comfere T, et al. Effects of enflurane, isoflurane, sevoflurane and desflurane on reperfusion injury after regional myocardial ischaemia in the rabbit heart in vivo. *Br J Anaesth* 1998; 81:905-912
- 296 Frassdorf J, De Hert S, Schlack W. Anaesthesia and myocardial ischaemia/reperfusion injury. *Br J Anaesth* 2009; 103:89-98

- 297 de Hert SG, Van der Linden PJ. Left ventricular function after cardiopulmonary bypass is related to the length-dependent regulation of myocardial function. *Anesth Analg* 2004; 99:311-312; author reply 312
- 298 De Hert SG, Van der Linden PJ, Cromheecke S, et al. Choice of primary anesthetic regimen can influence intensive care unit length of stay after coronary surgery with cardiopulmonary bypass. *Anesthesiology* 2004; 101:9-20
- 299 De Hert S, Vlasselaers D, Barbe R, et al. A comparison of volatile and non volatile agents for cardioprotection during on-pump coronary surgery. *Anaesthesia* 2009; 64:953-960
- 300 Cohen MV, Yang XM, Downey JM. Nitric oxide is a preconditioning mimetic and cardioprotectant and is the basis of many available infarct-sparing strategies. *Cardiovasc Res* 2006; 70:231-239
- 301 Duranski MR, Greer JJ, Dejam A, et al. Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. *J Clin Invest* 2005; 115:1232-1240
- 302 Kanno S, Lee PC, Zhang Y, et al. Attenuation of myocardial ischemia/reperfusion injury by superinduction of inducible nitric oxide synthase. *Circulation* 2000; 101:2742-2748
- 303 Yusuf S, Collins R, MacMahon S, et al. Effect of intravenous nitrates on mortality in acute myocardial infarction: an overview of the randomised trials. *Lancet* 1988; 1:1088-1092
- 304 Kukreja RC, Salloum FN, Das A, et al. Emerging new uses of phosphodiesterase-5 inhibitors in cardiovascular diseases. *Exp Clin Cardiol* 2011; 16:e30-35
- 305 Reffelmann T, Kloner RA. Phosphodiesterase 5 inhibitors: are they cardioprotective? *Cardiovasc Res* 2009; 83:204-212
- 306 Croom KF, Curran MP. Sildenafil: a review of its use in pulmonary arterial hypertension. *Drugs* 2008; 68:383-397
- 307 Bremer YA, Salloum F, Ockaili R, et al. Sildenafil citrate (viagra) induces cardioprotective effects after ischemia/reperfusion injury in infant rabbits. *Pediatr Res* 2005; 57:22-27
- 308 Das A, Salloum FN, Xi L, et al. ERK phosphorylation mediates sildenafil-induced myocardial protection against ischemia-reperfusion injury in mice. *Am J Physiol Heart Circ Physiol* 2009; 296:H1236-1243
- 309 Kukreja RC, Ockaili R, Salloum F, et al. Sildenafil-induced cardioprotection in rabbits. *Cardiovasc Res* 2003; 60:700-701; author reply 702-703

- 310 Ockaili R, Salloum F, Hawkins J, et al. Sildenafil (Viagra) induces powerful cardioprotective effect via opening of mitochondrial K(ATP) channels in rabbits. *Am J Physiol Heart Circ Physiol* 2002; 283:H1263-1269
- 311 Bell RM, Yellon DM. Atorvastatin, administered at the onset of reperfusion, and independent of lipid lowering, protects the myocardium by up-regulating a pro-survival pathway. *J Am Coll Cardiol* 2003; 41:508-515
- 312 Ikeda Y, Young LH, Lefer AM. Rosuvastatin, a new HMG-CoA reductase inhibitor, protects ischemic reperfused myocardium in normocholesterolemic rats. *J Cardiovasc Pharmacol* 2003; 41:649-656
- 313 Vilahur G, Casani L, Pena E, et al. Induction of RISK by HMG-CoA reductase inhibition affords cardioprotection after myocardial infarction. *Atherosclerosis* 2009; 206:95-101
- 314 Nusca A, Melfi R, Patti G, et al. Statin loading for acute coronary syndromes. *Curr Opin Cardiol* 2010; 25:373-378
- 315 Kim JS, Kim J, Choi D, et al. Efficacy of high-dose atorvastatin loading before primary percutaneous coronary intervention in ST-segment elevation myocardial infarction: the STATIN STEMI trial. *JACC Cardiovasc Interv* 2010; 3:332-339
- 316 Hausenloy DJ, Maddock HL, Baxter GF, et al. Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning? *Cardiovasc Res* 2002; 55:534-543
- 317 Piot C, Croisille P, Staat P, et al. Effect of cyclosporine on reperfusion injury in acute myocardial infarction. *N Engl J Med* 2008; 359:473-481
- 318 Cung TT, Morel O, Cayla G, et al. Cyclosporine before PCI in Patients with Acute Myocardial Infarction. *N Engl J Med* 2015; 373:1021-1031
- 319 Allen DG, Xiao XH. Role of the cardiac Na⁺/H⁺ exchanger during ischemia and reperfusion. *Cardiovasc Res* 2003; 57:934-941
- 320 Cropper JR, Hicks M, Ryan JB, et al. Cardioprotection by cariporide after prolonged hypothermic storage of the isolated working rat heart. *J Heart Lung Transplant* 2003; 22:929-936
- 321 Ryan JB, Hicks M, Cropper JR, et al. Cariporide (HOE-642) improves cardiac allograft preservation in a porcine model of orthotopic heart transplantation. *Transplantation* 2003; 75:625-631
- 322 Ryan JB, Hicks M, Cropper JR, et al. Sodium-hydrogen exchanger inhibition, pharmacologic ischemic preconditioning, or both for extended cardiac allograft preservation. *Transplantation* 2003; 76:766-771

