The role of myocardial membrane proteins and myocardial oedema in postoperative myocardial dysfunction

Jonathan Rogers Egan

MBBS BSci(Med) FRACP FJFICM

This thesis is submitted to the University of Sydney in fulfillment of the requirements for the degree of Doctor of Philosophy

Kids Heart Research,

The Children's Hospital at Westmead, Westmead, NSW, Australia,

Institute for Neuromuscular Research,

The Children's Hospital at Westmead, Westmead, NSW, Australia,

Discipline of Paediatrics and Child Health, Faculty of Medicine,

The University of Sydney, Sydney, NSW, Australia

February 2009

STATEMENT OF ORIGINALITY

The contents of this thesis have not been presented for the award of a degree or diploma at this or any other university. The data presented are the original work of the author except where specifically indicated in the text.

Jonathan Rogers Egan

ACKNOWLEDGMENTS

I would like to thank Associate Professor David Winlaw for his support and encouragement throughout my candidature. The project has been extremely rewarding and enlightening in terms of what is required to undertake basic science research, I have greatly appreciated the opportunity and experience.

I would also like to thank my co–supervisors Professor Kathryn North and Dr Tanya Butler for their guidance and support. Kathy has always been welcoming, enthusiastic and supportive throughout my candidature. Tanya has essentially taught me everything molecular that I have ever done, she has been a great teacher and I owe an enormous debt to her in terms of what she has taught me.

I am indebted to all of the laboratory members of Kids Heart Research and the Institute for Neuromuscular Research. I am very thankful to Rachel Peat, who kindly proofread the final thesis draft. I am especially grateful to Carol Au who has also taught me a great deal of science and helped me trouble-shoot laboratory issues. The laboratory assistance of Yee-Mun Tan and Fabian Graf was also much appreciated. Alison Compton, Nan Yang and Sandra Cooper have also given me much assistance. Our collaboration with Professor Alan Verkman was of enormous assistance experimentally. The laboratory of John Headrick and in particular Melissa Reichelt, greatly assisted in fast tracking our isolated heart capabilities. Ju Chiat Tan and Aisling McMahon, formerly of the Victor Chang Cardiac Research Institute were also of great assistance in facilitating our initial investigations using isolated cardiomyocytes.

The sheep experimental work would not have been possible without the assistance of Anne O'Neill, Leanne Mills, Ross Matthews, Craig Godfrey and in particular the logistic and planning support of Andrew Cole who always happily worked above and beyond what was expected. Thanks to the Children's Hospital animal handling staff for all their assistance and hardwork, to Hao Xu Lu, Agnes Ashley and Megan Cameron. Behind the scenes Trish McGregor, Murray Hinder, Aysen Yuksel, Jacqueline Mills and the staff

from inhalational therapy at the Children's Hospital at Westmead were invaluable. With equipment and laboratory needs Jim Pouliopoulos, Pramesh Kovoor, Gary Sholler, John Dittmer, Greg Craig, Gary Ma, Stephen Li, Boyd Webster and John Coakley were very helpful and supportive. The anaesthetic department at the Children's Hospital at Westmead were particularly supportive and instrumental in allowing us to look after the sheep so well. Thank-you to David Baines, Neil Street, Manchula Navaratnam, Sarah Johnston and Lance Thompson. Likewise the perfusion department of the Adolph Basser Cardiac Institute proved integral to the undertaking of these experiments, thank-you to John Dittmer, Oliver Beiker, Carla Zazulak and Alexander Guecheff. The cardiothoracic surgical staff were also of invaluable assistance under the guidance of David Winlaw, thank-you to Avetis Aharonyan, Smartin Abraham and John Murala. Thank-you also for the support of Cheryl Jones, Russell Dale, Sandra Harris and Denise Yuille throughout my candidature. Associate Professor Jenny Peat was a wonderful and reliable resource, guiding me through the statistical aspects of the research – despite her abhorrence of animal research, she was always patient and most helpful. I would also like to thank my colleagues in the paediatric intensive care unit for their patience whilst I have been undertaking the PhD.

Thank-you to the NHMRC for the financial support during my candidature.

Finally I am entirely indebted to the unending encouragement, love and support shown to me by my wife Justine and our wonderful children – Rachael, Kate and Henry.

ABSTRACT

The vast majority of children undergoing surgical repair of cardiac lesions do spectacularly well. However a significant proportion, ~ 25%, struggle to progress in the early postoperative period and require additional pharmacological and occasionally mechanical circulatory support. All children typically have some degree of postoperative myocardial dysfunction, with the severe spectrum termed the low cardiac output state (LCOS). LCOS is clinically defined as the requirement for new or escalated inotrope therapy, a widened arteriovenous oxygen difference, cardiac arrest or the need for reinstitution of mechanical circulatory support. LCOS is largely responsible for the morbidity and mortality involved in paediatric cardiac surgery. Despite the predictability of LCOS in the initial postoperative hours, the underlying pathophysiology remains unclear. The period of decline in cardiac function that typifies LCOS is temporally associated with the development of oedema in the tissues of the body, including the heart. This relationship between oedema and dysfunction has increasingly become blurred, with a tendency to elevate the temporal association to a causal link. We sought to explore the causes and contributions to myocardial dysfunction in this setting, including the roles of oedema and ischaemia within the heart. In focusing on oedema and ischaemia we also examined the effects of these insults on relevant myocardial membrane proteins, including those that permit rapid water transport – aquaporins (AQPs), and those involved in membrane mechanics – dystrophin, and membrane repair – dysferlin. Experimental settings which enabled the *in vitro* dissection of these insults and proteins of interest were combined with a clinically accurate *in vivo* model.

This thesis describes a series of thematically linked experiments that examined LCOS, myocardial oedema and the role of various membrane proteins. We performed isolated cardiomyocyte studies, isolated heart studies as well as a clinically relevant large animal (lamb) cardiopulmonary bypass (CPB) model. Across these models we also explored the role of therapeutically protecting myocardial membranes with Poloxamer 188 (P188) and assessed any influence on myocardial function, oedema and membrane proteins.

The results from these three models suggest that the clinically accepted dogma of a causative link between myocardial oedema and dysfunction overstates the contribution of myocardial oedema to LCOS. We found that ischaemia/reperfusion was of primary importance in causing myocardial dysfunction. Myocardial oedema without ischaemia had a mild and reversible contribution to myocardial dysfunction, but this was minor in comparison to the gross dysfunction attributable to ischaemia. Isolated cardiomyocytes, with induced oedema, functioned well. Whilst ischaemic cardiomyocytes, with less swelling still had severe contractile dysfunction. Isolated hearts, perfused with an oedema inducing crystalloid perfusate developed myocardial oedema and had minimal reversible systolic and diastolic dysfunction. Isolated hearts which experienced global ischaemia had comparable degrees of myocardial oedema, and significantly greater degrees of myocardial dysfunction that increased in severity with increasing duration of ischaemia. In the lamb CPB model, only those lambs which underwent aortic cross clamping and had a period of ischaemia had poor myocardial function. These lambs also had swollen hearts, raised myocardial AQP1 mRNA and reduced membrane dysferlin protein expression. Membrane dystrophin protein expression was not altered, somewhat unexpectedly with CPB with or without ischaemia. Lambs placed on CPB without ischaemia had good myocardial function, minimal oedema and unchanged membrane protein expression during the survival period. In a blinded lamb CPB trial of P188 there were improved haemodynamics and indicies of myocardial function associated with its use. This was also associated with preservation of dysferlin expression and reduced membrane injury. In parallel isolated heart trials of this therapy, there was a reduction in myocardial oedema associated with its use in non-ischaemic experiments. There was also a suggestion of improved diastolic function in ischaemic experiments, but no change in myocardial water content.

In conclusion, we have highlighted the primacy of ischaemia/reperfusion over oedema in contributing to LCOS. We have refuted the accepted dogma that myocardial oedema causes significant dysfunction in itself, with important oedema likely to result from ischaemia. We have shown that AQP1 may be involved in the pathogenesis of the capillary leak syndrome. Finally we have hinted at a role for prophylactic P188 in the

setting of LCOS, possibly highlighting the role of membrane repair in recovery after surgery. Isolated heart trials of P188 further support a non-rheological mechanism of action and also lend support to the causal separation of myocardial oedema and dysfunction. The integral membrane protein dysferlin, rather than dystrophin, is relevant in the setting of LCOS in the current era.

PUBLICATIONS

Butler TL, Egan JR, Graf FG, Au CG, McMahon AC, North KN, Winlaw DS. Ischemia and not oedema is the key to cardiac dysfunction – analysis using isolated cardiomyocytes and Langendorff-perfused hearts. **J Thorac Cardiovasc Surg. 2009 *in press*.**

Egan JR, Butler TL, Cole AD, Abraham S, Murala J, Baines D, Street N, Thompson L, Biecker O, Dittmer J, Cooper S, Au CG, North KN, Winlaw DS. Myocardial membrane injury in pediatric cardiac surgery – an animal model. **J Thorac Cardiovasc Surg. 2009 May;137(5):1154-62.**

Egan JR, Butler TL, Cole AD, Aharonyan A, Baines D, Street N, Navaratnam M, Biecker O, Zazulak C, Au CG, Tan YM, North KN, Winlaw DS. Myocardial ischemia is more important than the effects of cardiopulmonary bypass on myocardial water handling and post operative dysfunction – pediatric animal model. **J Thorac Cardiovasc Surg. 2008 Nov;136(5):1265-73, 1273.e1–2.**

Au CG, Butler TL, Egan JR, Cooper ST, Lo HP, Compton AG, North KN, Winlaw DS, Changes in skeletal muscle expression of AQP1 and AQP4 in dystrophinopathy and dysferlinopathy. **Acta Neuropathologica 2008 116(3):235-46.**

T. L. Butler, C. G. Au, B. Yang, J. R. Egan, Y-M. Tan, E. S. Hardeman, K. N. North, A. S. Verkman, and D. S. Winlaw, Cardiac aquaporin expression in human, rat and mouse. **Am J Physiol (Heart Circ Physiol) (2006) 291(2):H705-13.**

Egan JR, Butler TL, Au CG, Tan Y-M, North KN and Winlaw DS. Myocardial water handling and the role of aquaporins(review). **BBA Biomembranes (2006) 1758(8):1043-52.**

PRESENTATIONS

J.R.Egan. Myocardial aquaporins – role in water handling and postoperative dysfunction. *Joint Children's Medical Research Institute and Children's Hospital at Westmead lunchtime Seminar Series, Children's Medical Research Institute, Aug 2006, Westmead, NSW, Australia*

J.R.Egan, T. L. Butler, C. G. Au, K. N. North, D. S. Winlaw. Myocardial Dysfunction after Pediatric Cardiac Surgery; Causes, Contributing Factors and a Potential Therapy – *presented in Kona Hawaii, at the 34th annual meeting of the Western Thoracic Surgical Association of the USA, June, 2008.*

J.R. Egan, T.L. Butler, A. Cole, D. Baines, O. Biecker, C. Zazulak, J. Dittmer, D.S.Winlaw, Loss of Membrane Dystrophin and Increased Aquaporin Expression After Bypass. Heart, Lung and Circulation 2007;16:S24 – *presented in Christchurch, NZ, August 2007, at the annual scientific meeting of the Australian and New Zealand cardiac society*

Egan JR, Butler TL, Graf FG, Au CG, Walizada G, North KN, Winlaw DS. Myocardial dysfunction after paediatric cardiac surgery; causes, contributing factors and a potential therapy. *Annual Postgraduate Student Conference, Discipline of Paediatrics and Child Health, Faculty of Medicine, University of Sydney, Australia, August 2007.*

Egan JR, Butler TL, Graf FG, Au CG, Walizada G, Winlaw DS. Aquaporins in the heart: implications for water handling after cardiac surgery – *presented in Nara, Japan at 5th World Congress of Aquaporins, July, 2007, finalist in the award session.*

Egan JR, Butler TL, Winlaw DS, The impact of ischaemia and reperfusion on membrane proteins aquaporin 1 and dystrophin – contribution to myocardial dysfunction in a lamb model of neonatal cardiopulmonary bypass surgery. Pediatr Crit Care Med 2007;8(3) Suppl. A105. *Presented in Geneva, June 2007, World congress of paediatric intensive care, finalist in the basic science award session.*

Egan JR, Butler TL, Au CG, Tan YM, Cooper S, Baines D, Street N, Navaratnam M, Cole A, Onikul E, Walker T, Aharonyan A, Biecker O, Zazulak C, Dittmer J, North KN, Winlaw DS. Upregulation of myocardial aquaporin 1 in response to ischaemia. *Annual Postgraduate Student Conference, Discipline of Paediatrics and Child Health, Faculty of Medicine, University of Sydney, Australia, August 2006. Awarded the CHW Medical Staff Council Prize for Excellence.*

Egan J.R, Butler T.L, Au C.G, North K.N, Winlaw D.S. Postoperative cardiac dysfunction – an isolated perfused heart model of myocardial stunning. *Annual Postgraduate Student Conference, Discipline of Paediatrics and Child Health, Faculty of Medicine, University of Sydney, Australia, August 2005.*

JR Egan, TL Butler, S Tandy, CG Au, JC Tan, A McMahon, KN North, DS Winlaw. Post–operative cardiac dysfunction–an *in vitro* model of cardiac oedema. "*From Cell to Society 4,"Research Conference, University of Sydney, College of Health Sciences, Leura, NSW, Australia, November 2004.*

JR Egan, TL Butler, S Tandy, CG Au, A McMahon, KN North, DS Winlaw. Cardiac dysfunction–an in vitro model of cardiac oedema. *Annual Postgraduate Student Conference, Discipline of Paediatrics and Child Health, Faculty of Medicine, University of Sydney, Australia, August 2004. Winner of the Discipline of Paediatrics and Child Health 1st year postgraduate student encouragement prize.*

RESEARCH SCHOLARSHIPS

National Health and Medical Research Council: postgraduate medical scholarship 2004–06.

TRAVEL SCHOLARSHIPS

Young Investigator Travel scholarship, 5th International conference of Aquaporins, Nara Japan, 2007.

National Heart Foundation travel grant: travel scholarship to attend and present at 5th International conference of Aquaporins, Nara, Japan, 2007.

Cardiac Society of Australia and New Zealand: travel scholarship to attend and present at Annual Scientific Conference, Christchurch New Zealand, 2007.

ABBREVIATIONS

ACT	Activated clotting time
AQP	Aquaporin
AQP1	Aquaporin 1
AXC	Aortic cross clamp
BTS	Blalock Tausig shunt
CK	Creatinine kinase
COP	Colloid osmotic pressure
CPB	Cardiopulmonary bypass
CT	Cycle threshold
DAP	Dystrophin-associated protein
DBP	Diastolic blood pressure
DHCA	Deep hypothermic circulatory arrest
DMD	Duchenne muscular dystrophy
Dp/Dt_{min}	Change in pressure over change in time – minimum
ECMO	Extracorporeal membrane oxygenation
Hct	Haematocrit
HLHS	Hypoplastic left heart syndrome
IHC	Immunohistochemistry
I/R	Ischaemia reperfusion
IPC	Ischaemic preconditioning
LCOS	Low cardiac output state
LGMD	Limb girdle muscular dystrophy
LVDP	Left ventricle developed pressure
LVDP-D	Left ventricle developed diastolic pressure
LVDP-S	Left ventricle developed systolic pressure
MBP	Mean blood pressure
mPTP	mitochondrial permeability transition pore
MRI	Magnetic resonance imaging
MUF	Modified ultrafiltration

MWC	Myocardial water content
MWU	Mann-Whitney U test
NO^{-}	Nitric oxide
NPA	Asparagine-Proline-Alanine
PBS	Phosphate buffered saline
P188	Poloxamer 188
PFTE	Polytetrafluoroethylene (Gore-Tex™)
qRT-PCR	Quantitative reverse transcriptase – polymerase chain reaction
RT-PCR	Reverse transcriptase – polymerase chain reaction
SBP	Systolic blood pressure
SIRS	Systemic inflammatory response syndrome
TGA	Transposition of the great arteries
VAD	Ventricular assist device
VCFS	Velocardiofacial syndrome
VSD	Ventricular septal defect
WCL	Whole cell lysate

TABLE OF CONTENTS

STATEMENT OF ORIGINALITY…….……....ii
ACKNOWLEDGMENTS…….……iii
ABSTRACT…….……...….v
PUBLICATIONS……..…...viii
PRESENTATIONS……...……..…...ix
SCHOLARSHIPS……...……..……xi
ABBREVIATIONS……..……xii
TABLE OF CONTENTS……...……xiv
LIST OF FIGURES……..……...xxi
LIST OF TABLES……...…..xxiii
CHAPTER 1: OVERVIEW & LITERATURE REVIEW 1
1.1 Overview 1
1.1.1 Background 1
1.1.2 Preoperative period 1
1.1.3 Operative period 2
1.1.4 Cardiopulmonary bypass 3
1.1.5 Deep hypothermic circulatory arrest 4
1.1.6 Postoperative period 5
1.2 Myocardial dysfunction 6
1.2.1 Reduced cardiac performance 6
1.2.2 Myocardial stunning 7
1.2.3 Association of dysfunction with oedema 7
1.3 Aquaporins 10
1.3.1 Background 10
1.3.2 Aquaporin function 10
1.3.3 Aquaporin structure 11
1.3.4 Aquaporin 1 12
1.4 Myocardial water handling 13
1.4.1 Background 13
1.4.2 Endothelium 15
1.4.3 Microvascular fluid balance 16
1.4.4 Water handling in pathological circumstances 16
1.4.5 Role of aquaporins 17
1.5 Myocardial oedema 19
1.5.1 Background 19

1.5.2 Functional consequences of myocardial oedema 19
1.5.3 Pathogenesis of myocardial oedema 20
1.5.3.1 Haemodilution 21
1.5.3.2 Ischaemia and reperfusion 22
1.5.4 Myocardial oedema and mitochondria 23
1.5.4.1 Mitochondria and aquaporins 23
1.6 Myocardial aquaporins 24
1.6.1 Background 24
1.6.2 Myocardial aquaporins and the three compartment model 24
1.6.3 Myocardial aquaporins and potassium 25
1.6.4 Aquaporin 1 and cardiac disease models 25
1.6.5 Other myocardial aquaporins 26
1.6.6 Other aquaporin roles 30
1.7 Other myocardial membrane proteins 31
1.7.1 Dystrophin 31
1.7.1.1 Dystrophin in ischaemic preconditioning 31
1.7.1.2 Dystrophin in ischaemic and dilated cardiomyopathy 32
1.7.2 Dysferlin 32
1.8 Poloxamer 188 35
1.8.1 Haemorheological studies 35
1.8.2 Membrane protection and repair studies 37
1.9 Summary 39
1.10 Specific aims of the thesis 39
CHAPTER 2: METHODS 41
2.1 Ethical approval 41
2.1.1 Human muscle specimens 41
2.1.2 Animal experiments 42
2.2 Molecular biology techniques 43
2.2.1 Neonatal cardiac myocyte preparations 43
2.2.2 Subcellular fractionation for mitochondrial preparations 43
2.2.3 Water permeability measurements 44
2.2.4 Western blot 44
2.2.5 Membrane separation technique for dystrophin, dysferlin and caspase 3 45
2.2.6 Immunohistochemistry 45
2.2.7 Quantitative RT-PCR 46
2.2.8 Apoptosis 46

2.2.9 Myocardial membrane integrity 47
2.2.9.1 Evans blue analysis 47
2.2.9.2 Creatinine kinase 47
2.2.9.3 Troponin 47
2.2.10 Myocardial water content – desiccation 48
2.2.11 Colloid osmotic pressure 48
2.3 Isolated cardiomyocytes 49
2.3.1 Introduction 49
2.3.2 Mouse cardiomyocytes at Victor Chang Cardiac Research Institute 49
2.3.2.1 Anaesthesia and perfusion 49
2.3.2.2 Digestion and preparation 50
2.3.2.3 Microscope and image capture 52
2.3.3 Rat cardiomyocytes at the Children's hospital at Westmead 54
2.3.3.1 Anaesthesia and perfusion 54
2.3.3.2 Digestion and preparation 57
2.3.3.3 Isolated cardiomyocyte functional analysis 57
2.3.3.4 Isolated cardiomyocyte experimental protocol 58
2.4 Isolated heart (Langendorff) experiments 60
2.4.1 Introduction 60
2.4.2 Isolated heart perfusion and ischaemia 60
2.4.3 Balloon design 64
2.4.4 Langendorff protocols 66
2.4.4.1 Twenty minute ischaemia experiments 66
2.4.4.2 Poloxamer 188 – low dose study 66
2.4.4.3 Poloxamer 188 – high dose study 67
2.4.4.4 Thirty minute ischaemia and hypotonic experiments 67
2.5 Lamb cardiopulmonary bypass model 69
2.5.1 Introduction 69
2.5.2 Obtaining blood for the cardiopulmonary bypass circuit 69
2.5.3 Experimental preparation 70
2.5.4 Cardiopulmonary bypass technique 72
2.5.4.1 Initial series 72
2.5.4.2 Lamb Poloxamer 188 trial 75
2.5.5 Postoperative management 76
2.5.5.1 Initial series 76
2.5.5.2 Lamb Poloxamer 188 trial 76

2.5.6 Tissue collection 77
2.5.6.1 Initial series 77
2.5.6.2 Lamb Poloxamer 188 trial 77
2.6 Statistical analysis 78
CHAPTER 3: MYOCARDIAL EXPRESSION OF AQUAPORINS 79
3.1 Myocardial aquaporins 81
3.1.1 Initial reports 81
3.1.2 Recent publications on cardiac aquaporins 81
3.2 Methods 83
3.2.1 Ischaemic rat heart study 83
3.3 Results 84
3.3.1 RT-PCR for aquaporins in the myocardium of various species 84
3.3.2 Aquaporin expression in rat tissues 85
3.3.2.1 Western blot for aquaporin 1 85
3.3.2.2 Immunohistochemistry for aquaporin 1 86
3.3.2.3 Western blot for aquaporin 4 87
3.3.2.4 Immunohistochemistry for aquaporin 4 88
3.3.2.5 Western blot for other aquaporins 89
3.3.3 Ischaemic rat heart study 90
3.3.3.1 RT-PCR and Western blot analysis 90
3.3.3.2 Quantitative real time PCR analysis 91
3.3.3.3 Relative cycle thresholds for aquaporin mRNA in rat myocardium 92
3.3.4 Aquaporin knockout tissue experiments 94
3.3.5 Sheep aquaporin expression 96
3.3.5.1 RT-PCR in sheep 96
3.3.5.2 Real time PCR in sheep 97
3.3.5.3 Western blot analysis of sheep myocardium for aquaporins 98
3.3.5.4 Immunohistochemistry of sheep muscle 100
3.3.6 Subcellular location of aquaporin 1 101
3.3.6.1 Endothelial expression 101
3.3.6.2 Mitochondrial membrane aquaporin 1 expression 102
3.3.7 Aquaporin function 102
3.4 Discussion 104
CHAPTER 4: ISOLATED CARDIOMYOCYTE EXPERIMENTS 107
4.1 Introduction 108
4.1.1 Modelling of hypotonic conditions and ischaemia 108

4.2 Methods 108
4.2.1 Statistics 108
4.3 Results 110
4.3.1 Isolated mouse cardiomyocytes 110
4.3.2 Isolated rat cardiomyocytes – hypotonic and ischaemic buffer trials 111
4.3.2.1 Cardiomyocyte size 111
4.3.2.2 Cardiomyocyte calcium handling 113
4.3.2.3 Cardiomyocyte contractility 115
4.4 Discussion 116
CHAPTER 5: ISOLATED HEART (LANGENDORFF) EXPERIMENTS 119
5.1 Introduction 120
5.2 Methods 122
5.2.1 Statistical analysis 122
5.2.2 Tissue analysis 122
5.3 Results 124
5.3.1 Twenty minute ischaemia experiments 124
5.3.1.1 Haemodynamics 124
5.3.1.2 Myocardial water 126
5.3.1.3 Molecular results 127
5.3.2 Thirty minute ischaemia experiments 128
5.3.2.1 Haemodynamics 128
5.3.2.2 Myocardial water 129
5.4 Discussion 131
CHAPTER 6: LAMB CARDIOPULMONARY BYPASS MODEL 134
6.1 Introduction 135
6.2 Methods 136
6.2.1 Study design 136
6.2.1.1 Non CPB controls 136
6.2.1.2 CPB groups 136
6.2.2 Tissue analysis 137
6.2.3 Statistical analysis 137
6.3 Results 138
6.3.1 Haemodynamics 139
6.3.2 Biochemistry 139
6.3.3 Myocardial oedema 141
6.3.4 Molecular results 142
6.3.5 Apoptosis 143

6.4 Discussion 146
6.4.1 Haemodynamics 146
6.4.2 Myocardial oedema 146
6.4.3 Ischaemia 147
6.4.4 Aquaporins 148
6.4.5 Apoptosis 149
6.4.6 Limitations 150
6.4.7 Conclusions 150
CHAPTER 7: MYOCARDIAL MEMBRANE INJURY AND POLOXAMER 188 151
7.1 Introduction 153
7.2 Methods 154
7.2.1 Isolated rat heart trials of P188 154
7.2.1.1 Low dose P188 trail 154
7.2.1.2 High dose P188 trial 154
7.2.2 Lamb CPB trial of P188 154
7.2.2.1 Tissue analysis 156
7.2.2.2 Statistical analysis 156
7.3 Results 157
7.3.1 Isolated heart trials 157
7.3.1.1 Low dose trial 157
7.3.1.2 Haemodynamics 157
7.3.1.3 Myocardial water 159
7.3.1.4 High dose trial 160
7.3.1.5 Haemodynamics 160
7.3.1.6 Myocardial water 162
7.3.2 Lamb CPB trial 162
7.3.2.1 Molecular results 165
7.3.2.2 Myocardial oedema 170
7.3.2.3 Biochemistry and haematology 172
7.2.2.4 Haemodynamics 172
7.4 Discussion 175
7.4.1 Isolated heart models 175
7.4.2 Lamb CPB model 176
7.4.3 Dystrophin 176
7.4.4 Dysferlin 177
7.4.5 Low cardiac output state 178
7.4.6 Dystrophin and P188 178

7.4.7 Dysferlin and P188 179
7.4.8 Other effects of P188 179
7.4.9 Aquaporins 180
7.4.10 Limitations 180
CHAPTER 8: CONCLUSIONS 182
8.1 Key findings and future work 182
REFERENCES: 185

LIST OF FIGURES

1.1	Myocardial compartments	14
2.1	VCCRI cardiomyocyte isolation set up	51
2.2	VCCRI IonOptix isolation cardiomyocyte set up	52
2.3	The Children's hospital at Westmead, isolated rat cardiomyocyte set up	56
2.4	The Children's hospital at Westmead, IonOptix set up for cardiomyocyte imaging	58
2.5	Langendorff isolated heart	62
2.6	Rat Langendorff set up	63
2.7	Crystalloid perfusate being gassed	64
2.8	A distended latex balloon	65
2.9	Rat heart, with cannula and left ventricle balloon in situ	65
2.10	Sheep blood cardioplegia being cooled to 4°C	70
2.11	Lamb anaesthesia	71
2.12	Invasive lines in the sheep	71
2.13	Lamb experiment utilising CPB	73
2.14	Open lamb chest with venous and arterial cannula in situ	74
2.15	The operating theatre setup at the Vivarium	74
3.1	Myocardial aquaporin expression by RT-PCR	84
3.2	Western blot for aquaporin 1	85
3.3	Rat muscle immunohistochemistry	86
3.4	Western blot for aquaporin 4	87
3.5	Rat muscle immunohistochemistry	88
3.6	Western blot for aquaporin 7	89
3.7	Western blot and RT-PCR of aquaporin 1 and aquaporin 4 in ischaemic myocardium	90
3.8	Aquaporin 4 mRNA cycle threshold output from qRT-PCR	91
3.9	Rat myocardial aquaporin 1 and aquaporin 4 mRNA	92
3.10	Western blot utilising knockout tissue for aquaporin 1	94
3.11	Western blot utilising knockout tissue for aquaporin 4	95
3.12	Sheep aquaporin RT-PCR	96
3.13	Western blot analysis of sheep organ tissues with aquaporin 1 antibody	98
3.14	Western blot analysis of sheep organ tissues with aquaporin 4 antibody	99
3.15	Immunohistochemistry of sheep muscle	100
3.16	Endothelial and non-endothelial aquaporin 1	101
3.17	Western blot of plasma and mitochondrial membranes	102

3.18 Myocardial membrane water permeability in mouse 103

4.1 Isolated cardiomyocyte experiment schema summary 109
4.2 Mouse cardiomyocyte hypotonic buffer trial 110
4.3 Rat cardiomyocyte size 112
4.4 Calcium and contractility in rat cardiomyocytes 114

5.1 Isolated heart experiments – protocol summary 123
5.2 Ischaemic treatment of rat heart 125
5.3 Myocardial water content for isolated rat hearts in various allocations 126
5.4 Aquaporin 1 expression 127
5.5 Left ventricular developed pressure 130
5.6 Heart water percentage increase 130

6.1 Haemodynamic and biochemical variables 140
6.2 Myocardial water 141
6.3 Myocardial aquaporin 1 transcript expression 142
6.4 Myocardial aquaporin 1 protein expression 143
6.5 Myocardial apoptosis 144

7.1 P188 experiments – protocol summary 155
7.2 Left ventricular developed pressure(LVDP) in non-ischaemic rat isolated hearts 158
7.3 Left ventricular developed pressure(LVDP) in ischaemic rat isolated hearts 158
7.4 Myocardial water content for isolated rat hearts 159
7.5 Left ventricular developed pressure(LVDP) – systolic ±P188 160
7.6 Left ventricular developed pressure(LVDP) – diastolic ±P188 161
7.7 Contractility 161
7.8 Myocardial membrane fraction densitometry 166
7.9 Myocardial membrane dystrophin and dysferlin expression 167
7.10 Myocardial aquaporin 1 protein expression 168
7.11 Active caspase 3 protein expression 169
7.12 Measures of colloid osmotic pressure and haemoglobin 171
7.13 Haemodynamic and biochemical variables 173

LIST OF TABLES

1.1	Myocardial aquaporin expression	28
3.1	Outline of *in vitro* rat myocardium ischaemia study	83
3.2	Cycle thresholds – rat.	93
3.3	Cycle thresholds – sheep.	97
6.1	Baseline biochemical variables	138
7.1	Various measurements pre and post CPB	163
7.2	Haemodynamic and laboratory variables post CPB	164

CHAPTER 1: OVERVIEW & LITERATURE REVIEW

1.1 Overview

1.1.1 Background

One percent of children born each year in Australia have congenital heart disease, e.g. a "hole in the heart," or ventricular septal defect (VSD). A smaller number of children develop acquired cardiac disease – such as rheumatic heart disease – which is more prevalent amongst Aboriginal children and in children from the neighbouring south-western pacific rim. A significant number of these infants and children require cardiac surgery to repair or palliate these congenital and acquired cardiac lesions. Typically such operations proceed well, the postoperative course is uncomplicated and children recover rapidly to lead essentially normal lives. For instance, about 450 children per year have cardiac surgery at The Children's Hospital at Westmead, Sydney, Australia and the mortality rate is ~1–2%.

1.1.2 Preoperative period

The field of paediatric cardiac surgery has developed alongside adult practice as a highly specialised and robust field in its own right (Freedom, 2000). Operative repair is the culmination of the work of a diverse team of skilled professionals. Initially paediatric physicians or cardiologists diagnose the cardiac condition – now often done in utero or antenatally to facilitate delivery in a tertiary centre capable of managing an ill neonate. Following clinical and echocardiographic (ultrasound) diagnosis and initial stabilisation, an operation may or may not be indicated at some stage. Further diagnostic information may be required via a cardiac catheter. Cardiac catheter insertion is performed under general anaesthetic and provides images that document anatomical blood flows or shunts, as well as pressure and biochemical measurements. This information is important in complex conditions before surgery to determine the best management. An important initial consideration which may arise is whether it is possible to repair the heart in the

usual two ventricle fashion, or whether a single ventricle repair will be required, typically culminating in a Fontan repair (Fontan and Baudet, 1971). Magnetic resonance imaging (MRI) is increasingly being used together with computerised tomography (CT) angiography to obtain further information on cardiac anatomical considerations. Following the collection and synthesis of diagnostic information it is typical that a period of medical therapy is instituted – this may be for weeks, months or years, prior to proceeding to an operation. Alternatively it may be necessary to proceed to operative repair relatively promptly (2–5 days), based on clinical and echocardiographic findings – for instance in the case of transposition of the great arteries (TGA) or in hypoplastic left heart syndrome (HLHS).

1.1.3 Operative period

Following the initial period of diagnosis and stabilisation, the patient may be referred for operative repair. Specialist paediatric anaesthetic staff provide a stable anaesthetic platform for the surgeon to conduct the operation. This includes sedative, analgesic and neuromuscular blocking drugs. Inotropes – medicines which help the heart contract and vasodilators – medicines which help the circulation relax and make it easier for the heart to pump are utilised. The child is mechanically ventilated via an endotracheal tube and also has central venous and arterial lines for monitoring and delivery of medications and fluids. Operations are broadly referred to in terms of open or closed – the heart is "opened" – e.g. VSD repair. Or the repair is "closed" or extracardiac – e.g. Blalock Tausig shunt (BTS) – which involves a modified plastic tube (PFTE) being placed between ipsilateral subclavian and branch pulmonary arteries. Open procedures invariably require CPB to be instituted to support the body's circulation whilst the heart is opened. This is what is referred to as the "heart lung machine." It is a circulatory support device that is instituted by the surgeon and together with a perfusionist is maintained during the operation. It has a number of features that enable safe support of the circulation – it temporarily replaces the native circulation taking venous deoxygenated blood from the patient, oxygenating it and then returning it at an acceptable

flow into the ascending aorta, the large blood vessel which supplies oxygenated blood to the body.

1.1.4 Cardiopulmonary bypass

In essence CPB involves an extracorporeal circuit of ~10mm plastic tubing which contains a mix of physiological fluid – crystalloid (saline) together with blood. The typical volume of this circuit is 300ml and given that a 3kg neonate has a 240ml blood volume, the circuit requires blood in order to partially support the red blood cell concentration or haematocrit (Hct) and minimise the degree of haemodilution. The tubing is connected to an oxygenator and pump, and both the temperature and chemistry of the perfusate are managed by the perfusionist. The tubing connects to the patient via cannulae in the right atrium – secured with purse string sutures. Blood enters the heart lung machine tubing from the right atrium, and then having passed through the oxygenator and pump, returns through a cannula in the proximal ascending aorta. The patient is anticoagulated prior to going onto CPB to ensure that blood does not clot within the circuit. Thus the circulation is supported and the heart can be repaired. The passage of the patient's blood via this extracorporeal circuit together with all its various pieces can create an inflammatory like reaction within the patient – raised temperature, activation of white cells and platelet dysfunction (Wan, 1997).

Typically a further important measure is involved – that is stopping or arresting the heart. This provides a non beating operative field, an empty heart and facilitates repair integrity. To achieve this the heart is arrested in diastole – the relaxed phase of the contraction cycle – as opposed to systole – the active contraction phase. A small cannula is placed in the aorta – in between the CPB aortic cannula and the aortic valve. The openings to the coronary arteries are just above the aortic valve within the aortic root. A clamp is then placed across the aorta (aortic cross clamp, AXC) – in between these two cannula. Thus the CPB circuit supplies the circulation and the heart is isolated by the clamp. Between the AXC and the aortic valve is the small cannula – into which cardioplegia (a mix of blood and crystalloid) is administered. Conventional cardioplegia has a high potassium

concentration to induce cardiac standstill or arrest. This solution is cooled and delivered into the coronary arteries via the aortic root, causing the heart to arrest within seconds. The heart is relaxed in diastole and cooled to 25–28°C to further try and preserve it whilst it is being operated upon. Cardioplegia is given by repeated small volume infusion every 20 minutes until the operative repair is complete. During this period the heart is essentially having planned and controlled ischaemia – i.e. it is not receiving the nutrients required for normal function. The inability to fully protect the heart from this planned ischaemic reperfusion injury is a major impediment to improving cardiac surgery (Dobson, 2004). At the end of the operation when the heart is restarted – by stopping the cardioplegia and rewarming – a reperfusion injury of varying degrees occurs with associated myocardial dysfunction. And although dysfunction follows reperfusion – it is ischaemia which initiates the processes within the myocardium that lead to the development of myocardial dysfunction (Kloner and Jennings, 2001).

Once the heart is rewarmed, restarted and the circulation is stable the CPB circuit can be removed from the patient. At this point anticoagulation is reversed, haemostasis achieved and if the haemodynamics permit the chest is closed. Occasionally the chest is left open if the haemodynamics are borderline or if there is appreciable chest wall oedema present in the operating theatre (Odim, 1989; Iyer, 1997; Tabbutt, 1997a). The patient is then taken to the paediatric intensive care unit for a period of recovery and restitution.

1.1.5 Deep hypothermic circulatory arrest

It is worth noting at this point that deep hypothermic circulatory arrest (DHCA) is an alternative circulatory support strategy to the combination of CPB and AXC. Aortic and right atrial cannulae as described for instituting CPB with AXC are utilised, but after establishing a core temperature of less than 20°C the heart is arrested and the circulation drained into the oxygenator. Thus the heart is empty and arrested, but the circulation is also empty permitting operations on the aortic arch. DHCA in the current era is reserved for aortic arch surgery now, rather than other operations as was previously the case. CPB has been shown to be superior to DHCA in terms of minimising neurological sequelae

and there are no important haemodynamic differences apparent in the postoperative period (Newburger, 1993; Bellinger, 1995; Wernovsky, 1995; Bellinger, 2003). Typically when DHCA is utilised selective carotid perfusion is employed using the CPB machine and appropriate cannulae.

1.1.6 Postoperative period

The postoperative cardiac surgery patient arrives in the paediatric intensive care unit with their heart now repaired, but having also undergone a significant stress. Preceding the operation were often other important factors; 1) A period of medical therapy – particularly important if there was cardiac failure which can impact growth, nutritional reserves and ability to cope postoperatively, 2) previous operations, 3) other factors such as related/unrelated syndromes e.g. Trisomy 21 or velocardiofacial syndrome (VCFS) which can affect endocrine and or immunological function. On this background is the recent operative stress. Important aspects of the operation include the occurrence and duration of CPB and aortic cross clamping – this determines the severity of any inflammatory and ischaemia/reperfusion insult respectively. Other contributions to the postoperative setting are myocardial surgical trauma and haemodilution as part of CPB.

1.2 Myocardial dysfunction

1.2.1 Reduced cardiac performance

The vast majority of patients do well following surgery. However all have a reduction or decline in cardiac performance to some degree – this is a period of recovery from the operative repair. A period of reduced myocardial performance is typified by reduced stroke volume (volume of blood ejected from the heart with each contraction), increased heart rate and lower perfusion pressure (blood pressure). A temporary and mild reduction in urine output can occur and the peripheries can be cool suggesting reduced perfusion and increased peripheral vasoconstriction (increased systemic vascular resistance – or afterload).

About 25% of patients have a more severe decline in myocardial performance loosely defined and termed the low cardiac output state (LCOS). Classically there is a 25% decline in cardiac output and a rise in systemic vascular resistance also of around 25% (Parr, 1975; Burrows, 1988; Wernovsky, 1995). These patients require careful attention and management necessitating optimisation of inotropic and vasodilator therapy and occasionally reinstitution of mechanical support (extracorporeal membrane oxygenation, ECMO or ventricular assist device, VAD) in order to maintain the circulation until the heart can recover (Wessel, 2001). LCOS is broadly defined clinically, based on the following signs and symptoms; tachycardia, oliguria, poor perfusion, or cardiac arrest with or without a widened arterial-venous oxygen saturation difference or metabolic acidosis. In addition the institution of ECMO or VAD, an increase in inotropes relative to baseline (≥100%) or the administration of a new inotrope completes the defining criteria for the LCOS (Hoffman, 2003). Onset of LCOS usually occurs 6–12hrs postoperatively and it causes the majority of morbidity and mortality in the current era. Although the impact of LCOS is lessened by early supportive measures, such as milrinone (Primacor, Sanofi-Aventis, Macquarie Park, NSW, Australia) – an inotrope which is used in the early recovery period, the precise aetiology of LCOS remains unclear (Hoffman, 2003).

1.2.2 Myocardial stunning

The temporary diminishment in cardiac performance is largely predictable after cardiac surgery. The specific decline in cardiac output should be referred to as myocardial stunning and is largely responsible for the myocardial component of LCOS. Other features of LCOS are essentially reactionary – i.e. the increase in systemic vascular resistance is likely a response to reduced perfusion pressure (Wernovsky, 1995; Hoffman, 2003; Stocker, 2007). The term myocardial stunning, has been used to describe a period of myocardial hypocontractility despite normal coronary perfusion (Braunwald and Kloner, 1982). It is well described in adults (Breisblatt, 1990; Royster, 1993), but poorly in paediatrics (Booker, 1998). The resulting hypoperfusion of important organs including kidney and brain accounts for the majority of morbidity in current adult and paediatric cardiac surgical practice (Parr, 1975; Wernovsky, 1995; O'Connor, 1998). Stunning is considered to be mainly the result of ischaemia reperfusion (I/R) injury – the duration and severity of ischaemia determining the degree of stunning which then becomes apparent following reperfusion. Stunning also involves a degree of myocardial oedema (swelling of the tissues due to water) within the heart (Bolli and Marban, 1999). Induction of apoptotic pathways has also been suggested as a mechanism of cell loss contributing to myocardial dysfunction in the paediatric setting (Caldarone, 2004). The key mediators of injury are currently felt to be oxygen derived free radicals and calcium overload resulting in injury to myocellular components of the contractile apparatus (Bolli and Marban, 1999). Myocardial stunning is associated with cell swelling, altered mitochondrial morphology and function (Bolli and Marban, 1999; Akao, 2003). Haemodilution, which results mainly from the use of CPB has also been temporarily associated with myocardial dysfunction following CPB and its minimisation has been associated with improved outcomes (Jonas, 2003).

1.2.3 Association of dysfunction with oedema

LCOS occurs during the early postoperative period and is temporally associated with increased total body water and generalised tissue oedema (Wernovsky, 1995;

Hamada, 2004). The increase in total body water and tissue oedema is coincident with the predictable decline in cardiac output, leading many to speculate that oedema and myocardial dysfunction are causally related (Maehara, 1991; Wernovsky, 1995; Hamada, 2004). This increase in tissue oedema is often termed "capillary leak syndrome". This syndrome is well described following cardiac surgery, particularly in neonates and infants undergoing corrective surgery for congenital heart disease. It has been described as an 'endotheliopathy' and attributed to an increase in vessel permeability, induced by a systemic inflammatory process initiated by exposure of blood to foreign surfaces of the CPB circuit (Edmunds, 1998). In adults, capillary leak accounts for a 2–5% gain in extracellular fluid volume peaking at four hours post CPB (Hamada, 2004), again corresponding to the peak of myocardial dysfunction (Mangano, 1985; Breisblatt, 1990). The occasional practice of delayed sternal (chest) closure in the setting of paediatric cardiac surgery is a result of concerns relating to a swollen (oedematous) myocardium exacerbating early postoperative dysfunction through relative "tamponade' within the closed chest (Odim, 1989; Tabbutt, 1997a).

The temporal association of LCOS related dysfunction and increased total body and myocardial water has led to an accepted clinical dogma that LCOS and the oedema that occurs in the myocardium and throughout the body are causally linked. Recent examples of this include the following;

> "Capillary leak and oedema associated with cardiopulmonary bypass continue into the postoperative period and can compromise myocardial function in infants and children" (Tabbutt, 1997a)
>
> "It is well understood that the capillary leak syndrome developing in the early postoperative course will aggravate myocardial oedema and myocardial dysfunction." (Kostelka, 2005)
>
> "Our study (on aprotinin) has important implications in the development of molecular strategies to prevent tissue oedema and myocardial dysfunction, particularly in the setting of cardiac surgery." (Khan, 2005)

Contrary to these views however, in a large neonatal trial of DHCA versus CPB it was shown that despite differences in postoperative water balance there were no differences in haemodynamic function;

> "Despite the increased total body oedema (associated with CPB), there was no significant effect on postoperative haemodynamics, laboratory studies, duration of mechanical ventilation, length of stay in the ICU or hospital." (Wernovsky, 1995)

We sought to unravel this linking of dysfunction and water accumulation, therefore we reviewed water transport in the heart and how this may vary in pathological circumstances. Concurrently we explored the proteins within the myocardial membranes and the potential role of transcellular water movement mediated by aquaporins (AQPs). Subsequently we explored AQP related proteins, dystrophin and dysferlin, which additionally have important functions related to their membrane location and have not been explored in this setting. Existing anti–inflammatory therapies, such as corticosteroids and aprotinin that seek to target the CPB related systemic inflammatory response syndrome (SIRS) do not prevent the extravasation of water (Seghaye, 2003; Farstad, 2004; Tassani, 2007), leading us to investigate other possible avenues for intervention, such as the role of myocardial water channels or AQPs (Farstad, 2004; Castle, 2005).

1.3 Aquaporins

1.3.1 Background

The body consists of approximately 70% water, and AQPs in part serve as the "plumbing for cells". They are responsible for the secretion and absorption of cerebral spinal fluid, tears, saliva and the impressive concentrating ability of the kidneys (Agre, 2004). AQPs are a family of proteins that form channels across cell membranes permitting high capacity rapid movement of water along osmotic gradients. There are 13 AQPs found in mammals (AQP0 – 12), most of which permit transcellular passage of water while some also allow passage of glycerol (AQP3, 7, 9 and 10). In some circumstances, heavy metal salts, chloride, CO_2 and ammonia may also be transported by AQPs. Most AQPs display organ and cell specific expression which sometimes differs between species. Highly specific regional expression of different AQPs combine to coordinate water movement in a number of organs. For instance AQP1 expression in the proximal tubule of the kidney facilitates the initial extraction of water to concentrate urine. This process is an example of near-isosmolar water movement driven by active solute transport. Later, in the principal cells of the collecting duct AQP2 is sequestered within vesicles which, in response to vasopressin receptor activation, insert into the apical membrane to facilitate water reabsorption into the tubular cell. Expression of AQP3 and AQP4 in the basolateral membrane then allows water to move into the hypertonic interstitium of the renal medulla and finally to return to the circulation. AQPs are specific, but passive transporters, they provide a water specific pore across the cell membrane. Water can diffuse across the cell membrane (lipid bi-layer) without accessing an AQP, but transit is less rapid.

1.3.2 Aquaporin function

Much of our understanding of AQP function comes from analysis of pathological situations. Within the brain, AQP4 is integral to the blood brain barrier and is primarily

responsible for permitting the development of cerebral oedema following cytotoxic (cellular oedema) injury (Manley, 2000). Thus, AQP4 knockout mice demonstrate significant survival benefits compared to wild type mice following stroke and traumatic brain injury, two different models of cytotoxic oedema (Manley, 2000; Agre, 2004; Manley, 2004). Conversely, AQP4 knockout mice suffer a worse outcome in models of vasogenic (or interstitial) oedema as this water channel facilitates removal of water from the brain (Papadopoulos, 2004). AQP4 is anchored to the blood vessel-encapsulating astrocytic foot processes through a protein complex involving syntrophin and dystrophin. In skeletal muscle where the role of AQP4 is less well defined it is similarly associated with the dystrophin protein complex. In the skeletal muscle disease Duchenne muscular dystrophy (DMD), AQP4 protein is reduced in association with absent dystrophin protein, although this does not appear to primarily contribute to the muscle weakness (Verkman, 2000).

1.3.3 Aquaporin structure

Information on mammalian AQP structure is mainly based on analysis of AQP1. Monomers consist of six transmembrane helices which form “hourglass” pores. Protein monomers combine within the cell membrane to form a tetrameric complex, although each monomer is independently functional. Due to the design of the channel, requiring reorientation of water molecules during transit, the pore is highly specific to water and excludes H^+ passage (King, 2004). Gating of mammalian water channels does not appear to be a consistent feature in studies thus far, however pH and phosphorylation have influenced permeability in some experimental settings e.g. pulmonary AQP3 permeability was reduced by low pH (Zelenina, 2003) and *Xenopus* oocytes containing AQP4 had reduced permeability when phosphorylated (Han, 1998). However overall AQP function is largely subject to transcriptional, rather than short term regulation by pH or other influences (Verkman, 2005).

1.3.4 Aquaporin 1

AQP1 is the ubiquitous water channel found in endothelial cell membranes of vascular tissues throughout the body. It is also found in the plasma membranes of the red blood cell, kidney, lung, brain and eye (King, 2004). It was the first AQP discovered after being demonstrated in erythrocytes and shown to permit rapid water permeability in response to an osmotic gradient (Preston and Agre, 1991; Preston, 1992), although others have contended that it was found earlier (Benga, 2003).

The study of AQP1 null individuals, arising as a result of a mutation in the AQP1 gene leading to little or no AQP1 expression has provided an insight into the function of AQP1 in humans. In these subjects, the permeability of pulmonary endothelium was studied by computerised tomography following a three litre intravenous volume load. Pulmonary venules of AQP1 null humans were engorged, compared to control subjects that showed imminent interstitial pulmonary oedema in the form of peribronchiolar oedema, the thickness of the airway walls increasing by 40–50% in control subjects (King, 2002). It is likely that this was due to a decrease in pulmonary vascular permeability. The study of AQP1 knockout mice, under different conditions, has not revealed defects in alveolar or pleural reabsorption, with no impairment in lung fluid or gas transport physiology (Song, 2000; Verkman, 2002a). Interestingly, the analysis of the role of AQPs within the heart has been limited in comparison to studies of other tissues and organs.

1.4 Myocardial water handling

1.4.1 Background

There are essentially three compartments within the heart – intravascular, interstitial and myocellular, (Figure 1.1). Typically the heart has a neutral fluid balance, with accumulation of excess interstitial fluid in the heart prevented by anatomical and physiological factors. As blood traverses the myocardial capillary bed, it may return to the circulation via either the coronary sinus or the thebesian veins, emptying into the right atrium. As the majority of fluid flux occurs in the post capillary venules, easy passage or 'run off' is important. This flux in response to osmotic and oncotic gradients occurs regardless of rate of flow (Kellen and Bassingthwaighte, 2003b). Fluid that accumulates in the interstitium is handled by myocardial lymphatics which return water to the circulation via the thoracic duct.

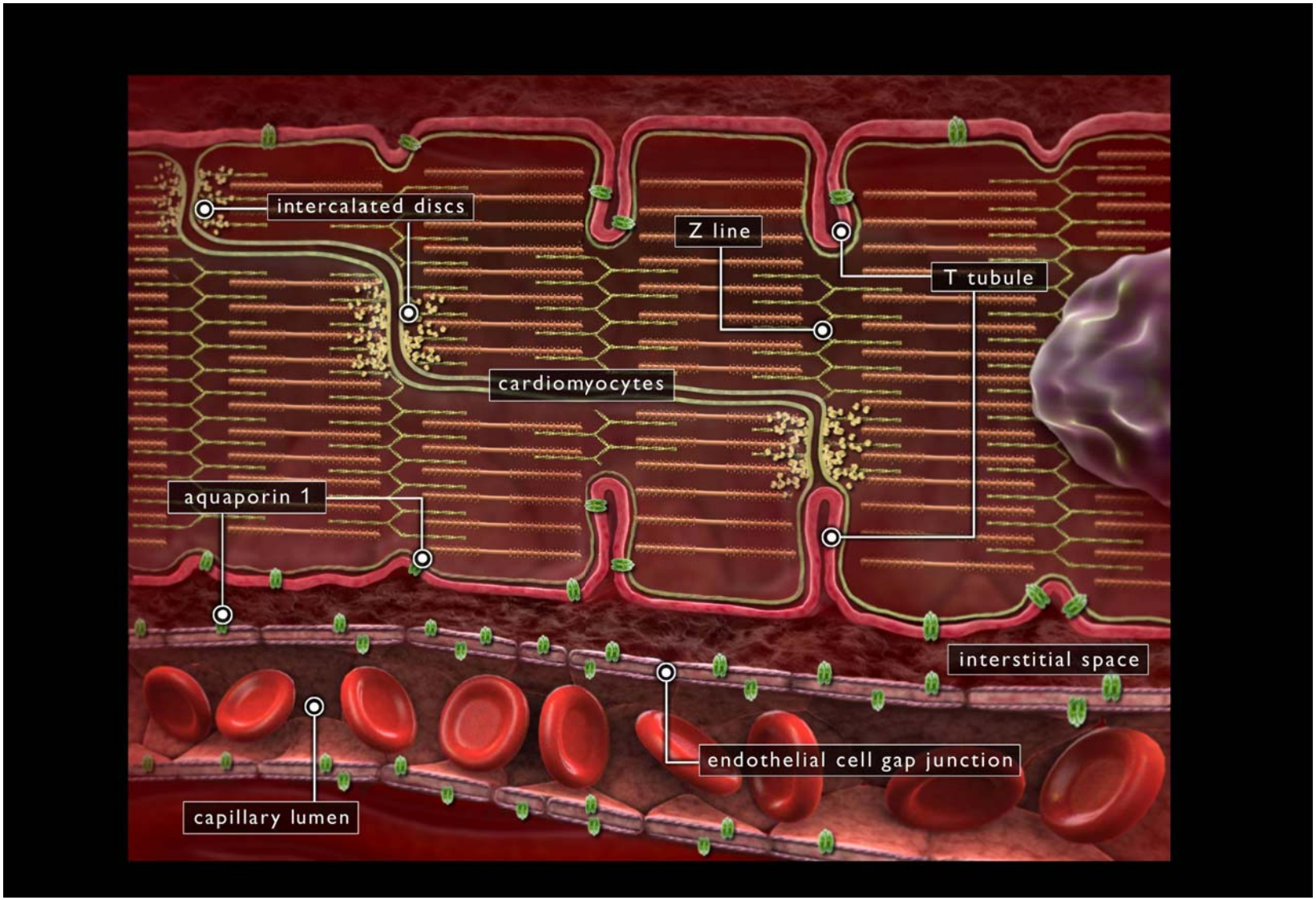

Figure 1.1: Myocardial compartments. Myocellular (cardiomyocytes), interstitial and vascular spaces shown. Aquaporin 1 protein also shown throughout figure. Graphic drawn by Animated Biomedical Productions, Westmead, NSW, Australia, under guidance.

