Bilirubin Induced Cardioprotection: From Endogenous Protection to Therapeutic Potential

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First and foremost, I would like to express my deepest gratitude to my primary supervisor, Dr. Andrew Bulmer. Without you, I'm not sure how I would have made it through a PhD, and I definitely would not have had the opportunities that you have given me. Thank you for the guidance and knowledge you shared with me over the past five years, from my research project to my PhD. I have been so fortunate to have your support, professionally and personally, through some of the most exciting and challenging times. I have the utmost respect for you, both as a person and a researcher, and I can't thank you enough for instilling in me a strong work ethic (60 hours/week minimum, I got there in the end) and a true passion for my research.

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helpful in collecting and analysing my data, and I am so grateful for that. Dr. Kevin Ashton, thank you for allowing me to into your lab at Bond University, not to mention your time and expertise. I was fortunate to have someone so knowledgeable to teach me the basics of gene expression analysis. I learnt so much in the short time that we spent together, and I will certainly take it all with me into my future research. Prof. Karl-Heinz Wagner, thank you for your support of my projects and the contributions to my manuscripts over the previous four years. In particular, thank you for welcoming me to Vienna, allowing me to work with your equipment and your lab and providing me with such an incredible experience.

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To my parents, Mum and Dad, I would not be who I am or where I am today without you. Thank you for your unwavering support and unconditional love, and for always allowing me to follow my dreams. I have an incredible amount of respect for you both, and the sacrifices you have made for my happiness. Nishma and Jayshree, thank you for always being sisters I can rely on, laugh with, cry with, and eat with. I don't know where I would be in this world without each one of you, your support and your love.

Patrick, this story is only just beginning but you have already shown me incredible support and so much love. Thank you for being, my confidant, the devil's advocate and my best friend. You have been there to share my happiness and made me smile during the more difficult times. I know that I grated on your very last nerve on so many occasions, and you have been nothing but supportive, respectful and beautiful. I simply cannot put into words how much I appreciate you and need you in my life. I love you. See you soon.

STATEMENT OF ORIGINALITY

I, **Bhavisha Bakrania**, hereby declare this thesis, entitled "*Bilirubin induced cardioprotection: From endogenous protection to therapeutic potential*" is my work and has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

_____

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03.07.2015

ABSTRACT

Over the past 30 years, knowledge concerning the *in vivo* relevance of bilirubin has evolved from being an inert waste product of haem catabolism to a physiologically important antioxidant and biomarker of cardiovascular disease (CVD). Elevated serum bilirubin concentrations, as observed in human Gilbert's syndrome (GS), are associated with reduced incidence of atherosclerosis, ischaemic heart disease (IHD), and a reduction in overall cardiovascular mortality. However, a comprehensive understanding of the mechanisms that might explain these associations remains to be delineated. Aside from its potent antioxidant capacity, bilirubin inhibits smooth muscle cell proliferation, intima-media thickening and influences vascular tone, all of which could represent additional mechanisms by which bilirubin could protect from CVD and associated mortality. This thesis addressed three aims; 1) to investigate whether endogenously elevated bilirubin affects cardiac structure and function in healthy rats; 2) to determine whether endogenously and exogenously elevated bilirubin impacts on cardiac stress resistance in aged and young rat hearts; and 3) to explore the effects of elevated endogenous bilirubin on expression of genes in left ventricular myocardium. If bilirubin was found to protect from myocardial ischaemia-reperfusion injury, the research within this thesis could underpin the development of therapies for myocardial infarction, for which there is currently no treatment. The first study of this thesis explored modifications of cardiac function and stress-resistance in female mutant Gunn (hyperbilirubinaemic) rats compared to littermate controls. Animals aged 12-13 months (n=8/group) underwent *in vivo* and *ex vivo* assessment of cardiac function and stress resistance. *Ex vivo* analysis revealed reduced intrinsic contractility in Gunn myocardium, which correlated positively with myocardial unconjugated bilirubin

(UCB) concentration. Gunn rats demonstrated a reduced rate of aortic pressure development and a reduced aortic pressure gradient *in vivo*, in association with significant aortic dilatation. Furthermore, Gunn hearts exhibited improved post-ischaemic left ventricular function. These findings suggested hyperbilirubinaemia modifies cardiac function by reducing after-load, and improves ischaemic stress-resistance in aged female myocardium. The second study considered whether the phenotypic effects of hyperbilirubinaemia were sex- and age-dependent. Cardiac structure and function was assessed in male Gunn and littermate control rats (n=10/group) at 3, 6 and 12 months of age. Myocardial ischaemic tolerance was then assessed and tissues assayed for oxidised lipids and proteins (n=8/group). No differences in baseline cardiac function were evident between groups at 3 months, however from 6 months onwards Gunn rats demonstrated significant aortic dilatation and reduced peak ejection velocities. Improved functional tolerance to myocardial ischaemia was observed in Gunn hearts and was accompanied by a significant reduction in infarct area, malondialdehyde and protein carbonyl content. Together with prior findings in female animals, these data indicate bilirubin exerts sex-independent effects on vascular structure, myocardial function and ischaemic tolerance. Cardioprotection is associated with reduced oxidative tissue injury, likely mediated a bilirubin's antioxidant properties. These data suggested that bilirubin might protect the heart from ischaemia-reperfusion injury in individuals with a mild endogenous hyperbilirubinaemia (i.e. Gilbert's syndrome). The third study in this thesis tested whether cardioprotection evident in hyperbilirubinaemic Gunn rats could be acutely recapitulated in myocardium from 'normobilirubinemic' Wistar rats, through either pre- or post-ischaemic bilirubin therapy. Water soluble bilirubin ditaurate was administered to Langendorff-perfused hearts 30 min prior to (Pre) or immediately after (Post) 30 mins of global ischaemia

(*n*=16/group). Post-ischaemic recoveries of left ventricular developed pressure were significantly enhanced in both treatment groups. Infarct size was significantly reduced in treated hearts, as well as tissue malondialdehyde and protein carbonyl content. These data collectively reveal significant cardioprotection with either pre- or post-ischaemic treatment of bilirubin ditaurate, with post-treatment being particularly effective. Significant reductions in infarct size and markers of oxidative damage support the potential utility of this endogenous molecule as a post-infarction treatment. The final study was designed to generate new hypotheses regarding the effects of endogenous hypebrilirubinaemia on the expression of genes within myocardium. These data may contribute to better understanding the functional and protective effects of hyperbilirubinaemia observed in the previous studies. Micro-array was used to determine genome-wide changes in left ventricular gene expression from Gunn and control animals. Micro-array analysis revealed significant changes in the expression of 304 transcripts, 97 with known functions. Pathway analysis revealed significant regulation of extracellular matrix, vascular development, response to signalling and olfactory sensory receptors. RT-qPCR validation confirmed modulation of seven selected genes in Gunn *vs.* control tissue. The extent to which these changes reflect direct impacts of unconjugated bilirubin *vs.* indirect modulation of 'oxidative' phenotype, and their importance to the structural remodelling evident in Gunn rats awaits further study. This thesis has contributed to the field, by revealing effects of endogenous hyperbilirubinaemia on cardiovascular function, resistance to cardiac ischaemic stress, via protection from lipid and protein oxidation and has generated new hypotheses to indicate that bilirubin may also regulate gene expression. These findings provide many new areas for investigation, which are expected to lead to a better appreciation of bilirubin in the context of cardiovascular health, disease and therapy.

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LIST OF CO-AUTHORED PUBLICATIONS INCLUDED IN THIS THESIS

Bakrania B, Du Toit, EF, Ashton KJ, Kiessling CJ, Wagner K-H, Headrick JP, Bulmer AC. Hyperbilirubinaemia modulates myocardial function, aortic ejection, and ischaemic stress resistance in the Gunn rat. *Am J Physiol Heart Circ Physiol* 307: H1142-9, 2014 (Chapter 3)

Bakrania B, Du Toit, EF, Wagner K-H, Headrick JP, Bulmer AC. Age-dependent cardiovascular modulation and myocardial stress-resistance in the male hyperbilirubinaemic Gunn rat. *Acta Physiologica.* Manuscript under review (Chapter 4)

Bakrania B, Du Toit EF, Wagner K-H, Headrick JP, Bulmer AC. Pre- or post-ischaemic bilirubin ditaurate treatment reduces oxidative tissue damage and improves cardiac function. *Int J Cardiol*. 202: 27-32, 2015 (Chapter 5)

Bakrania B, Ashton KJ, Du Toit EF, Wagner K-H, Headrick JP, Bulmer AC. Modulation of extracellular matrix, anti-apoptotic pathways and olfactory receptor expression in left ventricular myocardium of hyperbilirubinaemic Gunn rats Manuscript in preparation (Chapter 6)

LIST OF CO-AUTHORED PUBLICATIONS NOT INCLUDED IN THIS THESIS

Boon A-C, Hawkins CL, Bisht K, Coombes JS, **Bakrania B**, Wagner K-H, Bulmer AC. Reduced circulating oxidised LDL is associated with hypocholesterolemia and enhanced thiol status in Gilbert's syndrome. *Free Radic Biol Med.* 52: 2120-7, 2012.

Bisht K, Tampe J, Shing C, **Bakrania B**, Wagner K-H, Bulmer A.C. Endogenous tetrapyrroles influence leukocyte responses to lipopolysaccharide in human blood: Pre-clinical evidence demonstrating the anti-inflammatory potential of biliverdin. *J Clin Cell Immunol.* 5: 1-12, 2014.

STATEMENT OF CONTRIBUTION
TO CO-AUTHORED MANUSCRIPTS

During my candidature I have co-authored six journal articles, for which I was the first author of four. These four articles can be found in Chapters 3-6 of this thesis and the contribution of all authors have been addressed below. The full text of the remaining articles can be found in the appendix of this thesis, and my contribution can be found at the end of this section.

CHAPTER 3 includes the article entitled 'Hyperbilirubinaemia modulates myocardial function, aortic ejection, and ischaemic stress resistance in the Gunn rat', which has been published in the *American Journal of Physiology – Heart and Circulatory Physiology* 307: H1142-9, 2014.

Authors: Bakrania B, Toit Du EF, Ashton KJ, Kiessling CJ, Wagner K-H, Headrick JP, Bulmer AC.

My contribution:

- Preparation and submission of animal ethics application
- Maintenance and care of animal colony
- Echocardiography analysis of all animals
- Langendorff heart perfusion of all animals
- Millar catheterisation of all animals
- Biochemical and tissue sample collection and preparation
- HPLC analysis for serum and tissue
- RT-qPCR of housekeeping genes and genes of interest
- Statistical analysis of all data
- Manuscript preparation and revision

Eugene F. Du Toit's contribution:

- Assistance in developing study design
- Provided training in experimental procedures
- Supplied apparatus for all animal experiments
- Contributed to revision of the manuscript

Kevin J. Ashton's contribution:

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- Designed primers for specific genes
- Provided apparatus to run PCR analysis
- Completed addition PCR analyses
- Contributed to revision of the manuscript

Can J. Kiessling's contribution:

- Completed addition PCR analyses

Karl-Heinz Wagner's contribution:

- Assistance in developing study design
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- Contributed to revision on the manuscript

John P. Headrick's contribution:

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- Developed figures for manuscript
- Contributed to revision of the manuscript

Andrew Bulmer's contribution:

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- Provided training in echocardiography analysis
- Provided training in biochemical and tissue analyses
- Provided training in statistical analysis
- Manuscript preparation and revision

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CHAPTER 4 includes the article entitled 'Age-dependant cardiovascular modulation and myocardial stress resistance in the male hyperbilirubinaemic Gunn rat', which has been submitted and is currently under review by *Acta Physiologica*

Authors: Bakrania B, Du Toit EF, Wagner K-H, Headrick JP, Bulmer AC.

My contribution:

- Preparation and submission of animal ethics application
- Maintenance and care of animal colony
- Langendorff heart perfusion of all animals
- Biochemical and tissue sample collection and preparation
- HPLC analysis for bilirubin ditarurate and malondialdehyde
- Biochemical sample analyses for biomarkers of tissue damage
- Tissue sample analyses for infarct size
- ELISA analysis for concentration of protein carbonyls
- Statistical analysis of all data
- Manuscript preparation and revision

Eugene F. Du Toit's contribution:

- Assistance in developing study design
- Provided training in experimental procedures
- Supplied Langendorff apparatus and Millar catheterisation
- Contributed to revision of the manuscript

Karl-Heinz Wagner's contribution:

- Assistance in developing study design
- Provided training in HPLC analysis of malondialdehyde content
- Secured funding for the project
- Contributed to revision on the manuscript

John P. Headrick's contribution:

- Assistance in developing study design
- Contributed to revision of the manuscript

Andrew Bulmer's contribution:

- Development of study design
- Secured funding for the project
- Preparation and submission of animal ethics application
- Provided training in biochemical and tissue analyses
- Provided training in statistical analysis
- Manuscript preparation and revision

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Dr. Andrew Bulmer, Supervisor

CHAPTER 5 includes the article entitled 'Pre- or post-ischaemic bilirubin ditaurate treatment reduces oxidative tissue damage and improves cardiac function', which has been published in the *International Journal of Cardiology.*

Authors: Bakrania B, Du Toit EF, Wagner K-H, Headrick JP, Bulmer AC.

My contribution:

- Preparation and submission of animal ethics application
- Maintenance and care of animal colony
- Langendorff heart perfusion of all animals
- Biochemical and tissue sample collection and preparation
- HPLC analysis for bilirubin ditarurate and malondialdehyde
- Biochemical sample analyses for biomarkers of tissue damage
- Tissue sample analyses for infarct size
- ELISA analysis for concentration of protein carbonyls
- Statistical analysis of all data
- Manuscript preparation and revision

Eugene F. Du Toit's contribution:

- Assistance in developing study design
- Provided training in experimental procedures
- Supplied apparatus for Langendorff heart perfusions and Millar catheterisation
- Contributed to revision of the manuscript

Karl-Heinz Wagner's contribution:

- Assistance in developing study design
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- Secured funding for the project
- Contributed to revision on the manuscript

John P. Headrick's contribution:

- Assistance in developing study design
- Contributed to revision of the manuscript

Andrew Bulmer's contribution:

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- Provided training in biochemical and tissue analyses
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CHAPTER 6 includes the article entitled 'Modulation of extracellular matrix, anti-apoptotic pathways and olfactory receptor expression in left ventricular myocardium of hyperbilirubinaemic Gunn rats' which has been prepared for future submission to the *American Journal of Physiology - Physiological Genomics*.

Authors: Bakrania B, Ashton, KJ, Du Toit, E Wagner K-H, Headrick JP, Bulmer AC.

This study includes an Affymetrix microarray of heart tissue, which was sourced to the Ramaciotti Centre for Genomics at the University of New South Wales.

My contribution:

- Preparation and submission of animal ethics application
- Harvest and preparation of heart tissue
- Preparation of tissue for microarray analysis
- Performed RNA extraction
- Performed cDNA synthesis
- PCR analysis
- Developed figures for manuscript
- Manuscript preparation and revision

Kevin Ashton's contribution:

- Development of study design
- Developed figures for manuscript
- Provided training in PCR
- PCR and microarray statistical analysis
- Contributed to revision of the manuscript

Eugene Du Toit's contribution:

- Contributed to study design
- Revised the manuscript

Karl-Heinz Wagner's contribution:

- Provided financial support for the study
- Revised the manuscript

John Headrick's contribution:

- Contributed to study design
- Revised the manuscript

Andrew Bulmer's contribution:

- Development of study design
- Secured funding for the project
- Preparation and submission of animal ethics application
- Manuscript preparation and revision

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Boon A-C, Hawkins CL, Bisht K, Coombes JS, **Bakrania B**, Wagner K-H, Bulmer AC. Reduced circulating oxidised LDL is associated with hypocholesterolemia and enhanced thiol status in Gilbert's syndrome. *Free Radic Biol Med.* 52: 2120-7, 2012.

My contribution:

- Preparation and submission of animal ethics application
- Maintenance and care of animal colony
- Harvest and preparation of blood samples from animals
- Revisions of methods section of manuscript

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Bisht K, Tampe J, Shing C, **Bakrania B**, Wagner K-H, Bulmer A.C. Endogenous tetrapyrroles influence leukocyte responses to lipopolysaccharide in human blood: Pre-clinical evidence demonstrating the anti-inflammatory potential of biliverdin. *J Clin Cell Immunol.* 5: 1-12, 2014.

My contribution:

- Preparation and submission of animal ethics application
- Maintenance and care of animal colony
- Harvest and preparation of blood samples from animals
- HPLC analysis of unconjugated bilirubin in Gunn rats
- Revisions of methods section of manuscript

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ABBREVIATIONS

+dP/dt	rate of contraction (change of pressure over change in time)
-dP/dt	rate of relaxation (change of pressure over change of time)
ABCC2	gene coding for multi-drug resistance protein 2
AoVTI	aortic velocity time integral
ATP	adenosine tri-phosphate
Atp2a1	sarcoplasmic/endoplasmic reticulum calcium ATPase 1
Atp2a2	sarcoplasmic/endoplasmic reticulum calcium ATPase 2
BCA	bicinchoninic Acid
B-mode	brightness mode
BRT	bilirubin ditaurate
BVR	biliverdin reductase
C5aR	complement receptor 5a
Ca^{2+}	calcium ions
CABG	coronary artery bypass graft
$CaCl_2$	calcium chloride
CAD	coronary artery disease
Cat	catalase
CF	coronary flow
CKD	chronic kidney disease
CK-MB	creatine kinase MB
CNS	Crigler-Najjar syndrome
Col3A1	collagen, type iii, alpha 1

CoPP	cobalt photoporphyrin
CORM	carbon monoxide releasing molecule
CVD	cardiovascular disease
DAMP	damage associated
DMSO	dimethyl sulfoxide
DOCA	deoxycorticosterone acetate
DP	diastolic pressure
ECM	extracellular matrix
EC-SOD	extracellular superoxide dismutase
ED	endothelial dysfunction
EDTA	ethylenediaminetetraacetic acid
ES	enrichment score
ET-1	endothelin-1
FDR	false discovery rate
Fmod	fibromodulin
FRAP	ferric reducing ability of plasma
GPCR	G-protein coupled receptor
GPx	glutathione peroxidase
GS	Gilbert's syndrome
GTPCH 1	guanosine 5’-triphosphate cyclohydrolase
H+	hydrogen ions
H2O2	hydrogen peroxide
HDL	high density lipoproteins
HO-1	haem oxygenase-1

HOCl	hypochlorous acid
HPLC	high performace liquid spectometry
HR	heart rate
HSF	heat shock factor
HSP	heat shock protein
Igf1	insulin-like growth factor
IGF1R	insulin-like growth factor 1 receptor
IHD	ischaemic heart disease
IL-6	interleukin 6
IMT	intima-media thickening
IPC	ischaemic pre-conditioning
I-R	ischaemia-reperfusion
IVCT	isovolumetric contraction time
IVRT	isovolumetric relaxation time
KCl	potassium chloride
KHS	Krebs-Henseleit solution
LAD	left anterior descending
LCSA	lumen cross-sectional area
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
Lp-PLA_2	lipoprotein-associated phospholipase A_2
LVAW;d	left ventricular anterior wall thickness during diastole
LVAW;s	left ventricular anterior wall thickness during systole
LVDP	left ventricular developed pressure

LVID;d	left ventricular internal dimension during diastole
LVID;s	left ventricular internal dimension during systole
LVOT	left ventricular outflow tract (aorta)
LVPW;d	left ventricular posterior wall thickness duing diastole
LVPW;s	left ventricular posterior wall thickness duing systole
MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
$MgSO_4$	magnesium sulphate
MI	myocardial infarction
M-mode	motion mode
Mmp2	matrix metallopeptidase 2
MPTP	mitrochondria permeability transition pore
MRP-2	multi-drug resistance protein 2
MV A	mitral valve active filling
MV E	mitral valve early filling
N_2	nitrogen
Na+/K+ pumps	sodium potassium pumps
NaCl	sodium chloride
$NaHCo_3$	sodium bicarbonate
NCD	non-communicable disease
NF-κB	nuclear factor-κB
NIH	National Institute of Health
NO	nitric oxide
Nrep	neuronal regeneration related protein

O2	superoxide
OH•	hydroxyl radical
ONOO-	peroxynitrite
oxLDL	oxidised low-density lipoprotein
PC	peak contracture
Pgk1	Phosphoglycerate kinase 1
PLA2	phospholipase 2
Pln	Phospholamban
Ppia	Peptidylprolyl isomerase A
RAAS	renin-aldosterone-angiotensin system
RMA	robust multi-array average
RONS	reactive oxygen and nitrogen species
ROS	reactive oxygen species
RPP	rate pressure product
RRRC	Rat Resource and Research Center
RT-qPCR	real time quantitative ploymerase chain reaction
Rxfp1	relaxin/insulin-like family peptide receptor 1
Ryr2	ryanodine receptor 2
SD	standard deviation
Sod1	superoxide dismutase 1
Sod2	superoxide dismutase 2
SP	systolic pressure
Sparc	secreted protein, acidic, cysteine-rich (osteonectin)
SWOP	second window of protection

TBARS	thiobarbituric acid reactive substances
TEAC	trolox equivalent antixodant capacity
TIMI	thrombolytic inhibition of myocardial ischaemia
TLR	toll like receptor
TNF-α	tumor necrosis factor α
TOC	time to onset of contraction
UCB	unconjugated bilirubin
UGT1A1	uridine glucuronosyl transferase
VSMC	vascular smooth muscle cell

CONFERENCE PRESENTATIONS DURING PHD CANDIDATURE

Oral Presentations

1. 8th International Conference on Haem Oxygenases, BioIron & Oxidative Stress
   Sydney, Australia, 2014

2. Experimental Biology
   San Diego, USA, 2014

3. Gold Coast Health and Medical Research Conference
   Gold Coast, Australia, 2013

4. Gold Coast Health and Medical Research Conference
   Gold Coast, Australia, 2012

Poster Presentations

1. The Society for Free Radical Research Australasia -
   Melbourne, Australia, 2014

2. 8th International Conference on Haem Oxygenases, BioIron & Oxidative Stress
   Sydney, Australia, 2014

3. Experimental Biology
   San Diego, USA, 2014

4. Experimental Biology
   San Diego, USA, 2012

5. Cardiac Society of Australia and New Zealand
   Brisbane, Australia, 2012

AWARDS AND SCHOLARSHIPS GRANTED DURING PHD CANDIDATURE

1. PhD scholarship, 2012

Australian Postgraduate Award

2. Travel Award, 2014

Awarded by the American Association of Anatomists, Experimental Biology, CA

3. Langman Graduate Student Platform Presentation Award, 2014

Awarded by the American Association of Anatomists, Experimental Biology, CA

4. Conference Travel Award, 2014

Awarded by Griffith Graduate Research School

5. Higher Degree Research Grant, 2014

Awarded by Griffith University, International Experience Incentive Scheme

6. Best Abstract Award, 2014

Awarded by 8th Int. Conference on Haem Oxygenases, BioIron & Oxidative Stress

7. Young Investigator Award, 2014

Awarded by the Society of Free Radical Research Australasia

CHAPTER 1

INTRODUCTION

1.1 Emergence of bilirubin in human pathophysiology

Birth records of 'yellow babies' date back thousands of years (89). However, it was not until the late 18th century that the condition neonatal jaundice was described in detail (90). Jean Baptiste Timothée Baumes was one of the most influential pioneers of research into neonatal jaundice, the colour of which is caused by the accumulation of bilirubin within peripheral tissues. Importantly, Baumes understood that jaundice did not always require treatment and was awarded by the University of Paris for 'describing neonatal jaundice and distinguishing between those circumstances in which jaundice needs professional help and those in which one only needs to await the course of nature' (89, 90). After Baumes' award-winning contribution in 1788, and a second edition of the same publication in 1806 (14), in which he described 10 cases of neonatal jaundice and potential therapies for these infants, he continued to explore the causes of this condition. In the years following, many doctoral theses were produced from this topical area of research (89). In 1847, Jaques François Édouard Hervieux made critical observations that the yellow colouration of bilirubin was found throughout the tissues of the body, including the brain. Furthermore, he reported that a physiological neonatal jaundice appeared during the first two to four days of life and lasted for one to two weeks, never reappearing in the following months, and that neonatal jaundice alone is not terminal (89). Over 100 years after the award-winning contribution of Baumes, Christian Georg Schmorl coined the term 'kernicterus', or bilirubin induced brain injury, after observing areas of highly pigmented yellow tissue in the brain and included these observations in his 1904 publication (Figure 1.1) (202). In addition, Schmorl noticed that if the brains in his investigations were not preserved immediately, the yellow colouration would disappear, leading to speculation that an oxidising enzyme existed in the brain, resulting in the break-down of accumulated pigment. Broderson

and Bartels later confirmed that bilirubin is oxidised by a number of compounds, including hydrogen peroxide, cytochrome c and xanthine oxidase in the brain and in other tissues, laying the basis of later critical experiments confirming bilirubin's antioxidant activity (33, 89).

Through hundreds of years of research it has been concluded that high concentrations of unconjugated bilirubin (UCB), the accumulating yellow bile pigment in neonatal jaundice, is partly a consequence of acutely reduced function of hepatic glucuronosyl transferase. Unconjugated bilirubin is produced during haem catabolism, as a byproduct of red blood cell degradation, and is excreted by conjugation to glucuronic acid in the liver, which is facilitated by uridine glucuronosyl transferase 1A1 (UGT1A1). During gestation, diminished UGT1A1 activity allows free movement of UCB through the placenta (158). In addition, the lifespan of red blood cells in the fetus are considerably shorter than in adults, which makes hepatic conjugation, leading to bilirubin excretion, a vital process to avoid pathological accumulation after birth (91). Neonatal jaundice is now commonly treated using phototherapy, which temporarily transforms the structure of bilirubin into a freely excretable water-soluble isomer, that does not require glucuronidation (168). Upon phototherapy, jaundice is usually resolved within two weeks and can prevent irreversible brain damage in neonates (168).

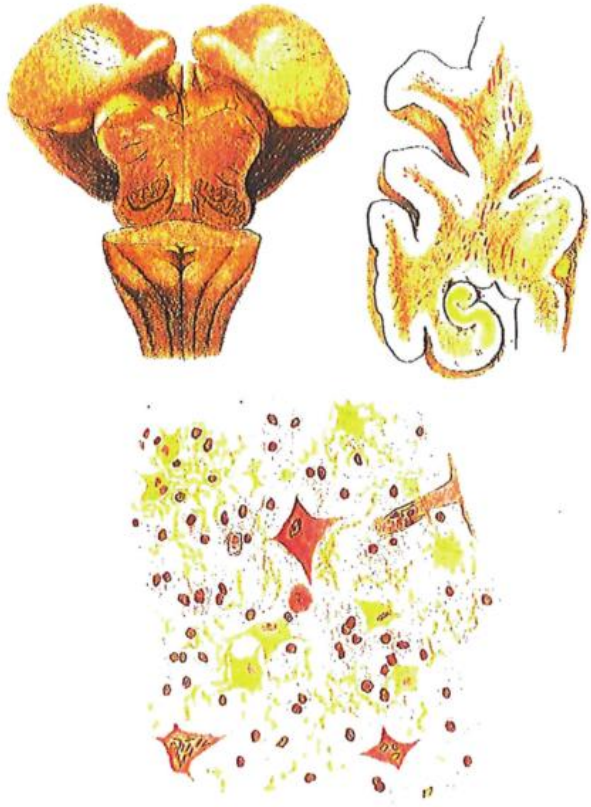


Figure 1.1: Christian Georg Schmorl's depiction of kernicteric brain tissue, observed in the hippocampus, as published in 1904 (202)

1.2 Haem catabolism: A protective role for bilirubin

In adults, haemoproteins, including haemoglobin, the oxygen carrying constituent of red blood cells, is the major source of haem catabolism, leading to bilirubin production (Figure 2.9) (39). Red blood cells are phagocytosed by splenic and hepatic macrophages (Kuppfer cells). Haemoglobin then undergoes catalysis into two main constituents, globin and haem, with haem being metabolised by haem oxygenases, including the stress inducible isoform, haem oxygenase 1 (HO-1). This enzyme is highly expressed in macrophages and the reticulo-endothelial system (i.e. spleen, liver) and liberates iron, biliverdin and carbon monoxide from haem (77). Biliverdin is rapidly chemically reduced by biliverdin reductase (BVR) and, therefore, is normally

undetectable in most cells and blood plasma (40). Unconjugated bilirubin is then transported to the liver bound to serum albumin and is actively and passively transported into hepatocytes (122). Here, bilirubin is conjugated to one or two glucuronic acid molecules by UGT1A1 and is actively transported by multi-drug resistance protein 2 (MRP-2) into the bile canaliculi. Bilirubin glucuronides are then directed into the bile and onwards into the small intestine. Within the gut, bilirubin is first deconjugated by bacterial β-glucuronidase to unconjugated pigment and is then further reduced to structurally related pigments (77). The most notable of these compounds are stercobilin, which is excreted in the faeces, and urobilinogen, which is excreted in the urine (221). A graphic demonstration of haem catatabolism exists in the process of bruising in mammals where initial haem release (red/black) is followed by green colouration (formation of biliverdin) and finally the appearance of yellow (unconjugated bilirubin) colouration. The lipophilicity of unconjugated bilirubin further explains the lingering colouration of bruises often days and sometimes weeks after trauma. Similar to neonatal jaundice, bilirubin's yellow/orange colour can also be seen in the skin/sclera in adult patients with hepatic insufficiency, which is often caused by elevated circulating bilirubin glucuronides, secondary to hepatitis or biliary obstruction (77).

Within clinical practice, bilirubin has been regarded as a potential neurotoxin due to its role in kernicterus, as mentioned previously. Additionally, increased bilirubin and pathological jaundice are often associated with hepatic dysfunction/disease; however, these signs and symptoms are in fact secondary to the underlying cause. A ground-breaking manuscript was published in 1987 by Stocker *et al* who conclusively demonstrated that bilirubin possessed antioxidant activity. Not surprisingly, this

publication generated a paradigm shift concerning the understanding of bilirubin biology, transforming bilirubin from being a neurotoxic compound to a one possessing physiological importance. For example, total serum antioxidant capacity of individuals with mildly elevated bilirubin was shown to be significantly increased and protected from serum oxidation (243). There is now strong evidence to indicate that bilirubin actively reduces the risk and progression of several diseases associated with 'oxidative stress' including atherosclerosis (25, 39, 173). For example, the incidence of Ischaemic Heart Disease (IHD) is reduced from 12% in the general population to 2% in persons with mildly elevated bilirubin (243). In addition, bilirubin may improve vascular compliance by inhibiting vascular smooth muscle cell proliferation (176), increasing nitric oxide bioavailability (156) and/or inhibiting NADPH oxidase activity (218, 240), which could in part explain the reduced risk of IHD seen in these patients. Bilirubin may also play a more general role in preventing cardiomyopathies due to their association with and exacerbation by the presence of reactive oxygen and nitrogen species (RONS).

Interestingly, unconjugated bilirubin concentrations in Wistar and mutant UGT1A1 (hyperbilirubinaemic) Gunn rats tissues were recently reported (266), revealing the deposition of UCB in blood (179nmol/g), liver (88nmol/g) and kidney (3nmol/g) in Gunn rats. These data are not surprising considering each of these organs is involved either in the formation, transport or excretion of bilirubin species. However, high concentrations were also reported in the myocardium (21 vs. 0.16 nmol/g in Gunn vs. controls), which could potentially affect cardiac function and protect the heart from RONS injury. A growing body of work implicating bilirubin as a protective agent against disease suggests that a 'U shaped' relationship exists, indicating that very low

and very high concentrations may be detrimental, with high-normal values protecting from disease (108). It should be noted that total bilirubin concentrations generally decrease during the later years of life, due to decreased haematopoiesis. Therefore, maintaining a mildly elevated bilirubin concentration later in life may confer resistance to disease, during periods when the body is normally most susceptible (e.g. to cardiovascular disease). If such protection could be induced by actively increasing circulating bilirubin concentrations, new therapies could be developed for the world's leading causes of death. .

This thesis sought to address four aims; 1) to determine whether life-long endogenous hyperbilirubinaemia in male and female animals effects cardiac development and baseline heart function; 2) to determine whether endogenously elevated cardiac bilirubin concentrations are associated with improved post-ischaemic function and attenuation of oxidative tissue damage; 3) to investigate whether protection from ischaemia-reperfusion injury can be induced by acutely treating hearts with a synthetic bilirubin analogue (when treated before ischaemia or during reperfusion); and 4) to investigate changes in cardiac gene expression in Gunn rat (endogenous hyperbilirubinaemic) and wild-type animals, to provide preliminary insight into possible genomic mechanisms influencing cardiac function and protection from ischaemia-reperfusion injury.

1.3 Study One

This study, entitled 'Hyperbilirubinaemia modulates myocardial function, aortic ejection, and ischaemic stress resistance in the Gunn rat' can be found in Chapter 3 of this thesis, and has been published in the *American Journal of Physiology – Heart and Circulatory Physiology*.

Unconjugated bilirubin is now regarded as a robust biomarker of cardiovascular health (245). However, despite evidence indicating a role for bilirubin in protecting from atherogenesis, the discrete effects of *endogenous* hyperbilirubinaemia on cardiac function and ischaemic tolerance have not been reported.