- 323 Mentzer RM, Jr., Bartels C, Bolli R, et al. Sodium-hydrogen exchange inhibition by cariporide to reduce the risk of ischemic cardiac events in patients undergoing coronary artery bypass grafting: results of the EXPEDITION study. *Ann Thorac Surg* 2008; 85:1261-1270
- 324 Gao L, Tsun J, Sun L, et al. Critical role of the STAT3 pathway in the cardioprotective efficacy of zoniporide in a model of myocardial preservation - the rat isolated working heart. *Br J Pharmacol* 2011; 162:633-647
- 325 Gao L, Hicks M, MacDonald PS. Improved preservation of the rat heart with celsior solution supplemented with cariporide plus glyceryl trinitrate. *Am J Transplant* 2005; 5:1820-1826
- 326 Hing AJ, Watson A, Hicks M, et al. Combining cariporide with glyceryl trinitrate optimizes cardiac preservation during porcine heart transplantation. *Am J Transplant* 2009; 9:2048-2056
- 327 Baxter K, Howden B, Saunderson A, et al. Improved cardiac preservation by the addition of nitroglycerine to colloid-free University of Wisconsin solution (MUW). *J Heart Lung Transplant* 1999; 18:769-774
- 328 Baxter K, Howden BO, Jablonski P. Cardiac preservation with nitroglycerine and cyclic adenosine monophosphate. *Transplant Proc* 1999; 31:1046-1047
- 329 Watson AJ, Gao L, Sun L, et al. Enhanced preservation of the rat heart after prolonged hypothermic ischemia with erythropoietin-supplemented Celsior solution. *J Heart Lung Transplant* 2013; 32:633-640
- 330 Watson AJ, Gao L, Sun L, et al. Enhanced preservation of pig cardiac allografts by combining erythropoietin with glyceryl trinitrate and zoniporide. *Am J Transplant* 2013; 13:1676-1687
- 331 du Toit EF, Rossouw E, Salie R, et al. Effect of sildenafil on reperfusion function, infarct size, and cyclic nucleotide levels in the isolated rat heart model. *Cardiovasc Drugs Ther* 2005; 19:23-31
- 332 Baum A, Pohlmeyer G, Rapp KG, et al. Lewis rats of the inbred strain LEW/Han: life expectancy, spectrum and incidence of spontaneous neoplasms. *Exp Toxicol Pathol* 1995; 47:11-18
- 333 Deerberg F. Age-associated versus husbandry-related pathology of aging rats. *Neurobiol Aging* 1991; 12:659-662
- 334 Feldman JD, Woda BA. Pathology and tumor incidence in aged Lewis and BN rats. *Clin Immunol Immunopathol* 1980; 15:331-343
- 335 Neely JR, Liebermeister H, Battersby EJ, et al. Effect of pressure development on oxygen consumption by isolated rat heart. *Am J Physiol* 1967; 212:804-814

- 336 Galinanes M, Hearse DJ. Assessment of ischemic injury and protective interventions: the Langendorff versus the working rat heart preparation. *Can J Cardiol* 1990; 6:83-91
- 337 Sutherland FJ, Hearse DJ. The isolated blood and perfusion fluid perfused heart. *Pharmacol Res* 2000; 41:613-627
- 338 Guidelines for the determination of death. Report of the medical consultants on the diagnosis of death to the President's Commission for the Study of Ethical Problems in Medicine and Biomedical and Behavioral Research. *JAMA* 1981; 246:2184-2186
- 339 Mascia L, Mastromauro I, Viberti S, et al. Management to optimize organ procurement in brain dead donors. *Minerva Anestesiol* 2009; 75:125-133
- 340 Schrader H, Hall C, Zwetnow NN. Effects of prolonged supratentorial mass expansion on regional blood flow and cardiovascular parameters during the Cushing response. *Acta Neurol Scand* 1985; 72:283-294
- 341 Novitzky D, Rose AG, Cooper DK. Injury of myocardial conduction tissue and coronary artery smooth muscle following brain death in the baboon. *Transplantation* 1988; 45:964-966
- 342 Novitzky D, Horak A, Cooper DK, et al. Electrocardiographic and histopathologic changes developing during experimental brain death in the baboon. *Transplant Proc* 1989; 21:2567-2569
- 343 Smith M. Physiologic changes during brain stem death--lessons for management of the organ donor. *J Heart Lung Transplant* 2004; 23:S217-222
- 344 Stiegler P, Sereinigg M, Puntschart A, et al. Oxidative stress and apoptosis in a pig model of brain death (BD) and living donation (LD). *J Transl Med* 2013; 11:244
- 345 Li J, Konstantinov IE, Cai S, et al. Systemic and myocardial oxygen transport responses to brain death in pigs. *Transplant Proc* 2007; 39:21-26
- 346 Baroldi G, Bigi R, Cortigiani L. Ultrasound imaging versus morphopathology in cardiovascular diseases. Myocardial cell damage. *Cardiovasc Ultrasound* 2005; 3:32
- 347 Kolin A, Norris JW. Myocardial damage from acute cerebral lesions. *Stroke* 1984; 15:990-993
- 348 Greenhoot JH, Reichenbach DD. Cardiac injury and subarachnoid hemorrhage. A clinical, pathological, and physiological correlation. *J Neurosurg* 1969; 30:521-531

- 349 Saat TC, Susa D, Roest HP, et al. A comparison of inflammatory, cytoprotective and injury gene expression profiles in kidneys from brain death and cardiac death donors. *Transplantation* 2014; 98:15-21
- 350 Barklin A. Systemic inflammation in the brain-dead organ donor. *Acta Anaesthesiol Scand* 2009; 53:425-435
- 351 Schuurs TA, Morariu AM, Ottens PJ, et al. Time-dependent changes in donor brain death related processes. *Am J Transplant* 2006; 6:2903-2911
- 352 Pratschke J, Wilhelm MJ, Kusaka M, et al. Activation of proinflammatory genes in somatic organs as a consequence of brain death. *Transplant Proc* 1999; 31:1003-1005
- 353 Wilhelm MJ, Pratschke J, Beato F, et al. Activation of the heart by donor brain death accelerates acute rejection after transplantation. *Circulation* 2000; 102:2426-2433
- 354 Pratschke J, Wilhelm MJ, Kusaka M, et al. Accelerated rejection of renal allografts from brain-dead donors. *Ann Surg* 2000; 232:263-271
- 355 Shah VR. Aggressive management of multiorgan donor. *Transplant Proc* 2008; 40:1087-1090
- 356 Szabo G, Hackert T, Sebening C, et al. Role of neural and humoral factors in hyperdynamic reaction and cardiac dysfunction following brain death. *J Heart Lung Transplant* 2000; 19:683-693
- 357 Chen JM, Cullinane S, Spanier TB, et al. Vasopressin deficiency and pressor hypersensitivity in hemodynamically unstable organ donors. *Circulation* 1999; 100:II244-246
- 358 Sutherland AJ, Ware RS, Winterford C, et al. The endothelin axis and gelatinase activity in alveolar macrophages after brain-stem death injury: a pilot study. *J Heart Lung Transplant* 2007; 26:1040-1047
- 359 Watts RP, Bilska I, Diab S, et al. Novel 24-h ovine model of brain death to study the profile of the endothelin axis during cardiopulmonary injury. *Intensive Care Med Exp* 2015; 3:31
- 360 Berman M, Ali A, Ashley E, et al. Is stress cardiomyopathy the underlying cause of ventricular dysfunction associated with brain death? *Journal of Heart and Lung Transplantation* 2010; 29:957-965
- 361 Santise G, D'Ancona G, Falletta C, et al. Donor pharmacological hemodynamic support is associated with primary graft failure in human heart transplantation. *Interact Cardiovasc Thorac Surg* 2009; 9:476-479