1.4.2 Endothelium

When describing myocardial water permeability, most authors are actually describing the net effect of water movement between the vascular compartment and the interstitium. This is partly determined by exposure of the interstitium to blood vessels, essentially capillary density or surface area available for water flux, which is very high in the heart compared to other organs (Wearn, 1928).

Kellen and Bassingthwaighte using a crystalloid perfused isolated rabbit heart model, have described the routes of water movement across capillaries within the heart (Kellen and Bassingthwaighte, 2003a, b). There are two important routes that water moves through, in response to osmotic and oncotic gradients. The majority of water (67%) passes through small pores (~7nmØ) which are formed by tight junctions within the interendothelial clefts – these are referred to as endothelial cell gap junctions in Figure 1.1. Most of the remainder of water (28%) passes through the endothelial cell directly. Water transits across an endothelial cell by i) diffusion through the lipid bi-layers, ii) coupled to ion–channels or substrate transporters, such as glucose, Na^+, K^+ or Ca^{2+} and iii) via AQPs (Wright and Rees, 1998). AQPs are considered to be the most important of these transendothelial cell routes. AQPs span both the luminal and abluminal lipid bi-layers as well as the sarcolemma of the cardiomyocytes. It is calculated that *in vivo* with blood perfusing the capillaries, rather than crystalloid perfusate (used for experimental modelling), that the contribution of AQPs in the transendothelial route would increase from 28% to 40-50% (Kellen and Bassingthwaighte, 2003b). Lastly there is a third and minor route by which water can leave the intravascular compartment and that is via infrequent large pores (24nmØ) in the endothelium that mainly exist to permit large proteins to egress into the lymphatic circulation (Kellen and Bassingthwaighte, 2003b).

1.4.3 Microvascular fluid balance

The balance of microvascular fluid filtration is modelled by the Starling forces, reviewed in detail by Mehlhorn *et al* (Mehlhorn, 2001). Many of the factors driving water movement are affected during cardiac surgery, including the plasma colloid osmotic pressure, which is reduced by haemodilution, and the compressive force of regular myocardial contractions, which cease when the heart is arrested in diastole (impairing myocardial lymphatic drainage) for the duration of the operation. These factors combine to produce altered water handling and a shift in the balance towards accumulation of water in the interstitium. On the other hand myocellular accumulation of lactate in the setting of I/R is likely to lead to intracellular accumulation of fluid, with the myocellular compartment experiencing a hypo-osmotic stress with ischaemia (Wright and Rees, 1998).

1.4.4 Water handling in pathological circumstances

Water movement between the interstitial and intracellular space has been relatively ignored compared to factors regulating microvascular permeability which are pertinent to vascular – interstitial flows. This may be because in normal circumstances, cardiomyocytes are not subject to variations in the extracellular osmotic environment. Intracellular water represents approximately 77% of total tissue water (Polimeni and Al-Sadir, 1975) and maintenance of interstitial and cardiomyocyte volumes are inextricably linked through osmotically obliged water movement. Water movement between the interstitial and intracellular compartments may become important in circumstances where i) interstitial fluid volumes are increased because of pathologically increased microvascular permeability, such as CPB (Hoffmann and Dunham, 1995), or reduced colloid osmotic pressure (Kellen and Bassingthwaighte, 2003b) – leading to an expanded interstitial space and so called interstitial or vasogenic oedema, and ii) when there is an inwardly directed osmotic gradient as a result of cellular events such as ischaemia, where

accumulation of lactate osmotically draws water into the cell during reperfusion, leading to cytotoxic or myocellular oedema (Garcia-Dorado and Oliveras, 1993; Wright and Rees, 1998).

1.4.5 Role of aquaporins

The contribution that AQPs make to myocardial water handling has been explored in a number of species with differing conclusions. The techniques used included isolated cardiomyocytes with video-microscopic observation of osmotically induced changes in cell volume and assessment of sarcolemmal water permeability by measuring the rate of swelling or shrinkage. Together with an assessment of the Arrhenius activation energy (*E*a), which is a measure of the energy barrier to water flux (normally high because diffusion of water is limited by the lipid bi-layer), the contribution of AQPs to net sarcolemmal water permeability can be estimated. On the basis of these measurements, Ogura *et al*. showed that AQPs represent a major route of water transport in guinea pig and rat heart cells (Ogura, 2002a; Ogura, 2002b). The inhibitory effect of adding mercury (a non-specific AQP inhibitor) on interstitial fluid volumes supported these findings. Similar techniques were used by Suleymanian and Baumgarten (Suleymanian and Baumgarten, 1996) in rabbit ventricular cardiomyocytes. They demonstrated similar sarcolemmal permeability, but a higher *E*a, and concluded that diffusion, not water channels, were the predominant path of water movement in the rabbit. Species differences in AQP expression may explain this discrepancy and the contribution of AQPs to human cardiomyocyte water movement is at present unknown. Although these isolated cardiomyocyte models suggest a role for AQPs of varying degrees in the heart, they have only modelled the movement of water into the cardiomyocyte and have disregarded the important vascular and interstitial components.

The vascular and interstitial compartments, both of which are lined by endothelial cells with AQPs, are key to water movements within the heart. Hence it is considered in more

developed recent work that the contribution of transcapillary water passage via the transendothelial AQPs is of the order of 40-50% *in vivo* (Kellen and Bassingthwaighte, 2003b). Furthermore as already alluded to, in several non-cardiac tissues, such as brain (Manley, 2000; Manley, 2004; Papadopoulos and Verkman, 2005) and lung (King, 2002) the involvement of AQPs in the development and resolution of tissue oedema has been demonstrated. This further supports a rational basis for postulating a role of AQPs in myocardial water handling and the development and resolution of myocardial oedema.

1.5 Myocardial oedema

1.5.1 Background

Accumulation of body water occurs in the context of cardiac surgery and whilst in adults this is 2–5% (Hamada, 2004), it appears greater in paediatrics, ranging from 11% (Maehara, 1991) to ~20–30% (Wernovsky, 1995). Human myocardial water gains in this setting have not been established, but comparable large animal studies have found gains in the order of 1 – 3% (Foglia, 1978; Mehlhorn, 1995a).

Myocardial oedema may develop through a combination of vasogenic and cytotoxic/myocellular pathways. Typically interstitial or vasogenic oedema is described (Mehlhorn, 1995b; Tassani, 2007), associated with increased capillary leak. In addition, the heart suffers the metabolic effects of ischaemia and reperfusion. This leads to the generation of intracellular lactate and creates a strong inwardly directed osmotic gradient that causes cardiomyocytes to swell and leads to myocellular or cytotoxic oedema (Wright and Rees, 1998; Kloner and Jennings, 2001). Myocardial oedema should thus be considered a secondary phenomenon and a downstream effect of the primary pathological processes involved in cardiac surgery requiring CPB.

1.5.2 Functional consequences of myocardial oedema

Relatively small increases in myocardial fluid content have been associated with significant systolic ventricular dysfunction (Cross, 1961; Utley, 1974; Foglia, 1978; Goto, 1991; Carlson, 1992; Weng, 1992; Spotnitz, 1995; Allen, 1997; Geissler and Allen, 1998; Albers, 2001; Jia, 2002). In one analysis, a 2.6% gain in myocardial water produced by hypo-osmotic priming solutions for CPB was associated with a 43% decline in left ventricular performance and a similar order of reduction in compliance (Foglia, 1978). A second study demonstrated similar increases in myocardial water (↑3%) content impacting significantly on myocardial function (↓30%) (Laine and Allen, 1991).

Diastolic function has also been shown to be compromised in association with myocardial oedema (Pogatsa, 1976; Mavroudis and Ebert, 1978; Garcia-Dorado and Oliveras, 1993; Spotnitz and Hsu, 1994; Geissler and Allen, 1998; Jia, 2002). This could be particularly relevant in the paediatric setting following cardiac surgery, as many infants already have impaired diastolic function as a result of hypertrophy related to pre-existing structural lesions.

However the associations between oedema and dysfunction have been temporal only, with varied models, confounding variables – particularly ischaemia, and no established mechanistic links.

1.5.3 Pathogenesis of myocardial oedema

Recently in an animal model of regional ischaemia it was postulated that oedema led to impaired myosin–actin interaction and resulted in stunning (Bragadeesh, 2008). This was an interesting finding and although it makes intuitive sense, it is still uncertain whether the oedema itself was responsible or merely a bystander event or process. Regional myocardial ischaemia was induced in pigs in short repeated bursts, with a duration totalling 50 minutes. Also associated with this was a decline in myocardial function measured by 2D echocardiography. Associated with this ischaemic insult was an increase in myocardial water of 10% and an increased distance between adjacent myosin contractile filaments on electron microscopy. After five days of observation there was no substantial improvement in myocardial function, but the extra myocardial water had declined to 5%. This divergence in the degree of dysfunction and degree of oedema suggests that other factors – namely the ischaemia, resulted in the contractile dysfunction and the oedema is merely a bystander phenomenon. Alternatively this 5% gain in myocardial water content (MWC) exceeded a threshold necessary for normal myocardial function. Ischaemia/reperfusion is a recognised cause of contractile injury leading to dysfunction through calcium excess and oxygen derived free radicals (Bolli and Marban, 1999). The study by Bragadeesh *et al* did demonstrate a temporal relationship between

the onset of oedema and dysfunction in the heart, but the induced ischaemia confounded any plausible causative link between oedema and dysfunction(Bragadeesh, 2008).

A recent study from a group who have done extensive work in the field appears to have been more helpful in elucidating the contribution of oedema to dysfunction. In an animal model of CPB with cardioplegia, it was found that oedema without injury to the myocardium – e.g. via I/R, did not result in dysfunction (Fischer, 2006). Myocardial oedema of the order of 2% was not associated with dysfunction when produced by either brief CPB ~ 12 minutes with cardioplegia or coronary sinus hypertension – a non-ischaemic insult. So myocardial oedema occurring on CPB – with only brief ischaemia, was not associated with detectable myocardial dysfunction. Hence it follows that oedema of this degree did not have important functional implications. Suggesting that other factors – such as those implicated in causing stunning, are responsible for dysfunction and that oedema is a secondary phenomenon (Bolli and Marban, 1999). The two main purported causes of myocardial oedema are discussed below.

1.5.3.1 Haemodilution

Haemodilution occurs in the context of CPB and cardiac surgery and has been shown to negatively impact on outcome (DeFoe, 2001; Jonas, 2003). Given the lowering of colloid osmotic pressure with CPB it is postulated that water transits into the interstitium of the tissues of the body and that interstitial oedema results (Rosenkranz, 1980; Mehlhorn, 2001). Furthermore the occurrence of the so called capillary leak syndrome is considered to result in increased interstitial volumes and interstitial or vasogenic oedema (Farstad, 2004). This has been attributed to loss of albumin via the endothelium (Seghaye, 1996) and perhaps mediated by complement activation (Zhang, 2005). Although there is a drop in colloid osmotic pressure (COP), this does not appear to be due to albumin loss and may reflect water movement instead (Tassani, 2001; Tassani, 2002b; Farstad, 2004; Tassani, 2007). It has also been previously shown that interstitial

pressure increases following CPB – consistent with increased interstitial fluid volumes (Menninger, 1980). Supporting the movement of water as being intrinsic to interstitial or vasogenic oedema is the utility of modified ultrafiltration (MUF) in rapidly reducing myocardial wall thickness and haemodilution in clinical studies (Davies, 1998; Chaturvedi, 1999). MUF in the setting of cardiac surgery is carried out at the completion of the operation just prior to removal of the CPB circuit from the patient. Essentially the patients blood is passed through an artificial kidney (a series of microscopic membranes within a hollow tube) and water is removed down a osmotic gradient. This has the effect of reversing to some extent the haemodilution which occurs during CPB.

1.5.3.2 Ischaemia and reperfusion

As in the recent work by Bragadeesh *et* al, myocardial oedema has been attributed to ischaemia and reperfusion injury (Garcia-Dorado and Oliveras, 1993; Spotnitz and Hsu, 1994; Palmer, 2004). In several species and models, myocellular oedema is evident if the ischaemic injury exceeds 15 – 20 minutes (Garcia-Dorado and Oliveras, 1993; Spotnitz and Hsu, 1994; Kloner and Jennings, 2001; Palmer, 2004). Swelling of organelles and in particular mitochondria is an early event, with mitochondria potentially having a role in myocardial stunning. Mitochondria are involved in preconditioning, liberation of reactive oxygen species and controlled cell death (apoptosis). The initial movement of water into mitochondria is thought to be part of the early protective response to ischaemia, countering the effect of mitochondrial matrix shrinkage that increases the inter-membrane space and is deleterious to respiration (Dos Santos, 2002). In this context, water is thought to move in response to an influx of K^+ ions, as a result of mitoK_{ATP} channel opening. Chemical openers of this channel, such as diazoxide, have a cardioprotective effect, however the pore-forming proteins underlying this protective effect have yet to be identified (O'Rourke, 2004).

1.5.4 Myocardial oedema and mitochondria

After reperfusion, swelling of cardiac mitochondria occurs due to increased permeability of the inner mitochondrial membrane and is probably driven by the osmotic effect of accumulation of anionic proteins, monovalent cations, intermediates of the Krebs cycle and other small organic molecules (Beavis, 1985). This occurs even with the major ion channels closed, and is reversible with long periods of reperfusion (Bosetti, 2004). Again, the route by which water travels in this setting is unknown. Excessive swelling may occur with opening of the mitochondrial permeability transition pore (mPTP) which allows non-selective movement of small molecules. This leads to irreversible loss of the mitochondrial membrane potential, uncoupling of the respiratory chain and release of cytochrome C, initiating apoptotic or necrotic cell death (Akao, 2003).

1.5.4.1 Mitochondria and aquaporins

In the kidney the inner mitochondrial membrane was found to contain AQPs, possibly AQP8, that allowed excessive swelling without mPTP opening (Lee, 2005). Chemical inhibitors of AQPs prevented mitochondrial swelling, organelle disruption and cell death. Thus it was considered that inwardly directed water movement into mitochondria may be beneficial early, then deleterious in subsequent stages of the mitochondrial response to ischaemia and reperfusion. Expression of mitochondrial AQPs has been reported in hepatic (Ferri, 2003; Calamita, 2005) and renal tissue (Lee, 2005). However more recently Yang *et al* suggested that AQPs in mitochondria have no functional relevance. Water permeability was similar in mitochondrial preparations from wild type and AQP knockout mice by stopped-flow light scattering. Furthermore the addition of mercury altered permeability similarly across groups and AQP8 was not expressed in hepatic mitochondrial preparations utilising wild-type/AQP8 null mice as positive/negative controls (Yang, 2006).

1.6 Myocardial aquaporins

1.6.1 Background

The myocardial expression of AQPs is poorly characterised. Soon after the initial description of AQP1, it was noted that the cardiac microvasculature and the endocardium expressed this protein (Bondy, 1993). The abundance of AQP1 protein in cardiac tissue was thought to reflect the dense vascular supply of this organ, however early reports suggested that water channels did not play a major role in microvascular filtration in the heart (Suleymanian and Baumgarten, 1996). In normal circumstances, cardiomyocytes do not experience osmotic imbalance and to date, cardiac AQPs have not been investigated to the same extent as renal, pulmonary and brain AQPs.

1.6.2 Myocardial aquaporins and the three compartment model

A contemporary view of myocardial AQP expression would include cells on both sides of the interstitial space, namely the endothelial barrier, as well as the sarcolemma of the cardiomyocyte. Evidence of a functional role for AQP1 expression in endothelial cells has been outlined earlier (Ogura, 2002b; Kellen and Bassingthwaighte, 2003a, b). Myocellular expression has been noted in rat cardiomyocytes, (Page, 1998), with functional interactions demonstrated with caveolae in response to changes in the osmotic environment. Our group has demonstrated AQP1 expression within t-tubules (Au, 2004), themselves invaginations of the sarcolemma extending into the myocardium within the Z line structures, (Figure 1.1). Ionic fluxes, particularly relevant to Ca^{2+} homeostasis are fundamental to electromechanical coupling and K^{+} flux is involved in attenuating the cardiac action potential. These ionic fluxes may require accompanying rapid osmotic equilibration, although the potential role of AQPs in this context has not been established.

1.6.3 Myocardial aquaporins and potassium

Links between potassium and water transport are evident in the glial cells of the brain, with the possibility that such interactions may also be relevant in the heart. AQP4 has been shown to co-localise with the Kir4.1, potassium channel in astrocyte end feet (Nagelhus, 2004) and this interaction involves the dystrophin-associated proteins (DAP) (Guadagno and Moukhles, 2004). Loss of DAP integrity reduces AQP4 expression and the clearance of extracellular potassium leading to seizures (Amiry-Moghaddam and Ottersen, 2003; Dudek and Rogawski, 2005). In the eye, co-localisation of Kir4.1, AQP4 and DAP has also been demonstrated (Connors and Kofuji, 2006) and reduced expression of AQP4 and Kir4.1 channels may contribute to proliferative retinopathy following disruption to the retinal blood supply, occurring with retinal detachment (Tenckhoff, 2005). Thus a link may exist between rapid potassium transport and water permeability at the sarcolemma, which impacts upon ionic balance and tolerance of ischaemia. AQP4, Kir4.1 potassium channels and the DAP complex are all present in the mouse heart although the role of cardiac AQPs in these processes has yet to be established.

1.6.4 Aquaporin 1 and cardiac disease models

Two reports describe myocardial AQP1 expression in cardiac disease models. Jonker *et al*. demonstrated up-regulation of myocardial AQP1 mRNA and protein expression in a model of chronic foetal haemodilution (Jonker, 2003). Immunohistochemistry showed AQP1 mostly within the vasculature and comparatively little within cardiomyocytes. It is not known whether the observed increase in AQP1 expression acts to increase interstitial oedema or facilitates clearance through movement of water back through endothelial cells into the vascular compartment.

The second study of AQP1 in a model of clinical practice focused on the effect of CPB in the lamb (Tabbutt, 1997b). In this study lambs underwent CPB and DHCA with six hours of reperfusion, following which AQP1 mRNA levels were unchanged. Of note in this study was the utilisation of an operative technique now not commonly employed (DHCA) and which is also associated with less tissue oedema (Wernovsky, 1995). AQP1 mRNA abundance was also not correlated with protein expression, myocardial oedema or myocardial function. It remains unclear what effect current CPB techniques might have on expression of AQP1 and how this may affect myocardial oedema and function. We viewed these two studies as preliminary in terms of investigating the mechanisms involved in this setting.

1.6.5 Other myocardial aquaporins

A number of AQPs in addition to AQP1 have been identified in myocardial tissue. Most reports have focused on the identification of relevant mRNA by PCR without correlation with protein expression or site of expression. While many AQP mRNAs are present in the heart, protein is detectable for only a few of them and AQP1 expression is the most prominent in immunohistochemical studies (Au, 2004). The expression of AQPs within the heart is summarised below, (Table 1.1).

There are limited published data on human myocardial AQP expression, other than AQP1. AQP3 transcript was identified by microarray, but without the positive staining by immunohistochemistry as shown for other tissues in the study (Mobasheri, 2005). In an earlier report AQP3 was not found in myocardium using Northern analysis (Ishibashi, 1995). AQP4 mRNA has also been identified by Northern analysis (Yang, 1995). AQP4 is of particular interest due to the noted loss of AQP4 expression in muscular dystrophies associated with the loss of dystrophin (Frigeri, 2004) and the cardiomyopathy occurring with such diseases (Muntoni, 2003). Human cardiac AQP7 mRNA expression has been identified (Kuriyama, 1997; Ishibashi, 1998b) and may be derived from cardiac

associated adipose tissue where AQP7 is well described. Screens of human myocardial tissue by PCR using degenerate primers based on the Asparagine-Proline-Alanine (NPA) motif common to many AQPs failed to demonstrate any novel AQPs in the heart (Au, 2004) although low levels of expression may limit the usefulness of this strategy.

In the hearts of other species, numerous AQPs have been found. AQP7 has been found within the capillaries of adipose tissue associated with cardiac tissue. AQP7 which also permits glycerol transport, has been shown to be important in avoiding obesity in mice, but its role in adipose tissue remains to be determined (Hibuse, 2005; Skowronski, 2007). AQP8 is reported within the mitochondrial fraction of rat heart by Western blot (Calamita, 2005), but is reportedly absent in this species by Northern analysis (Ishibashi, 1997b; Koyama, 1997). AQP8 would be interesting, if it indeed was myocardial and mitochondrial in location, but in functional and expression analysis using knockout tissue there is no apparent expression or function of any AQPs in mitochondria (Yang, 2006).

Table 1.1: Myocardial aquaporin expression

	Human	**Sheep**	**Rat**	**Mouse**
AQP0				
AQP1	PRESENT i)RT-PCR, IHC, Western – endothelial & myocellular distribution (Au, 2004) ii)Northern (Hasegawa, 1994)	PRESENT i)RNAse protection assay (Tabbutt, 1997b) ii)Northern, Western, IHC – endothelial and minimal myocellular distribution (Jonker, 2003)	PRESENT i) IHC – endothelial and myocellular distribution (Page, 1998) ii)RT-PCR, IHC, Western – endothelial and myocellular distribution (Au, 2004) iii)Western, Northern and autoradiography – endothelial (Bondy, 1993) iv)Northern (Hasegawa, 1994) v)RNAse Protection Assay (Umenishi, 1996) vi)IHC – endothelial (Nielsen, 1993)	PRESENT i) Western (Nejsum, 2000)
AQP2	ABSENT i)Microarray and IHC (Mobasheri, 2005)		ABSENT i) RNAse protection assay (Umenishi, 1996)	ABSENT i)Northern (Yang, 1999)
AQP3	CONFLICTING REPORTS i)Microarray and IHC – present (Mobasheri, 2005) ii)Northern – absent (Ishibashi, 1995)		ABSENT i & ii)Northern (Echevarria, 1994; Ishibashi, 1994) iii) RNAse protection assay (Umenishi, 1996)	
AQP4	PRESENT i)Northern (Yang, 1995)		ABSENT i) RNAse Protection Assay (Umenishi, 1996)	PRESENT i)Northern (Ma, 1996b)
AQP5			ABSENT i & ii)RNAse protection assay (Umenishi, 1996; Funaki, 1998)	ABSENT i)Northern (Krane, 1999)

Table 1.1: Myocardial aquaporin expression *continued*

	Human	**Sheep**	**Rat**	**Mouse**
AQP6	ABSENT i)Northern (Ma, 1996a)			
AQP7	PRESENT i & ii)Northern (Kuriyama, 1997; Ishibashi, 1998b)		PRESENT i & ii)Northern (Ishibashi, 1997a; Ishibashi, 1998b)	PRESENT i)RT-PCR, western, IHC(Nejsum, 2000; Skowronski, 2007)
AQP8	ABSENT i)Northern (Koyama, 1998)		CONFLICTING REPORTS i & ii)Northern – absent (Ishibashi, 1997b; Koyama, 1997) iii)Western – present (Calamita, 2005)	PRESENT i)Northern (Ma, 1997b)
AQP9	ABSENT i)Northern (Ishibashi, 1998a)			
AQP10	ABSENT i) RNAse Protection assay (Hatakeyama, 2001) ii)Northern (Ishibashi, 2002)			ABSENT i)Northern (Morinaga, 2002)
AQP11				PRESENT i)Northern (Morishita, 2005)
AQP12				ABSENT i) RNA dot blot (Itoh, 2005)

1.6.6 Other aquaporin roles

AQP1 may transport CO_2 as well as water and this could benefit the heart during metabolic stress (Nakhoul, 1998; Cooper, 2002; Miller, 2004). AQP1 expression in *Xenopus* oocytes increased CO_2 permeability by 40% over controls and this increase was inhibited by blocking AQP1 function with mercury (Nakhoul, 1998). However physiologically relevant CO_2 transport via AQPs could not be demonstrated during both normal and stressed states in erythrocytes, lung and kidney cells utilising relevant knockout mouse materials, despite water permeability being altered by more than 100 fold (Yang, 2000; Fang, 2002; Verkman, 2002a, b; Miller, 2004). Thus physiologically important CO_2 transport by AQPs within the heart seems unlikely, but has not been directly studied.

Somewhat similarly, AQP1 is purported to transport nitric oxide (NO^-). In isolated cell constructs, NO^- transit was found to be increased in the presence of AQP1 and reduced in the presence of mercurials or with RNA interference. However, true controls in the form of knockout tissue were not available and the results remain intriguing at this stage, (Herrera, 2006).

1.7 Other myocardial membrane proteins

1.7.1 Dystrophin

Given the membrane location of AQPs we sought to determine the contribution to LCOS of other myocardial membrane relevant proteins. Specifically we focused on dystrophin. Dystrophin was isolated in both skeletal and cardiac muscle in 1987 by Eric Hoffman and colleagues. It is absent in sufferers of Duchenne muscular dystrophy and was named after its isolation from the genetic locus of that inherited disease (Hoffman, 1987). It is a large (427kDa) membrane imbedded protein which is important in mechanotransduction (force transfer) in muscle, linking the intracellular contractile apparatus with the extracellular attachments (McNally and MacLeod, 2005). The absence or loss of function of dystrophin is felt principally to affect muscle through loss of membrane integrity (Glover and Brown, 2007). Dystrophin has been shown to be abnormal (termed dystrophinopathy) in various more severe settings, i.e. 1) ischaemic cardiomyopathy, 2) Coxsackie B viral myocarditis and 3) Duchenne muscular dystrophy (Vatta, 2002; McNally and MacLeod, 2005; Lee, 2006).

The role of dystrophin following CPB and in the setting of LCOS is unknown. Dystrophin has been shown in some animal models to be upregulated as a result of ischaemic preconditioning (Kido, 2004) and therefore may have a role in ameliorating LCOS.

1.7.1.1 Dystrophin in ischaemic preconditioning

Ischaemic preconditioning (IPC) is the practice of training cardiac muscle to cope with ischaemia. Essentially I/R injury is lessened following a series of brief sequential ischaemic insults ~ four periods of 5 minutes, prior to a more sustained planned ischaemic event (Murry, 1986). This has been verified in a number of models over the last 20 years, although the exact mechanism remains to be determined and it has not become part of conventional practice (Peart and Headrick, 2008). Nevertheless, in two

small animal models IPC has been shown to be associated with both improved function and restoration of membrane dystrophin (Kido, 2004; Kyoi, 2006).

1.7.1.2 Dystrophin in ischaemic and dilated cardiomyopathy

Investigators have found that dystrophin was important in the contractile dysfunction of end stage ischaemic and dilated cardiomyopathy (Vatta, 2002). In 18 of 20 patients with severe cardiomyopathy, the amino terminus of dystrophin was affected. Interestingly this change was reversed in a smaller group of patients who had ventricular assist devices inserted (Vatta, 2004). The other two commonly tested domains of dystrophin – rod and carboxy-terminal, were not affected in this study. However in other comparable models, others have found the amino terminus and rod domains were both affected (Toyo-Oka, 2004). Hence it was concluded that impaired dystrophin was a common cytoskeletal mechanism for the resulting myocardial dysfunction seen in end stage cardiomyopathy. Such cardiomyopathies represent severe pathology and the degree to which abnormalities of dystrophin contribute to contractile dysfunction in the setting of post CPB cannot be inferred.

1.7.2 Dysferlin

Whilst dystrophin is important to maintaining membrane integrity in response to injury, dysferlin has been shown to be a key protein in the restoration or repair of injured membranes in skeletal and cardiac muscle (Han, 2007; Han and Campbell, 2007). Thus dysferlin absence also leads to a dystrophy because of a diminished capacity for rapid muscle membrane repair after injury and or normal muscle use (Glover and Brown, 2007). Dysferlin protein (230kDa) was discovered by Jing Liu and colleagues during their investigations using skeletal muscle and DNA from patients suffering from Miyoshi myopathy and limb girdle muscular dystrophy (LGMD). It was named because of its dystrophy association and similarities to a previously found protein called fer–1. Fer–1 is a similarly large protein which is involved in membrane fusion and mobility in spermatozoa of *C. elegans* (Liu, 1998). A partial or complete absence of dysferlin in

humans is responsible for Miyoshi myopathy and LGMD2B. Both diseases present with weakness and atrophy of proximal skeletal muscles, elevated creatinine kinase (CK) and have slowly progressive courses (Glover and Brown, 2007). Both conditions have generally been accepted as not having a cardiac phenotype – until recently. A case report of cardiomyopathy in a patient with LGMD2B (Kuru, 2004) was followed by a case series in which two of seven patients had congestive cardiac failure (Wenzel, 2007). Wenzel *et al* have also shown in a mouse model that dysferlin deficient mice have a poor response to an inotrope induced stress, exhibit deficiencies in cardiomyocyte membrane integrity and develop a cardiomyopathy – in agreement with the work by Han *et al* (Han, 2007).

Dysferlin has been demonstrated to mediate the sealing of breaches in muscle cell membranes. Dysferlin facilitates the docking and fusion of a membrane patch into a membrane defect and has been found to have important protein interrelationships with Caveolin-3, Calpain 3 and Affixin (Glover and Brown, 2007). In dystrophin deficient mice, skeletal muscle membrane injury occurred when they were exercised, and dysferlin laden vesicles were shown to be vital in repairing these membrane defects. It was demonstrated that dysferlin was integral to membrane repair mechanisms in skeletal muscle (Bansal, 2003). A similar response to myocardial membrane injury was seen – with dysferlin having a role in repairing defects in cardiac cell membranes. It was remarked that dysferlin mediated membrane repair was indispensable for maintaining the sarcolemma integrity of cardiomyocytes (Han, 2007). Dysferlin has been shown to be expressed in endothelial cells within the brain and associated with increased permeability in inflamed areas of the brain in multiple sclerosis (Hochmeister, 2006). Interestingly this endothelial expression could be induced by tumour necrosis factor, which incidentally is known to be increased in the setting of CPB and I/R (Wan, 1997; Hochmeister, 2006). Although dysferlin has not been described in muscle endothelium, the response of dysferlin to the potentially membrane injuring effects of CPB and I/R has not been determined.

Given that dysferlin is considered an important membrane reparative protein and whilst studying the impact of CPB and I/R on membrane integrity, we also tested the role of a pharmacological membrane protecting agent – Poloxamer 188, in this setting.

1.8 Poloxamer 188

Poloxamer 188 (P188), with a MW of 8400 is a non–ionic copolymer surfactant. It has hydrophilic and hydrophobic moieties which insulate hydrophobic substances such as fibrin, reducing aggregation, blood viscosity and improving blood flow (Hunter, 1990; Fatkin, 1997). This insulating attribute also appears to confer membrane sealing or protecting properties (Maskarinec, 2002). It has been used in a number of human clinical settings on the basis of its effects. The main proposed effects of P188 can be categorised into 2 groups. Firstly, haemorheological effects have seen it used to improve viscosity and improve small vessel blood flow in the setting of myocardial ischaemia, thromboembolic disease and sickle cell anaemia (Schaer, 1994; Adams-Graves, 1997; Fatkin, 1997). Secondly, membrane protection or repair effects have seen it used in models of electrocution, cerebral ischaemia, myocardial membrane fragility and muscular dystrophy (Colbassani, 1989; Lee, 1999; Yasuda, 2005; Ng, 2008). The impressively short timeframe over which myocardial function improved *in vivo* in dystrophin deficient mice in the study by Yasuda *et al* prompted us to explore it in LCOS. We further considered it was a reasonable therapy to trial given its myriad of potential beneficial effects in the setting of CPB and its safety profile in human studies (Adams-Graves, 1997; Yusuf, 1997).

1.8.1 Haemorheological studies

P188 has been demonstrated *in vitro* to improve blood viscosity following coronary artery bypass surgery using CPB. Uniquely it appears able to reduce viscosity, without causing haemodilution. This has been attributed to its lubrication of endothelial cells and its ability to reduce adhesiveness of fibrin, platelets and other cellular elements without causing haemodilution (Hunter, 1990; Fatkin, 1997). Early experimental use was in the setting of frost bite where sludging and rouleaux formation is problematic and tissue viability is reduced (Knize, 1969). Fatkin *et al* in an *in vitro* study of blood echogenicity demonstrated reduced red blood cell rouleaux formation with P188, confirming this proposed mechanism of action (Fatkin, 1997). Because of these attributes

P188 has progressed to phase three human trials in the setting of sickle cell disease. It appears particularly beneficial in reducing the duration and pain of sickle crises in younger patients (Gibbs and Hagemann, 2004). In an initial trial of 50 patients it was found to be well tolerated. It significantly reduced the duration and pain of a sickle crises as well as reduced the total analgesic use and duration of hospitalisation. It was considered to potentially offer benefit in this difficult setting (Adams-Graves, 1997).

Because P188 may reduce red blood cell aggregation, it is considered to have some benefits in reducing thromboembolic phenomena (Fatkin, 1997). It has been trialled as an adjunctive agent in an animal model of myocardial infarction (Schaer, 1994). P188 was administered during a focal myocardial ischaemic insult – prior to reperfusion in a series of dogs. P188 administration was associated with a significant reduction (42%) in infarct size. There was also an associated improvement in renal function as well as improvements in global (ejection fraction was 10% better) and regional ventricular function compared with controls. The authors also confirmed *in vitro* work that found neutrophil adherence was inhibited by P188 and this was considered an important element of the mechanism of P188 in this setting (Lane and Lamkin, 1984). Somewhat concerningly, the associated effect of P188 on reducing neutrophil activity was supported by later work, where it was found to increase sepsis in mice receiving Fluosol – an artificial blood substitute of which P188 is a component (Lane and Lamkin, 1986). Nevertheless Schaer *et al* proceeded with a clinical trial based on their large animal study and randomised 114 patients with acute myocardial infarction to P188 or placebo in addition to conventional thrombolytic therapy (Schaer, 1996). The results of this study demonstrated a significant associated benefit with P188 in terms of reduced infarct size (38%) based on nuclear medicine studies and improved function (ejection fraction 8% better). It was speculated that haemorheological mechanisms were the most likely explanation for its benefit. Subsequently an expanded large placebo controlled trial of P188 was undertaken (Yusuf, 1997). In this multicentre trial involving almost 3000 patients, no benefits associated with P188 were found, when administered after the ischaemic event. There was no difference in infarct size or myocardial function. There was also some concern with regard to renal toxicity with measures of renal function being

worse in a subset of older patients with pre-existing renal impairment. It was concluded that P188 did not have any demonstrable benefits in this setting and that there were risks associated with its administration. We note however the heterogeneity of subjects and that treatment was post I/R injury.

Whilst P188 continues to be explored in the clinical setting of sickle cell disease (Gibbs and Hagemann, 2004) its haemorheological effects have not been studied in the cardiac setting since it was found not to have benefit following acute myocardial infarction. However in parallel, work has been occurring on the membrane protecting attributes of P188 in various cardiac settings. Interestingly in one of the earliest myocardial infarction animal models Justicz *et al* administered P188 and Mannitol following regional infarction and demonstrated ~50% reduction in infarct size and improved function. The attributed mechanism was haemorheological, but because there were no differences in the blood flow between treatment groups, this was questioned. The authors speculated that because the Evans blue staining of the infarcted and adjacent regions was significantly reduced in the P188 treated animals, that P188 may have been associated with improving the integrity of the damaged myocardial membranes (Justicz, 1991).

1.8.2 Membrane protection and repair studies

P188 has been shown *in vitro* to squeeze into defects in lipid monolayers and then to be extruded by the repaired membrane (Maskarinec, 2002). It has been used in experimental studies of electrical injury where electroporation of cell membranes occurs and it appears to improve membrane integrity in this setting (Lee, 1992). In animal skeletal muscle that was electropermeabilised there was improved functional and histological findings associated with P188 administration. In the P188 treated muscles there was also less oedema. The muscle cells performed particularly well if they were pretreated with P188 prior to electropermeabilisation.

P188 was administered to dogs prior to 2½ hours of DHCA with brief CPB (Mezrow, 1992). Because of the neurological concerns related to DHCA this was the focus of the

outcome measures, and those animals that received P188 prior to DHCA had an improved neurological outcome. It is unclear if there were any haemodynamic differences in the study. The authors speculated that mechanisms involving the protection of membranes may have resulted in these improvements(Mezrow, 1992).

In an isolated heart model of sepsis induced dysfunction, membrane injury was associated with systolic and diastolic dysfunction. P188 was found to prevent the membrane induced injury as well as the associated contractile dysfunction. Importantly P188 was administered prior to the injury. It was considered that P188 had a role in sepsis induced cardiac injury, but to our knowledge it has not been trialled in humans in this context (Watanabe and Okada, 2003).

Yasuda *et al* published the use of P188 in a dystrophin deficient mouse model and found *in vivo* and *in vitro* that it inferred a number of benefits. In isolated cardiomyocytes there was improved cell compliance and less stretch induced membrane damage and cell loss. Whilst *in vivo* there was rapidly improved diastolic function in the setting of inotrope induced myocardial stress, measured with pressure–volume loops. It was concluded that P188 may have a role as a chemical sealant in the setting of cardiac failure related to dystrophinopathy (Yasuda, 2005). Subsequently there have been two conflicting reports using P188 in the setting of skeletal muscle injury in dystrophin deficient mice. Soon after Yasuda *et al*, there was a report of exercise induced skeletal muscle injury in dystrophic mice being pretreated with P188 (Quinlan, 2006). There was no associated benefit in function or membrane leakiness with P188. This lack of effect was considered to possibly be due to excessive exercise, insufficient P188 availability/dose or perhaps differences between cardiac and skeletal muscle. Recently isolated skeletal muscles from dystrophin deficient mice were found *in vitro* to have restored force transfer and function with P188 therapy (Ng, 2008). The rapidity of effect and improved functional result was similar to that observed in the isolated cardiomyocytes by Yasuda *et al* and it also suggested benefit in skeletal muscle.

1.9 Summary

The occurrence and importance of the LCOS in the postoperative setting, together with the perceived causal association with total body and myocardial water accumulation warrants further investigation. The pathogenesis of myocardial oedema and its relationship to the premier perioperative insults of CPB and I/R also deserves study. Myocardial membrane proteins may have a role in this setting, in particular a group of water specific proteins – AQPs – have not been explored adequately. In other comparable settings membrane proteins – such as dystrophin and dysferlin – have roles and so merit investigation. Poloxamer 188 has been shown in an animal model of dystrophinopathy to rapidly improve myocardial function and its utility in the setting of CPB and I/R is not known.

1.10 Specific aims of the thesis

1. To evaluate the relative importance of myocardial oedema and ischaemia in causing myocardial dysfunction.
2. To define the role of myocardial AQPs in the setting of myocardial oedema and dysfunction.
3. Determine the role of dystrophin and dysferlin in the setting of myocardial dysfunction.
4. Assess the effect of a membrane sealant (P188) in clinically relevant settings of myocardial dysfunction.

The aim – to determine the relationship between function and oedema, was examined in three models – isolated cardiomyocytes (single isolated heart cells), isolated hearts (*ex vivo* rodent hearts with retrograde aortic perfusion) and a clinically relevant lamb CPB model. Incorporated into this was a study of the proteins of interest – AQPs, dystrophin and dysferlin. Finally, *in vivo* trials of a potential therapy – P188, were conducted, in

which we studied the functional responses to P188, as well as the effects of P188 upon oedema, ischaemia and the proteins of interest.

CHAPTER 2: METHODS

Summary

Three related models were used to study dysfunction, ischaemia, oedema and membrane proteins in the myocardium. These were isolated cardiomyocytes, isolated hearts and a large animal CPB model. Preceding these models was a period of confirmation and characterisation of myocardial AQP expression. These methods are outlined in detail in this chapter, together with details of the techniques used in assessing myocardial water content (MWC) and the molecular biology techniques used.

All projects were conceived as part of meetings involving the Kids Heart Research group. Experiments and figures were performed and prepared by myself unless noted in the figure legend as otherwise. The initial characterisation of myocardial aquaporins was greatly enhanced by collaboration with Professor Alan Verkman and the knockout mouse experiments conducted in his laboratory at the University of California, San Francisco by Dr Tanya Butler. Dr Tanya Butler took over the isolated cell work once it was established locally at the Children's Hospital at Westmead. Dr Tanya Butler also performed the last two series of isolated heart experiments, following a period of observation and training. Having assisted in the development and establishment of these techniques locally, my main focus became conducting and analysing the two series of sheep experiments.

2.1 Ethical approval

2.1.1 Human muscle specimens

The Human Ethics Committee at The Children's Hospital at Westmead approved the use of tissue for these studies. Human ventricular myocardium was obtained fresh from the operating theatre. Tissue normally discarded as part of corrective surgery for children with structural heart disease was used. Ventricular myocardium was most commonly obtained from the right ventricular outflow tract in children undergoing repair

for Tetralogy of Fallot. Samples from left ventricular outflow tract and inter-ventricular septum were also available. The tissue was obtained while the patient was on CPB after cardioplegic arrest of the heart. Specimens were immediately frozen in liquid nitrogen after resection, with a warm ischaemia time of around 30 seconds. Additional cardiac tissue was obtained from donor hearts that were not used for transplantation. Human skeletal muscle was obtained from a bank of tissue that included muscle with normal histology derived from children undergoing amputation for osteosarcoma.

2.1.2 Animal experiments

The Animal Ethics Committees of The Children's Hospital at Westmead and Western Sydney Area Health Service approved these studies and all animals received humane care in compliance with National Health and Medical Research Council animal care guidelines (NHMRC, 2004). The initial pilot isolated cardiomyocyte experiments performed at Victor Chang Cardiac Research Institute (VCCRI) with Dr Aisling McMahon were done under the auspices of the St Vincent's Hospital and Garvan Institute of Medical Research Ethics Committee, and were in compliance with National Health and Medical Research Council animal care guidelines (NHMRC, 2004).

2.2 Molecular techniques

2.2.1 Neonatal cardiac myocyte preparations

Primary cultures of cardiac myocytes were prepared from the hearts of neonatal Wistar rats using the Worthington Cardiomyocyte Isolation System (Worthington Biochemical Corp', NJ, USA). Cells were prepared from two day old rats following the manufacturer's instructions. The cells were plated onto plastic culture dishes coated with Type IV Collagen (Sigma-Aldrich, St Louis, MO, USA) at 7.5µg/ml and harvested at various times between zero and four days of culture. Spontaneous beating of the cells was observed from day one of culture. Isolation and culture of cardiac myocytes using this method produces cultures that are more than 90% pure with the major cellular contaminant being fibroblasts (Wagner, 1998).

2.2.2 Subcellular fractionation for mitochondrial preparations

Mitochondrial and sarcolemmal membrane fractions were prepared from wild-type mouse skeletal muscle and heart using OptiPrep gradients (Axis-Shield, Oslo, Norway) following the manufacturer's methods. Fresh tissue was finely minced and then dispersed in ice-cold buffer [250 mM sucrose, 10 mM HEPES pH7.4, 1 mM EDTA, protease inhibitor cocktail] with a dounce homogeniser. Nuclei and whole cells were removed by centrifugation at 700*g* for 10 minutes (x2). The supernatant was then centrifuged for 10 minutes at 16,000*g* to produce a mitochondrial pellet. The supernatant of this spin was retained as the cytoplasmic/membrane fraction. The mitochondrial pellet was washed twice with homogenisation buffer. Each fraction was separately added to 20% iodixanol in homogenizing buffer and centrifuged at 300,000*g* for two hours at 15°C to produce a continuous gradient. Fractions were retained and the membranes concentrated with further centrifugation at 100,000*g* for 30 minutes. Proteins were solubilised in equal concentration of 5x SDS sample buffer [240 mM Tris-HCl (pH6.8), 6 mM EDTA, 6% SDS, 0.01% bromophenol blue, 3% β-mercaptoethanol] prior to Western analysis.

2.2.3 Water permeability measurements

Hearts from three mice were pooled, homogenised and membrane fractions purified by centrifugation through a discontinuous sucrose gradient as described previously (Yang, 1996). Membrane fractions were pelleted, resuspended at approximately 1 mg protein/ml in PBS and passed ten times through a 27-gauge needle. Stopped-flow measurements of vesicle osmotic water permeability were carried out on a Hi-Tech Sf-51 (Hi-Tech Co, Salisbury, UK) instrument with measurements repeated five to 10 times per group and repeated in two independent experiments. Membrane vesicles were subjected to a 140 mosM osmotic gradient by mixing with an equal volume of 280 mM mannitol in PBS. The kinetics of decreasing vesicle volume was measured from the time course of 90 degree scattered light intensity at 530nm. The data were fitted to a single exponential function. The osmotic water permeability coefficient (Pf) was calculated as described previously by Verkman *et al* (Verkman, 1989).

2.2.4 Western blot

For Western analysis of whole cell lysates, frozen tissue was homogenised in ice-cold lysis buffer [50 mM Tris (pH8.0), 150 mM NaCl, 0.1% sodium *n*-dodecyl sulfate (SDS), 1% NP-40, 1 mM phenylmethyl sulfonyl fluoride (PMSF)] containing protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA) using a dounce homogeniser on ice. The homogenate was briefly spun at 1,000*g* to pellet cell debris. Protein concentrations were determined with Bio-Rad protein assay dye reagent concentrate (Hercules, CA, USA). The homogenates were mixed with an equal volume of 5x SDS sample buffer before loading onto 12% SDS-PAGE gels.

Electrophoresis was followed by transfer to PVDF Immobilon-P membranes (Millipore Australia, Sydney, NSW, Australia). Membranes were blocked then incubated with AQP1 (AQP11-A, 1:1000 dilution, Alpha Diagnostics, San Antonio, TX, USA), AQP3 (sc-9885, 1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), AQP4 (sc-9888, 1:1000 dilution, Santa Cruz Biotechnology), AQP5 (1:500 dilution, MERCK,

Boronia, VIC, Australia), AQP7 (sc-28625, 1:500 dilution, Santa Cruz Biotechnology), mitochondrial porin (1:3000 dilution, Molecular Probes, Invitrogen, Carlasbad, CA, USA), β-dystroglycan (1:250 dilution, Novocastra Laboratories, Newcastle upon Tyne, UK), cardiac/skeletal actin (5c5, 1:3000 dilution, Sigma-Aldrich) or VE-cadherin (sc-6458, 1:1000 dilution, Santa Cruz Biotechnology) antibodies. Secondary antibodies were horseradish peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology), donkey anti-rabbit IgG (Amersham Biosciences, Rydalmere, NSW, Australia) or sheep anti-mouse IgG (Amersham Biosciences) and the signal was visualised using ECL (Amersham Biosciences).

2.2.5 Membrane separation technique for dystrophin, dysferlin and caspase 3

Membrane preparations were prepared according to the published method of Kido *et al* (Kido, 2004) following ultracentrifugation and then run as previously described for Western blots. Membranes were probed with the following primary antibodies for dystrophin: MANDRA-1 (1:250); (Sigma-Aldrich) for the C terminus, Dys1 (1:200); (Novocastra) for the rod domain and Dys3 (1:20); (Novocastra) for the N-terminus, with appropriate positive and negative controls (Vatta, 2002; Kido, 2004; Rodriguez, 2005). Dysferlin was detected with the antibody Hamlet1(1:200); (Novocastra). Loading was controlled with an antibody to α-actinin-2 (4B3; affinity-purified rabbit; 1:200,000; kindly provided by Alan Beggs, Boston, MA, USA).

2.2.6 Immunohistochemistry

Cryosections 8μm thick were fixed with 3% formaldehyde in PBS, washed and incubated in blocking buffer (PBS containing 2% serum). AQP4 (sc-20812; 1:50 dilution, Santa Cruz Biotechnology), AQP1 (AQP11-A, 1:1000 dilution, Alpha Diagnostics) or α-sarcomeric actinin (EA53, 1:400 dilution, Sigma-Aldrich) antibodies were applied for one hour. Samples were washed then incubated with Cy3-conjugated donkey anti-goat or donkey anti-rabbit IgG (1:250 dilution, Jackson ImmunoResearch Laboratories, West

Grove, PA, USA). Samples were washed and mounted onto glass slides using Immu-Mount (Shandon, Pittsburgh, PA, USA).

2.2.7 Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed with LUX primers (Invitrogen, Carlsbad, CA, USA) for rat AQP1 (5'-CAACGTGTGTGGGAGCCATCG[JOE]TG-3'; 5'-CCAGGTCATTTCGGCCAAG-3'), for sheep AQP1 (5'-CGA GAT CGC CAC TGT CAT CCT CT[FAM]G-3'; 5'-CAT TGA GGC CAA GCG AGT TG-3'). For rat AQP4 (5'-CAC TTA GAG GCG GTG GGG TAA G[JOE]G-3'; 5'-TTG AGT CCA GAC GCC TTT GA-3'), for sheep AQP4 (5'-GAC AGA AGA AAA GCC ATT ACC TGT[FAM]G-3'; 5'-GAT GCT GAG TCC AAA GCA GAG G-3') and r18S (5'-GAC CTG CCG AGA TTG AGC AAT AAC AGG[FAM]C-3'; 5'-GTA GGG TAG GCA CAC GCT GAG-3') using the Platinum PCR SuperMix-UDG kit. All samples were run in duplicate. AQP levels were quantified during 45 cycles using a Corbett Research Rotor-Gene RG 3000A (Mortlake, NSW, Australia) and analysis was performed using Rotor-Gene Real Time Analysis Version 6.0 (Corbett Research, 2004). mRNA levels were quantified using r18S RNA to normalise the raw AQP signals, this is the preferred house keeping gene in the setting of ischaemia (Jenner, 2004). Quantification of transcript was achieved through use of appropriate standard curves for mRNA of r18S and gene of interest. The Delta delta method of quantification was then utilised – essentially this calculates a fold change in gene of interest after first measuring the change in gene of interest transcript from baseline tissue (e.g. control) less the change in r18S RNA in these 2 tissue samples (Jenner, 2004).

2.2.8 Apoptosis

Assessment of apoptotic cell death was performed with a TUNEL assay (TdT-mediated dUTP Nick-End Labelling) using the In Situ Cell Death Detection Kit (Roche, Basal, Switzerland). Positively labelled cells vs. total cells were counted to quantify cell death. The entire slide was scanned at high power magnification by an observer blinded

to animal allocation. Haematoxylin and eosin (H+E) stained slides were also reviewed in a blinded fashion to review tissue integrity and exclude necrosis. Western blots for active caspase 3 were prepared using cytoplasmic fractions obtained as part of the separation technique (Kido, 2004). These were then probed using the caspase 3 antibody (active rabbit anti-caspase 3, 1:2000, BD Pharmingen, BD Biosciences, North Ryde, NSW, Australia).

2.2.9 Myocardial membrane integrity

2.2.9.1 Evans blue analysis

Paraformaldehyde immersed sections were cut and mounted on slides by Aysen Yuksel of histopathology at the Millennium institute, (Westmead, NSW, Australia). Evans Blue staining of the myocardium was assessed as an estimation of extracellular water accumulation and membrane integrity (blinded to allocation) by reviewing 5 – 100X fields and staining rated as abundant – (2), present – (1) or absent – (0).

2.2.9.2 Creatinine kinase

Blood was drawn from lambs having CPB post anaesthetic induction and then 6hr post CPB for total CK. This was transported at 4°C to the Biochemistry department at the Children's Hospital at Westmead and CK measured by the production of ATP in a coupled enzyme reaction using the Vitros CK Slide method on a Vitros Fusion 5.1 (Ortho-Clinical Diagnostics, Beerse, Belgium).

2.2.9.3 Troponin

Plasma troponin T was measured in lambs having CPB post anaesthetic induction and then 6 hr post CPB. Collected blood was immediately spun for 15 minutes at 5000*g* and stored briefly at 4°C, prior to being frozen at 80°C prior to measurement. Measurement was performed at the Institute of Clinical Pathology & Medical Research,

Westmead Hospital, (Westmead, NSW, Australia). Measurement of troponin T was by an electrochemiluminescence immunoassay conducted on the Roche E170 machine, (Roche, Mannhein, Germany).