The aim of this study was to determine whether endogenously elevated bilirubin modifies cardiac structure, function and ischaemic stress resistance in the female Gunn rat, compared to normobilirubinaemic wild-type littermate controls. *Ex vivo* cardiac function and stress resistance was measured using Langendorff perfusion, where each heart was excised from the animal, then underwent 30 mins of aerobic perfusion followed by 35 mins of global ischaemia followed by 90 mins of reperfusion. *In vivo* cardiac structure and function were also assessed using transthoracic echocardiography and Millar catheterisation. After catheterisation, hearts were removed and assessed for modifications in the expression of specific genes regulating antioxidant defences and calcium homeostasis, using RT-qPCR. Baseline left ventricular developed pressure (LVDP) was significantly reduced *ex vivo* (Gunn, 100±13mmHg vs. control, 114±12mmHg, P=0.047), however, a similar effect was not noted *in vivo* (Gunn, 155±30mmHg vs. control, 148±26mmHg, P=0.77). Interestingly, echocardiography analysis revealed that mean aortic blood flow velocity (Gunn, 439±64mL/s vs. control,

644±62mL/s, P<0.001) and rate of aortic pressure development (Gunn, 3008±461mmHg/s vs. control, 4452±644mmHg/s, *P*=0.02) were significantly reduced in Gunn animals, implicating a role for bilirubin in modifying vascular compliance. No changes in cardiac structure were noted *in vivo* between groups, however the aorta of Gunn hearts were significantly dilated compared to controls during systole (Gunn, 2.42±0.15mm vs. control, 2.72±0.2mm, P=0.02) and diastole (Gunn, 1.70±0.27mm vs. control, 2.11±0.17mm, P=0.01), suggesting altered systolic function was due to aortic remodelling. *Ex vivo* Gunn hearts also demonstrated improved left ventricular developed pressure (LVDP) after 90 mins of reperfusion, following 35 min ischaemia (63±14 *vs.* 35±12%, *P*<0.01). Analysis of cardiac gene expression demonstrated increased glutathione peroxidase and reduced superoxide dismutase/phospholamban gene expression in Gunn rats. Reduced contractility *ex vivo* was compensation for *in vivo*, in order to maintain systemic pressures, in the face of reduced afterload. Furthermore, changes in gene expression suggest that mechanisms responsible for ischaemic cardioprotection may be related to improved antioxidant defense mechanisms and altered calcium handling.

1.4 Study Two

This study, entitled 'Age-dependent cardiovascular modulation and myocardial stress-resistance in the male hyperbilirubinemic Gunn rat' is detailed in Chapter 4 of this thesis. This study has been submitted to the *American Journal of Physiology – Heart and Circulatory Physiology* and is currently under review.

Although post-ischaemic cardiac function was demonstrated to be improved in female Gunn hearts (in study 1), whether protection would be extended to male

hyperbilirbinaemic hearts was unknown. Furthermore, it was hypothesised that the antioxidant effects of bilirubin might contribute to reduced cardiac injury and improved post-ischaemic function. In addition, it was unknown if the effects of life-long exposure to bilirubin exerted its effects on the heart during development.

The aim of this study was to determine whether cardiac function is modified in male Gunn rats, and to explore whether such an effect would manifest itself in a time-dependant manner over the lifespan. In addition, stress resistance was assessed and lipid and protein oxidation markers quantified after reperfusion to determine whether bilirubin's antioxidant properties might contribute to cardioprotection. Hyperbilirubinemic rats and littermate controls underwent echocardiography at 3, 6 and 12 months of age, with hearts subsequently assessed for resistance to 30 min of ischaemia. Hearts were prepared to measure infarct size and oxidative tissue damage (malondialdehyde, protein carbonyl) after reperfusion. While no difference in baseline cardiac function was evident in Gunn *vs*. control rats at 3 months of age, from 6 months onwards Gunn rats demonstrated aortic dilatation during systole and reduced peak ejection velocities. In addition, duration of ventricular ejection increased progressively from 6 months, indicating a negative inotropic effect of bilirubin *in vivo*. *Ex vivo* analysis of baseline function supported these findings, demonstrating reduced LVDP and contractility in hyperbilirubinaemic rats. Furthermore, stress-resistance was improved in Gunn hearts: post-ischaemic recoveries of LVDP (76±22% vs. 29±17% Control, $P<0.01$) and coronary flow (96±9% vs. 86±16% Control, $P<0.01$) were all improved in Gunn hearts, accompanied by reduced infarct size (21±5% vs. 47±15% Control, $P<0.01$), and ventricular malondialdehyde (0.14±0.02 vs. 0.22±0.07 nmol/mg Control, $P<0.01$) and protein carbonyl content (0.11±0.05 vs. 0.17±0.04 nmol/mg

Control, P<0.01). These data reveal life-long hyperbilirubinaemia induces age-dependent hypo-contractility in male Gunn rats, and improved stress resistance. Together with prior findings in female animals, these data indicate bilirubin exerts sex-independent effects on vascular structure, myocardial function and ischaemic tolerance, the latter likely mediated via anti-oxidant mechanisms.

1.5 Study Three

This study entitled 'Pre- or post-ischaemic bilirubin ditaurate treatment reduces oxidative tissue damage and improves cardiac function' is detailed within Chapter 5 of the thesis, has been submitted to the *International Journal of Cardiology* and is currently under review.

The devastating social and economic impact of cardiovascular disease clearly indicates the need for effective preventative and therapeutic agents. Currently, very limited treatment options exist to salvage myocardium after myocardial infarction in humans. Several trials have determined whether new drugs or interventions can reduce infarct size, including pre- and post-conditioning stimuli. Although numerous investigations have been performed in laboratory settings, effective compounds rarely reach or pass through stringent clinical trials (83). Three reports have previously investigated whether bilirubin reduces cardiac ischaemia-reperfusion injury, but not without limitations and drawbacks. Therefore, this study provides a comprehensive investigation into the efficacy of bilirubin treatment. Furthermore, the previous studies in this thesis suggested an important role of *endogenous* bilirubin in protection from ischaemia-reperfusion. Therefore, the current study sought to test whether this protective effect could be induced by pre- or post-treating hearts with a novel bilirubin

analogue.

This study aimed to determine whether exogenous bilirubin ditaurate (BRT, a synthetic form of bilirubin) administered at physiologically attainable and relevant concentrations before (pre-treatment) or after (post-treatment) ischaemic stress, could reduce cardiac damage and improve functional outcomes. Hearts from 16 male Wistar rats were excised and underwent Langendorff heart perfusion. Hearts were aerobically perfused for 45 min (normalisation period), followed by 30 min of no-flow ischaemia and 120 min of reperfusion. Hearts were either untreated or received 50 μM BRT for 30 min before (Pre) or after (Post) ischaemia. At the end of experiments hearts were removed for analysis infarct size and oxidative damage. Ischaemia induced contractile dysfunction and cellular injury, with both BRT treatments improving I-R outcomes. Final post-ischaemic recoveries for left ventricular diastolic and developed pressures were significantly enhanced in the treatment groups: end-diastolic pressure (Control, 78±14; Pre, 51±15; Post, 51±13 mmHg; $P<0.01$); left ventricular developed pressure, (LVDP; Control 44±15; Pre, 71±19; Post, 84±13 mmHg; $P<0.01$). Similarly, tissue damage measured via biomarkers released into the coronary effluent was reduced by ~30% ($P<0.01$) with BRT Pre treatment and ~50% ($P<0.01$) by Post treatment. Interestingly, function was improved and tissue damage reduced more significantly in the Post group. This was also confirmed in infarct size data, which was 67±17% in controls vs. 37±16% and 23±12% in the Pre and Post groups, respectively (both $P<0.01$). Correlations between myocardial BRT content (atrial) post-ischaemic function support the role of cardiac bilirubin in protection. Oxidative tissue damage was also significantly reduced; lipid peroxidation (malondialdehyde) by ~25% in the Pre group ($P=0.2$) and ~40% in the Post group ($P<0.05$); and protein oxidation by over 60% in

both Pre and Post ($P<0.05$) groups.This study demonstrated that bilirubin reduces post-ischaemic cellular damage and improves contractile function when applied at physiologically relevant concentrations. Importantly, BRT significantly reduced infarct size when applied after an ischaemic event, which is a critical finding in the context of developing a potential therapeutic agent.

1.6 Study Four

This study entitled 'Modulation of gene expression in left ventricular tissue of hyperbilirubinaemic Gunn rats' is detailed within Chapter 6 of the thesis, and has been prepared for submission to the *American Journal of Physiology - Physiological Genomics* in future.

The potent antioxidant capacity of bilirubin is most widely acknowledged as being the protective mechanism by which it reduces the incidence of cardiovascular disease. However, the findings of Study 1 and Study 2 in this thesis, suggest an effect on cardiac function and vascular structure, which may be influenced by transcriptional modifications, in addition to changes in reduction-oxidation status.

The aim of this study was to conduct a preliminary investigation into differential gene expression in left ventricular myocardium from Gunn (an animal model of hyperbilirubinaemia) and wild-type rats (n=6/group). Microarray was performed on all tissue samples followed by PCR validation and pathway analysis from microarray data. The microarray revealed significant changes in the expression of 304 genes, of which 97 were known genes. PCR of 7 randomly selected genes indicated that *Col3A1* (2.19-fold), *Igf1* (1.45-fold), *Mmp2* (1.48-fold), *Nrep* (2.06-fold), *Rxfp1* (1.93-fold), and *Sparc*

(1.49-fold), were upregulated, and *Fmod was* downregulated (-2.02-fold) in Gunn animals. In addition, these data reveal significant modulation of pathways responsible for extracellular martrix (ECM) remodelling, vascular development and olfactory signalling. These data provide preliminary evidence of differential gene expression in hyperbilirubinaemic myocardium rats, and support previous findings of altered cardiac function and improved stress resistance.

CHAPTER 2

LITERATURE REVIEW

2.1 The challenge of cardiovascular disease

Cardiovascular disease (CVD) is recognised as all clinical conditions pertaining to the heart and vasculature. Atherosclerosis is the most common cause of CVD, which is characterised by the narrowing of blood vessels, leading to a lack of oxygen delivery to distal tissues. Atherosclerotic plaque rupture and subsequent thrombus/embolus formation is the primary cause of coronary artery occlusion and leads to cardiac reperfusion injury upon surgical intervention or natural dissipation of the plaque/clot (196).

Cardiovascular disease, cancer, chronic respiratory disease and diabetes were responsible for 82% of deaths worldwide in 2012 (255), of which 37%, and more than 17 million deaths, were attributed to cardiovascular disease (Figure 2.1) (254). Importantly, in Australia, CVD caused 31.7% of total deaths in 2010 (11). Males over 85 years of age were at the highest risk, with 47.1% suffering some form of CVD. In addition, 42% of total deaths related to chronic disease worldwide occurred in individuals below 70 years of age in low- and middle-income countries, however, only 28% of deaths were in this age group for high-income countries, highlighting the necessity of accessible and affordable treatments and preventative interventions (255). Although therapeutic agents are of particular interest, the value of education should not be under-estimated because CVD risk is greatly influenced by modifiable risk factors/behaviours including smoking, leading a sedentary lifestyle, consuming a diet high in saturated fats and excessive alcohol consumption.

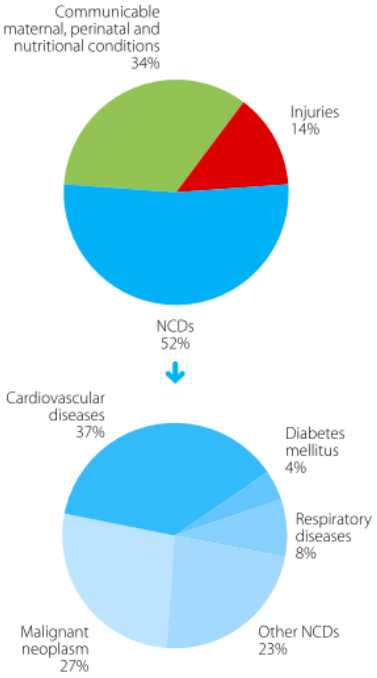


Figure 2.1: Proportion of global deaths under the age of 70 years, comparable estimates, 2012. NCD, Non-communicable diseases (255).

2.1.1 Co-morbidities of cardiovascular disease

Co-morbidities are clinical conditions that occur in conjunction with others, in this case cardiovascular disease. Preventable CVD is often a result of a pre-existing condition such as chronic kidney disease (CKD), obesity, diabetes mellitus and even psychological conditions, such as depression (166). In 2005, 60% of people in Australia with diabetes also had CVD, and 13.2% of deaths were attributed a combination of two or more conditions (CVD, CKD, Diabetes mellitus) (235). Many risk factors are prevalent in today's society (smoking, sedentary lifestyle, increased dietary saturated fat intake) and contribute to each of these chronic conditions. Therefore, it is not surprising that in 2014 39% of the global population was classed as overweight (255), with an estimated 50% of adults in the United States being overweight (167). Unfortunately, in 2005, more than 60% of Australians were classed as either overweight or obese (10), with almost 25% of children classed as obese (102). Chronic kidney disease increases

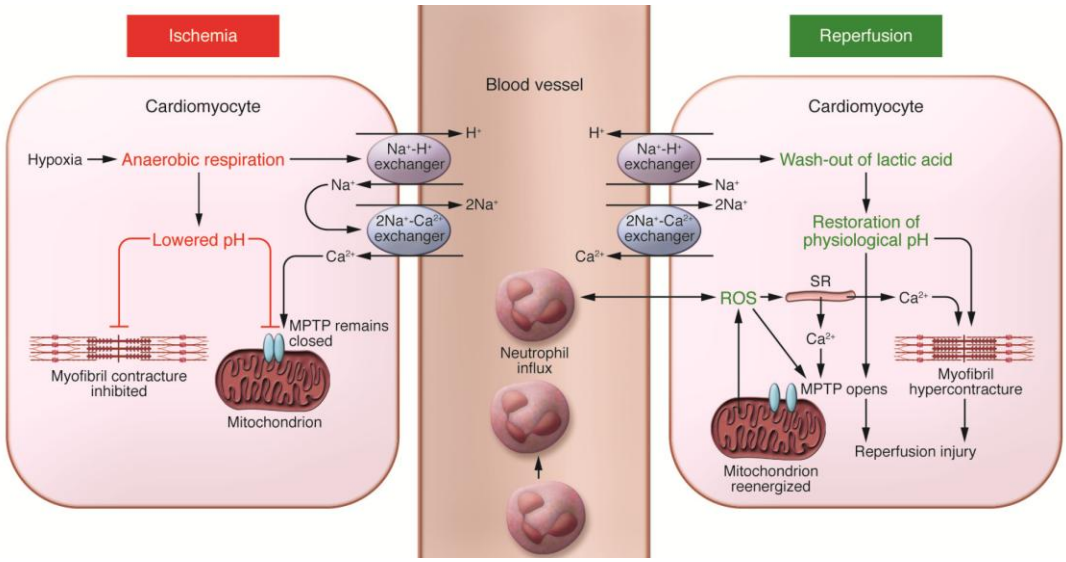
the risk of cardiovascular disease 10-30 fold in these individuals (250). Specifically, reduced glomerular filtration rate in patients increases the likelihood of cardiovascular events (86). Furthermore, studies show that individuals with CKD are more likely to succumb to CVD than renal disease (199). Metabolic syndrome is characterised by the co-occurrence of three of the following symptoms; abdominal obesity, hypertension, elevated fasting serum glucose and/or triglycerides, and high high-density lipoprotein (HDL) concentrations (125). Individuals with this condition, are at three to four times the risk of myocardial infarction alone (125). The presence of these disease states significantly increases the occurrence of cardiovascular disease in society. They may also exacerbate the impacts of CVD, for example worsening outcomes from IHD and myocardial infarction. Therefore, early diagnosis and continuous monitoring of individuals at high risk of CVD is important, and undoubtedly saves lives.

2.1.2 Myocardial infarction and reperfusion injury

Ischaemia is defined as an insufficient supply of oxygen, caused by impaired blood flow to an organ/tissue, with infarction (MI, myocardial infarction) the death of tissue as a result of this reduction in tissue perfusion and oxygenation. Reperfusion refers to the reintroduction of blood flow and oxygen to the previously ischaemic tissue (163). Reperfusion is essential to the potential revival or salvage of ischaemic tissue, however, this does not come without risk and can be more harmful than the initial ischaemic insult (101). An overview of the processes involved in ischaemia reperfusion are shown in Figure 2.2. Due to lack of oxygen, ATP synthesis via mitochindrial oxidative phosphorylation decreases/ceases and anearobic glycolysis increases to partly compensate for reduced ATP supply. Anaerobic glycolysis yeilds hydrogen ions (H^+) as a by-product, causing cellular pH to decrease, and ultimately inhibiting enzyme activity

required for this metabolic process. Simultaneously, the mitochondria permeability transition pore (MPTP) closes, inhibiting the movements of solutes into the mitochondria, which would cause dysfunction and its collapse (94, 96). The combined effects of H^+ and insufficent ATP synthesis disrupt the function of the passive and active ion transport systems, usually triggering arrhythmias (37, 193). Intercellular calcium concentrations generally begin to increase at this time, causing contractile dysfunction. Reactive oxygen and nitrogen species are simultaneosly produced, inducing oxidative damage to cell membrane phospholipids. Proteases activated by elevated calcium cleave cytoskeleton filaments, increase membrane permeability, and further promote cellular swelling, and ultimately cell death.

As noted previously, reperfusion is neccesary for reviving ischaemic tissue, however not without inducing additional oxidative stress and damage. This injury process involves generation of a large number of oxidants which trigger an inflammatory response. The predominant molecules/processes responsible for inducing reperfusion injury are; calcium overload, which is a result of oxidative membrane damage allowing extracellular Ca^{2+} influx, endothelial cells and neutrophils, which are recruited to the area in response to the initial ischaemic insult (155, 180). During reperfusion, when pH is rapidly restored, the MPTP reopens, leading to ATP depletion and cell death (65, 87, 94). A promising area of research revolves around inhibitors of the MPTP (94). More broadly, research shows that intervention during reperfusion can reduce injury, however the timing of such a treatment is crucial to limiting damage and maximising recovery (37).



ROS, Reactive oxygen species; MTPT, mitochondria permeability transition pore

Figure 2.2: Processes affecting ischaemia-reperfusion cellular dysfunction and injury (94)

Interestingly, reperfusion injury is exacerbated in patients with hypercholesterolaemia and/or hypertension (241). Although the mechanisms involved are unknown, it is speculated that the existing oxidative stress and endothelial dysfunction predispose the tissue to more severe injury. Creatine-kinase MB (CK-MB) and troponin are proteins released from the cytosol during cell rupture, and thus are employed as biomarkers of MI (64, 205). Clinically, the circulating concentration of these compounds can be tested in patients, and are indicative of the severity and timing of an MI. Interestingly patients undergoing revascularisation procedures also have increased levels of circulating CK-MB. Furthermore, the concentration of CK-MB released after coronary artery bypass graft (CABG) has been correlated with the likelihood of recurrent MI and mortality rate in patients (64).

2.1.3 Cell death resulting from ischaemia-reperfusion injury

Myocardial cell death is believed to occur by two major pathways: apoptosis, necrosis or a combination of the two (131). Apoptosis, or programmed cell death, is usually mediated by external factors such growth factor deficiency and nutrient deprivation. Cells that undergo apoptosis usually experience mitochondrial dysfunction (e.g. due to RONS mediated damage) inducing mitochondrial cytochrome C release and downstream caspase activation. These events then lead to DNA fragmentation, cellular blebbing and ultimately cellular destruction (131). The remaining cell fragments are removed by mononuclear phagocytic cells including macrophages (68). Necrosis, however, is a form of forced cell death that is usually caused by extreme perturbation in cellular function (ie. ATP depletion resulting in loss of ionic equilibrium and cellular swelling), often resulting in permanent tissue dysfunction, as seen after severe myocardial ischaemia (131). Necrosis usually occurs within masses of cells, which release their contents into the interstitium and leak into the circulation (131). The presence of cellular enzymes and proteins (referred to as damage associated molecular patterns; DAMP) within the blood/interstitium represents a powerful inflammatory stimulus bringing neutrophils to the affected area, causing further cellular breakdown (of potentially viable cells), thus acutely exacerbating cell damage (80). Infiltration of monocyte (that differentiate into macrophages) follows, stimulating wound healing responses, including deposition of collagen, leading to fibrosis and eventually scar formation, as seen in the myocardium of MI survivors (68). Although ischaemia-reperfusion injury is more often associated with necrosis and cellular swelling, cellular shrinkage and apoptosis also occurs. The infarct area in MI is generally surrounded by a much larger 'area at risk', which consists of reversibly ischaemic tissue. However, as the oxygen depletion continues, the infarcted area grows into the surrounding tissue.

Apoptosis is an ATP-dependant process and therefore the availability of ATP is a major determinant of how cells will ultimately die (35–37). Since apoptosis is triggered by oxygen deprivation, anti-apoptic and growth promoting compounds are a target for reducing tissue damage and increasing post-MI recovery.

2.1.4 Myocardial stunning

Not all injury in ischaemia-reperfusion is irreversible, and 'stunning' reflects a temporary dysfunction within cardiomyocytes. The first evidence of myocardial stunning was observed after coronary artery occlusion in dogs (104). Stunning is defined as continued contractile dysfunction that occurs post-ischeamia, in reversibly damaged tissue salvaged by reperfusion (32, 129, 241). Stunning is induced in part by tissue becoming sufficiently ischaemic to influence the conduction of action potentials (ie. function of Na^+/K^+ pumps) without causing cellular necrosis. For this reason, stunned tissue is usually found in close proximity to necrotic tissue (24). During an extended period of time, varying from hours to days, this dysfunction corrects itself, demonstrating that stunning is indeed a reversible form of ischaemic injury (101). Xanthine oxidase may be responsible for myocardial stunning as reported by Ovize *et al* (179), and is present in high concentrations in dog hearts, which experience severe stunning after ischaemic insult. Furthermore, increasing evidence suggests that patients who experience myocardial stunning are at increased risk of developing heart failure, after repeated periods of myocardial ischaemia (128), making an intervention which attenuates stunning equally important as treatments for myocardial infarction/necrosis.

2.2 Significance of ischaemic heart disease

Ultimately, CVD often leads to ischaemic heart disease (IHD), also known as

coronary artery disease (CAD), which is a group of conditions characterised by stable and unstable angina, and myocardial infarction (254). More than 17 million deaths are attributed to CVD, of which IHD is responsible for 7 million fatalities, each year. The demographic of patients who are hospitalised due to acute myocardial infarction (AMI) has increased from 63.5 years of age (1975) to 70.8 years of age (2005), based on a 30-year retrospective study in Worchester, MA (79). Interestingly, this study also reported that in 2005, patients were also more likely to be female with a history of obesity, diabetes, hypertension and/or stroke (79). However, the incidence of hospitalisations steadily decreased from 2001 to 2005, and survival rates improved from 81% in 1975 to 91% in 2005 after non-lethal MI, specifically. This is likely a result of newer technologies to analysing cardiac function and medications, hence the importance of monitoring patients who are at a higher risk of CVD. Still, a major failing remains in the absence of effective cardioprotective therapies to counter the damage arising in the heart with IHD and specifically infarction. Therefore, this thesis largely focuses on this area of research, and subsequent discussion addresses: the roles of ROS; development of occlusive ischaemia via atherosclerosis; infarction and IR injury, and the concept of 'cardioprotection'.

2.3 Monitoring cardiovascular function

In vivo non-invasive methods of cardiac function analysis are usually preferred in a clinical setting. Furthermore, the ever-rising prevalence of cardiovascular disease (CVD) demands earlier screening for such conditions. Early indications of CVD include thickening of vessel walls, hypertrophy of the left ventricle, stenosis, cardiac function and haemodynamics, all of which can be investigated using echocardiography. Echocardiography is widely used both clinically and in a laboratory setting, for diagnosis and to monitor structure and function, and recovery after ischaemia. Catheterisation is an invasive procedure whereby, in a clinical setting, a catheter is inserted through the radial or femoral artery and fed through the vasculature to the heart. In a laboratory, this procedure can be mimicked for rats (and other small animals) using a small Millar catheter, which can be useful to determine modifications in cardiac and vascular function.

2.3.1 Echocardiography

Echocardiography is a clinically relevant and accurate tool that can be used for the assessment of heart structure and function. The assessment of heart function is accomplished by tracking the movement of cardiac tissue and blood velocity, and can be vital in revealing cardiovascular disease (111). This tool is also important in measuring physiological changes in cardiac function. For example, the heart undergoes cardiac remodelling to achieve increased demands in athletes, and some unrelated conditions can affect baseline heart function, such as in individuals with Gilbert's syndrome. Gilbert's syndrome is an asymptomatic condition typified by elevated levels of bilirubin, a by-product of haem catabolism. Hypertension is another common CVD, the incidence of which is reduced in the presence of elevated bilirubin in humans and

animals (50, 170). Furthermore, these individuals show significantly reduced intima-media thickness in the aorta and carotid artery, reducing the risk of atherosclerosis and stenosis of the vessels (61, 67). Echocardiography can therefore, be used both descriptively and diagnostically.

Echocardiography is an ultrasound designed specifically for assessment of the heart; therefore the mechanisms are similar. Briefly, ultrasounds require high frequency sound waves to be transmitted through tissue (106). These waves travel for a given time (and depth) until they are reflected back by tissues and/or red blood cells. The speed of ultrasound waves in the tissues remains constant therefore; a simple relationship between roundtrip travel time and the depth of tissues relative to the transducer face exists. The area of tissue that ultrasound focuses on is called the sample volume, therefore, the distance to the sample volume (and target tissue) equals the ultrasound speed divided by round trip travel time (111). Using this technology, basic images of tissue density can be created in one (M-mode) and two dimensions (B-mode). Technology called pulsed wave Doppler, relies on the same principle in addition to the "Doppler effect" which assumes that structures moving towards the transducer compress sound when it is reflected, the extent of which is proportional to the velocity of the moving object. In biology, doppler echocardiography can assess the movement of red blood cells and therefore blood flow, non-invasively. A brief description of each of these applications is given below.

i) Motion mode (M-Mode)

Motion mode, or M-mode, has a high temporal resolution (1000 Hz) and it used to show motion of the heart tissue in one dimension. To conceptualise m-mode, one

must imagine the movement of the heart structures through a pinprick beam within a particular plane. Diagnostically, this type of ultrasound presents temporal changes in echoes, where the depths of echo-producing interfaces are displayed along the Y axis, against time (69) (Figure 2.3). M-Mode also allows for the measurement of heart structure (e.g. anterior wall thicknesses during diastole and systole). M-mode can be used to assess four views in particular; parasternal long axis and parasternal short axis of the heart, and to obtain left ventricular anterior wall and posterior wall thicknesses during systole and diastole (LVAW;s LVAW;d, LVPW;s, LVPW;d). In addition, the left ventricular internal dimension (distance between endocardial borders) during systole and diastole (LVID;s, LVID;d) can be measured. In obtaining views in both long and short axis, images are captured at the point where papillary muscles transect the M-mode beam in an attempt to assess these variables at a specific and consistent anatomical position.

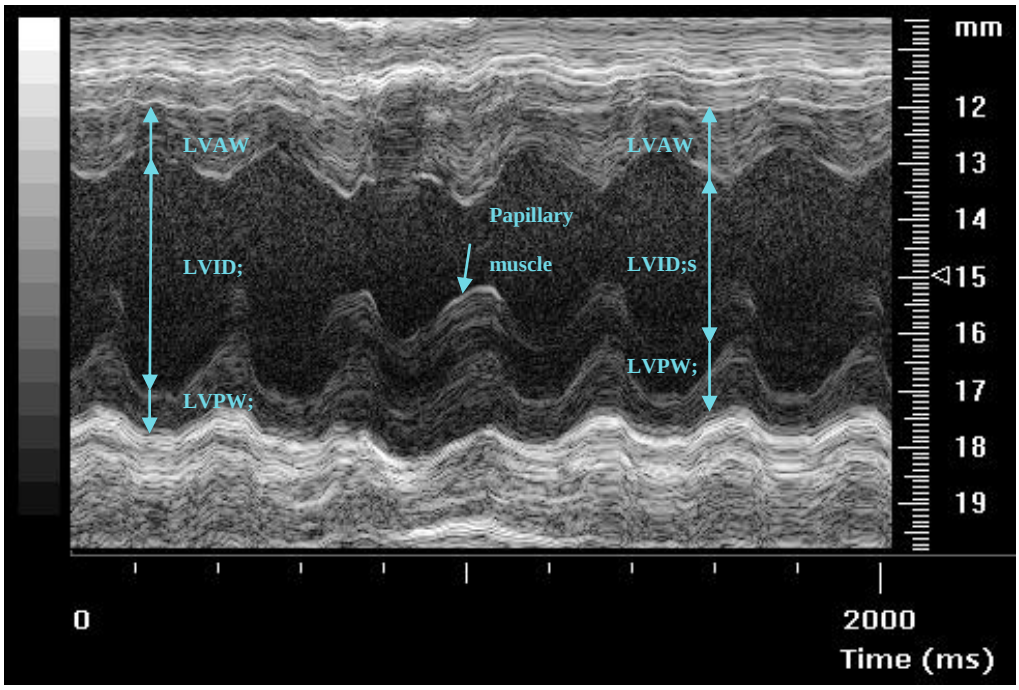


Figure 2.3: M-mode long axis view of aged male mouse heart

ii) Brightness mode (B-mode)

Brightness mode, B-mode, produces an image of bright dots on an oscilloscope screen that represent echoes transmitted back to the transducer in two dimensions, where brighter dots indicate denser tissue and a stronger echo being received. This particular mode receives echoes from a 2D single plane as opposed to movement in one dimension in M-mode.(106). This view allows measurement of both structural and functional parameters, as well as aortic dilatation (LVOT, left ventricular outflow tract).

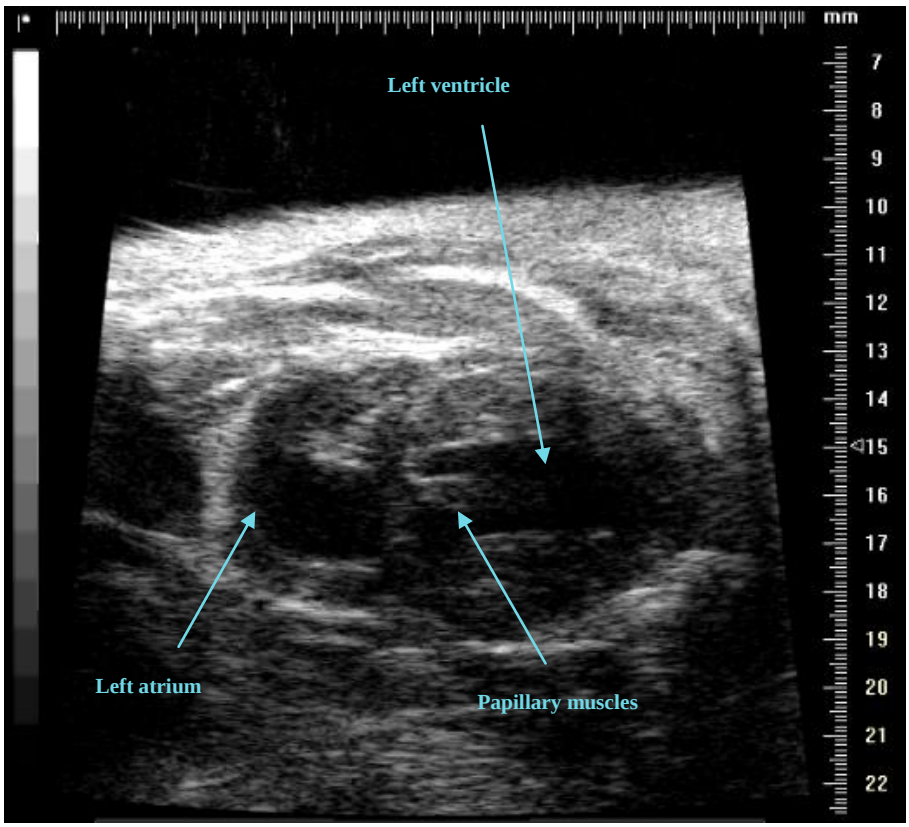


Figure 2.4: B-mode long axis view of young male mouse heart

iii) Pulsed wave Doppler

Pulsed wave Doppler emits ultrasound in pulses, based on a specified sample volume along the ultrasound beam, which the operator chooses; the frequency shift data for this volume is used and the rest is essentially "ignored". Although pulsed wave Doppler can accurately determine the depth of tissue from where it is receiving signals from, it is limited to its ability in determine blood flow velocities (111). Pulsed wave Doppler can be used to assess peak velocity of blood flow within the left ventricle during filling (diastolic function). From this output mitral valve early filling and active filling peak velocities (MV E, MV A) can be calculated as well as the isovolumetric relaxation and contraction times (IVRT, IVCT). In addition, pulsed wave Doppler can be used in the suprasternal view to measure peak velocity of blood flow (aortic velocity time integral [AoVTI] parameters) leaving the left ventricle (in the aorta) as a marker of systolic function.

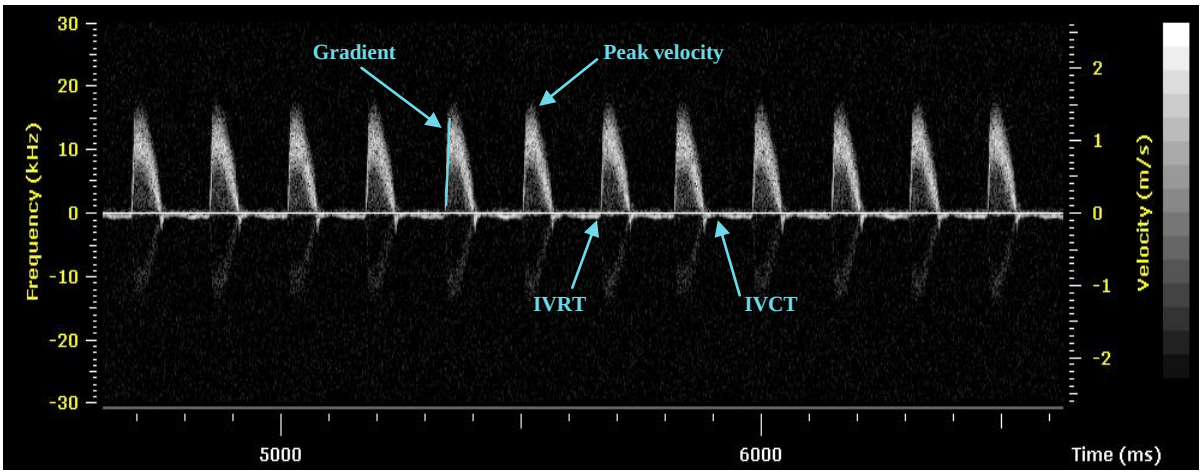


Figure 2.5: Pulsed wave Doppler output at the root of the aorta in young male mouse heart

2.3.2 Millar catheterisation

Catheters have been used to monitor and deliver treatment to the coronary circulation for many years, in the clinic, although they are more invasive than echocardiography. Once the catheter has been inserted, either through the carotid, radial or femoral artery, pressure measurements can be taken. In addition, this procedure allows administration of an x-ray contrast compound for a cardiac angiography, which maps the vessels of the coronary circulation revealing injury or disruption. The use of similar catheterisation can be achieved in a laboratory using Millar catheters. For pressure measurements, a catheter that is attached to a pressure transducer is inserted into the carotid artery, fed into the aortic arch and the ascending aorta to obtain vascular pressure fluctuations in the large arteries. Furthermore, Millar catheterisation has the benefit of being able to measure ventricular pressure, by movement of the transducer through the aortic valve and into the left ventricle. Measurement of ventricular pressures is quite important, providing information on the rate of pressure development (systolic function) and relaxation (diastolic function) in addition to the calculation of systolic pressures required for ejection (and therefore, afterload). Although catheterisation is primarily used clinically in the presence of CVD, changes in these parameters can be a result of physiological or pathological impact on the heart.