- 362 Galinanes M, Hearse DJ. Brain death-induced impairment of cardiac contractile performance can be reversed by explantation and may not preclude the use of hearts for transplantation. *Circ Res* 1992; 71:1213-1219
- 363 Zhang SJ, Wang T. The influence of brain death on donor liver and the potential mechanisms of protective intervention. *Front Med* 2011; 5:8-14
- 364 Dujardin KS, McCully RB, Wijdicks EF, et al. Myocardial dysfunction associated with brain death: clinical, echocardiographic, and pathologic features. *J Heart Lung Transplant* 2001; 20:350-357
- 365 Gao L, Kwan JC, Macdonald PS, et al. Improved poststorage cardiac function by poly (ADP-ribose) polymerase inhibition: role of phosphatidylinositol 3-kinase Akt pathway. *Transplantation* 2007; 84:380-386
- 366 Jabbour A, Gao L, Kwan J, et al. A recombinant human neuregulin-1 peptide improves preservation of the rodent heart after prolonged hypothermic storage. *Transplantation* 2011; 91:961-967
- 367 Kwan JC, Gao L, Macdonald PS, et al. Cardio-protective signalling by glyceryl trinitrate and cariporide in a model of donor heart preservation. *Heart Lung Circ* 2015; 24:306-318
- 368 Wijdicks EF, Varelas PN, Gronseth GS, et al. Evidence-based guideline update: determining brain death in adults: report of the Quality Standards Subcommittee of the American Academy of Neurology. *Neurology* 2010; 74:1911-1918
- 369 Oto T, Calderone A, Li Z, et al. p38 Mitogen-activated protein kinase inhibition reduces inflammatory cytokines in a brain-dead transplant donor animal model. *Heart Lung Circ* 2009; 18:393-400
- 370 Di Padova F, Pozzi C, Tondre MJ, et al. Selective and early increase of IL-1 inhibitors, IL-6 and cortisol after elective surgery. *Clin Exp Immunol* 1991; 85:137-142
- 371 Cremer J, Martin M, Redl H, et al. Systemic inflammatory response syndrome after cardiac operations. *Ann Thorac Surg* 1996; 61:1714-1720
- 372 Ellstrom M, Bengtsson A, Tylman M, et al. Evaluation of tissue trauma after laparoscopic and abdominal hysterectomy: measurements of neutrophil activation and release of interleukin-6, cortisol, and C-reactive protein. *J Am Coll Surg* 1996; 182:423-430
- 373 Tanguay JF, Krucoff MW, Gibbons RJ, et al. Efficacy of a novel P-selectin antagonist, rPSGL-Ig for reperfusion therapy in acute myocardial infarction: The RAPSODY trial. *Journal of the American College of Cardiology* 2003; 41:404A-405A

- 374 Faxon DP, Gibbons RJ, Chronos NA, et al. The effect of blockade of the CD11/CD18 integrin receptor on infarct size in patients with acute myocardial infarction treated with direct angioplasty: the results of the HALT-MI study. *J Am Coll Cardiol* 2002; 40:1199-1204
- 375 Baran KW, Nguyen M, McKendall GR, et al. Double-blind, randomized trial of an anti-CD18 antibody in conjunction with recombinant tissue plasminogen activator for acute myocardial infarction: limitation of myocardial infarction following thrombolysis in acute myocardial infarction (LIMIT AMI) study. *Circulation* 2001; 104:2778-2783
- 376 Deswal A, Bozkurt B, Seta Y, et al. Safety and efficacy of a soluble P75 tumor necrosis factor receptor (Enbrel, etanercept) in patients with advanced heart failure. *Circulation* 1999; 99:3224-3226
- 377 Seok J, Warren HS, Cuenca AG, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci U S A* 2013; 110:3507-3512
- 378 Iyer A, Gao L, Doyle A, et al. Increasing the Tolerance of DCD Hearts to Warm Ischemia by Pharmacological Postconditioning. *Am J Transplant* 2014
- 379 Colvin-Adams M, Smithy JM, Heubner BM, et al. OPTN/SRTR 2012 Annual Data Report: heart. *Am J Transplant* 2014; 14 Suppl 1:113-138
- 380 Del Rizzo DF, Menkis AH, Pflugfelder PW, et al. The role of donor age and ischemic time on survival following orthotopic heart transplantation. *J Heart Lung Transplant* 1999; 18:310-319
- 381 Boucher F, Tanguy S, Besse S, et al. Age-dependent changes in myocardial susceptibility to zero flow ischemia and reperfusion in isolated perfused rat hearts: relation to antioxidant status. *Mech Ageing Dev* 1998; 103:301-316
- 382 Wojtovich AP, Nadtochiy SM, Brookes PS, et al. Ischemic preconditioning: the role of mitochondria and aging. *Exp Gerontol* 2012; 47:1-7
- 383 Boengler K, Konietzka I, Buechert A, et al. Loss of ischemic preconditioning's cardioprotection in aged mouse hearts is associated with reduced gap junctional and mitochondrial levels of connexin 43. *Am J Physiol Heart Circ Physiol* 2007; 292:H1764-1769
- 384 Boengler K, Schulz R, Heusch G. Loss of cardioprotection with ageing. *Cardiovasc Res* 2009; 83:247-261
- 385 Dai DF, Rabinovitch PS, Ungvari Z. Mitochondria and cardiovascular aging. *Circ Res* 2012; 110:1109-1124
- 386 Sengupta P. The Laboratory Rat: Relating Its Age With Human's. *Int J Prev Med* 2013; 4:624-630

- 387 Hardy AR, Quay RJ, Huson LW. Estimation of Age in the Norway Rat (*Rattus-Norvegicus Berkenhout*) from the Weight of the Eyelens. *Journal of Applied Ecology* 1983; 20:97-102
- 388 Quinn R. Comparing rat's to human's age: how old is my rat in people years? *Nutrition* 2005; 21:775-777
- 389 Andreollo NA, Santos EF, Araujo MR, et al. Rat's age versus human's age: what is the relationship? *Arq Bras Cir Dig* 2012; 25:49-51
- 390 Tolnai S, Beznak M. Studies of lysosomal enzyme activity in normal and hypertrophied mammalian myocardium. *J Mol Cell Cardiol* 1971; 3:193-208
- 391 Walker EM, Jr., Nillas MS, Mangiarua EI, et al. Age-associated changes in hearts of male Fischer 344/Brown Norway F1 rats. *Ann Clin Lab Sci* 2006; 36:427-438
- 392 Farhat F, Loisanse D, Garnier JP, et al. Norepinephrine release after acute brain death abolishes the cardioprotective effects of ischemic preconditioning in rabbit. *Eur J Cardiothorac Surg* 2001; 19:313-320
- 393 Kirsch M, Bertrand S, Garnier JP, et al. Pretreatment with a potassium-channel opener before prolonged cardiac storage: an evaluation in an experimental brain death model. *The Annals of thoracic surgery* 1999; 67:1623-1629; discussion 1630
- 394 Kirsch M, Farhat F, Garnier JP, et al. Acute brain death abolishes the cardioprotective effects of ischemic preconditioning in the rabbit. *Transplantation* 2000; 69:2013-2019
- 395 Chiari P, Piriou V, Hadour G, et al. Preservation of ischemia and isoflurane-induced preconditioning after brain death in rabbit hearts. *Am J Physiol Heart Circ Physiol* 2002; 283:H1769-1774
- 396 Ebrahim Z, Yellon DM, Baxter GF. Ischemic preconditioning is lost in aging hypertensive rat heart: independent effects of aging and longstanding hypertension. *Exp Gerontol* 2007; 42:807-814
- 397 Schulman D, Latchman DS, Yellon DM. Effect of aging on the ability of preconditioning to protect rat hearts from ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* 2001; 281:H1630-1636
- 398 Fenton RA, Dickson EW, Dobson JG, Jr. Inhibition of phosphatase activity enhances preconditioning and limits cell death in the ischemic/reperfused aged rat heart. *Life Sci* 2005; 77:3375-3388
- 399 Parsa CJ, Matsumoto A, Kim J, et al. A novel protective effect of erythropoietin in the infarcted heart. *J Clin Invest* 2003; 112:999-1007
- 400 Lu MJ, Chen YS, Huang HS, et al. Erythropoietin alleviates post-ischemic injury of rat hearts by attenuating nitrosative stress. *Life Sci* 2012; 90:776-784