2.2.10 Myocardial water content – desiccation

Samples of intraventricular septum, left and right free walls were weighed after blotting. They were then dried at ~80°C for 48 hours in an oven (Hybaid limited, Hampshire, UK) or until their weight was static. Myocardial water content was then calculated by [weight(wet)–weight(dry)]/weight(wet) and expressed as a proportion (Jia, 2002).

2.2.11 Colloid osmotic pressure

Colloid osmotic pressure (COP) was measured using plasma from lambs having CPB. About 3ml of blood was placed in a glass heparinised test tube and spun at room temperature for 10 minutes at 5000*g*. Plasma (150-200µl) was pipetted into a plastic test tube and COP measured in mmHg, using the OSMOMAT 050 (Gonotec, Berline, Germany).

2.3 Isolated cardiomyocytes

2.3.1 Introduction

Isolated cardiomyocytes – obtained by digestion of the extracellular myocardial mass – allowed the manipulation and study of the unfettered contractile apparatus. This model enabled us to study the contractile response of the cell to oedema and ischaemia inducing insults. In conjunction we were also able to determine any change in cell size in response to these stresses. Thus we gained an insight into the effects of these insults on the contractile machinery of the heart. The limitations of this model relate to its remoteness from the *in vivo* state and that it also requires a substantial amount of capital investment to establish. Consequently our initial pilot experiments were undertaken at the VCCRI to establish proof of principle, prior to establishing the model at The Children's Hospital at Westmead.

2.3.2 Mouse cells at Victor Chang Cardiac Research Institute

Mice were wild type C57B16 x 129/Sv and housed at the Garvan Institute of Medical Research in Sydney, Australia. Animals were caged with up to four other mice. They were fed standard feed (irradiated mouse pellets, Gordon's Specialty Stock feed, Yanderra, NSW, Australia) and acidified water. Facility temperature was maintained at 19-20°C, humidity was 50% and there was a split 12hr light day cycle.

2.3.2.1 Anaesthesia and perfusion

The mice received intraperitoneal heparin 5U/g (1000U/ml, AstraZeneca, North Ryde, NSW, Australia). Anaesthesia was then given 20 minutes later by intraperitoneal injection (0.0025ml/g) of a xylazine (20mg/ml) and ketamine (100mg/ml) mix. This mix consisted of 1mg of xylazine (Ilium Xlazil-20, Troy Lab. P/L, Smithfield, NSW, Australia), 4.35g of ketamine (Ilium Ketamil, Troy Lab. P/L, Smithfield, Australia) and

was reconstituted to 0.5ml with heparinised saline (Heparin Sodium, 50U/5ml, AstraZeneca, North Ryde, NSW, Australia).

2.3.2.2 Digestion and preparation

The heart was removed via sternotomy from the anaesthetised mouse. The heart was placed in semi-frozen Krebs buffer in a plastic dish. The aorta was identified under a dissecting microscope and cannulated – with the tip of the cannula secured by 3.0 silk suture, just distal to the aortic valve, (Figure 2.5B).

The cannulated heart was attached to the Langendorff apparatus and the heart perfused at 70mmHg with Krebs buffer containing 2mM[Ca^{2+}] at 37°C. Steady flow of ~3ml/min was maintained for five minutes. The perfusion was then switched for five minutes to the low calcium buffer inducing arrest. The protease medium (Protease type XXIV(P-8038, 8.8 U/mg) was perfused for one minute – initiating tissue breakdown. The final collagenase/ hyaluronidase (Collagenase type II(CLS-2, 34.2U/mg)(Worthington Biochemical Corp., NJ, USA) and (Hyaluronidase Type I-S(H-3506,999U/mg) solution was then run for 10 minutes, (Figure 2.2). The ventricles were removed, finely cut up and placed in a small flask containing 5ml fresh collagenase/ hyaluronidase. This was shaken for five minutes at 37°C. The medium was strained via a 200µm gauze into a tube. This supernatant was then centrifuged at 15*g* for one minute. A loose cell pellet was collected and resuspended in Krebs buffer with increasing concentrations of calcium, from 200µM[Ca^{2+}], 500µM[Ca^{2+}] and finally 1mM[Ca^{2+}]. This solution was then placed in the microscope stage dish of the IonOptix setup (IonOptix, Milton, MA, USA), (Figure 2.2).

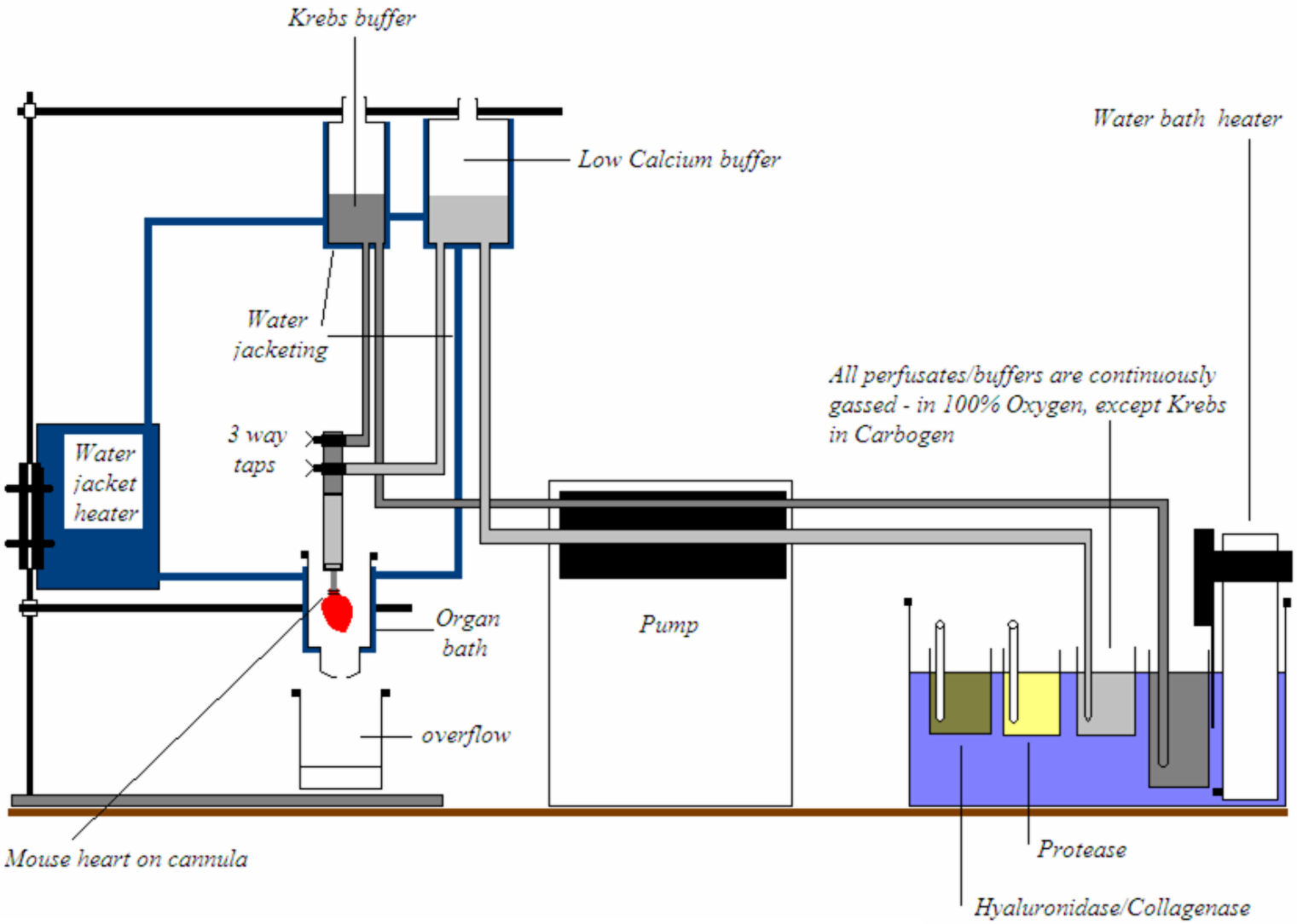

Figure 2.1 : VCCRI cardiomyocyte isolation set up. *Modified from the thesis of JC Tan.*

See http://www.library.unsw.edu.au/~thesis/adt-NUN/uploads/approved/adt-NUN20071010.103345/public/02whole.pdf

2.3.2.3 Microscope and image capture

The cells were placed in a shallow cell chamber (University NSW) on an inverted microscope (Eclipse TE200, Nikon, Japan) and after five minutes were viewed at 40X. Cells were perfused with gassed Krebs solution, pH 7.4, 1.25mM Ca^{2+} , 32°C and perfusate inflow/outflow were controlled by 505DU (Watson Marlow, UK) and Minipuls 3 (Gilson, France) pumps respectively. Perfusing solutions were kept at 32°C in a water bath (Type 05, R.A.P, Montgomery, UK). Solutions used were a baseline isotonic Krebs buffer (sodium chloride 119mM, potassium chloride 4.7mM, magnesium sulphate 0.94mM, potassium phosphate 1.2mM, sodium bicarbonate 25mM, glucose 11.5mM and calcium chloride 1mM) and then a hypotonic Tyrodes (sodium chloride 70mM, potassium chloride 5.4mM, magnesium chloride 1mM, Hepes 5mM, glucose 10mM, sucrose 70mM and calcium chloride 1.8mM (Ogura, 2002b)– they were substituted 10 minutes each as the inflow perfusate.

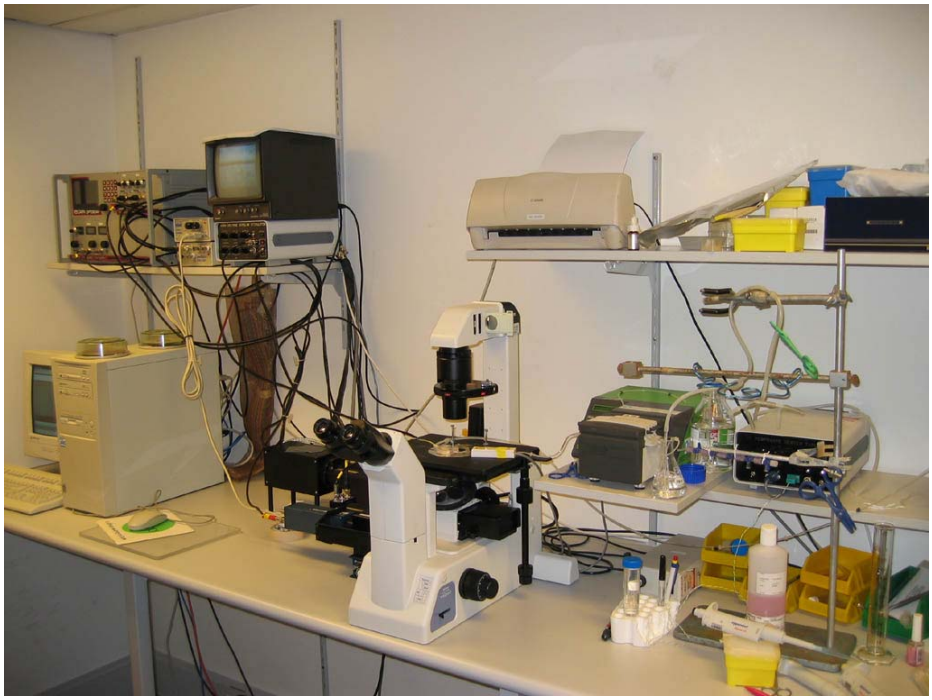

Figure 2.2: The VCCRI IonOptix isolated cardiomyocyte set up.

Cell images illuminated by the microscope red light (>620λ) were transmitted to the charge-coupled device (CCD)-camera, Myocam (IonOptix, Milton, MA, USA), sampling at 240Hz. Digital images of 20 cells per heart were captured at 60Hz and stored using an image capture program (Rapview, InoOptix, Milton, MA, USA). Cell length and width were measured using Image tool software (UTHSCSA, USA).

Information pertaining to the methodology of studies performed at VCCRI was confirmed by reference to the PhD thesis of Ju Chiat Tan – who also assisted in these pilot experiments. His thesis is accessible at http://www.library.unsw.edu.au/~thesis/adt-NUN/uploads/approved/adt-NUN20071010.103345/public/02whole.pdf

2.3.3 Rat cardiomyocytes at Children's Hospital at Westmead

Adult male Wistar rats (200–400g) were housed, maximum four per cage in the Westmead Hospital Dept of Animal Care, Research Holding. They were fed a commercial balanced diet from Glenn Forest Specialty Feeds (Perth, WA, Australia). They had 12hr light/dark cycles, experienced ambient humidity and room temperature was maintained at 22 – 24°C.

2.3.3.1 Anaesthesia and perfusion

Wistar rats (200–350g) were anaesthetised with inhaled isoflurane (Aerrane, Baxter, Old Toongabbie, NSW, Australia). Sternotomy was performed, the heart excised and immediately placed in ice-cold Krebs buffer (Sigma-Aldrich, St Louis, MO, USA). All solutions were made with deionised and filtered (0.04μm) water (Merck, Kilsyth, VIC, Australia). Hearts were then cannulated on a modified Langendorff setup, (Figure 2.3). In essence this consisted of custom made glassware (University of Queensland glassblowing services, Brisbane, QLD, Australia), tubing (Tygon Lab tubing, Cole-Parmer Instrument Co, Ill, USA), tubing pump (Masterflex combo drive and easy load head, Cole-Parmer instrument, Barnant Co, Burlington, Ill, USA) and a water bath heater (Ratek thermoregulator, Ratek instruments, Boronia, VIC, Australia). Hearts were perfused at 6-8ml/min with oxygenated solutions warmed to 37°C. Perfusion for five minutes with Krebs buffer was followed by five minutes perfusion with nominally calcium-free Tyrode's buffer containing (in mmol/L) 140 NaCl, 5.4 KCl, 1$MgCl_2$, 5 HEPES, 20 taurine, 10 glucose. Digestion was then achieved by perfusion for 20 minutes with Tyrode's buffer containing 1mg/ml collagenase type II (Worthington Biomedical, Lakewood, NJ, USA), 1mg/ml hyaluronidase type I (Sigma-Aldrich, St Louis, MO, USA) and 175μM $CaCl_2$.

Hearts were then removed from the apparatus, coarsely minced and subject to three times five minute rounds of digestion at 37°C with gentle mechanical agitation. Cells were

filtered through a 180μm filter, pelleted by gravity and washed in Tyrode's buffer containing increasing amounts of calcium to a final concentration of 1mM.

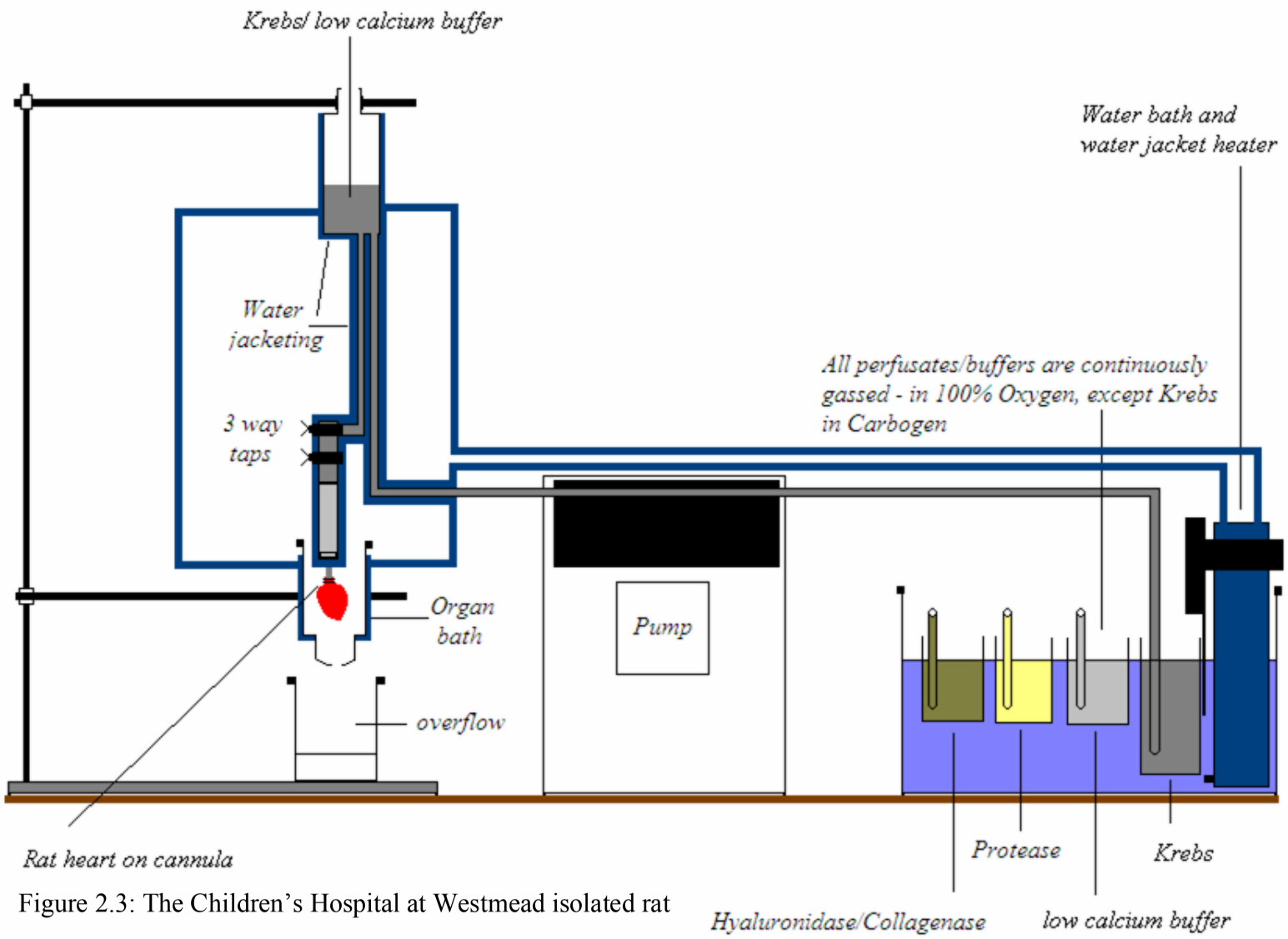

Figure 2.3: The Children's Hospital at Westmead isolated rat cardiomyocyte set up. Modified from the thesis of JC Tan.

See http://www.library.unsw.edu.au/~thesis/adt-NUN/uploads/approved/adt-NUN20071010.103345/public/02whole.pdf

2.3.3.2 Digestion and preparation

Cardiomyocytes were placed in a custom chamber (IonOptix, Milton, MA, USA) on an inverted Nikon Eclipse TE2000U microscope (Coherent Scientific, Hilton, SA, Australia) and perfused at 1-2mL/min with buffers warmed in-line (Cell MicroControls, Norfolk, VA, USA) to 25°C. Buffers for swelling studies were taken from the work of Ogura (Ogura, 2002b) with some modification. The primary buffer was Tyrode's buffer containing (in mmol/L) 140 NaCl, 5.4 KCl, 1$MgCl_2$, 5 HEPES, 10 glucose, 1 $CaCl_2$, referred to as Isotonic (=1T). Tyrode's buffer in which 35mM NaCl was replaced with 70mM sucrose was referred to as LowNa Isotonic and Tyrode's buffer with 35mM NaCl removed to make the solution hypotonic was referred to as 0.75T. This degree of hypotonicity was chosen as an estimate of the hypo-osmotic stress associated with ischaemia and modified from the extremes used by others (Suleymanian and Baumgarten, 1996; Ogura, 2002b) to more closely reflect the subtle changes occurring during surgery and recovery in infants. Buffers for ischaemia experiments were taken from Lu *et al* (Lu, 2005) and contained (in mmol/L) 140 NaCl, 5.4 KCl, 1$MgCl_2$, 5 HEPES, 1 $CaCl_2$, 2.5 Na-lactate. Isotonic, LowNa Isotonic and 0.75T buffers were adjusted to pH 7.4 and oxygenated while the ischaemic buffer was adjusted to pH 6.8 and bubbled with 100% N_2.

2.3.3.3 Isolated cardiomyocyte functional analysis

Cardiomyocytes were loaded with Fura-2-AM (Invitrogen, Carlsbad, CA, USA) by incubation in a 5μM solution at room temperature for 10 minutes, followed by washing. Intracellular calcium was measured by exciting cardiomyocytes alternately at 340 and 380nm and reading emission at 505nm. Function was measured using video-based detection and calculation of sarcomere length (IonOptix, Milton, MA, USA), (Figure 2.4). Unloaded cells were paced (MyoPacer, IonOptix, Milton, MA, USA) to contract at a frequency of 1Hz while sarcomere length and Fura-2 transients were recorded. A minimum of 10 transients were averaged from each cell and treatment. Measures of sarcomere length and intracellular calcium analysed included resting

(diastolic) and peak (systolic) values, maximum departure and return velocities, the time of transient onset, peak time, and the constant tau describing calcium removal. Cells were excluded from analysis if they displayed membrane blebs, resting sarcomere lengths of less than 1.6μm, frequent spontaneous contractions without stimulation or less than 5% shortening.

Figure 2.4: The Children's Hospital at Westmead, IonOptix set up for cardiomyocyte imaging.

2.3.3.4 Isolated cardiomyocyte experimental protocol

Cells were separated into three treatment groups. The numbers of cardiomyocytes per group ranged from 9-21 and were derived from four to six individual rats. Each treatment group consisted of a baseline, test, and recovery phase with functional parameters measured at the end of each phase.

For swelling studies, cells were first perfused with LowNa Isotonic buffer, followed by eight minutes in 0.75T buffer and recovery in LowNa Isotonic buffer for eight minutes. Control cells were perfused continuously with LowNa Isotonic buffer and recordings made at equivalent times with the test group.

In ischaemic experiments, cells were perfused with Isotonic buffer followed by 10 minutes in ischaemic buffer. Recovery was measured at three and 10 minutes of reperfusion in either LowNa isotonic buffer or 0.75T buffer.

Cell area was calculated for all cells during diastole, using images captured at the end of each treatment time (baseline, test and recovery). Cell borders were manually traced and area in μm^2 calculated using Image J (NIH, Bethesda, MD, USA).

2.4 Isolated heart (Langendorff) experiments

2.4.1 Introduction

The Langendorff technique is an ingenious method of studying myocardial function, see Skrzypiec-Spring *et al* (Skrzypiec-Spring, 2007) for a recent review. A series of figures purpose drawn under our guidance by Animated Biomedical Productions (Westmead, NSW, Australia) below further outlines the technique, (Figure 2.5).

2.4.2 Isolated heart perfusion and ischaemia

Rats were anaesthetised with inhaled isoflurane (Aerrane, Baxter, Old Toongabbie, NSW, Australia) and oxygen mixture and injected with 0.5ml heparin (Heparin sodium 5000U/5ml, Pharmacia, Bentley, WA, Australia) into the left renal vein. Thoracotomy was performed and the hearts rapidly excised and plunged into ice-cold Krebs-Helseleit perfusate solution [118 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11 mM glucose, 1.2 mM $CaCl_2$]. Baseline control hearts (no perfusion) were processed immediately after removal from the animal. For perfused hearts, the aorta was cannulated and the hearts perfused via the ascending aorta using a Langendorff apparatus (Radnoti Glass Technology Inc., Monrovia, CA, USA) at a constant pressure of 80mmHg, (Figure 2.6). The perfusate was gassed with 95% O_2/5% CO_2 and maintained at 37°C, (Figure 2.7). Thebesian vein drainage was vented with a polyethylene (PE) tubing, 0.90 x 0.50mm (Microtube extrusions, North Rocks, NSW, Australia) placed through the left ventricular apex. The left atrial appendage was removed and a latex balloon was placed in the left ventricle via the atrium. The balloon was connected to a pressure transducer (Capto, Skoppum, Norway) and filled with saline to produce an end-diastolic pressure of 5mmHg. The system was calibrated using a tracheal tube cuff pressure manometer (Mallinckrodt Medical, Athlone, Ireland). Measurements of left ventricular developed pressure (systolic and diastolic), contractility – change in pressure over change in time, minimum – (dP/dt_{min}) and heart rate were

collected online in real time PowerLab 8/30 (ADInstruments, Sydney, Australia), (Figure 2.5 and 2.6).

Individual hearts were only accepted if the intrinsic heart rate was above 200 beats/min and achieved a developed pressure > 100mmHg systolic pressure within twenty minutes of perfusion.

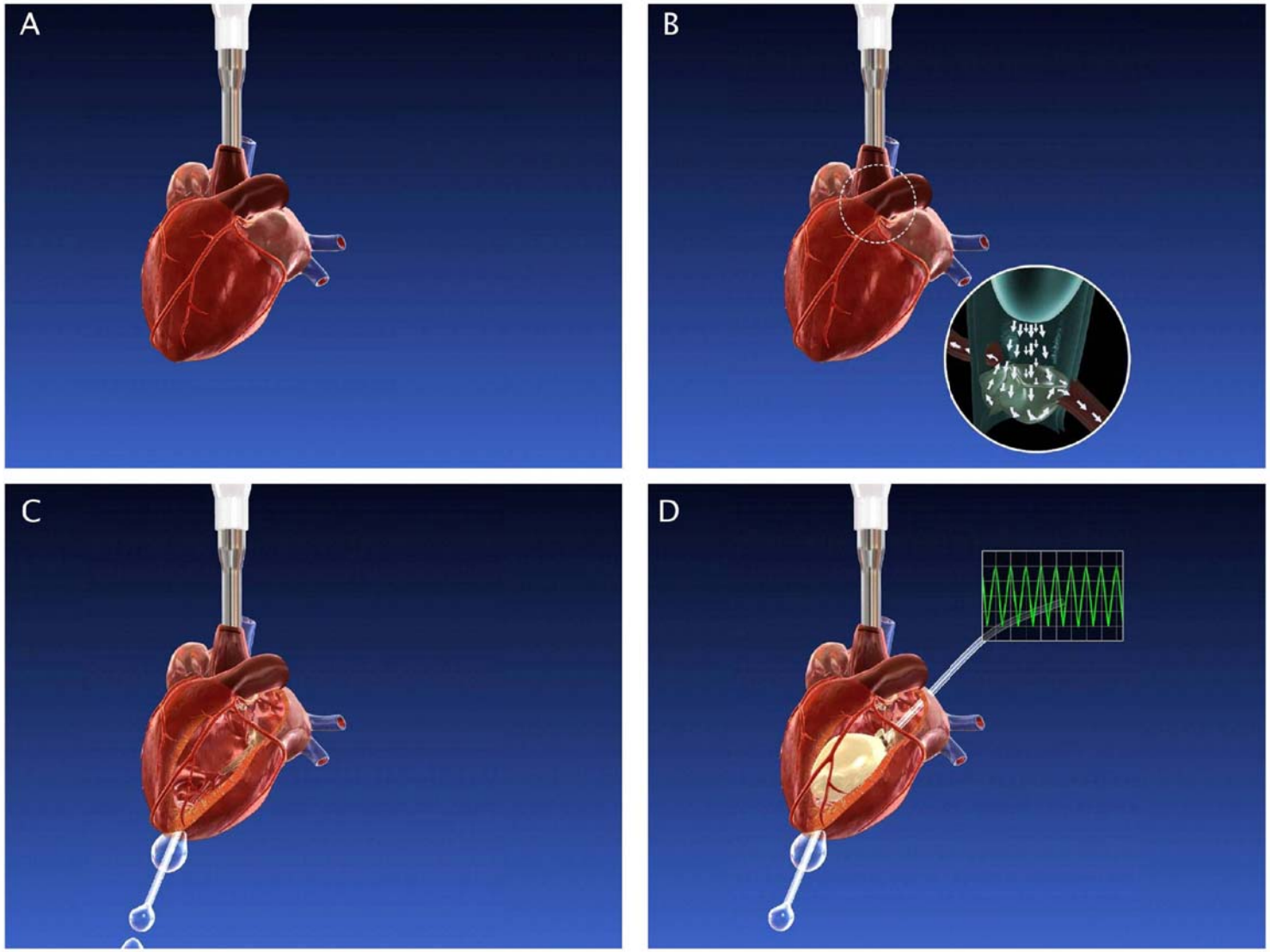

Figure 2.5: Langendorff isolated heart; A – cannulated rat heart with sutured aortic cannula. B - A cannulated rat heart, the cutaway shows the perfusate ejecting against the closed aortic valve and perfusing the coronary arteries. C – a drainage tube is passed via the left atrium (LA) via the apex of the heart. D – a latex fluid filled balloon is then placed via the LA into the left ventricular cavity – this generates a pressure waveform. Under guidance by Animated Biomedical Productions(Westmead, NSW, Australia)

Figure 2.6: Rat Langendorff set up.

Figure 2.7: Crystalloid perfusate being gassed.

2.4.3 Balloon design

Design was as per Dr Tamsin Jenner of Griffith University, (Gold Coast, Queensland, Australia).

Latex from the thin end of a condom – with the lubricant rinsed off in soap and water was used. Silk suture – 3.0 (Sofsilk, Syneture, Norwalk, CT, USA), 21-guage x 1¼ needle (Terumo, Tokyo, Japan), 3ml syringe (Terumo, Elkton, MD, USA), and polyethylene (PE) tubing, 1.45 x 0.75mm (Microtube extrusions, North Rocks, NSW, Australia) were used in construction. A small 1-2mm wide flange was formed at one end of a 10cm length of PE tubing, the other end was carefully threaded over the 21-guage needle tip (this was carefully blunted prior by snipping off and filing the sharpest and most distal needlepoint). The tubing was placed flange first into the distal 3cm of the condom (excess latex having been discarded). A 15cm length of suture was tied around the condom/tubing about 8mm from the end of the condom – this was the approximate final length of the balloon and was suitable for a 200-400g rat. A half knot was tied on the front and a full knot secured on the back. The tubing was pulled back until the flange abutted the suture. The balloon was filled with saline via the needle/syringe, and bubbles

removed. Excess suture and latex were removed. The balloon was stored in water, in the dark and was used for ~ 1 month, (Figure 2.8 and 2.9).

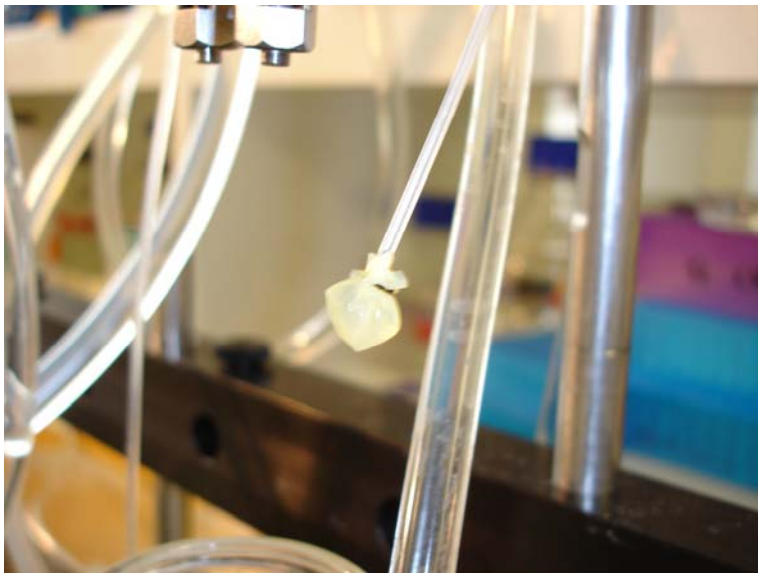

Figure 2.8: A distended latex balloon.

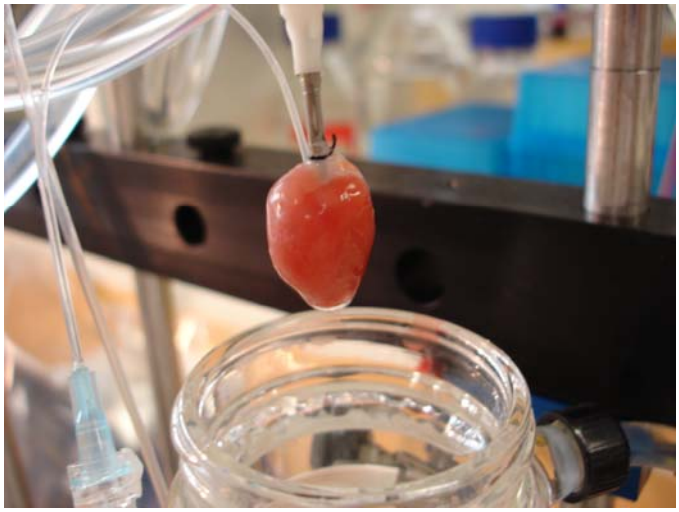

Figure 2.9: Rat heart, with cannula and LV balloon in situ. Note heart is sitting above organ bath for the image.

2.4.4 Langendorff protocols

2.4.4.1 Twenty minute ischaemia experiments

Hearts were removed from the anaesthetised animal, plunged into 4°C buffer and then processed without being perfused to form the non perfused control group. The next group of animal hearts were first stabilised on the Langendorff set up for 20 minutes and processed immediately after this time to produce the 20 minute control group. An additional control group was produced by maintaining perfusion for an additional 100 minutes.

Hearts in the ischaemic treatment group received 20 minutes of perfusion followed by 20 minutes of zero-flow ischaemia and 70 minutes of reperfusion.

2.4.4.2 Poloxamer 188 – low dose study

The dose of P188 used was calculated to minimise free Poloxamer 188 (P188) and was based on work which suggested a lower dose ~50μM reduced free micelle formation (Maskarinec, 2002). Maskarinec *et al* found that P188 only inserted into damaged membranes at or below this concentration and that higher concentrations impeded this effect. They also found that non-specific sealing did not occur – only sealing of defects at relatively low doses. We chose this concentration of 50μM or 0.42mg/ml – which was significantly less (~350 times) than that used clinically – 150mg/ml (Adams-Graves, 1997). The P188 (3.36g) was mixed with the Krebs-Henseleit perfusate (8L) and delivered at constant pressure into the heart. Gassing of the perfusate in the presence of P188 led to bubble formation – this led to a gradual loss of perfusate – necessitating additional aliquots being made. It also meant that we were not blinded to the solution being delivered. Ischaemic duration for these hearts was 30 minutes.

2.4.4.3 Poloxamer 188 – high dose study

The dose used here was based on the conventional weight based human doses used in the various sickle cell (Adams-Graves, 1997) and myocardial infarction studies of 300mg/kg using a concentration of P188 of 150mg/ml. The weight of the rat was used to calculate the dose/rate at 300mg/kg pre ischaemia. P188 was reconstituted in phosphate buffered saline (PBS) which was given without P188 for the placebo/ control arm. It was delivered as a constant infusion from an infusion pump into the perfusate line via a T piece connector. Infusion syringes were made independently, wrapped in foil and then the experiments were performed blinded to allocation. The allocation code was broken once analysis was complete. Ischaemic time for these hearts was 30 minutes.

2.4.4.4 Thirty minute ischaemia and hypotonic experiments

Control non-perfused hearts were removed from the animals as for cannulated hearts but immediately processed without perfusion. For all other treatment groups, hearts were first stabilised for 20 minutes.

Two main experimental designs were used to investigate the effect of i) osmotic or oncotic pressure changes, and ii) ischaemia.

Mild osmotic and oncotic changes were produced following the methodology of Kellen and Bassingthwaighte (Kellen and Bassingthwaighte, 2003b). Hearts were perfused with Krebs buffer plus an additional infusion of 15mM NaCl + 30mM sucrose or 0.5% dextran. Hypotonicity was achieved by removing 35mM NaCl from the Krebs buffer. This produced a 0.75T hypotonic equivalent to that used in the cardiomyocyte experiments. Infusion of 70mM sucrose to the 0.75T Krebs produced isosmotic Krebs.

For ischaemia experiments, hearts were perfused with Krebs buffer and baseline function was measured for 20 minutes followed by 30 minutes of zero-flow ischaemia and 50 minutes of reperfusion. These were matched by a perfusion control group, produced by

maintaining perfusion for 100 minutes. At the end of perfusion, all hearts had MWC determined by desiccation.

2.5 Lamb cardiopulmonary bypass model

2.5.1 Introduction

Time-mated Poll Dorset cross ewes from the University of NSW farms, (Hay, Riverina, NSW, Australia) were transported by road whilst 80-90 days gestation to holding pastures at Little Bay, Sydney, NSW. Shortly after they were transported by road to The Westmead Hospital Department of Animal Care, Vivarium. Ewes grazed on a large predominantly kikuyu pasture with shelter available and a fresh water river frontage. Water was supplied *ad libitum* and Lucerne hay was provided twice daily. Natural delivery of lambs occurred around term (~147days). Lambs were aged between three to 10 days of age for the first series of experiments and were 14–36 days of age for the subsequent P188 series. Animal weight was measured preoperatively and groups arranged to maintain statistically similar weight ranges.

2.5.2 Obtaining blood for the cardiopulmonary bypass circuit

The maternal ewe was anaesthetised with isoflurane (Aerrane, Baxter, Old Toongabbie, NSW, Australia) and a 20-guage cannula (Terumo, Tokyo, Japan) inserted into a shaved forelimb vein. Ketamine 100mg, midazolam 1mg, 5000U heparin (Heparin sodium 5000U/5ml, Pharmacia, Bentley, WA, Australia) was given. Isoflurane was maintained and ketamine and midazolam given as required to maintain anaesthesia. The ewe was nursed in the lateral decubitus position, slightly head up on a mobile lift trolley. The trolley was raised to one metre from the ground. The neck was shaved and a 12-guage cannula (Terumo, Tokyo, Japan) was secured in an internal jugular vein. The cannula was connected to a 450ml blood collection bag (Baxter, Old Toongabbie, NSW, Australia). Venous blood drained via the cannula serially into three blood collection bags. A terminal dose, 150mg/kg of pentobarbitone (Lethobarb, 325mg/ml, Virbac Animal Health, Peakhurst, NSW, Australia) was then given into the jugular vein. The three units of blood was utilised to create the blood cardioplegia (one unit) and to prime the CPB

circuit (two units), (Figure 2.10). Any remaining blood was used for colloid replacement post CPB for chest drain losses mainly.

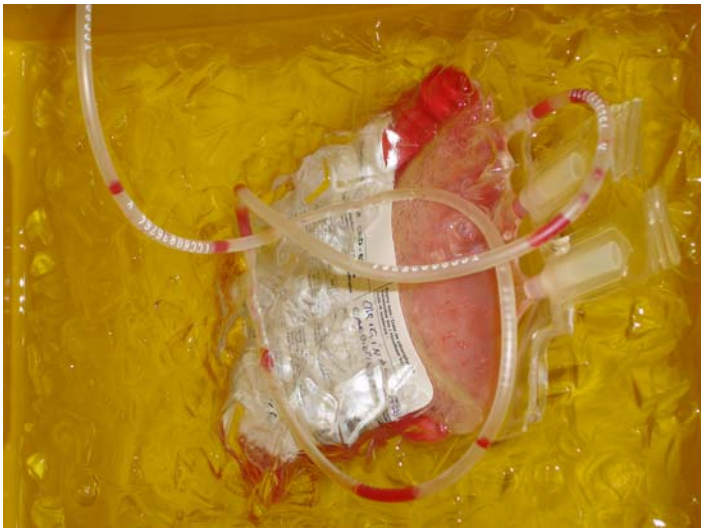

Figure 2.10: Sheep blood cardioplegia being cooled to 4°C.

2.5.3 Experimental preparation

Neonatal lambs were anaesthetised by spontaneously breathing isoflurane (Aerrane, Baxter, Old Toongabbie, NSW, Australia) and given IVI ketamine (1mg/kg), (Ketalar, Pfizer, West Ryde, NSW, Australia) and midazolam (100mcg/kg), (Hypnovel, Roche, Dee Why, NSW, Australia). The lamb was intubated with a 5.5mm cuffed endotracheal tube (Mallinckrodt Medical, Athlone, Ireland). Ventilation was maintained with a Campbell ventilator (ULCO, Marrickville, NSW, Australia) aiming for physiologically normal oxygen saturations (100%) and $paCO_2$ (40–45mmHg) – typical ventilation parameters were 20–25/5cmH_2O in 100% oxygen, (Figure 2.11). General anaesthesia was maintained with inhaled isoflurane (0.1–1%) continuously via the circuit, intermittent ketamine, midazolam and pancuronium (Faulding Pharmaceuticals, Melbourne, VIC, Australia), were also administered as required. Intravenous flucloxacillin (25mg/kg), (Faulding Pharmaceuticals, Melbourne, VIC, Australia), was given sixth hourly.

A three lumen 5.5Fr 13cm central line (Arrow, Reading, PA, USA) was placed percutaneously into the right internal jugular vein. A single lumen 20cm 3Fr catheter (Cook, Bloomington, IN, USA) was inserted percutaneously into the right femoral artery, (Figure 2.12). Cutaneous three lead electrocardiograph (ECG), rectal temperature probe, central venous pressure and intra–arterial pressure were monitored continuously. Venous and arterial blood gases were taken regularly.

Figure 2.11: Lamb anaesthesia. Left, is a lamb being breathed down with a mask and circuit. On the right is an intubated lamb, with monitoring in situ.

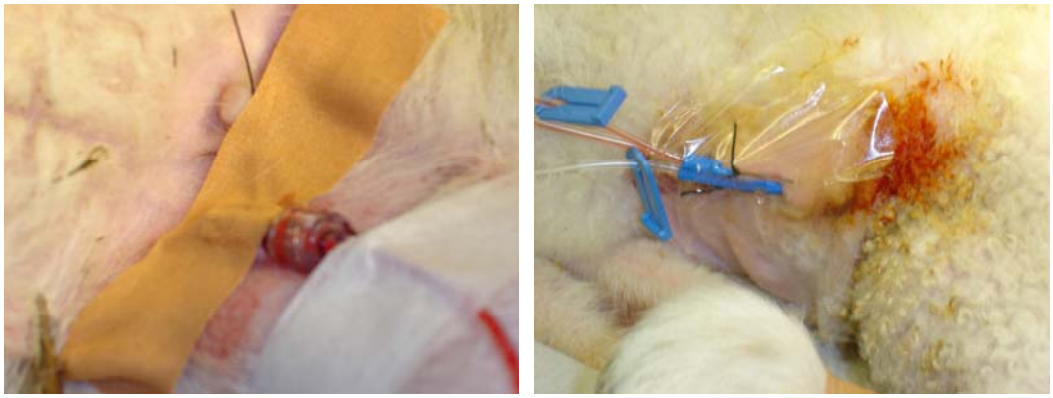

Figure 2.12: Invasive lines in the sheep. Left is a 3Fr femoral (right) arterial line. On the right is the jugular (right) triple lumen central venous line.

2.5.4 Cardiopulmonary bypass technique

2.5.4.1 Initial series

Midline sternotomy was performed and the pericardium opened. CPB was established after heparin (Heparin sodium 5000U/5ml, Pharmacia, Bentley, WA, Australia) administration (400U/kg) with right atrial and ascending aorta cannulation. The activated clotting time (ACT) was checked 20-30 minutely and maintained over 400 seconds with bolus doses of heparin. ACT was checked on a Hemochron Jr. Signature (ITC, Edison, NJ, USA), using Hemochron ACT+ cuvettes (ITC, Edison, NJ, USA). The extracorporeal circuit was established using a heart lung machine (Cobe, Arvada, CO, USA). A standard ¼-¼ or ¼-⅜ inch bypass circuit was used connected to a Terumo RX5 or SX10 oxygenator with an open venous reservoir and Terumo Capiox AF02 arterial filter (Terumo, Tokyo, Japan). About 90% of the bypass circuit prime volume comprised of maternal sheep blood and about 10% of Baxter Plasma Lyte-148-Replacement fluid (Baxter, Old Toongabbie, NSW, Australia) buffered with sodium bicarbonate and about three units of heparin per ml of prime fluid. Nonpulsatile flow rates were adjusted to maintain a flow rate of about 150ml/kg/min and the mean systemic pressures between 30–40mmHg**.** If necessary the isoflurane dose was also varied to maintain the desired blood pressure range. The lambs body core temperature was slowly lowered to 28–30°C, via a heater cooler unit (Conair-Churchill, Pittsburgh, PA, USA), (Figures 2.13, 2.14 and 2.15).

In the CPB+AXC group, following establishment of CPB, the aorta was cross clamped and blood cardioplegia at 4°C was administered 20ml/kg into the proximal ascending aorta. The cardioplegia comprised of a 4:1 blood to crystalloid mix. The average composition was pH 7.4, pCO_2 30mmHg, pO_2 70mmHg, K 19mmol/L, Ca 0.8mmol/L, bicarbonate 17mmol/L and haematocrit 12%. This was delivered 20 minutely into the aortic root for 90 minutes at which point bypass was weaned and the native circulation re–established. The average composition of the initial CPB machine prime, which did not

significantly differ between treatment groups was pH 7.6, pCO_2 24mmHg, pO_2 202mmHg, haematocrit 21%, K 4.4mmol/L, Ca 0.4mmol/L and bicarbonate 21mmol/L.

Figure 2.13: Lamb experiment utilising CPB.

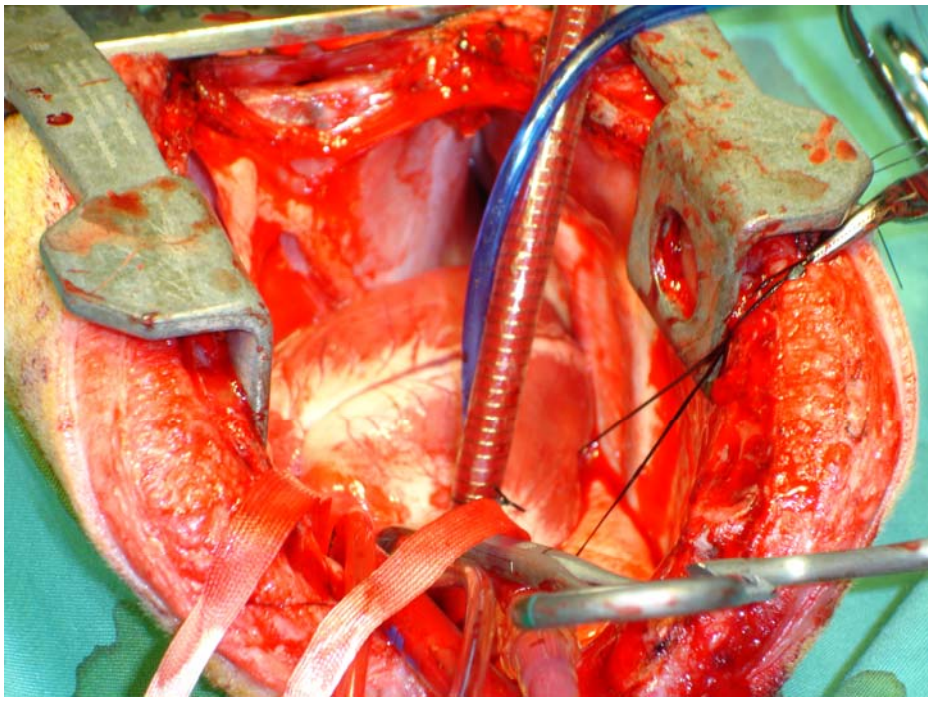

Figure 2.14: Open lamb chest with venous and arterial cannula in situ.

Figure 2.15: The operating theatre set up at the Vivarium. Anticlockwise from left are two pefusionists, surgical fellow, surgeon and two anaesthetic/ ICU staff.

Modified ultrafiltration (MUF) was performed on all lambs having CPB. Internal defibrillation (0.5–1.0J/kg) and or lignocaine (1mg/kg), (Xylocaine, AstraZeneca, North Ryde, NSW, Australia) were administered as required. Routine surgical techniques in maintenance and weaning from CPB were used, including venting of the left atrial appendage in all cases.

2.5.4.2 Lamb Poloxamer 188 trial

The protocol was the same apart from the following three differences:

Lambs received a blinded solution (2ml/kg) as a bolus load over one hour, immediately prior to CPB and then an infusion (0.2ml/kg/hr) of the same solution for the remainder of the experiment (11hrs). Administration was through the central line via an opaque extension (Codan, Santa Ana, CA) and a dedicated syringe driver (Graseby 3200, Graseby Medical, Watford, UK). The solution was prepared immediately prior to the experiment. It was filtered via a 0.22µm filter unit (Millipore, Co. Cork, Ireland) and wrapped in foil. Phosphate buffered saline (PBS) was the carrier solution administered without P188 to placebo animals. In treatment animals P188 also called Pluronic F-68 (Sigma-Aldrich, St Louis, MO, USA), was added to give a concentration of 150mg/ml in PBS and thus a one hour loading dose of 300mg/kg and then a maintenance infusion of 30mg/kg/hr – as per previous reports (Adams-Graves, 1997). Staff involved in animal experiments were unaware of the animal allocation until all data had been analysed.

Cardioplegia was repeated 20 minutely for 120 minutes.

Baseline full blood count, coagulation, liver function, urea, creatinine, creatinine kinase (CK) were measured prior to CPB. These were repeated six hours after CPB.

2.5.5 Postoperative management

2.5.5.1 Initial series

Following the reestablishment of the native circulation protamine (1–3mg/kg) was given and haemostasis achieved. The sternotomy was closed over two 28Fr intrathoracic drains which were placed on low pressure wall suction. Dopamine (~5mcg/kg/min), (CSL, Parkville, VIC, Australia) and sodium nitroprusside (~1mcg/kg/min), (Faulding Pharmaceuticals, Melbourne, VIC, Australia), were commenced after CPB and kept at these rates. Crystalloid (0.9% saline, Baxter, Old Toongabbie, NSW, Australia), or maternal blood was given to maintain an adequate preload – (central venous pressure, CVP, CVP>4mmHg) and (haemoglobin, Hb, Hb>8g/dL) and isoflurane was continued together with intermittent intravenous agents(ketamine and midazolam) and pancuronium. Ventilation was maintained and adjusted according to blood gas parameters. All animals were managed by a paediatric intensive care consultant (JRE) using conventional techniques relevant to the care of human infants.

2.5.5.2 Lamb Poloxamer 188 trial

The protocol was the same apart from the following differences:

The period of post CPB support was nine hours.

Seven hours after completion of CPB 0.2mg/kg of 0.1% Evans blue was given intravenously (Zhang, 2005).

2.5.6 Tissue collection

2.5.6.1 Initial series

After a period of reperfusion following bypass (range 3–6hrs), the sternotomy was re–opened and the aorta cross clamped and a further dose of ~4°C cardioplegia was delivered. Following electrical and cardiac standstill ~ 60 seconds, the heart was removed and placed on an ice cooled board whilst being dissected for further analysis. The heart was transversely sectioned into atria, great vessels and ventricles. The ventricles were sectioned for MWC and together with other organ tissues were either frozen at –80°C or placed in 4% paraformaldehyde for sectioning.

2.5.6.2 Lamb Poloxamer 188 trial

Nine hours following bypass the sternotomy was re–opened and an intravenous dose of pentobarbitone was delivered.

2.6 Statistical analysis

Data were expressed as means±SEM unless otherwise stated. Statistical significance was determined by the Mann-Whitney U test (MWU). Repeated measures ANOVA and linear mixed models using covariance type AR–1 when appropriate – typically for longitudinal continuous haemodynamic data – e.g. in the Langendorf experiments or in the sheep experiments. A p value of less than or equal to 0.05 was considered statistically significant (Kusuoka and Hoffman, 2002). Microsoft Excel (Microsoft Corp, Seattle, WA, USA) was used for the collection of raw data, it was also used to produce graphs and tables. The statistical package SPSSv15.0 for Windows, (SPSS, Chicago, Ill, USA) was used for analysis. Power calculations were performed for the two series of sheep experiments in view of their small sample sizes. This was done using Sample Power 2.0, (SPSS, Chicago, Ill, USA).

Statistical issues relevant to the particular models used were included in the methods sections of the following chapters.

CHAPTER 3: MYOCARDIAL EXPRESSION OF AQUAPORINS

Abstract

In studying AQPs in the setting of LCOS, we sought to refine the characterisation of AQP expression in the hearts of mammals.

We focused on the species relevant to the planned initial animal models – rats and sheep, with reference to mice and keeping in mind the basic relevance of human expression. We focused on the AQPs that had been previously described in the myocardium – AQP1 and AQP4. Immunohistochemistry, Western blot, semi-quantitative and quantitative RT-PCR were conducted on whole cell preparations and subcellular fractions to define more precisely the expression and localisation of AQPs in the myocardium. Additionally a trial of *in vitro* ischaemia was undertaken to determine if there was a change in AQP expression with ischaemia in rat myocardium. Ambiguities in expression were resolved through use of AQP knockout mouse tissue. An osmotic equilibrium study using myocardial membrane vesicles was performed to determine the functional contribution of AQPs in mouse myocardium.