2.4 Experimental analysis of cardiac function

Ex vivo function of the heart represents a valuable tool in research. In this case, bilirubin can be infused to assess both baseline function and resistance to ischaemia-reperfusion injury. Furthermore, this method complements the use of echocardiography, because assessment of heart function can be conducted in the absence of neuro-hormonal input, therefore providing an assessment of intrinsic heart function and

resistance to ischaemia-reperfusion injury. The Langendorff perfused heart model allows assessment of the isolated heart, both with and without intervention.

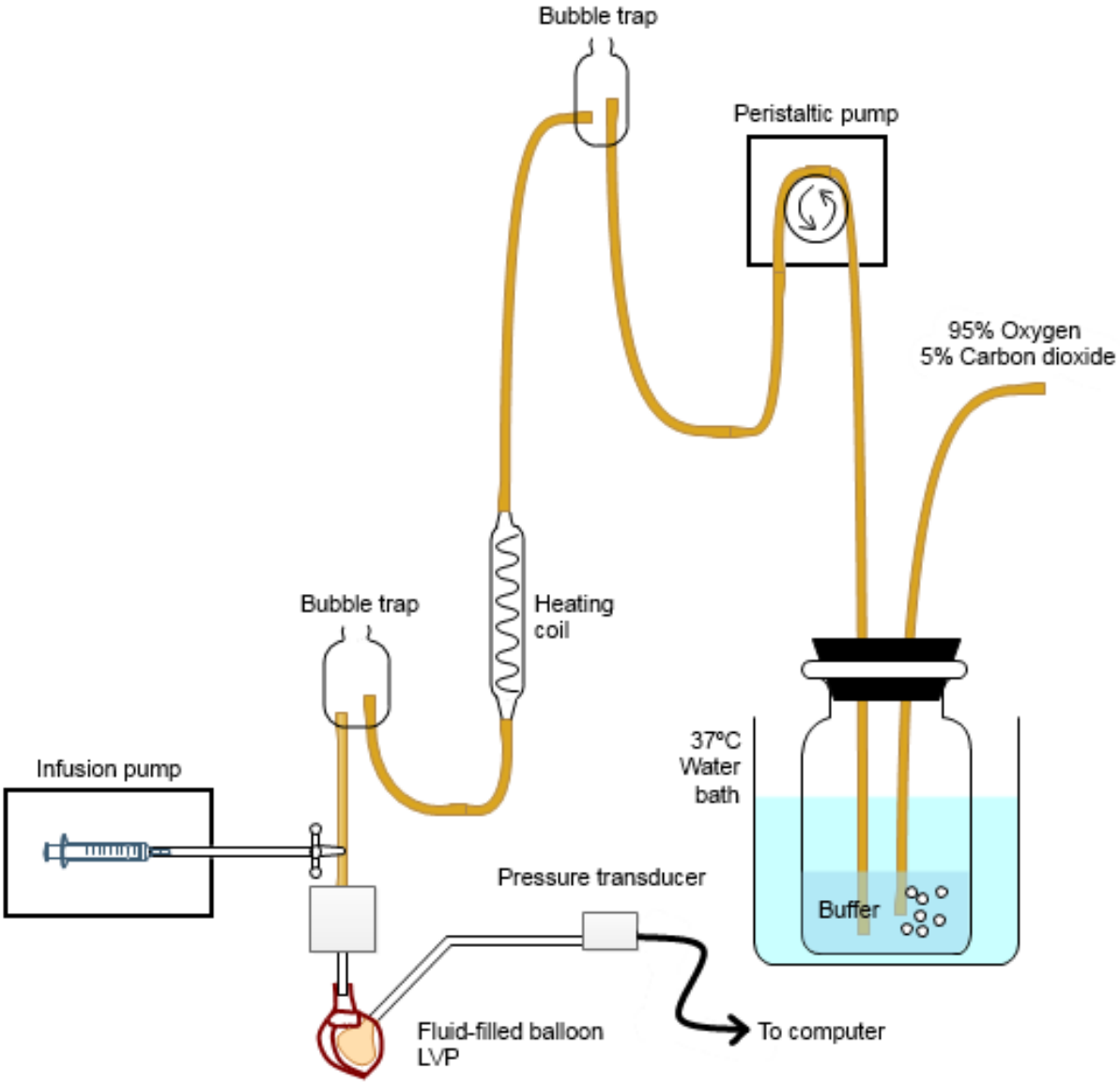
2.4.1 Langendorff perfused heart

The Langendorff perfused heart model was first developed by Oskar Langendoff in 1895, with the aim of assessing isolated heart function (271). This perfusion model has numerous advantages including the ability to assess many pharmacological, biochemical, physiological or morphological effects on cardiac function. The technique requires the insertion of a cannula through the ascending aorta, and it is here that a physiological perfusion solution (Krebs-Henseleit solution) enters the coronary circulation of the heart (192). Typically, the heart is perfused via either constant flow or pressure, with the oxygenated Krebs-Henseleit solution at 37ºC. This perfused heart model can determine diastolic pressure (DP), systolic pressure (SP), left ventricular developed pressure (LVDP), rate of pressure development during contraction (+dP/dT), rate of pressure reduction during relaxation (-dP/dT), rate pressure product (RPP) and coronary perfusion rate. The RPP of a heart is an indicator of the oxygen consumption based on the beats per minute and the pressure development). Hearts can also be stained using this apparatus to allow quantification of infarct size. Clinically, maximising cardiac function and minimising infarct size post-ischaemia are the primary objectives to target therapy.

Table 2.1: Compounds used in preparing Krebs-Henseleit buffer

	MW	mM	For 5 L (g)
NaCl	58.4	118	34.48
Glucose	180.16	11	9.91
$NaHCO_3$	84.01	25	10.50
KCl	74.55	4.7	1.75
$MgSO_4$	246.48	1.2	1.48
EDTA	372.24	0.5	0.93
$CaCl_2$	147.02	1.75	1.29

NaCl, sodium chloride; $NaHCO_3$, sodium bicarbonate; KCl, potassium chloride; $MgSO_4$, magnesium sulphate; EDTA, Ethylenediaminetetraacetic acid; $CaCl_2$, calcium chloride



LVP, left ventricular pressure

Figure 2.6: Schematic image of Langendorff perfused heart model

2.5 Role of reactive oxygen species in chronic disease

Reactive oxygen and nitrogen species (RONS) include molecules known as 'free radicals' such as superoxide ($O_2^{\bullet}$), the hydroxyl radical ($OH^{\bullet}$) and oxidants including hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and hypochlorous acid (HOCl) (222). These molecules are generally defined by the presence of unpaired valence shells making them highly reactive (free radicals), or a capacity to liberate free radicals

(oxidants). These compounds are implicated as major contributors to the development of chronic disease. Under normal physiological conditions RONS are produced continuously as a consequence of mitochondrial respiration, and these molecules are neutralised by an array of simple and enzymatic antioxidant defences (Stocker, 2004). However when the production of RONS exceeds the capacity of biological systems to neuralise them, a condition of 'oxidative stress' develops, which can lead to free radicals damaging proteins, lipids and DNA within cells. Therefore, the extent of oxidative stress can be assessed by measuring markers of oxidant damage in various matrices. Furthermore, when RONS production is increased, they may damage mitochondria, which then uncouples the synthesis of adenosine tri-phostphate (ATP).

Reactive oxygen and nitrogen species also initiate the extrinsic apoptotic pathway (252), triggering cell death, and other processes that are part of an immune response. Oedema has also been cited as a characteristic of inflammation (252), which increases endothelial permeability. Adenosine tri-phosphate (ATP) is a critical source of energy that powers many cellular processes, the absence or deficiency of which causes massive cellular disruption and death (252). Reactive oxygen and nitrogen species are present, or produced, as a result of certain lifestyle behaviours (for example, consuming a diet high in sugar/saturated fatty acids). In recent years, studies and reviews have highlighted the benefits of consuming antioxidants (21, 31, 142), although the relationship between the consumption of a nutritious diet and good health dates back to at least 500 BC (21). Cardiovascular disease is just one of the chronic diseases that are prevented/reduced by the consumption of antioxidants. For example, animals fed lyophilised apples (a source of vitamin C and several other compounds that have antioxidant properties) show signs of cholesteral-lowering properties (6). Furthermore,

the authors suggest reduced lipid peroxidation due to excretion of reduced malondialdehyde (MDA; a metabolite of lipid peroxidation) (6). Research investigating effects of consumption of antioxidants have become tainted due to a number of poorly structured clinical trials, however the benefits of administering such compounds in the presence of events which produce a sudden peak of RONS (such as ischaemia) is still promising.

During ischaemia, oxygen delivery to an organ/limb is decreased or ceased; therefore anaerobic respiration predominates within the ischaemic region and is ultimately unable to meet the metabolic requirements of the tissue. A number of processes during ischaemia lead to the formation of RONS production, including the metabolism of adenosine through to hypoxanthine and its oxidation to xanthine, liberating superoxide radicals (186, 256), which then inflicts oxidative damage on the cell. The appearance of this damage, which can be measured biochemically, is associated with impaired cellular function and cell death, when biological systems are exposed to oxidative stress (223). Reestablishment of blood flow (and oxygenation) - reperfusion - is vital to salvaging tissue, however a major excess of free radical production occurs as a result (223). Due to the high concentration of oxygen radicals introduced, an inflammatory cascade is activated leading to disruption of cell membranes, calcium influx, and ultimately, arrhythmias, dysfunction and cell death (37, 155). Ischaemia-reperfusion is typically associated with massive and permanent cellular damage and subsequent cell death (necrosis). Such a phenomenon can be observed histologically in the heart, after reperfusion, by the presence of an infarct and reduced myocardial systolic function (274).

2.5.1 Endothelial dysfunction leading to CVD

Diseases of the vasculature can produce peripheral dysfunction, damage to other organs (such as chronic kidney disease) or lead to myocardial infarction and increase strain on the heart. Unimpeded function of the vasculature, particularly of the coronary circulation which perfuses the heart, is critical to the functioning of the heart. Lining the vessels of both the circulatory and lymphatic systems are endothelial cells. These cells create a semi-permeable barrier, as well as contain proteins and signalling capabilities to initiate inflammation, angiogenesis, platelet coagulation, vascular tone and several other processes to maintain haemostasis. Endothelial dysfunction (ED), defined by an imbalance in vasoconstricting and vasodilating factors, is speculated to be an early determinant and trigger of chronic disease, leading to CVD and hypertension (60). Nitric oxide (NO) is an important endothelium-derived vasodilator, the deficiency and inhibition of which has been associated with chronic kidney disease leading to CVD (15). In addition, NO reduces platelet and leukocyte adhesion and migration, and vascular smooth muscle cell proliferation, all of which are contributing factors to the development of atherosclerosis (78). Reactive oxygen species (ROS) are often implicated in contributing to endothelial dysfunction (60, 78, 162), by inducing the action of nuclear factor-κB (NF-κB) and reducing NO synthase activity (78), in addition to directly interacting with and reducing the bioavailability of NO. Due to a decrease in NO synthesis/bioavailability, NF-κB remains uninhibited resulting in the upregulation of endothelial adhesion molecules (60), another contributing factor to atherogenesis. Since ROS induce pro-inflammatory actions of the endothelium, it is not surprising that antioxidants reduce the expression of adhesion molecules in endothelial cells both *in vitro* and *in vivo* (60). Although NO is a powerful mediator of ED, in the presence of superoxide, it forms peroxynitrite, a potent oxidant. Even in the presence of superoxide

dismutase, an antioxidant enzyme responsible for the dismutation of superoxide, the reaction between NO and superoxide occurs 3-4 times more faster than SOD's ability to quench superoxide (162). Therefore, production of peroxinitrite from NO is favoured over dismutation to H_2O_2. In addition, peroxynitrite has inhibitory effects on prostacyclin synthase (273), another endothelium-derived vasodilator, and NO synthase (132, 272). Like most ROS, peroxynitrite can cause oxidative damage to macromolecules and DNA, which has the potential to impair cellular function (16).

Endothelin-1 (ET-1) is a potent vasoconstrictor and pro-inflammatory peptide and may be responsible for the development of ED, which exerts its actions in reciprocity with NO (23). In conditions where NO synthesis has been inhibited, ET-1 often causes hypertension and vascular injury (60). Not surprisingly, ET-1 is elevated in patients suffering from atherosclerosis (136) and cardiovascular disease (75). Furthermore, smooth muscle cell proliferation, as present in atherosclerosis, is induced by ET-1, and improved with antioxidant treatment (133). Animal models of hypertension, such as the DOCA-salt rat model, express high levels of ET-1 in vessel walls (201). More recent studies have implicated ET-1 as a key factor in pre-eclampsia, a disease in pregnant women, typified by hypertension and proteinuria (113). This condition predisposed both mother and baby to cardiovascular risk later in life (113). Reducing endothelial dysfunction, with antioxidants or other compounds, could play an important role in effectively reducing the prevalence of cardiovascular disease.

2.5.2 Atherosclerosis and coronary occlusion in IHD

Atherosclerosis is the most prevalent form of CVD, and is a common precursor to myocardial ischaemia and infarction. This condition has afflicted humans for many

thousands of years, and has been found in the aorta of Egyptian mummies, with exactly the same characteristics as in patients today (211). Atherogenesis is hypothesised to be initiated by 3 different factors, namely; lipid retention, lipid oxidation and endothelial dysfunction, resulting in chronic inflammation and sclerotic plaque development, leading to narrowing of the vessels and stenosis (109). A primary risk factor for the development of atherosclerosis is the total and low-density lipoprotein (LDL) cholesterol concentration (60, 80, 109). During the early stages of atheroscerolsis LDL, a lipid carrying protein, can be oxidised (oxLDL) and penetrate the intima of the vessel, binding to the proteoglycan matrix. Low-density lipoproteins (LDL) stimulate a powerful inflammatory response once oxidised, which can be a pathological result of increased free radicals or enzyme modification. For example, phospholipase 2 (PLA2) triggers this oxidation (72), and is found in sclerotic plaque as well as in normal vessels (80). In addition, malondialdehyde (MDA) is also a by-product produced during LDL oxidation, which can bind to macromolecules and DNA (160).

The presence of oxLDL triggers the activation of endothelial cells, leading to the secretion of adhesion molecules and chemokines, such as tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6). This inflammatory response is responsible for recruiting macrophages, lymphocytes and neutrophils to the area (109). As macrophages attempt to endocytose and break down oxLDL, they transform into foam cells, which stimulate local inflammatory responses, leading to recruitment of additional inflammatory cells to the area. The smooth muscle cells of the normal vessel also secrete proteoglycans, collagen and elastin, which all contribute to development of fibrous connective tissue in the local area (109). It is important to note that during the early stages of atherosclerosis, these lesions may be minute, not becoming visible until years later

(109). As the condition progresses, smooth muscle cells proliferate and marcophages necrose (provoking an inflammatory response), ultimately forming a necrotic core, usually constituting 30-50% of the total plaque volume (109). Large vessels undergo vascular remodelling when plaques become prominent, however when 40% of the vessel becomes occluded its haemodynamic capacity is reduced, limiting vascular flow distal to the area affected (109). Such plaques eventually become clinically significant, and at risk of rupture, resulting in down-stream ischaemia. A thin fibrous or eroded plaque cap is at a higher risk of rupturing, revealing the thromobogenic core (109). It is generally accepted that the process of atherogenesis is relatively slow. In fact, a recent article has suggested that its development begins during pre-pubescence, with the patients suffering this disease over 55 years of age (109, 227, 228). Incidentally, the >55 year old cohort is also more susceptible to myocardial infarction (109). These studies highlight the importance of maintaining cardiovascular health during adolescence as well as adulthood, by lowering cholesterol and increasing exercise (232).

2.6 Cardioprotection

Cardioprotection is a phenomenon that is not completely understood, but is thought to be an evolutionary response to free radical and toxins (80), which trigger protective mechanisms and pathways during an ischaemic or other injurious insult. Resistance to ischaemia is in part determined by the availability of nutrient substrate reserves within the tissue. Cardiac and renal tissue have the capacity to withstand longer time periods (ie. 20-25 minutes) of ischaemia compared to the brain, which undergoes irreversible damage within a few minutes (213).

Ischaemic pre-conditioning (IPC) is perhaps the most widely understood form of

cardioprotection and has been comprehensively described in several reports (83, 101, 103, 183, 205, 242, 260). Furthermore, this form of cardioprotection may represent a clinically relevant therapeutic tool and is evident in persons experiencing angina (70). Ischaemic preconditioning is a process where the blood supply to an organ is deliberately and acutely occluded for less than 5 minutes, followed by reperfusion. This process can be completed in several cycles to enhance the protective effect. In other words, this response reflects homeostasis, whereby a mild non-injurious stress provides enhanced tolerance to subsequent potentially injurious stress (164). However, with increased duration of ischaemia (ie. >20 minutes), patients experience significant risk of losing viable tissue through cell death (241). The preconditioning effect is nullified if the subsequent ischaemic insult exceeds 3 hours in duration (164). Interestingly, several reports have found that hearts remain in a 'classic' preconditioned state for between 1-4 hours, depending on species and the degree of consciousness (42, 165, 197). A Second Window Of Protection (SWOP) exists between 12-72 hours post-ischaemia, which is less protective and is believed to be mediated by transcription and translation of genes regulating cell survival pathways (147, 260). Although these protective pathways have not been entirely characterised, this protective process consists of 4 main targets; initial triggers, transduction pathways, memory mechanisms, and end-effectors (260). The triggers stimulate protective pathways, while the transduction signal (eg. PI3K, Akt) and memory targets (eg. protein kinase C) keep the heart in a preconditioned state, until the end effectors (eg. heat shock proteins) instigate protection during the severe ischaemic event (260).

In 1990, Thornton *et al* administered a protein synthesis inhibitor, and noted that the preconditioning effect was still present, suggesting this phenomenon does not

require the modification of protein expression (233). These findings have since been expanded; protein synthesis may not be involved in 'classic' preconditioning, considering these mechanisms are initiated after ~10 minutes of ischaemia and protein expression is unlikely to be altered during this time. However the SWOP can be suppressed using a protein inhibitor, and therefore involves a change in protein expression. This would also explain why SWOP is delayed (119). Several studies have concluded that preconditioning the heart improves tolerance to ischaemia by delaying cell death by an additional ~20-30 minutes, rather than avoiding it all together (212, 260).

A number of G-protein coupled receptors (GPCRs) are implicated in triggering preconditioning, including opioid and adenosine receptors. Adenosine appears to play a particularly important role in cardioprotection, and was thought to act via binding to A_1- and A_3-receptors (44, 234). However, it is now clear the adenosine A_{2A} and potentially A_{2B} receptors play critical roles. Adenosine has also been shown to couple and interact directly with protein kinase C (PKC) (65), which is suggested to be a mediator of cardioprotection. The therapeutic efficacy of adenosine administration in models of cardiac I-R has led to the administration of pharmacological A_1/A_3 receptor agonists in several studies (119, 192). Although results have yielded generally positive results, they remain inconclusive, perhaps due to the instability of the compound itself (119). The activation of these receptors stimulates the activity of potassium channels, resulting in hyperpolerisation of the sarcolemma and inhibition of calcium influx (188, 242). This response effectively reduces energy consumption, prevents mitochondrial dysfunction and protease activation, which in turn preserves available energy stores to maintain ionic equilibrium (242). Applying A_1/A_3 receptor antagonists negate these protective

effects, however only in higher concentrations (50μM) (119). Liu *et al* (143) first described the translocation of PKC from cytosol to the sarcolemma in rabbit myocardium in response to ischaemia, providing evidence of 'memory', since PKC then remains in the sarcolemma until the time of an severe ischaemic episode. Although the initial study by Liu *et al* has since been replicated in small animals, the effect appears to be lost in larger animals and it is still unclear which isoenzymes of PKC, if any, are actually involved in the preconditioning process. Interestingly, ROS play an integral part in initiating a preconditioned state and may occur through direct activation of PKC. Furthermore, preconditioning can be completely abolished through administration of free radical scavengers such as superoxide dismutase (259). Although cardioprotection appears to be inducible, the intrinsic mechanisms remain poorly characterised. Therefore, the discovery of novel cardioprotective agents and their mechanisms of action remain popular targets of research.

2.7 Current therapeutic areas of research

Current drug treatment for patients who have experienced myocardial infarction (MI) primarily involves 'reperfusion therapy. Thrombolytic agents are used to facilitate dissolution of occlusions and reduce risk of subsequent clot formation (260). The efficiency of thrombolytics was extensively investigated through the Thrombolytic Inhibition of Myocardial Ischaemia (TIMI) program funded by the NIH and were shown to be successful in reducing infarct size, however remained ineffective in limiting the actual ischaemic event (5). In addition, β-blockers, which block the action of stress responses mediated by adrenaline, are used to manage angina, as well as potentially benefiting MI (13). Although these drugs have been successful in reducing the recurrence of ischaemia, both compounds are used to manage symptoms and neither relieves the underlying conditions that could cause a MI, nor do they treat the actual MI. Cardioprotective therapy to ameliorate the cardiac damage arising during ischaemia-reperfusion remains a highly sought after yet unrealised goal.

Since the first characterisation of non-lethal ischaemia, and associated pre-conditioning described by Murry *et al* in (1986), there has been considerable curiosity regarding the potential to therapeutically mimic IPC (164). Recently, the Cyclosporine to ImpRove Clinical oUtcome in ST-elevation myocardial infarction (CIRCUS) trial attempted the use of cyclosporine (a drug commonly used to reduce the risk of organ rejection) as a pre-treatment in the moments before the MI. However, it was not able to improve the clinical outcomes of the patients in the 12 months following (58). Although very few investigated compounds induce a clinically significant effect, published studies have been critical to our understanding of intrinsic protective pathways and mechanisms within the heart. As a result, additional targets inducing cardioprotection

have been discovered (205). For example, heat shock proteins (HSPs) which improve cellular defences against stress may represent an important therapeutic target (18). A recurring hurdle in developing cardioprotective agents includes the delivery of such agents into the intracellular space, where ROS are being produced (55). These heat shock proteins are induced physiologically by stress, therefore require hours to accumulate within the cytosol, and are able to repair misfolded proteins or promote degradation while minimising collateral damage (18). Several HSPs have been considered to have protective effects during myocardial ischaemia, including HSP90, HSP84, HSP27, HSP70 and HSP32. Marber and colleagues (148, 149) have demonstrated that transgenic HSP70 expression reduces contractile dysfunction (Figure 2.4) and infarct size after 20 minutes ischaemia. Haem oxygenase-1, also a heat shock protein (formally known as HSP32), reduces infarct size and improves cardiac performance when administered (55, 153). Unfortunately, HSPs are upregulated physiologically in the presence of stress, therefore inducing their activity can reflect the progression of disease, which can confounds some research. With this in mind, it is important to note that upregulation of heat shock factor (HSFs), which are transcriptional factors responsible for HSP expression, remain plausible methods for further increasing the activity of HSPs without introducing additional stress. To provide an example, heat shock factor-1(HSF1) lays dormant and bound to HSPs. Under stress conditions such as ischaemia, HSF1 is phosphorylated, ultimately allowing it to move to the nucleus and bind DNA (152, 187). Although findings in patients experiencing myocardial infarction are limited, upregulation of HSF1 in animal models is protective in the brain (152, 237). Furthermore, HSF1 abundance is increased by 150% in left ventricular tissue in rats after coronary artery ligation (152), further suggesting a role in modulating cellular I-R tolerance. Clinically, the effect of remote ischaemic

preconditioning on clinical outcomes (ERICCA) trial aimed to improve the outcomes after a coronary artery bypass (93). After the patient was under anaesthesia, four 5 minute inflations and deflations of a blood pressure cuff were performed. Unfortunately, there was no significant difference in the end-points hypothesised (cardiovascular death, non-fatal myocardial infarction, coronary revascularisation and stroke). Furthermore, troponin T (a marker of cardiovascular death) was not significantly different between groups (92).

Unfortunately, MI usually occurs spontaneously, which makes most 'preconditioning' therapies impractical, and emphasises the need for treatment options during reperfusion, after the ischaemic event has occurred. Previous experimental studies (99, 100) have shown that administering agents in the first few minutes of reperfusion can assist organ recovery by decreasing the magnitude of oxidative stress and reducing the incidence of reperfusion arrhythmias. Hearse *et al.* (101) argued that oxidative stress, which occurs primarily during reperfusion, plays an important role in myocardial dysfunction and the administration of cardioprotective agents can reduce the severity of ischaemic injury (101). No post-treatment compounds have been trialled in the clinic. However, postconditioning, by briefly allowing reperfusion before impeding blood flow again, has seen some success in clinical trials (30, 216, 231). In 2005, Staat *et al.* investigated the postconditioning effect of four episodes of 1-minute inflation (occlusion) and 1-minute deflation (reperfusion) cycles in coronary angioplasty patients, and found ~36% reduction in infarct size and significantly reduced creatine kinase release, a biomarker of tissue damage (216). In another study, an experimental group underwent remote ischaemic postconditioning (ie. transient ischaemia-reperfusion of the upper arm) in a cycle of four bouts of 5-minutes ischaemia and 5-minutes reperfusion

(applied by blood pressure cuff inflation and deflation) in the ambulance en route to hospital. All patients then received angioplasty upon arrival at the hospital, leading to salvage of 16% more tissue than in the non-intervention group (30). However, a more recent meta-analysis performed by Zhang *et al*, claimed that remote preconditioning does not significantly improve outcomes in CABG patients (267).

2.7.1 Antioxidant therapy for cardioprotection

Considering the major role that ROS play in major forms of CVD, including IHD/myocardial infarction, a rational basis exists for the investigation of antioxidants as potential cardioprotective agents. In addition to HO-1 (see Section 2.5), several other endogenous antioxidants appear to improve characteristics of cardiovascular disease. Extracellular superoxide dismutase (EC-SOD) is involved to the dismutation of superoxide to oxygen (O_2) and hydrogen peroxide (H_2O_2), and is proposed to be present in vasculature to improve the bioavailability of NO (178). Chu *et al* showed that gene transfer therapy of EC-SOD in spontaneously hypertensive rats reduced arterial pressure by 25% (53). Superoxide dismutases also protect against myocardial infarction when up-regulated in rats (3) and rabbits (139). Catalase (*Cat*) and glutathione peroxidise (*GPx*) are involved in detoxifying H_2O_2 to water and oxygen, and have shown beneficial effects in attenuating ischaemia-reperfusion injury in mouse hearts overexpressing these antioxidants (138, 264). In addition to Cat and GPx, which are involved in dissociating damaging ROS, other antioxidants such as vitamin E and bilirubin act by binding ROS, making them unable to bind to macromolecules such as DNA, lipids and proteins. For example, lipoprotein-associated phospholipase A_2 (Lp-PLA_2) hydrolyses oxidised phospholipids, which inhibits their pro-inflammatory effects. Similarly, guanosine 5'-triphosphate cyclohydrolase (GTPCH 1) indirectly promotes NO synthesis for

vasodilatation. Vitamin A, C and E are also antioxidants which could improve post-ischaemic outcomes. For example, Sagach *et al.* showed that Trolox, a water-soluble analogue of Vitamin E, improves post-ischaemic cardiac functional recovery and reduces Thiobarbituric acid reactive substances (TBARS; a by-product of lipid peroxidation) when administered *ex vivo* (198). Antioxidants which can ultimately lower free oxidants and their actions represent promising avenues of cardioprotective therapy (Figure 2.7). Haem oxygenase-1 (HO-1) and its metabolites present an intriguing target in cardiovascular therapy.

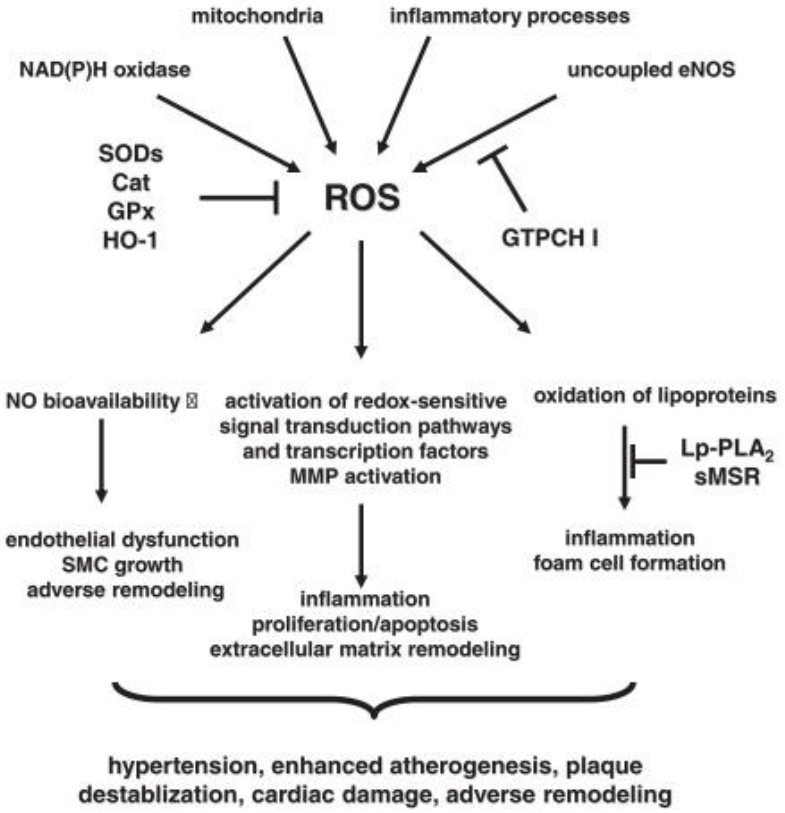


Figure 2.7: Overview of the sources and effects of ROS and targets for antioxidant therapy. eNOS, endothelial NO synthase; SODs, superoxide dismutases; Cat, Catalase; GPx, Glutathione peroxidises; HO-1, Haem oxygenase 1; GTPCH 1, Guanosine 5'-triphosphate cyclohydrolase 1; MMP, matrix metalloproteinases; Lp-PLA_2, Lipoprotein-associated phospholipase A_2; sMSR, soluble macrophage scavenger receptor (137).

2.8 Clinical studies investigating cardiovascular health and bilirubin

The cardioprotective effects of bilirubin in animal models have been translated in clinical studies for over 2 decades. Since Schwertner *et al* (206) associated low bilirubin with increased risk of coronary artery disease (CAD), many other reports have implicated bilirubin as a biomarker of cardiovascular health (245). In 2000, Vitek *et al* reported that IHD prevalence approximated 12% in the general population and ~2% in individuals with Gilbert's syndrome (>40 years old) (243). In addition to these individual retrospective studies, the risk of ischaemic heart disease was reported to be decreased in men in a landmark meta-analysis. Novotný *et al*. reported that the association between serum bilirubin and prevalence of IHD was significantly negatively correlated ($r = -0.31$, $P<0.01$) (173). More specifically, studies show that higher serum bilirubin is linked with reduced intima media thickness (IMT) of the aorta (120) and carotid artery (73). Interestingly, IMT is also associated with hypertension which causes vascular remodelling and stenosis, and could in part explain reduced blood pressure in GS individuals. This hypothesis has been confirmed in publications reporting reduced risk of hypertension in individuals with higher serum bilirubin, particularly in females (50). Furthermore, recent reports link elevated bilirubin to reduced vascular stiffness and improved augmentation index, further supporting previous reports linking bilirubin with pre-clinical atherosclerosis (7). These findings present a mechanism by which the risk of atherosclerosis may be reduced under hyperbilirubinaemic conditions. In otherwise healthy patients, Rantner *et al.* reported reduced risk of peripheral artery disease in patients with high bilirubin, which supports these findings (191).

The accumulating evidence demonstrating a protective role for bilirubin in

attenuating CVDs has led to several investigations exploring similar relationships in other chronic human diseases, such as diabetes and kidney disease. Fukui *et al.* reported that serum bilirubin concentrations were higher in patients with type 2 diabetes, who are not undergoing haemodialysis compared to patients undergoing dialysis 3 times per week (82). Furthermore, this data suggests that free radicals, which are produced as the blood reacts with external dialysis membranes, may also react with bilirubin. Interestingly, for patients who suffer from type 2 diabetes, the risk of lower limb amputation is increased in patients with low circulating bilirubin levels (47). Amputation in diabetic patients is largely influenced by impaired microcirculatory perfusion at the level of the capillaries, suggesting that bilirubin preserves endothelial function, perhaps imparting its vasodilatory properties. Glycation is the bonding of proteins or lipids to sugar molecules, which are generally in higher concentrations in diabetic patients (215). Kalousová *et al* (121) reported reduced end-stage glycation products in GS individuals, delaying stenosis and atherogenesis (215).