- 401 Cai Z, Semenza GL. Phosphatidylinositol-3-kinase signaling is required for erythropoietin-mediated acute protection against myocardial ischemia/reperfusion injury. *Circulation* 2004; 109:2050-2053
- 402 Bullard AJ, Govewalla P, Yellon DM. Erythropoietin protects the myocardium against reperfusion injury in vitro and in vivo. *Basic Res Cardiol* 2005; 100:397-403
- 403 Stromer H, de Groot MC, Horn M, et al. Na(+)/H(+) exchange inhibition with HOE642 improves postischemic recovery due to attenuation of Ca(2+) overload and prolonged acidosis on reperfusion. *Circulation* 2000; 101:2749-2755
- 404 Cohen MV, Yang XM, Downey JM. The pH hypothesis of postconditioning: staccato reperfusion reintroduces oxygen and perpetuates myocardial acidosis. *Circulation* 2007; 115:1895-1903
- 405 Csont T, Szilvassy Z, Fulop F, et al. Direct myocardial anti-ischaemic effect of GTN in both nitrate-tolerant and nontolerant rats: a cyclic GMP-independent activation of KATP. *Br J Pharmacol* 1999; 128:1427-1434
- 406 Broillet MC. S-nitrosylation of proteins. *Cell Mol Life Sci* 1999; 55:1036-1042
- 407 Doulias PT, Tenopoulou M, Greene JL, et al. Nitric oxide regulates mitochondrial fatty acid metabolism through reversible protein S-nitrosylation. *Sci Signal* 2013; 6:rs1
- 408 Beleslin-Cokic BB, Cokic VP, Yu X, et al. Erythropoietin and hypoxia stimulate erythropoietin receptor and nitric oxide production by endothelial cells. *Blood* 2004; 104:2073-2080
- 409 Muller-Strahl G, Kottenberg K, Zimmer HG, et al. Inhibition of nitric oxide synthase augments the positive inotropic effect of nitric oxide donors in the rat heart. *J Physiol* 2000; 522 Pt 2:311-320
- 410 Kojda G, Kottenberg K. Regulation of basal myocardial function by NO. *Cardiovasc Res* 1999; 41:514-523
- 411 Knight DR, Smith AH, Flynn DM, et al. A novel sodium-hydrogen exchanger isoform-1 inhibitor, zoniporide, reduces ischemic myocardial injury in vitro and in vivo. *J Pharmacol Exp Ther* 2001; 297:254-259
- 412 Karmazyn M. NHE-1: still a viable therapeutic target. *J Mol Cell Cardiol* 2013; 61:77-82
- 413 Karmazyn M. The role of the myocardial sodium-hydrogen exchanger in mediating ischemic and reperfusion injury. From amiloride to cariporide. *Ann N Y Acad Sci* 1999; 874:326-334
- 414 Yellon DM, Baxter GF. Sodium-hydrogen exchange in myocardial reperfusion injury. *Lancet* 2000; 356:522-523

- 415 Matsui Y, Takagi H, Qu X, et al. Distinct roles of autophagy in the heart during ischemia and reperfusion: roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy. *Circulation research* 2007; 100:914-922
- 416 Xuan YT, Guo Y, Han H, et al. An essential role of the JAK-STAT pathway in ischemic preconditioning. *Proc Natl Acad Sci U S A* 2001; 98:9050-9055
- 417 Kumarasinghe G, Iyer A, Watson A, et al. Clinical experience supplementing celsior preservation solution with pro-survival kinase agents glyceryl trinitrate and erythropoietin demonstrates excellent myocardial recovery post cardiac transplantation. *Transplantation* 2013; 96:S259-S260
- 418 Nilsson J, Ohlsson M, Stehlik J, et al. Prediction of Primary Graft Dysfunction After Heart Transplantation. *Journal of Heart and Lung Transplantation* 2015; 34:S35-S35
- 419 Esmailian F, Patel J, Kittleson M, et al. Shorter Cold Ischemic Time in Older Donors Post-Heart Transplant Appears to Be Protective. *Journal of Heart and Lung Transplantation* 2015; 34:S17-S17
- 420 Hausenloy DJ, Yellon DM. Preconditioning and postconditioning: new strategies for cardioprotection. *Diabetes Obes Metab* 2008; 10:451-459
- 421 Marasco SF, Vale M, Pellegrino V, et al. Extracorporeal membrane oxygenation in primary graft failure after heart transplantation. *Ann Thorac Surg* 2010; 90:1541-1546
- 422 Fudim M, Davis ME, Jenkins C, et al. Marginal Donor Use in Patients Undergoing Heart Transplantation With Left Ventricular Assist Device Explantation. *Ann Thorac Surg* 2015
- 423 D'Alessandro C, Aubert S, Golmard JL, et al. Extra-corporeal membrane oxygenation temporary support for early graft failure after cardiac transplantation. *Eur J Cardiothorac Surg* 2010; 37:343-349
- 424 Boolell M, Gepi-Attee S, Gingell JC, et al. Sildenafil, a novel effective oral therapy for male erectile dysfunction. *Br J Urol* 1996; 78:257-261
- 425 Boolell M, Allen MJ, Ballard SA, et al. Sildenafil: an orally active type 5 cyclic GMP-specific phosphodiesterase inhibitor for the treatment of penile erectile dysfunction. *Int J Impot Res* 1996; 8:47-52
- 426 Porst H, Rosen R, Padma-Nathan H, et al. The efficacy and tolerability of vardenafil, a new, oral, selective phosphodiesterase type 5 inhibitor, in patients with erectile dysfunction: the first at-home clinical trial. *Int J Impot Res* 2001; 13:192-199
- 427 Prasad S, Wilkinson J, Gatzoulis MA. Sildenafil in primary pulmonary hypertension. *N Engl J Med* 2000; 343:1342