AQP1, AQP4, AQP7 and AQP11 mRNA were present in myocardium from mouse, rats and humans. Sheep myocardium contained AQP1 and AQP4 as well as AQP0, AQP3 and AQP9. AQP1 protein was expressed in the endothelium of myocardial vessels in rat and sheep. Myocellular expression of AQP1 on immunohistochemistry was supported by expression in rat cardiomyocytes which had negligible endothelial tissue present. AQP4 mRNA was found in rat, sheep, mouse and human heart. AQP4 protein was present in mouse and sheep heart on Western blot, although immunohistochemistry in sheep myocardium revealed non-specific nuclear staining. Western blots of rat myocardium revealed a protein of expected size, although only non-specific staining was present on immunohistochemistry. A trial of *in vitro* ischaemia, using quantitative RT-PCR demonstrated a six fold increase in AQP4 compared to baseline, but baseline levels of AQP4 were negligible. There was no change in AQP1. Western blot comparison of

human and rat myocardium with wild type and AQP4 knockout mouse myocardial tissue revealed that humans and rats did not in fact have AQP4 protein in their hearts. However sheep and mice did have low levels of AQP4 protein in their myocardium. AQP7 protein using available antibodies was present in human and mouse heart, but not in rat heart, although it was also shown to be negative in rat adipose tissue. Antibodies to AQP0, AQP3, AQP6, AQP9 and AQP11 did not reliably demonstrate AQP protein expression in a range of rat, mouse and sheep tissues, including myocardium. Myocardial AQP1 was specifically located within the plasma membrane fractions and not within mitochondrial membranes. Myocardial membrane vesicles derived from AQP1 knockout mice were equivalent in function to wild type and AQP4 and AQP8 knockout vesicles in the presence of mercurials; i.e. despite the mouse myocardium expressing a myriad of AQPs, only AQP1 appeared to have a functional role in osmotic equilibration.

AQP1 was thus found to be the importantly expressed water channel within rat, sheep, mouse and human hearts. Endothelial and myocellular membranes contained AQP1. Of all the AQPs expressed in mouse hearts, including AQP7, AQP1 was found to be the only functionally relevant AQP. AQP1 mRNA in rat heart was unaltered following a brief period of *in vitro* ischaemia. AQP4 protein exists in relatively small amounts in sheep and mouse hearts. A six fold increase in AQP4 mRNA in rat hearts following brief ischaemia is almost certainly physiologically irrelevant given the low levels of baseline transcript and absence of protein. AQP1 is likely to be the sole important water channel in terms of expression and osmotically obligated water movement in mammals and served as the primary AQP focus in the animal models of LCOS.

3.1 Myocardial Aquaporins

3.1.1 Initial reports

Soon after AQP1 was first described it was noted to be present within the heart – particularly within the endothelium and endocardium of the heart and its vessels (Bondy, 1993). Its functional role in water handling has been alluded to earlier and was demonstrated by several authors. Based on these studies, the role of endothelial AQP1 in permitting osmotically obligated water movement also appears to be relatively greater than its role within cardiomyocyte membranes (Suleymanian and Baumgarten, 1996; Ogura, 2002b; Kellen and Bassingthwaighte, 2003b).

The effect of haemodilution and reduced oncotic pressure on AQP1 expression was studied in a foetal lamb model (Jonker, 2003). Over a five day duration the foetus had a ~50% reduction in Hct. In response to this there was a two fold increase in AQP1 mRNA and protein expression in both right and left ventricular tissue. AQP1 was demonstrated principally in the endothelial membranes of the myocardium, using immunohistochemistry (IHC). The authors concluded that AQP1 was important in cardiac fluid balance and speculated about the involvement of other AQPs in the heart (Jonker, 2003).

3.1.2 Recent publications on cardiac aquaporins

Subsequently work from our lab further refined the location of AQP1 within the myocardium. AQP1 was confirmed as being mainly endothelial and in addition a myocellular pool was present and expression was also seen in the t-tubules of the cell membrane (Au, 2004). The t-tubular system is specific to cardiac muscle as opposed to skeletal muscle. It consists of a series of shallow tortuous invaginations of the sarcolemma into the cardiomyocyte volume – (Figure 1.1). The system is considered to be important in increasing the surface area of the cardiomyocyte and improving ionic exchange, thus facilitating efficient excitation contraction coupling. On the basis of a

screen of human cardiac tissue, no novel AQP sequences were present. AQP1 was considered to be present in both myocellular and endothelial membrane pools, but this remained to be confirmed (Au, 2004).

AQP1 continues to be the main AQP studied within the heart, although most other AQPs have been found to some degree in the heart in most species as discussed in Chapter 1 and summarised in Table 1.1. Warth *et al* reported that AQP4 was increased at a transcript level following I/R in a mouse model of focal ischaemia (Warth, 2007). It was found that mRNA was raised six fold after 45 minutes ischaemia, but only two fold after 60 minutes ischaemia. Protein expression was not examined. AQP1 was not investigated in this model and nor were there measurements of oedema or dysfunction within the mouse hearts. Although this was a potentially intriguing result, AQP4 protein is not present in human hearts. We sought to develop and confirm the characterisation of AQPs in mammalian hearts of interest.

3.2 Methods

The methods for these experiments are detailed in Chapter 2, apart from the following.

3.2.1 Ischaemic rat heart study

A study utilising RT-PCR, qRT-PCR and Western blot analysis of AQP1 and AQP4 in ischaemic rat hearts was undertaken.

Rats were anaesthetised with isoflurane (Aerrane, Baxter, Old Toongabbie, NSW, Australia) and the hearts from four animals were removed via sternotomy. Four individual pieces of ventricular tissue from each animal were placed in a small weigh dish and exposed to four graduations of ischaemic insult, (Table 3.1). All myocardial tissue was *ex vivo* and not reperfused.

To quantify the response of rat myocardial AQPs to ischaemia, quantitative RT-PCR primers were developed for rat mRNA, and were used to initially examine AQP1 and AQP4. Ribosomal 18S RNA was used as an internal house keeping gene. The response of AQP1 and AQP4 to a moderate period of warm ischaemia, without reperfusion was studied.

Table 3.1. Outline of *in vitro* rat myocardium ischaemia study.

Allocation	Ischaemic insult prior to processing for mRNA and protein
1	Nil
2	30 minutes at room temperature
3	60 minutes at 4°C
4	60 minutes at 4°C , then 30 minutes at room temperature

3.3 Results

3.3.1 RT-PCR for aquaporins in the myocardium of various species

A screening RT-PCR experiment was undertaken utilising the myocardial tissues from mouse, rat and humans. Neonatal rat derived cardiomyocytes (containing negligible endothelial tissue) were also screened.

The RT-PCR screen demonstrated mRNA for AQP1, AQP4, AQP7 and AQP11 in mouse, rat and human myocardial tissue. Neonatal cardiomyocytes additionally contained AQP3 and AQP5, but lacked AQP6 and AQP9 mRNA compared with adult rat myocardium. Mouse myocardium contained all AQPs apart from AQP2, AQP3, AQP5, AQP9 and AQP10. Whilst human myocardium had mRNA for all AQPs apart from AQP2, AQP6 and AQP8, (Figure 3.1).

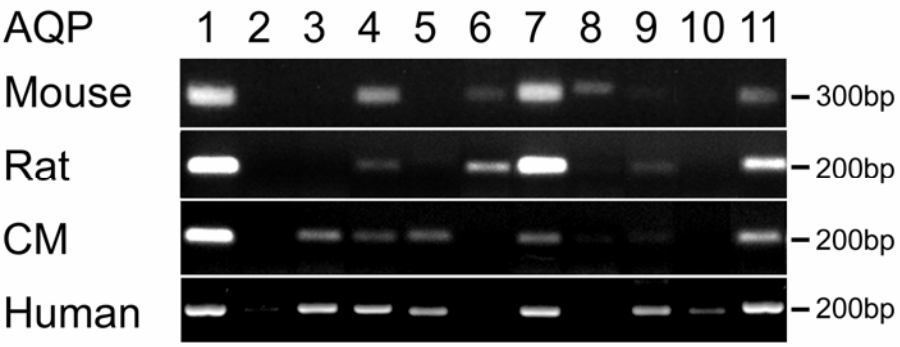

Figure 3.1. Myocardial AQP expression by RT-PCR; in Mouse, Rat, neonatal cardiomyocytes from rat (CM) and human. AQP1, AQP4, AQP7 and AQP11 transcript are present in all tissues studied. *This figure was prepared by Dr Tanya Butler.*

3.3.2 Aquaporin protein expression in rat tissues

3.3.2.1 Western blot for aquaporin 1

AQP1 protein was demonstrated in the well vascularised tissues of rat, including lung, left ventricle, right ventricle, atrium, liver, kidney, spleen and skeletal muscle. There were variable levels of the glycosylated fraction in these rat tissues. AQP1 was not demonstrated in the small intestine of the rat, (Figure 3.2).

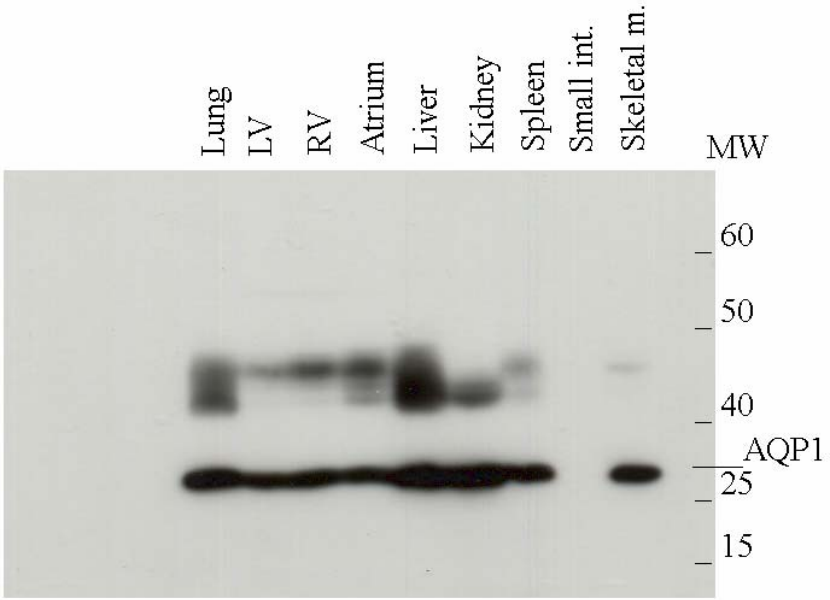

Figure 3.2. Western blot for AQP1. Whole cell lysates from rat organ tissues were probed for AQP1 (28kDa), with a larger glycosylated band. AQP1 is present in the lung, left ventricle (LV), right ventricle (RV), atrium, liver, kidney, spleen and skeletal muscle (Skeletal m). It was absent from the small intestine (Small int.).

3.3.2.2 Immunohistochemistry for aquaporin 1

IHC was performed on rat cardiac and skeletal muscle. For AQP1 this demonstrated a dominant endothelial staining in both tissues as well as possible myocellular expression in the muscles, (Figure 3.3).

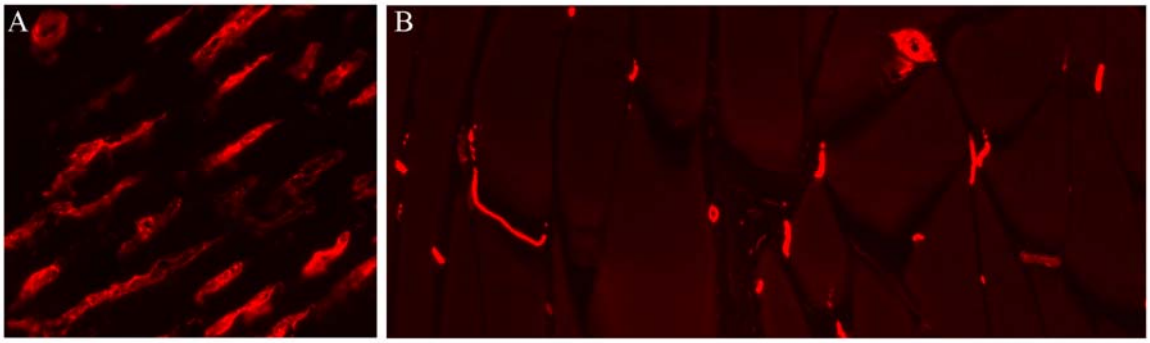

Figure 3.3. Rat muscle immunohistochemistry. Rat cardiac muscle (A) and skeletal muscle (B) were stained for AQP1, which demonstrated a strong endothelial signal, 100X.

3.3.2.3 Western blot for aquaporin 4

A protein of the expected size of AQP4 was found in rat left and right ventricular tissue, atrium, kidney and skeletal muscle. It was not seen in the lung, liver, spleen or small intestine tissue of the rat. A smaller molecular weight protein was also seen in the skeletal muscle of rat, but with reduced signal, (Figure 3.4).

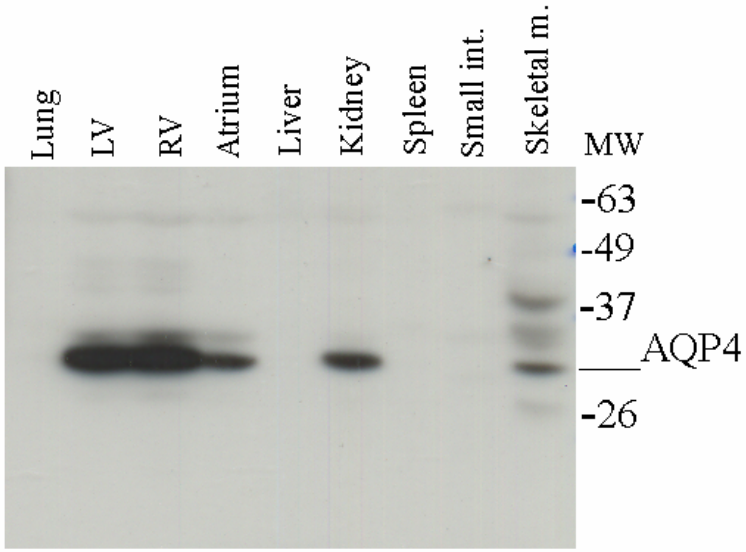

Figure 3.4. Western blot for AQP4. Whole cell lysates of rat organ tissues, probed with AQP4 antibody. Left ventricle (LV), right ventricle (RV), atrium, kidney and skeletal muscle (Skeletal m.) expressed a ~32 kDa protein, the expected size of AQP4. Lung, liver, spleen and small intestine (Small int.) did not contain this protein.

3.3.2.4 Immunohistochemistry for aquaporin 4

Rat cardiac and skeletal muscle were stained with AQP4 antibody. Skeletal muscle demonstrated expected and typical homogenous sarcolemmal staining. Cardiac muscle however had non specific nuclear staining, and a myocellular staining pattern that was also present in the absence of the primary antibody, (Figure 3.5).

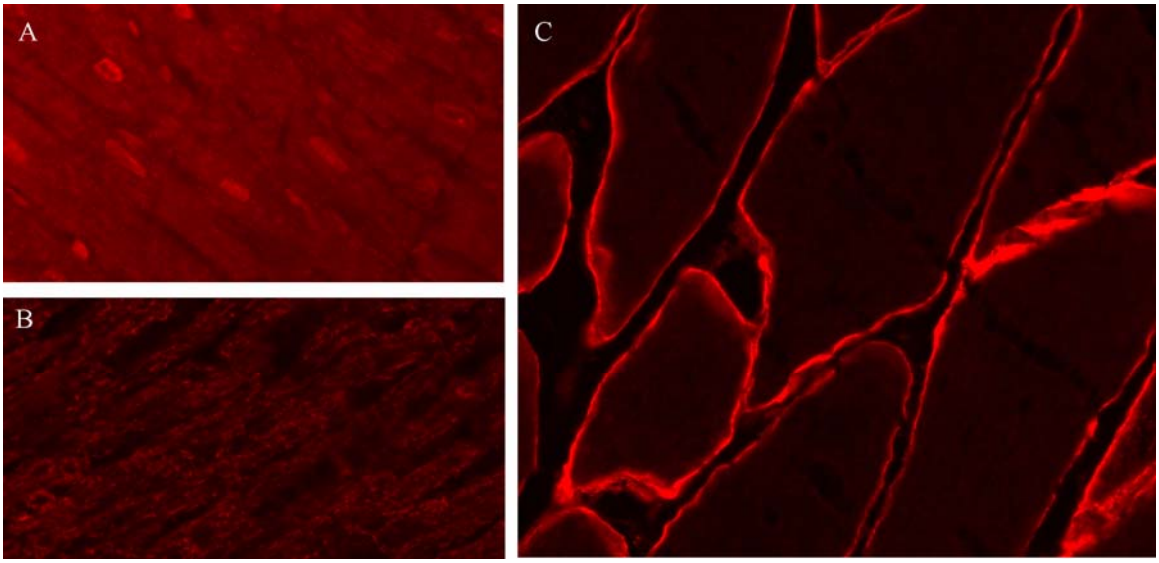

Figure 3.5. Rat muscle immunohistochemistry. AQP4 antibody of rat cardiac muscle (A), without primary (B) and skeletal muscle (C). Cardiac muscle revealed a generalised non specific staining and nuclear staining. Skeletal muscle showed expected sarcolemmal membrane staining, 100X.

3.3.2.5 Western blot for other aquaporins

A protein the size of aquaporin 7 ~(26 kDa) was detected in human and mouse myocardium, but not in rat myocardium. This protein was also seen in rat testis. It was unexpectedly absent from rat adipose tissue, (Figure 3.6).

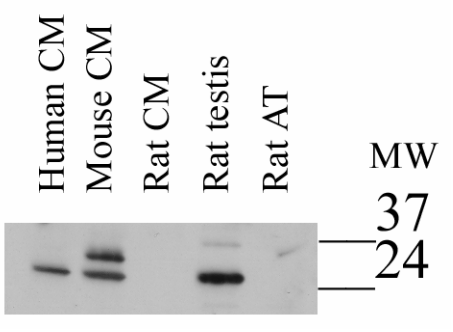

Figure 3.6. Western blot for AQP7. Whole cell lysates from human cardiac muscle (CM), mouse cardiac muscle (CM), rat cardiac muscle (CM), rat testis and rat adipose tissue (AT). Both rat testis and adipose tissue typically express AQP7. *This figure was prepared by Dr Tanya Butler.*

Western blots were also performed using available antibodies to AQP6 and AQP9 in the rat. No AQP11 antibody was available. No reliable protein expression data was available with these antibodies in a range of rat tissues, including myocardium.

3.3.3 Ischaemic rat heart study

The response of myocardial AQP1 and AQP4 to moderate periods of ischaemia, without reperfusion were studied.

3.3.3.1 RT-PCR and Western blot analysis

AQP1 mRNA was present in all myocardial samples from all four rats, and there was no consistent pattern of variation with ischaemic insult. AQP1 protein by Western blot was consistently present and did not vary appreciably with insult, (Figure 3.7).

AQP4 mRNA was present in only one of four baseline rat samples and had variable expression. AQP4 mRNA was present after 30 minutes room temperature ischaemia in three out of four rats. AQP4 protein appeared to be present and did not vary across groups, (Figure 3.7).

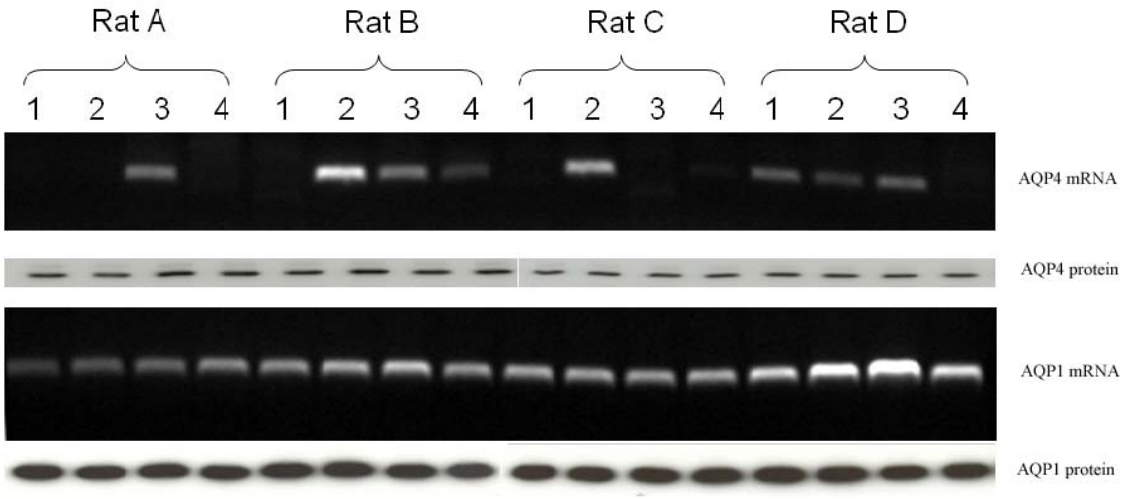

Figure 3.7. Western blot and RT-PCR of aquaporin 1 and aquaporin 4 in ischaemic myocardium. The myocardium of four rats (A–D) was exposed to graduated ischaemic insults, without reperfusion; 1=nil, 2=30 minutes room temperature, 3=60 minutes at 4°C and 4=60 minutes at 4°C and then 30 minutes at room temperature. mRNA was assessed by using RT-PCR and was compared with protein analysed by Western blot. *The Western blots used in this figure were prepared by Dr Tanya Butler*

3.3.3.2 Quantitative real time PCR analysis

AQP4 real time PCR demonstrated a late rise in transcript after at least 35 cycles and no transcript was apparent in the baseline samples. The samples that were exposed to 30 minutes of room temperature ischaemia had greater AQP4 mRNA expression, (Figure 3.8).

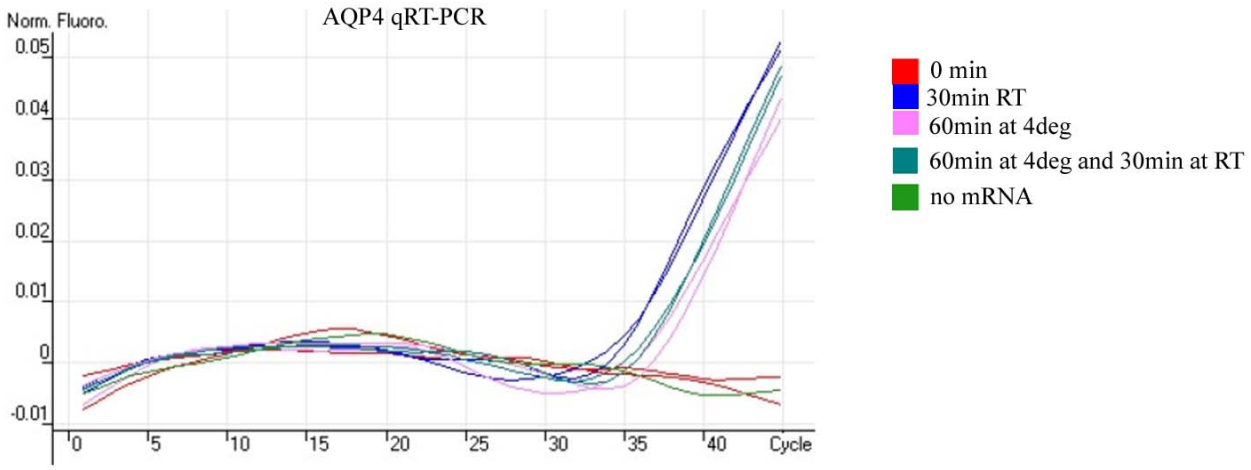

Figure 3.8. Aquaporin 4 mRNA cycle threshold output from qRT-PCR. Myocardium was exposed to 0 minute ischaemia, 30 minutes room temperature ischaemia (RT), 60 minutes at 4°C ischaemia, or 60 minutes at 4°C ischaemia followed by 30 minutes of room temperature (RT) ischaemia. A no mRNA sample was also run for 45 cycles.

AQP1 real time PCR showed no significant differences in mRNA from baseline tissue compared with all other timepoints, (Figure 3.9).

In the 30 minute room temperature ischaemia group, AQP4 demonstrated a significant increase compared to baseline, with a six fold increase in mRNA, (i.e. a relative change in cycle threshold compared to baseline and r18S RNA of ~ –3), (Figure 3.9). There was also a significant increase in AQP4 transcript after one hr at 4°C and 30 minutes at room temperature.

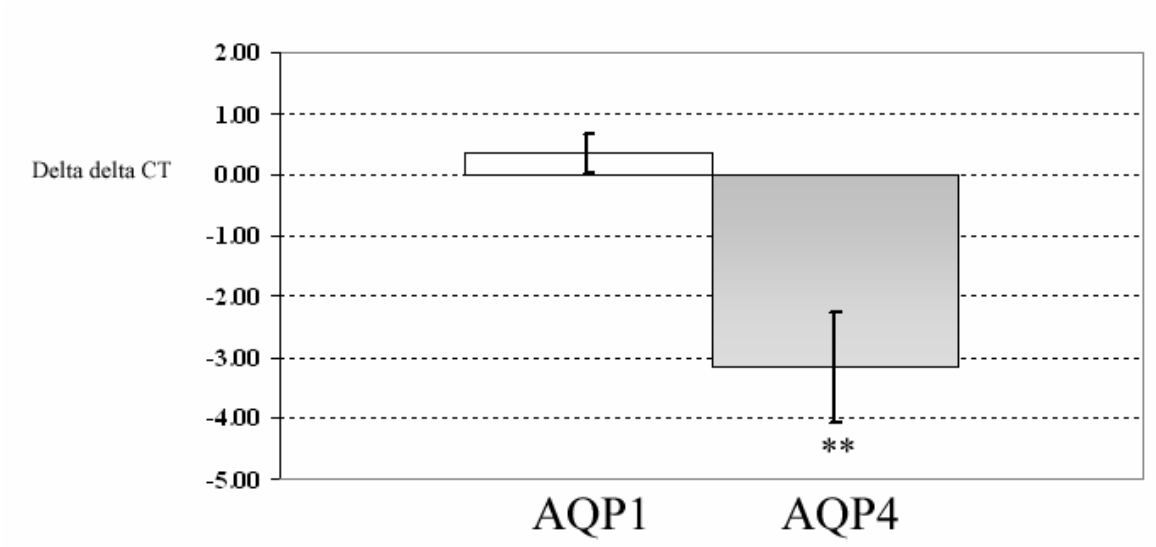

Figure 3.9. Rat myocardial aquaporin 1 and aquaporin 4 mRNA. Delta delta cycle threshold (CT) controlled by r18S RNA, mean±SEM shown. The 30 minutes room temperature ischaemic group tissue was utilised for this figure, n=4, (**p value <0.001) using Mann-Whitney U test (MWU) compared to baseline expression.

3.3.3.3 Relative cycle thresholds for aquaporin mRNA in rat myocardium

The cycle thresholds for AQP1 and AQP4 mRNA in rat myocardium were compared with other rat organ tissues of interest. A high cycle threshold means that there is very little transcript of interest present in the tissue.

Cycle thresholds for AQP1 in skeletal and cardiac muscle were essentially the same, although both were lower than in spleen. Cycle thresholds for AQP4 were higher in skeletal muscle, but comparable to brain expression. Rat cardiac tissue had high cycle thresholds, over 40, i.e. there was negligible AQP4 mRNA in rat myocardium, (Table 3.2).

Table 3.2 Cycle thresholds in rat. CT of aquaporin 1 and aquaporin 4 in various rat tissues in a 45 cycle run.

Transcript of interest	Tissue	Typical cycle threshold(CT)
AQP1	Skeletal muscle	27
AQP1	Cardiac muscle	26
AQP1	Spleen	21
AQP4	Skeletal muscle	32
AQP4	Cardiac muscle	42
AQP4	Brain	26

3.3.4 Aquaporin knockout tissue experiments

To clarify and confirm the expression of AQPs in rat myocardium, tissue from AQP knockout mice and litter matched wild type controls, was used as positive and negative controls. Western blots were probed with the AQP antibodies of interest.

AQP1 was absent in skeletal muscle, cardiac muscle and brain tissue from $AQP^{-/-}$ knockout mice, but present in comparable wild type control tissues. AQP1 was present in human cardiac and skeletal muscle. It was also present in rat cardiac and skeletal muscle, (Figure 3.10).

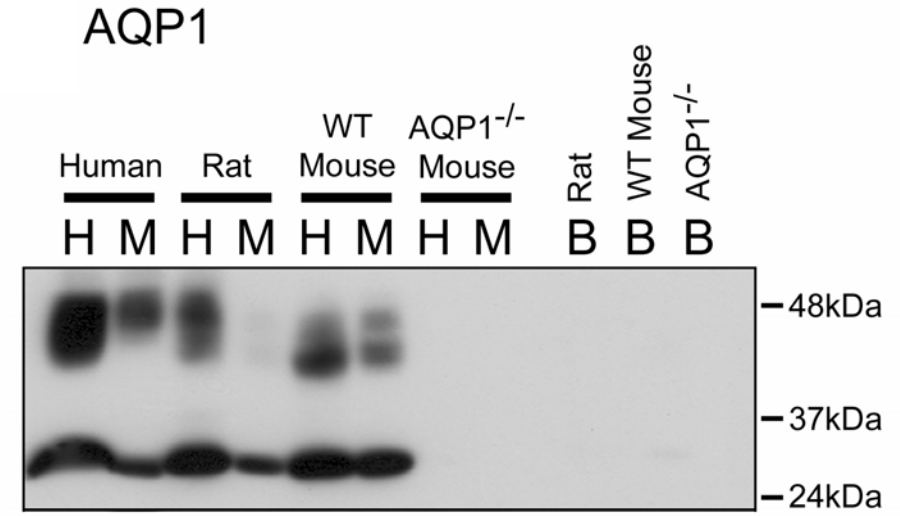

Figure 3.10. Western blot utilising knockout tissue for aquaporin 1. Whole cell lysates from human, rat and mouse heart (H), muscle (M) and brain (B). AQP1 is absent from heart and muscle in the knockout ($AQP1^{-/-}$) and not present in the brain tissue of rat or WT mouse. AQP1 is present in cardiac and skeletal muscle of human, rat and wild type mouse (WT Mouse). *This figure was prepared by Dr Tanya Butler.*

AQP4 was absent from $AQP4^{-/-}$ knockout mice brain, heart and skeletal muscle, but present in comparable wild type mouse tissues. Expression was relatively greater in mouse skeletal than cardiac muscle. AQP4 was present in human skeletal muscle only – not in cardiac muscle. In rats, AQP4 was present in skeletal muscle at low levels, absent in cardiac muscle and highly expressed in brain. An unknown higher molecular weight

protein was expressed in all muscle tissues of humans, rats and mice – this had previously been mistakenly assumed to be AQP4. This unknown protein was relatively more abundant than AQP4 in rat skeletal muscle, (Figure 3.11).

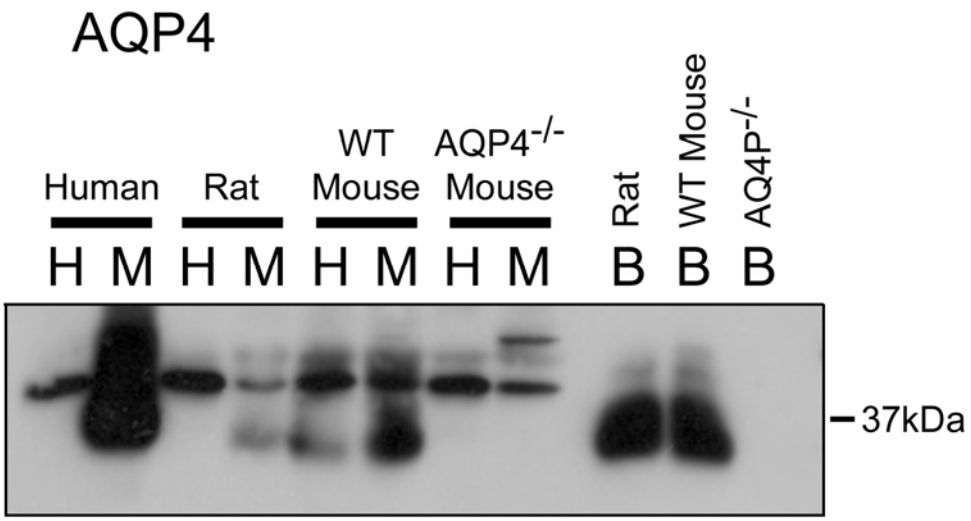

Figure 3.11. Western blot utilising knockout tissue for aquaporin 4. Whole cell lysates from human, rat and mouse heart (H), muscle (M) and brain (B). AQP4 is absent from heart, muscle and brain in the knockout mouse ($AQP4^{-/-}$). AQP4 is present in the mouse (WT Mouse) and rat brain. AQP4 is present in the skeletal muscle of human, rat and mouse. AQP4 protein is present in mouse heart, but not in rat or human myocardial tissue. *This figure was prepared by Dr Tanya Butler.*

3.3.5 Sheep aquaporin expression

Expression of AQP was studied in sheep myocardium, by qRT-PCR and Western blot using available antibodies.

3.3.5.1 RT-PCR in sheep

Sheep myocardium had AQP1 mRNA present on RT-PCR. There appeared to be relatively less AQP4 mRNA. AQP0, AQP3 and AQP9 mRNA were also apparent at low levels, (Figure 3.12).

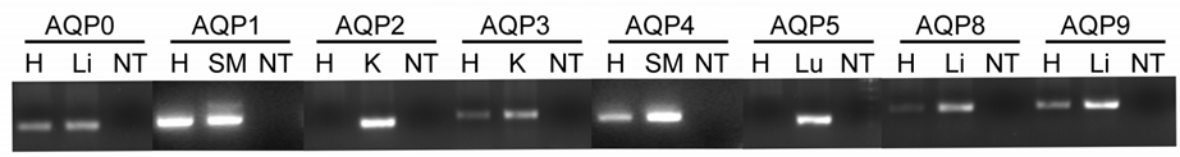

Figure 3.12. Sheep aquaporin RT-PCR. A screen using published primers for all or part of AQP0, AQP1, AQP2, AQP3, AQP4, AQP5, AQP8 and AQP9 mRNA was conducted on sheep tissue. Tissues were studied in comparison to known positive controls and no template controls (NT). Tissues studied included Heart (H), Liver (Li), Skeletal muscle (SM), Kidney (K) and Lung (Lu). *This figure was prepared by Yee Mun Tan.*

3.3.5.2 Real time PCR in sheep

Real time primers were developed for AQP0, AQP1, AQP3, AQP4 and AQP9 for the sheep. The internal housekeeping transcript r18SRNA was utilised. The relative cycle thresholds were compared for the various AQP mRNAs from sheep heart and other tissues. AQP1 was most abundant, with AQP4 transcript detectable at low levels and the mRNA from other AQPs detected only at low to negligible levels, (Table 3.3).

Table 3.3. Cycle thresholds in sheep. CT of AQP0, AQP1, AQP3, AQP4 and AQP9 in various sheep tissues in a 45 cycle run.

Transcript of interest	Sheep tissue	Typical cycle threshold(CT)
AQP0	Cardiac muscle	33
AQP1	Kidney	18
AQP1	Cardiac muscle	21
AQP3	Cardiac muscle	33
AQP4	Lung	26
AQP4	Skeletal muscle	28
AQP4	Cardiac muscle	36
AQP9	Cardiac muscle	38*
* Arbitrary CT as standard curves not reliable.		

3.3.5.3 Western blot analysis of sheep myocardium for aquaporins

AQP1 and AQP4 protein were studied using Western blot and IHC in the sheep heart.

AQP1 was present in sheep cardiac muscle. Skeletal muscle and lung also expressed AQP1 protein. AQP1 was relatively less expressed in the liver of sheep, (Figure 3.13).

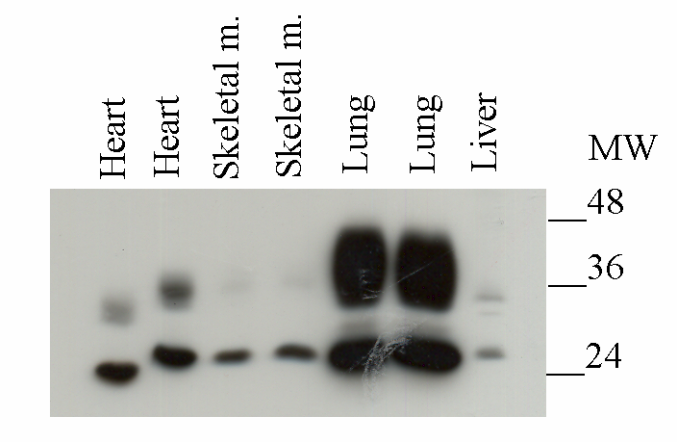

Figure 3.13. Western blot analysis of sheep organ tissues with aquaporin 1 antibody. AQP1 is just above the 24kDa marker, with its larger glycosylated band above. Tissues included heart, skeletal muscle (skeletal m.), lung and liver.

AQP4 protein was found to be present in sheep cardiac muscle, skeletal muscle, lung and liver. However only a relatively low level of AQP4 protein expression was found in sheep organs in comparison to rat brain and rat skeletal muscle, (Figure 3.14).

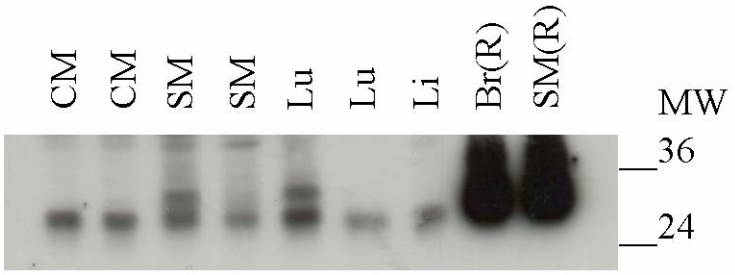

Figure 3.14. Western blot analysis of sheep organ tissues with aquaporin 4 antibody. Whole cell lysates were probed for AQP4 protein by Western blot analysis. Sheep tissues analysed included cardiac muscle (CM), skeletal muscle (SM), Lung (Lu) and Liver (Li). For positive controls rat brain [Br(R)] and skeletal muscle [SM(R)] were utilised.

3.3.5.4 Immunohistochemistry of sheep muscle

AQP1 was expressed in the endothelium of cardiac and skeletal muscle in the sheep. Additionally AQP1 protein expression was also apparent within the myocellular tissue of both cardiac and skeletal muscle. AQP4 was present in the sarcolemma of skeletal muscle. There was non specific and faint uptake in cardiac muscle using this same AQP4 antibody (AQP4r). Non specific AQP4 staining was seen in both muscle types with the other AQP4 antibody utilised (AQP4g), (Figure 3.15).

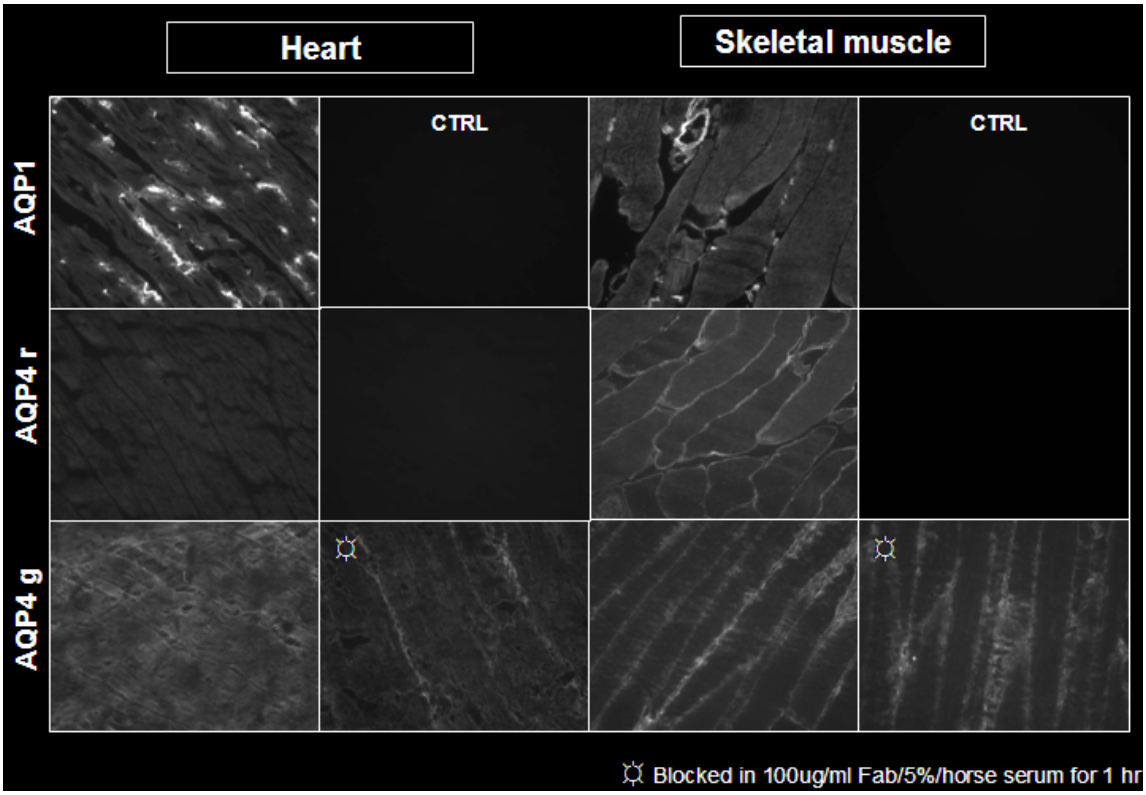

Figure 3.15. Immunohistochemistry of sheep muscle. Cardiac and skeletal muscle probed for AQP1 and AQP4 protein – using AQP1, AQP4r (rabbit) and AQP4g (goat) antibodies. Control (CTRL) images did not have primary antibody present. AQP1 is present in both muscle types and has a dominant endothelial expression pattern. AQP4 is present in the sarcolemma of skeletal muscle using the AQP4r antibody. This antibody reveals faint cardiac muscle uptake. There is non specific staining of both muscle types using the AQP4g antibody, 60X magnification. *This figure was prepared by Yee Mun Tan.*

3.3.6 Subcellular location of aquaporin 1

3.3.6.1 Endothelial expression

Protein samples from rat derived cardiomyocytes – which are relatively endothelium poor, myocardium, liver and skeletal muscle were examined for AQP1 and endothelial cell specific protein expression. Endothelial cells – as seen by cadherin expression – was present in heart, liver and skeletal muscle tissue. Endothelial cells were relatively less prevalent in rat derived neonatal cardiomyocytes. The cardiomyocytes had AQP1 expression comparable to skeletal muscle and relatively less than cardiac muscle, (Figure 3.16).

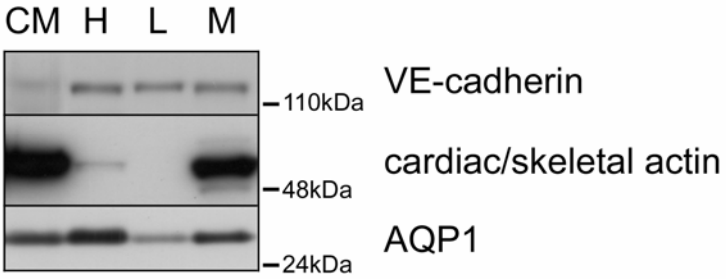

Figure 3.16. Endothelial and non-endothelial aquaporin 1. Isolated cardiomyocytes were probed for vascular endothelial (VE)-cadherin, actin and AQP1. Cardiomyocytes (CM), heart (H), liver (L) and skeletal muscle (M) tissue were probed. Endothelial poor cardiomyocytes demonstrate AQP1, whilst AQP1 is expressed to a relatively greater extent in the endothelial rich myocardial tissue. *This figure was prepared by Dr Tanya Butler.*

3.3.6.2 Mitochondrial membrane aquaporin 1 expression

Mouse plasma and mitochondrial membrane fractions were analysed to determine the expression of AQP1 in mitochondrial membranes. AQP1 was abundantly expressed in plasma membrane fractions, but only minimally expressed in mitochondrial membrane fractions, (Figure 3.17).

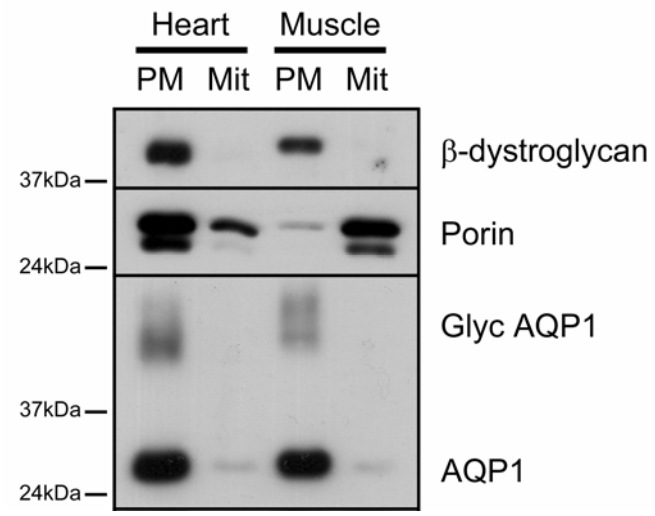

Figure 3.17. Western blot of plasma and mitochondrial membranes. Mouse cardiac and skeletal muscle was separated into plasma membrane (PM) and mitochondrial (Mit) fractions and then probed with AQP1 antibody. Fractions were also probed with porin – a mitochondrial specific protein – and β-dystroglycan, a plasma membrane specific protein. Glycosylated (Glyc) AQP1 is also shown. AQP1 is essentially found within plasma membranes and not mitochondrial fractions. *This figure was prepared by Dr Tanya Butler.*

3.3.7 Aquaporin function

AQP1 is the predominantly expressed AQP across the species studied. We sought to determine the relative contribution of various AQPs in the mouse as a guide to the contributions of AQPs in the mouse and other species. The mouse was chosen because of

the existence of knockouts, but it also expresses AQP4 and AQP7 at the protein level in addition to AQP1.

Membrane vesicles formed from mouse myocardial tissue were subjected to an osmotic stress in order to determine the water permeability of the tissue derived vesicles. Tissue was obtained from wild type and AQP1$^{-/-}$, AQP4$^{-/-}$ and AQP8$^{-/-}$ knockout mice Myocardial membrane vesicles from AQP4$^{-/-}$ and AQP8$^{-/-}$ knockout mice performed similarly to those from wild type mice when subjected to an osmotic challenge. Wild type mice in the presence of mercurials performed similarly to AQP1 knockout mice studies. AQP1 was found to be the functionally relevant AQP in the mouse, on the basis of osmotic permeability studies performed on myocardial membrane vesicle preparations , (Figure 3.18).

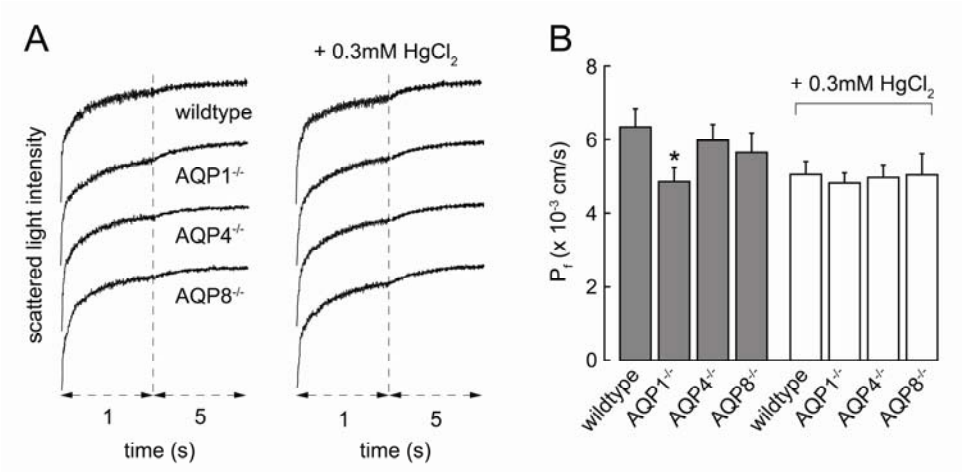

Figure 3.18. Myocardial membrane water permeability in mouse. Wild type (WT), AQP1 null, AQP4 null and AQP8 null mice were compared. AQP1 null membranes were significantly slower and displayed a lower permeability coefficient, (A,B, $p<0.01$). Addition of mercury inhibited AQP function and reduced rate of equilibration and permeability of all membranes to AQP1 null equivalent values. *This figure was prepared by Dr Tanya Butler.*

3.4 Discussion

Consistent with earlier findings (Au, 2004), AQP1 was found to be the water channel expressed in myocardial tissue of all the mammalian species studied. AQP1 was found to be expressed in rat and sheep myocardium, enabling its study in the proposed models of LCOS. Fundamentally it was also the only water channel confirmed, through comparison with knockout mouse tissue, as being present within human myocardial tissue. Hence AQP1 is of central relevance to studying the role of water channels in the obligatory movement of water along osmotic gradients within the myocardium in the setting of LCOS.

AQP1 was mainly expressed within the endothelial membranes of the heart. A smaller, myocellular pool of AQP1 protein was confirmed in endothelial poor isolated cardiomyocytes. The myocellular expression of AQP1 was less apparent though, compared with the abundance of AQP1 in the endothelium. The predominant endothelial expression of AQP1 supports the findings of Kellen *et al*. The inclusion of the endothelial components as part of an isolated heart model demonstrated a significant contribution (28–50%) to osmotic water movement by AQP1 (Kellen and Bassingthwaighte, 2003b). Whilst in the isolated cardiomyocyte studies, the absence of endothelial AQP1 led to a more modest contribution from AQP1 (Suleymanian and Baumgarten, 1996; Ogura, 2002b). Thus the demonstrated non endothelial AQP1 presumably facilitates interstitial, myocellular compartment water transport in the rare circumstances that an osmotic gradient develops, e.g. ischaemia. However osmotic gradients are considerably more likely to occur across the endothelial, interstitial compartments and hence there are relatively more AQP1 channels to cope with fluxes in plasma osmolality, e.g. dehydration.

There has been speculation that AQPs may be involved in water transport across mitochondrial membranes (Ferri, 2003; Calamita, 2005; Lee, 2005). We found that AQP1 was minimally expressed in mitochondrial membrane fractions and was principally found in plasma membrane fractions. Thus it is unlikely that AQPs have a role in mitochondrial

water transport. This confirmed what has been recently shown in a series of experiments utilising knockout tissue as controls which found equivalent mitochondrial permeability regardless of AQP expression (Yang, 2006).

Although AQPs don't have a role in mitochondria, there is a suggestion that AQPs may be involved in response to ischaemia (Warth, 2007). We found that AQP1 was unaltered in response to graduated ischaemic insults in the rat heart. This supported the theory that AQPs are constitutively expressed and are not subject to alteration within a short timeframe (Ma, 1997a). AQP4 however underwent a six fold increase in transcript in response to ischaemia. This fold change was identical to that seen in a mouse model of myocardial infarction by Warth *et al*. However we subsequently found that AQP4 mRNA is minimally expressed in rat heart and that AQP4 protein is absent, suggesting that the ischaemia associated AQP4 mRNA fold change was physiologically irrelevant. In the following animal models we planned to measure AQP1 mRNA and protein over longer timeframes, where expression changes have been demonstrated in response to haemodilution (Jonker, 2003).

The alterations seen in AQP4 mRNA with ischaemia, the subsequent review of AQP4 protein expression in the heart, and the eventual finding that AQP4 protein was absent from rat and human heart highlights the important role of knockout tissues. Others have suggested that AQP4 was present in human heart (Birkenkamp-Demtroeder, 2003), but it clearly isn't. Furthermore in those animals where AQP4 protein does exist in the heart, such as the mouse, it does not appear to have a functional role.

We confirmed AQP4 was present in sheep and mouse heart, but from a functional perspective only AQP1 had a role in mouse heart. Osmotic equilibration occurred fastest in the presence of AQP1. Wild type mice had equivalent permeability to $AQP4^{-/-}$ and $AQP8^{-/-}$ knockout mouse tissues. Only in the presence of mercury, did $AQP1^{-/-}$ knockout tissue demonstrate equivalent permeability to wild type mice and $AQP4^{-/-}$ and $AQP8^{-/-}$ mice. The difference in permeability between wild type and $AQP1^{-/-}$ knockout myocardial membranes was ~25%, similar to what Kellen *et al* demonstrated in the

whole heart of rabbits using crystalloid perfusates. AQP1 was thus considered to be the sole important water channel in terms of expression and osmotically obligated water movement in mammals. Therefore, AQP1 has served as the primary AQP focus in the following animal models of LCOS.

CHAPTER 4: ISOLATED CARDIOMYOCYTE EXPERIMENTS

Abstract

To determine the role of oedema in myocardial dysfunction we studied the cardiomyocyte. In parallel we investigated the relative contribution of ischaemia to cell swelling and function. This permitted the study of the impact on the contractile apparatus – albeit without the extracellular framework, but permitted detailed modelling.

The effects of hypo-osmotic stress and ischaemia upon cell size and function were modelled. We determined the dimensional and functional responses of the contractile apparatus to acute alterations in cell conditions. The benefit of this technique was that the contractile effect was isolated. However this limited the external validity of these studies, in that there were no extracellular components. Ischaemic stress was modelled by exposing the cells to a metabolic milieu which included an acidic (pH 6.8), raised lactate and simulated ischaemia with a hypoxic environment.