Chronic kidney disease (CKD) is commonly associated with atherosclerosis, reducing renal perfusion and inducing the activation of the Renin-Aldosterone-Angiotensin system (RAAS) to promote hypertension. Whether renal artery atherosclerosis/stenosis represents the primary cause of hypertension or not, hypertension independently induces glomerular injury and progressive kidney dysfunction, leading to failure. Progressive deterioration of kidney function, leading to the development of azotemia, uraemia and requiring dialysis further encourages the atherosclerotic process (25), resulting in these patients ultimately succumbing to severe cardiovascular events. Elevated bilirubin could thus contribute to protection from both inter-related renal and cardiovascular disease. Persuasive data published by Chen *et al.*

demonstrated the role of high serum bilirubin in reducing cardiovascular events and all-cause mortality in 661 long-term dialysis patients (48). In this article, UGT1A1*28 polymorphic patients (possessing bilirubin concentrations indicative of Gilbert's Syndrome) showed ~90% fewer cardiovascular events during the 12 year duration of the study. Furthermore, all cause mortality was reduced by over 30% in high bilirubin patients (Figure 2.8) (48). Despite these surprisingly positive results, perhaps the most convincing evidence suggesting a bilirubin protective effect stems from a study performed by Horsfall *et al.*, who demonstrated all-cause mortality of Gilbert's syndrome individuals was reduced by >50%, compared to controls in a cohort of over 504 206 individuals.

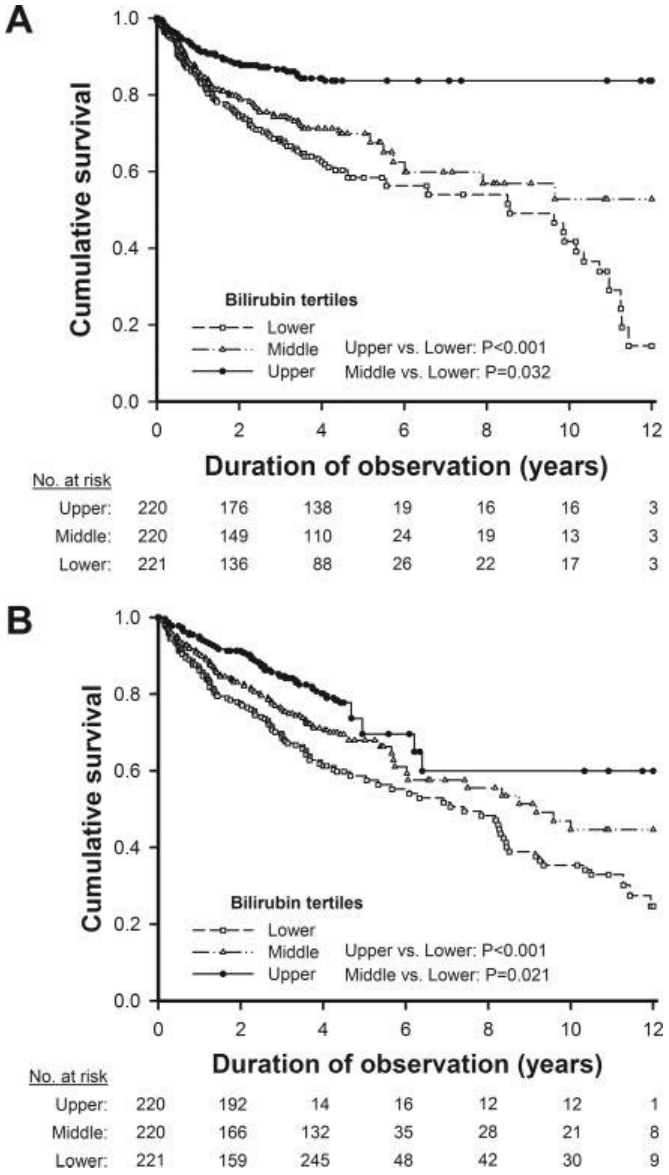


Figure 2.8: Kaplan-Meier survival analysis curves for endpoints of (A) the first cardiovascular event and (B) all cause mortality among 661 haemodialysis patients in relation to tertiles of serum bilirubin. Tertiles were distributed as follows; lower, 0.59±0.09 mg/dl; middle, 0.76±0.04 mg/dl; upper, 0.99±0.25 mg/dl (48).

2.9 Protective effects of haem oxygenase-1and its metabolites

Haem oxygenase-1 is a stress induced antioxidant enzyme responsible for the breakdown of haemoglobin. Haem is catabolised into biliverdin, carbon monoxide and iron by biliverdin reductase. Biliverdin is then rapidly reduced by biliverdin reductase (BVR) to bilirubin (Figure 2.9). Although bilirubin was initially considered a waste

product and neurotoxin, a growing body of work has implicated it (and its metabolites) as cytoprotective compounds.

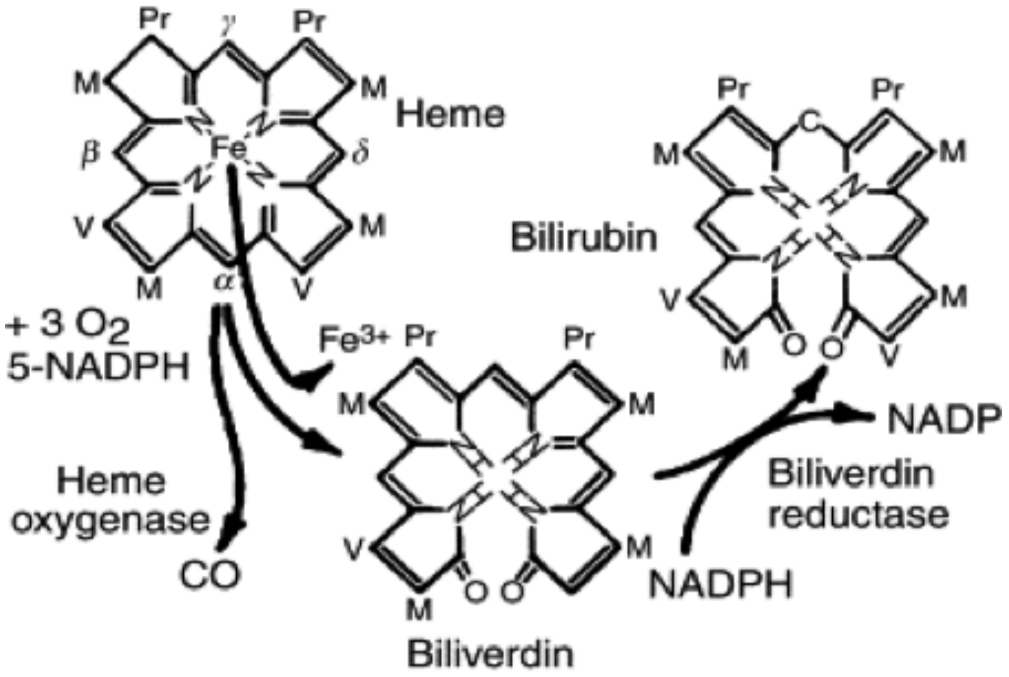


Figure 2.9: Schematic representing the biochemistry of enzymatic bilirubin formation (247)

As indicated previously, HO-1 is a heat stress protein (HSP) and an antioxidant enzyme. Haem oxygenase-1 is particularly important enzyme because heam, the substrate for HO-1, is accessible for catabolism during stress and trauma. Haem itself is a pro-oxidant molecule; therefore, HOs act as antioxidant molecules by removing pro-oxidants and producing antioxidant compounds. In 1994, Schwertner *et al.* first reported that low circulating bilirubin concentrations were associated with increased risk of coronary artery disease (CAD) in humans (206). These data also revealed that a 50% decrease in bilirubin was associated with a 47% increase in risk of CAD (206). Following this finding, Yet *et al.* have also demonstrated improvement in myocardial ischaemia-reperfusion injury in mice with cardiac specific overexpression of HO-1

(220, 262), in addition to increased right ventricular infarct development in HO-1 null mice (263). In addition, Masini *et al.* administered hemin (4 mg/kg), a HO-1 inducing compound (153), to Wistar rats and demonstrated reduced post-ischaemic infarct size, malondialdehyde production and arrhythmias. Furthermore, these effects were reversed by co-administration of a HO-1 inhibitor (153). The role of HO-1 has also been widely investigated in association with atherosclerosis, where elevated HO-1 consistently ameliorates atherosclerotic processes, including endothelial cell injury (257) and intimal thickening (220, 238, 239). While HO appears to induce protection through its antioxidant properties, the products carbon monoxide and biliverdin also demonstrate strong anti-inflammatory effects.

2.9.1 Carbon monoxide and inflammation

Carbon monoxide (CO), acknowledged as an anti-inflammatory molecule, is easily transported throughout tissues, allowing pervasive protection (137). Mechanisms potentially contributing to the protective effects of CO includes induction of ROS formation, which may lead to the induction of protective pathways (51, 169). Carbon monoxide has also been widely implicated in reducing endothelial injury and inflammation, particularly during haemorrhagic shock (169). Nassour *et al.* (169) investigated the effects of carbon monoxide releasing molecule (CORM) administration in mice after a 120 minutes haemorrhagic period (femoral arterial blood withdrawn until a mean arterial pressure of 25 mmHg). Administration of CORM reduced hepatic and renal injury and was accompanied by inhibition of pro-inflammatory cytokines and adhesion molecules (169).

2.9.2 Biliverdin and inflammation

Biliverdin possesses antioxidant and anti-inflammatory capacities (Table 2.2), and is rapidly reduced to bilirubin via the action of biliverdin reductase in mammals. Interestingly, in most species, biliverdin represents the terminal product of haem catabolism and is excreted intact, however it is possible that evolutionary selective pressure led to the reduction of biliverdin to bilirubin in humans (221). Increasing interest has been directed towards understanding the anti-inflammatory properties of biliverdin, given the clear presence of haem catabolism after trauma, which is accompanied by a pronounced inflammatory response. Biliverdin inhibits the expression and/or action of toll like receptor (TLR)-4 (249), complement receptor 5a (C5aR) (20) and other cytokine signalling pathways. There is growing interest in biliverdin reductase (BVR), involved in converting biliverdin to bilirubin (114), based upon its potential role in recycling biliverdin to bilirubin, once bilirubin is oxidised. In particular, Sedlak *et al* proposed that BVR plays an integral role in this 'antioxidant cycle', in the presence of H_2O_2, which converts bilirubin back to biliverdin (208). This redox cycle allows the improved availability of antioxidants (biliverdin) into the system to denature additional ROS, increasing the antioxidant outcome.

2.10 Genetic mutations linked to hyperbilirubinaemia

Hyperbilirubinaemia, a condition of elevated bilirubin concentrations in the circulation, can be associated with increased haem catabolism (ie. acute haemolytic events), genetic mutations in the UGT1A1 (glucuronosyl transferase 1A1 (56)) or ABCC2 [multidrug resistance protein 2 (203)] genes, and in patients experiencing hepatobiliary disease. While mutations in UGT1A1 prevent conjugation of bilirubin to glucuronic acid, with excess unconjugated bilirubin regurgitated back into the systemic circulation, ABCC2 gene mutations reduce expression of the MRP-2 (multi-drug resistance protein 2) transporter, reducing movement of bilirubin glucuronides into the biliary canaliculus, and reflux of conjugated bilirubin into the systemic circulation. Conditions of genetic aetiology are generally characterised by UCB concentrations greater than 17.1 µM (1 mg/dL) in serum, where conjugated bilirubin accounts for only 15-25% of the total bilirubin concentration (56). In the absence of genetic mutation in these genes, the concentrations of unconjugated to total bilirubin can also be used to differentiate between primary hepatic injury (elevated circulating unconjugated bilirubin) and biliary obstruction (elevated circulating conjugated bilirubin).

In humans, the gene encoding UGT1A1, is located on q37 of chromosome 2. A defect in this gene, caused by an additional TA repeat in the gene promoter (56), is associated with the development of Gilbert's syndrome (GS). Gilbert's syndrome is an autosomal recessive condition, characterised by mildly elevated bilirubin (>17.1 µM) due to a 70-80% reduction in hepatic activity of glucuronosyl transferase 1A1. Exclusion criteria for the diagnosis of this condition include the presence of haemolytic disorders (increased reticulocyte count/gross haemolysis) and liver disease (elevated hepatic transaminase activities in serum), and depending on ethnicity it is present in 3-

17% of the population (76). Although patients with GS have reported varied symptoms including fatigue, nausea and weight loss, clinical studies have yet to determine whether elevated bilirubin could induce such symptomology. However, an earlier study found that GS patients who also possessed hereditary spherocytosis (haemolytic anaemia), had increased risk of developing gall stones, which is often accompanied by abdominal pain and nausea (85). Crigler-Najjar syndrome (CNS) is an autonomic recessive condition, characterised by extremely elevated concentrations of unconjugated bilirubin. The condition exists in two forms: Type 1 (340-850μM), the more severe form due to complete absence of glucuronsyl transferase 1A1 activity; and Type 2 (120-340 μM), where extremely low, but still detectable UGT1A1 activity exists (226). Since the characterisation of this condition in 1952 (57), different mutations in the UGT1A1 gene have been shown to induce these syndromes. For example in CNS Type 1, genetic lesions are commonly accepted to be the cause, while point mutations are thought to be responsible for CNS Type 2 (52). Although this condition is severe, it is also considered rare with an estimated prevalence of 1:1000000 live births for both types (52). Unfortunately, most patients die within the first 18 months of birth, as a result of bilirubin-induced neurotoxication (kernicterus). Individuals with CNS that live beyond puberty and do not receive a liver transplant, ultimately die due to bilirubin-related encephalopathies (49, 57). Although the outcomes of CNS deficiencies are severe, mildly elevated concentrations of bilirubin, as seen in Gilbert's syndrome are associated with protection from chronic disease in several studies (41, 48, 141, 173, 243, 244). Gilbert's syndrome is associated with a 70-80% deficiency of glucuronosyltrasferase activity due to reduced transcription (ie. mutation occurs within the gene promoter, which drives transcription), therefore GS individuals possess functional UGT1A1 which differentiates it from Crigler-Najjar syndrome (226). This hyperbilirubinaemia affords

individuals improved antioxidant capacity to potentially combat chronic disease.

2.11 Antioxidant properties of bilirubin

In 1987, Stocker *et al* first reported that bilirubin may have physiologically important antioxidant effects (222). In this article, unconjugated bilirubin was shown to scavenge peroxyl radicals more potently than α-tocopherol. Furthermore, when bilirubin is bound to albumin, it retains its antioxidant capacity and contributes importantly to the antioxidant capacity of human plasma (219). Oxidative stress has been implicated within the pathogenesis of most chronic diseases, including cardiovascular diseases. Bilirubin's potent antioxidant capacity thus suggests it may present a promising cardioprotective agent. Interestingly, both unconjugated and conjugated forms of bilirubin contain multiple double bonds and a pair of hydrogen atoms at its C10 bridge, making it highly reactive to incumbent radical species (209, 223).

The enhanced antioxidant capacity of hyperbilirubinaemic patients has been well documented. In particular GS individuals, who only express mildly elevated levels of bilirubin, still possess significantly improved total antioxidant status compared to non-GS individuals (243). Interestingly, increasing unconjugated bilirubin concentration improved the total antioxidant capacity (243). Similarly, Bulmer *et al.* measured the Trolox equivalent antioxidant capacity (TEAC) and ferric reducing ability of plasma (FRAP) of GS serum and found they were significantly improved by 4.6% and 17.7%, respectively. Furthermore, this group demonstrated that GS serum had an improved resistance to copper oxidation, a process which may contribute to CVD (41). More recently, GS individuals have been shown to possess reduced oxLDL concentrations and an improved oxidative stress status based on the ratio of reduced:oxidised

glutathione and protein carbonyl concentrations (26). These data suggest that the elevated antioxidant capacity of hyperbilirubinaemic plasma may confer physiological resistance to systemic oxidative stress and thus protect from disease (245).

Table 2.2: Reactivity of the antioxidants bilirubin and biliverdin with different oxidants (adapted from Stocker, 2004) (223)

Radical/Oxidant	**Scavenging**
Superoxide anion radical (221)	No
Copper (26, 39, 261)	Yes
Hydrogen peroxide, alkyl hydroperoxides (221)	No
Quinones (71)	Yes
Peroxyl radicals (222)	Yes
alpha-tocopheroxyl radical (253)	Yes
Mixed function oxidase/ hydroxyl radical (98)	Yes
Hypochlorous acid (221)	Yes
Nitric oxide (124)	Yes
Peroxynitrate (124)	Yes
Nitroxyl radical (124)	Yes

2.12 Cytoprotective effects of bilirubin

Bilirubin is clearly cyto-protective (127, 223), however excessive accumulation of the pigment can cause cell death and brain damage in neonates (116). Despite these observations, bilirubin rarely causes toxicity in adults. The protective and neurotoxic effects of bilirubin are both related to the concentrations of unconjugated bilirubin present *in vivo*. Concentrations below 100 μM are generally considered safe (266). The key to this finding is that bilirubin is normally bound to albumin and must be kept below the circulating albumin concentration, ensuring that it does not dissociate from its carrier protein and accumulate within lipid rich organs, including the brain (177). Therefore, circulating concentrations below the albumin binding capacity of the blood (600 μM) are generally non toxic and may be protective (against CVD, cancer and all-cause mortality) (107). This protection is clinically demonstrable in the condition of Gilbert's syndrome, involving a mild, unconjugated hyperbilirubinaemia (39, 204, 266, 274). In addition to CVD, atherosclerotic plaque formation is reduced in hyperbilirubinaemic patients (67, 126). The mechanisms behind this finding remain to be fully defined but likely involve the antioxidant capacity of bilirubin and inhibition of smooth muscle cell proliferation. An integral component of atherogenesis is vascular smooth muscle cell (VSMC) proliferation. Ollinger *et al* reported reduced VSMC proliferation following carotid arterial balloon injury in Gunn compared to wild-type Wistar rats (176). In addition, the authors reported that VSMC inhibition *in vitro* was not the result of apoptosis, but rather arrest of the cell cycle at G_0/G_1, via inhibition of p38, a mitogen-activated protein kinase (MAPK) that promotes cell proliferation (176). Arrest of the cell cycle was also confirmed by correlation to bilirubin serum levels. These findings, coupled with the potent antioxidant capacity of bilirubin (39), provide mechanisms that may underpin protection from atherosclerosis, and ultimately

myocardial infarction.

Interestingly, hypertension is also reduced in the deoxycorticosterone acetate (DOCA)-salt hyperbilirubinaenic (Gunn) rats. Nath *et al.* reported that Gunn rats and littermate Wistar controls were placed on a high salt diet, and that systemic hypertension after 3 weeks was significantly reduced in Gunn rats compared to wild-type controls (170). Furthermore, the concentration of superoxide anions, which quench NO and reduces its bioavailability, was increased in wild-type DOCA-salt animals. It was speculated that bilirubin could therefore inhibit superoxide-induced NO quenching to improve NO bioavailability and vasodilatation. Nath *et al.* also reported that after 4 weeks of DOCA-salt treatment, HO-1 -/- mice developed hypertension while HO-1 +/+ animals maintained normal systolic arterial pressure. Although the results observed in HO-$1^{-/-}$ mice do not directly implicate bilirubin in regulating blood pressure, these data were the first to demonstrate that HO-1 (and its metabolites) could inhibit the development of hypertension.

*2.12.1 Protection associated with the UGT1A1*28 polymorphism*

Hyperbilirubinaemia in Gilbert's syndrome individuals is influenced by a UGT1A1*28 polymorphism (207). This polymorphism, like bilirubin, is clearly associated with protection from several chronic diseases. More recently, several studies have sought to determine whether a relationship exists between the UGT1A1*28 polymorphism and disease. Lin *et al.* reported data from the Framingham Heart Study demonstrating that individuals with both mutated alleles of the UGTA1 gene promoter (UGT1A1*28) and higher serum bilirubin levels had reduced risk of CVD, (141). Interestingly, individuals who possess the UGT1A1*28 alleles are not at reduced risk of coronary heart disease and myocardial infarction (28). Total bilirubin concentrations in these individuals (~15 μM) are lower than concentrations classified as Gilbert's syndrome (17.1 μM). The authors concede that concentrations were not available for all individuals, nor was serum collection standardised, which resulted in considerable variation in the bilirubin concentrations (28). In addition to CVD, endometrial cancer (66) and Hodgkin's lymphoma (194) are reportedly reduced in homozygous UGT1A1*28 individuals. Conversely, UGT1A1 polymorphisms have also been associated with increased cancer risk, such as breast cancer (1), colorectal cancer (105) and chronic lymphatic leaukemia (123), in some cohorts. These studies all suffer limitations since bilirubin was not accurately measured. This would explain why another study (117) reported that colorectal cancer correlated with lower concentrations of serum bilirubin. These results further confirm the association between bilirubin concentration specifically, and chronic disease, opposed to the actual genetic mutation

2.12.2 Bilirubin and ischaemia-reperfusion injury

Although bilirubin is negatively associated with the prevalence of chronic diseases as indicated above, the role of bilirubin in the acute manifestation (ie. heart attacks/stokes) of cardiovascular disease has been less well explored. Within the past 10 years bilirubin has been shown to improve recovery of organs from acute ischaemia-reperfusion injury in experimental studies. This protection is primarily attributed to bilirubin's antioxidant and anti-inflammatory properties (134), which decrease reperfusion-induced oxidative stress. In 2001, Ceran *et al.* investigated the effects of elevated bilirubin on ischaemia-reperfusion injury in the small intestine of the rat and concluded that elevated levels of (exogenous) bilirubin could protect the gut against malondialdehyde production (45). This discovery was followed by those of Hammerman *et al.* (2002), who tested similar effects of bilirubin on the gut, assessing levels of thiobarbituric acid reducing substances (a non-specific marker of lipid peroxidation) and tissue histology (88). During this study unconjugated bilirubin was administered i.p., prior to ischaemia. This treatment reduced the severity of injury associated with lipid peroxidation compared to control animals (88). The results of additional studies further corroborate the protective effects of bilirubin in organs including the kidney (2), liver (265) and brain (185), however limited studies have investigated the protective effects of bilirubin on the heart.

2.12.3 Experimental investigations of bilirubin and myocardial ischaemia-reperfusion injury

Relatively few reports have investigated the influence of haem-oxygenase and bilirubin on post-ischaemic function and recovery in the heart. Masini *et al* (153) treated hearts with hemin, a HO-1 inducing compound, and attributed the post-ischaemic

protective effects to carbon monoxide (CO), iron, biliverdin and/or bilirubin, but did not measure these compounds specifically in tissue or blood. Clark *et al* (55) reported improved cardiac functional recovery after up-regulating HO-1 expression in male Lewis rats (via administration of 75 µmol/kg hemin) 24 hours prior to 30 minutes of global ischaemia. In addition, myocardial infarct area was reduced in treated animals. In another experiment, the authors added 0.1 µM unconjugated bilirubin to perfusion medium prior to *ex vivo* cardiac I/R and demonstrated significantly improved post-ischaemic function (54). However, the bilirubin concentrations applied were not physiologically relevant, and were at least 100 fold lower than circulating concentrations seen in humans, potentially limiting the relevance of these results (ie. such effects would already be activated in humans with much higher normal levels of endogenous bilirubin). In another report, Ben-Amotz *et al* administered bilirubin (10mg/kg) to Sprague Dawley rats one hour prior to left anterior descending (LAD) occlusion, and demonstrated a reduced cardiac infarct size (17). Interestingly, left ventricular function was improved during *ischaemia* in bilirubin-treated hearts; however, improved functional recovery was lost during reperfusion (17). Although the authors implicate bilirubin in this protection, tissue or serum concentrations were not measured, limiting the authors' conclusions regarding bilirubin induced cardioprotection. More recently, Issan *et al* (110) administered diabetic mice with cobalt photoporphyrin (CoPP, 5 mg/kg), which induces HO-1, 48 hrs prior to LAD ligation. In addition to reduced oxidative tissue damage, this study reported elevation of AKT, a pro-survival protein. Similarly, Sodhi *et al* (214) administered CoPP (3 mg/kg) 5 days after LAD ligation, which reduced myocardial fibrosis and myocyte death. Although increasing haem-oxygenase in hearts appears to provide cardioprotection, the effects of elevated bilirubin remains inconclusive, with only scattered research findings in *ex vivo*

and *in vivo* models in mice and rats. In addition, some evidence exists to implicate bilirubin as more than just an antioxidant, with effects on contractility. Furthermore, a conclusive mechanism of action explaining protective effects in cardiac tissue remains to be confirmed and published, despite the strong likelihood that bilirubin's antioxidant effects might be critical to protecting the heart.

CHAPTER 3

HYPERBILIRUBINAEMIA MODULATES MYOCARDIAL FUNCTION, AORTIC EJECTION AND ISCHAEMIC STRESS-RESISTANCE IN THE GUNN RAT

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Key words: bilirubin; myocardium; Gilbert's syndrome; ischaemia-reperfusion; ischaemic heart disease, vascular function

3.1 Abstract

Mildly elevated circulating unconjugated bilirubin (UCB) is associated with protection against hypertension and ischaemic heart disease. We assessed whether endogenously elevated bilirubin in Gunn rats modifies cardiovascular function and resistance to ischaemic insult. Hearts were assessed *ex vivo* (Langendorff perfusion) and *in vivo* (Millar catheterisation, echocardiography), and left ventricular myocardial gene expression was measured via quantitative real time PCR (RT-qPCR). *Ex vivo* analysis revealed reduced intrinsic contractility in Gunn myocardium (+dP/dt, 1976±622 *vs*. 2907±334 mmHg/s, P<0.01; -dP/dt, -1435±372 *vs*. -2234±478, P<0.01), which correlated positively with myocardial UCB concentration (P<0.05). *In vivo* analyses showed no changes in left ventricular contractile parameters and ejection (fractional shortening, ejection fraction). However, Gunn rats exhibited reductions in rate of aortic pressure development (3008±461 *vs*. 4452±644 mmHg/s, P<0.02), mean aortic velocity (439±64 *vs*. 644±62 ml/s, P<0.01) and the aortic volume time integral pressure gradient (2.32±0.65 *vs.* 5.72±0.74 mmHg, P<0.01), in association with significant aortic dilatation (12-24% increase in aortic diameter, P<0.05). *Ex vivo* Gunn hearts exhibited improved ventricular function after 90 minutes of reperfusion, following 35 minutes ischaemia (63±14 *vs*. 35±12%, P<0.01). These effects were accompanied by increased glutathione peroxidase and reduced superoxide dismutase and phospholamban gene expression in Gunn myocardium (P<0.05). These data collectively indicate that hyperbilirubinaemia in Gunn rats: i) reduces intrinsic cardiac contractility, which is compensated for *in vivo*; ii) induces aortic dilatation that may beneficially influence aortic ejection velocities and pressures; and iii) may improve myocardial stress-resistance in association with beneficial transcriptional changes. These effects may contribute to protection from CVD with elevated bilirubin.

3.2 Introduction

Human Gilbert's Syndrome (GS) is a condition prevalent in 3-5% (225) of the general Caucasian population and is characterised by mildly elevated circulating unconjugated bilirubin (UCB) concentrations (39) due to hepatic uridine glucuronosyl transferase 1A1 (UGT1A1) deficiency. Vitek *et al.* (243) reported that patients with GS are protected from ischaemic heart disease (IHD), with a 2% incidence of IHD *vs*. 12% in the general population. Reduced IHD incidence may be related to improved antioxidant status and a hypolipidemic state in GS (26, 41), limiting development of atherosclerosis (243) and potentially influencing the resistance of myocardium to ischaemia-reperfusion (I-R). Bulmer *et al.* (38) revealed that GS plasma is less susceptible to copper-induced lipid oxidation, providing the first mechanistic insight into decreased risk of atherosclerosis development in this cohort. Bilirubin's antioxidant effects may also improve vascular compliance via prevention of vascular smooth muscle proliferation (176), increased NO production (156) and/or inhibition of NADPH activity/oxidative stress (218, 240). However, despite current evidence indicating an 'anti-atherogenic' role for bilirubin, the discrete effects of *endogenous* bilirubin on cardiac function and ischaemic tolerance have not been reported. The effects of *exogenous* bilirubin on I-R injury have been investigated in organs including the gut (45), kidney (2), liver (265) and brain (146), and generally support beneficial and antioxidant actions of bilirubin. Two reports have assessed effects of *exogenous* bilirubin on myocardial I-R injury (17, 55). However, the physiological applicability of these findings is questionable due to the low bilirubin concentrations studied (0.05-0.10 μM) (55) and the absence of reported UCB concentrations following administration (17). The current investigation is the first to test whether elevation in *endogenous* bilirubin (1-50 μM), as seen in individuals with low *vs.* mildly elevated UCB (human

GS), modify myocardial structure/function, blood ejection velocities and cardiac stress-resistance. We adopt a mechanistic approach by utilising the hyperbilirubinaemic Gunn rat (spontaneous UGT1A1 mutant model), hypothesising that elevated cardiac bilirubin content may beneficially modify cardiac functional parameters and intrinsic resistance to ischaemic insult by inducing shifts in ventricular gene expression.

Table 3.1: Calculations for parameters measured

	Definition	Equation
%EF	Percentage Ejection Fraction	$\frac{LV\ vol;d - LV\ vol;s}{LV\ vol;d} \times 100$
%FS	Percentage Fraction Shortening	$\frac{LVID;d - LVID;s}{LVID;d} \times 100$
LV mass	Left ventricular mass	$1.053 \times ((LVID;d + LVPW;d + IVS)^3 - LVID;d^3)$
AoVTI	Aortic Velocity time integral	Absolute value
SV	Stroke volume	$7.85 \times LVOT^2 \times AoVTI$
CO	Cardiac output	$(AoVSV) \times HR \times LVOT/1000$
AoD	Aortic distensibility	ΔLCSA / (Aortic Pressure Pulse x LCSA)

LV, left ventricular; LVIDd, LV internal dimension at diastole; LVIDs, LV internal dimension at systole; LVPWd, LV posterior wall thickness at diastole; IVS, interventricular septal thickness; LVOT, LV outflow tract; HR, heart rate; LCSA, lumen cross-sectional area

3.3 Methods

The Griffith University Animal Ethics Committee (MSC/04/09) approved the conduct of experiments prior to their commencement. Two breeding pairs of heterozygote Gunn rats were obtained from the Rat Resource and Research Center (RRRC), University of Missouri, Columbia, USA. Animals were bred to form either homozygote Gunn (jaundiced) or heterozygote/wild-type (non-jaundiced) offspring. All animals possessing normal bilirubin concentrations were pooled and termed 'wild-type' while those expressing hyperbilirubinaemia were phenotypically defined as 'Gunn rats'. All animals were provided with standard laboratory rodent food pellets (Speciality Feeds, Glen Forrest, Australia) and fresh water daily. All studies were undertaken in 12-13 month old female rats (owing to offspring gender distribution from breeding pairs). Eight Gunn and eight age-matched wild-type rats were assessed for baseline cardiovascular function *in vivo* using echocardiography before *ex vivo* analysis of cardiac function and intrinsic resistance to I-R using the isolated Langendorff heart model (see below). Another group of six female Gunn rats and age matched wild-type littermates were assessed for cardiovascular function via Millar catheterisation and left ventricular gene expression (see below).

3.3.1 Echocardiography

Echocardiographic analysis was undertaken using a Vevo 770 high resolution *in vivo* imaging system and RMV710B scan-head for small rodents (VisualSonics Inc., Toronto, Canada). A total of four cardiac views were obtained per animal; parasternal long-axis, parasternal short-axis, four chamber apical, and the suprasternal views (focused on the aorta). For each analysis rats were anesthetized with ~2% isoflurane administered in 2 L/min O_2. During anaesthesia, rats were placed on a heating pad

(37.5˚C) with rectal temperature measured continuously and electrocardiograms/heart rate monitored using a surface electrocardiogram in order to maintain animals within normal temperature and heart rate ranges. Body weight was recorded prior to each assessment and used in calculation (Table 3.1) of cardiac structural and functional parameters, as indicated within the results section.

3.3.2 Langendorff perfused heart model

One week after echocardiographic analysis hearts were removed for Langendorff perfusion. Animals were injected with pentobarbitone sodium (60 mg/ml; 1 μl/g), with an additional 50 μl injected where necessary to eliminate pain reflexes. Hearts were then excised, arrested in cold Krebs-Henseleit solution and the aorta cannulated on the Langendorff apparatus within 90 s. Hearts were then perfused at a mean constant pressure of 74.3 mmHg with Krebs-Henseleit buffer containing (in mM): 118 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.75 $CaCl_2$, 1.2 $MgSO_4$, 11 D-glucose and 0.5 EDTA. The buffer was heated to 37˚C and gassed with 95% O_2 and 5% CO_2, to give a pH of 7.4. A small fluid-filled balloon, connected to a pressure transducer (PowerLab, ADInstruments, Castle Hill, Australia), was introduced into the left ventricle and pressure recorded using LabChart software (ADInstruments, Castle Hill, Australia). Diastolic pressure was set at 4 mmHg, and then enclosed in a water-jacked chamber to maintain external temperature. Each heart underwent 30 minutes of baseline functional assessment, which was followed by 35 minutes of global ischaemia and 90 minutes of aerobic reperfusion. During the ischaemic period hearts were immersed in warmed Krebs-Henseleit buffer to maintain temperature at 37°C. At the onset of reperfusion the chamber was drained. All animals were investigated within a three day period to reduce potential variability caused by the oestrous cycle.

Cardiac function was continuously recorded throughout experiments, and is reported after 15 and 30 minutes of normoxic (pre-ischaemic) perfusion, and 10, 30, 60 and 90 minutes of reperfusion. Time to onset of contracture (TOC), defined as the time for diastolic pressure to rise by 4 mmHg, and the peak contracture pressure (PC) achieved during ischaemia were measured. Functional variables assessed included heart rate (HR), coronary flow (CF), and left-ventricular diastolic pressure (DP), systolic pressure (SP), developed pressure (LVDP), rate of pressure change during contraction (+dP/dT) and relaxation (-dP/dT). The rate pressure product (RPP) was calculated as the product of LVDP and HR.