- 428 Watanabe H, Ohashi K, Takeuchi K, et al. Sildenafil for primary and secondary pulmonary hypertension. *Clin Pharmacol Ther* 2002; 71:398-402
- 429 Michelakis E, Tymchak W, Lien D, et al. Oral sildenafil is an effective and specific pulmonary vasodilator in patients with pulmonary arterial hypertension: comparison with inhaled nitric oxide. *Circulation* 2002; 105:2398-2403
- 430 Giordano D, De Stefano ME, Citro G, et al. Expression of cGMP-binding cGMP-specific phosphodiesterase (PDE5) in mouse tissues and cell lines using an antibody against the enzyme amino-terminal domain. *Biochim Biophys Acta* 2001; 1539:16-27
- 431 Salloum F, Yin C, Xi L, et al. Sildenafil induces delayed preconditioning through inducible nitric oxide synthase-dependent pathway in mouse heart. *Circ Res* 2003; 92:595-597
- 432 Das S, Maulik N, Das DK, et al. Cardioprotection with sildenafil, a selective inhibitor of cyclic 3',5'-monophosphate-specific phosphodiesterase 5. *Drugs Exp Clin Res* 2002; 28:213-219
- 433 Garcarena CD, Fantinelli JC, Caldiz CI, et al. Myocardial reperfusion injury: reactive oxygen species vs. NHE-1 reactivation. *Cell Physiol Biochem* 2011; 27:13-22
- 434 Kukreja RC, Ockaili R, Salloum F, et al. Cardioprotection with phosphodiesterase-5 inhibition--a novel preconditioning strategy. *J Mol Cell Cardiol* 2004; 36:165-173
- 435 Gross GJ. The role of mitochondrial KATP channels in cardioprotection. *Basic Res Cardiol* 2000; 95:280-284
- 436 Fryer RM, Eells JT, Hsu AK, et al. Ischemic preconditioning in rats: role of mitochondrial K(ATP) channel in preservation of mitochondrial function. *Am J Physiol Heart Circ Physiol* 2000; 278:H305-312
- 437 Takashi E, Wang Y, Ashraf M. Activation of mitochondrial K(ATP) channel elicits late preconditioning against myocardial infarction via protein kinase C signaling pathway. *Circ Res* 1999; 85:1146-1153
- 438 Das A, Ockaili R, Salloum F, et al. Protein kinase C plays an essential role in sildenafil-induced cardioprotection in rabbits. *Am J Physiol Heart Circ Physiol* 2004; 286:H1455-1460
- 439 Salloum F, Xi L, Ockaili R, et al. Delayed pharmacological preconditioning with Viagra (Sildenafil) against myocardial infarction is independent of p38 MAPK signaling pathway. *Circulation* 2002; 106:378-378
- 440 Xi L, Jarrett NC, Hess ML, et al. Essential role of inducible nitric oxide synthase in monophosphoryl lipid A-induced late cardioprotection: evidence from

- pharmacological inhibition and gene knockout mice. *Circulation* 1999; 99:2157-2163
- 441 Wang X, Fisher PW, Xi L, et al. Essential role of mitochondrial Ca²⁺-activated and ATP-sensitive K⁺ channels in sildenafil-induced late cardioprotection. *J Mol Cell Cardiol* 2008; 44:105-113
 - 442 Das A, Xi L, Kukreja RC. Phosphodiesterase-5 inhibitor sildenafil preconditions adult cardiac myocytes against necrosis and apoptosis. Essential role of nitric oxide signaling. *J Biol Chem* 2005; 280:12944-12955
 - 443 Nagy O, Hajnal A, Parratt JR, et al. Sildenafil (Viagra) reduces arrhythmia severity during ischaemia 24 h after oral administration in dogs. *Br J Pharmacol* 2004; 141:549-551
 - 444 Perez NG, Piaggio MR, Ennis IL, et al. Phosphodiesterase 5A inhibition induces Na⁺/H⁺ exchanger blockade and protection against myocardial infarction. *Hypertension* 2007; 49:1095-1103
 - 445 Reffelmann T, Kloner RA. Effects of sildenafil on myocardial infarct size, microvascular function, and acute ischemic left ventricular dilation. *Cardiovasc Res* 2003; 59:441-449
 - 446 James JF, Hewett TE, Robbins J. Cardiac physiology in transgenic mice. *Circ Res* 1998; 82:407-415
 - 447 Christensen G, Wang Y, Chien KR. Physiological assessment of complex cardiac phenotypes in genetically engineered mice. *Am J Physiol* 1997; 272:H2513-2524
 - 448 Patten RD, Hall-Porter MR. Small animal models of heart failure: development of novel therapies, past and present. *Circ Heart Fail* 2009; 2:138-144
 - 449 Nahrendorf M, Hiller KH, Hu K, et al. Cardiac magnetic resonance imaging in small animal models of human heart failure. *Med Image Anal* 2003; 7:369-375
 - 450 Slama M, Susic D, Varagic J, et al. Echocardiographic measurement of cardiac output in rats. *Am J Physiol Heart Circ Physiol* 2003; 284:H691-697
 - 451 Wan SY, Ritman EL, Higgins WE. Multi-generational analysis and visualization of the vascular tree in 3D micro-CT images. *Comput Biol Med* 2002; 32:55-71
 - 452 Smit H, Guridi RP, Guenoun J, et al. T1 mapping in the rat myocardium at 7 tesla using a modified CINE inversion recovery sequence. *J Magn Reson Imaging* 2014; 39:901-910
 - 453 Badea CT, Fubara B, Hedlund LW, et al. 4-D micro-CT of the mouse heart. *Mol Imaging* 2005; 4:110-116

- 454 Liu J, Rigel DF. Echocardiographic examination in rats and mice. *Methods Mol Biol* 2009; 573:139-155
- 455 Edler I, Hertz CH. The use of ultrasonic reflectoscope for the continuous recording of the movements of heart walls. 1954. *Clin Physiol Funct Imaging* 2004; 24:118-136
- 456 Wild JJ, Reid JM. Diagnostic use of ultrasound. *The British journal of physical medicine : including its application to industry* 1956; 19:248-257; passim
- 457 Wild JJ, Crawford HD, Reid JM. Visualization of the Excised Human Heart by Means of Reflected Ultrasound or Echography - Preliminary Report. *American Heart Journal* 1957; 54:903-906
- 458 Feigenbaum H, Feigenbaum H. Evolution of echocardiography. *Circulation* 1996; 93:1321-1327
- 459 Segal BL, Likoff W, Kingsley B. Echocardiography - Application in Mitral Valve Disease. *American Journal of Cardiology* 1966; 17:137-&
- 460 Segal BL, Likoff W, Kingsley B. Echocardiography - an Aid in Difficult Diagnostic Problems. *Circulation* 1966; 34:1211-&
- 461 Popp RL, Wolfe SB, Hirata T, et al. Estimation of Right and Left Ventricular Size by Ultrasound - a Study of Echoes from Interventricular Septum. *American Journal of Cardiology* 1969; 24:523-&
- 462 Fortuin NJ, Hood WP, Sherman ME, et al. Determination of Left Ventricular Volumes by Ultrasound. *Circulation* 1971; 44:575-&
- 463 American College of Cardiology Foundation Appropriate Use Criteria Task F, American Society of E, American Heart A, et al. ACCF/AHA/ASNC/HFSA/HRS/SCAI/SCCM/SCCT/SCMR 2011 Appropriate Use Criteria for Echocardiography. A Report of the American College of Cardiology Foundation Appropriate Use Criteria Task Force, American Society of Echocardiography, American Heart Association, American Society of Nuclear Cardiology, Heart Failure Society of America, Heart Rhythm Society, Society for Cardiovascular Angiography and Interventions, Society of Critical Care Medicine, Society of Cardiovascular Computed Tomography, and Society for Cardiovascular Magnetic Resonance Endorsed by the American College of Chest Physicians. *J Am Coll Cardiol* 2011; 57:1126-1166
- 464 Porter TR, Shillcutt SK, Adams MS, et al. Guidelines for the Use of Echocardiography as a Monitor for Therapeutic Intervention in Adults: A Report from the American Society of Echocardiography. *Journal of the American Society of Echocardiography* 2015; 28:40-56