In a series of isolated cardiomyocyte studies we found that hypotonic buffer induced changes in cell size of ~ 7% that were not associated with contractile dysfunction. Ischaemia, despite being associated with less cell swelling (~2%), was associated with significant contractile dysfunction. Both insults altered calcium handling, but only following ischaemia was this associated with dysfunction.

4.1 Introduction

4.1.1 Modelling of hypotonic conditions and ischaemia

Others have sought to study isolated cardiomyocytes in terms of swelling, ischaemia and contractility, but as yet all three parameters have not been assessed in a single experimental series. We sought to specifically assess the impact made by oedema and by ischaemia to cardiomyocyte dysfunction.

4.2 Methods

Methodology was as per Chapter 2, an additional schema of the experimental protocols is provided here, (Figure 4.1).

4.2.1 Statistics

Analysis was as per Chapter 2 apart from the following.

For the isolated rat cardiomyocyte studies, the Kruskal-Wallis test followed by Mann-Whitney U test was used to compare treatment and control groups. Within group comparison of baseline, test and recovery values used the Friedman test followed by Wilcoxon signed rank test. In isolated cardiomyocyte analyses, to account for variability in data and time-dependent changes in cardiomyocyte function, significance was only accepted if both within-group significance and between-group significance from the isotonic control group was found.

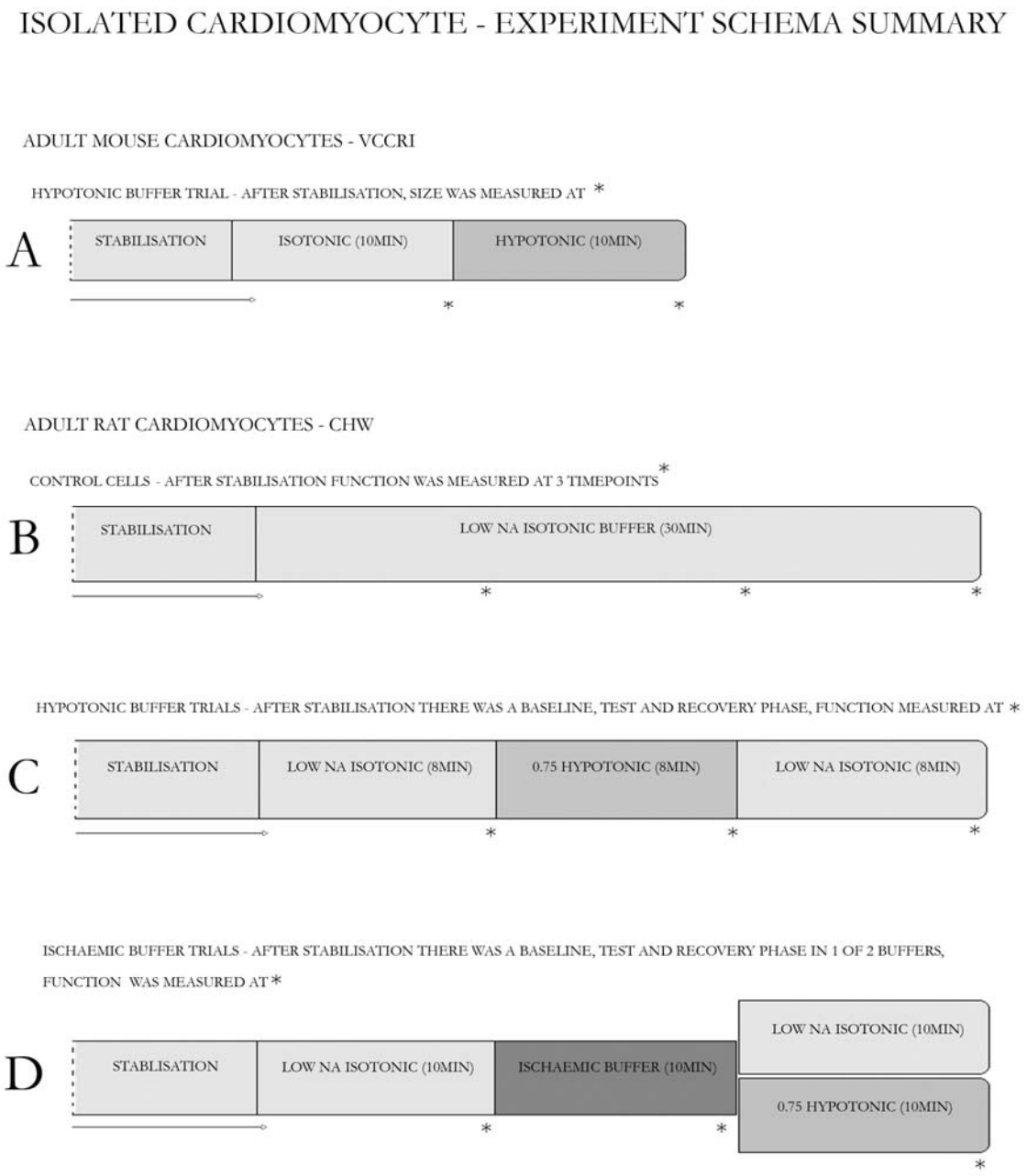

Figure 4.1. Isolated cardiomyocyte experiment schema summary. An experimental schema of the isolated cell trials at the Victor Chang Cardiac Research Institute (VCCRI) and at the Children's Hospital at Westmead (CHW). Following the stabilisation periods cell area ± calcium handling and cell contractility were measured at the end of two – three intervals within the protocols. For C and D these intervals were termed basal, test and recovery.

4.3 Results

4.3.1 Isolated mouse cardiomyocytes

Cardiomyocytes from adult mice were placed in an isotonic Kreb's solution and their size was measured in diastole after 10 minutes. The solution was then changed to a hypotonic Tyrode's solution and the size was again measured, (Figure 4.1.A). These groups of cells were then compared in terms of area. Area increased ~25% in the hypotonic solution. There was a non significant decrease in cell length of ~5% and a significant increase in cell width of ~30%, (Figure 4.2).

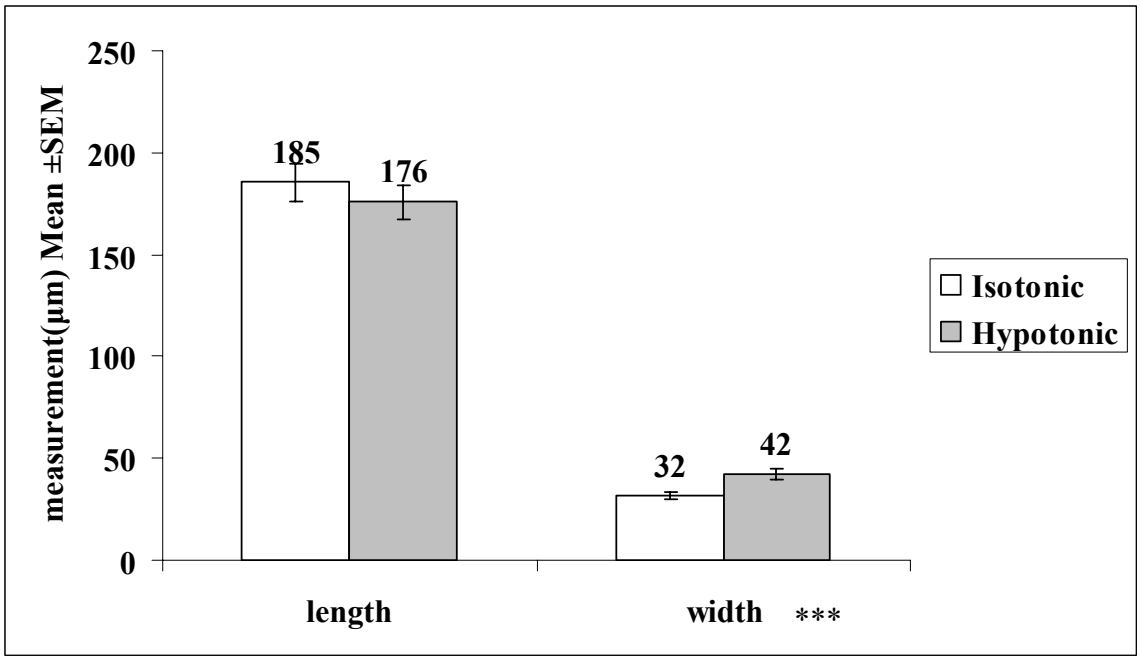

Figure 4.2. Mouse cardiomyocyte hypotonic buffer trial. Length and width of cells exposed to isotonic (clear column) and hypotonic (grey column) solutions. Length was not different, (p=0.5), whilst width varied with treatment, (p=0.001***). *This data was produced from an experiment conducted with Dr Aisling McCahon*.

4.3.2 Isolated rat cardiomyocytes – hypotonic and ischaemic buffer trials

4.3.2.1 Cardiomyocyte size

Cardiomyocyte size was studied in relation to exposure to isotonic, hypotonic and ischaemic buffers, (Figure 4.1 B, C and D).

Size was unchanged in isotonic conditions. In hypotonic buffer, cardiomyocyte area significantly increased by 7%. With re-exposure to isotonic buffer there was a shrinkage of cells to ~1% less than their initial size. In ischaemic buffer, cell area significantly increased by 2% and then returned to baseline in isotonic buffer. Alternatively, when ischaemic cells were recovered in hypotonic buffer, area increased 4% further to ~6% above baseline. This was larger compared to both baseline, ($p<0.01$) and ischaemic, ($p<0.01$) cell size, (Figure 4.3 A, B and C).

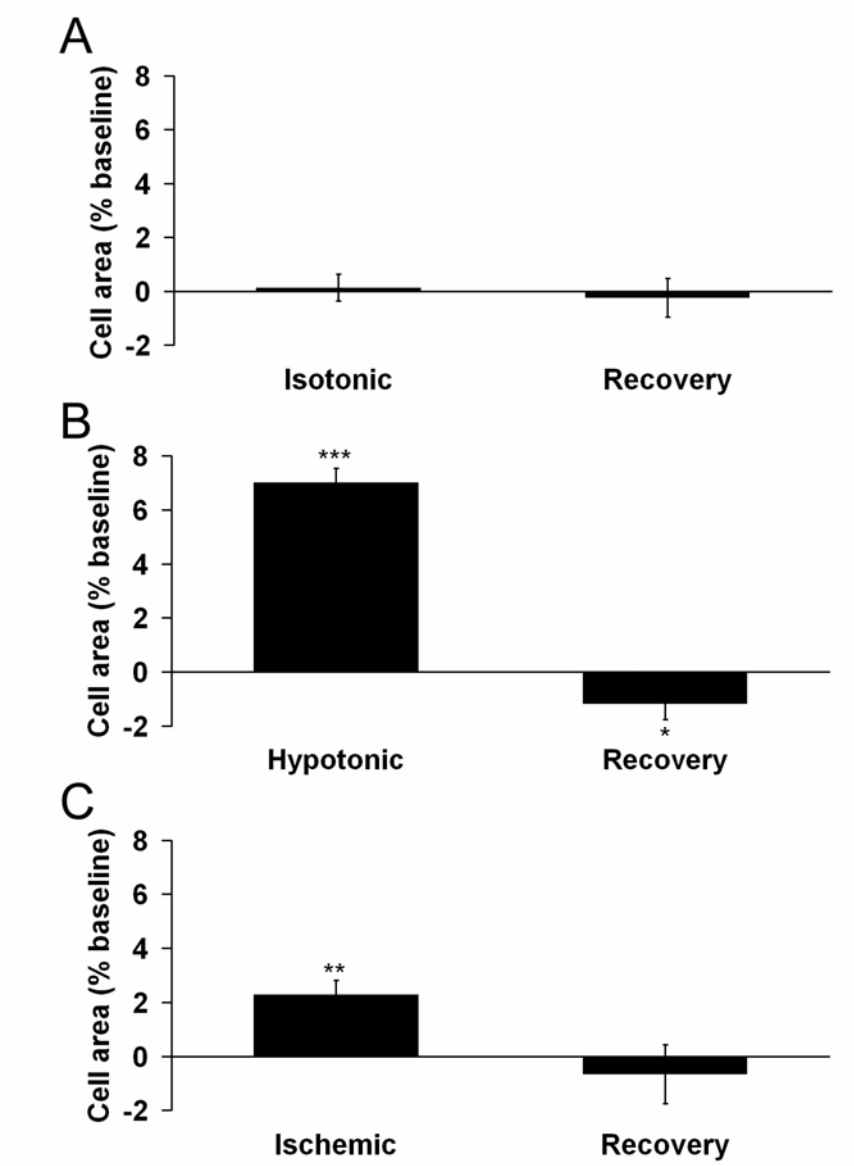

Figure 4.3. Rat cardiomyocyte size. Cardiomyocyte size relative to baseline, percentage change with 8–10 minutes exposure to buffers. Cardiomyocyte dimensions were recorded during diastole. A) Cells perfused continuously in isotonic buffer had static size. B) Cells in hypotonic buffer swelled, (***$p<0.001$). Recovery in isotonic buffer resulted in shrinking of the cells to less than their original size, (*$p<0.05$). Ischaemia resulted in cardiomyocyte swelling, (**$p<0.01$) with return to baseline size following reperfusion in isotonic buffer. *This figure was prepared by Dr Tanya Butler.*

4.3.2.2 Cardiomyocyte calcium handling

Cardiomyocyte calcium handling was studied at three time-points (designated basal, test and recovery) in each of the three experimental protocols, (Figure 4.1 B, C and D). Mean transient data from cells of the three experimental protocols are shown, (Figure 4.4 A, B and C). Comparison was made within each protocol i.e. variation within the three timepoints, and also between different protocols i.e. variation in calcium transients during hypotonic vs. ischaemic stress.

Resting calcium transient measurements of control cardiomyocytes demonstrated no change across the time-course of the experiments. Resting levels of intracellular calcium were unchanged in the cells exposed to hypotonic buffers. Cells exposed to ischaemic buffers however had increased calcium in diastole, which resolved in the recovery phase, (Figure 4.4 A).

Calcium clearance was unaltered across isotonic and hypotonic protocols. However slower calcium clearance was seen in ischaemic cells as an increase in tau, compared to baseline and isotonic controls, (Figure 4.4 B).

Peak calcium levels were unchanged with time in the isotonic cells. The hypotonic cells however had a lower peak intracellular calcium level compared to baseline and isotonic controls. Peak calcium in ischaemic cells was not significantly different from baseline and isotonic controls, (Figure 4.4 C).

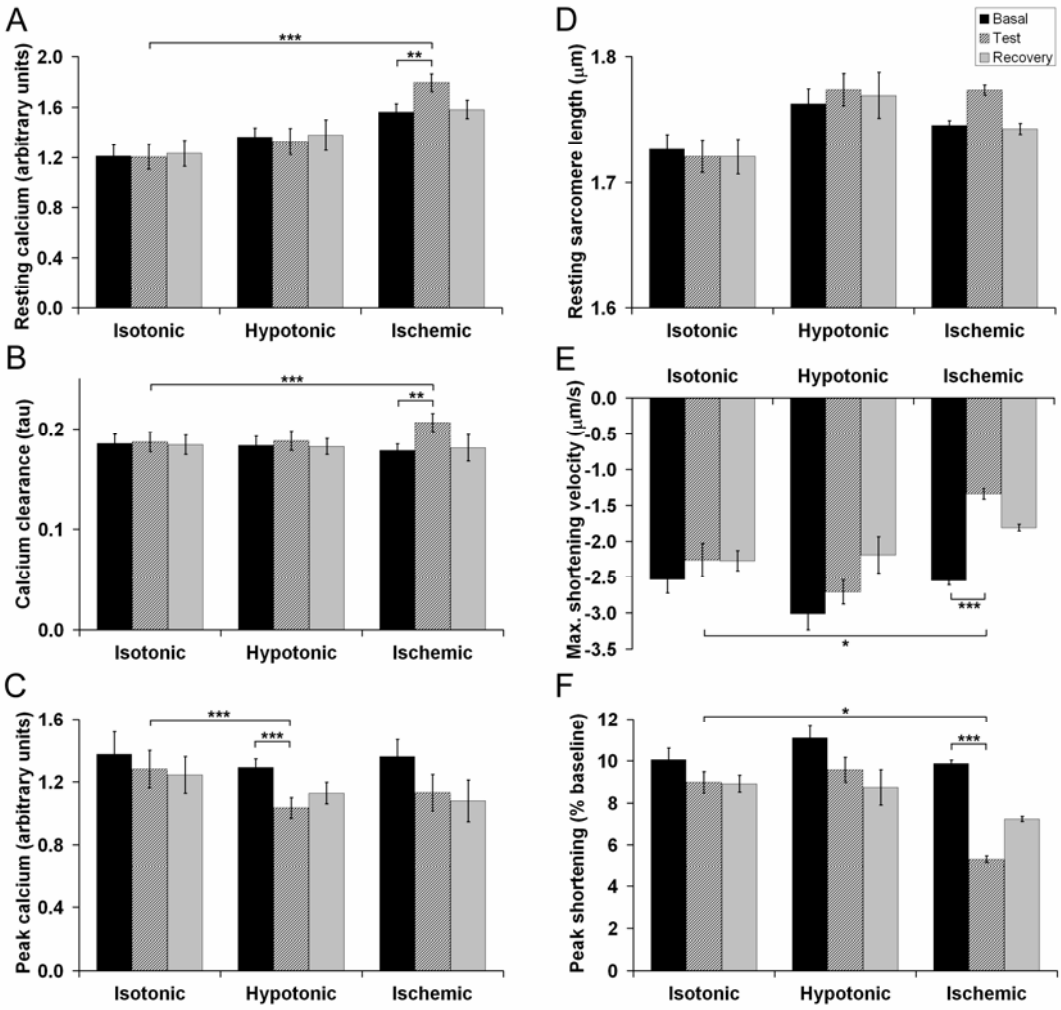

Figure 4.4. Calcium and contractility in rat cardiomyocytes – treatment effects. Intracellular calcium transient data (left panel, A,B,C) and sarcomere shortening (right panel, D,E,F). Baseline measurements (black column), test measurements (hatched columns) and recovery measurements (grey columns) are shown. The isotonic control group was produced by continuous perfusion of cells with isotonic buffer. A) Diastolic intracellular calcium was significantly increased in ischaemic cells, (***$p<0.001$) and returned to baseline values with isotonic reperfusion. B) The calcium clearance was significantly increased during ischaemic exposure, (**$p<0.01$). C) Peak intracellular calcium was reduced in the hypotonic group, (***$p<0.001$). D) Diastolic sarcomere length was not significantly altered by treatment. E) Maximum velocity of shortening was significantly reduced by ischaemia, (**$p<0.01$), but not by hypotonic swelling. F) Peak shortening was significantly reduced by ischaemia, (**$p<0.01$), but not by hypotonic challenge. *This figure was prepared by Dr Tanya Butler.*

4.3.2.3 Cardiomyocyte contractility

Cardiomyocyte contractility was studied in relation to these stresses, (Figure 4.1 B, C and D).

Similarly to cardiomyocyte calcium handling, contractility was studied at three time-points (designated basal, test and recovery) in each of the three experimental protocols. Mean values for cells from the three experimental protocols are shown, (Figure 4.4 D, E and F). Comparison was made within and between protocols in order to confirm significant variation.

Resting sarcomere length was measured on three occasions within each protocol and there were no significant differences within or between the isotonic, hypotonic or ischaemic cells, (Figure 4.4 D).

The maximal shortening velocity of cardiomyocytes were not significantly different in isotonic or hypotonic cells. In the Ischaemic cells though there was a significant reduction compared to baseline and isotonic controls, (Figure 4.4 E).

The peak shortening of cardiomyocytes was also not significantly different in isotonic or hypotonic cells. The ischaemic cells however displayed a significant reduction in peak shortening, with some recovery after the insult, (Figure 4.4 F).

4.4 Discussion

Isolated cardiomyocytes were assessed functionally in their response to ischaemic and hypotonic insults. We found that cells swelled in response to hypotonic stress (7%), more so than they did in response to ischaemia (2%). Cell function however was severely impacted by ischaemia and not by swelling induced by hypotonic buffers. Hence in terms of the hierarchy of myocardial insults, ischaemia out ranked oedema. Interestingly if a hypotonic stress followed the ischaemic insult there was an additional degree of oedema seen (increase from 2 to 6%), but no additional contractile dysfunction. So even when oedema was added to an already injured and dysfunctional cell it did not contribute to additional dysfunction. This supports the assertion that ischaemia is of primary import in terms of contributing to contractile dysfunction.

There was a gross decline in diastolic and systolic function in the cardiomyocytes which experienced ischaemia. Alterations in calcium handling associated with ischaemia – i.e. reduced calcium clearance and increased diastolic/resting calcium could have resulted from increased calcium influx seen with ischaemia (Otani, 1989). This influx in calcium is likely to be the result of injury to the cardiomyocyte membrane (Otani, 1989). There were no changes seen in contractility in cells exposed to hypotonic stress and this was despite lower peak calcium levels. This alteration in calcium levels could be attributed to swelling induced alterations in ion channels as described by Vandenberg *et al* (Vandenberg, 1996). Alternatively this uncoupling of calcium concentration and contractility has been described in similar settings previously (Mangano, 1993).

A reduction in contractility in association with swelling of cardiomyocytes has been asserted previously, by Mizutani *et al* (Mizutani, 2005). There was a 30% decline in cardiomyocte shortening in association with 0.6T and 0.9T buffers, although there was also a 20% decline with the 1.0T or isotonic buffers – which suggests a lack of stability in the model. Because of the reduced contractility in the 1.0T buffer, significantly reduced contractility in the 0.6T and 0.9T groups was only demonstrated when compared with the 2.6T or hypertonic group. So upon review there was no significant difference in cell

shortening between 0.6T, 0.9T and isotonic buffers. The cells exposed to 0.6T buffer – swelled 30%, whilst the cells exposed to 0.9T had a 7% increase in area – of similar magnitude to our findings using 0.75T buffers. If we compare the function between 0.6T cells and 0.9T cells there is absolutely no difference, both are ~ 30% reduced from baseline. So despite a 23% difference in size there was no difference in function between these two groups. This suggests that there is not a direct causal relationship between swelling and dysfunction.

A possible explanation for the lack of dysfunction seen in our study was the duration of the hypotonic insult. Askenasy *et al* in whole isolated heart studies found that a hypotonic stress was tolerated without functional decline for five to eight minutes (Askenasy and Navon, 1997). In the study by Mizutani *et al* (Mizutani, 2005) there was a 20 minute exposure to hypotonic stress, compared to our eight minute duration hypotonic test. However Askenasy's work was following ischaemia. This was similar to our study of exposure of ischaemic cells to hypotonic buffers. In these cells we did not demonstrate dysfunction over a 10 minute interval, thus it is unlikely that duration of exposure was responsible for the difference in swelling related dysfunction.

There are limitations to isolated cardiomyocyte work in translation to clinical work. We subsequently utilised the isolated heart model in order to deal with the more obvious deficits – namely short experimental time-course and lack of extracellular matrix/interstitial compartment. Despite these limitations, isolated cardiomyocyte studies provide a glimpse of the isolated myocellular apparatus and its response to swelling and ischaemia. The contractile apparatus seemed least capable of tolerating an ischaemic insult, as opposed to an oedema inducing insult.

If myocellular swelling is not responsible for dysfunction, but rather ischaemic injury, what are the likely mechanisms? Direct damage to the contractile machinery has been found in the setting of ischaemia/reperfusion as a result of numerous insults, with the predominant causes increased calcium – leading to activation of proteases (Bolli and Marban, 1999) and oxygen derived free radicals (Bulkley, 1994). Unfortunately methods

of attempting to ameliorate these pathways have not proven successful (Bolli and Marban, 1999; Becker, 2004). Thus avoiding ischaemia or at least avoiding the effects of ischaemia reaching the myocellular compartment would appear worthy of investigation.

So in conclusion isolated cardiomyocytes were affected by both hypo-osmotic induced oedema and ischaemia induced oedema. Both these insults affected the size of the cells and calcium handling, but only ischaemia resulted in severe and significant dysfunction. These findings are potentially relevant to the postoperative clinical setting and warrant assessment in a whole organ model.

CHAPTER 5: ISOLATED HEART (LANGENDORFF) EXPERIMENTS

Abstract

As in the isolated cardiomyocyte, the isolated heart model provided the ability to study function, oedema and ischaemia. Additionally in the whole heart model it was possible to study the global contribution of the interstitial and vascular compartments.

A purpose built Langendorff isolated heart perfusion system was used – with retrograde aortic perfusion. Rat hearts were perfused with a standard crystalloid perfusate, which was temporarily suspended to model global ischaemia. Global ischaemia was conventionally modelled at 37°C with suspension of aortic root flow, this resulted in reversible myocardial contracture. Two series of experiments focused on the contribution of oedema and ischaemia to myocardial dysfunction.

Hyposmotic stressed hearts had a 9% increase in water and a < 5mmHg increase in diastolic pressure. This was compared with ischaemic hearts that had a 6% increase in water and a 25–50mmHg increase in diastolic pressure. Both ischaemic and non-ischaemic oedematous hearts had mild reductions in systolic function, which resolved rapidly in only the non-ischaemic hearts. There were no appreciable changes in AQP1 mRNA or protein expression.

In conclusion, ischaemia was of great importance functionally. In the whole heart, oedema without ischaemia had much less impact, being associated with mild reversible dysfunction. Ischaemia induced dysfunction was mainly diastolic, with systolic dysfunction occurring with longer durations of ischaemia. During this timeframe no measurable changes were seen in AQP1 expression.

5.1 Introduction

A necessary extension from the isolated cardiomyocyte model was the isolated heart or Langendorff perfused heart. In the whole organ model it is possible to perform experiments of longer duration and to study the contribution of the interstitial and vascular compartments (Aliev, 2002). The inclusion of these compartments improves the correlation of the model with the clinical setting. The extracellular compartments are also relevant as sites of water accumulation and because of the high expression of AQPs in endothelial cell membranes. Experiments utilising the Langendorff technique, originally described in 1897 by Oscar Langendorff, have contributed greatly to the current understanding of mammalian cardiac physiology (Skrzypiec-Spring, 2007). It provides a robust, reproducible model with the potential to study function, oedema and proteins of interest.

Typically it is accepted that in settings analogous to CPB with or without I/R that water accumulates in the interstitial space as a result of endothelial leakiness (Garcia-Dorado and Oliveras, 1993; Mehlhorn, 2001; Kellen and Bassingthwaighte, 2003b). However contention surrounds the importance and degree of intracellular oedema – as discussed previously (Bolli and Marban, 1999; Askenasy, 2001; Kloner and Jennings, 2001). In an isolated heart model, Palmer *et al* showed that progressive increases in duration of ischaemia resulted in progressive increases in dysfunction – particularly diastolic. It was found that at after 20 minutes ischaemia there was a shift from reversible to irreversible injury. This was also associated with increased MWC and increased mitochondrial swelling/ oedema. Hence it was concluded that ischaemia was the primary cause of myocardial and mitochondrial oedema and that the threshold for irreversible injury was 20 minutes – this correlates with the standard interval between cardioplegic doses in the clinical setting (Palmer, 2004).

Curiously in the work by Palmer *et al,* myocardial oedema did not occur to any significant degree in non-ischaemic hearts, despite a lack of colloid in the perfusate (Palmer, 2004). This lack of oedema in non-ischaemic hearts has not been described by

others, even when colloid is added to the conventional perfusate (Apstein, 1977). For instance Kellen *et al* describe the accumulation of 1 – 2g of water (~10%) during the initial stabilisation phase (30 minutes), despite the inclusion of low concentrations of albumin in the perfusate (Kellen and Bassingthwaighte, 2003b). Palmer *et al* modelled ischaemia in the accepted manner, by stopping perfusate flow at room temperature. This results in a significant insult – no flow, globally at room temperature (Palmer, 2004). The resulting injury and myocardial dysfunction is more severe than with conventional planned myocardial ischaemia. In the setting of CPB for instance relative hypothermia and blood cardioplegia are utilised to minimise ischaemia induced injury.

We sought to define the relative contributions of ischaemia and oedema in a whole heart model of myocardial dysfunction. AQPs have not been studied in this setting either so their role was simultaneously studied by looking at myocardial mRNA and protein for AQP1 expression.

5.2 Methods

These are outlined in Chapter 2, a supplementary schema of experimental protocols is included here, (Figure 5.1).

In the second series of experiments, an additional group of experiments were conducted specifically to determine the water content of hearts. Water content of three groups (n=4 per group) were compared; i) 20 minutes isosmotic Krebs perfusion, ii) 20 minutes perfusion with isosmotic Krebs followed by 20 minutes with hyposmotic Krebs, and iii) successive perfusion in isosmotic, hyposmotic and isosmotic Krebs for 20 minutes each MWC, was then measured.

5.2.1 Statistical analysis

Statistical analysis was as discussed in Chapter 2 apart from the following comments.

For physiological data obtained from Langendorff experiments, values were averaged over 10 minutes epochs. Statistical significance was determined by analysis of variance with repeated measures. The Mann-Whitney U test was used to compare unrelated groups in terms of MWC, analysis of densitometry and real-time RT-PCR experiments.

5.2.2 Tissue Analysis

This included assessment of MWC, myocardial AQP1 mRNA and protein, as discussed in Chapter 2. Only MWC was performed on the second series of experiments.

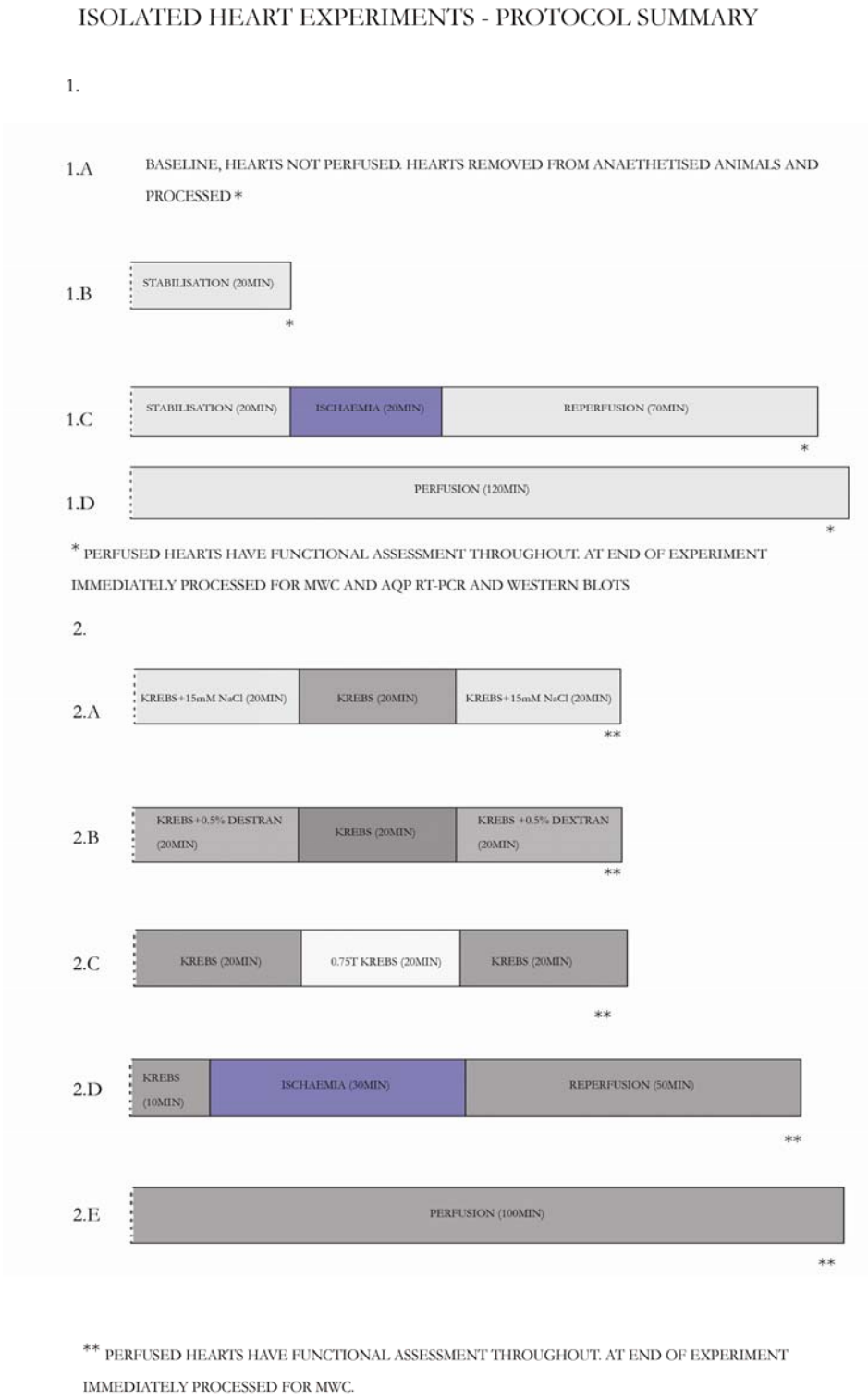

Figure 5.1. Isolated heart experiments – protocol summary. An outline of the experimental protocols 1) 20 minute ischaemia experiments and 2) 30 minute ischaemia experiments used with the isolated heart experiments.

5.3 Results

5.3.1 Twenty minute ischaemia experiments

All experiments proceeded as planned. Four treatment groups were compared with six animals in each group; baseline (hearts excised and not perfused), 20 minute perfusion controls, 20 minutes perfusion followed by 20 minutes ischaemia and 70 minutes reperfusion and finally a 120 minute perfusion control group (without ischaemia), (Figure 5.1 – 1).

5.3.1.1 Haemodynamics

Heart function was assessed as LVDP measured at systole (Figure. 5.2A) and diastole (Figure. 5.2B). The ischaemic insult did not affect the systolic LVDP, but resulted in a significant increase in diastolic LVDP during the reperfusion phase, ($p=0.002$). This increase in diastolic pressure confirmed that the hearts suffered a significant ischaemic insult. Other indexes of heart function, including heart rate, recovered to control levels in ischaemic hearts during the reperfusion phase.

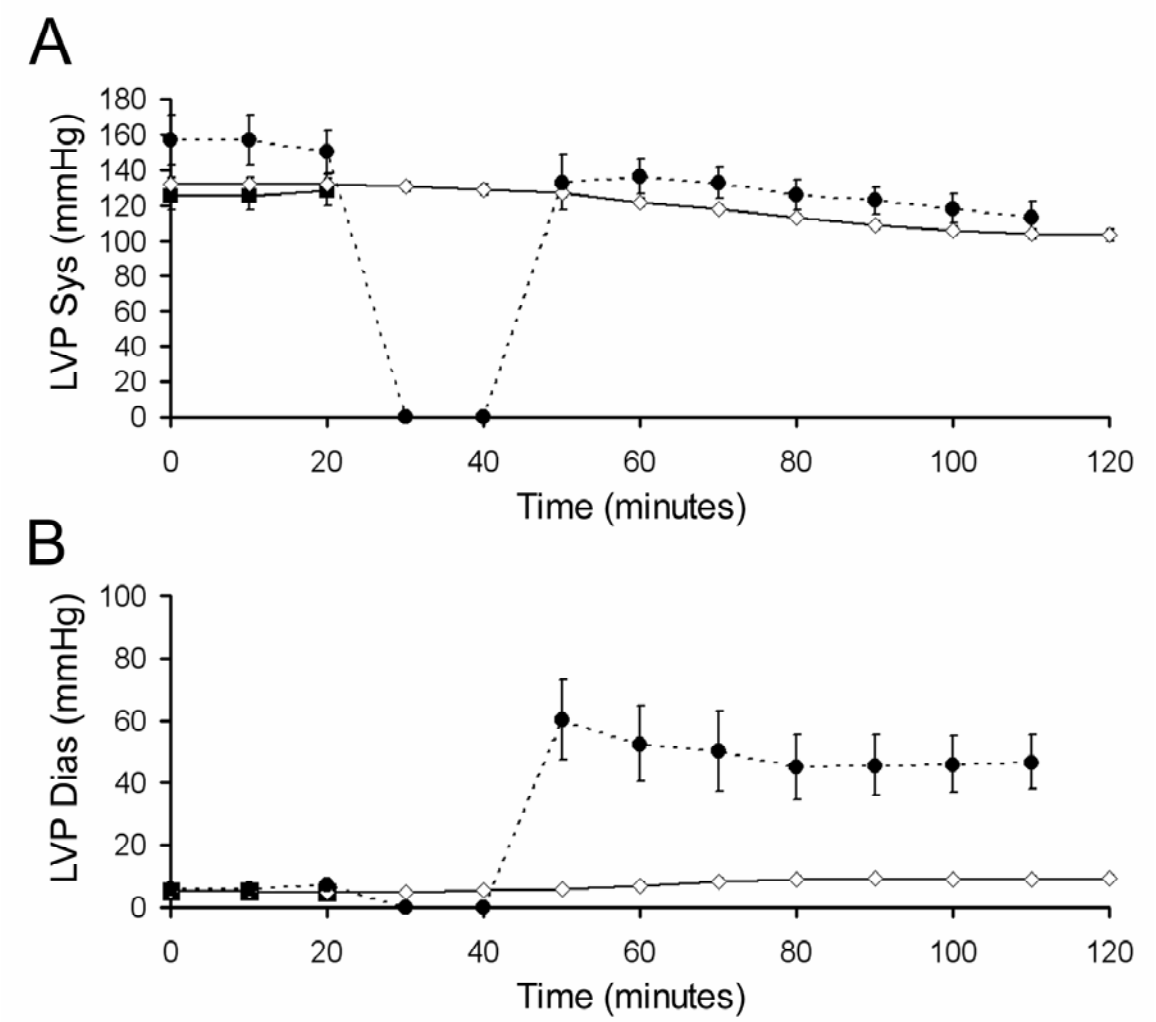

Figure 5.2. Ischaemic treatment of rat heart. (A-B) Rat hearts were perfused as controls (-■-), or made ischaemic through cessation of perfusate flow (-●-) for 20 minutes before reperfusion, or continuously perfused without ischaemia (-◊-). Left ventricular systolic pressure (LVP Sys) recovered well after ischaemia (A) whilst diastolic pressure (LVP Dias) was significantly increased, ($p=0.002$). Mean±SEM is shown.

5.3.1.2 Myocardial water

The baseline control hearts had a MWC of 0.768±(0.0008). The 20 minute control perfused hearts had a MWC of 0.823±(0.003) which was significantly greater than baseline, (p=0.03). The hearts perfused for 120 minutes without ischaemia had a similar MWC to the 20 minutes perfused group of 0.819±(0.0037), and were statistically different from baseline control hearts, (p=0.025). The 20 minute ischaemia hearts were the wettest 0.832±(0.0013) and were statistically different from the baseline control group, (p=0.05) and 20 minute control perfused, (p=0.03) groups and with respect to the 120 minutes perfused group, (p=0.05), (Figure 5.3).

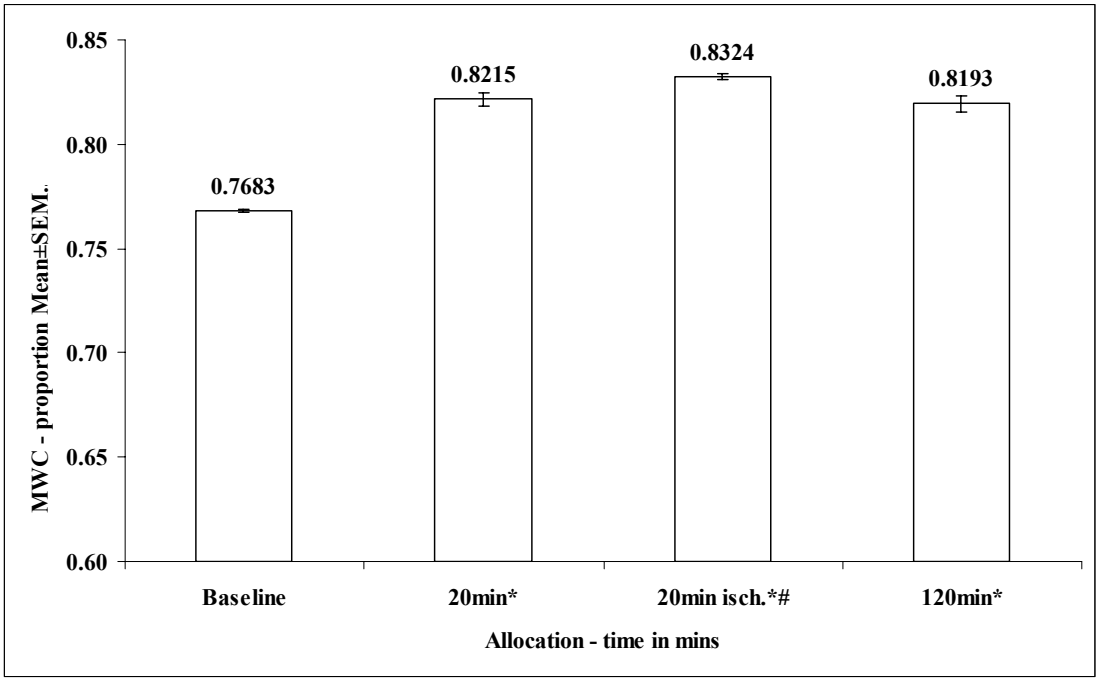

Figure 5.3: Myocardial water content for isolated rat hearts in various allocations. See text for details, 20 minute ischaemia hearts were the wettest compared to all other allocations, (*#p<0.05). Mean±SEM is shown.

5.3.1.3 Molecular results

AQP1 and AQP4 mRNA and protein levels were measured by quantitative RT–PCR and Western blotting, respectively. AQP1 transcript, (Figure. 5.4A) and protein, (Figure. 5.4B) were not significantly different across all treatment groups. AQP4 protein was not evident in the hearts, regardless of treatment (data not shown). Although AQP4 transcript was detectable, it was several fold less than the amounts measured in skeletal muscle and AQP4 protein was absent from rat myocardium.

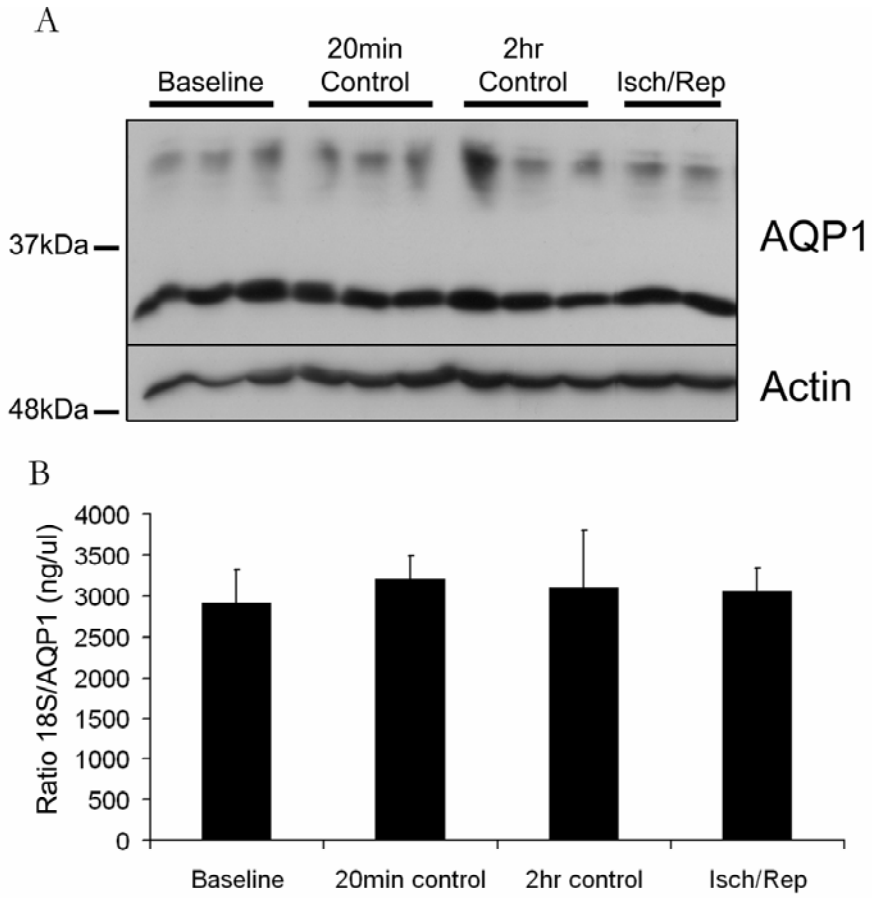

Figure 5.4. Aquaporin 1 expression. (A) Measured by Western blotting and normalised to the expression of cardiac actin, was not affected by osmotic or ischaemic stress. (B) AQP1 transcript levels were also unchanged. Mean±SEM is shown. *This Figure was prepared by Dr Tanya Butler.*

5.3.2 Thirty minute ischaemia experiments

All experiments proceeded as planned, (Figure 5.1 – 2). Groups 2A–C related to osmotic/oncotic alterations. The hearts were perfused 20 minutes with infusion, 20 minutes without infusion and 20 minutes with infusion. These infusions were 2A; 15mM NaCl and 30mM sucrose (to measure the effects of mild hyperosmolarity), 2B; 0.5% dextran (to measure the effects of mild increases in oncotic pressure) and, 2C; 0.75T Krebs (to measure the effects of hypotonic stress). The remaining experiments focused on ischaemia. Groups 2D; 10 minute baseline stabilisation, 30 minutes ischaemia, 50 minutes reperfusion and, 2E; 100 minute perfusion control group.

5.3.2.1 Haemodynamics

Measured pressures were not significantly different with infusion of NaCl with sucrose, (Figure 5.5A). Diastolic pressure in the presence of 0.5% dextran was significantly higher than without it, ($p<0.01$, Figure 5.5B) , but systolic pressure was not significantly altered.

Alterations from isosmotic to 0.75T hyposmotic perfusate led to a mild, but significant increase in diastolic pressure, ($p<0.001$), which was reversed by return to isosmotic solution, (Figure 5.5C). Systolic pressure reduced and then also recovered with return to isosmotic perfusion 1.0T to 0.75T, ($p<0.01$).

Ischaemia induced severe diastolic dysfunction with an increase in diastolic pressure compared to baseline, ($p<0.05$, Figure 5.5D). Systolic pressure was also significantly lower in the reperfusion phase compared to baseline, ($p<0.05$). Although this systolic pressure was not significantly different from non-ischaemic controls.

5.3.2.2 Myocardial water

All hearts became oedematous at the completion of experiments ~6%, compared to baseline and there were no significant differences for those receiving 15mM NaCl and 30mM sucrose, 0.5% dextran or those hearts exposed to ischaemia.

In additional experiments focused on 20 minute intervals of perfusion, perfusion with 0.75T Krebs induced additional swelling. Hearts perfused with 0.75T hyposmotic Krebs took on 3.3% more water than hearts perfused with isosmotic Krebs, ($p<0.05$, Figure 5.6). Isosmotic recovery allowed resolution of this swelling to baseline values.

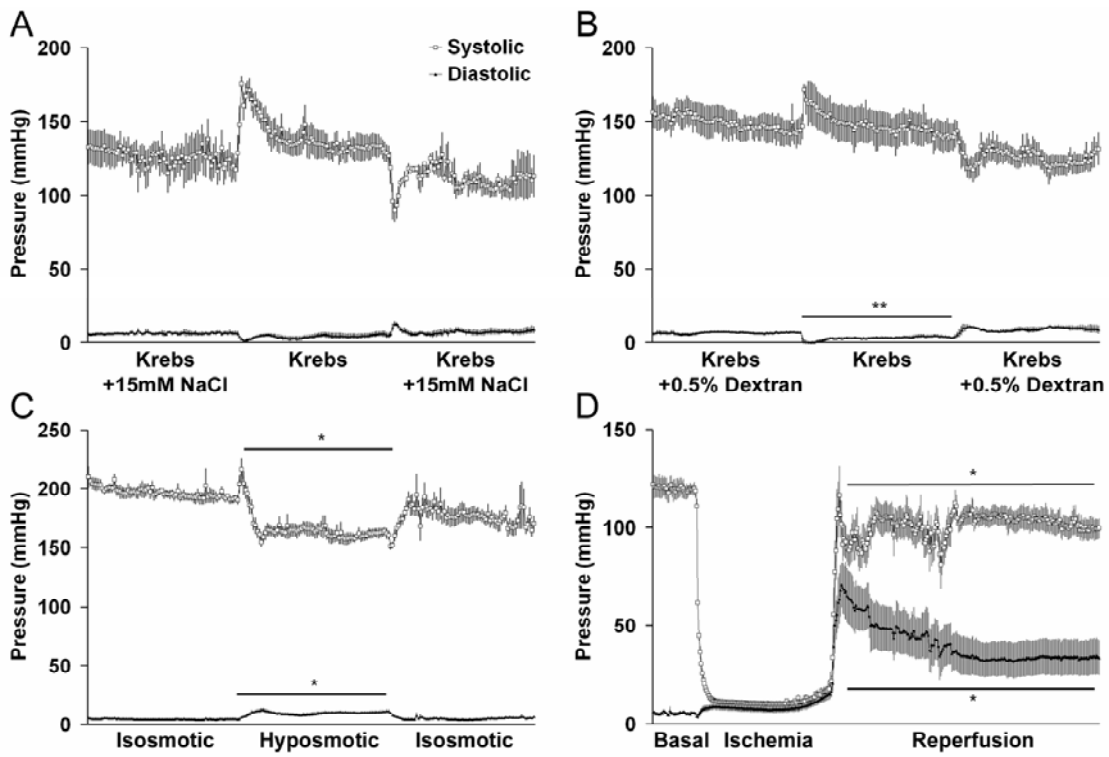

Figure 5.5. Left ventricular developed pressure; for A – C osmotic challenges and D, 30 minute ischaemic challenge – see text for details. Mean±SEM is shown. *This figure was prepared by Dr Tanya Butler.*

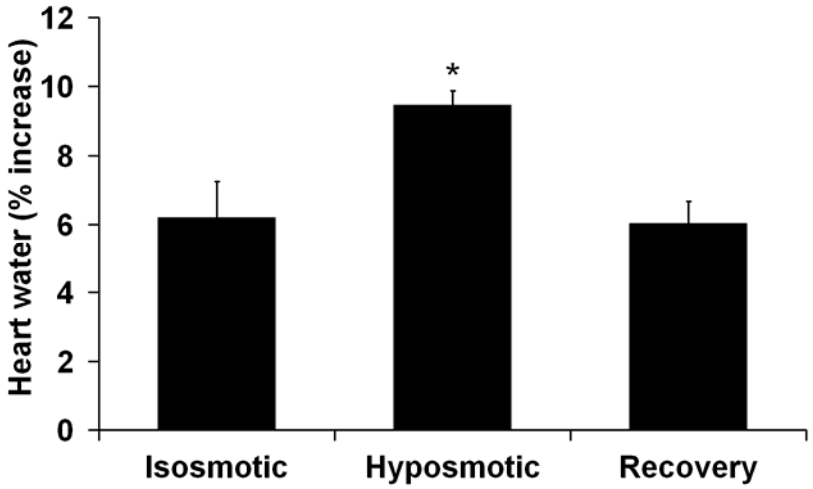

Figure 5.6. Heart water percentage increase; from baseline after perfusion in isosmotic, hyposmotic and isosmotic Krebs for 20 minutes each. Hearts perfused in hyposmotic 0.75T Krebs gained an additional 3.3% water, (*$p<0.05$). Mean±SEM is shown. *This figure was prepared by Dr Tanya Butler.*

5.4 Discussion

The Langendorff system extended our previous isolated cardiomyocyte work to the whole organ level. Although it did not model CPB it provided a recognised oedema inducing environment – as a result of the crystalloid perfusate (Rubboli, 1994). It allowed global I/R to be modelled and for functional and MWC measurements to be taken (Apstein, 1977). Molecular studies could also be undertaken, although the time course was still relatively short.

Ischaemia was found to be of principle importance in causing myocardial dysfunction, particularly diastolic dysfunction. Systolic dysfunction was only seen with the longer duration of global ischaemia, 30 minutes, in the second series of experiments. This supports a dose dependent effect of ischaemia upon myocardial dysfunction, similar to what others have described (Palmer, 2004). No additional oedema was found in the hearts arrested for 30 minutes compared with control non-ischaemic hearts. However in the initial series utilising 20 minutes ischaemia, an additional one percent increase in MWC was associated with ischaemia. The 20 minute ischaemia hearts with additional oedema had better performance overall compared with the subsequent 30 minute ischaemic group. So despite the burden of additional oedema, the duration of ischaemia was of greater importance in determining the severity of myocardial dysfunction.

In these studies we did not see a relationship between degree of oedema and degree of dysfunction. Hyposmotic hearts had a 9% increase in water and a < 5mmHg increase in diastolic pressure. This compares with ischaemic hearts that had a six percent increase in water and a 25–50mmHg increase in diastolic pressure. Thus despite 30% less oedema, ischaemic hearts had a five to 10 fold greater degree of dysfunction that persisted throughout the experiment. This further supports a divergence in association between important dysfunction and oedema.