3.3.3 Millar catheterisation

Each animal was anaesthetised using pentobarbitone sodium (60 mg/ml; 1 μl/g). After loss of pain reflexes, animals were placed on a heat pad, intubated and ventilated (Harvard Apparatus, MA, USA) at 10 ml/kg (80 breaths/min). A small incision was made lateral to the trachea for isolation of the carotid artery via blunt dissection. The superior section of the carotid was ligated with silk suture (Ethicon Inc., NJ, USA). The inferior section was clamped and an 18G needle was inserted into the isolated artery. The Millar catheter (Millar Instruments, TX, USA) was then inserted inferiorly. A ligature was tied around both the carotid and the catheter line to prevent bleeding on clamp removal. The Millar catheter was fed inferiorly from the carotid, into the ascending aorta and then into the left ventricle. Once in the ventricle, the animal was positioned onto its left side and data was collected for a period of 10 minutes using LabChart software. The catheter was then removed from the left ventricle and placed in the aorta to record aortic pressures for a further 10 minutes. A minimum of 10 sequential beats at the end of each data collection period were used in analyses.

3.3.4 Biochemical analyses

Approximately 5 ml of whole blood was collected immediately after removal of hearts. All samples were centrifuged (22000 G; 5 minutes) and serum aliquots were transferred into Eppendorf tubes and frozen at -80˚C. Serum UCB was assessed, which involved adding 160 µl of HPLC mobile phase (0.1 M n-dioctylamine acetate in 95:5 methanol:H2O) to 40 µl of serum, which precipitated serum proteins and extracted bilirubin from the sample (40). This solution was vortexed for 20 s, then centrifuged at 22000 G for 5 minutes. 150 µl of supernatant was transferred into HPLC vials and 50 µl was injected onto the HPLC column. The column (Phenomenex Australia, reverse phase, C18, 150x4.5 mm) was perfused isocratically (Separations module 2960, Waters 996, MA, USA) using the same mobile phase indicated above at 0.7 ml/min. Standard curves (0.5-100 µM) were generated using commercially available UCB (Frontier Scientific, UT, USA). The bilirubin concentration of all serum samples were expressed relative to the area under the curve, integrated at 450 nm (Photo diode array, Waters 996, MA, USA).

For analysis of cell damage in ischaemia studies, coronary effluent was collected from the heart at baseline (immediately prior to ischaemia) and at 5 and 15 minutes of reperfusion, with samples immediately frozen at -80˚C. These samples were assayed for lactate dehydrogenase (LDHI2) content, a measure of cellular damage, via a Cobas Integra 400 chemical analyser (Roche Diagnostics, Switzerland).

3.3.5 Atrial bilirubin content

The left atrium was collected from each Langendorff perfused heart during baseline equilibration in order to determine myocardial bilirubin concentration in non-

ischaemic cardiac tissue. The left atrium was blotted dry and frozen at -80˚C. The atria were broken down using CellLytic solution (Sigma-Aldrich, Castle Hill, Australia), using scalpel blades and needle homogenisation (18-25 G) and centrifuged (22000 *g*, 5 minutes). 40 µl of supernatant was used to assess the tissue UCB content using HPLC, as previously described. Estimation of the extraction efficiency for UCB in myocardial protein lysates was determined in triplicate samples by addition of exogenous UCB to protein lysates from Wistar rats (0.1 µM final concentration in 0.1% v/v DMSO) and extracting/analysing bilirubin as for blood analysis. Extraction efficiency approximated 70±19% (mean±SD). Bilirubin content was expressed relative to protein content as determined using a BCA protein kit (Pierce, Thermo Scientific, IL, USA).

3.3.6 Gene expression analysis via RT-qPCR

Transcript levels for antioxidant enzymes and determinants of Ca2+ handling and contraction were assessed. RNA extraction, cDNA synthesis and two-step RT-qPCR was performed, as previously described (9), in left ventricular myocardial samples from Gunn and control hearts. Hearts were excised, the left ventricle removed and placed in RNAlater solution (Qiagen, Melbourne, Australia), and frozen at -80°C. Differential expression of the following 8 transcripts were assessed: *Cat* (Catalase), *Gpx1* (Glutathione peroxidase 1), *Sod1* and *Sod2* (Superoxide dismutase 1 and 2), *Pln* (Phospholamban), *Ryr2* (Ryanodine receptor 2), *Atp2a1*, *Atp2a2* (Sarcoplasmic/endoplasmic reticulum calcium ATPase 1, 2; see Table 3.2 for primer details), *Ppia* (Peptidylprolyl isomerase A) and *Pgk1* (Phosphoglycerate kinase 1) were evaluated among several potential reference genes and validated as the most stably expressed genes (stability M-value = 0.26) in all analysed samples. Quantitative PCR data were normalised to these two reference genes and expressed relative to wild-type

controls.

3.3.7 Statistical analysis

All data are presented as a mean ± standard deviation (SD) and were assessed for normal distribution followed by appropriate parametric (unpaired *t*-test) or non-parametric (Mann-Whitney *U*-test) statistical testing using Sigmaplot V11.0 (Systat Inc., IL, USA). For group comparisons (Gunn rat *vs*. wild-type control), unpaired *t*-tests were used to assess potential differences. Bivariate comparisons were tested using Pearson's correlation. Significance was set at $P<0.05$.

Table 3.2: Quantitative real-time PCR primer sequences for reference and target genes coding antioxidant enzyme and proteins influencing Ca^{2+} and contractility

Gene Name	Gene Symbol	GeneID	Forward Primer (5'-3')	Reverse Primer (5'-3')
ATPase, Ca^{2+} transporting, cardiac muscle, fast twitch 1	*ATP2A1*	116601	AGGTGGTCTGTATCTTCTTGAC	ACCAAGTTCACCCATAGCAG
ATPase, Ca^{2+} transporting, cardiac muscle, slow twitch 2	*ATP2A2*	29693	GGCTATTGGCTGTTATGTTGG	GGGTTGTCCTCCTTACACTG
Catalase	*CAT*	24248	TGCGGACATTCTATACGAAGG	GAAATTCTTGACCGCTTTCCTC
Glutathione peroxidase 1	*GPX1*	24404	ACACCGAAATGAATGATCTGC	TCTTGCCATTCTCCTGATGTC
Peptidylprolyl isomerase A	*PPIA*	25518	CAAGACTGAGTGGCTGGA	GAGATGGTGATCTTCTTGCTG
Phosphoglycerate kinase 1	*PGK1*	24644	AGCTCCTGGAAGGTAAAGTC	CTGCACTAACACCAAATGGA
Phospholamban	*PLN*	64672	CACGATTAAGAGTGAGACTGATGG	AGACATTTATGAGCCACACTGAG
Ryanodine receptor 2, cardiac	*RYR2*	689560	CATGGCTTTGAAACCCATACTC	ACATAAGATTCCTGTCCCGTG
Superoxide dismutase 1, soluble	*SOD1*	24786	TACACAAGGCTGTACCACTG	CACACGATCTTCAATGGACAC
Superoxide dismutase 2, mitochondrial	*SOD2*	24787	AGAACCCAAAGGAGAGTTGC	CTTATTGAAGCCAAGCCAGC

3.4 Results

To assess whether UCB might modify intrinsic contractile function, normoxic function was assessed in Langendorff perfused cardiac tissue (Table 3.3). *Ex vivo* left ventricular systolic and developed pressures were significantly reduced in Gunn hearts ($P<0.05$). In addition, Gunn hearts exhibited significant reductions (~35%) in left ventricular +dP/dT and -dP/dT ($P<0.01$; Table 3.3). Measures of baseline *ex vivo* contractile function correlated significantly with myocardial tissue UCB content (Figure 3.1A, $P<0.05$; Figure 3.1B, $P<0.05$).

Table 3.3: Ex vivo cardiovascular parameters at baseline in aged female Gunn hearts vs. controls (n=8/group)

Parameters	**Control**	**Gunn**	***P*-Value**
Atrial [UCB], nmoles/mg protein	0.012±0.012	0.378±0.128*	<0.001
CF, ml/min	13±3	11±2	0.15
HR, bpm	319±58	302±40	0.50
DP, mmHg	3.4±1.7	3.5±1.6	0.874
SP, mmHg	118±11	103±14*	0.046
LVDP, mmHg	114±12	100±13*	0.047
+dP/dt, mmHg/s	2907±334	1976±622*	0.002
-dP/dt, mmHg/s	-2234±478	-1435±372*	0.002
RPP, mmHg/min	36649±7934	29996±3698	0.05

Data are means ± SD; n = 8 rats/group. *P<0.05 vs. control. Ex vivo measures were obtained in Gunn and wild-type rats under normoxic conditions. *P* values are shown for measures in Gunn vs. control hearts. UCB, unconjugated bilirubin; CF, coronary flow; HR, heart rate; DP, diastolic pressure; SP, systolic pressure; LVDP, left ventricular developed pressure; +dP/dt, rate of ventricular contraction; -dP/dt, rate of ventricular relaxation; RPP, rate-pressure product.

Table 3.4: In vivo structural and functional cardiovascular parameters in aged female Gunn rats vs. controls (n=8/group)

Parameters	**Control**	**Gunn**	**P-Value**
Morphologic	*n = 8*	*n = 8*	
Body weight, g	259±30	200±32*	0.002
Blood [UCB], µmol/L	1.4±0.6	46.4±11.4*	<0.001
Echocardiography	*n = 8*	*n = 8*	
HR, bpm	329±29	319±35	0.617
CO, ml/min/g	0.19±0.39	0.20±0.03	0.797
FS, %	49±14	49±8	0.947
EF, %	77±14	74±10	0.68
SV, µL/g	190±33	180±42	0.669
Mean Aortic V, mm/s	644±62	438±64*	<0.001
Peak Aortic V, mm/s	1239±108	733±58*	<0.001
AoVTI gradient, mmHg	5.72±0.74	2.31±0.65*	<0.001
AoVTI duration, ms	90±5	108±13*	0.01
MV E, mm/s	884.9±261.6	730.81±113.2	0.21
MV A, mm/s	647.9±188	540.53±120.9	0.27
MV E/A	1.3±0.08	1.4±0.2	0.9
Isovolumetric relaxation time, ms	22.9±6.6	32.8±7.9*	0.04
Isovolumetric contraction time, ms	22.4±10.5	25.4±4.2	0.53
LV mass, g	0.70±0.05	0.58±0.11*	0.03
LV mass/body weight	0.0028±0.0003	0.0029±0.0007	0.857
Aortic diameter			
LVOT at systole, mm	2.42±0.15	2.72±0.2*	0.02
LVOT at diastole, mm	1.70±0.27	2.11±0.17*	0.01
Millar catheterization	*n = 4*	*n = 6*	
Ventricular			
HR, bpm	330±36	312±61	0.979
Diastolic pressure, mmHg	3±1	2±1	0.712
Systolic pressure, mmHg	151±24	156±31	0.772
LVDP, mmHg	148±26	155±30	0.77
+dP/dt, mmHg/s	11992±2084	11610±3624	0.88
-dP/dt, mmHg/s	-9806±1681	-10750±3262	0.96
Aortic			
HR, bpm	332±35	309±65	0.835
Diastolic pressure, mmHg	98±33	116±21	0.77
Systolic pressure, mmHg	151±24	153±29	0.71
Aortic pulse pressure, mmHg	53±10	37±10	0.09
+dP/dt, mmHg/s	4452±644	3008±461*	0.02
-dP/dt, mmHg/s	-1432±333	-1349±312	0.18

Data are means ± SD; *n* = number of rats/group. *P<0.05 vs. control. In vivo measures were obtained in Gunn and wild-type rats under normoxic conditions. *P* values are shown for measures in Gunn vs. control hearts. [UCB], concentration of unconjugated bilirubin; HR, heart rate; CO, cardiac output; FS, fractional shortening; EF, ejection fraction; SV, stroke volume; V, velocity; AoVTI gradient, peak pressure gradient of blood entering the aorta (from AoVTI); AoVTI duration, duration of aortic ejection (from AoVTI); MV *E*, peak mitral valve (MV) early filling; MV *A*, peak mitral valve active filling; MV *E/A*, mitral valve early-to-active filling ratio; LVOT, left ventricular outflow tract; LVDP, left ventricular developed pressure; +dP/dt, rate of pressure development; -dP/dt, rate of relaxation

Circulating UCB was increased significantly and body weight reduced in Gunn *vs.* wild-type rats (Table 3.4). Structural cardiac parameters were not different between groups, with the exception of left ventricular anterior wall thickness during systole (3.0±0.5mm *vs.* 2.2±0.2mm in Gunn hearts, P<0.05). *In vivo* echocardiographic analyses revealed no alterations in left ventricular contractile function in Gunn rats (Table 3.4), contrasting depressed mechanical function in isolated myocardium (Table 3.3). Specifically, measures of cardiac output, FS, EF and SV remained unaltered, while the aortic velocity time integral (AoVTI) was reduced together with a marked (35-40%) reduction in peak and mean aortic blood velocities (Table 3.4). Furthermore, a significant ~60% reduction in the aortic pressure gradient existed in Gunn animals (Table 3.4), which was negatively correlated with circulating bilirubin concentration (Figure 3.1C; P<0.05). Stroke volume was maintained, with a significantly increased systolic duration (~20 ms increase in AoVTI duration; *P*<0.05) in Gunn hearts (Table 3.4). In addition, Gunn rats possessed a dilated left ventricular outflow tract (~24 % increase in diameter during diastole, and 12% during systole), while aortic distensibility itself did not differ between groups (Figure 3.2). Millar catheter analysis showed that *ventricular* pressure development and systolic and diastolic *aortic* pressures did not differ between Gunn and wild-type rats. However, a ~30% reduction in the rate of

aortic pressure development (+dP/dt) was detected in Gunn animals (Table 3.4).

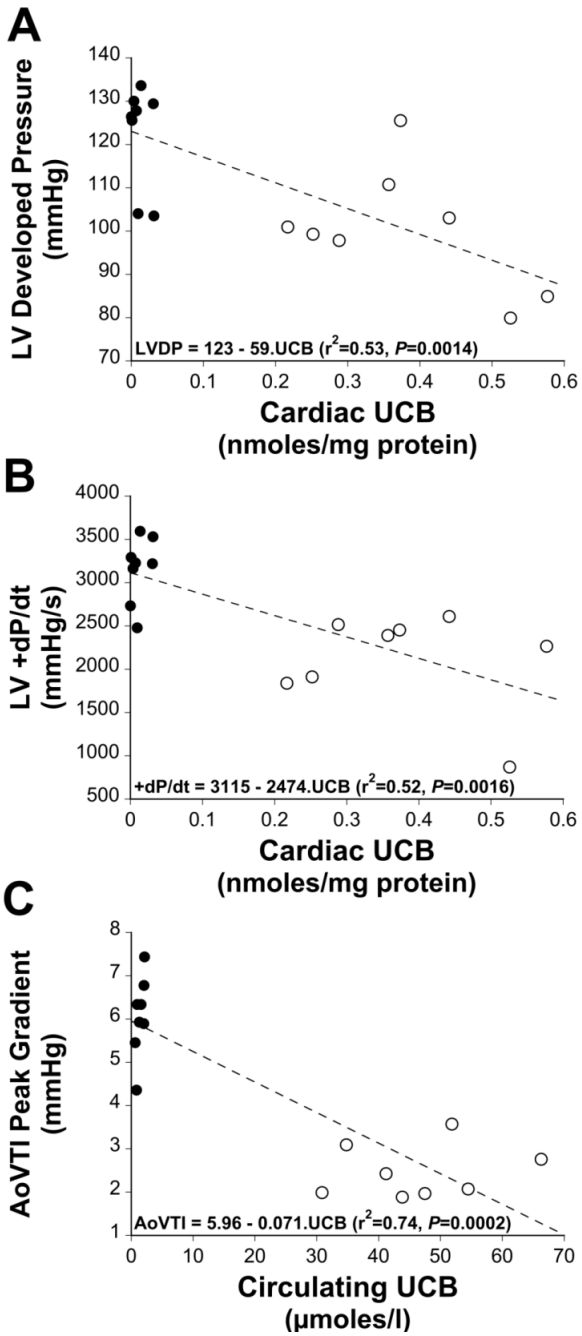

Figure 3.1: *In vivo and ex vivo cardiac functional parameters versus circulating unconjugated bilirubin (UCB) concentrations.* A) and B) data for left ventricular (LV) developed pressure (LVDP) ex vivo (A; $P<0.01$) and LV dP/dt ex vivo (B; $P<0.01$) versus tissue UCB concentrations. C: aortic velocity time integral (AoVTI) pressure gradient of blood entering the aorta in vivo ($P<0.01$) versus circulating UCB concentrations for female aged Gunn ($n = 8$) and wild-type (control) rats ($n = 8$). Lines are linear regression best fits, with regression equations, r^2 values, and P values as shown.

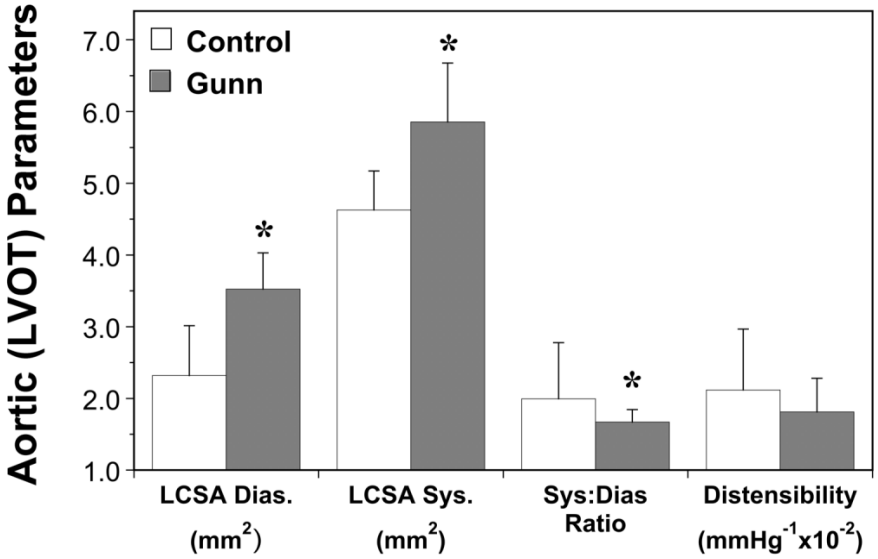

Figure 3.2: *Aortic cross-sectional area and distensibility.* Data are shown for lumen cross-sectional area (LCSA) during diastole (Dias) and systole (Sys) and the distensibility of the aorta for female aged Gunn (n = 8) and wild-type (control) rats (n = 8). LVOT, LV outflow tract. Data are means ± SD. *P<0.05 vs. control rats.

The myocardial response to *ex vivo* ischaemic insult itself differed across groups, with significantly delayed and reduced contracture development in Gunn *vs.* control hearts (Figure 3.3A). Post-ischaemic LDH efflux over the initial five minutes of reperfusion also tended to be reduced in Gunn hearts, although this failed to achieve significance (Figure 3.3A, P=0.21). Left ventricular developed pressure differed between groups at baseline (Table 3.3), and, recovery of function was thus normalised to baseline function. Percent recoveries for left ventricular developed pressure, rate pressure product, +dP/dt and -dP/dt were all significantly improved in Gunn hearts (Figure 3.3B).

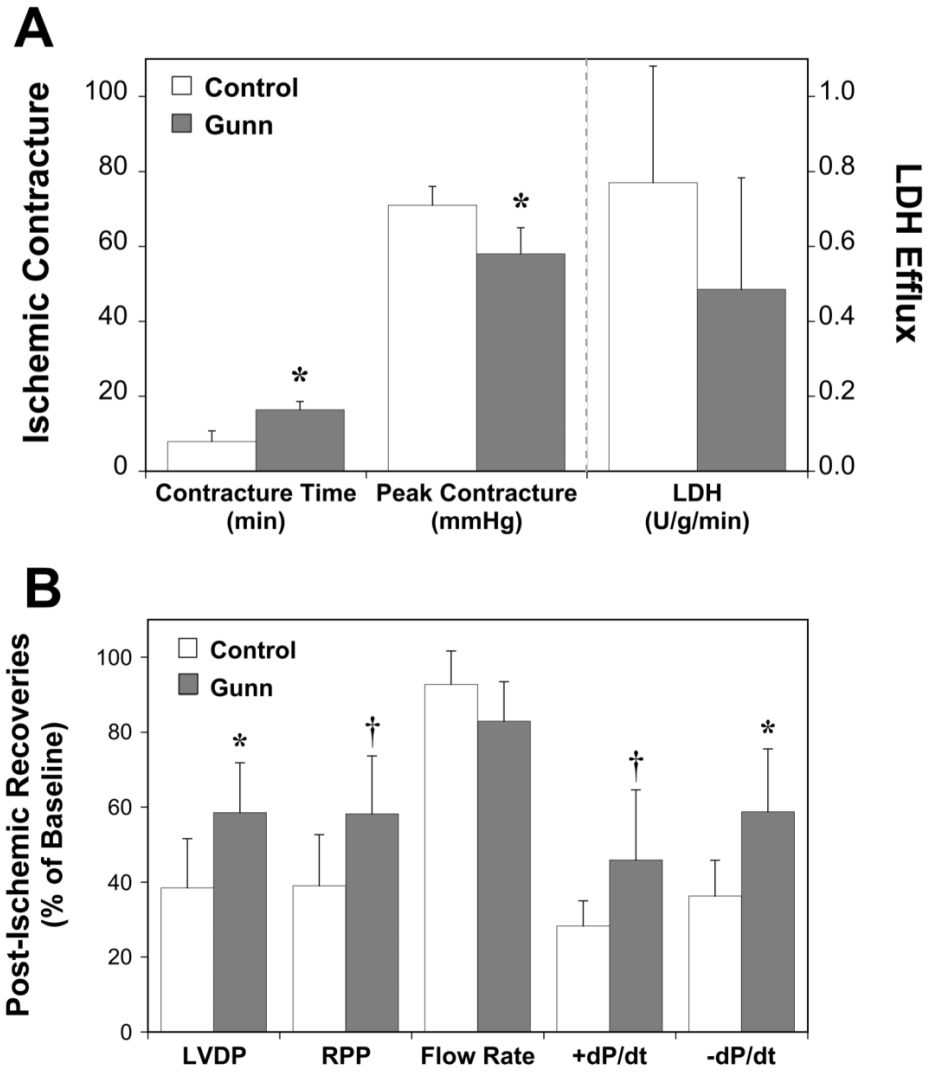

Figure 3.3: *Ischaemic contracture and post-ischaemic outcomes in ex vivo hearts.* Data are shown for isolated hearts from female aged Gunn (n = 8) and wild-type (control) rats (n = 8) subjected to 35 minutes of global ischaemia and 90 minutes of reperfusion. A) Rate and extent of ischaemic contracture and post-ischaemic lactate dehydrogenase (LDH) efflux. B) Ventricular mechanical and coronary flow recoveries from ischaemia-reperfusion. LVDP, left ventricular developed pressure; RPP, rate-pressure product; +dP/dt, rate of pressure development; -dP/dt, rate of relaxation. Data are means ± SD. *P<0.05 vs. control rats. †P<0.1 vs. control rats.

Gene expression was analysed in left ventricular myocardium (Figure. 3.4). Of the genes transcribing antioxidant enzymes (*Sod1, Sod2, Cat* and *Gpx1*), *Sod1* was significantly reduced while *Gpx1* expression was significantly increased (P<0.05). Of the genes regulating Ca^{2+} dynamics and contractility (*Atp2a1, Atp2a2, Pln, Ryr2*), *Pln* was significantly down-regulated (P<0.05).

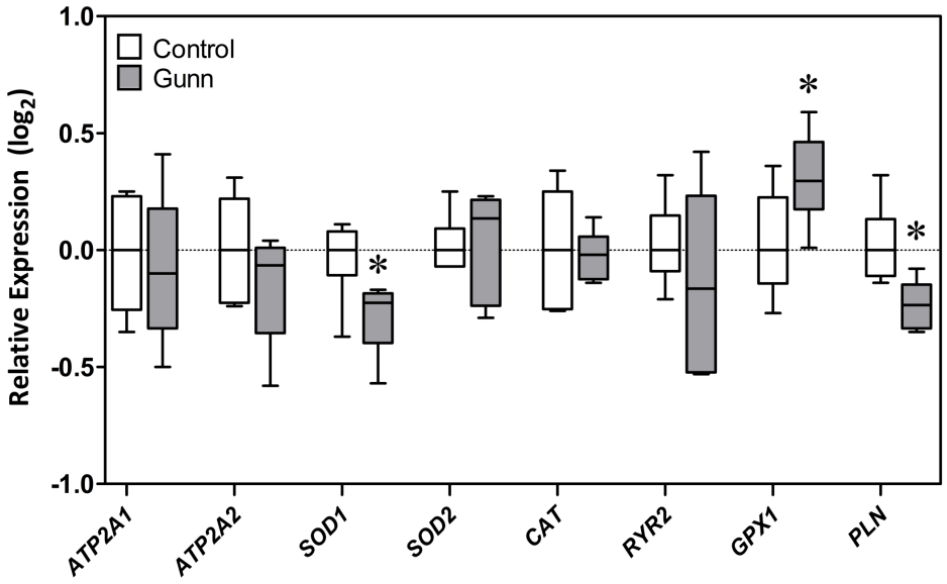

Figure 3.4: *Gene expression in the LV*. The expression of genes regulating cardiac contractility and antioxidant enzyme expression in LVs from naive female aged Gunn (*n* = 6) and wild-type (*n* = 6) rats is shown. ATP2A1, sarco(endo-)plasmic reticulum; Ca^{2+}-ATPase (SERCA)1; ATP2A2, SERCA2; SOD, superoxide dismutase; CAT, catalase; RYR2, ryanodine receptor 2; GPX1, glutathione peroxidase 1; PLN, phospholamban. *P<0.05 vs. control rats.

3.5 Discussion

This study investigated the effects of congenitally elevated UCB - in mutant UGT1A1 Gunn rats - on myocardial function, gene expression and intrinsic resistance to I-R. *Ex vivo* analysis revealed significant depression of left ventricular dP/dt in hyperbilirubinaemic hearts, supporting a hypo-contractility that correlates with myocardial UCB content (Figure 3.1B). In contrast, *in vivo* Millar catheterisation revealed no significant change in ventricular pressure development, indicating effective compensation for this cardiac hypo-contractility *in vivo*. Echocardiography identified reductions in the velocity of aortic ejection (and the AoVTI/pressure gradient) in Gunn animals with otherwise preserved ventricular function, consistent with reduced aortic +dP/dt measured via Millar catheter analysis. These changes likely arise due to significant aortic dilatation detected in Gunn animals, which may beneficially modify left ventricular afterload. Gunn hearts also demonstrated improved tolerance to I-R *ex vivo*, together with shifts in left ventricular expression of genes for antioxidant enzymes and phospholamban. These data provide mechanistic insight into the regulatory effects of elevated bilirubin within the heart. The basis of these cardiac and vascular alterations, and their relevance to CVD susceptibility in humans, remain to be delineated.

3.5.1 Ex vivo ventricular function

Ex vivo perfused myocardium from Gunn rats exhibited intrinsically reduced rates of contraction and relaxation (Table 3.3). These changes, which correlate with myocardial tissue UCB content, indicate that elevated bilirubin directly influences cardiac contractility. Elevations in myocardial UCB concentrations are consistent with evidence of cardiac and extra-vascular accumulation of bilirubin (266). The mechanistic basis of this negative inotropy is unclear, although bilirubin may influence Ca^{2+} levels

and expression of related proteins. The Ca^{2+} -binding properties of bilirubin itself (46) provides extra- and intra-cellular Ca^{2+} sinks, while reduced expression of parvalbumin reported in Gunn rats (albeit for neuronal tissue) (210) could limit contractility if arising in myocardium. On the other hand, bilirubin can increase smooth muscle permeability to Ca^{2+} (236). Antioxidant actions of bilirubin might also modify redox-dependent control of contraction (229), while Malik *et al.* (146) show bilirubin negatively influences mitochondrial energy production, thus potentially energy-dependent contraction. It is noteworthy that acute obstructive jaundice in humans also significantly inhibits myocardial inotropic responses (145).

In terms of molecular determinants of contraction, transcripts for proteins influencing Ca^{2+} handling and contraction were not substantially modified in Gunn hearts, except for repression of *Pln*. Dephosphorylated phospholamban inhibits sarcoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA) activity and contractility, an effect reversed by phosphorylation (via β-adrenergic stimulation). Thus, repression of phospholamban cannot mediate depressed contractility in Gunn myocardium, though may well reflect an important molecular adaptation. Compensatory mechanisms that effectively counter cardiac hypo-contractility *in vivo* (revealed in well maintained contraction *in vivo vs*. depression *ex vivo*) likely include autonomic stimulation, and chronic ß-adrenergic activity which can depress cardiac phospholamban (195). As has been theorised, repression (and phosphorylation) of phospholamban may reflect an adaptation, which attempts to maintain contractile function via enhanced SR Ca^{2+} uptake/loading, albeit at the expense of normal regulatory control of SR Ca^{2+} (195).

3.5.2 Myocardial stress-resistance

Very few studies have assessed the impact of UCB on myocardial I-R injury (17, 55). Clark *et al.* (55) tested the effect of non-physiological bilirubin levels (0.1 μM in perfusing fluid) on function in *ex vivo* mounted hearts exposed to I-R, demonstrating reduced infarct size and modest improvements in post-ischaemic ventricular function after 60 minutes reperfusion. These data largely agree with those presented here for ischaemic outcomes (*ie.* improvements in pressure development, rate-pressure product, and contracture). A recent study in a small cohort (17) reports that i.p. administration of UCB to rats 1 hr prior to coronary artery ligation and reperfusion reduces infarct size, and improves ischaemic but not post-ischaemic ventricular function. These data for bilirubin treatment generally support our findings for *endogenous* bilirubin, though circulating and myocardial bilirubin levels were not assessed (17), and we report improved post-ischaemic function. Our data indicate that Gunn rats exhibiting extracellular UCB levels similar to those in GS (39), and perfused in the absence of circulating bilirubin are resistant to I-R, implicating effects of tissue *vs.* circulating bilirubin on intrinsic determinants of stress-resistance. Both ischaemic contracture development and post-ischaemic contractile dysfunction were improved (Figure 3.3). Cellular disruption/death in Gunn hearts also tended to be reduced, with a ~40% (though insignificant) reduction in LDH release *vs.* wild-types (Figure 3.3A). How moderate chronic hyperbilirubinaemia modifies the capacity of myocardial tissue to withstand I-R is unclear, though reductions in contracture development and diastolic dysfunction (Figure 3.3A) point to alterations in Ca^{2+} homeostasis and energy state (192), consistent with influences of bilirubin on Ca^{2+} levels/regulatory proteins (46) and mitochondrial respiration (146). Antioxidant actions of bilirubin are likely to confer benefit in I-R, while elevated NO levels with hyperbilirubinaemia may additionally

contribute (240). Ben-Amotz *et al.* speculate that antioxidant effects of bilirubin inhibit the peroxidase activity of cytochrome *c*, oxidation of lipids such as cardiolipin, and thereby apoptosis (17).

We assessed gene expression for antioxidant enzyme expression because bilirubin activates NRF2 and induces antioxidant response element activation (189). Superoxide dismutase, catalase and glutathione peroxidase all possess NRF2 promoter sequences. Expression of Gpx1 was elevated in naive Gunn hearts, providing a potential mechanism whereby bilirubin could protect from oxidative damage in I-R (*e.g.* by neutralising hydrogen/lipid peroxides). These data are intriguing in light of increased free thiol and glutathione levels in hyperbilirubinemic humans (26), which may be driven in a NRF2-dependent manner (84). Divergent effects on *Gpx1 vs*. *Sod1* (Cu-Zn SOD) highlight the complexity of antioxidant regulation (154). Decreased *Sod1* expression could reflect reduced H_2O_2 formation (which normally induces superoxide dismutase) via induction of glutathione peroxidase 1. Increased *Gpx1* and resultant repression of *Sod1* may reflect a strategy to limit H_2O_2 bioavailability (251), with increased bilirubin additionally neutralising superoxide radical cation directly (172). Such a theory would support a hypothesis that bilirubin prevents lipid peroxidation during cardiac reperfusion injury (17), which could result from direct radical scavenging via bilirubin and induction of antioxidant enzymes such as glutathione peroxidase 1.

3.5.3 In vivo ventricular and aortic functional parameters in Gunn rats

Despite significantly reduced intrinsic contractility in isolated cardiac tissue, *in vivo* ventricular function (assessed via echocardiography and Millar catheterisation) was comparable in Gunn and control animals (Table 3.4). As noted above, absence of

contractile differences *in situ* indicates adequate neurohumoral (*eg*. autonomic) compensation for depressed cardiac contractility. On the other hand, *aortic* ejection dynamics (velocities and the AoVTI pressure gradient) and rate of aortic pressure development were all significantly reduced in Gunn animals (Table 3.4). Prior work indicates no difference in systolic arterial pressures in male Gunn rats (170), consistent with similar aortic pressures documented here. Increased afterload therefore cannot explain altered velocity of aortic ejection in Gunn animals (Table 3.4). Both the rate of aortic pressure development and duration of ejection (AoVTI duration) were increased in Gunn rats (Table 3.4), a prolongation of ejection which may maintain SV and cardiac output in the face of reduced ejection velocities. Reductions in aortic velocities and AoVTI pressure gradient in the absence of differences in ventricular contraction indicate a systemic effect of bilirubin within the arterial circulation. Measurement of aortic diameter confirms a dilated left ventricular outflow tract (Table 3.4), without changes in arterial compliance or distensibility (Figure 3.2). A dilated arterial tree is consistent with evidence of elevated NO levels in hyperbilirubinaemic animals (240), which may not only reduce vascular tone but influence aortic structure/remodeling. This structural change may also reflect inhibitory effects of bilirubin on vascular smooth muscle proliferation (176).