- 465 Beigel R, Cercek B, Siegel RJ, et al. Echo-Doppler hemodynamics: an important management tool for today's heart failure care. *Circulation* 2015; 131:1031-1034
- 466 Foppa M, Rao SN, Manning WJ. Doppler echocardiography in the evaluation of a heart murmur. *JAMA* 2015; 313:1050-1051
- 467 Lang RM, Badano LP, Tsang W, et al. EAE/ASE recommendations for image acquisition and display using three-dimensional echocardiography. *J Am Soc Echocardiogr* 2012; 25:3-46
- 468 Shiota T. Role of modern 3D echocardiography in valvular heart disease. *Korean J Intern Med* 2014; 29:685-702
- 469 Sun YJ, Wang F, Zhang RS, et al. Incremental value of resting three-dimensional speckle-tracking echocardiography in detecting coronary artery disease. *Exp Ther Med* 2015; 9:2043-2046
- 470 Biswas M, Sudhakar S, Nanda NC, et al. Two- and three-dimensional speckle tracking echocardiography: clinical applications and future directions. *Echocardiography* 2013; 30:88-105
- 471 Calleja A, Poulin F, Woo A, et al. Quantitative Modeling of the Mitral Valve by Three-Dimensional Transesophageal Echocardiography in Patients Undergoing Mitral Valve Repair: Correlation with Intraoperative Surgical Technique. *J Am Soc Echocardiogr* 2015
- 472 Flachskampf FA, Wouters PF, Edvardsen T, et al. Recommendations for transoesophageal echocardiography: EACVI update 2014. *Eur Heart J Cardiovasc Imaging* 2014; 15:353-365
- 473 Mintz GS. Clinical utility of intravascular imaging and physiology in coronary artery disease. *J Am Coll Cardiol* 2014; 64:207-222
- 474 Han JK, Koo BK, Park KW, et al. Optimal intravascular ultrasound criteria for defining the functional significance of intermediate coronary stenosis: an international multicenter study. *Cardiology* 2014; 127:256-262
- 475 de Simone G, Wallerson DC, Volpe M, et al. Echocardiographic measurement of left ventricular mass and volume in normotensive and hypertensive rats. Necropsy validation. *Am J Hypertens* 1990; 3:688-696
- 476 Inaba Y, Davidson BP, Kim S, et al. Echocardiographic evaluation of the effects of stem cell therapy on perfusion and function in ischemic cardiomyopathy. *J Am Soc Echocardiogr* 2014; 27:192-199
- 477 Schwarz ER, Pollick C, Dow J, et al. A small animal model of non-ischemic cardiomyopathy and its evaluation by transthoracic echocardiography. *Cardiovasc Res* 1998; 39:216-223

- 478 Vildbrad MD, Andersen A, Andersen TK, et al. Limitations and pitfalls in measurements of right ventricular stroke volume in an animal model of right heart failure. *Physiol Meas* 2015; 36:925-937
- 479 Richardson JD, Bertaso AG, Frost L, et al. Cardiac magnetic resonance, transthoracic and transoesophageal echocardiography: a comparison of in vivo assessment of ventricular function in rats. *Lab Anim* 2013; 47:291-300
- 480 Jones EF, Harrap SB, Calafiore P, et al. Development and validation of echocardiographic methods for estimating left ventricular mass in rats. *Clin Exp Pharmacol Physiol* 1992; 19:361-364
- 481 Kiatchoosakun S, Restivo J, Kirkpatrick D, et al. Assessment of left ventricular mass in mice: comparison between two-dimensional and m-mode echocardiography. *Echocardiography* 2002; 19:199-205
- 482 Bonow RO, Maurer G, Lee KL, et al. Myocardial viability and survival in ischemic left ventricular dysfunction. *N Engl J Med* 2011; 364:1617-1625
- 483 Underwood SR, Bax JJ, vom Dahl J, et al. Imaging techniques for the assessment of myocardial hibernation. Report of a Study Group of the European Society of Cardiology. *Eur Heart J* 2004; 25:815-836
- 484 Willerson JT. Technetium-99m MIBI as a myocardial perfusion agent. *Circulation* 1990; 82:1067-1069
- 485 Taillefer R. Technetium-99m sestamibi myocardial imaging: same-day rest-stress studies and dipyridamole. *Am J Cardiol* 1990; 66:80E-84E
- 486 Rohatgi R, Epstein S, Henriquez J, et al. Utility of positron emission tomography in predicting cardiac events and survival in patients with coronary artery disease and severe left ventricular dysfunction. *Am J Cardiol* 2001; 87:1096-1099, A1096
- 487 Tamaki N, Kawamoto M, Tadamura E, et al. Prediction of reversible ischemia after revascularization. Perfusion and metabolic studies with positron emission tomography. *Circulation* 1995; 91:1697-1705
- 488 Won KS, Song BI. Recent trends in nuclear cardiology practice. *Chonnam Med J* 2013; 49:55-64
- 489 Todica A, Boning G, Lehner S, et al. Positron emission tomography in the assessment of left ventricular function in healthy rats: a comparison of four imaging methods. *J Nucl Cardiol* 2013; 20:262-274
- 490 Stegger L, Heijman E, Schafers KP, et al. Quantification of left ventricular volumes and ejection fraction in mice using PET, compared with MRI. *J Nucl Med* 2009; 50:132-138

- 491 Vanhove C, Lahoutte T, Defrise M, et al. Reproducibility of left ventricular volume and ejection fraction measurements in rat using pinhole gated SPECT. *Eur J Nucl Med Mol Imaging* 2005; 32:211-220
- 492 Hendel RC, Patel MR, Kramer CM, et al. ACCF/ACR/SCCT/SCMR/ASNC/NASCI/SCAI/SIR 2006 appropriateness criteria for cardiac computed tomography and cardiac magnetic resonance imaging: a report of the American College of Cardiology Foundation Quality Strategic Directions Committee Appropriateness Criteria Working Group, American College of Radiology, Society of Cardiovascular Computed Tomography, Society for Cardiovascular Magnetic Resonance, American Society of Nuclear Cardiology, North American Society for Cardiac Imaging, Society for Cardiovascular Angiography and Interventions, and Society of Interventional Radiology. *J Am Coll Cardiol* 2006; 48:1475-1497
- 493 Jeffery NS, Stephenson RS, Gallagher JA, et al. Micro-computed tomography with iodine staining resolves the arrangement of muscle fibres. *J Biomech* 2011; 44:189-192
- 494 Jarvis JC, Stephenson R. Studying the microanatomy of the heart in three dimensions: a practical update. *Front Pediatr* 2013; 1:26
- 495 Friedrich MG. Myocardial edema--a new clinical entity? *Nat Rev Cardiol* 2010; 7:292-296
- 496 Rudolph A, Abdel-Aty H, Bohl S, et al. Noninvasive detection of fibrosis applying contrast-enhanced cardiac magnetic resonance in different forms of left ventricular hypertrophy relation to remodeling. *J Am Coll Cardiol* 2009; 53:284-291
- 497 Vallee JP, Ivancevic MK, Nguyen D, et al. Current status of cardiac MRI in small animals. *MAGMA* 2004; 17:149-156
- 498 Gilson WD, Kraitchman DL. Cardiac magnetic resonance imaging in small rodents using clinical 1.5 T and 3.0 T scanners. *Methods* 2007; 43:35-45
- 499 Nahrendorf M, Wiesmann F, Hiller KH, et al. Serial cine-magnetic resonance imaging of left ventricular remodeling after myocardial infarction in rats. *J Magn Reson Imaging* 2001; 14:547-555
- 500 Schneider JE, Lanz T, Barnes H, et al. Ultra-fast and accurate assessment of cardiac function in rats using accelerated MRI at 9.4 Tesla. *Magn Reson Med* 2008; 59:636-641
- 501 Schneider JE, Cassidy PJ, Lygate C, et al. Fast, high-resolution in vivo cine magnetic resonance imaging in normal and failing mouse hearts on a vertical 11.7 T system. *J Magn Reson Imaging* 2003; 18:691-701