In the isolated rat heart, oedema was found to cause minimal and reversible dysfunction. The rapid and complete reversal in dysfunction would support the role of the reduced peak calcium found in our isolated cell work, secondary to stretch induced alterations in ion permeability (Vandenberg, 1996). Potentially the amelioration of calcium availability, through the administration of additional calcium or novel inodilators which can increase free calcium may overcome this mild oedema associated dysfunction (Brixius, 2005). Such inodilators have been shown to be beneficial in small clinical studies (Brixius, 2005; Egan, 2006; Namachivayam, 2006; Osthaus, 2008). Although there is no evidence that inodilators such as Levosimendan (Abbott, Botany, NSW, Australia), improve non-ischaemic or ischaemic oedema, more recent work in isolated hearts suggests beneficial effects only when given before ischaemia (Brendt, 2008).

Isolated hearts that had additional swelling as a result of hyposmotic stress showed immediate mild dysfunction throughout a 20 minute test interval. This differed from the isolated cardiomyocytes that did not demonstrate altered contractility during eight minutes exposure. In the isolated heart, water could cause swelling in both the myocellular and interstitial compartments. Given the isolated cell data, the most likely explanation is that oedema within the interstitial compartment of the isolated heart caused a restrictive functional impairment – reduced ventricular distensibility. Alternatively the reduced peak calcium levels in swollen cardiomyocytes may have had greater functional implications in the whole heart – reduced ventricular contractility, not seen in the isolated cell. A trial of supplementary calcium or inodilator could potentially unravel the underlying mechanism of oedema induced myocardial dysfunction.

Whatever the cause, the dysfunction associated with oedema was mild. Oedema occurring as part of the conventional crystalloid perfusate in isolated hearts has been studied by Rubboli *et al* and shown not to be associated with dysfunction (Rubboli, 1994). Hearts perfused at conventional pressures for 105 minutes had 12.5% gains in water and no significant changes in either developed pressure or change in pressure over change in time (Rubboli, 1994). Only when perfusion pressure was escalated to supranormal levels did an association occur between MWC and developed pressure. It is

likely that normal membrane permeability was breached by excessive perfusion pressures (100 – 140mmHg), given the method of inducing oedema which exceeded 30%. Apstein *et al* also showed stability of function and water content in the isolated heart, following an initial and rapid gain in water. In this series of experiments it was also demonstrated that there was no difference in MWC between ischaemic and non-ischaemic rat hearts (Apstein, 1977).

In the isolated heart we also analysed the myocardium for AQP1 mRNA and protein to determine if there was any change in expression with the induction of ischaemia and oedema. Over this timeframe there were no changes in AQP1 mRNA or protein expression. This was in keeping with animal models of ischaemia in other settings of relatively short duration, and agrees with the dogma that AQPs do not display rapid expression changes (Verkman, 2002a). AQPs tend to undergo slow transcription mediated changes in expression as demonstrated in a longer timeframe model of anaemia in a foetal sheep model (Jonker, 2003). As a consequence of this finding we deferred further molecular work to the large animal, longer timeframe setting.

The use of conductance catheters *in vivo* would have allowed the capture of pressure volume loops and potentially more detailed functional analysis. Nevertheless for the significantly large insults studied it is likely that the methods used were adequately sensitive. Translating these isolated heart findings to the clinical setting is problematic. Recently in a large animal model small degrees of CPB associate oedema ~ 1 – 2% were not found to be associated with myocardial dysfunction (Fischer, 2006). Our finding that 6 – 9% water gains caused mild and reversible dysfunction suggest that myocardial oedema, even of this degree clinically, may not have important functional implications.

Thus ischaemia was confirmed as being of primary importance in causing myocardial dysfunction – principally diastolic dysfunction. Oedema was shown to be capable of causing mild, reversible dysfunction of a significantly lesser degree than that associated with ischaemia. The clinical importance of ischaemia and oedema in a whole animal model however remains to be established.

CHAPTER 6: LAMB CARDIOPULMONARY BYPASS MODEL

Abstract

To assist in translation of our earlier *in vitro* work into the clinical environment, we established a clinically relevant large animal model to examine relationships between myocardial ischaemia, oedema and cardiac dysfunction, and to also assess the role of AQPs.

Sixteen lambs were studied. Seven were non-bypass controls and nine underwent CPB. Six had 90 minutes of aortic cross clamping with blood cardioplegia and moderate hypothermia. The remaining three underwent CPB without aortic cross clamping. Haemodynamic and biochemical data were recorded, myocardial oedema, apoptotic markers and AQP expression were determined.

The CPB with aortic cross clamp (AXC) group suffered global myocardial ischaemia, reperfusion and demonstrated reduced myocardial performance, with early postoperative tachycardia, hypotension, elevated serum lactate and impaired tissue oxygen delivery, compared to the CPB without AXC group. The CPB with aortic cross clamp lambs had increased myocardial water compared with non CPB lambs and a two fold increase in AQP1 mRNA expression compared to non CPB and CPB without aortic cross clamp lambs.

A temporal association between haemodynamic dysfunction, myocardial oedema and increased AQP1 expression was found. CPB without ischaemia was associated with minimal oedema, negligible myocardial dysfunction and static AQP expression. Ischaemic reperfusion injury was the main cause of myocardial oedema and myocardial dysfunction.

6.1 Introduction

A large animal model of CPB was examined for two main reasons. Firstly in order to incorporate and extend the findings of the isolated cell and heart studies into the near clinical realm. Secondly to examine the effects of CPB ± ischaemia on myocardial function, oedema and membrane proteins over a longer duration in a whole animal comparable to the human infant.

A similar albeit porcine model of CPB had been previously established locally. We decided upon an ovine model because AQP DNA sequences were published for the sheep, but not for the pig and because of physiological similarities between lambs and human infants.

We replicated CPB with and without ischaemia in the form of aortic cross clamping (AXC) and we measured clinically relevant functional indices. We measured oedema, apoptosis and proteins of interest terminally as interval samples were considered potentially functionally detrimental. Our original intention was to use conductance catheter technology, but logistically this was not achievable.

6.2 Methods

As discussed in Chapter 2.

6.2.1 Study design

Sixteen lambs of either sex were utilised weighing 7.4 ± 0.4kg.

6.2.1.1 Non CPB controls

There were seven 'controls' that underwent cardiectomy without receiving cardiopulmonary bypass (non CPB controls). Lambs were prepared and monitored as for the CPB groups and maintained under anaesthesia for one hour prior to cardiectomy. Cardiac standstill was achieved following a terminal dose of cardioplegia in four of the seven controls. Blood was obtained from the ewe for the purpose of making blood cardioplegia. The other three non CPB controls were euthenased with pentobarbitone, also whilst under anaesthesia with the sternum open, allowing rapid cardiectomy and specimen preservation. These two approaches were used to account for the effect of cardioplegia on myocardial water, which is recognised to potentially increase MWC (Spotnitz, 1995).

6.2.1.2 CPB groups

Of the nine CPB lambs, three underwent 90 minutes of bypass without aortic cross clamping (CPB-AXC) and were maintained for three hours after separation from bypass. The remaining six had AXC (CPB+AXC) and were maintained for either three (n=3) or six (n=3) hours after separation from bypass.

6.2.2 Tissue Analysis

As per Chapter 2 this included assessment of MWC, myocardial AQPs and apoptosis.

6.2.3 Statistical Analysis

This was as per Chapter 2, apart from the following comments.

Statistical significance for haemodynamic data was determined by both the Mann-Whitney U test (MWU) and linear mixed models using covariance type AR–1. The animals receiving CPB±AXC were compared in terms of haemodynamic variables–both functional and biochemical. Haemodynamic variables were analysed over the complete survival period and also by comparison of 30 minute epochs. Our sample size gave us 80% power to demonstrate that a 2.3 standard deviation effect size difference between groups was significant at $p \leq 0.05$.

For measures of myocardial water, AQP1 expression and apoptosis, comparison was made between non CPB control animals and CPB±AXC, using MWU.

6.3 Results

All experiments were completed as intended and there were no significant differences in pre CPB haemodynamic values. There were no inotropes or vasodilators administered prior to CPB and baseline biochemical indices were not significantly different, (Table 6.1). The animals received similar weight-based doses of sedatives, analgesics, anaesthetics and muscle relaxants.

Table 6.1. Baseline biochemical variables

	n	Pre CPB indices (mean±SEM)				
		Haemoglobin (g/dL)	Glucose (mmol/L)	Lactate (mmol/L)	Venous Saturation (%)	COP (mmHg)
Control	4	10.5±0.8	6.5±1.0	1.5±0.3	78±14.9	13.8±1.3
CPB–AXC	3	10.3±0.2	5.8±2.3	1.4±0.5	72.5±16.9	12.3±1.5
CPB+AXC	6	10.2±0.4	5.7±0.4	2±0.7	76.8±4.7	14.6±0.5

AXC – Aortic cross clamp
COP – colloid osmotic pressure
CPB – cardiopulmonary bypass

6.3.1 Haemodynamics

The haemodynamic picture of reduced cardiac performance was seen in those animals that had CPB+AXC, (Figure 6.1). The CPB+AXC lambs were more hypotensive post CPB. Mean blood pressure (MBP) was significantly less by mixed model analysis, ($p<0.01$) following CPB. Diastolic blood pressure (DBP) was also significantly less using mixed model analysis, ($p<0.05$) after CPB. The differences remained at the end of the survival period. Systolic blood pressure (SBP) was lower in the CPB+AXC group and this was significantly so using MWU for the early and mid epochs post CPB. Heart rate was higher in the CPB+AXC group, also by MWU, in all but the last post CPB epoch. Central venous pressure, fluid requirements and doses of vasoactive medications did not differ between the groups.

6.3.2 Biochemistry

The CPB+AXC group had a higher lactate (6.5mmol/L vs 3.6, $p<0.05$) after CPB. However by three hrs post CPB the differences were not significant, 3.1mmol/L in the CPB+AXC group and 2.1mmol/L in the CPB–AXC lambs. The CPB+AXC group had a persistently lower venous saturation after CPB. The initial post CPB venous saturation was 61% in the CPB+AXC group, compared with 76.7%, ($p<0.05$), in the CPB–AXC lambs. The venous saturations at three hrs post CPB were 70.0% and 83.8%, ($p<0.05$), respectively, (Figure 6.1). The glucose and haemoglobin levels remained similar in the two CPB groups. The COP was not significantly different between the two CPB groups at any time following CPB. COP showed the expected increase following MUF. In the CPB–AXC group it rose from 12.3mmHg to 14.5mmHg and in the CPB+AXC group from 13.5mmHg to 15.7mmHg. Following a further three hrs the COP values were 13mmHg and 13.6mmHg respectively.

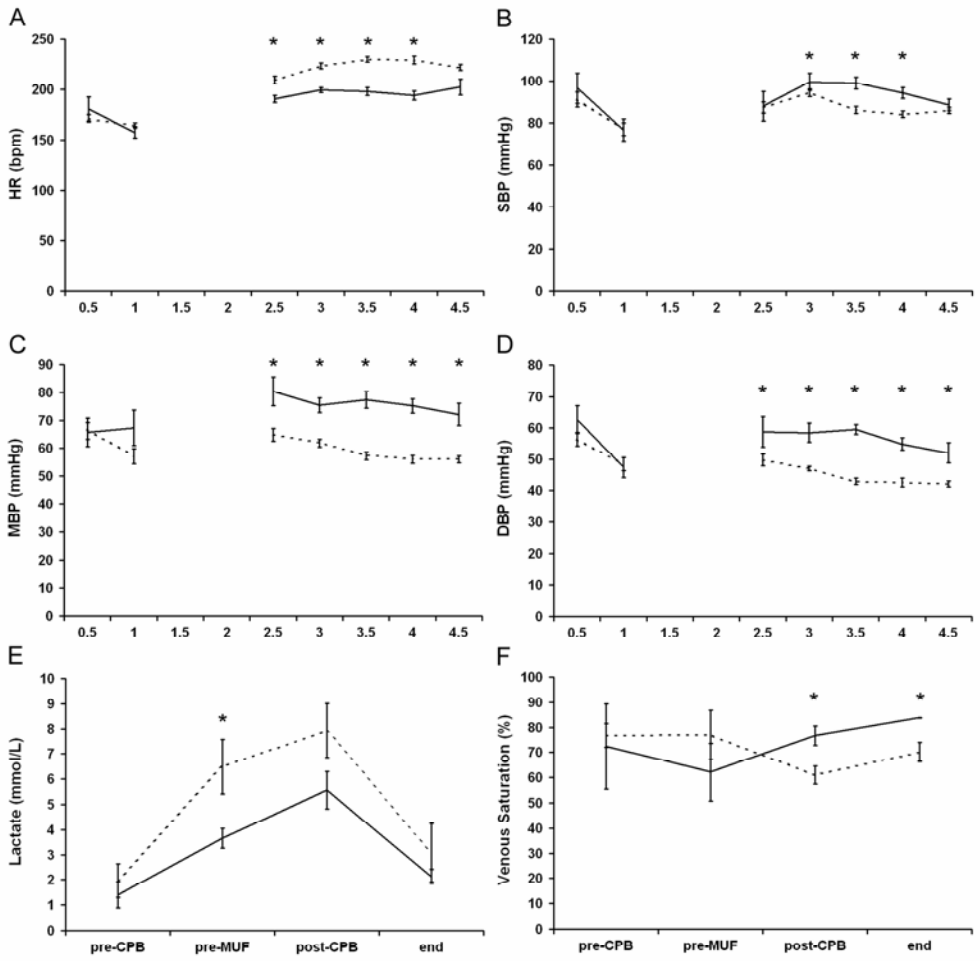

Figure 6.1. Haemodynamic and biochemical variables. In A–F, lambs are grouped as CPB without AXC**,** (solid line) and CPB+AXC (dashed line). A shows similar heart rates between groups, but after CPB the CPB+AXC group have a significantly higher heart rate until the last 30 minute epoch of postoperative analysis**,** (MWU). B displays systolic blood pressure which is significantly lower in the CPB+AXC group in the mid epochs following CPB**,** (MWU). C and D show mean blood pressure and diastolic blood pressure respectively, being significantly lower in CPB+AXC animals throughout the postoperative period**,** (mixed model analysis). E demonstrates a higher lactate in the CPB+AXC just prior to the completion of CPB**,** (MWU). In F, there is a corresponding fall in venous saturation in this group which remains throughout the postoperative period**,** (MWU), (*$p<0.05$). Mean ± SEM is shown.

6.3.3 Myocardial oedema

Myocardial water was increased (but, not significantly) in the control lamb tissue that was sacrificed following terminal cardioplegia; 0.785 as opposed to the control lambs which received pentobarbitone, 0.781. Myocardial water content in the CPB–AXC group was 0.787, whilst in the CPB+AXC group it was 0.796, which was significantly greater than cardioplegia control tissue, ($p<0.05$), but not different to CPB–AXC. In summary, there was a 1% increase in myocardial water associated with CPB+AXC with only a 0.2% increase in myocardial water in the CPB–AXC group, (Figure 6.2).

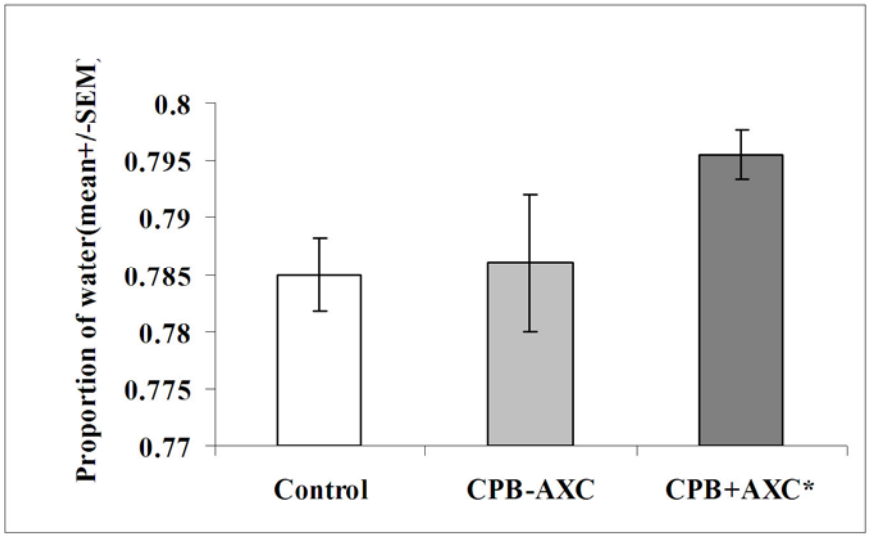

Figure 6.2. Myocardial water. Proportion of heart as water in □non CPB controls, □CPB without AXC and ■CPB+AXC lambs. Significant myocardial oedema was present in CPB+AXC lambs and a non–significant increase in myocardial water occurred in CPB–AXC lambs, (MWU), (*$p<0.05$). Mean ± SEM is shown.

5.3.4 Molecular results

Myocardial AQP1 mRNA by quantitative real time qRT-PCR was increased two fold in the CPB+AXC, compared with control tissue, ($p<0.05$). AQP1 mRNA in the CPB+AXC group was also significantly higher when compared to the CPB–AXC group, ($p<0.05$), (Figure 6.3). There was no associated increase in AQP1 on western blot analysis in either of the CPB groups, (Figure 6.4). AQP4 transcript was not altered compared to controls in either CPB group and low protein levels were also unchanged by experimental group (data not shown). AQP0, AQP3 and AQP9 transcripts were detectable at low levels. No appreciable changes were seen in these with qRT-PCR (data not shown). Protein for these three AQPs were not demonstrable by Western blot despite appropriate antibodies and control tissue (data not shown).

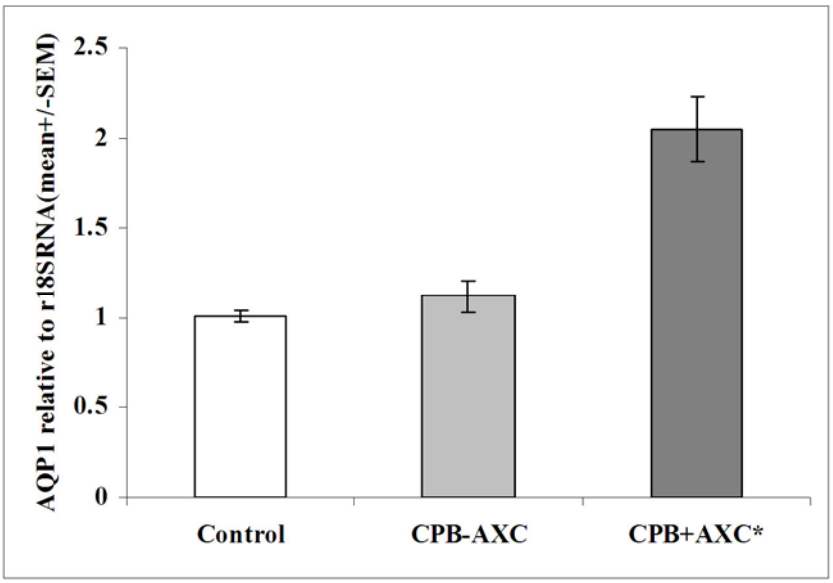

Figure 6.3. Myocardial aquaporin 1 transcript expression. RNA was extracted from the myocardium of □non CPB controls, □CPB without AXC and □CPB+AXC lambs. The quantitative real time PCR results shown are representative of at least three individual animals in each group and are representative of three independent sets of experiments. AQP1 mRNA was normalised to ribosomal 18S RNA(r18S RNA), (MWU), (*$p<0.05$) versus non CPB controls and CPB–AXC. Mean ± SEM is shown.

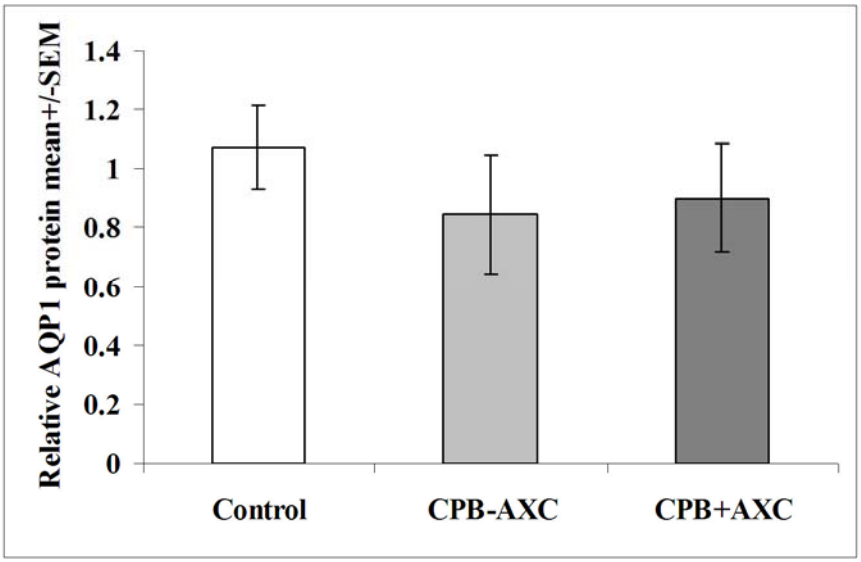

Figure 6.4. Myocardial aquaporin 1 protein expression. AQP1 protein was obtained from the myocardium of □non CPB controls, □CPB without AXC and □CPB+AXC lambs. AQP1 protein was measured by Western blotting, analysed by densitometry and normalised to the expression of cardiac actin. Representative results demonstrate no significant differences between groups, (MWU). Mean ± SEM is shown.

6.3.5 Apoptosis

Completed apoptosis was not demonstrable by TUNEL staining in any of the preparations to a significant degree. H+E stained slides also did not demonstrate necrosis, although tissue oedema was more apparent in the CPB+AXC group. Active caspase 3 protein, an early marker of apoptotic pathway induction (Rodriguez and Schaper, 2005), was significantly increased in the CPB+AXC group. The control and CPB–AXC groups had comparable levels of caspase 3, (Figure 6.5).

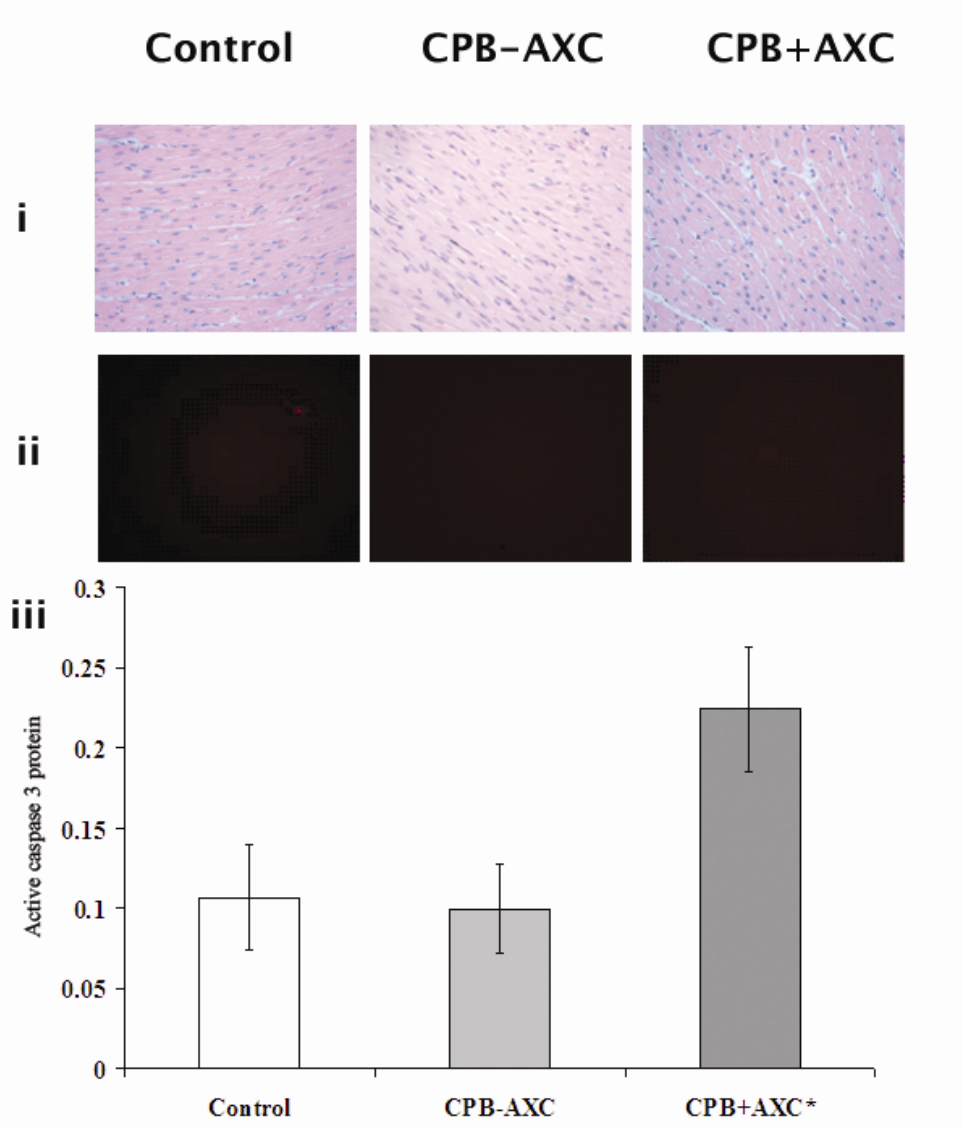

Figure 6.5. Myocardial apoptosis. Representative results of □non CPB controls, □CPB without AXC and □CPB+AXC lambs. i) H+E micrographs of ventricular myocardium taken at 100X magnification. The CPB+AXC images are suggestive of more tissue oedema, however there was no apparent necrosis. ii) Micrographs of TUNEL stained ventricular myocardium at 100X magnification. Appropriate positive and negative controls were performed (data not shown). No significant differences were seen in TUNEL staining, *these images were prepared by Dr Tanya Butler*. iii) Graph of the results of active caspase 3 densitometry for the 3 groups from Western blot analysis, loading controlled with cardiac actin. The CPB+AXC animals demonstrated a significant

increase in active caspase 3 protein, suggestive of active early apoptotic pathways, (MWU), (*$p<0.05$). Mean ± SEM is shown.

6.4 Discussion

6.4.1 Haemodynamics

Haemodynamic changes consistent with reduced myocardial performance were seen following CPB. In the CPB+AXC animals there was significant tachycardia and systolic dysfunction. Impaired tissue oxygen delivery and lower diastolic pressure also occurred in those animals following CPB+AXC. CPB alone did not result in significant haemodynamic or biochemical derangements, it was I/R that was primarily associated with haemodynamic dysfunction and impaired tissue oxygen delivery. The primacy of I/R in precipitating reduced myocardial performance has been alluded to previously however in some well controlled animal experiments, this has not been demonstrated (Addetia, 1986; Blatchford, 1994). In our series, CPB alone was well tolerated and only when coupled with I/R did significant perturbations in circulatory function result. Consequently measures to minimise the duration and impact of I/R should be the focus of ongoing research into postoperative reduced myocardial performance or LCOS.

6.4.2 Myocardial oedema

The greatest degree of myocardial oedema was seen in those animals following CPB with I/R. CPB without I/R was associated with development of some myocardial oedema, although not significantly different from baseline. Together with the isolated cell and isolated heart studies this supports a re-evaluation of the dogma surrounding myocardial oedema and its contribution to the development of myocardial dysfunction following CPB. Ischaemia is a potential confounder in several of the sentinel papers involved in asserting the link between oedema and dysfunction (Mehlhorn, 1995a; Mehlhorn, 1995b), and there have been no previous attempts to experimentally differentiate the contribution of ischaemia to oedema-associated dysfunction. Oedema does occur with CPB alone – in our study it was of the order of 0.2%, this was not associated with significant myocardial dysfunction. It is likely that oedema in this group was mainly interstitial or vasogenic in nature, resolved quickly and was not associated

with important dysfunction. Others have shown that such oedema should have resolved within six hours (Mehlhorn, 1995b). And in other studies MWC has increased up to 2% at two hours, in animal models of CPB, without dysfunction (Fischer, 2006). Hence it may have been possible to demonstrate greater myocardial oedema following CPB alone if we had looked earlier in the postoperative period, but such a timeframe would not correlate with LCOS and dysfunction that is seen 6–12 hours postoperatively.

6.4.3 Ischaemia

Global ischaemia with aortic cross clamping resulted in raised lactate, reduced venous saturations and tended to increase CK. In our study, ischaemia was associated with a greater degree of oedema formation compared to CPB alone, and was associated with important dysfunction. The 1% increase in myocardial water that we observed following I/R could be expected to associated with a 10% reduction in myocardial function based on work by Laine *et al* (Laine and Allen, 1991). Interestingly in our isolated heart work there was also an additional 1% of myocardial oedema, compared with non-ischaemic hearts associated with a 20 minute ischaemia interval. Similarly diastolic dysfunction was more apparent than systolic dysfunction as seen in the CPB with I/R lambs. However, 2% water gains in animal CPB models did not result in any myocardial dysfunction using ultrasonic crystals and left ventricular micromanometers (Fischer, 2006), so it is unlikely that the moderate dysfunction we observed was caused by oedema.

We did not measure cardiac output or load independent measures of myocardial function, but the average difference in mean blood pressure following CPB, when comparing those with or without I/R in our study, was 17mmHg (22%). This suggests that other factors, not oedema alone, were responsible. It is likely that I/R results in a greater degree of interstitial or vasogenic oedema which persisted, together with cytotoxic or myocellular oedema due to accumulation of lactate. In isolated cells, we have established that myocellular injury and dysfunction occurs following ischaemia with a degree of associated oedema. Cytotoxic oedema has been shown to be short lasting (Askenasy,

2001), but may reflect important injury of the contractile apparatus that persists for the duration of the LCOS, e.g. partial troponin I degradation as a consequence of calcium influx and oxygen derived free radicals (Bolli and Marban, 1999).

6.4.4 Aquaporins

The increase in myocardial water occurred without a significant reduction in COP. This is in keeping with other reports modelling the capillary leak syndrome after CPB (Farstad, 2004; Tassani, 2007). Hence there was movement of free water down osmotic gradients across the endothelium into the myocardial tissue, as occurs in ischaemia (Wright and Rees, 1998). Water moves via AQPs which exist abundantly within the endothelium and sarcolemma, to leak into the intracellular space of the myocardium if osmotic gradients mandate, and as may occur early during reperfusion as a result of intracellular lactate accumulation (Wright and Rees, 1998; Au, 2004).

AQP1 transcript was increased following CPB with ischaemia within the myocardium. This increase was specific to those animals that had I/R and not CPB alone. This was a two fold increase in transcript, but without an associated protein rise. AQP1 has been studied in a neonatal lamb heart previously, in a deep hypothermic circulatory arrest (DHCA) model – no change in AQP1 expression was found in this study (Tabbutt, 1997b). Potentially AQP1 expression may have been modified by inducing deep hypothermia as has been described in other tissues and settings (Fujita, 2003). Such modifications may impact on oedema formation and function as reported elsewhere (Wernovsky, 1995; Tassani, 2002a), however the impact of DHCA vs. continuous flow on AQP expression was not examined in our study. There is an intriguing discrepancy in AQP1 mRNA following CPB + AXC and DHCA, perhaps underlying the differential in water accumulation with these two modalities of support.

The finding that AQP1 transcript increased in the lamb after CPB+AXC extends our earlier results utilising a rat isolated heart model. In the rat, a brief period of global ischaemia with reperfusion was not associated with changes in AQP1 transcript or protein

levels. Species differences, duration of ischaemia and post ischaemic observation as well as utilisation of CPB may explain these differences. On the basis of our earlier work, we do not feel that species uniqueness adequately explain this difference since the AQP expression profile of the sheep is similar to rat and human. Duration of ischaemia may be important as up-regulation of AQP1 has been shown in interventions lasting hours or days rather than minutes. In a foetal sheep model, anaemia induced myocardial AQP1 increase over five days, suggesting that longer timeframes permitted adjustments in myocardial AQP1 protein levels (Jonker, 2003). As longer duration experiments have permitted changes in AQP transcript to be uncovered, it is likely that 9–12hr experiments would be necessary to determine if protein changes occur. We cannot determine the significance of the demonstrated two fold rise in AQP1 transcript and this finding warrants further study. Interestingly though, the two fold increase we found was identical to the increase described by Jonker *et al* after a more prolonged insult. A two fold increase could represent the maximal upscaling of AQP1 expression.

6.4.5 Apoptosis

The suggestion by Calderone *et al* that induction of apoptotic pathways may cause post-ischaemic dysfunction, as well as later cell loss, is an intriguing hypothesis (Caldarone, 2004). We sought to corroborate these findings, but did not demonstrate completed apoptosis as an important factor in LCOS over the early time period. We did however demonstrate increased expression of active caspase 3 in the CPB+AXC animals suggestive of early apoptotic activation (Rodriguez and Schaper, 2005). Mitochondrial function was not measured in our study. The negative predictive value of TUNEL is greater than its positive predictive value, especially because it can be positive during tissue regeneration and recovery (Rodriguez and Schaper, 2005). We support the possibility that apoptosis may be a contributing factor to LCOS and this may be particularly important in young infants having multiple operations with progressive cell loss over time. Since ischaemia is the likely pro–apoptotic trigger, the findings reinforce the importance of better managing the myocardium during ischaemia.

6.4.6 Limitations

These experiments were limited by their small size and that they were conducted in animals. The animals also did not have structural heart disease and hence no preoperative volume or pressure loading. Measurements of vascular/ventricular coupling as well as load independent measurements of systolic and diastolic function would be preferable in future experiments. The haemodynamic and functional monitoring used to support our findings were somewhat rudimentary, but equivalent to techniques relied upon in the clinical setting.

6.4.7 Conclusions

Ischaemia and reperfusion were associated with a significant degree of myocardial oedema, clinically relevant dysfunction and increased expression of AQP1. Oedema formation is mostly related to ischaemia and not CPB. The main focus in LCOS research should be the prevention of post-ischaemic dysfunction rather than the systemic inflammatory response to CPB and generalised water accumulation.

CHAPTER 7. MYOCARDIAL MEMBRANE INJURY AND POLOXAMER 188

Abstract

Poloxamer 188 (P188) was studied in isolated heart models, focusing on oedema, ischaemia and function. P188 was then studied in an extended duration lamb CPB model together with an assessment of membrane injury and the membrane proteins dystrophin, dysferlin and AQP1.

Low and high dose P188 trials in isolated rat hearts were undertaken with and without global ischaemia, with measurement of function (LVDP and Dp/Dt_{min}) and MWC. In a lamb CPB model, eight lambs were randomised to saline±P188. Lambs underwent two hours of CPB and aortic cross clamping. After a further nine hours of monitoring the hearts were assessed for water content, capillary leak and protein expression.

In the isolated rat heart with low dose P188, there was a significant reduction in non-ischaemia associated myocardial oedema, ($p=0.03$). Whilst in the high dose trial, P188 was associated with a trend towards improved diastolic function following ischaemia, ($p=0.06$), and no difference in myocardial water. In the lambs, P188 was associated with an improved haemodynamic profile (higher blood pressure, higher venous saturation and lower lactate), although the heart rate tended to be higher. Dystrophin expression was unaffected by ischaemia/reperfusion and dysferlin expression was reduced. AQP1 protein increased following ischemia/reperfusion. P188 administration was associated with supra-normal levels of dystrophin, preservation of dysferlin expression and normalisation of AQP1 expression. P188 was associated with less capillary leak, maintained colloid osmotic pressure and less haemodilution.

Isolated heart trials of P188 support a non-rheological mechanism of action. Low micelle doses appear to reduce endothelial leakiness in non-ischaemic settings, whilst higher doses are associated with a trend towards improved diastolic function in the setting of

ischaemia. In the lamb CPB model, indicators of reduced myocardial performance such as lower blood pressure and lower oxygen delivery, were lessened in association with the administration of P188. Changes in protein expression within the myocardial membrane were found in a clinically relevant model of paediatric cardiac surgery. P188 was also associated with potentially beneficial changes in membrane protein expression, reduced capillary leakage and less haemodilution. P188 is a potentially useful therapy in the setting of planned ischaemia/reperfusion.

7.1 Introduction

Given the primacy of ischaemic injury seen in the isolated cell, isolated heart and initial lamb CPB studies, we focused our investigations on the myocardial membranes. Markers of injury and a therapy to potentially protect the membranes of the heart were investigated. P188 was an acceptable candidate therapy – as discussed in Chapter 1, and therefore was initially trialled at a low dose to avoid free micelle formation in an isolated heart model. Subsequently at a higher, more clinically relevant dose – as discussed in Chapter 2, P188 was tested in isolated rat hearts and then in the lamb. The lamb CPB experiments utilised an extended recovery period to determine if AQP1 protein as well as mRNA changes occurred. Dystrophin and dysferlin, which both have roles in membrane integrity and repair, were also examined. The focus was to determine the impact of ischaemia/reperfusion on dystrophin and dysferlin, as well as whether P188 may reduce ischaemic injury related dysfunction in a clinically relevant model.

7.2 Methods

As per Chapter 2, an additional schema of the isolated rat heart trials and lamb CPB model are included here, (Figure 7.1).

7.2.1 Isolated rat heart trials of P188

7.2.1.1 Low dose P188 trial

Five treatment groups with at least six animals in each were compared. Ischaemic and non-ischaemic groups were randomised to P188 or conventional buffer, (Figure 7.1 – 1A – D). Tissue analysis and statistics was as per Chapter 2.

7.2.1.2 High dose P188 trial

Two groups of five animals were randomised to P188 or placebo, (Figure 7.1 – 2A). Tissue analysis and statistics were as per Chapter 2.

7.2.2 Lamb CPB trial of P188

Eight lambs of either sex were utilised weighing 20.3±1.7kg. Three lambs received placebo (CPB–P188) and five received P188 (CPB+P188), (Figure 7.1 – 3).

In earlier experiments, (see Chapter 6), lambs underwent CPB±AXC. Myocardial tissue from these animals was assessed for dystrophin and dysferlin expression. Also in these earlier experiments, three lambs were anaesthetised, received no PBS±P188 and were sacrificed prior to CPB, for non CPB data (non CPB controls).

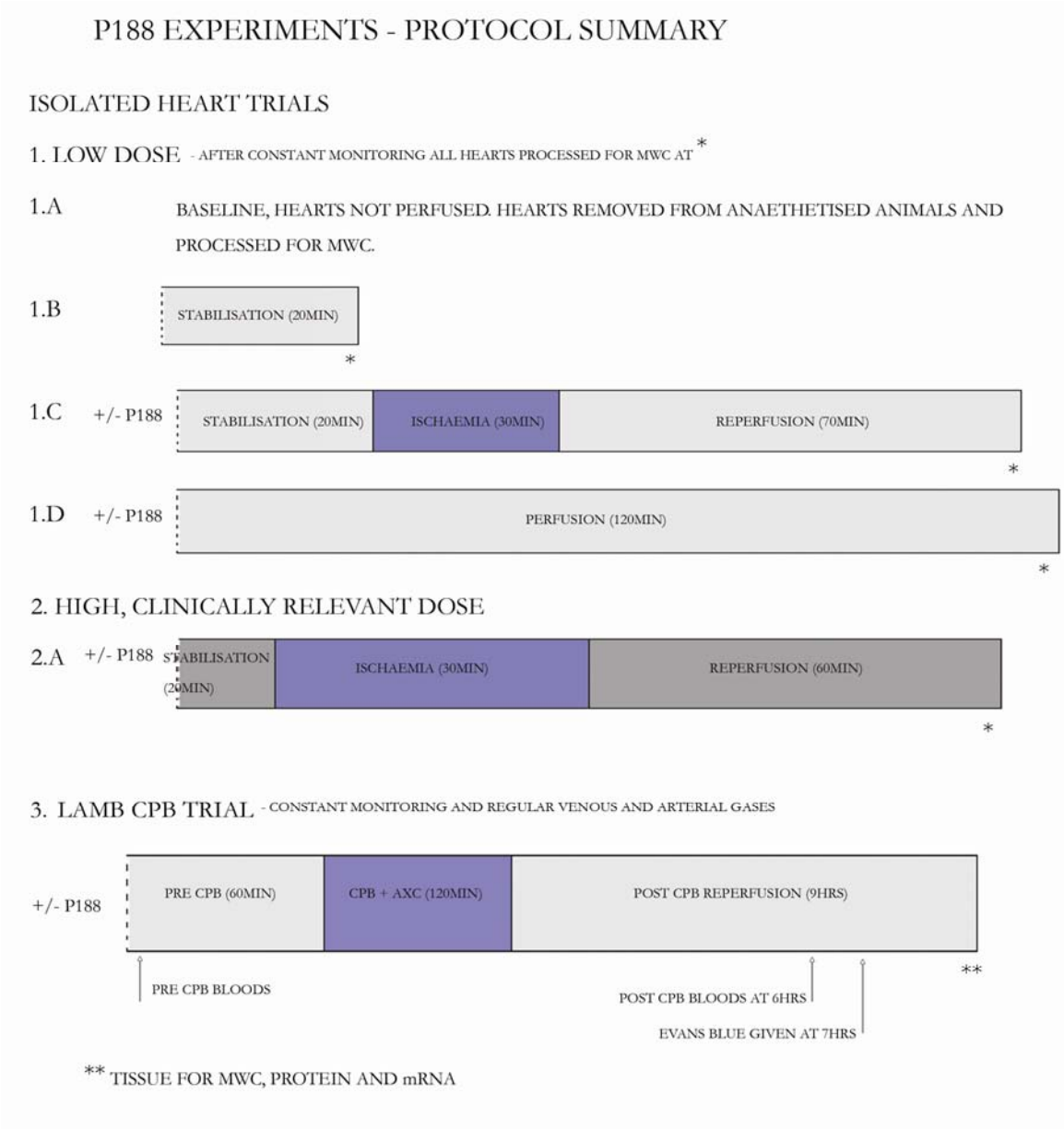

Figure 7.1. P188 experimental protocol summary, including 1). low and 2). high dose isolated heart trials and the 3). lamb CPB P188 trial.

7.2.2.1 Tissue analysis

This was as per Chapter 2 and included assessment of MWC, myocardial AQPs, dystrophin, dysferlin and Evans blue staining.

7.2.2.2 Statistical Analysis

This was as per Chapter 2, apart from these comments.

The lambs ± P188 were compared in terms of haemodynamic variables–both functional and biochemical. Haemodynamic data variables were analysed over the complete survival period and also by comparison of 60 minute epochs. Our sample size gave us 80% power to demonstrate that a 0.7 standard deviation effect size difference between groups was significant at a p value of 0.05. For measures of protein expression, myocardial water and blood tests, comparison was made between control animals and nine hour post CPB ± P188, using MWU.

7.3 Results

7.3.1 Isolated heart trials

7.3.1.1 Low dose trial

All experiments proceeded as planned. Five treatment groups were compared with at least six animals in each group; baseline (hearts excised and not perfused), 20 minute perfusion controls, 120 minute perfusion controls (without ischaemia) ± P188, and 20 minute perfusion followed by 30 minutes ischaemia, followed by 80 minutes reperfusion ± P188, (Figure 7.1 A – D).

7.3.1.2 Haemodynamics

Heart function was assessed as LVDP measured at systole (LVDP–S) and diastole (LVDP–D) without ischaemia ± P188 (Figure. 7.2). Hearts that were perfused for 120 minutes were stable, with a slight decline in systolic and slight rise in diastolic pressures. Animals that received P188 had a trend towards a higher diastolic pressure, ($p=0.116$), whilst the systolic pressure of P188 perfused animals was similar, ($p=0.473$) to standard perfusate animals, (Figures 7.2). Ischaemia for 30 minutes principally affected diastolic function, there was no significant difference between groups in terms of systolic, ($p=0.313$) or diastolic function, ($p=0.684$), (Figure 7.3).

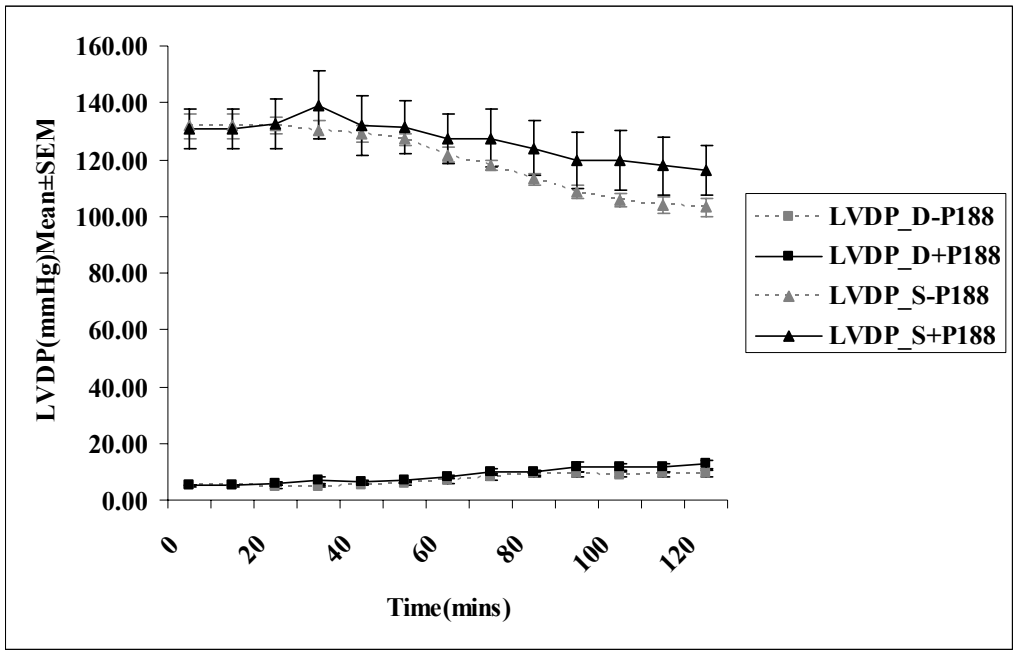

Figure 7.2. Left ventricular developed pressure (LVDP) in non-ischaemic rat isolated hearts. These hearts were perfused for 120 min ± P188. See text for details.

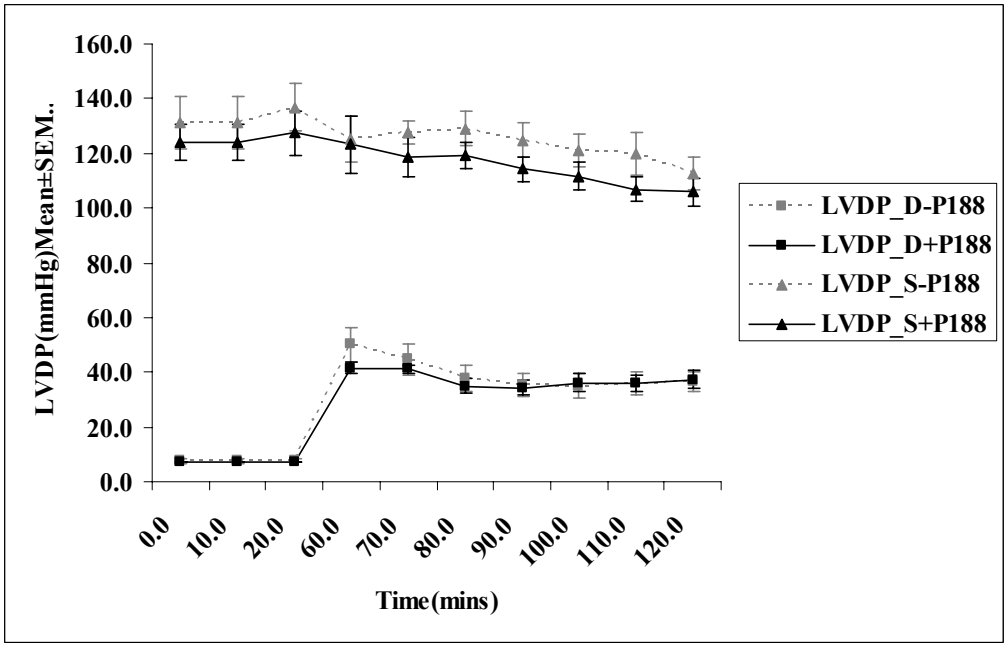

Figure 7.3. Left ventricular developed pressure (LVDP) in ischaemic rat isolated hearts. These hearts were perfused ± P188, initially for 20 minutes, then after 30 minutes global ischaemia were again perfused for 70 minutes. See text for details.

7.3.1.3 Myocardial water

The baseline hearts had a MWC of 0.768 ± 0.0008. The MWC of non-ischaemic hearts perfused for 120minutes was 0.819 ± 0.0037, (p=0.025 compared with baseline) and for those with P188 was 0.78± 0.0069, (p=0.05 compared to baseline and p=0.025 between groups). The MWC of ischaemic hearts was 0.827 ± 0.0055, (p=0.025 compared with baseline) and for those with P188, 0.827 ± 0.0035, (p=0.02 compared to baseline and p=0.58 between groups), (Figure 7.4).

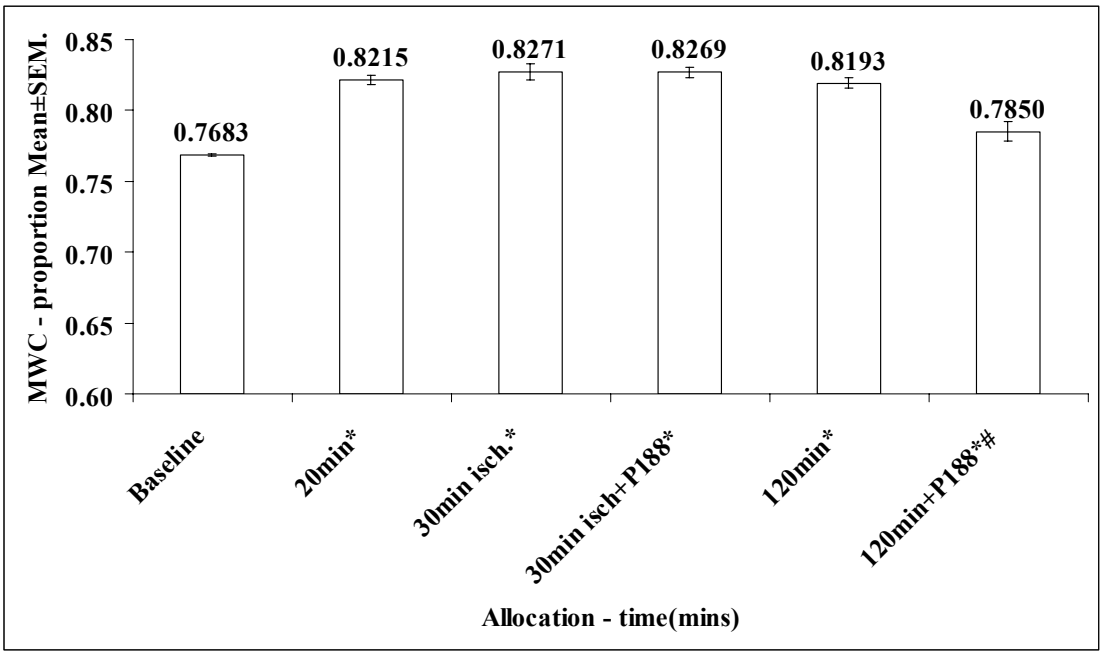

Figure 7.4. Myocardial water content for isolated rat hearts; allocations including ± P188. See text for details, 120 min+P188 hearts were the driest perfused hearts, compared with baseline and 120 min–P188, (*#p<0.05).

7.3.1.4 High dose trial

All experiments proceeded as planned. Two treatment groups were compared with five animals in each group; 20 minutes perfusion followed by 30 minutes ischaemia, followed by 60 minutes reperfusion±P188, (Figure 7.1 – 2A).

7.3.1.5 Haemodynamics

Heart function was assessed as LVDP and analysed in systole and diastole. There were no appreciable differences in systolic pressures between groups, (p=0.557), (Figure 7.5). There was a trend towards lower diastolic pressures in the P188 group, (p=0.128), (Figure 7.6). Improved diastolic function was supported by a trend towards Dp/Dt_{min} being lower in the P188 group, (p=0.064), (Figure 7.7).