Aortic dilatation could reduce afterloading and ejection dynamics. Aortic dilatation reported here is also consistent with reductions in intima-media thickness (IMT) in the thoracic aorta/carotid arteries of individuals with elevated bilirubin (120, 244). Such changes, increasing unstressed luminal volume and altering outflow tract dynamics, could limit development of hypertension and the negative impacts of afterload *in vivo*. To the best of our knowledge this is the first report of aortic dilatation

in the Gunn rat. The basis and impact(s) of this vascular change requires further investigation. An important limitation of this study concerns the difference in UCB concentrations in Gunn rats (and their litter-mate controls) *vs.* concentrations in human GS and healthy controls. Bilirubin levels in GS exist in a spectrum, rising to as much as 80-100 μM (25, 26, 243, 246). Mean concentrations approximate 35 μM in GS (26, 243, 246), 10 μM in healthy controls) (26, 243, 246) and <10 μM in individuals at risk of IHD (173). Concentrations of UCB are moderately higher in Gunn rats *vs.* human GS patients, and also vary (50-110 μM) depending on source, sex and age of animals (25, 41, 246). While there is considerable overlap in bilirubin levels between GS individuals and Gunn rats, it might be argued the Gunn rat does not specifically replicate the condition of GS. The moderately higher levels are explained by the differing etiology of unconjugated hyperbilirubinaemia in these settings, with Gunn rats completely lacking UGT1A1 activity due to a frame shift mutation (112), whereas a gene promoter polymorphism in the UGT1A1 gene reduces UGT1A1 and thus activity by ~70% in GS (29).

We also acknowledge that control rats' UCB concentrations (1-2 μM) are below those normally seen in healthy, non-GS individuals (~10 μM) (246), more closely approximating circulating biliruibin concentrations in persons at risk of IHD (173). Thus, outcomes in Gunn *vs.* control rats might reflect effects of elevated bilirubin on cardiovascular phenotype within a 'sensitised' baseline or comparator group (ie. individuals at increased risk of CVD).

3.6 Conclusions

This initial characterisation of cardiac phenotype in hyperbilirubinaemic Gunn rats reveals significantly reduced myocardial contractility *ex vivo* (associated with potentially compensatory repression of *Pln*), an inhibitory effect that is effectively compensated for *in vivo*. Aortic ejection velocities and pressure gradient are reduced *in vivo* (with a compensatory increase in ejection duration), likely as a result of significant aortic dilatation. These vascular changes may beneficially influence afterloading, cardiac function and remodeling processes. Preliminary evidence also supports a stress-resistant phenotype in middle-aged Gunn hearts, associated with increased expression of *Gpx1*. These changes may be relevant to protection against IHD apparent in GS (243). Further work is warranted to elucidate the molecular mechanisms underlying bilirubin's effects on stress-resistance, contraction, vascular ejection mechanics and determinants of afterload. Such information not only advances our understanding of the basis of protection from CVD in GS, but assists in developing novel means of manipulating myocardial function and I-R resistance (40).

CHAPTER 4

AGE-DEPENDANT CARDIOVASCULAR CHANGES AND MYOCARDIAL STRESS RESISTANCE IN GUNN RATS

This chapter has been submitted to the Acta Physiologica as a research article. The formatting and referencing style of the original manuscript has been changed to coincide with this thesis.

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Keywords: Bilirubin, cardioprotection, Gilbert's syndrome, infarction, ischaemia-reperfusion injury, oxidative stress

4.1 Abstract

Bilirubin, an antioxidant and by-product of haem catabolism, substantially reduces risk of cardiovascular disease, as evidenced from studies of mild hyperbilirubinaemia in Gilbert's Syndrome. Little is known regarding myocardial stress-resistance in hyperbilirubinemic conditions or whether life-long exposure modifies cardiac function, potentially protecting from cardiovascular disease. Hyperbilirubinemic rats and littermate controls underwent echocardiography at 3, 6 and 12 months of age, with hearts subsequently assessed for resistance to 30 minutes of ischaemia. While no difference in baseline cardiac function was evident in Gunn *vs.* control rats at 3 months of age, from 6 months onwards Gunn rats demonstrated aortic dilatation during systole and reduced peak ejection velocities. In addition, duration of ventricular ejection increased progressively from 6 months, indicating a negative inotropic effect of bilirubin *in vivo*. *Ex vivo* analysis of baseline function supported these findings, demonstrating reduced left ventricular pressure development (LVDP) and contractility in hyperbilirubinemic rats. Furthermore, stress-resistance was improved in Gunn hearts: post-ischaemic recoveries of LVDP (76±22% vs. 29±17% Control, $P<0.01$) and coronary flow (96±9% vs. 86±16% Control, $P<0.01$) were all improved in Gunn hearts, accompanied by reduced infarction (21±5% vs. 47±15% Control, $P<0.01$), and ventricular malondialdehyde (0.14±0.02 vs. 0.22±0.07 nmol/mg Control, $P<0.01$) and protein carbonyl contents (0.11±0.05 vs. 0.17±0.04 nmol/mg Control, $P<0.01$). These data reveal life-long hyperbilirubinaemia induces age-dependent hypo-contractility in male Gunn rats, and improved stress resistance. Together with prior findings in female animals, these data indicate bilirubin exerts sex-independent effects on vascular structure, myocardial function and ischaemic tolerance, the latter likely mediated via anti-oxidant mechanisms.

4.2 Introduction

Over the past two decades, bilirubin has emerged as a powerful biomarker of cardiovascular health (245). Total bilirubin is negatively related with pre-clinical and overt atherosclerosis (61), CVD (157) and all-cause mortality (108) in the general population. Gilbert's syndrome (GS), a benign condition observed in 5-10% of Caucasian populations (226) characterised by mildly elevated bilirubin due to glucuronosyl transferase 1A1 (UGT1A1) deficiency, profoundly reduces risk of ischaemic heart disease (IHD) (243). The antioxidant capacity of bilirubin (222) may contribute to these beneficial cardiovascular effects (206), though other factors may also participate. Bilirubin protects against events leading to atherosclerosis and thus IHD (173, 244), including serum oxidation (39), low-density lipoprotein (LDL) oxidation (26), and smooth muscle cell proliferation (176, 184). In addition, vascular intima-media thickening, an early indicator of atherosclerosis, is reduced in individuals with higher serum bilirubin levels (120). We recently reported shifts in left ventricular ejection and aortic diameter in *female* hyperbilirubinemic Gunn rats that may improve afterloading, together with evidence of enhanced cardiac stress-resistance and up-regulation of ventricular antioxidant gene expression. Whether similar changes arise in males is untested.

Despite evidence supporting anti-atherogenic effects of bilirubin, few studies have addressed its potential benefits in the event of organ ischaemia/myocardial infarction, which would additionally contribute to protection from CVD mortality. Bilirubin can protect from acute ischaemia-reperfusion injury in the gut (45, 88) and kidney (2). Two preliminary studies implicate bilirubin treatment as a protective intervention in cardiac ischaemia-reperfusion injury (17, 55), and we recently showed

that female Gunn rats with endogenous hyperbilirubinaemia are protected from post-ischaemic cardiac dysfunction (12). Furthermore, it's not clear whether the favourable effects of bilirubin are a result of chronic exposure, and, whether this has an effect on cardiac structure and function. The current study explores whether endogenously elevated bilirubin (in Gunn rats) protects, middle-aged *male* hearts from ischaemia-reperfusion injury (extending evidence of benefit in female Gunn rats), and undertakes the first assessment of post-ischaemic lipid and protein oxidation. Focus on the ischaemic tolerance of middle-aged hearts is particularly relevant given the age-dependence of IHD, and evidence age not only limits myocardial ischaemic tolerance but impairs responses to the most widely studied cardioprotective interventions (22, 183).

4.3 Methods

The Griffith University Animal Ethics Committee approved all experiments prior to commencement (MSC/06/12). All animals were housed in a temperature and light controlled facility, and were provided with standard rat pellets (Specialty Feeds, WA, Australia) and fresh water for the duration of the study. A group of 10 male Gunn (jaundiced) Wistar rats and heterozygous (non-jaundiced; termed wild-type hereon) littermate controls were used for this study. Each animal was weighed weekly from 3-12 weeks of age, followed by echocardiography assessment at 3, 6 and 12 months of age. An additional 6 rats from the same litter (aged to 12 months old) were added to each group (Control, n = 16, Gunn, n = 16) and all animals were sacrificed for Langendorff heart perfusion 1 week after final echocardiography analysis.

4.3.1 Echocardiography

Echocardiography (Vevo 770, VisualSonics, Toronto, ON) assessments were carried out on each animal at 3, 6 and 12 months, as previously described (12). Briefly, animals were weighed and anesthetized using ~2% isoflurane in 2 L/min oxygen, and images from 4 views were obtained; long axis, short axis, suprasternal and four chamber view.

4.3.2 Langendorff heart perfusion

Rats were anesthetised using pentobarbitone sodium (60 mg/ml), administered i.p. at 1 µL/g body weight. Up to an additional 50 µL was administered until pedal and tail pinch reflexes ceased. Hearts were then excised, arrested in cold Krebs-Henseleit solution (KHS), mounted onto a Langendorff heart perfusion apparatus via the aorta, and perfused at constant pressure (73.4 mmHg) with KHS (containing: NaCl, 118 mM; glucose, 11 mM; $NaHCO_3$, 25 mM; KCl, 4.7 mM; $MgSO_4$, 1.2 mM; EDTA, 0.5 mM; $CaCl_2$, 1.75 mM) maintained at 37˚C and gassed with 95% O_2/5% CO_2 (pH 7.4). Blood was also collected from the chest cavity for later analysis, centrifuged at 20000x*g* for 10 minutes and stored at -80ºC. A small fluid filled balloon was inserted into the left ventricle, connected to a pressure transducer (PowerLab, ADInstruments, Castle Hill, Australia) for functional measurements using LabChart Pro (ADInstruments, Castle Hill, Australia). Diastolic pressure was set at 4mmHg at baseline and then a pressure trace was recorded. Each heart was stabilized for 30 minutes, followed by 30 minutes global ischaemia and a 120 minutes recovery period. For subsequent functional analysis, mean values were determined from 30 seconds recordings at each selected time point using LabChart software. Baseline function was assessed immediately prior to ischaemia, and at 10, 30, 60, 90 and 120 minutes of reperfusion. Data was collected for heart rate, diastolic pressure, systolic pressure, left ventricular developed pressure,

+dP/dt and –dP/dt. Coronary flow was manually determined at each time point. The RPP was calculated as the product of heart rate x LVDP. After reperfusion, hearts were prepared for either infarct size quantification or analysis of oxidative damage (*n*=8 per analysis).

4.3.3 Coronary efflux of myocardial enzymes

Coronary effluent was collected throughout the reperfusion period for each heart, and stored at -80˚C until enzyme content analysis. Samples were assessed for creatine kinase (CK) and lactate dehydrogenase (LDH) contents using a Cobas Integra 400+ chemistry analyzer (Roche Diagnostics, NSW, Australia).

4.3.4 Tissue protein concentration

Left atrial and ventricular tissue samples were homogenized in 1 mL CellLytic (Sigma-Aldrich, Castle Hill, NSW) using a glass homogenizer and protein concentrations determined using a BCA protein kit (Pierce, Thermo Scientific, IL, USA). All reactions were carried out per manufacturer's instructions in a 96 well plate, with absorbance read at 540 nm. Unconjugated bilirubin (UCB), MDA and protein carbonyl contents were normalized to tissue protein concentration.

4.3.5 Unconjugated Bilirubin Content in Blood and Myocardium

Unconjugated bilirubin (UCB) content was measured in serum and left atrial tissue from each heart (the latter removed immediately prior to ischaemia). Atrial tissue was immediately frozen at -80ºC. For analysis, 20 mg tissue was pulverized in 1 mL CellLytic (Sigma-Aldrich, Castle Hill, NSW) using a glass homogenizer. After a further 5 minutes on ice, homogenate was centrifuged at 22000x*g* for 5 minutes. For HPLC, 40

*u*L of resultant supernatant, or 40 *u*L of serum was used to assay UCB content, as described previously (12).

4.3.6 Myocardial MDA content

Following 120 minutes of reperfusion, 8 hearts from each group were prepared for MDA analysis via HPLC, as previously described (130). Left ventricular tissue was isolated and immediately frozen in liquid N_2. A mortar and pestle was used to pulverize tissue, from which 50 mg was removed and degraded in a glass homogenizer with 1 mL CellLytic. The sample was centrifuged (22000x*g*, 5 minutes) and supernatant removed and stored at -80˚C. For analysis, samples were diluted (1:3) in deionized water and 50 µL of the diluted sample added to 700 µL orthophosphoric acid (0.44 M), 250 µL 2-thiobarbituric acid (41.6 mM; Sigma-Aldrich, Austria) and 500 µL deionized water in Pyrex glass test tubes. The tubes were heated at 100˚C for 1 hr, cooled on ice for 10 minutes, and 200 µL of sample was added to 200 µL methanol:NaOH. Samples were centrifuged for 5 minutes (3000x*g* at 4˚C) and 20 µL of final supernatants injected onto a HPLC column (LiChroCART, Merck, Germany) for analysis. The column was perfused at 1.3 mL/min using an initial mobile phase of 50 mM phosphate buffer (KH_2PO_4 in deionized water, pH adjusted to 6 using 1M KOH) and a final mobile phase of 60:40 phosphate buffer:methanol. Absorbance was monitored at 563 nm. Standard curves were prepared using 1,1,3,3-tetraethoxypropane, which yields MDA when hydrolyzed (Sigma-Aldrich, Austria). Concentrations of MDA in each sample were determined by comparison of peak areas to those from routinely performed standard curves.

4.3.7 Myocardial protein carbonyl content

Myocardial protein carbonyl concentration was measured using an ELISA kit (Enzo Life Sciences, Lause, Switzerland). Left ventricular tissue (75 mg) stored at -80˚C was homogenized using a 1 mL detergent-free buffer (20 mM phosphate buffer containing 20 µM butylated hydroxytoluene and 100 µM diethylene triamine penta-acetic acid) in a glass homogenizer. Samples were centrifuged for 5 minutes (22000x*g*) and the supernatant assayed via ELISA as per manufacturer's instructions, with slight modification: 35 µg protein was derivatized using DNP and 75 uL of this solution was added to 1 mL of EIA buffer before adding 200 µL of this solution to each ELISA well.

4.3.8 Infarct size quantification

After the 120 minutes of reperfusion, the remaining eight hearts from each group were perfused for a further 20 minutes with 1% 2, 3, 5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, NSW, Australia) dissolved in Krebs-Henseleit buffer. Recovery data was not recorded for this period. Hearts were then placed in 10% formalin (Sigma-Aldrich, NSW, Australia) for 24 hours before being sliced into 2 mm sections from apex to base and returned to formalin for a further 24 hrs. Images were obtained after blotting dry the slices and scanning both sides. Infarct area (white) was compared to viable tissue (brick red) using a color selection application on Adobe Photoshop (Version CS6, Adobe Systems, CA, USA), and all values were expressed as percentages of the whole tissue area.

4.3.9 Statistical analysis

All data have been presented as a mean±standard deviation. Data were assessed for normality of distribution and homogeneity of variance and appropriate parametric or non-parametric analysis applied. Weight gain (Fig 1), age-dependent echocardiography

(Figure 2) and time-dependent I-R outcome (Figure 3) data were analyzed using repeated measured two-way ANOVA to determine if, and where, changes occurred between Gunn and control groups, over time. Between group post-hoc comparisons were made using Tukey's test for all parametric data and the Holm-Sidak test for all non-parametric data. For paired data (tissue and oxidative injury, and infarct size) student's T-test was performed. Pearson's correlation was performed to determine relationships between variables. A significance of $P<0.05$ was considered significant.

4.4 Results

Gunn rats and littermate controls exhibited similar birth weights. However, at approximately 10 weeks of age body weights became consistently and significantly different, with wild-type animals ultimately being >100 g heavier at 12 months of age ($P<0.05$) (Figure 4.1). All animals underwent echocardiography assessment to determine potential age-dependent effects of hyperbilirubinaemia on cardiovascular structure and function. Circulating and tissue concentrations of UCB were confirmed as significantly increased in Gunn animals ($P<0.05$; Table 4.1). No differences in indices of overall cardiac ejection were noted (EF, $P=0.19$; FS, $P=0.29$; Table 4.1). In contrast, aortic velocity-time integral, mean gradient and mean velocity of blood exiting the left ventricle were 25-40% lower in Gunn rats (Table 4.1, $P<0.05$). Echocardiographic assessment revealed systolic aortic diameter was increased by 25%, at 12 months of age (Fig 4.2A; $P<0.05$). Furthermore, age-dependent reductions in aortic peak velocity and increased ejection time were also observed, which developed gradually from 3-12 months (Figure 4.2C, 4.2D). *Ex vivo* cardiac assessment demonstrated a reduction in baseline LVDP and +dP/dt in hearts from 12 months Gunn *vs.* control rats, maintained under identical loading, substrate and Ca^{2+} conditions (Table 4.1, $P<0.05$).

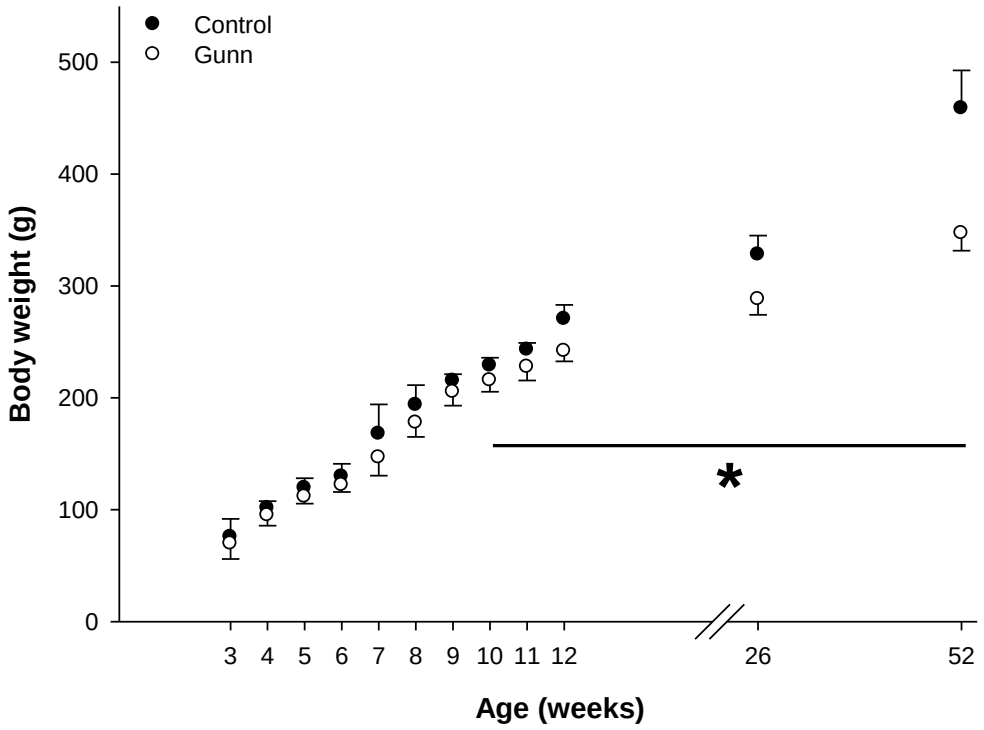


Figure 4.1: *Weight gain.* Body weight (P<0.05) of male Control (n = 10) and Gunn (n = 10) animals from 3 weeks to 12 months of age. *P<0.05 at specific time points vs. Control.

Table 4.1: Baseline in vivo (n=10/group) and ex vivo (n=16/group) cardiac function of aged male Gunn hearts and littermate controls at 12 months of age.

	Control	**Gunn**	***P* value**
In vivo			
Circulating [UCB], µmol/L	1.8±0.77	61.9±0.27.3*	<0.01
Tissue [UCB], nm/mg protein	0.014±0.007	0.191±0.07*	<0.01
Body mass, g	442±40	337±19*	<0.01
Heart mass, g	1.65±0.21	1.40±0.36*	0.02
Heart/body, %	0.38±0.06	0.42±0.11	0.22
Ejection fraction, %	74.7±3.8	79.9±10.5	0.15
Fraction shortening, %	51.3±5.0	55.4±10.8	0.29
IVRT, ms	22.3±4.2	25.6±4.4	0.11
IVCT, ms	26.0±3.9	31.04±6.2*	0.04
MV E/A	1.3±0.1	1.4±0.2	0.52
AoVTI, cm^2	8.8±1.1	5.4±1.9*	<0.01
Mean velocity, mm/s	1682±419	1246±326*	<0.01
Mean gradient, mmHg	3.6±1.7	2.3±0.9*	<0.01
Cardiac output, ml/min/g body weight	0.25±0.02	0.32±0.02*	<0.01
Ex vivo			
Heart Rate, bpm	245±25.2	261±22.3	0.07
Diastolic pressure, mmHg	3.86±1.2	4.07±1.18	0.63
Systolic pressure, mmHg	153±26.5	132±29.7*	0.04
LVDP, mmHg	150±26.7	127±29.6*	0.04
RPP	36762±7391	33250±7584	0.19
+dP/dt, mmHg/s	4613±1371	3659±1217	0.05
-dP/dt, mmHg/s	-2820±573	-2627±768	0.43
Coronary flow, ml/min/g	10.7±1.84	12.1±3.15	0.15

*P<0.05 vs. control. [UCB], unconjugated bilirubin concentration; LV, left ventricular; IVRT, Isovolumetric relaxation time; IVCT, Isovolumetric contraction time, AoVTI, Aortic velocity-time integral, LVDP, left ventricular developed pressure; RPP, rate pressure product; +dP/dt, rate of pressure development, -dP/dt, rate of relaxation

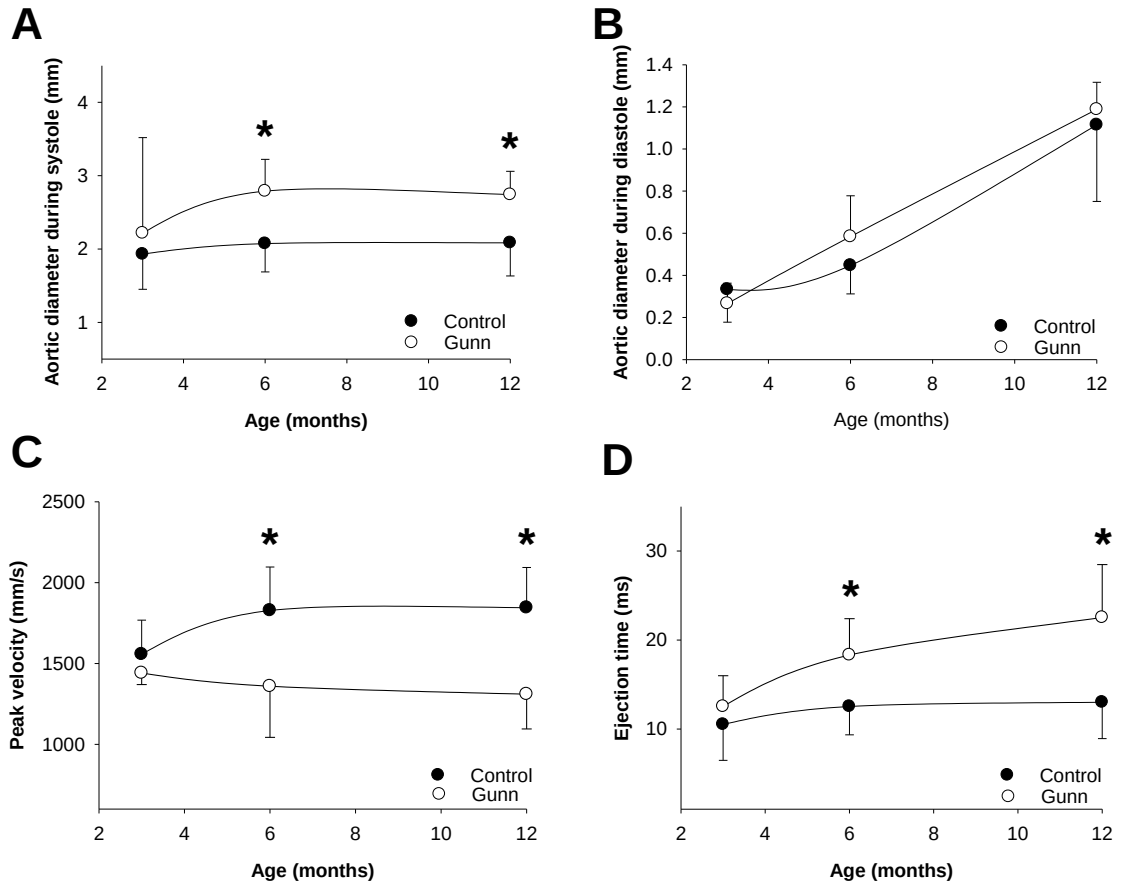


Figure 4.2: *Echocardiographic parameters in young to middle-aged rats*. Parameters of cardiac function and vascular structure including A) aortic diameter during systole, B) aortic diameter during diastole, C) peak aortic blood velocity and D) ejection time in male Gunn (n = 10) and wild-type controls (n = 10) hearts at 3, 6 and 12 months. *P<0.05 at specific time points vs. Control

The ischaemic tolerance of *ex vivo* myocardium was significantly improved in Gunn hearts (Fig 4.3A), with >2-fold greater recovery of LVDP and RPP after 120 minutes reperfusion (Figure 4.3A, 4.3C; $P<0.05$). This was associated with a decline in post-ischaemic diastolic pressure and significantly improved coronary reflow in Gunn hearts (Figure 4.3D, 4.3E; $P<0.05$). Interestingly, the rate of ischaemic contracture development during the ischaemic period was also significantly delayed in Gunn (17.2±2.9 minutes for diastolic pressure to rise by 4 mmHg, $P<0.01$) *vs*. control hearts (13.5±2.2 minutes), and peak contracture reached during ischaemia was much greater in

Control hearts (59±8 mmHg *vs*. 76±6 mmHg in controls, $P<0.01$). Functional protection in Gunn hearts was associated with significant reductions in cardiac enzyme efflux, a marker of myocardial death: Gunn hearts released ~30% less CK (Figure 4.4A, $P<0.05$) and LDH (Figure 4.4B, $P<0.05$) into the coronary effluent throughout the reperfusion period. Moreover, myocardial infarct size was markedly reduced by ~55% in Gunn *vs*. control rats (Figure 4.4C, $P<0.05$). The final infarct size was negatively correlated with unconjugated bilirubin concentration in cardiac tissue ($r=-0.62$, $P<0.05$) (Figure 4.5). Importantly, post-ischaemic left ventricular myocardium from Gunn rats displayed 30-40% lower levels of the oxidative damage markers MDA and protein carbonyl concentration (Figure 4.6).

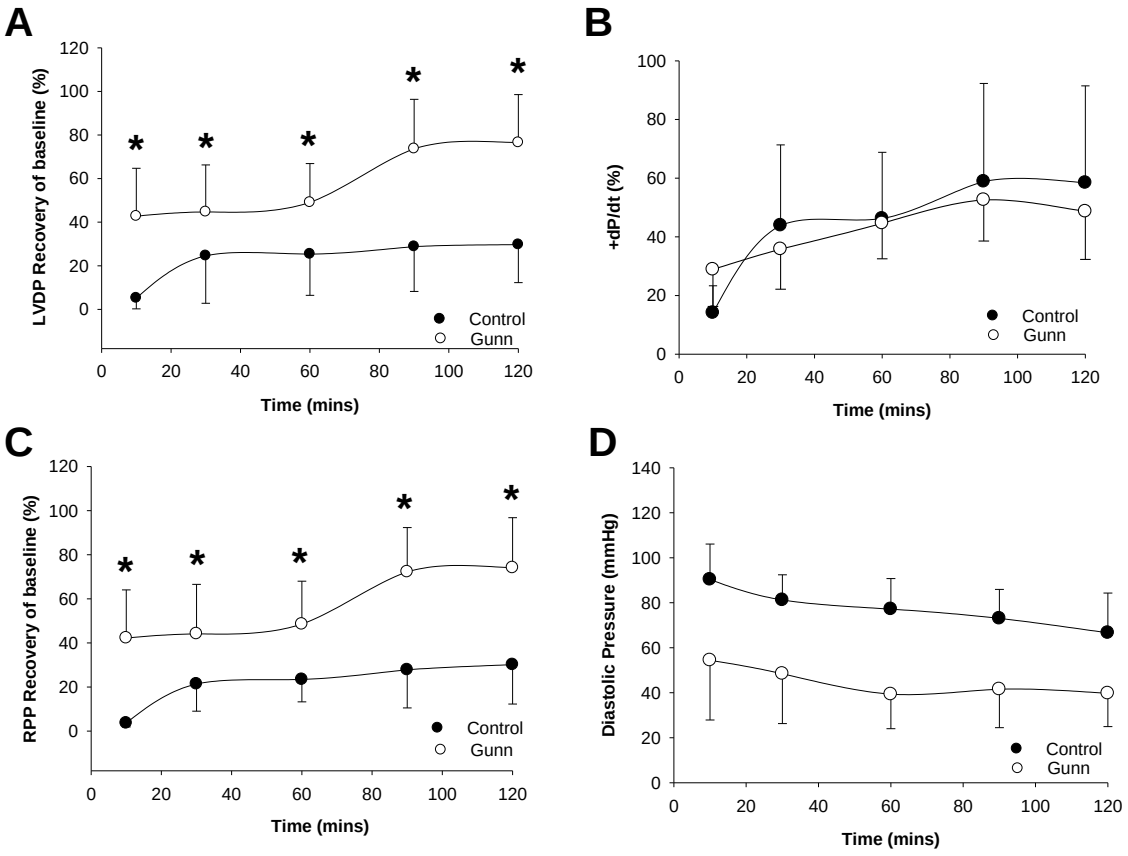


Figure 4.3: *Post-ischaemic functional outcomes.* Relative (%) recoveries are shown for: A) Left ventricular developed pressure (LVDP) B) Rate pressure product (RPP), C) rate of pressure development (+dP/dt), and D) diastolic pressure .Outcomes assessed in aged male control (n = 16) and Gunn (n = 16) hearts subjected to 30 minutes ischaemia. Data are mean±SD. *P<0.05 at specific time points vs. Control

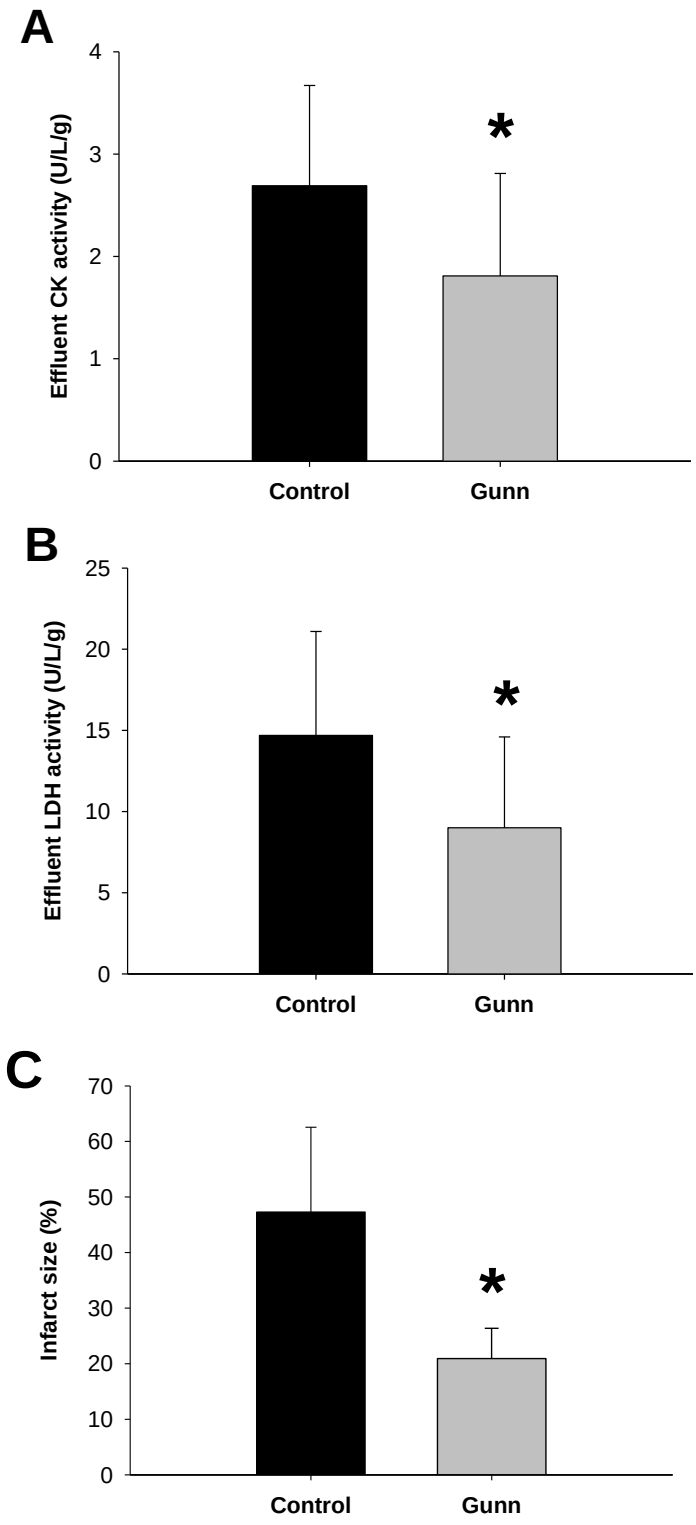


Figure 4.4: *Tissue injury and infarct size*. Data showing A) total post-ischaemic creatine kinase (CK), release, B) total post-ischaemic lactate dehydrogenase (LDH) release, and C) infarct size in aged male Control and Gunn hearts (n = 8 per group). Data are mean±SD *P<0.05 vs. Control.