- 502 Price AN, Cheung KK, Cleary JO, et al. Cardiovascular magnetic resonance imaging in experimental models. *Open Cardiovasc Med J* 2010; 4:278-292
- 503 Bellenger NG, Marcus NJ, Rajappan K, et al. Comparison of techniques for the measurement of left ventricular function following cardiac transplantation. *J Cardiovasc Magn Reson* 2002; 4:255-263
- 504 Bellenger NG, Marcus NJ, Davies C, et al. Left ventricular function and mass after orthotopic heart transplantation: a comparison of cardiovascular magnetic resonance with echocardiography. *J Heart Lung Transplant* 2000; 19:444-452
- 505 Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986; 1:307-310
- 506 Saleh MG, Sharp SK, Alhamud A, et al. Long-term left ventricular remodelling in rat model of nonreperfused myocardial infarction: sequential MR imaging using a 3T clinical scanner. *J Biomed Biotechnol* 2012; 2012:504037
- 507 Ebert SN, Taylor DG, Nguyen HL, et al. Noninvasive tracking of cardiac embryonic stem cells in vivo using magnetic resonance imaging techniques. *Stem Cells* 2007; 25:2936-2944
- 508 Jones JR, Mata JF, Yang Z, et al. Left ventricular remodeling subsequent to reperfused myocardial infarction: evaluation of a rat model using cardiac magnetic resonance imaging. *J Cardiovasc Magn Reson* 2002; 4:317-326
- 509 Franco F, Dubois SK, Peshock RM, et al. Magnetic resonance imaging accurately estimates LV mass in a transgenic mouse model of cardiac hypertrophy. *Am J Physiol* 1998; 274:H679-683
- 510 Sechtem U, Pflugfelder PW, Gould RG, et al. Measurement of right and left ventricular volumes in healthy individuals with cine MR imaging. *Radiology* 1987; 163:697-702
- 511 Bellenger NG, Davies LC, Francis JM, et al. Reduction in sample size for studies of remodeling in heart failure by the use of cardiovascular magnetic resonance. *J Cardiovasc Magn Reson* 2000; 2:271-278
- 512 Katz J, Milliken MC, Stray-Gundersen J, et al. Estimation of human myocardial mass with MR imaging. *Radiology* 1988; 169:495-498
- 513 Soliman OI, Kirschbaum SW, van Dalen BM, et al. Accuracy and reproducibility of quantitation of left ventricular function by real-time three-dimensional echocardiography versus cardiac magnetic resonance. *Am J Cardiol* 2008; 102:778-783

- 514 Pattynama PM, Lamb HJ, van der Velde EA, et al. Left ventricular measurements with cine and spin-echo MR imaging: a study of reproducibility with variance component analysis. *Radiology* 1993; 187:261-268
- 515 Arias T, Chen J, Fayad ZA, et al. Comparison of echocardiographic measurements of left ventricular volumes to full volume magnetic resonance imaging in normal and diseased rats. *J Am Soc Echocardiogr* 2013; 26:910-918
- 516 Stuckey DJ, Carr CA, Tyler DJ, et al. Cine-MRI versus two-dimensional echocardiography to measure in vivo left ventricular function in rat heart. *NMR Biomed* 2008; 21:765-772
- 517 Nosir YF, Fioretti PM, Vletter WB, et al. Accurate measurement of left ventricular ejection fraction by three-dimensional echocardiography. A comparison with radionuclide angiography. *Circulation* 1996; 94:460-466
- 518 Dawson D, Lygate CA, Saunders J, et al. Quantitative 3-dimensional echocardiography for accurate and rapid cardiac phenotype characterization in mice. *Circulation* 2004; 110:1632-1637
- 519 Reffelmann T, Kloner RA. Transthoracic echocardiography in rats. Evaluation of commonly used indices of left ventricular dimensions, contractile performance, and hypertrophy in a genetic model of hypertrophic heart failure (SHHF-Mcc-facp-Rats) in comparison with Wistar rats during aging. *Basic Res Cardiol* 2003; 98:275-284
- 520 Tournoux F, Petersen B, Thibault H, et al. Validation of noninvasive measurements of cardiac output in mice using echocardiography. *J Am Soc Echocardiogr* 2011; 24:465-470
- 521 Semelka RC, Tomei E, Wagner S, et al. Normal left ventricular dimensions and function: interstudy reproducibility of measurements with cine MR imaging. *Radiology* 1990; 174:763-768
- 522 Grothues F, Smith GC, Moon JC, et al. Comparison of interstudy reproducibility of cardiovascular magnetic resonance with two-dimensional echocardiography in normal subjects and in patients with heart failure or left ventricular hypertrophy. *Am J Cardiol* 2002; 90:29-34
- 523 Jenkins C, Bricknell K, Chan J, et al. Comparison of two- and three-dimensional echocardiography with sequential magnetic resonance imaging for evaluating left ventricular volume and ejection fraction over time in patients with healed myocardial infarction. *Am J Cardiol* 2007; 99:300-306
- 524 Tremoleda JL, Kerton A, Gsell W. Anaesthesia and physiological monitoring during in vivo imaging of laboratory rodents: considerations on experimental outcomes and animal welfare. *EJNMMI Res* 2012; 2:44