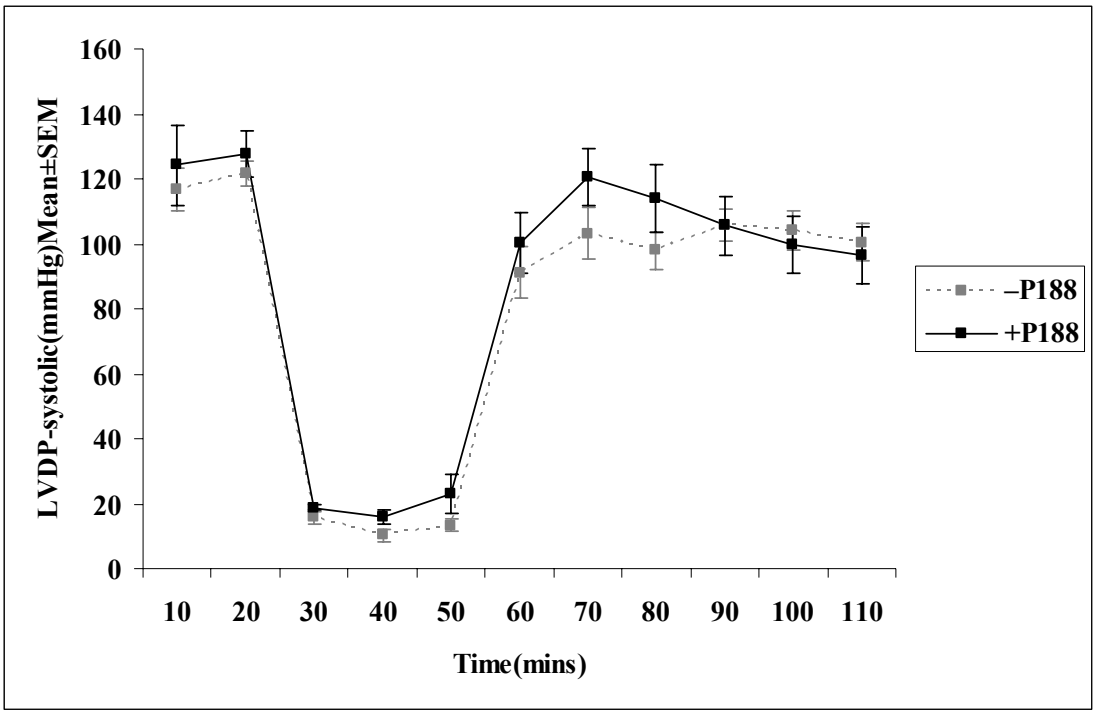

Figure 7.5. Left ventricular developed pressure (LVDP) – systolic ± P188. See text for details. *This figure was prepared by Dr Tanya Butler.*

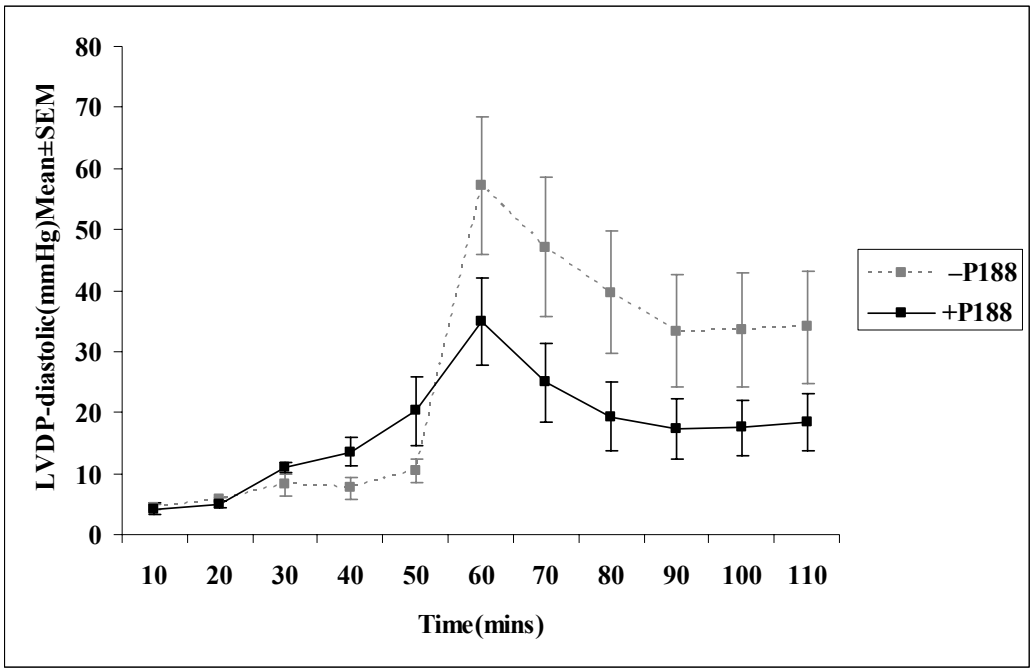

Figure 7.6. Left ventricular developed pressure (LVDP) – diastolic ± P188. See text for details, there was a trend to lower diastolic pressures with P188, (p=0.1). *This figure was prepared by Dr Tanya Butler.*

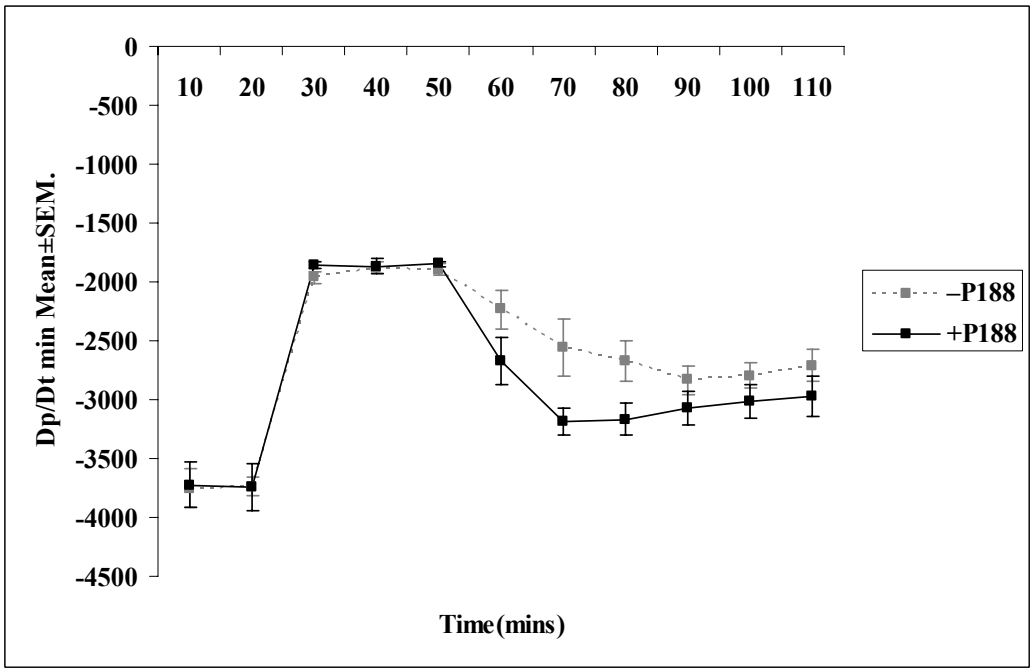

Figure 7.7. Contractility; Dp/Dt_{min}. See text for details, there was a trend to lower Dp/Dt_{min} with P188, (p=0.06). *This figure was prepared by Dr Tanya Butler.*

7.3.1.6 Myocardial water

There was no differences between the MWC of these two groups. P188 hearts had an MWC of 0.827 ± 0.008 and non P188 hearts were 0.827 ± 0.023, ($p=0.917$).

7.3.2 Lamb CPB trial

All experiments were completed as intended. No inotropes or vasodilators were administered prior to CPB and rates of administration following CPB were similar, (Table 7.1). There were no significant differences in pre CPB haemodynamic values, apart from a lower heart rate in the CPB+P188 group, (Table 7.2).

Table 7.1. Various measurements pre and post CPB.

		Mean(±SEM)	
		pre CPB	post CPB
Total CK(40-240 IU)	9hr	223(44)	2092(1388)
	9hr+P188	204(67)	1781(415)
	p value	0.5	0.5
Troponin T(<0.05ug/L)	9hr	0.01(0)	1.83(1.23)
	9hr+P188	0.01(0)	0.99(0.24)
	p value	1	0.65
Dopamine (mcg/kg/min)	9hr		5.2(0.2)
	9hr+P188		4.8(0.5)
	p value		0.4
Sodium Nitroprusside (mcg/kg/min)	9hr		1(0)
	9hr+P188		1.1(0.1)
	p value		1
Input(crystalloid) (ml)	9hr	767(145)	1080(205)
	9hr+P188	808(105)	1052(225)
	p value	1	1
Input(colloid) (ml)	9hr		477(46)
	9hr+P188		554(57)
	p value		0.4
Input(total) (ml)	9hr		1557(197)
	9hr+P188		1606(211)
	p value		0.9
Chest drain loss (ml)	9hr		667(133)
	9hr+P188		420(68)
	p value		0.13
Platelets x 10^9/L	9hr	315(46)	249(24)
	9hr+P188	382(12)	255(61)
	p value	0.16	0.45
APTT (23-34seconds)	9hr	33.8(5)	36.9(6)
	9hr+P188	35(3)	40.2(5)
	p value	0.7	0.5
Fibrinogen (1.5-6g/L)	9hr	2.5(0.5)	2.2(0.3)
	9hr+P188	2.3(0.3)	2.3(0.1)
	p value	0.7	0.5
Mean ACT (on CPB)	9hr	560(80)	
	9hr+P188	622(39)	
	p value	0.7	

CPB – cardiopulmonary bypass
CK – creatinine kinase
APTT - activated partial thromboplastin time
ACT – activated clotting time
p value – Mann Whitney U test

Table 7.2. Haemodynamic and laboratory variables post CPB

	Hours post CPB									
	Baseline	1	2	3	4	5	6	7	8	9
Mean(±SEM)										
CVP(mmHg)										
9hr	2.0(0.5)	3.3(0.6)	2.6(0.4)	2.9(0.4)	3.1(0.4)	2.6(0.4)	1.9(0.4)	2.5(0.6)	2.6(0.5)	2.7(0.6)
9hr+P188	2.5(0.5)	4.2(0.6)	3.5(0.5)	2.8(0.4)	2.7(0.4)	2.7(0.4)	2.8(0.4)	2.7(0.4)	2.1(0.5)	2.3(0.4)
p value*	0.5	0.37	0.52	0.17	0.14	0.49	0.91	0.51	0.66	0.75
p value #										0.88
HR(bpm)										
9hr	131(6)	148(4)	153(3)	152(5)	147(3)	157(5)	156(4)	145(3)	135(3)	127(2)
9hr+P188	107(4)	147(3)	153(3)	162(3)	163(2)	158(2)	159(3)	161(2)	155(3)	144(3)
p value*	<0.01	0.75	0.62	0.2	<0.01	0.54	0.64	<0.01	<0.01	<0.01
p value #										0.15
SBP(mmHg)										
9hr	93(5)	94(3)	93(3)	101(3)	101(2)	91(2)	84(1)	79(2)	74(2)	75(2)
9hr+P188	89(3)	96(3)	102(2)	102(1)	95(1)	91(2)	95(2)	93(2)	87(2)	84(2)
p value*	0.6	0.47	0.01	0.54	0.02	0.99	<0.01	<0.01	<0.01	<0.01
p value #										0.12
MBP(mmHg)										
9hr	75(4)	66(4)	72(3)	76(2)	75(2)	69(2)	62(1)	58(2)	55(1)	56(2)
9hr+P188	74(3)	76(3)	84(2)	81(1)	75(1)	73(2)	75(2)	73(1)	68(2)	65(1)
p value*	0.8	0.04	<0.01	0.05	0.97	0.08	<0.01	<0.01	<0.01	<0.01
p value #										<0.01
DBP(mmHg)										
9hr	62(3)	60(3)	55(3)	58(2)	57(2)	51(1)	46(1)	43(2)	41(1)	43(2)
9hr+P188	62(3)	63(3)	70(1)	63(1)	56(1)	56(2)	58(1)	54(1)	51(1)	49(1)
p value*	0.8	0.43	<0.01	0.02	0.78	0.03	<0.01	<0.01	<0.01	<0.01
p value #										<0.01
COP(mmHg)					Δ from baseline					
9hr	13.3(0.8)	-2(0.3)		-1.2(0.2)			-1.8(0.5)			-3.5(0.5)
9hr+P188	12.6(0.5)	0.4(1.8)		-0.5(0.8)			-1.4(1.4)			-1.5(0.6)
p value*	0.30	0.46		0.37			0.55			0.02
Hct(%)					Δ from baseline					
9hr	29.4(2.1)	-3.9(2.9)		-5.0(1.8)			-3.2(1.2)			-2.9(2.1)
9hr+P188	23.8(1.8)	7.1(2.3)		5.8(1.4)			7.7(1.7)			5.6(3.0)
p value*	0.1	0.05		0.03			0.03			0.1
Hb(g/dL)					Δ from baseline					
9hr	9.5(0.7)	-1.3(0.9)		-1.7(0.6)			-1.1(0.4)			-1.0(0.7)
9hr+P188	8.0(0.6)	2.0(0.8)		1.6(0.4)			2.2(0.5)			2.1(0.7)
p value*	0.18	0.05		0.03			0.03			0.05
Venous sat(%)					Δ from baseline					
9hr	92.5	-8.5(6.3)		-0.5(3.4)			-6.7(1.5)			-9.4(2.5)
9hr+P188	82.8	3.8(14.3)		13.3(7.3)			8.6(7.1)			9.6(7)
p value*	0.65	0.72		0.30			0.05			0.05
Lactate(mmol/L)					Δ from baseline					
9hr	1.23(0.26)	3.83(0.72)		0.83(0.28)			-0.10(0.2)			-0.23(0.2)
9hr+P188	2.14(0.42)	1.26(0.67)		-0.56(0.5)			-1.08(0.4)			-1.18(0.4)
p value*	0.10	0.04		0.07			0.17			0.09

Cardiopulmonary bypass (CPB), Central venous pressure (CVP), heart rate (HR), systolic blood pressure (SBP), mean blood pressure (MBP), diastolic blood pressure (DBP), colloid osmotic pressure (COP), haematocrit (Hct), haemoglobin (Hb).
* p value calculated by Mann Whitney U test, # p value calculated post CPB by linear mixed models using covariance type AR–1

7.3.2.1 Molecular results

Dystrophin measured in membrane fractions by Western blot was unchanged in the CPB–P188 group compared with non CPB controls, ($p=0.3$). Membrane dystrophin expression was higher in the CPB+P188 group (using antibodies to both C-terminus and rod domain) compared to CPB–P188 and non CPB control groups, ($p<0.05$, Figure 7.8A). Antibodies to the N-terminus of the human form of dystrophin did not cross-react with sheep dystrophin.

Dystrophin in membrane fractions by Western blot was also examined in our earlier work, from Chapter 6. No significant differences were seen with and without AXC or following various durations post CPB, (Figure 7.9).

Dysferlin expression was significantly reduced in membrane fractions following CPB in the CPB–P188 group compared with non CPB controls by Western blot, ($p<0.05$). In the CPB+P188 group there was maintenance of dysferlin expression at levels similar to those in the non CPB control animals, ($p=0.8$, Figure 7.8B).

Dysferlin expression in membrane fractions was also examined in our earlier work by Western blot. In these experiments we found a reduction in dysferlin with AXC and an increased reduction with a longer duration of reperfusion following CPB, (Figure 7.9).

AQP1 protein levels were increased nine hours following CPB in the CPB–P188 group compared with non CPB controls by Western blot, ($p<0.05$). The CPB+P188 animals displayed less of a rise in AQP1 protein expression compared with non CPB controls, ($p=0.28$, Figure 7.10).

Active caspase 3 protein was analysed by Western blot. There were no significant differences between groups and compared with baseline levels, (Figure 7.11).

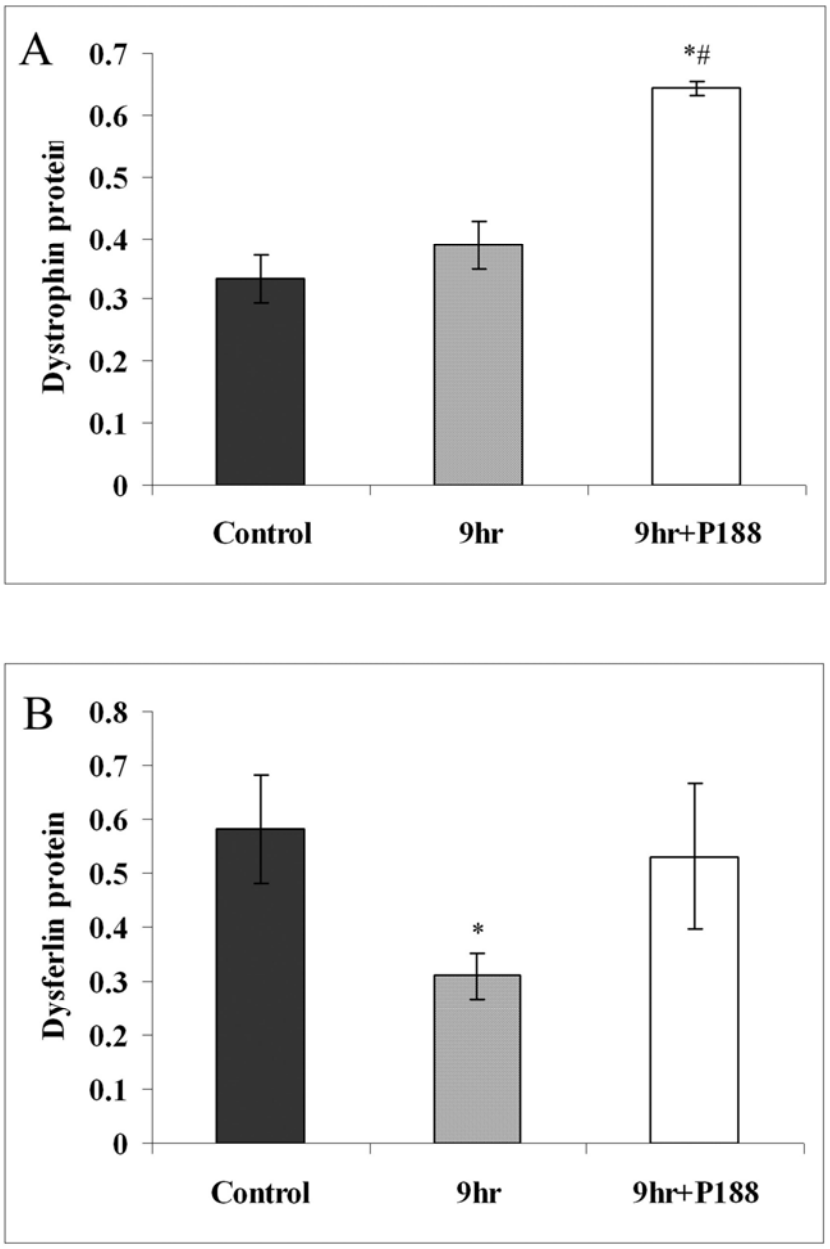

Figure 7.8. Myocardial membrane fraction densitometry. Membrane fractions were measured by densitometry of Western blots (controlled for protein loading by α actinin-2 measurement). Lambs were grouped as non CPB controls (dark column), CPB–P188 (grey column) and CPB+P188 (open column). Protein levels were compared between groups using the Mann-Whitney U test. A. The CPB+P188 group showed higher membrane dystrophin levels compared with non CPB controls, (*$p<0.05$) and CPB–P188 (#$p<0.05$). B. The CPB–P188 group showed a significant decrease in dysferlin expression compared to non CPB controls, (*$p<0.05$). Mean ± SEM is shown.

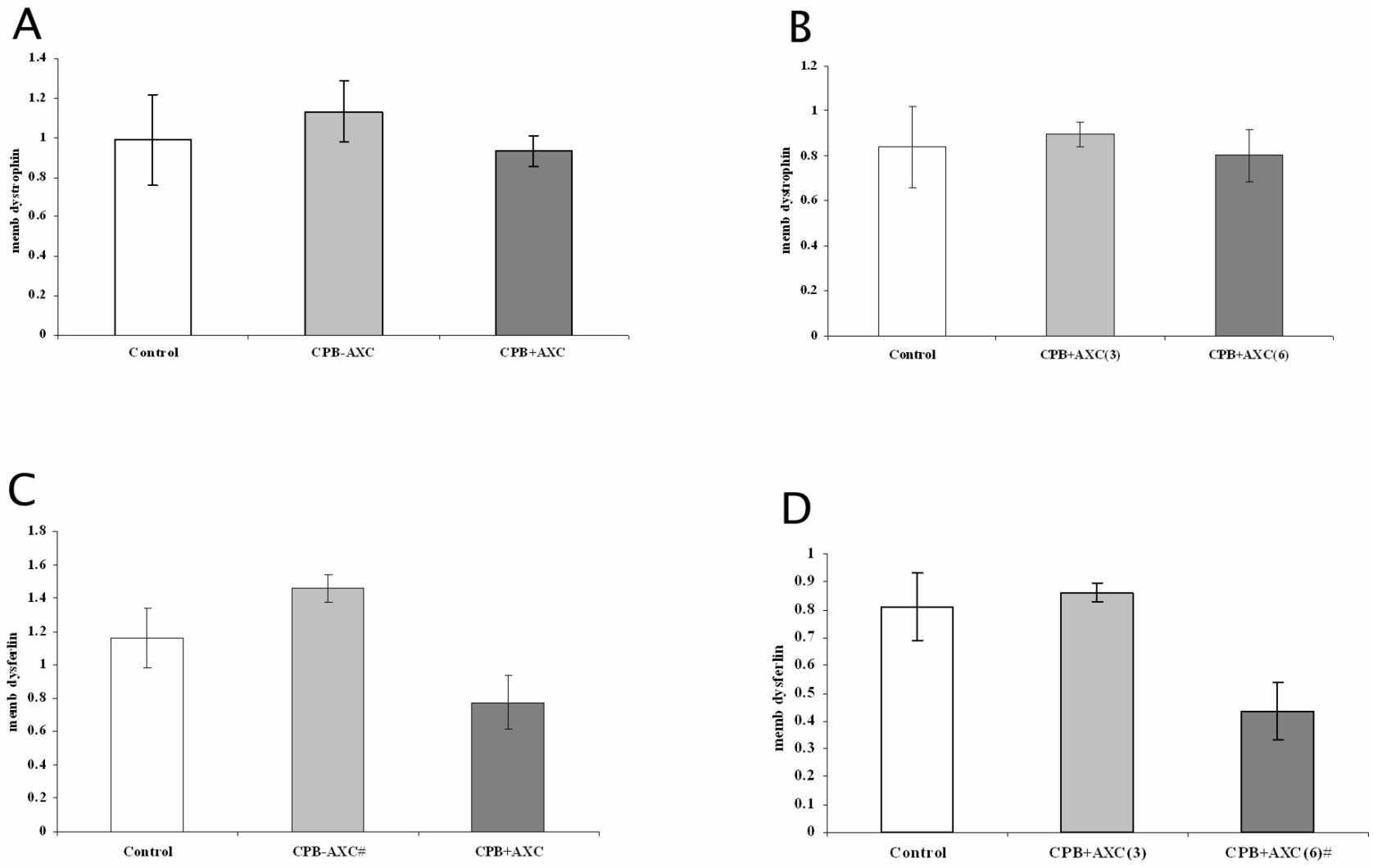

Figure 7.9. Myocardial membrane (memb) dystrophin (A,B) and dysferlin (C,D) expression. Groups of at least three animals, from previous work are(A,C) Control vs CPB-AXC vs CPB+AXC. And in (B,D) control vs CPB+AXC 3hrs vs CPB +AXC 6hrs. Dystrophin densitometry shows no difference between allocations. Dysferlin however was significantly higher without AXC vs with AXC (C) and was significantly lower after 6hrs following CPB+AXC versus 3hrs.(# $p < 0.05$). Mean ± SEM is shown.

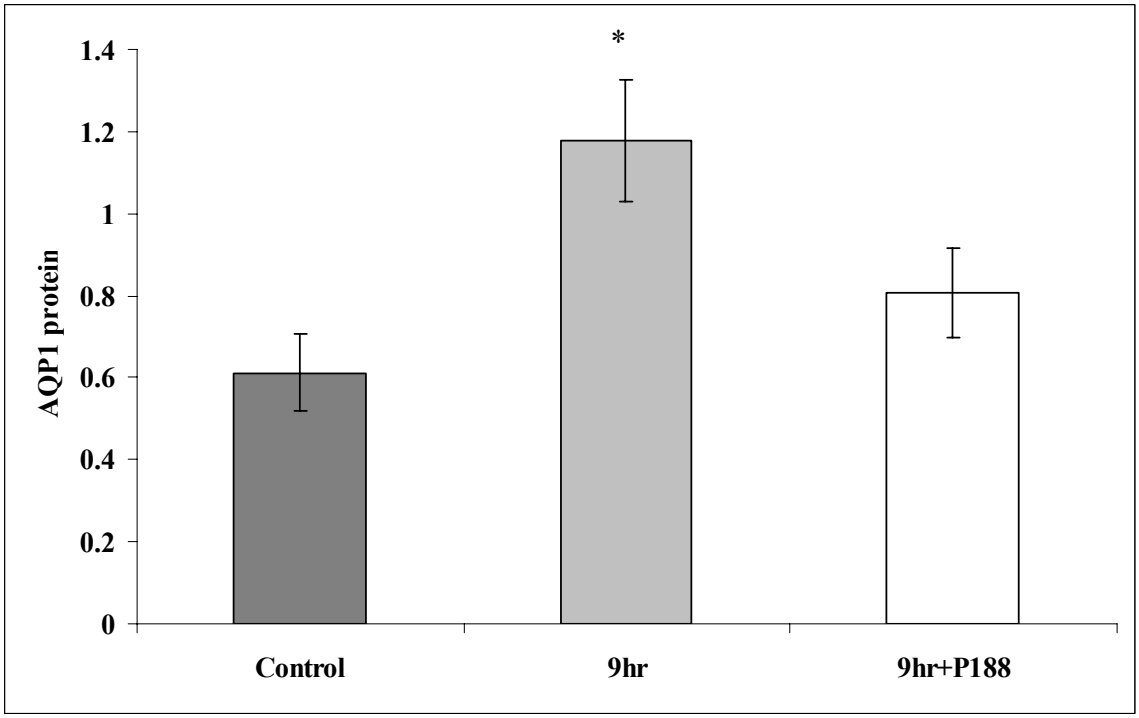

Figure 7.10. Myocardial aquaporin 1 protein expression. Western blot analysis of total AQP1 protein was normalised to cardiac actin. Lambs were grouped as non CPB controls (dark column), CPB–P188 (grey column) and CPB+P188 (open column). Protein levels were compared between groups using the Mann-Whitney U test. Representative results demonstrate a significant increase at 9hrs after CPB in the CPB–P188 group, compared with non CPB controls, (*$p<0.05$). Mean ± SEM is shown.

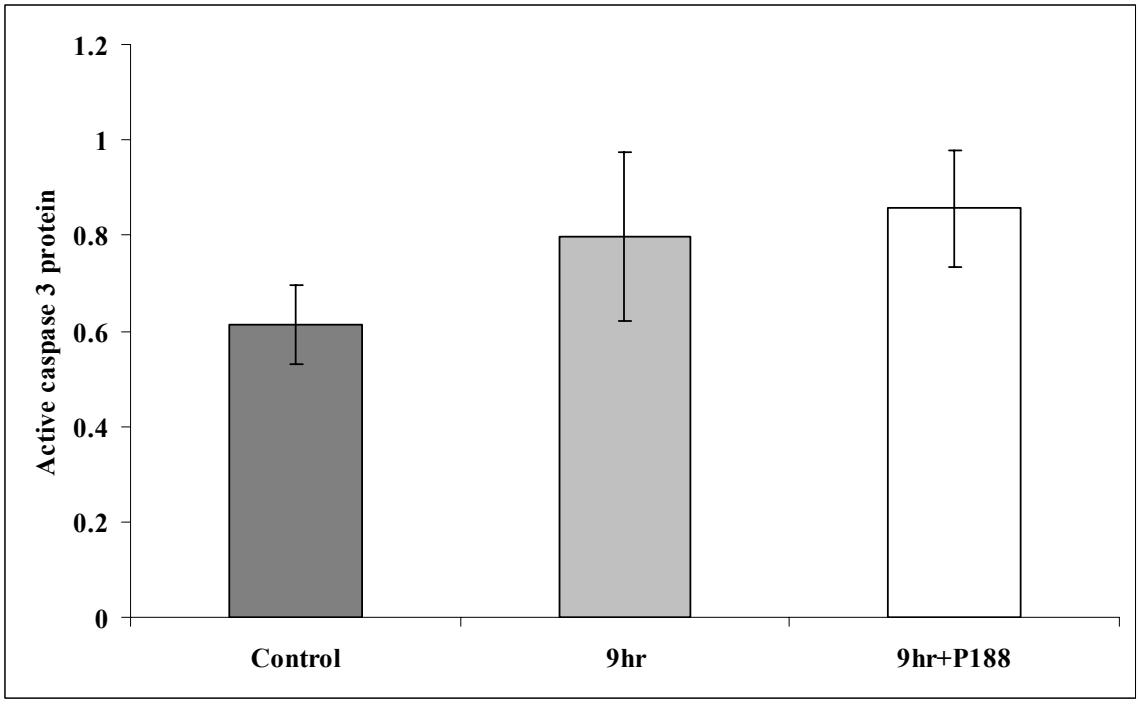

Figure 7.11. Active caspase 3 protein expression. Western blot analysis was normalised to cardiac actin. Lambs were grouped as non CPB controls (dark column), CPB–P188 (grey column) and CPB+P188 (open column). Protein levels were compared between groups, using the Mann-Whitney U test, there were no differences. Mean ± SEM is shown.

7.3.2.2 Myocardial oedema

The myocardium of CPB–P188 animals tended to be more oedematous 0.78 ± 0.005 compared to the hearts of the CPB+P188 group 0.77 ± 0.003 although non-significantly, ($p=0.08$). Evans Blue uptake was greater in the CPB–P188 group, (2.6±0.2) compared to the CPB+P188 group, (1.7±0.2, $p<0.05$). Albumin tended to fall by more in the CPB–P188 animals than the CPB+P188 group (3.33 ± 0.67g/L, vs 0.8 ± 1.7g/L), although non-significantly, ($p=0.17$). Colloid osmotic pressure (COP) was significantly reduced by 3.5 ± 0.46mmHg in the CPB–P188 animals at nine hours compared to 1.46 ± 0.58mmHg in the CPB+P188 animals, ($p<0.05$), (Figure 7.12A and Table 7.2). There were no significant differences in volumes of crystalloid or colloid fluid administered, (Table 7.1). There was a trend towards greater chest drain losses in the CPB–P188 group, ($p=0.13$, Table 7.1).

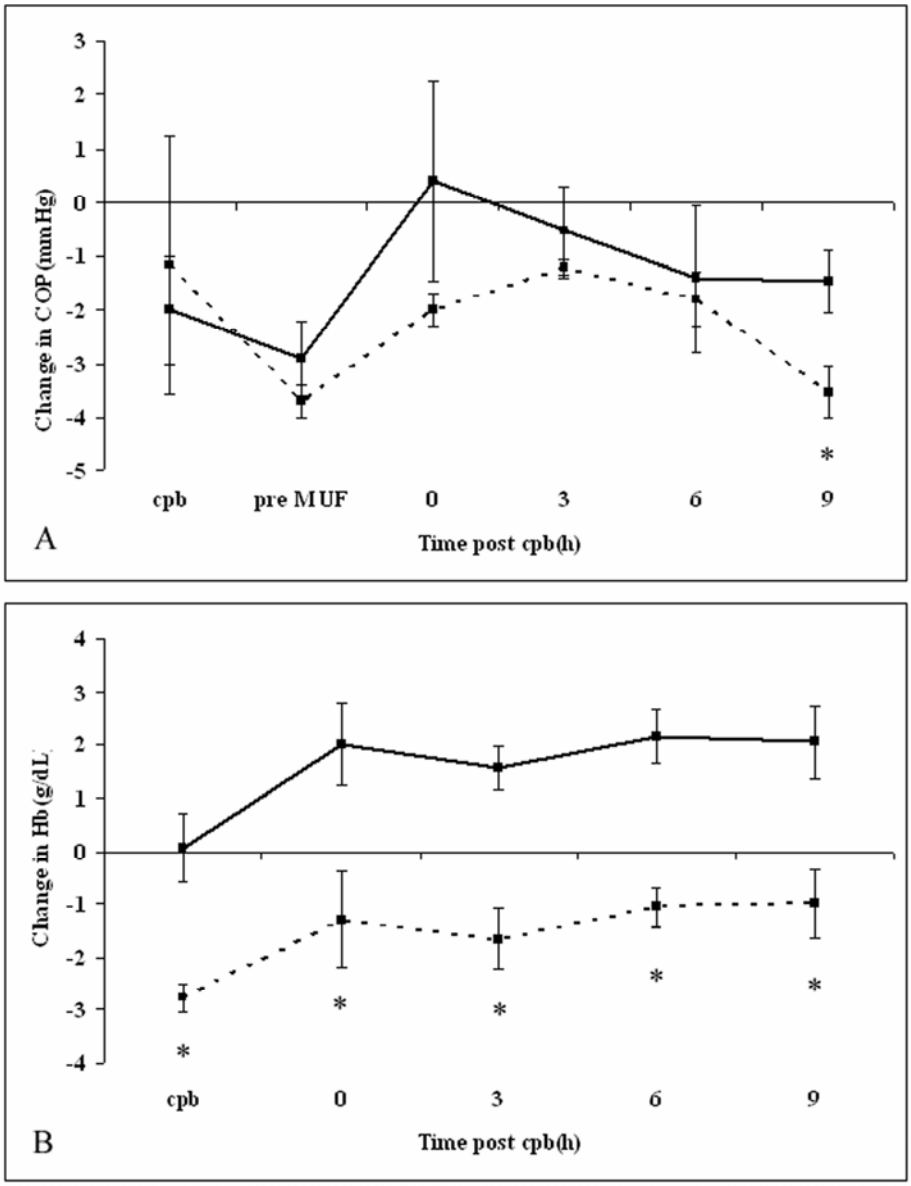

Figure 7.12. Measures of colloid osmotic pressure and haemoglobin. Lambs were grouped as CPB–P188 (dashed line) and CPB+P188 (solid line). Comparison between groups was by the Mann-Whitney U test. A. Reduction in Colloid osmotic pressure (COP) was greater at nine hours in the CPB–P188 group 3.5 ± 0.46mmHg compared to a drop of 1.46 ± 0.58mmHg in the CPB+P188 animals, (*$p<0.05$). B. Haemoglobin dropped in the CPB–P188 group following CPB compared with the CPB+P188 animals, (*$p<0.05$).

7.2.2.3 Biochemistry and haematology

Creatinine increased in the CPB–P188 group by 10.7 ± 4.4μmol/L, compared with a reduction in the CPB+P188 animals of 11 ± 6.9μmol/L, ($p<0.05$). Urea tended to increase not significantly in the CPB–P188 group 1.78 ± 0.49mmol/L, whilst in the CPB+P188 animals it rose by 0.72 ± 0.67mmol/L, ($p=0.18$). Total CK and troponin T did not differ significantly between groups, (Table 7.1). The CPB–P188 group initially had a higher lactate following reperfusion compared with the CPB+P188 animals, ($p<0.05$, Figure 7.13E and Table 7.2). Venous saturation was increased from baseline in those receiving P188 at six and nine hours post CPB, ($p=0.05$, Figure 7.13F and Table 7.2). P188 was associated with a maintenance of Hb and Hct compared to baseline, (Figure 7.12B and Table 7.2). There were no significant differences in other haematology and coagulation tests, (Table 7.1).

7.2.2.4 Haemodynamics

Heart rate and systolic blood pressure (SBP) tended to be lower in the CPB–P188 animals in the later epochs, ($p<0.01$ at 7–9 hrs post CPB), but overall were not different, ($p=0.15$ and $p=0.12$ respectively), (Figures 7.13A&B and Table 7.2). The CPB–P188 animals had significantly lower mean blood pressure (MBP) and diastolic blood pressure (DBP) throughout the nine hour period following CPB, ($p<0.01$), (Figures 7.13C&D and Table 7.2). Central venous pressure (CVP) was similar overall between groups, ($p=0.88$, Table 7.2).

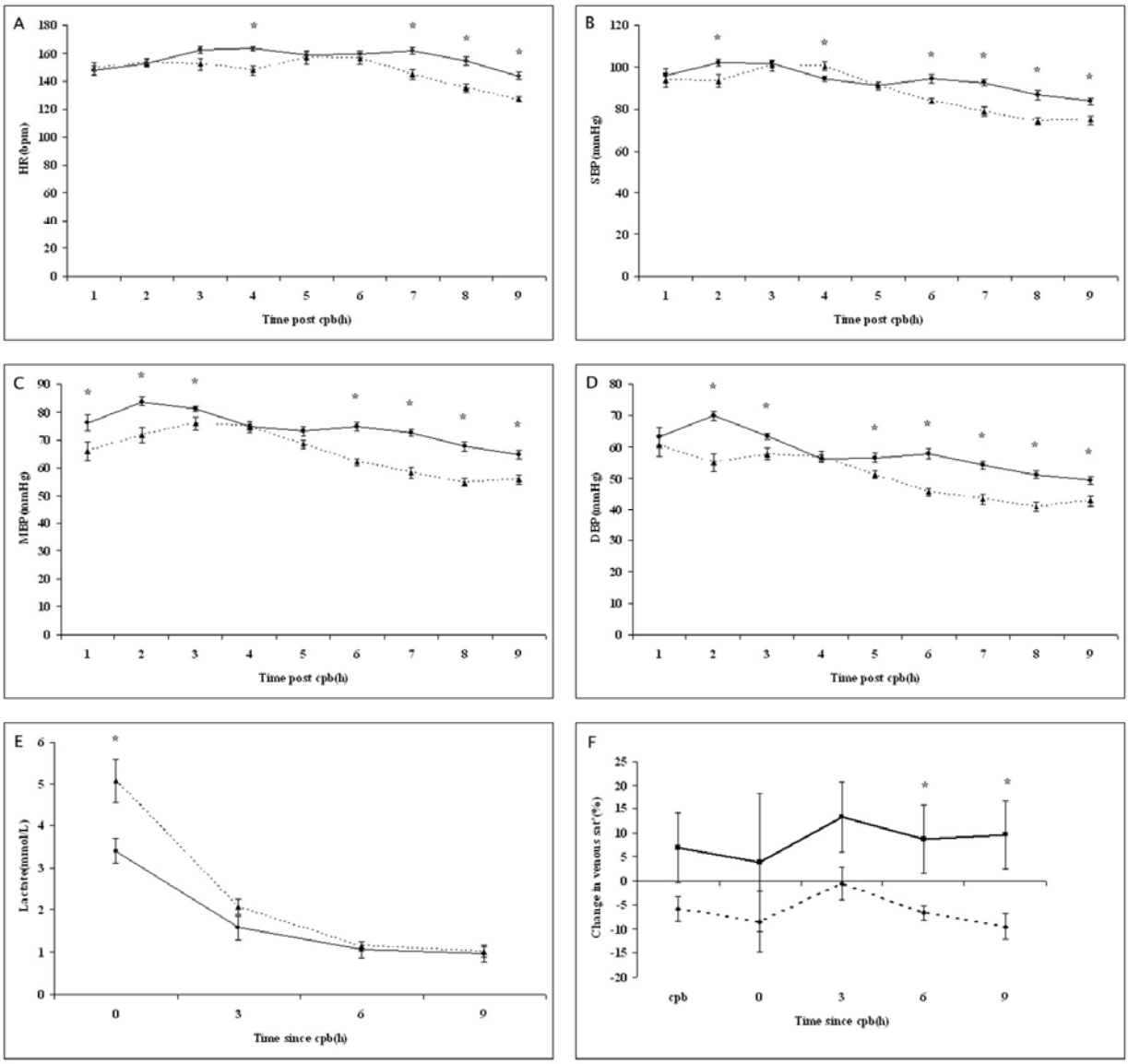

Figure 7.13. Haemodynamic and biochemical variables. Lambs were grouped as CPB–P188 (dashed line) and CPB+P188 (solid line). Comparison between groups was by both the Mann-Whitney U test and linear mixed models using covariance type AR–1 as appropriate. A. Heart rate (HR) was higher in the CPB+P188 group during the later epochs, but overall the difference was not significant. B. Systolic blood pressure (SBP) was significantly higher in the CPB+P188 group during the later epochs, but overall not different. C and D show mean blood pressure (MBP) and diastolic blood pressure (DBP) respectively. Both are significantly higher overall in the CPB+P188 group, (*p<0.01). E.

Demonstrates a lower lactate in the CPB+P188 group following reperfusion, (*$p<0.05$), but similar levels subsequently. F. The change in venous saturation after CPB compared with baseline, was significantly higher in the CPB+P188 group at six and nine hours post CPB, with a final increase of 10 ± 7% compared with a drop in the CPB–P188 group of 9 ± 3%, (*$p=0.05$). Mean ± SEM is shown.

7.4 Discussion

7.4.1 Isolated heart models

The ischaemic isolated heart model produced diastolic dysfunction which was not ameliorated by low dose P188. At this dose where P188 has been shown to fill endothelial defects (Maskarinec, 2002) there was no appreciable effect on function or myocardial water gain in the setting of ischaemia. However at this dose in non-ischaemic hearts P188 was associated with significantly drier hearts, ~ 4% drier. This is a substantial difference in myocardial oedema. Interestingly, this degree of oedema reduction had no functional impact and these hearts trended towards slightly worse diastolic dysfunction than the non P188 treated hearts. Thus a therapy associated with a 4% reduction in MWC had no measurable functional benefit in the isolated heart. This was an interesting and novel finding suggesting that P188 was able to block the hypo–oncotic induction of oedema that is an inherent anomaly of the crystalloid Langendorff perfusate (Apstein, 1977). A potential mechanism would be by blocking endothelial leakiness, perhaps by endothelial clefts or AQPs.

The subsequent trial of P188 at higher doses was improved by blinding, and demonstrated a trend towards improved diastolic function associated with P188 administration. The developed diastolic pressure was ~ 15–20mmHg less than placebo. The lower Dp/Dt_{min} in the P188 group was also supportive of improved diastolic function in this small trial. No changes in systolic function or MWC were seen. Given the lack of blood in the study it suggested that non-rheological mechanisms, such as membrane protective mechanisms may be at work. The absence of differences in MWC further suggested that the blocking of endothelial leakiness was not effective in the setting of ischaemia and that other mechanisms were present. Again the discrepancy between oedema and function was seen, no difference in oedema was found despite differential function, this time in the setting of ischaemia. This finding was supportive of subsequent work and of extrapolation to the large animal model to determine if there was any benefit in the setting of the combined insults of CPB and I/R.

7.4.2 Lamb CPB model

A clinically accurate animal model of reduced myocardial performance was studied with a blinded trial of P188. Previously undescribed changes in expression of proteins within the myocardial membrane were found. Hallmarks of reduced performance, such as lower blood pressure and lower oxygen delivery were lessened in association with the administration of P188. P188 treatment was also associated with potentially beneficial changes in membrane protein expression, reduced capillary leakage and reduced haemodilution. However this was a small study and consequently its findings can at best suggest avenues for further analysis. Our use of clinically relevant surgical, perfusion and anaesthetic techniques provided a clinically valid model of the milder spectrum of LCOS.

7.4.3 Dystrophin

Dystrophin is a candidate protein in the contractile failure which occurs in the setting of LCOS and has not been previously investigated in this context. Regional ischaemia by coronary occlusion in animals, has been shown to reduce both its membrane location and overall expression (Kido, 2004; Rodriguez, 2005). Ischaemic cardiomyopathy has also been associated with reduced dystrophin expression (Vatta, 2002). In several ways, regional ischaemia and end stage ventricular failure represent more severe forms of pathology than that experienced with present day infant heart surgery. In our clinical model of LCOS we found no change in dystrophin following CPB with I/R. This was supported by earlier work which showed no difference with respect to CPB±AXC or duration of reperfusion. Evidence from others suggests it is possible that changes occurred only at the N-terminus which we were not able to study with the antibodies available for sheep. However we consider this unlikely, given that we observed changes in dystrophin expression associated with P188 therapy that were identical with both the rod domain and C-terminus antibodies. Others have similarly shown ischaemia related changes in dystrophin that were not specific to the N terminus (Toyo-Oka, 2004). CPB with I/R represents a planned insult and current myocardial

protection techniques appear adequate in preventing an acute dystrophinopathy occurring in this setting.

7.4.4 Dysferlin

Dysferlin has been shown to have a specific and critical role in membrane repair in animal models of both skeletal and cardiac muscle (Bansal, 2003; Han, 2007), but its response to CPB and I/R has not previously been determined. In earlier work, membrane dysferlin was found to be reduced in association with ischaemia (AXC) and further reduced with longer reperfusion durations. Membrane dysferlin protein expression was again confirmed to be reduced following CPB and I/R in this current study. Thus we have shown reduced dysferlin expression in association with ischaemia and increased duration of reperfusion, but not with CPB alone. Dysferlin loss was also associated with evidence of increased membrane damage and a greater reduction in COP. Available studies suggest dysferlin-mediated membrane repair is indispensable for the maintenance of cellular integrity in models of myocellular stress (Han, 2007). Thus, the reduction in dysferlin associated with I/R may represent an important aspect of the pathophysiology contributing to LCOS.

Our findings are in keeping with the role of dysferlin in maintaining membrane integrity and reducing membrane leakiness in skeletal and cardiac muscle models (Bansal, 2003; Han and Campbell, 2007). In the endothelium of the brain, dysferlin has been shown to be present in areas of inflammation and to increase perivascular leakiness. Dysferlin expression was also increased by tumour necrosis factor in brain endothelial tissue (Hochmeister, 2006). Dysferlin has not been described in the blood vessels of muscle. However this is an intriguing interaction worthy of further exploration, as tumour necrosis factor is generally accepted as having a role in postoperative myocardial dysfunction (Wan, 1997).

7.4.5 Low cardiac output state

LCOS in this study was mild and characterised by lower blood pressure and impaired oxygen delivery. Lactate was raised following CPB whilst venous saturations and blood pressure were reduced after CPB in the control group. Post CPB venous saturations, blood pressure and lactate were improved in association with P188. Haemoglobin was preserved following CPB in association with P188 administration and this is likely to have partly contributed to improved oxygen delivery and haemodynamics in this group. Large studies have shown an association between reduced haemodilution and improved outcome. Interestingly Jonas *et al* in the Boston Haemodilution study also reported an initially raised lactate following CPB in the more haemodiluted group (Jonas, 2003). The control animals in our study had a drop in Hb on CPB, and following CPB were more haemodiluted than the CPB+P188 animals. These control animals also had an initially raised lactate following CPB. This pattern of haemodilution was also seen in our previous large animal experiments – outlined in Chapter 6. In the P188 group of animals however there was a stable haemoglobin on CPB and the lactate was not as elevated as in the control group following CPB. This rise in haemoglobin was maintained throughout the postoperative period. Given that there were no substantive differences in coagulation, P188 may be responsible for minimising red cell loss and minimising haemodilution through reducing membrane injury.

7.4.6 Dystrophin and P188

Animals in the control group had unaltered dystrophin, reduced dysferlin expression and increased membrane damage. P188 has been shown by others to rapidly correct abnormalities in dystrophin (Yasuda, 2005), although in the post cardiac surgery setting an acute dystrophinopathy was not apparent in our study. Interestingly, P188 therapy was associated with an increase in membrane dystrophin to supra-normal levels. Increased membrane dystrophin has been shown to occur in response to ischaemic preconditioning in an animal model, suggesting increased expression of dystrophin may have beneficial functional effects (Kido, 2004). The role of this increase in dystrophin

expression cannot at this stage be linked to the apparent improvements seen with this treatment.

7.4.7 Dysferlin and P188

It has been suggested that P188 would also be beneficial in the setting of abnormalities in dystrophin-related proteins such as dysferlin (Yasuda, 2005). P188 administration prior to CPB and I/R was associated with maintenance of normal dysferlin levels. Maintenance of dysferlin levels has been shown in other settings to be associated with less membrane injury (Han, 2007). There was evidence of less membrane injury in the animals receiving P188 and these animals also had higher dysferlin expression levels. Hence dysferlin warrants further study in this post CPB setting.

7.4.8 Other effects of P188

P188 may have other haemodynamic effects. The prophylactic use of P188 has shown benefit in improving blood viscosity (Hunter, 1990; Fatkin, 1997). P188 was also shown to have benefits on neurological outcome after deep hypothermic circulatory arrest in a large animal CPB model (Mezrow, 1992). Despite these benefits in planned ischaemia, it was not beneficial in a large clinical trial as adjunctive therapy following acute myocardial infarction (Yusuf, 1997). There were no beneficial effects on infarct size or haemodynamics and concerns arose regarding renal impairment from P188. It is possible that benefits associated with P188 were obscured by patient heterogeneity and the failure of P188 to treat, rather than prevent ischaemia related injury. In studies of membrane integrity following electrical injury, membrane integrity was enhanced only by prophylactic treatment with P188 (Lee, 1992). In our study the use of MUF with CPB may have removed excess P188 and perhaps minimised any renal injury. We did not demonstrate a rise in creatinine associated with P188, creatinine was in fact reduced in those animals, as it also was in an earlier animal myocardial infarct study (Justicz, 1991). We found a maintenance of haemoglobin levels and no coagulopathy associated with its use, therefore P188 may have reduced red cell fragility.

7.4.9 Aquaporins

The role of oedema and water transport proteins in the setting of LCOS has not been established. We have previously demonstrated that AQP1 mRNA increased two fold in the setting of LCOS following CPB with I/R, but did not observe an associated rise in AQP1 protein at three hours, Chapter 6. Following a longer period (nine hours) of observation both AQP1 mRNA and protein were increased two fold. Whilst a two fold increase was modest, the associated changes in capillary leak suggested that AQP1 may have a role in capillary leak. To determine whether this change in AQP1 expression was a pathological or beneficial compensatory response will require direct modulation of the channel. Increased AQP1 expression could be part of the explanation for the transudate which occurs into the tissues of the body following CPB and I/R (Farstad, 2004). It is likely that AQP1 has a role in permitting water to meet its osmotic obligations in this setting. Because AQP1 is so prevalent already in the endothelial membrane and because increased AQP1 transcript was primarily seen after I/R, perhaps the increased AQP1 expression is just at the myocellular membrane to permit water movement across this membrane, which is particularly vulnerable to ischaemia.

7.4.10 Limitations

This study was limited by its small size and that it was conducted in animals. A range of clinically relevant refinements such as inotropes, vasodilators, cardioplegia and MUF were used to improve the clinical validity of the model. These therapies are likely to have reduced the underlying severity of myocardial dysfunction and may have had other effects, but given their place in current clinical practice they were included. The haemodynamic and functional monitoring used to support our findings was simple, but equivalent and relevant to techniques relied upon in the clinical setting. All animals were managed in the post CPB period by one individual in the same way with a view to maintaining critical parameters within acceptable limits. This best reflects clinical practice but added a potentially confounding variable in assessing outcomes, blinding to therapy should effectively have minimised any impact from this variable.

The molecular changes which occurred in dysferlin and AQP proteins are novel and warrant further investigation. Load-independent measurements of systolic and diastolic function would be beneficial in future studies to completely define the functional effects related to P188. Although we have not sought to define the mechanistic action of P188 in this study, we have generated data to support further study of P188 in this setting. The demonstration of less oedema and better functional indices associated with P188 was in agreement with the initial isolated heart studies. Whether the insults of CPB and or I/R are principally protected against by P188, can only be speculated upon at this stage.

CHAPTER 8: CONCLUSIONS

8.1 Key findings and future work

Ischaemia was of primary importance in causing myocardial dysfunction in models analogous with the postoperative LCOS setting. CPB without ischaemia was well tolerated and not associated with myocardial dysfunction or oedema compatible with contributing to early postoperative dysfunction. Ischaemia of increasing duration initially resulted in greater degrees of diastolic dysfunction and eventually in associated systolic dysfunction. Ischaemic injury to the myocardium resulted in increased acidosis, reduced myocardial oxygen delivery and higher ischaemic markers. This injury was also associated with increased intracellular calcium, membrane injury and myocardial oedema.

In comparison, oedema as a result of CPB or non-ischaemic hypo–osmotic exposure was generally greater than that seen with ischaemia, but associated with mild and reversible dysfunction at most. There was no evidence of membrane injury, apoptosis or calcium excess in the non-ischaemic setting. Thus myocardial ischaemia was of significantly greater relative importance in its contribution to myocardial dysfunction, compared with myocardial oedema.

Ischaemia clearly needs to be the focus of myocardial protective strategies, rather than oedema or CPB which are not associated with significant dysfunction. Insulating the contractile apparatus from reperfusion injury with novel agents – such as P188 may have a role. Alternatively providing better myocardial protection during planned ischaemia by utilising new myocardial protection strategies – such as adenosine/lignocaine based cardioplegia (Dobson and Jones, 2004; Jin, 2008), may potentially improve the myocellular metabolic environment during I/R. Associated oedema in response to osmotic obligated movement via AQPs is apparent, but does not contribute to important dysfunction.

AQP1 is the only water channel responsible for water movement within mammalian hearts. It permits rapid water flux across endothelial membranes and slightly slower transit across the myocellular membrane. It is not important in mitochondrial membranes and expression does not rapidly alter in response to ischaemia or other severe osmotic insults. AQPs permit osmotically obligated water transit and over lengthy durations will increase expression, but probably only to the extent of doubling their protein expression. With ischaemia associated increases in AQP expression, it is likely that most of the increased expression is at the myocellular membrane, given the abundant endothelial expression that already exists. In this setting, AQPs are passive and subject to the gradients generated by ischaemia, haemodilution and albumin leakage. There is no apparent role for their modulation in the postoperative setting. In the preoperative setting however, some infants become particularly oedematous whilst receiving prostaglandin infusions to maintain ductal patency. The diuretic acetazolamide is known to inhibit AQPs (Tanimura, 2008) and this could potentially minimise preoperative oedema which tends to contribute further to postoperative fluid accumulation in the soft tissues. Examination of the $AQP1^{-/-}$ knockout mouse cardiac phenotype is nearing completion and it will be interesting to see if there is an improved phenotype in the setting of ischaemia and reperfusion.