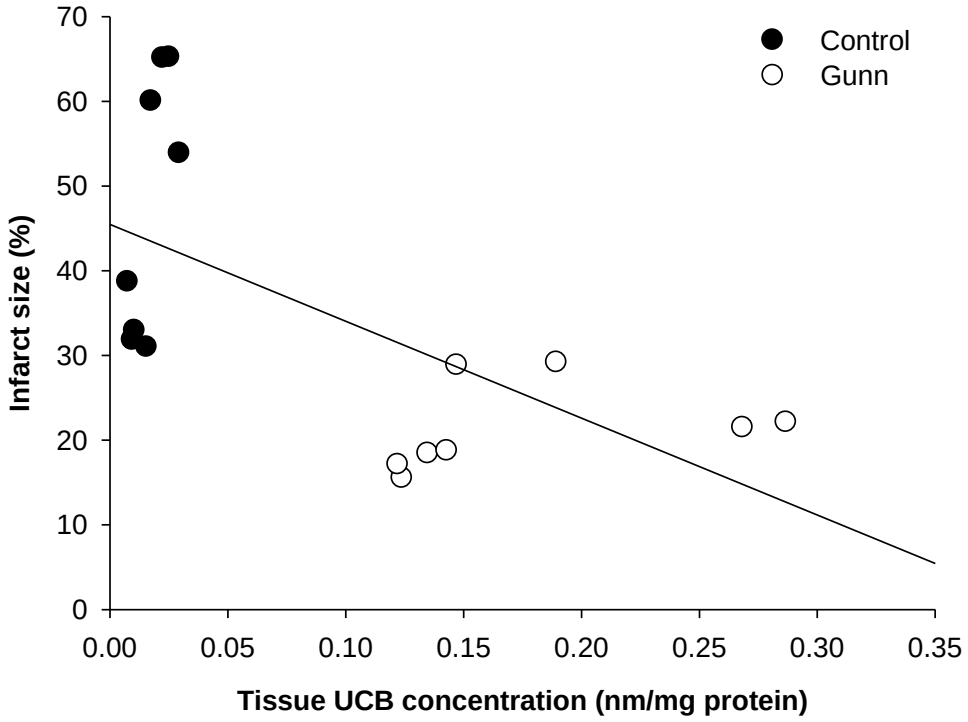


Figure 4.5: *Correlation between infarct size and UCB content.* Correlation shown for UCB tissue concentration vs. infarct size (R=-0.62, P=0.01) in post-ischaemic aged male Control (n = 8) and Gunn (n = 8) hearts.

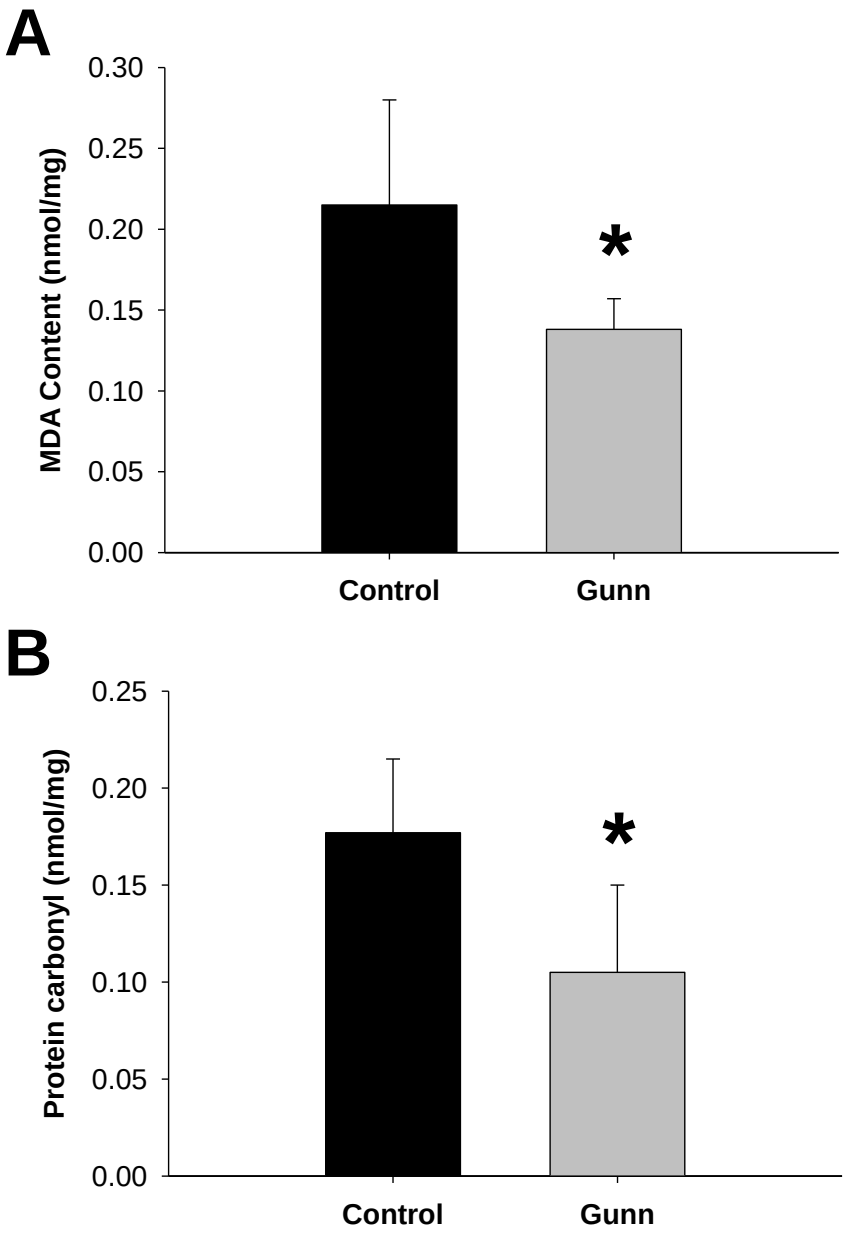


Figure 4.6: Oxidative tissue injury. Left ventricular A) malondialdehyde (MDA), and B) protein carbonyl content in post-ischaemic hearts from aged male Control (n = 8) and Gunn (n = 8) rats. Data are mean±SD. *P<0.05 vs. Control.

4.5 Discussion

In a recent analysis of female Gunn rats we reported structural and functional changes to the heart in association with evidence of improved stress-resistance (12). The current analysis extends these observations: confirming similar shifts in aortic structure and ejection kinetics in males; characterizing age-dependent evolution of these changes; and providing clear evidence of protection against contractile dysfunction, cell death/infarction, and oxidative stress during myocardial I-R. These results, together with prior analysis of females (12), indicate the cardioprotective effects of bilirubin are sex-independent (not requiring, for example, sex-specific elevations in estrogen due to UGT1A1 deficiency). This is also the first confirmation that endogenous hyperbilirubinaemia is linked to reduced oxidative damage in post-ischaemic myocardium: improved stress-resistance in middle-aged Gunn hearts is accompanied by reduced protein and lipid oxidation. Observed changes in cardiovascular structure and function, evident *in vitro* and *in vivo*, implicate modulatory effects of chronic bilirubin on left ventricular contractility, aortic ejection kinetics and large artery structure. It remains unknown whether this reflects unappreciated (and sex-independent) impacts of bilirubin itself on cardiovascular modeling and function *vs.* indirect effects through ROS-dependent modulation of such processes.

4.5.1 Reduced Growth Rate in Gunn Rats

Mature Gunn rats experience reduced weight compared to wild-type littermate (12). The current data regarding lower age-dependent growth in Gunn rats is consistent with recent data for body weight in 3-20 months Gunn *vs.* wild-type rats (217). Gunn animals commence at birth with normal body weights, yet diverge as control animals grow at a significantly faster rate (apparent from ~5 weeks of age), resulting in 25-30%

lower body weight (~100 g) at 12 months (Figure 4.1). This is paralleled by reduced left ventricular mass in Gunn rats, with no change in heart:body weight ratios. Although the exact mechanism for this reduced weight (which is also seen in Gilbert's syndrome patients), several compounds which are associated with obesity, metabolic syndrome and diabetes when elevated, are reduced with hyperbilirubinaemia. These include remnant lipoprotein cholesterol (122), oxidised LDL (26), LDL, triglycerides, total cholesterol (59).

4.5.2 Altered Cardiovascular Structure and Function in Gunn Rats

While indexes of global cardiac function, including cardiac output, ejection fraction and fractional shortening, are unaltered to 12 months of age in Gunn animals, the kinetics (and pressure gradient) of aortic ejection are significantly reduced compared with wild-type controls. These ejection parameters are comparable at 3 months of age, diverging from ~6 months onwards (Figure 4.2). The changes may reflect shifts in afterload, and/or ventricular contractility. As reported for females, aortic diameter was significantly greater (by ~25%) in Gunn rats from 6 months onwards, which may reduce afterload consisted with reduced velocities of blood exiting the left ventricle. This structural change may be related to observed reductions in intima-media thickness in GS patients, potentially involving reduced smooth muscle cell proliferation (and effectively limiting stenosis in these individuals) (67, 120).

Inhibitory effects of hyperbilirubinaemia on intrinsic cardiac contractility in male rats are similar to those observed recently in female Gunn rats (12), confirming the effects are not sex-dependent and emerge with age. Hearts from middle-aged male Gunn rats exhibit reduced *ex vivo* contractility with lower LVDP and +dP/dt (Table

4.1), generally consistent with findings in females. Thus, bilirubin appears to exert negative inotropic effects in a sex-independent manner, which may contribute to observed reductions in peak ejection velocity *in vivo*.

4.5.3 Cardiac Protection and Injurious Oxidative Outcomes in Hearts from Gunn Rats

Hearts from male Gunn rats were highly resistant to I-R injury *ex vivo*. During ischaemia, time to onset of contracture and peak contracture were significantly improved (Table 4.1), both of which are indicators if ischaemic tolerance. Functional outcomes after 120 minutes reperfusion were up to 2-fold greater in Gunn hearts, accompanied by modestly improved coronary reflow (facilitating both metabolite washout and nutrient delivery). In addition cellular damage and infarct size were reduced by up to 55%, in association with 30-40% reductions in left ventricular MDA and protein carbonyl formation. Relative reductions in MDA and protein carbonyl contents were similar (~30-40%), suggesting relatively broad anti-oxidant specificity of bilirubin. Bilirubin protects against protein oxidation in serum of GS individuals, supporting a role in improving protein function via anti-oxidant actions (26, 27). The impact of such effects of bilirubin is highlighted in the profound reduction in infarct size, a cardioprotection that correlates with tissue bilirubin content. This high degree of protection in middle-aged hearts is significant given evidence of age-dependent inhibition of cardiac ischaemia tolerance and cardioprotective efficacies, including ischaemic pre- and postconditioning (22, 183). Indeed, there is considerable evidence poor ischaemic tolerance of middle-aged or aged myocardium stems from reductions in antioxidant status (182, 230), a change that might be effectively countered by elevations

in bilirubin. It is therefore, not unreasonable to conclude that mild hyperbilirubinaemia may protect myocardium in the human condition of Gilbert's Syndrome, improving survival after MI and reducing cardiovascular mortality as documented very recently (107).

4.6 Conclusions

These data provide further information regarding the physiological importance of elevations in unconjugated hyperbilirubinaemia, which results in potentially beneficial shifts in cardiovascular structure and function and substantial protection against myocardial ischaemic injury. Interestingly, cardiac function appears to be modified as a result of chronic life-long exposure to bilirubin, potentially explaining why cardiovascular diseases are less likely to develop Gilbert's syndrome patients. These findings are important and demonstrate that bilirubin exert its effects within multiple systems to protect from CVD mortality. In addition, we document powerful cardioprotective effects of bilirubin in male Gunn rats, consistent with effects in females and suggesting that targeted manipulation of bilirubin metabolism, particularly before or after ischaemia, might present a viable therapeutic avenue for cardiac protection in the clinically relevant middle-aged male.

4.7 Supplemental data

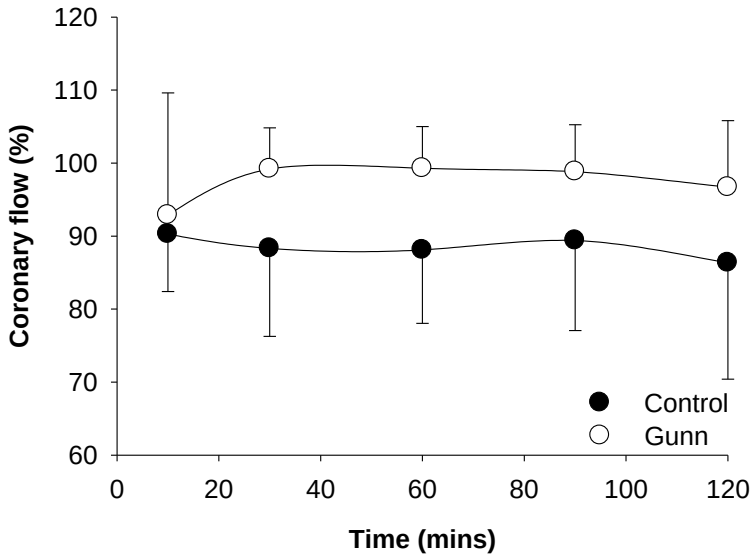


Fig 4.1s: *Post-ischaemic functional outcomes.* Relative (%) recovery of coronary flow in control (n = 16) and Gunn (n = 16) hearts subjected to 30 minutes ischaemia. Data are mean±SD.

CHAPTER 5

PRE- OR POST-ISCHAEMIC BILIRUBIN DITAURATE TREATMENT REDUCES OXIDATIVE TISSUE DAMAGE AND IMPROVES CARDIAC FUNCTION

This chapter has been published in the International Journal of Cardiology as a research article. The formatting and referencing style of the original publication have been changed to coincide with this thesis.

Bhavisha Bakrania, Eugene F Du Toit, Karl-Heinz Wagner, John P Headrick, Andrew C Bulmer

Keywords: Myocardial ischaemia-reperfusion; haem oxygenase; antioxidant; oxidative stress, Gilbert's syndrome

5.1 Abstract

Unconjugated bilirubin (UCB), an endogenous antioxidant, may protect the heart against ischaemia-reperfusion (I-R) injury. However, the 'cardioprotective' potential of bilirubin therapy remains unclear. We tested whether pre- or post-ischaemic treatment of *ex vivo* perfused hearts with bilirubin ditaurate (BRT) improves post-ischaemic functional outcomes and myocardial oxidative damage. Isolated Langendorff perfused hearts (male, Wistar rats) were treated with 50 μM BRT for 30 minutes before (Pre) or after (Post) 30 minutes of zero-flow ischaemia. Functional outcomes were monitored, with myocardial damage estimated from creatine kinase efflux, infarct size, and left ventricular lipid/protein oxidation assessed by measuring malondialdehyde and protein carbonyls. Ischaemia induced contractile dysfunction and cellular injury, with both BRT treatments improving I-R outcomes. Final post-ischaemic recoveries for left ventricular diastolic/developed pressures were significantly enhanced in treated groups: end-diastolic pressure (Control, 78±14, Pre, 51±15*, Post, 51±13 mmHg*); left ventricular developed pressure, (LVDP; Control 44±15, Pre, 71±19*, Post, 84±13 mmHg*). Myocardial injury/infarction (MI) was also significantly reduced with BRT treatment: post-ischaemic creatine kinase efflux (Control, 1.24±0.41, Pre, 0.86±0.31*, Post, 0.51±0.29 U/g/ml*; infarct size, Control, 67±17, Pre, 39±15*, Post, 22±11 %*). These changes were accompanied by significantly reduced malondialdehyde and protein carbonyl content in Pre and Post treated hearts (*P<0.05 vs. Control). These data collectively reveal significant cardioprotection upon BRT treatment, with post-treatment being particularly effective. Significant reductions in infarct size and lipid and protein oxidation indicate a mechanism related to protection from oxidative damage and indicate the potential utility of this molecule as a post MI treatment.

5.2 Introduction

Braunwald and colleagues historically established that acute myocardial infarction could be limited by treatments prior to and upto three hours post event (150). Therefore, a global research effort has focused on 'cardioprotective' therapies to limit myocardial damage with infarction or surgical ischaemia (adjunctive to timely reperfusion). However, despite four decades of research, clinically effective cardioprotection remains elusive (103). While trials suggest some benefit is achievable via ischaemic pre- or post-conditioning interventions, these widely studied candidates induce modest clinical benefits relative to profound outcomes in diverse pre-clinical models (103). This poor translation reflects, among other factors, a 'comorbidity conundrum': aging (most infarct patients being >65 yrs) and comorbidities including diabetes, obesity and hypertension appear to substantially impair myocardial sensitivity to pre- and post-conditioning and related stimuli engaging receptor-coupled cytoprotective signalling cascades (74, 183). Alternate approaches, by-passing conventional survival signalling pathways for example, are therefore warranted in the quest for efficacious cardioprotection.

Clinical and experimntal evidence suggests bilirubin, a relatively under-appreciated endogenous antioxidant generated from haem catabolism, may induce significant cardioprotection in humans and animal models. Haem is oxidized by haem oxygenase-1 (HO-1), yielding iron, CO and biliverdin, with the latter rapidly reduced to unconjugated bilirubin (UCB) via biliverdin reductase. Unconjugated bilirubin is bound to albumin in the blood and transported to the liver where it is glucuronidated, facilitating its excretion. Bilirubin and its metabolites are either excreted or reabsorbed via the enterohepatic circulation (40). An elevated blood UCB concentration can be

indicative of increased haem catabolism, impaired conjugation and/or excretion (41, 246). For example, mild hyperbilirubinaemia in Gilbert's syndrome (GS) is associated with a deficiency in uridine glucuronosyl transferase 1A1 (UGT1A1) and thus excretion of bilirubin from the blood. Individuals are diagnosed with GS when UCB concentrations exceed 17.1 μM compared to normal values approximating 10 μM (224). While mild hyperbilirubinaemia (and potential mild jaundice) in GS are generally considered benign, comparison of GS and matched controls represents a novel approach to investigating the effects of elevated bilirubin on oxidative stress, lipid metabolism and inflammation in humans (12, 19, 25, 26). Early reports suggest individuals with GS are protected from systemic oxidative stress, which may explain associated protection from cardiovascular disease and all-cause mortality (107), because reactive oxygen species are implicated in atherosclerosis, hypertension, and heart failure/cardiomyopathies, together with influencing recovery from heart surgery (137).

The potential of bilirubin to reduce myocardial I-R injury is supported by observations that HO-1, which is required for bilirubin formation, is protective when up-regulated prior to I-R (110). However, therapeutic induction of HO-1 itself may require hours to days of pre-ischaemic intervention, consistent with evidence for HO-1 involvement in more delayed protective responses (144, 269, 270). Alternatively, treatment with HO-1 metabolites responsible for cardioprotection offers a potentially more rapid means of inducing I-R tolerance. HO-1 metabolites - biliverdin/bilirubin, and CO - have been implicated in cardioprotection (137). Although antioxidant effects of bilirubin have been investigated in other organs subjected to I-R (2, 45, 88), the ability of physiological concentrations of bilirubin (or bilirubin analogues) to protect the myocardium from reperfusion induced oxidant injury is not yet reported. The current

study investigates whether a novel synthetic analogue of bilirubin (BRT, bilirubin ditaurate) improves the functional recovery of hearts subjected to I-R injury and determines whether such protection is accompanied by protection from lipid and protein oxidation.

5.3 Methods

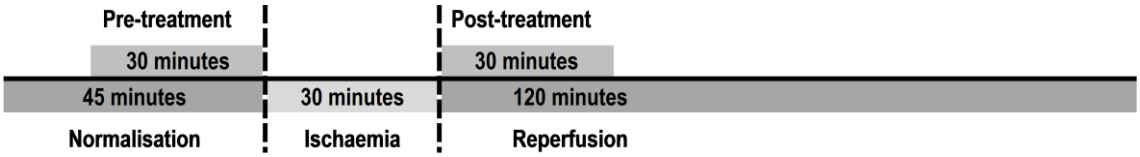
The Griffith University Animal Ethics Committee granted approval (MSC/06/12) for all experiments. Male Wistar rats were housed at the Griffith University Animal Facility, and were provided with standard laboratory rodent pellets (Specialty Feeds, WA, Australia) and fresh water daily. Animals were sacrificed for cardiac experimentation at 12 weeks of age.

5.3.1 Langendorff heart perfusion model and experimental protocol

Animals were anesthetised using pentobarbitone sodium (60 mg/dL administered i.p. at 1 µL/g body weight), with an additional 50 µL of anesthetic administered as required until pain reflexes had ceased. Hearts were excised during euthanasia and mounted on the Langendorff apparatus as previously described (12). Briefly, hearts were perfused at 73.4mmHg constant pressure, and were maintained at 37ºC for the duration of the experiment. Diastolic pressure was set at 4mmHg. After 45 minutes of aerobic stabilisation all hearts were subjected to 30 minutes of no-flow global ischaemia followed by 120 minutes of reperfusion. Hearts were untreated (Control, n=16), or received 50 µM BRT (Frontier Scientific, UT, USA) for 30 minutes prior to ischaemia (Pre, n=16) or for the initial 30 minutes of reperfusion (Post, n=16). A 30 minutes treatment period was established to allow BRT to accumulate within the tissue based on the pharmacokinetic evaluation of BRT infusion as reported by Bulmer

et al. (40). Our preliminary studies demonstrated the treatment above yielded similar cardiac bilirubin content as those observed in hyperbilirubinemic Gunn heart atria, providing a sound rationale for the BRT dose administered (12). Bilirubin ditaurate is light-sensitive, therefore, all syringes were sealed with foil and lighting was dimmed during administration. Left ventricular pressures were measured using a fluid-filled balloon connected to a pressure transducer and PowerLab (ADInstruments, NSW, Australia) data-acquisition system. Left ventricular diastolic and systolic pressures, developed pressure, +dP/dt and -dP/dt were continuously monitored, with data reported at the end of stabilisation (baseline), and at 10, 30, 60, 90 and 120 minutes of reperfusion. Coronary flow was manually recorded at these time points. On completion of studies, eight hearts were assessed for infarct size, and the remaining eight hearts from each group were frozen in liquid N_2, pulverized and protein extracted for assessment of oxidative damage (malondialdehyde and protein carbonyl content).

Figure 5.1: Schematic time course of Langendorff heart perfusion



5.3.2 Myocardial creatine kinase and lactate dehydrogenase efflux

Coronary effluent was pooled from each heart for analysis of lactate dehydrogenase (LDH) and creatine kinase (CK) efflux (measures of myocardial injury), prior to ischaemia (normoxic efflux values) and a sample taken at 120 minutes of reperfusion. Samples were stored at -80˚C until analysis. Lactate dehydrogenase (LDHI2) and creatine kinase (CK-MB) activity was subsequently measured using a

Cobas Integra 400 plus chemistry analyzer (Roche Diagnostics, NSW, Australia), according to manufacturer's directions.

5.3.3 Atrial bilirubin analysis

The left atrium was removed after each treatment period (10-15 seconds after bilirubin-free perfusion), and immediately prior to ischaemia in Control hearts. Atrial tissue was blotted dry and stored at -80˚C. For analysis, tissue was degraded in CellLytic Solution (Sigma-Aldrich, NSW, Australia), using a glass homogeniser, until intact tissue was no longer visible. The homogenate was allowed to stand for 10 minutes in ice before centrifugation at 22000x*g* for 5 minutes. A 40 µL supernatant sample was removed and BRT concentration determined using HPLC, as described previously (12). Pure BRT (Frontier Scientific, UT, USA) was used as an external standard.

5.3.4 Myocardial MDA content

Malondialdehyde concentrations were assessed in left ventricular tissue samples (*n*=8 per group) after 120 minutes of reperfusion. Ventricular homogenates were prepared as for atria, as described above. Final supernatant samples were removed and stored at -80˚C until analysis. Samples were diluted 1-in-4 in deionized water and MDA concentration quantified using HLPC (130). Samples were prepared for analysis by adding 700 µL orthophosphoric acid (0.44 M), 250 µL 2-thiobarbituric acid (41.6 mM; Sigma-Aldrich, Austria), 500 µL deionized water with 50 µL of the diluted sample in Pyrex glass test tubes. All samples were then heated at 100˚C for 1 hr, cooled on ice for 10 minutes, and 200 µL of sample added to 200 µL methanol:NaOH. Samples were centrifuged for 5 minutes (3000x*g* at 4˚C) and 20 µL samples of the final supernatants

were injected onto a HPLC column (LiChroCART, Merck, Germany) for analysis. The column was perfused at 1.3 mL/min using an initial mobile phase of 50 mM phosphate buffer (KH_2PO_4 in deionised water, pH adjusted to 6 using 1M KOH) and a final mobile phase of 60:40 phosphate buffer:methanol. Absorbance was monitored at 563 nm. Standard curves were prepared using 1,1,3,3-tetraethoxypropane, which yields MDA when hydrolyzed (Sigma-Aldrich, Austria). Concentrations of MDA in each sample were determined by comparison of peak areas to those obtained from standard curves.

5.3.5 Myocardial protein carbonyl content

Protein carbonyl concentration was measured in myocardial tissue using ELISA (Enzo Life Sciences, Lause, Switzerland). Left ventricular tissue (75mg) that had been stored at -80˚C, was homogenised using a 1mL detergent-free buffer (20mM phosphate buffer containing 20µM butylated hydroxytoluene and 100µM diethylene triamine penta-acetic acid) using a glass homogeniser. Samples were centrifuged for 5 minutes (22000x*g*) and the supernatant was used to carry out the ELISA assay as per the manufacturer's instructions, with slight modification. 35 µg protein was derivatiatized using DNP and 75 uL of this solution was added to 1 mL of EIA buffer. Finally, 200 uL of this solution was added to each ELISA well. Further ELISA analysis was conducted according the manufacturer's instruction.

5.3.6 Tissue protein concentration

Bilirubin (atrial), MDA and protein carbonyl concentrations (ventricular) were expressed relative to protein content, which were determined using a BCA protein kit (Pierce, Thermo Scientific, IL, USA) as per the manufacturer's instruction. Sample absorbance was read on a 96 well plate reader (at 540 nm).

5.3.7 Infarct size quantification

Recovery was recorded during the 120 minute reperfusion period. For the remaining eight hearts (from each group) a further 20 minute perfusion was performed with 1% 2, 3, 5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, NSW, Australia) dissolved in Krebs-Henseleit buffer. Hearts were then placed in 10% formalin (Sigma-Aldrich, NSW, Australia) for 24 hrs. Fixed hearts were sliced into 2 mm sections from apex to base, and returned to formalin for a further 24 hrs. After this time, slices were blotted dry and both sides scanned with images stored digitally. Infarct area (white in colour) was compared to viable tissue (brick red in colour) using a colour selection application on Adobe Photoshop (Version CS6, Adobe Systems, CA, USA), and all values were expressed as percentages of the whole tissue area.

5.3.8 Statistical analysis

All data were tested for normality of distribution and equality of variance. All parametric data was analysed using one-way ANOVA (followed by Holm-Sidak post hoc test) and all non-parametric data were analysed using ANOVA on ranks (followed by the Tukey's post hoc test; Sigmaplot, version 11.0; Systat Inc., IL, USA). Pearson's correlation was used to determine relationships between post-ischaemic outcomes and myocardial BRT content. A $P<0.05$ was considered significant. Data are presented as a mean±standard deviation.

5.4 Results

Heart rate, coronary flow and cardiac function were similar across groups and were unaltered by 30 minutes BRT treatment (Table 5.1). Since baseline function was comparable across groups, functional recovery data during post-ischaemic reperfusion are reported in absolute values.

Table 5.1: Baseline function, time to onset of ischaemic contracture and peak ischaemic contracture in control (n=16), Pre (n=16) and Post (n=16) hearts. Data are mean±SD. Statistical analysis performed using one-way ANOVA.

	Control	**Pre**	**Post**	***P-value***
HR, bpm	257±34	237±61	265±46	0.771
CF, ml/min/g	7.4±1.2	7.2±1.3	7.3±1.8	0.978
DP, mmHg	4±2	4±2	4±1	0.640
SP, mmHg	134±27	125±10	134±17	0.084
LVDP, mmHg	130±26	122±9.	130±18	0.157
+dP/dt, mmHg/s	3824±819	3343±779	3643±1031	0.309
-dP/dt, mmHg/s	-2885±543	-2435±951	-2538±729	0.061
RPP, mmHg/min	34017±7470	28568±6997	34416±8473	0.087
TOC, min	13.3±2.4	13.1±3.1	14.0±2.5	0.630
PC, mmHg	82.3±18.8	83.7±31.3	73.9±18.8	0.452

HR, Heart rate; CF, Coronary flow; DP, Diastolic pressure; SP, Systolic pressure; LVDP, Left ventricular developed pressure; +dP/dt, Rate of pressure development; -dP/dt, Rate of relaxation; RPP, Rate pressure product; TOC, Time to onset of contracture; PC, Peak contracture

The extent and rate of ischaemic contracture development was unaltered by BRT treatment (Table 5.1). However, post-ischaemic functional outcomes were significantly improved by both pre- and post-ischaemic BRT treatment (Figure 5.2 and 5.3). Left ventricular developed pressure, diastolic pressure and +dP/dt were significantly

($P<0.05$) improved in Pre and Post vs. Control groups, between 60 and 120 minutes of reperfusion (Figure 5.2B, 5.2D, 5.2E). However, the rate pressure product (RPP) was improved by post-treatment from 60 minutes onwards and only at 30 minutes in the pre-treatment group (Figure 5.2C). As highlighted in Figure 5.2D, the impact of both Pre and Post BRT on LVDP was largely a consequence of improvements in diastolic pressure during reperfusion.

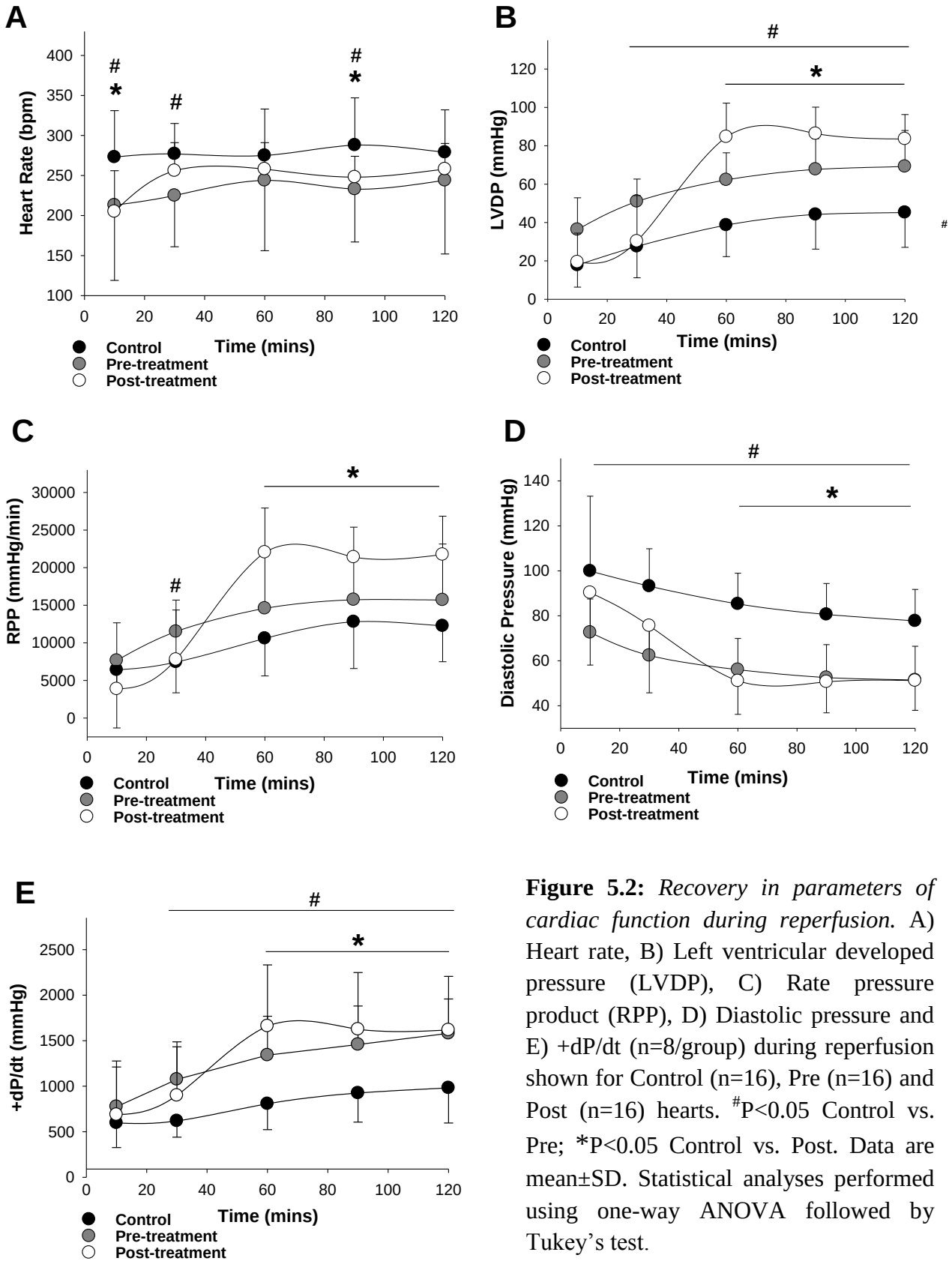


Figure 5.2: *Recovery in parameters of cardiac function during reperfusion.* A) Heart rate, B) Left ventricular developed pressure (LVDP), C) Rate pressure product (RPP), D) Diastolic pressure and E) +dP/dt (n=8/group) during reperfusion shown for Control (n=16), Pre (n=16) and Post (n=16) hearts. $^{\#}P<0.05$ Control vs. Pre; $^{*}P<0.05$ Control vs. Post. Data are mean±SD. Statistical analyses performed using one-way ANOVA followed by Tukey's test.