- 525 Myerson SG, Montgomery HE, World MJ, et al. Left ventricular mass: reliability of M-mode and 2-dimensional echocardiographic formulas. *Hypertension* 2002; 40:673-678
- 526 Myerson SG, Bellenger NG, Pennell DJ. Assessment of left ventricular mass by cardiovascular magnetic resonance. *Hypertension* 2002; 39:750-755
- 527 Germain P, Roul G, Kastler B, et al. Inter-study variability in left ventricular mass measurement. Comparison between M-mode echography and MRI. *Eur Heart J* 1992; 13:1011-1019
- 528 Mor-Avi V, Sugeng L, Weinert L, et al. Fast measurement of left ventricular mass with real-time three-dimensional echocardiography: comparison with magnetic resonance imaging. *Circulation* 2004; 110:1814-1818
- 529 Manning WJ, Wei JY, Fossel ET, et al. Measurement of left ventricular mass in rats using electrocardiogram-gated magnetic resonance imaging. *Am J Physiol* 1990; 258:H1181-1186
- 530 Pouleur AC, le Polain de Waroux JB, Pasquet A, et al. Assessment of left ventricular mass and volumes by three-dimensional echocardiography in patients with or without wall motion abnormalities: comparison against cine magnetic resonance imaging. *Heart* 2008; 94:1050-1057
- 531 Matheijssen NA, Baur LH, Reiber JH, et al. Assessment of left ventricular volume and mass by cine magnetic resonance imaging in patients with anterior myocardial infarction intra-observer and inter-observer variability on contour detection. *Int J Card Imaging* 1996; 12:11-19
- 532 Baur LH, Schipperheyn JJ, van der Velde EA, et al. Reproducibility of left ventricular size, shape and mass with echocardiography, magnetic resonance imaging and radionuclide angiography in patients with anterior wall infarction. A plea for core laboratories. *Int J Card Imaging* 1996; 12:233-240
- 533 Lorenz CH, Walker ES, Morgan VL, et al. Normal human right and left ventricular mass, systolic function, and gender differences by cine magnetic resonance imaging. *J Cardiovasc Magn Reson* 1999; 1:7-21
- 534 Abbott CP, Lindsey ES, Creech O, Jr., et al. A Technique for Heart Transplantation in the Rat. *Arch Surg* 1964; 89:645-652
- 535 Ono K, Lindsey ES. Improved technique of heart transplantation in rats. *J Thorac Cardiovasc Surg* 1969; 57:225-229
- 536 Heron I. A technique for accessory cervical heart transplantation in rabbits and rats. *Acta Pathol Microbiol Scand A* 1971; 79:366-372
- 537 Rao VK, Lisitza M. Accessory heart transplantation to groin in the rat. A new model for retransplantation experiments. *Transplantation* 1985; 40:567-569

- 538 Ma Y, Wang G. Comparison of 2 heterotopic heart transplant techniques in rats: cervical and abdominal heart. *Exp Clin Transplant* 2011; 9:128-133
- 539 Schmid C, Binder J, Heemann U, et al. Successful heterotopic heart transplantation in rat. *Microsurgery* 1994; 15:279-281
- 540 Capuano LG, Vellucci R, Toppi L, et al. [Heterotopic heart transplantation in the rat: a preliminary study]. *Ann Ital Chir* 1998; 69:351-356
- 541 Demirsoy E, Arbatli H, Korkut AK, et al. A new technique for abdominal heart transplantation in rats. *J Cardiovasc Surg (Torino)* 2003; 44:747-750
- 542 Araneda D, Padberg WM, Tilney NL. Refined techniques for heterotopic cardiac transplantation in the rat. *Transplant Proc* 1989; 21:2562
- 543 Wang D, Opelz G, Terness P. A simplified technique for heart transplantation in rats: abdominal vessel branch-sparing and modified venotomy. *Microsurgery* 2006; 26:470-472
- 544 Shan J, Huang Y, Feng L, et al. A modified technique for heterotopic heart transplantation in rats. *J Surg Res* 2010; 164:155-161
- 545 Wu YJ, Sato K, Ye Q, et al. MRI investigations of graft rejection following organ transplantation using rodent models. *Methods Enzymol* 2004; 386:73-105
- 546 Wu YL, Ye Q, Sato K, et al. Noninvasive evaluation of cardiac allograft rejection by cellular and functional cardiac magnetic resonance. *JACC Cardiovasc Imaging* 2009; 2:731-741
- 547 Baxter K, Howden BO, Jablonski P. Pretransplant rinse of hearts preserved with colloid-free UW solution and more effective heart preservation: studies in a rat abdominal heart transplant model. *Transplantation* 2002; 73:23-31
- 548 Bellenger NG, Grothues F, Smith GC, et al. Quantification of right and left ventricular function by cardiovascular magnetic resonance. *Herz* 2000; 25:392-399
- 549 Bellenger NG, Gatehouse PD, Rajappan K, et al. Left ventricular quantification in heart failure by cardiovascular MR using prospective respiratory navigator gating: comparison with breath-hold acquisition. *J Magn Reson Imaging* 2000; 11:411-417
- 550 Ledingham SJ, Braimbridge MV, Hearse DJ. The St. Thomas' Hospital cardioplegic solution. A comparison of the efficacy of two formulations. *J Thorac Cardiovasc Surg* 1987; 93:240-246
- 551 Cordell AR. Milestones in the development of cardioplegia. *Ann Thorac Surg* 1995; 60:793-796

- 552 Wahlberg JA, Southard JH, Belzer FO. Development of a cold storage solution for pancreas preservation. *Cryobiology* 1986; 23:477-482
- 553 Ploeg RJ, van Bockel JH, Langendijk PT, et al. Effect of preservation solution on results of cadaveric kidney transplantation. The European Multicentre Study Group. *Lancet* 1992; 340:129-137
- 554 Mahaffey KW, Granger CB, Nicolau JC, et al. Effect of pexelizumab, an anti-C5 complement antibody, as adjunctive therapy to fibrinolysis in acute myocardial infarction: the COMPLEMENT inhibition in myocardial infarction treated with thromboLYtics (COMPLY) trial. *Circulation* 2003; 108:1176-1183

APPENDIX A – Other publications during candidature

Published manuscripts

Kumarasinghe G, Lavee O, Parker A, Nivison-Smith I, Milliken S, Dodds A, Joseph J, Fay K, Ma DD, Malouf M, Plit M, Havryk A, Keogh AM, Hayward CS, Kotlyar E, Jabbour A, Glanville AR, Macdonald PS, Moore JJ. Post-transplant lymphoproliferative disease in heart and lung transplantation: defining risk and prognostic factors. *J Heart Lung Transplant*. 2015 Jun 11. pii S1053-2498(15)01295-4. doi:10.1016/j.healun.2015.05.021 [Epub ahead of print]

Published abstracts

Kumarasinghe G, Lavee O, Nivison-Smith I, Parker A, Malouf A, Keogh S, Milliken A, Dodds M, Plit M, Hayward C, Ma K, Fay K, Joseph J, Kotlyar E, Havryk A, Jabbour A, Glanville A, Macdonald P, Moore J. Defining prognostic markers in heart and lung transplant recipients with PTLD. *J Heart Lung Transplant*. 2014;33(4):S84

Kumarasinghe G, Lavee O, Parker A, Keogh A, Hayward C, Kotlyar E, Jabbour A, Havryk M, Malouf M, Plit M, Glanville P, Macdonald P, Moore J. Risk factors and response to therapy in heart and lung transplant recipients with post-transplant lymphoproliferative disease. *J Heart Lung Transplant*. 2013;32(4):S254

Kumarasinghe G, Hunter T, Robson D, Prichard R, Kotlyar E, Granger E, Jansz P, Dhital K, Spratt P, Keogh A, Macdonald P, Jabbour A, Hayward C. Continuous flow left ventricular assist devices improve survival in reactive but not fixed type 2 pulmonary hypertension compared with medical therapy. *J Heart Lung Transplant*. 2013;32(4):S232-233

Kumarasinghe G, Hayward C, Macdonald P, Keogh A, Kotlyar E, Granger E, Dhital K, Jansz P, Spratt P, Jabbour A. Continuous flow left ventricular assist devices improve survival in reactive but not fixed type 2 pulmonary hypertension. *Heart Lung Circulation*. 2013;22, Supp 1:S73