The membrane expression of dystrophin was not altered in the setting of myocardial dysfunction following CPB and varying durations of ischaemia and reperfusion. The insults involved in CPB and aortic cross clamping are obviously not severe enough and or the current methods of supporting the myocardium are adequate in preventing an acute dystrophinopathy. Dystrophin is not a key target protein in the setting of LCOS.

Whilst dystrophin was not overtly affected, membrane expression of dysferlin was reduced in response to increasing durations of ischaemia. Its reduction was associated with increased tissue damage, increased oedema and reduced contractility. Given the association of tumour necrosis factor and endothelial expressed dysferlin in the blood brain barrier (Hochmeister, 2006), it would be intriguing to examine any such interaction in the heart, given the role of tumour necrosis factor in ischaemia and CPB (Wan, 1997).

Interestingly P188 appeared to interact with the endothelium and was associated with normalisation of dysferlin following CPB with ischaemia.

Poloxamer 188 was associated with reductions in the severe diastolic dysfunction seen with ischaemia. It was associated with less membrane injury, tissue oedema and reduced haemodilution. In association with improved haemodynamics there was no reduction in renal function. Previous animal work in the setting of myocardial infarction had also been favourable, only for large human trials to prove otherwise. What remains to be seen is whether pre-emptively administering P188 to a larger animal CPB series justifies further trials of P188 in humans. Given the previous failures in treating myocardial infarction, there will need to be a strong positive animal experience prior to proceeding. Mechanistically P188 certainly appears to have membrane insulating attributes in an asanguinous model as well as likely effects on viscosity that additionally make it an attractive therapy.

In conclusion, the experiments outlined in this thesis have contributed to our understanding of how ischaemia and oedema impact on postoperative myocardial dysfunction. They have also clarified the role of water channels, dysferlin and dystrophin. P188 has potential as a membrane protecting therapy which may offer dysferlin and other membrane proteins protection from ischaemia in particular. Hopefully an increased understanding of the pathophysiological processes involved in LCOS will led to reductions in both its occurrence and severity.

REFERENCES

Adams-Graves P, Kedar A, Koshy M, Steinberg M, Veith R, Ward D, Crawford R, Edwards S, Bustrack J, Emanuele M. 1997. RheothRx (poloxamer 188) injection for the acute painful episode of sickle cell disease: a pilot study. Blood 90(5):2041-6.

Addetia AM, Lichtenstein SV, Ko AS, Sikkonen M, Sainani M, Salerno TA. 1986. Study on myocardial contractility after cardiopulmonary bypass versus cardioplegic arrest in an air-ejecting in vivo heart model. Ann Thorac Surg 41(3):260-4.

Agre P. 2004. Aquaporin water channels (Nobel Lecture). Angew Chem Int Ed Engl 43(33):4278-90.

Akao M, O'Rourke B, Teshima Y, Seharaseyon J, Marban E. 2003. Mechanistically distinct steps in the mitochondrial death pathway triggered by oxidative stress in cardiac myocytes. Circ Res 92(2):186-94.

Albers J, Schroeder A, de Simone R, Mockel R, Vahl CF, Hagl S. 2001. 3D evaluation of myocardial edema: experimental study on 22 pigs using magnetic resonance and tissue analysis. Thoracic and Cardiovascular Surgery 49(4):199-203.

Aliev MK, Dos Santos P, Hoerter JA, Soboll S, Tikhonov AN, Saks VA. 2002. Water content and its intracellular distribution in intact and saline perfused rat hearts revisited. Cardiovasc Res 53(1):48-58.

Allen SJ, Geissler HJ, Davis KL, Gogola GR, Warters RD, de Vivie ER, Mehlhorn U. 1997. Augmenting cardiac contractility hastens myocardial edema resolution after cardiopulmonary bypass and cardioplegic arrest. Anestheisa & Analgesia 85(5):987-92.

Amiry-Moghaddam M, Ottersen OP. 2003. The molecular basis of water transport in the brain. Nature Reviews Neuroscience 4(12):991-1001.

Apstein CS, Mueller M, Hood WB, Jr. 1977. Ventricular contracture and compliance changes with global ischemia and reperfusion, and their effect on coronary resistance in the rat. Circ Res 41(2):206-17.

Askenasy N. 2001. Is cytotoxic cellular edema real? The effect of calcium ion on water homeostasis in the rat heart. Cardiovasc Toxicol 1(1):21-34.

Askenasy N, Navon G. 1997. Continuous monitoring of intracellular volumes in isolated rat hearts during normothermic perfusion and ischemia. J Magn Reson 124(1):42-50.

Au CG, Cooper ST, Lo HP, Compton AG, Yang N, Wintour EM, North KN, Winlaw DS. 2004. Expression of aquaporin 1 in human cardiac and skeletal muscle. J Mol Cell Cardiol 36(5):655-62.

Bansal D, Miyake K, Vogel SS, Groh S, Chen CC, Williamson R, McNeil PL, Campbell KP. 2003. Defective membrane repair in dysferlin-deficient muscular dystrophy. Nature 423(6936):168-72.

Beavis AD, Brannan RD, Garlid KD. 1985. Swelling and contraction of the mitochondrial matrix. I. A structural interpretation of the relationship between light scattering and matrix volume. J Biol Chem 260(25):13424-33.

Becker LB. 2004. New concepts in reactive oxygen species and cardiovascular reperfusion physiology. Cardiovasc Res 61(3):461-70.

Bellinger DC, Jonas RA, Rappaport LA, Wypij D, Wernovsky G, Kuban KC, Barnes PD, Holmes GL, Hickey PR, Strand RD, et al. 1995. Developmental and neurologic status of children after heart surgery with hypothermic circulatory arrest or low-flow cardiopulmonary bypass. N Engl J Med 332(9):549-55.

Bellinger DC, Wypij D, duDuplessis AJ, Rappaport LA, Jonas RA, Wernovsky G, Newburger JW. 2003. Neurodevelopmental status at eight years in children with dextro-transposition of the great arteries: the Boston Circulatory Arrest Trial. Journal of Thoracic & Cardiovascular Surgery 126(5):1385-96.

Benga G. 2003. Birth of water channel proteins-the aquaporins. Cell Biol Int 27(9):701-9.

Birkenkamp-Demtroeder K, Bongartz S, Kupfer C, Schmitt M, Gams E. 2003. Expression of water channels in the human heart. J Clin Basic Cardiol 6:77-9.

Blatchford JW, 3rd, Barragry TP, Lillehei TJ, Ring WS. 1994. Effects of cardioplegic arrest on left ventricular systolic and diastolic function of the intact neonatal heart. Journal of Thoracic and Cardiovascular Surgery 107(2):527-35.

Bolli R, Marban E. 1999. Molecular and cellular mechanisms of myocardial stunning. Physiol Rev 79(2):609-34.

Bondy C, Chin E, Smith BL, Preston GM, Agre P. 1993. Developmental gene expression and tissue distribution of the CHIP28 water-channel protein. Proc Natl Acad Sci U S A 90(10):4500-4.

Booker PD. 1998. Myocardial stunning in the neonate. Br J Anaesth 80(3):371-83.

Bosetti F, Baracca A, Lenaz G, Solaini G. 2004. Increased state 4 mitochondrial respiration and swelling in early post-ischemic reperfusion of rat heart. FEBS Letters 563(1-3):161-4.

Bragadeesh T, Jayaweera AR, Pascotto M, Micari A, Le DE, Kramer CM, Epstein FH, Kaul S. 2008. Post-ischaemic myocardial dysfunction (stunning) results from myofibrillar oedema. Heart 94(2):166-71.

Braunwald E, Kloner RA. 1982. The stunned myocardium: prolonged, postischemic ventricular dysfunction. Circulation 66(6):1146-9.

Breisblatt WM, Stein KL, Wolfe CJ, Follansbee WP, Capozzi J, Armitage JM, Hardesty RL. 1990. Acute myocardial dysfunction and recovery: a common occurrence after coronary bypass surgery. J Am Coll Cardiol 15(6):1261-9.

Brendt P, Behrends M, Peters J. 2008. Myocardial stunning following no flow ischaemia is diminished by levosimendan or cariporide, without benefits of combined administration. Resuscitation 76(1):95-102.

Brixius K, Hoyer HK, Schwinger RH. 2005. Ca2+-sensitisers--a promising option to treat heart failure? Cardiovasc Drugs Ther 19(6):423-8.

Bulkley GB. 1994. Reactive oxygen metabolites and reperfusion injury: aberrant triggering of reticuloendothelial function. Lancet 344(8927):934-6.

Burrows FA, Williams WG, Teoh KH, Wood AE, Burns J, Edmonds J, Barker GA, Trusler GA, Weisel RD. 1988. Myocardial performance after repair of congenital cardiac defects in infants and children. Response to volume loading. J Thorac Cardiovasc Surg 96(4):548-56.

Calamita G, Ferri D, Gena P, Liquori GE, Cavalier A, Thomas D, Svelto M. 2005. The inner mitochondrial membrane has aquaporin-8 water channels and is highly permeable to water. J Biol Chem 280(17):17149-53.

Caldarone CA, Barner EW, Wang L, Karimi M, Mascio CE, Hammel JM, Segar JL, Du C, Scholz TD. 2004. Apoptosis-related mitochondrial dysfunction in the early postoperative neonatal lamb heart. Annals of Thoracic Surgery 78(3):948-55.

Carlson RE, Aisen AM, Buda AJ. 1992. Effect of reduction in myocardial edema on myocardial blood flow and ventricular function after coronary reperfusion. American Journal of Physiology 262(3 Pt 2):H641-H8.

Castle NA. 2005. Aquaporins as targets for drug discovery. Drug Discov Today 10(7):485-93.

Chaturvedi RR, Shore DF, White PA, Scallan MH, Gothard JW, Redington AN, Lincoln C. 1999. Modified ultrafiltration improves global left ventricular systolic function after open-heart surgery in infants and children. Eur J Cardiothorac Surg 15(6):742-6.

Colbassani HJ, Barrow DL, Sweeney KM, Bakay RA, Check IJ, Hunter RL. 1989. Modification of acute focal ischemia in rabbits by poloxamer 188. Stroke 20(9):1241-6.

Connors NC, Kofuji P. 2006. Potassium channel Kir4.1 macromolecular complex in retinal glial cells. Glia 53(2):124-31.

Cooper GJ, Zhou Y, Bouyer P, Grichtchenko II, Boron WF. 2002. Transport of volatile solutes through AQP1. J Physiol 542(Pt 1):17-29.

Cross CE, Rieben PA, Salisbury PF. 1961. Influence of coronary perfusion and myocardial edema on pressure-volume diagram of left ventricle. American Journal of Physiology 201:102-8.

Davies MJ, Nguyen K, Gaynor JW, Elliott MJ. 1998. Modified ultrafiltration improves left ventricular systolic function in infants after cardiopulmonary bypass. Journal of Thoracic and Cardiovascular Surgery 115(2):361-70.

DeFoe GR, Ross CS, Olmstead EM, Surgenor SD, Fillinger MP, Groom RC, Forest RJ, Pieroni JW, Warren CS, Bogosian ME, Krumholz CF, Clark C, Clough RA, Weldner PW, Lahey SJ, Leavitt BJ, Marrin CA, Charlesworth DC, Marshall P, O'Connor GT. 2001. Lowest hematocrit on bypass and adverse outcomes associated with coronary artery bypass grafting. Northern New England Cardiovascular Disease Study Group. Ann Thorac Surg 71(3):769-76.

Dobson GP. 2004. Organ arrest, protection and preservation: natural hibernation to cardiac surgery. Comp Biochem Physiol B Biochem Mol Biol 139(3):469-85.

Dobson GP, Jones MW. 2004. Adenosine and lidocaine: a new concept in nondepolarizing surgical myocardial arrest, protection, and preservation. J Thorac Cardiovasc Surg 127(3):794-805.

Dos Santos P, Kowaltowski AJ, Laclau MN, Seetharaman S, Paucek P, Boudina S, Thambo JB, Tariosse L, Garlid KD. 2002. Mechanisms by which opening the mitochondrial ATP- sensitive K(+) channel protects the ischemic heart. American Journal of Physiology Heart and Circulation Physiology 283(1):H284-H95.

Dudek FE, Rogawski MA. 2005. Regulation of brain water: is there a role for aquaporins in epilepsy? Epilepsy Curr 5(3):104-6.

Echevarria M, Windhager EE, Tate SS, Frindt G. 1994. Cloning and expression of AQP3, a water channel from the medullary collecting duct of rat kidney. Proc Natl Acad Sci U S A 91(23):10997-1001.

Edmunds LH, Jr. 1998. Inflammatory response to cardiopulmonary bypass. Annals of Thoracic Surgery 66(5 Suppl):S12-6; discussion S25-8.

Egan JR, Clarke AJ, Williams S, Cole AD, Ayer J, Jacobe S, Chard RB, Winlaw DS. 2006. Levosimendan for low cardiac output: a pediatric experience. J Intensive Care Med 21(3):183-7.

Fang X, Yang B, Matthay MA, Verkman AS. 2002. Evidence against aquaporin-1-dependent CO2 permeability in lung and kidney. J Physiol 542(Pt 1):63-9.

Farstad M, Heltne JK, Rynning SE, Onarheim H, Mongstad A, Eliassen F, Husby P. 2004. Can the use of methylprednisolone, vitamin C, or alpha-trinositol prevent cold-induced fluid extravasation during cardiopulmonary bypass in piglets? Journal of Thoracic and Cardiovascular Surgery 127(2):525-34.

Fatkin D, Loupas T, Low J, Feneley M. 1997. Inhibition of red cell aggregation prevents spontaneous echocardiographic contrast formation in human blood. Circulation 96(3):889-96.

Ferri D, Mazzone A, Liquori GE, Cassano G, Svelto M, Calamita G. 2003. Ontogeny, distribution, and possible functional implications of an unusual aquaporin, AQP8, in mouse liver. Hepatology 38(4):947-57.

Fischer UM, Cox CS, Jr., Stewart RH, Laine GA, Allen SJ. 2006. Impact of acute myocardial edema on left ventricular function. J Invest Surg 19(1):31-8.

Foglia RP, Lazar HL, Steed DL, Follette DM, Manganaro AJ, Deland E, Buckberg GD. 1978. Iatrogenic myocardial edema with crystalloid primes: effects on left ventricular compliance, performance, and perfusion. Surgical Forum 29:312-5.

Fontan F, Baudet E. 1971. Surgical repair of tricuspid atresia. Thorax 26(3):240-8.

Freedom RM, Lock J, Bricker JT. 2000. Pediatric cardiology and cardiovascular surgery: 1950-2000. Circulation 102(20 Suppl 4):IV58-68.

Frigeri A, Nicchia GP, Balena R, Nico B, Svelto M. 2004. Aquaporins in skeletal muscle: reassessment of the functional role of aquaporin-4. FASEB Journal 18(7):905-7.

Fujita Y, Yamamoto N, Sobue K, Inagaki M, Ito H, Arima H, Morishima T, Takeuchi A, Tsuda T, Katsuya H, Asai K. 2003. Effect of mild hypothermia on the expression of aquaporin family in cultured rat astrocytes under hypoxic condition. Neurosci Res 47(4):437-44.

Funaki H, Yamamoto T, Koyama Y, Kondo D, Yaoita E, Kawasaki K, Kobayashi H, Sawaguchi S, Abe H, Kihara I. 1998. Localization and expression of AQP5 in cornea, serous salivary glands, and pulmonary epithelial cells. Am J Physiol 275(4 Pt 1):C1151-7.

Garcia-Dorado D, Oliveras J. 1993. Myocardial oedema: a preventable cause of reperfusion injury? Cardiovascular Research 27(9):1555-63.

Geissler HJ, Allen SJ. 1998. Myocardial fluid balance: pathophysiology and clinical implications. Thoracic and Cardiovascular Surgery 46 Suppl 2:242-5; discussion 6-7.

Gibbs WJ, Hagemann TM. 2004. Purified poloxamer 188 for sickle cell vaso-occlusive crisis. Ann Pharmacother 38(2):320-4.

Glover L, Brown RH, Jr. 2007. Dysferlin in membrane trafficking and patch repair. Traffic 8(7):785-94.

Goto R, Tearle H, Steward DJ, Ashmore PG. 1991. Myocardial oedema and ventricular function after cardioplegia with added mannitol. Can J Anaesth 38(1):7-14.

Guadagno E, Moukhles H. 2004. Laminin-induced aggregation of the inwardly rectifying potassium channel, Kir4.1, and the water-permeable channel, AQP4, via a dystroglycan-containing complex in astrocytes. Glia 47(2):138-49.

Hamada Y, Kawachi K, Tsunooka N, Nakamura Y, Takano S, Imagawa H. 2004. Capillary leakage in cardiac surgery with cardiopulmonary bypass. Asian Cardiovascular and Thoracic Annals 12(3):193-7.

Han R, Bansal D, Miyake K, Muniz VP, Weiss RM, McNeil PL, Campbell KP. 2007. Dysferlin-mediated membrane repair protects the heart from stress-induced left ventricular injury. J Clin Invest 117(7):1805-13.

Han R, Campbell KP. 2007. Dysferlin and muscle membrane repair. Curr Opin Cell Biol 19(4):409-16.

Han Z, Wax MB, Patil RV. 1998. Regulation of aquaporin-4 water channels by phorbol ester-dependent protein phosphorylation. Journal of Biological Chemistry 273(11):6001-4.

Hasegawa H, Lian SC, Finkbeiner WE, Verkman AS. 1994. Extrarenal tissue distribution of CHIP28 water channels by in situ hybridization and antibody staining. Am J Physiol 266(4 Pt 1):C893-903.

Hatakeyama S, Yoshida Y, Tani T, Koyama Y, Nihei K, Ohshiro K, Kamiie JI, Yaoita E, Suda T, Hatakeyama K, Yamamoto T. 2001. Cloning of a new aquaporin (AQP10) abundantly expressed in duodenum and jejunum. Biochem Biophys Res Commun 287(4):814-9.

Herrera M, Hong NJ, Garvin JL. 2006. Aquaporin-1 Transports NO Across Cell Membranes. Hypertension.

Hibuse T, Maeda N, Funahashi T, Yamamoto K, Nagasawa A, Mizunoya W, Kishida K, Inoue K, Kuriyama H, Nakamura T, Fushiki T, Kihara S, Shimomura I. 2005. Aquaporin 7 deficiency is associated with development of obesity through activation of adipose glycerol kinase. Proc Natl Acad Sci U S A 102(31):10993-8.

Hochmeister S, Grundtner R, Bauer J, Engelhardt B, Lyck R, Gordon G, Korosec T, Kutzelnigg A, Berger JJ, Bradl M, Bittner RE, Lassmann H. 2006. Dysferlin is a new marker for leaky brain blood vessels in multiple sclerosis. J Neuropathol Exp Neurol 65(9):855-65.

Hoffman EP, Brown RH, Jr., Kunkel LM. 1987. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell 51(6):919-28.

Hoffman TM, Wernovsky G, Atz AM, Kulik TJ, Nelson DP, Chang AC, Bailey JM, Akbary A, Kocsis JF, Kaczmarek R, Spray TL, Wessel DL. 2003. Efficacy and safety of milrinone in preventing low cardiac output syndrome in infants and children after corrective surgery for congenital heart disease. Circulation 107(7):996-1002.

Hoffmann EK, Dunham PB. 1995. Membrane mechanisms and intracellular signalling in cell volume regulation. Int Rev Cytol 161:173-262.

Hunter RL, Papadea C, Gallagher CJ, Finlayson DC, Check IJ. 1990. Increased whole blood viscosity during coronary artery bypass surgery. Studies to evaluate the effects of soluble fibrin and poloxamer 188. Thromb Haemost 63(1):6-12.

Ishibashi K, Kuwahara M, Gu Y, Kageyama Y, Tohsaka A, Suzuki F, Marumo F, Sasaki S. 1997a. Cloning and functional expression of a new water channel abundantly expressed in the testis permeable to water, glycerol, and urea. J Biol Chem 272(33):20782-6.

Ishibashi K, Kuwahara M, Gu Y, Tanaka Y, Marumo F, Sasaki S. 1998a. Cloning and functional expression of a new aquaporin (AQP9) abundantly expressed in the peripheral leukocytes permeable to water and urea, but not to glycerol. Biochem Biophys Res Commun 244(1):268-74.

Ishibashi K, Kuwahara M, Kageyama Y, Tohsaka A, Marumo F, Sasaki S. 1997b. Cloning and functional expression of a second new aquaporin abundantly expressed in testis. Biochem Biophys Res Commun 237(3):714-8.

Ishibashi K, Morinaga T, Kuwahara M, Sasaki S, Imai M. 2002. Cloning and identification of a new member of water channel (AQP10) as an aquaglyceroporin. Biochim Biophys Acta 1576(3):335-40.

Ishibashi K, Sasaki S, Fushimi K, Uchida S, Kuwahara M, Saito H, Furukawa T, Nakajima K, Yamaguchi Y, Gojobori T, et al. 1994. Molecular cloning and expression of a member of the aquaporin family with permeability to glycerol and urea in addition to water expressed at the basolateral membrane of kidney collecting duct cells. Proc Natl Acad Sci U S A 91(14):6269-73.

Ishibashi K, Sasaki S, Saito F, Ikeuchi T, Marumo F. 1995. Structure and chromosomal localization of a human water channel (AQP3) gene. Genomics 27(2):352-4.

Ishibashi K, Yamauchi K, Kageyama Y, Saito-Ohara F, Ikeuchi T, Marumo F, Sasaki S. 1998b. Molecular characterization of human Aquaporin-7 gene and its chromosomal mapping. Biochim Biophys Acta 1399(1):62-6.

Itoh T, Rai T, Kuwahara M, Ko SB, Uchida S, Sasaki S, Ishibashi K. 2005. Identification of a novel aquaporin, AQP12, expressed in pancreatic acinar cells. Biochem Biophys Res Commun 330(3):832-8.

Iyer RS, Jacobs JP, de Leval MR, Stark J, Elliott MJ. 1997. Outcomes after delayed sternal closure in pediatric heart operations: a 10-year experience. Ann Thorac Surg 63(2):489-91.

Jenner TL, Mellick AS, Harrison GJ, Griffiths LR, Rose'Meyer RB. 2004. Age-related changes in cardiac adenosine receptor expression. Mech Ageing Dev 125(3):211-7.

Jia CX, Rabkin DG, Hart JP, Dean DA, Cabreriza SA, Weinberg AD, Spotnitz HM. 2002. Regional variation in myocardial water content in the edematous pig heart. Journal of Surgical Research 106(1):70-5.

Jin ZX, Zhang SL, Wang XM, Bi SH, Xin M, Zhou JJ, Cui Q, Duan WX, Wang HB, Yi DH. 2008. The myocardial protective effects of a moderate-potassium adenosine-lidocaine cardioplegia in pediatric cardiac surgery. J Thorac Cardiovasc Surg 136(6):1450-5.

Jonas RA, Wypij D, Roth SJ, Bellinger DC, Visconti KJ, du Plessis AJ, Goodkin H, Laussen PC, Farrell DM, Bartlett J, McGrath E, Rappaport LJ, Bacha EA, Forbess JM, del Nido PJ, Mayer JE, Jr., Newburger JW. 2003. The influence of hemodilution on outcome after hypothermic cardiopulmonary bypass: results of a randomized trial in infants. J Thorac Cardiovasc Surg 126(6):1765-74.

Jonker SS, Davis LE, van der Bilt JDW, Hadder B, Hohimer AR, Giraud GD, Thornburg KL. 2003. Anaemia stimulates aquaporin 1 expression in the fetal sheep heart. Exp Physiol 88(6):691-8.

Justicz AG, Farnsworth WV, Soberman MS, Tuvlin MB, Bonner GD, Hunter RL, Martino-Saltzman D, Sink JD, Austin GE. 1991. Reduction of myocardial infarct size by poloxamer 188 and mannitol in a canine model. Am Heart J 122(3 Pt 1):671-80.

Kellen MR, Bassingthwaighte JB. 2003a. An integrative model of coupled water and solute exchange in the heart. Am J Physiol Heart Circ Physiol 285(3):H1303-16.

Kellen MR, Bassingthwaighte JB. 2003b. Transient transcapillary exchange of water driven by osmotic forces in the heart. Am J Physiol Heart Circ Physiol 285(3):H1317-31.

Khan TA, Bianchi C, Araujo E, Voisine P, Xu SH, Feng J, Li J, Sellke FW. 2005. Aprotinin preserves cellular junctions and reduces myocardial edema after regional ischemia and cardioplegic arrest. Circulation 112(9 Suppl):I196-201.

Kido M, Otani H, Kyoi S, Sumida T, Fujiwara H, Okada T, Imamura H. 2004. Ischemic preconditioning-mediated restoration of membrane dystrophin during reperfusion correlates with protection against contraction-induced myocardial injury. Am J Physiol Heart Circ Physiol 287(1):H81-90.

King LS, Kozono D, Agre P. 2004. From structure to disease: the evolving tale of aquaporin biology. Nat Rev Mol Cell Biol 5(9):687-98.

King LS, Nielsen S, Agre P, Brown RH. 2002. Decreased pulmonary vascular permeability in aquaporin-1-null humans. Proc Natl Acad Sci U S A 99(2):1059-63.

Kloner RA, Jennings RB. 2001. Consequences of brief ischemia: stunning, preconditioning, and their clinical implications: part 1. Circulation 104(24):2981-9.

Knize DM, Weatherley-White RC, Paton BC. 1969. Use of antisludging agents in experimental cold injuries. Surg Gynecol Obstet 129(5):1019-26.

Kostelka M. 2005. Invited commentary. Ann Thorac Surg 80(2):684-5.

Koyama N, Ishibashi K, Kuwahara M, Inase N, Ichioka M, Sasaki S, Marumo F. 1998. Cloning and functional expression of human aquaporin8 cDNA and analysis of its gene. Genomics 54(1):169-72.

Koyama Y, Yamamoto T, Kondo D, Funaki H, Yaoita E, Kawasaki K, Sato N, Hatakeyama K, Kihara I. 1997. Molecular cloning of a new aquaporin from rat pancreas and liver. J Biol Chem 272(48):30329-33.

Krane CM, Towne JE, Menon AG. 1999. Cloning and characterization of murine Aqp5: evidence for a conserved aquaporin gene cluster. Mamm Genome 10(5):498-505.

Kuriyama H, Kawamoto S, Ishida N, Ohno I, Mita S, Matsuzawa Y, Matsubara K, Okubo K. 1997. Molecular cloning and expression of a novel human aquaporin from adipose tissue with glycerol permeability. Biochem Biophys Res Commun 241(1):53-8.

Kuru S, Yasuma F, Wakayama T, Kimura S, Konagaya M, Aoki M, Tanabe M, Takahashi T. 2004. [A patient with limb girdle muscular dystrophy type 2B (LGMD2B) manifesting cardiomyopathy]. Rinsho Shinkeigaku 44(6):375-8.

Kusuoka H, Hoffman JI. 2002. Advice on statistical analysis for Circulation Research. Circ Res 91(8):662-71.

Kyoi S, Otani H, Hamano A, Matsuhisa S, Akita Y, Fujiwara H, Hattori R, Imamura H, Kamihata H, Iwasaka T. 2006. Dystrophin is a possible end-target of ischemic preconditioning against cardiomyocyte oncosis during the early phase of reperfusion. Cardiovasc Res.

Laine GA, Allen SJ. 1991. Left ventricular myocardial edema. Lymph flow, interstitial fibrosis, and cardiac function. Circ Res 68(6):1713-21.

Lane TA, Lamkin GE. 1984. Paralysis of phagocyte migration due to an artificial blood substitute. Blood 64(2):400-5.

Lane TA, Lamkin GE. 1986. Increased infection mortality and decreased neutrophil migration due to a component of an artificial blood substitute. Blood 68(2):351-4.

Lee RC, Hannig J, Matthews KL, Myerov A, Chen CT. 1999. Pharmaceutical therapies for sealing of permeabilized cell membranes in electrical injuries. Ann N Y Acad Sci 888:266-73.

Lee RC, River LP, Pan FS, Ji L, Wollmann RL. 1992. Surfactant-induced sealing of electropermeabilized skeletal muscle membranes in vivo. Proc Natl Acad Sci U S A 89(10):4524-8.

Lee WK, Bork U, Gholamrezaei F, Thevenod F. 2005. Cd2+-induced cytochrome c release in apoptotic proximal tubule cells: role of mitochondrial permeability transition pore and Ca2+ uniporter. American Journal of Physiology Renal Physiology 288(1):F27-F39.

Lee YT, Sung K, Shin JO, Jeon ES. 2006. Images in cardiovascular medicine. Disruption of dystrophin in acute fulminant coxsackieviral B4 infection. Circulation 113(6):e76-7.

Liu J, Aoki M, Illa I, Wu C, Fardeau M, Angelini C, Serrano C, Urtizberea JA, Hentati F, Hamida MB, Bohlega S, Culper EJ, Amato AA, Bossie K, Oeltjen J, Bejaoui K, McKenna-Yasek D, Hosler BA, Schurr E, Arahata K, de Jong PJ, Brown RH, Jr. 1998. Dysferlin, a novel skeletal muscle gene, is mutated in Miyoshi myopathy and limb girdle muscular dystrophy. Nat Genet 20(1):31-6.

Lu J, Zang WJ, Yu XJ, Chen LN, Zhang CH, Jia B. 2005. Effects of ischaemia-mimetic factors on isolated rat ventricular myocytes. Exp Physiol 90(4):497-505.

Ma T, Yang B, Gillespie A, Carlson EJ, Epstein CJ, Verkman AS. 1997a. Generation and phenotype of a transgenic knockout mouse lacking the mercurial-insensitive water channel aquaporin-4. J Clin Invest 100(5):957-62.

Ma T, Yang B, Kuo WL, Verkman AS. 1996a. cDNA cloning and gene structure of a novel water channel expressed exclusively in human kidney: evidence for a gene cluster of aquaporins at chromosome locus 12q13. Genomics 35(3):543-50.

Ma T, Yang B, Verkman AS. 1996b. Gene structure, cDNA cloning, and expression of a mouse mercurial-insensitive water channel. Genomics 33(3):382-8.

Ma T, Yang B, Verkman AS. 1997b. Cloning of a novel water and urea-permeable aquaporin from mouse expressed strongly in colon, placenta, liver, and heart. Biochem Biophys Res Commun 240(2):324-8.

Maehara T, Novak I, Wyse RK, Elliot MJ. 1991. Perioperative monitoring of total body water by bio-electrical impedance in children undergoing open heart surgery. Eur J Cardiothorac Surg 5(5):258-64; discussion 65.

Mangano DT. 1985. Biventricular function after myocardial revascularization in humans: deterioration and recovery patterns during the first 24 hours. Anesthesiology 62(5):571-7.

Mangano DT. 1993. Myocardial stunning: an overview. J Card Surg 8(2 Suppl):204-13.

Manley GT, Binder DK, Papadopoulos MC, Verkman AS. 2004. New insights into water transport and edema in the central nervous system from phenotype analysis of aquaporin-4 null mice. Neuroscience 129(4):983-91.

Manley GT, Fujimura M, Ma T, Noshita N, Filiz F, Bollen AW, Chan P, Verkman AS. 2000. Aquaporin-4 deletion in mice reduces brain edema after acute water intoxication and ischemic stroke. Nature Medicine 6(2):159-63.

Maskarinec SA, Hannig J, Lee RC, Lee KY. 2002. Direct observation of poloxamer 188 insertion into lipid monolayers. Biophys J 82(3):1453-9.

Mavroudis C, Ebert PA. 1978. Hemodilution causes decreased compliance in puppies. Circulation 58(3 Pt 2):I155-9.

McNally EM, MacLeod H. 2005. Therapy insight: cardiovascular complications associated with muscular dystrophies. Nat Clin Pract Cardiovasc Med 2(6):301-8.

Mehlhorn U, Allen SJ, Adams DL, Davis KL, Gogola GR, de Vivie ER, Laine GA. 1995a. Normothermic continuous antegrade blood cardioplegia does not prevent myocardial edema and cardiac dysfunction. Circulation 92(7):1940-6.

Mehlhorn U, Davis KL, Burke EJ, Adams D, Laine GA, Allen SJ. 1995b. Impact of cardiopulmonary bypass and cardioplegic arrest on myocardial lymphatic function. Am J Physiol 268(1 Pt 2):H178-83.

Mehlhorn U, Geissler HJ, Laine GA, Allen SJ. 2001. Myocardial fluid balance. Eur J Cardiothorac Surg 20(6):1220-30.

Menninger FJ, 3rd, Rosenkranz ER, Utley JR, Dembitsky WP, Hargens AR, Peters RM. 1980. Interstitial hydrostatic pressures in patients undergoing CABG and valve replacement. J Thorac Cardiovasc Surg 79(2):181-7.

Mezrow CK, Mazzoni M, Wolfe D, Shiang HH, Litwak RS, Griepp RB. 1992. Poloxamer 188 improves neurologic outcome after hypothermic circulatory arrest. J Thorac Cardiovasc Surg 103(6):1143-6.

Miller RT. 2004. Aquaporin in the heart--only for water? J Mol Cell Cardiol 36(5):653-4.

Mizutani S, Prasad SM, Sellitto AD, Schuessler RB, Damiano RJ, Jr., Lawton JS. 2005. Myocyte volume and function in response to osmotic stress: observations in the presence of an adenosine triphosphate-sensitive potassium channel opener. Circulation 112(9 Suppl):I219-23.

Mobasheri A, Wray S, Marples D. 2005. Distribution of AQP2 and AQP3 water channels in human tissue microarrays. J Mol Histol 36(1-2):1-14.

Morinaga T, Nakakoshi M, Hirao A, Imai M, Ishibashi K. 2002. Mouse aquaporin 10 gene (AQP10) is a pseudogene. Biochem Biophys Res Commun 294(3):630-4.

Morishita Y, Matsuzaki T, Hara-chikuma M, Andoo A, Shimono M, Matsuki A, Kobayashi K, Ikeda M, Yamamoto T, Verkman A, Kusano E, Ookawara S, Takata K, Sasaki S, Ishibashi K. 2005. Disruption of aquaporin-11 produces polycystic kidneys following vacuolization of the proximal tubule. Mol Cell Biol 25(17):7770-9.

Muntoni F. 2003. Cardiomyopathy in muscular dystrophies. Current Opinion in Neurology 16(5):577-83.

Murry CE, Jennings RB, Reimer KA. 1986. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 74(5):1124-36.
Nagelhus EA, Mathiisen TM, Ottersen OP. 2004. Aquaporin-4 in the central nervous system: cellular and subcellular distribution and coexpression with KIR4.1. Neuroscience 129(4):905-13.

Nakhoul NL, Davis BA, Romero MF, Boron WF. 1998. Effect of expressing the water channel aquaporin-1 on the CO2 permeability of Xenopus oocytes. Am J Physiol 274(2 Pt 1):C543-8.

Namachivayam P, Crossland DS, Butt WW, Shekerdemian LS. 2006. Early experience with Levosimendan in children with ventricular dysfunction. Pediatr Crit Care Med 7(5):445-8.

Nejsum LN, Elkjaer M, Hager H, Frokiaer J, Kwon TH, Nielsen S. 2000. Localization of aquaporin-7 in rat and mouse kidney using RT-PCR, immunoblotting, and immunocytochemistry. Biochem Biophys Res Commun 277(1):164-70.

Newburger JW, Jonas RA, Wernovsky G, Wypij D, Hickey PR, Kuban KC, Farrell DM, Holmes GL, Helmers SL, Constantinou J, et al. 1993. A comparison of the perioperative neurologic effects of hypothermic circulatory arrest versus low-flow cardiopulmonary bypass in infant heart surgery. N Engl J Med 329(15):1057-64.

Ng R, Metzger JM, Claflin DR, Faulkner JA. 2008. Poloxamer 188 reduces the contraction-induced force decline in lumbrical muscles from mdx mice. Am J Physiol Cell Physiol 295(1):C146-50.

NHMRC. 2004. Australian code of practice for the care and use of animals for scientific purposes. 7th ed

Nielsen S, Smith BL, Christensen EI, Agre P. 1993. Distribution of the aquaporin CHIP in secretory and resorptive epithelia and capillary endothelia. Proc Natl Acad Sci U S A 90(15):7275-9.

O'Connor GT, Birkmeyer JD, Dacey LJ, Quinton HB, Marrin CA, Birkmeyer NJ, Morton JR, Leavitt BJ, Maloney CT, Hernandez F, Clough RA, Nugent WC, Olmstead EM, Charlesworth DC, Plume SK. 1998. Results of a regional study of modes of death associated with coronary artery bypass grafting. Northern New England Cardiovascular Disease Study Group. Ann Thorac Surg 66(4):1323-8.

O'Rourke B. 2004. Evidence for mitochondrial K+ channels and their role in cardioprotection. Circulation Research 94(4):420-32.

Odim JN, Tchervenkov CI, Dobell AR. 1989. Delayed sternal closure: a lifesaving maneuver after early operation for complex congenital heart disease in the neonate. J Thorac Cardiovasc Surg 98(3):413-6.

Ogura T, Imanishi S, Shibamoto T. 2002a. Osmometric and water-transporting properties of guinea pig cardiac myocytes. Jpn J Physiol 52(4):333-42.

Ogura T, Matsuda H, Imanishi S, Shibamoto T. 2002b. Sarcolemmal hydraulic conductivity of guinea-pig and rat ventricular myocytes. Cardiovasc Res 54(3):590-600.

Osthaus WA, Boethig D, Winterhalter M, Huber D, Goerler H, Sasse M, Sumpelmann R. 2008. First experiences with intraoperative Levosimendan in pediatric cardiac surgery. Eur J Pediatr.

Otani H, Prasad MR, Jones RM, Das DK. 1989. Mechanism of membrane phospholipid degradation in ischemic-reperfused rat hearts. Am J Physiol 257(1 Pt 2):H252-8.

Page E, Winterfield J, Goings G, Bastawrous A, Upshaw-Earley J, Doyle D. 1998. Water channel proteins in rat cardiac myocyte caveolae: osmolarity-dependent reversible internalization. Am J Physiol 274(6 Pt 2):H1988-H2000.

Palmer BS, Hadziahmetovic M, Veci T, Angelos MG. 2004. Global ischemic duration and reperfusion function in the isolated perfused rat heart. Resuscitation 62(1):97-106.

Papadopoulos MC, Manley GT, Krishna S, Verkman AS. 2004. Aquaporin-4 facilitates reabsorption of excess fluid in vasogenic brain edema. Faseb J 18(11):1291-3.

Papadopoulos MC, Verkman AS. 2005. Aquaporin-4 gene disruption in mice reduces brain swelling and mortality in pneumococcal meningitis. J Biol Chem 280(14):13906-12.

Parr GV, Blackstone EH, Kirklin JW. 1975. Cardiac performance and mortality early after intracardiac surgery in infants and young children. Circulation 51(5):867-74.

Peart JN, Headrick JP. 2008. Sustained cardioprotection: Exploring unconventional modalities. Vascul Pharmacol 49(2-3):63-70.

Pogatsa G, Dubecz E, Gabor G. 1976. The role of myocardial edema in the left ventricular diastolic stiffness. Basic Research in Cardiology 71(3):263-9.

Polimeni PI, Al-Sadir J. 1975. Expansion of extracellular space in the nonischemic zone of the infarcted heart and concomitant changes in tissue electrolyte contents in the rat. Circ Res 37(6):725-32.

Preston GM, Agre P. 1991. Isolation of the cDNA for erythrocyte integral membrane protein of 28 kilodaltons: member of an ancient channel family. Proceedings of the National Academy of Sciences U S A 88(24):11110-4.

Preston GM, Carroll TP, Guggino WB, Agre P. 1992. Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. Science 256(5055):385-7.

Quinlan JG, Wong BL, Niemeier RT, McCullough AS, Levin L, Emanuele M. 2006. Poloxamer 188 failed to prevent exercise-induced membrane breakdown in mdx skeletal muscle fibers. Neuromuscul Disord 16(12):855-64.

Rodriguez M, Cai WJ, Kostin S, Lucchesi BR, Schaper J. 2005. Ischemia depletes dystrophin and inhibits protein synthesis in the canine heart: mechanisms of myocardial ischemic injury. J Mol Cell Cardiol 38(5):723-33.

Rodriguez M, Schaper J. 2005. Apoptosis: measurement and technical issues. J Mol Cell Cardiol 38(1):15-20.

Rosenkranz ER, Utley JR, Menninger FJ, 3rd, Dembitsky WP, Hargens AR, Peters RM. 1980. Interstitial fluid pressure changes during cardiopulmonary bypass. Ann Thorac Surg 30(6):536-42.

Royster RL. 1993. Myocardial dysfunction following cardiopulmonary bypass: recovery patterns, predictors of inotropic need, theoretical concepts of inotropic administration. J Cardiothorac Vasc Anesth 7(4 Suppl 2):19-25.

Rubboli A, Sobotka PA, Euler DE. 1994. Effect of acute edema on left ventricular function and coronary vascular resistance in the isolated rat heart. Am J Physiol 267(3 Pt 2):H1054-61.

Schaer GL, Hursey TL, Abrahams SL, Buddemeier K, Ennis B, Rodriguez ER, Hubbell JP, Moy J, Parrillo JE. 1994. Reduction in reperfusion-induced myocardial necrosis in dogs by RheothRx injection (poloxamer 188 N.F.), a hemorheological agent that alters neutrophil function. Circulation 90(6):2964-75.

Schaer GL, Spaccavento LJ, Browne KF, Krueger KA, Krichbaum D, Phelan JM, Fletcher WO, Grines CL, Edwards S, Jolly MK, Gibbons RJ. 1996. Beneficial effects of RheothRx injection in patients receiving thrombolytic therapy for acute myocardial infarction. Results of a randomized, double-blind, placebo-controlled trial. Circulation 94(3):298-307.

Seghaye MC. 2003. The clinical implications of the systemic inflammatory reaction related to cardiac operations in children. Cardiol Young 13(3):228-39.

Seghaye MC, Grabitz RG, Duchateau J, Busse S, Dabritz S, Koch D, Alzen G, Hornchen H, Messmer BJ, Von Bernuth G. 1996. Inflammatory reaction and capillary leak syndrome related to cardiopulmonary bypass in neonates undergoing cardiac operations. Journal of Thoracic and Cardiovascular Surgery 112(3):687-97.

Skowronski MT, Lebeck J, Rojek A, Praetorius J, Fuchtbauer EM, Frokiaer J, Nielsen S. 2007. AQP7 is localized in capillaries of adipose tissue, cardiac and striated muscle: implications in glycerol metabolism. Am J Physiol Renal Physiol 292(3):F956-65.

Skrzypiec-Spring M, Grotthus B, Szelag A, Schulz R. 2007. Isolated heart perfusion according to Langendorff---still viable in the new millennium. J Pharmacol Toxicol Methods 55(2):113-26.

Song Y, Ma T, Matthay MA, Verkman AS. 2000. Role of aquaporin-4 in airspace-to-capillary water permeability in intact mouse lung measured by a novel gravimetric method. J Gen Physiol 115(1):17-27.

Spotnitz HM. 1995. Effects of edema on systolic and diastolic function in vivo. Journal of Cardiothoracic Surgery 10(4 Suppl):454-9.

Spotnitz HM, Hsu DT. 1994. Myocardial edema: importance in the study of left ventricular function. Advances in Cardiac Surgery 5:1-25.

Stocker CF, Shekerdemian LS, Norgaard MA, Brizard CP, Mynard JP, Horton SB, Penny DJ. 2007. Mechanisms of a reduced cardiac output and the effects of milrinone and levosimendan in a model of infant cardiopulmonary bypass. Crit Care Med 35(1):252-9.

Suleymanian MA, Baumgarten CM. 1996. Osmotic gradient-induced water permeation across the sarcolemma of rabbit ventricular myocytes. J Gen Physiol 107(4):503-14.

Tabbutt S, Duncan BW, McLaughlin D, Wessel DL, Jonas RA, Laussen PC. 1997a. Delayed sternal closure after cardiac operations in a pediatric population. Journal of Thoracic and Cardiovascular Surgery 113(5):886-93.

Tabbutt S, Nelson DP, Tsai N, Miura T, Hickey PR, Mayer JE, Neufeld EJ. 1997b. Induction of aquaporin-1 mRNA following cardiopulmonary bypass and reperfusion. Mol Med 3(9):600-9.

Tanimura Y, Hiroaki Y, Fujiyoshi Y. 2008. Acetazolamide reversibly inhibits water conduction by aquaporin-4. J Struct Biol.

Tassani P, Barankay A, Haas F, Paek SU, Heilmaier M, Hess J, Lange R, Richter JA. 2002a. Cardiac surgery with deep hypothermic circulatory arrest produces less systemic inflammatory response than low-flow cardiopulmonary bypass in newborns. J Thorac Cardiovasc Surg 123(4):648-54.

Tassani P, Kunkel R, Richter JA, Oechsler H, Lorenz HP, Braun SL, Eising GP, Haas F, Paek SU, Bauernschmitt R, Jochum M, Lange R. 2001. Effect of C1-esterase-inhibitor on capillary leak and inflammatory response syndrome during arterial switch operations in neonates. J Cardiothorac Vasc Anesth 15(4):469-73.

Tassani P, Schad H, Schreiber C, Zaccaria F, Haas F, Mossinger H, Altmeyer S, Kohler R, Seghaye MC, Lange R. 2007. Extravasation of albumin after cardiopulmonary bypass in newborns. J Cardiothorac Vasc Anesth 21(2):174-8.

Tassani P, Schad H, Winkler C, Bernhard A, Ettner U, Braun SL, Eising GP, Kochs E, Lange R, Richter JA. 2002b. Capillary leak syndrome after cardiopulmonary bypass in elective, uncomplicated coronary artery bypass grafting operations: does it exist? J Thorac Cardiovasc Surg 123(4):735-41.

Tenckhoff S, Hollborn M, Kohen L, Wolf S, Wiedemann P, Bringmann A. 2005. Diversity of aquaporin mRNA expressed by rat and human retinas. Neuroreport 16(1):53-6.

Toyo-Oka T, Kawada T, Nakata J, Xie H, Urabe M, Masui F, Ebisawa T, Tezuka A, Iwasawa K, Nakajima T, Uehara Y, Kumagai H, Kostin S, Schaper J, Nakazawa M, Ozawa K. 2004. Translocation and cleavage of myocardial dystrophin as a common pathway to advanced heart failure: a scheme for the progression of cardiac dysfunction. Proc Natl Acad Sci U S A 101(19):7381-5.

Umenishi F, Verkman AS, Gropper MA. 1996. Quantitative analysis of aquaporin mRNA expression in rat tissues by RNase protection assay. DNA Cell Biol 15(6):475-80.

Utley JR, Michalsky GB, Bryant LR, Mobin-Uddin K, McKean HE. 1974. Determinants of myocardial water content during cardiopulmonary bypass. J Thorac Cardiovasc Surg 68(1):8-16.

Vandenberg JI, Rees SA, Wright AR, Powell T. 1996. Cell swelling and ion transport pathways in cardiac myocytes. Cardiovasc Res 32(1):85-97.

Vatta M, Stetson SJ, Jimenez S, Entman ML, Noon GP, Bowles NE, Towbin JA, Torre-Amione G. 2004. Molecular normalization of dystrophin in the failing left and right ventricle of patients treated with either pulsatile or continuous flow-type ventricular assist devices. J Am Coll Cardiol 43(5):811-7.

Vatta M, Stetson SJ, Perez-Verdia A, Entman ML, Noon GP, Torre-Amione G, Bowles NE, Towbin JA. 2002. Molecular remodelling of dystrophin in patients with end-stage cardiomyopathies and reversal in patients on assistance-device therapy. Lancet 359(9310):936-41.

Verkman AS. 2000. Physiological importance of aquaporins: lessons from knockout mice. Current Opinion in Nephrology and Hypertension 9(5):517-22.

Verkman AS. 2002a. Aquaporin water channels and endothelial cell function. Journal of Anatomy 200(6):617-27.

Verkman AS. 2002b. Does aquaporin-1 pass gas? An opposing view. Journal of Physiology 542(Pt 1):31.

Verkman AS. 2005. More than just water channels: unexpected cellular roles of aquaporins. J Cell Sci 118(Pt 15):3225-32.

Verkman AS, Weyer P, Brown D, Ausiello DA. 1989. Functional water channels are present in clathrin-coated vesicles from bovine kidney but not from brain. J Biol Chem 264(34):20608-13.

Wagner DR, Combes A, McTiernan C, Sanders VJ, Lemster B, Feldman AM. 1998. Adenosine inhibits lipopolysaccharide-induced cardiac expression of tumor necrosis factor-alpha. Circ Res 82(1):47-56.

Wan S, LeClerc JL, Vincent JL. 1997. Inflammatory response to cardiopulmonary bypass: mechanisms involved and possible therapeutic strategies. Chest 112(3):676-92.

Warth A, Eckle T, Kohler D, Faigle M, Zug S, Klingel K, Eltzschig HK, Wolburg H. 2007. Upregulation of the water channel aquaporin-4 as a potential cause of postischemic cell swelling in a murine model of myocardial infarction. Cardiology 107(4):402-10.

Watanabe M, Okada T. 2003. Lysophosphatidylcholine-induced myocardial damage is inhibited by pretreatment with poloxamer 188 in isolated rat heart. Mol Cell Biochem 248(1-2):209-15.

Wearn JT. 1928. The extent of the capillary bed of the heart. J Exp Med 47:273-91.

Weng ZC, Nicolosi AC, Detwiler PW, Hsu DT, Schierman SW, Goldstein AH, Spotnitz HM. 1992. Effects of crystalloid, blood, and University of Wisconsin perfusates on weight, water content, and left ventricular compliance in an edema-prone, isolated porcine heart model. J Thorac Cardiovasc Surg 103(3):504-13.

Wenzel K, Geier C, Qadri F, Hubner N, Schulz H, Erdmann B, Gross V, Bauer D, Dechend R, Dietz R, Osterziel KJ, Spuler S, Ozcelik C. 2007. Dysfunction of dysferlin-deficient hearts. J Mol Med 85(11):1203-14.

Wernovsky G, Wypij D, Jonas RA, Mayer JE, Jr., Hanley FL, Hickey PR, Walsh AZ, Chang AC, Castaneda AR, Newburger JW, et al. 1995. Postoperative course and hemodynamic profile after the arterial switch operation in neonates and infants. A comparison of low-flow cardiopulmonary bypass and circulatory arrest. Circulation 92(8):2226-35.

Wessel DL. 2001. Managing low cardiac output syndrome after congenital heart surgery. Critical Care Medicine 29(10 Suppl):S220-S30.

Wright AR, Rees SA. 1998. Cardiac cell volume: crystal clear or murky waters? A comparison with other cell types. Pharmacology and Therapeutics 80(1):89-121.

Yang B, Brown D, Verkman AS. 1996. The mercurial insensitive water channel (AQP-4) forms orthogonal arrays in stably transfected Chinese hamster ovary cells. J Biol Chem 271(9):4577-80.

Yang B, Fukuda N, van Hoek A, Matthay MA, Ma T, Verkman AS. 2000. Carbon dioxide permeability of aquaporin-1 measured in erythrocytes and lung of aquaporin-1 null mice and in reconstituted proteoliposomes. J Biol Chem 275(4):2686-92.

Yang B, Ma T, Verkman AS. 1995. cDNA cloning, gene organization, and chromosomal localization of a human mercurial insensitive water channel. J Biol Chem 270(39):22907-13.

Yang B, Ma T, Xu Z, Verkman AS. 1999. cDNA and genomic cloning of mouse aquaporin-2: functional analysis of an orthologous mutant causing nephrogenic diabetes insipidus. Genomics 57(1):79-83.

Yang B, Zhao D, Verkman AS. 2006. Evidence against functionally significant aquaporin expression in mitochondria. J Biol Chem 281(24):16202-6.

Yasuda S, Townsend D, Michele DE, Favre EG, Day SM, Metzger JM. 2005. Dystrophic heart failure blocked by membrane sealant poloxamer. Nature 436(7053):1025-9.

Yusuf S. 1997. Effects of RheothRx on mortality, morbidity, left ventricular function, and infarct size in patients with acute myocardial infarction. Collaborative Organization for RheothRx Evaluation (CORE). Circulation 96(1):192-201.

Zelenina M, Bondar AA, Zelenin S, Aperia A. 2003. Nickel and extracellular acidification inhibit the water permeability of human aquaporin-3 in lung epithelial cells. J Biol Chem 278(32):30037-43.

Zhang S, Wang S, Li Q, Yao S, Zeng B, Ziegelstein RC, Hu Q. 2005. Capillary leak syndrome in children with C4A-deficiency undergoing cardiac surgery with cardiopulmonary bypass: a double-blind, randomised controlled study. Lancet 366(9485):556-62.