Release of CK into the coronary effluent supported a significant reduction in I-R dependent tissue damage in Pre and Post groups (Figure 5.3A). Creatine kinase efflux was reduced by ~30% in the Pre and ~50% in Post group, at 120 minutes of reperfusion ($P<0.01$). Lactate dehydrogenase efflux was also reduced in the Pre ($P=0.1$) and Post group ($P<0.05$; Figure 3B) by ~30%. Equally, infarct quantification showed significantly reduced area of cell death (Figure 5.4A and 5.4B) representing 67±17% in Controls, in comparison to 37±16% and 23±12% in the Pre and Post groups, respectively (both $P<0.01$).

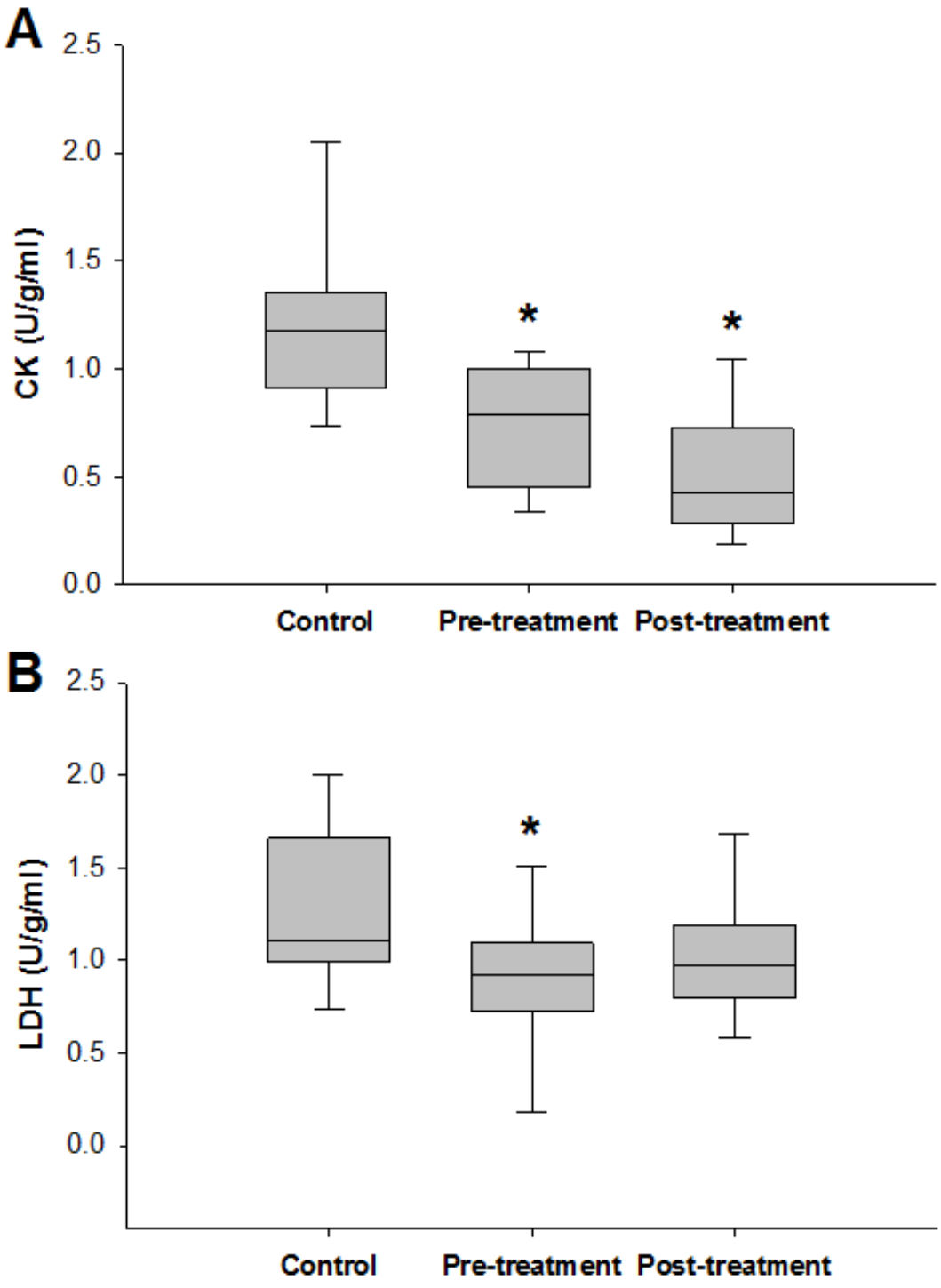


Figure 5.3: *Biomarkers of tissue damage during reperfusion.* Data shown for A) Creatine kinase (CK) activity (C vs. Pre P<0.05, C vs. Post P<0.05, Pre vs. Post P>0.05) B) Lactate dehydrogenase (LDH) activity (C vs. Pre P>0.02, C vs. Post P>0.05, Pre vs. Post P>0.05) measured in total coronary effluent at 120 minutes (n=8/group; *P<0.05 vs. Control). Statistical analysis performed using one-way ANOVA followed by Tukey's (for CK) and Holm-Sidak (for LDH) post hoc tests.

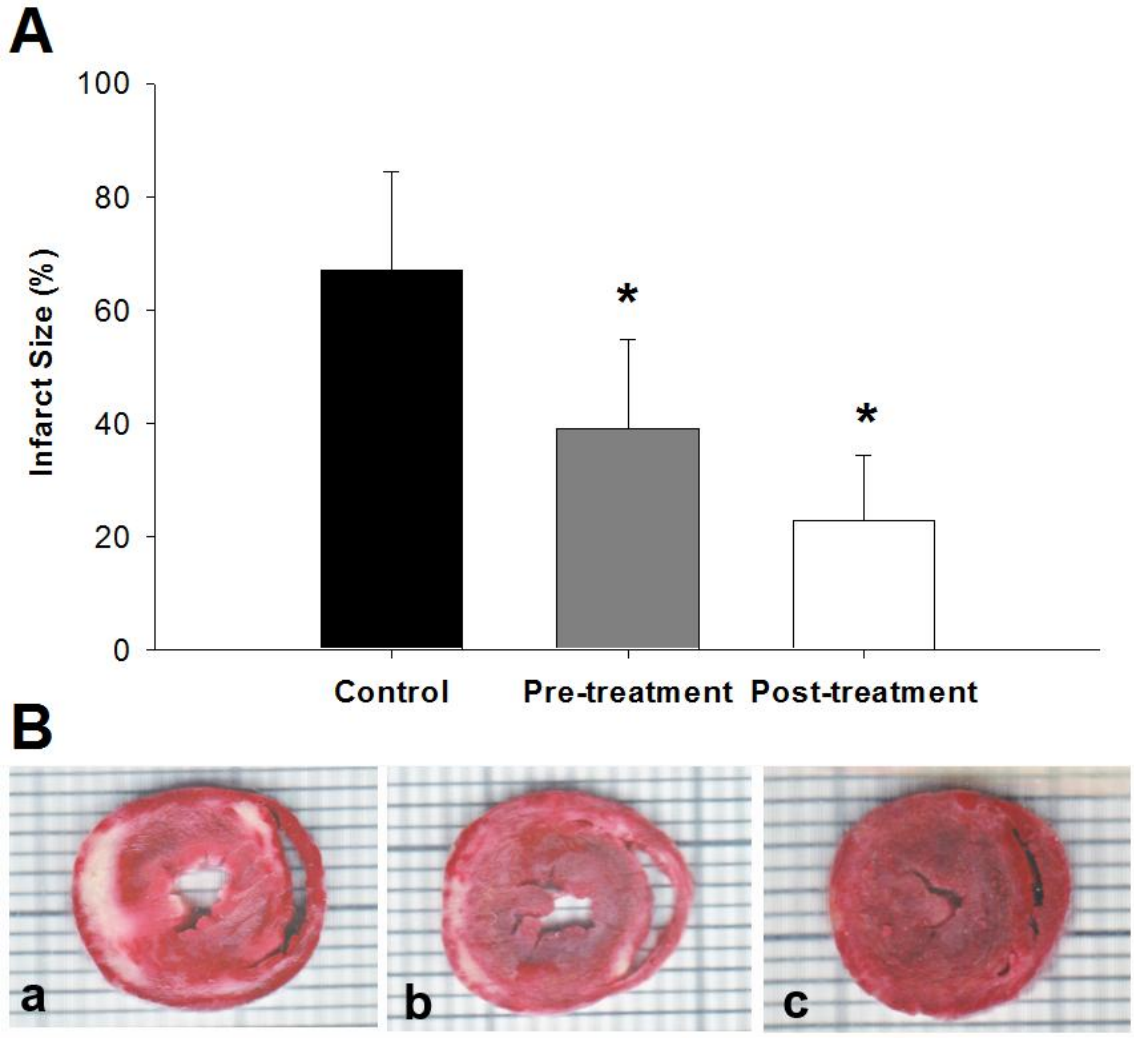


Figure 5.4: *Infarct area quantification*. Data shown for A) Infarct size quantification (presented as a percentage of total area) after 120 minutes of reperfusion (C vs. Pre P<0.01, C vs. Post P<0.01, Pre vs. Post P=0.06) for Control, Pre and Post groups (n=8 per group; *P<0.05 vs. Control) and B) Representative images of a) Control, b) Pre and c) Post hearts, after 120 minutes of reperfusion and staining with 1% TTC. Data are mean±SD. Statistical analysis performed using one-way ANOVA followed by Holm-Sidak post hoc test.

A positive correlation between post-ischaemic recovery of LVDP (at 120 minutes reperfusion) and myocardial (atrial) BRT concentration was observed (Figure 5.5A; R=0.58, P<0.001). In addition, a negative correlation between infarct size and (atrial) BRT concentration existed (Figure 5.5B; R=0.63, P<0.001).

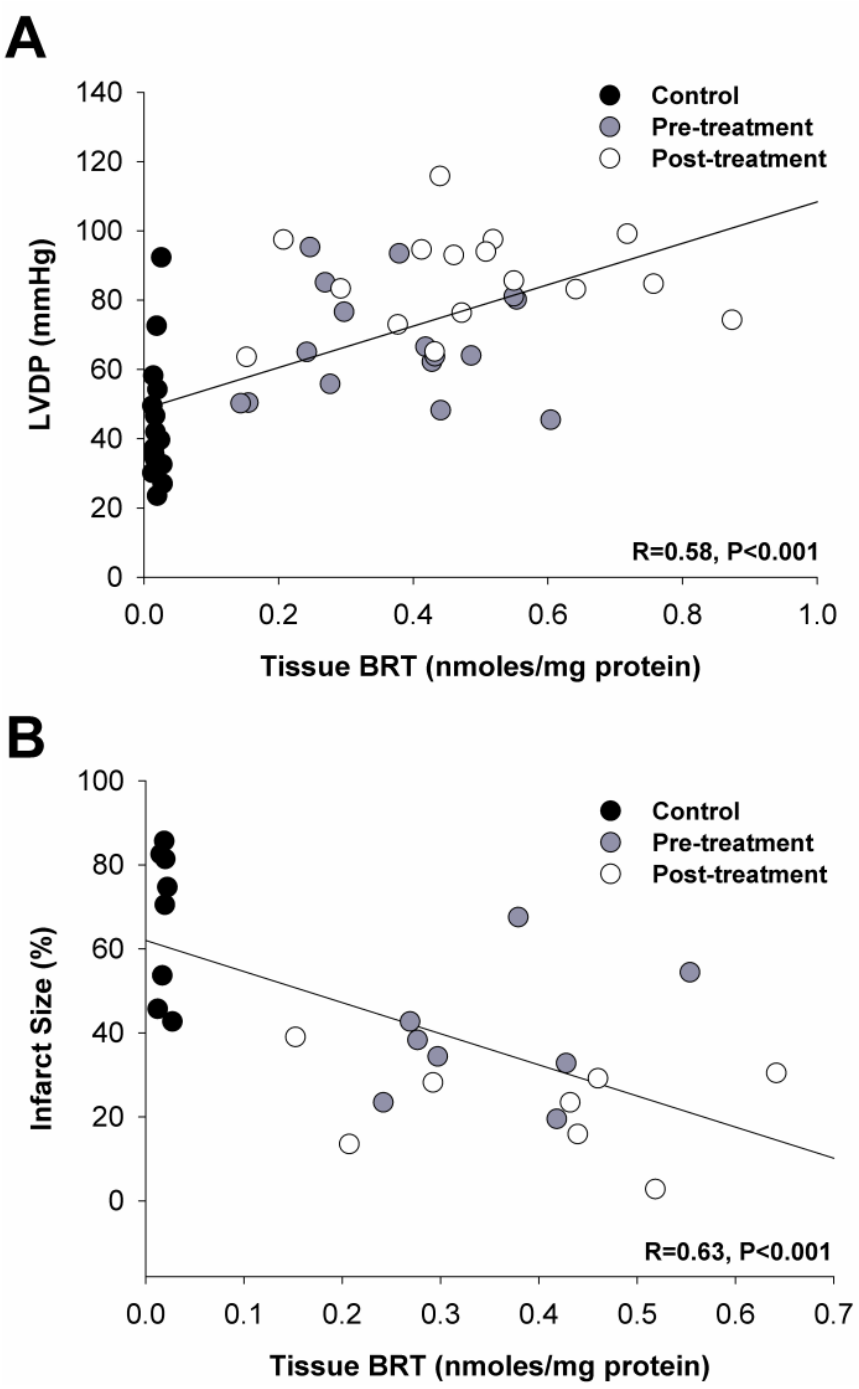


Figure 5.5: *Relationship between functional recovery, infarct size and myocardial (atrial) BRT content.* Pearson's correlation shown for bilirubin ditaurate (BRT) content in left atrial tissue versus A) Left ventricular developed pressure at 120 minutes of reperfusion (n=16/group; r=0.58, P<0.001) and B) Infarct size (n=8/group; r=-0.64, P<0.001)

To test whether antioxidant effects of BRT influenced myocardial protection from I-R, left ventricular MDA (Figure 5.6; lipid peroxidation) and protein carbonyl (Figure 5.7; protein oxidation) content were assessed. Lipid oxidation was reduced by ~25% in the Pre group (P=0.2) and ~40% in the Post group (P<0.05) hearts (Figure 5.6). Protein oxidation was significantly reduced by over 60% in both the Pre and Post (both P<0.05) groups, respectively (Figure 5.7).

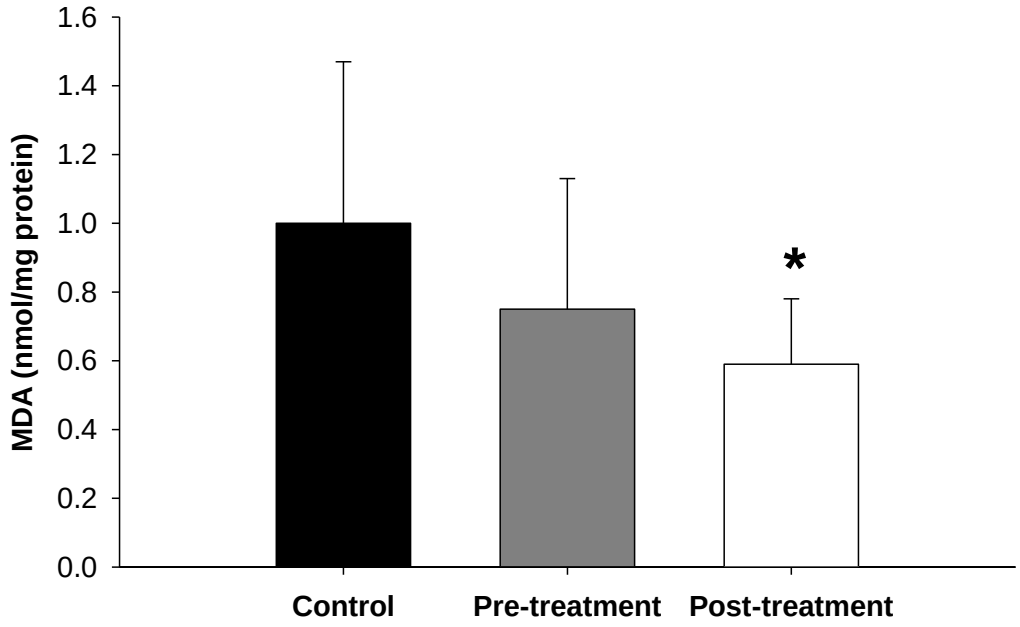


Figure 5.6: *Left ventricular MDA content after 120 minutes of reperfusion.* Data shown for lipid peroxidation/malondialdehyde (MDA) content in left ventricular tissue (presented relative to protein concentration) for Control, Pre (P>0.05) and Post (P<0.05) hearts (n=8/group). Data are mean±SD. Statistical analysis performed using one-way ANOVA followed by Tukey's post hoc test, *P<0.05 vs. Control.

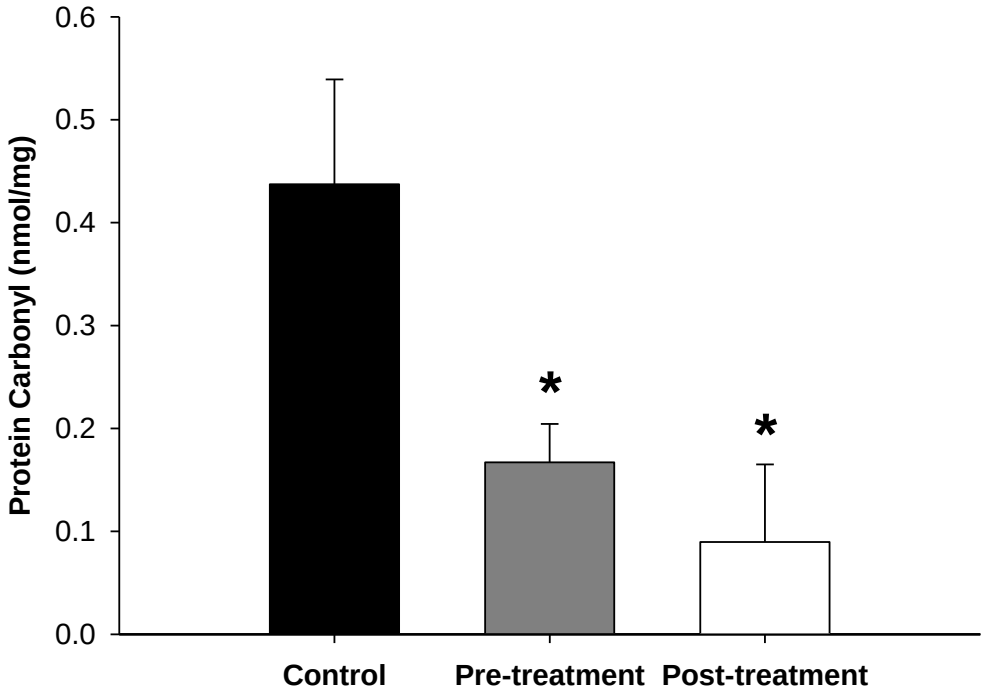


Figure 5.7: *Left ventricular protein carbonyl content after 120 minutes of reperfusion.* Data shown for protein carbonyl content in left ventricular tissue for Control, Pre ($P<0.05$) and Post ($P<0.05$) hearts (n=8/group). Data are mean±SD .Statistical analysis performed using one-way ANOVA followed by Tukey's post hoc test, *$P<0.05$ vs. Control.

5.5 Discussion

This study is the first to show that treatment of *ex vivo* perfused hearts with ~50 µM of the water-soluble bilirubin analogue BRT, immediately prior to (Pre) or following ischaemia (Post), significantly improves cardiac function, and reduces multiple markers of tissue damage upon reperfusion. Interestingly, the relative protective efficacy of post-ischaemic BRT appeared greater than pre-ischaemic BRT. These observations suggest that events occurring within post-ischaemic myocardium may be more critical to I-R outcomes and/or sensitive to BRT, consistent with a protective effect of BRT on oxidative tissue injury particularly during reperfusion.

Bilirubin ditaurate did not significantly affect cardiac function prior to ischaemia-reperfusion (Table 5.1), though a tendency towards a modest reduction in contractile function existed (DP; +dP/dt; Table 5.1). This is in agreement with recent work identifying a potential negative inotropic effect of chronically elevated unconjugated hyperbilirubinaemia in the Gunn rat (12). Interestingly, time to onset of contracture and peak contracture were not reduced in the pre-treatment group, however, are significantly improved by chronic hyperbilirubinaemia in association with ischaemic tolerance (12). These data indicate that differences in the physico-chemical properties of endogenous unconjugated bilirubin, which is hydrophobic, versus that of the synthetic, water soluble bilirubin ditaurate likely influence their biological effects, including their antioxidant properties (161) and physical interactions with molecular targets (38).

Bilirubin exhibits potent antioxidant activity, which would be beneficial under circumstances of excessive free radical production, as experienced during ischaemia-reperfusion. *In vitro* studies show that bilirubin is a strong cytoprotective agent, and that

low concentrations (in the μM range) of unconjugated bilirubin efficiently scavenge lipid peroxyl radicals (223). The antioxidant capacity of bilirubin surpasses that of α-tocopherol, another potent lipophilic antioxidant, at physiologically relevant O_2 concentrations (222). In addition, bile pigments including bilirubin can efficiently scavenge 1*e*- and 2*e*-oxidants (43), whereas most antioxidants typically scavenge only one class of reactive oxygen species efficiently whilst reacting slowly with others (97).

Unconjugated bilirubin protects tissues from I-R injury and tissue damage, linking its antioxidant effects to cytoprotective activity (2, 45, 88). For example, Ceran *et al.* demonstrated that rats infused with UCB (1 mg/mL) for 2 hrs prior to 45 minutes intestinal I-R exhibited reduced malondialdehyde content and tissue damage in the gut (45). Adin *et al.* also showed that a UCB flush prior to 20 minutes of warm renal ischaemia improves glomerular filtration rate, renal vascular resistance and mitochondrial integrity, however, did not report markers of oxidant damage (2). Three reports currently implicate bilirubin in cardioprotection (17, 55, 153) . Clark *et al.* assessed myocardial tolerance to I-R injury, after injecting hemin (i.p.) into rats prior to *ex vivo* ischaemia-reperfusion. Hemin administration induces HO-1 and consequent CO and bilirubin production, and this study reported improved cardiac function and reduced infarct size upon reperfusion. In addition, Clark *et al.* perfused naive hearts with 0.05 or 0.1 μmol unconjugated bilirubin, and then delivered a 30 minute ischaemic insult to hearts. Although functional improvements were observed, the concentrations of bilirubin administered, or of that found in cardiac tissue of hemin treated animals, fell well below normal concentrations. Therefore, the physiological relevance of these findings remain unclear (55). Furthermore, markers of oxidative injury were not investigated, which may have demonstrated protection via an antioxidant mechanism of

action. Masini and colleagues also demonstrated a possible role of bilirubin in protecting the heart from oxidant injury and administered hemin (4 mg/kg) up to 24 hrs prior to *in vivo* focal I-R injury in rats (30 minutes). The authors reported reduced infarct size, MDA production, reperfusion arrhythmias and Ca^{2+} overload. Unfortunately, however, this article did not directly implicate bilirubin induced protection, because CO, biliverdin/bilirubin and iron are liberated upon haem catabolism by HO. Therefore, HO-1 or any of its products, which induce an array of cyto-protective mechanisms, could have contributed to protection, greatly complicating the interpretation of these findings (153). The previous study by Ben-Amotz *et al.* reported that UCB administration (10 mg/kg i.p.) 1 hr prior to a 30 minutes *in vivo* left anterior descending artery occlusion significantly reduced infarct size and improved fractional shortening during ischaemia, although no change in post-ischaemic function was noted. Furthermore, endpoints of tissue oxidation were not reported (17). Finally, the previous two studies did not document bilirubin concentrations in blood or tissue; therefore, it remains unknown whether bilirubin was directly responsible for the protective effects documented. In summary, the existing published research in the field has not adequately and comprehensively investigated whether bilirubin, *per se*, protects from cardiac I-R injury, or the possible mechanisms responsible for its protection.

We have demonstrated that MDA, produced as a result of lipid peroxidation and widely used as a marker of oxidative damage, was reduced in BRT post-treated hearts (Figure 5.6). In addition, we have demonstrated efficacious protection against protein oxidation in both Pre and Post hearts (Figure 5.7). These data suggest that BRT could preserve cellular integrity by protecting from lipid peroxidation and protect cellular function, by inhibiting protein oxidation. These data further support bilirubin's broad

capacity to neutralise 1*e*- and 2*e*-oxidants that target lipids and proteins for oxidation. Furthermore, this is the first report that clearly demonstrates bilirubin treatment *after ischaemia* also, if not more effectively, protects from cardiac dysfunction and reduces tissue damage. To implicate a role for delivery of BRT into tissue in protecting from cardiac dysfunction and infarct size, tissue BRT concentrations were strongly and significantly correlated with these parameters. Importantly, this study aimed to mimic physiologically relevant concentrations of bilirubin approximating ~50 μm, which we previously showed protected Gunn rats from myocardial ischaemia-reperfusion injury (12), and is associated with reduced cardiovascular mortality in humans with Gilbert's syndrome (107).

Based upon these results we conclude that BRT administration protects hearts from ischaemia-reperfusion via an antioxidant mechanism of action. The less pronounced antioxidant effect (ie. prevention of MDA formation) in Pre group may reflect the oxidation and/or washout of BRT during ischaemia/early reperfusion reducing concentrations available throughout reperfusion when radical production is at its greatest (241). Post-ischaemic BRT, delivered during the critical reperfusion period, appeared most effective in limiting I-R mediated infarction, and lipid and protein oxidation. The presence and continuous replenishment of BRT during the period of key radical production likely explains the greater reduction in MDA and protein carbonyl production in the Post group. Despite protection from lipid peroxidation only occurring upon post-treatment, pre- and post-treatment of hearts significantly inhibited protein oxidation and thus likely protects regulatory and structural contractile proteins from damage. In addition to reducing oxidative stress, vasodilatation of vessels, due to improved nitric oxide bioavailability, could be responsible for improved recovery

during reperfusion (156). However, as shown here, coronary flow did not differ between groups (Table 5.1), suggesting improved function occurred independently of nutrient supply/waste product removal.

Antioxidant therapies for cardiac I-R have been comprehensively studied in recent years, due to their potentially efficacious impact on myocardial function and infarct size (137, 174). Despite their success in the laboratory, these studies seldom translate to a clinical setting, largely because post-treatment remains ineffective in the setting of unpredictable MI, or is not published (83, 118, 135). Bilirubin ditaurate represents a viable therapeutic target, given its rapid hepatic excretion and thus is unlikely to be toxic (40). The cardioprotective effect of BRT is further supported by reports that mild accumulation of unconjugated bilirubin in humans (as in GS, and at concentrations similar to those used here) is associated with reduced incidence of cardiac events in hemodialysis patients (48), cardiovascular (157) and all-cause mortality in the general population (107, 108). These data strongly indicate that bilirubin's effects are targeted at multiple steps in the pathophysiological process of cardiovascular disease and associated mortality, with this investigation specifically demonstrating protection of the heart from ischaemia-reperfusion (245). Furthermore, this report is the first to demonstrate the utility of BRT as a potential cardioprotective therapy, with the capacity to improve myocardial outcomes from MI when applied *after* the ischaemic event.

5.6 Conclusion

This study demonstrates that BRT protects cardiac tissue from I-R injury at concentrations attainable upon acute i.v. infusion *in vivo* (40). Multiple endpoints of oxidative damage were reduced in the presence of BRT, likely a consequence of scavenging free radicals and providing an effective mechanism of tissue protection. Bilirubin ditaurate treatment during *reperfusion* (ie. delivered after myocardial ischaemia) improved post ischaemic function, reduced infarct size, lipid peroxidation and protein oxidation to the greatest extent. Furthermore, these data support the potential utility of bilirubin ditaurate by first responders as a post-conditioning agent in conjunction with the current treatment of anti-thrombolytics, particularly within the first few minutes of reperfusion.

5.7 Supplementary data

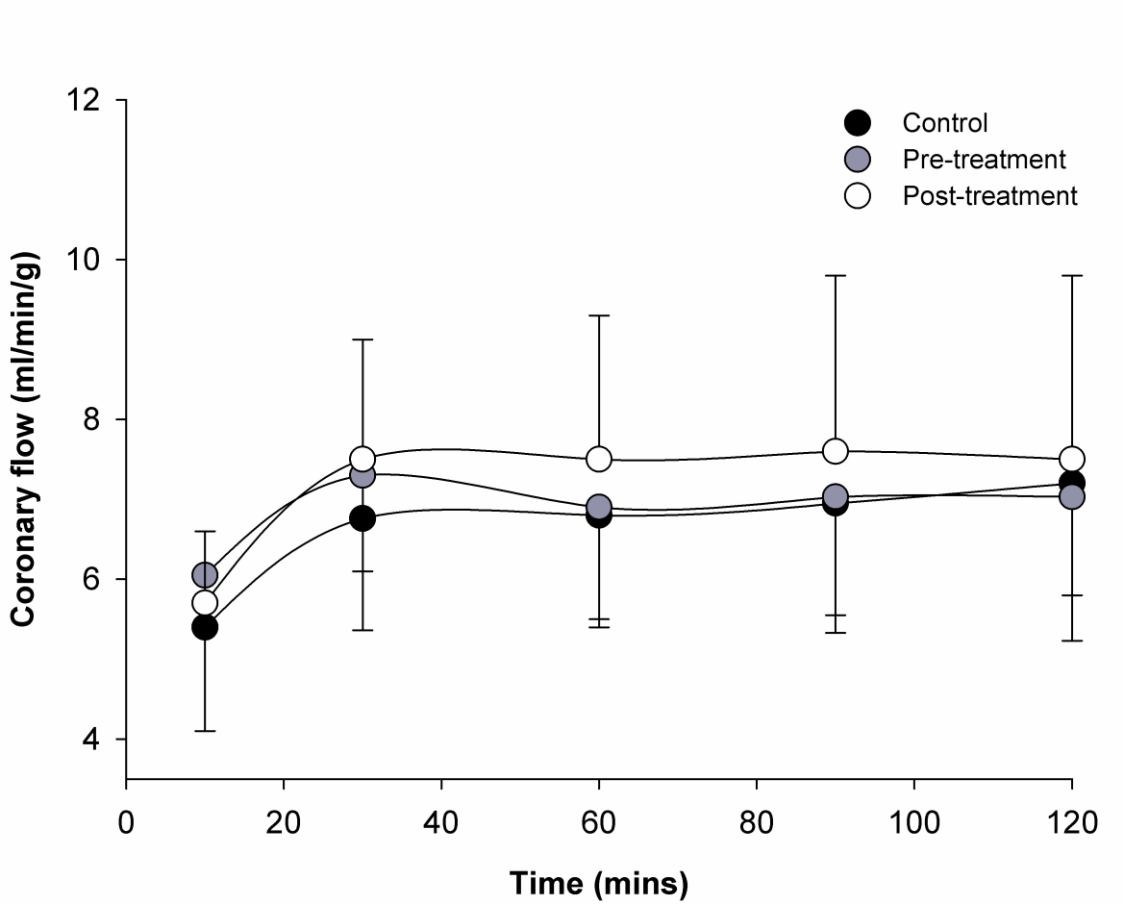


Figure 5.1s: *Coronary flow recovery*. Data shown for coronary flow recovery during reperfusion expressed relative to heart mass for Control, Pre and Post groups (n=16/group). Data are mean±SD. Statistical analysis performed using one-way ANOVA.

CHAPTER 6

MODULATION OF EXTRACELLULAR MATRIX, ANTI-APOPTOTIC PATHWAYS AND OLFACTORY RECEPTOR EXPRESSION IN LEFT VENTRICULAR MYOCARDIUM OF HYPERBILIRUBINAEMIC GUNN RATS

This chapter has been prepared for submission to the American Journal of Physiology - Physiological Genomics. The formatting and referencing style of the manuscript have been changed to coincide with this thesis.

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Keywords: Myocardial ischaemia-reperfusion; haem oxygenase; antioxidant; oxidative stress, Gilbert's syndrome

6.1 Abstract

While mild elevations in circulating bilirubin (as in Gilbert's syndrome [GS]) are strongly associated with reduced risk of cardiovascular disease (CVD) risk, an understanding of the mechanisms responsible for protection remain poorly-defined. The antioxidant capacity of bilirubin may contribute to protection, however, bilirubin may also affect cardiac function in isolated hearts, implying effects on regulatory mechanisms, including gene expression. This study aimed to interrogate differences in the left ventricular transcriptome of hyperbilirubinaemic Gunn *vs.* control rats. Gene microarray analysis revealed significant differences in the expression of 304 transcripts, 97 with known functions. Pathway analysis revealed differential regulation of extracellular matrix (ECM) organisation, vascular development and response to extracellular stimuli. RT-qPCR validation confirmed modulation of seven selected genes in Gunn *vs.* control tissue. The extent to which these changes reflect direct effects of unconjugated bilirubin *vs.* indirect modulation of the myocardial 'oxidative' phenotype, and their importance to altered myocardial function evident in Gunn rats awaits further study.

6.2 Introduction

Mild unconjugated hyperbilirubinaemia, as observed in Gilberts syndrome (226), is associated with profound reductions in cardiovascular disease (CVD) prevalence and mortality (126, 207, 243). The potent antioxidant effects of bilirubin (222), and improved antioxidant capacity of serum from hyperbilirubinaemic humans and rodents (26, 27), is likely to be a critical factor contributing to protection from CVD (25, 243). However, bilirubin also appears to reduce vascular intima-media thickening (67, 73, 120), inhibiting smooth muscle cell proliferation (176, 184), increasing nitric oxide (NO) bioavailability (156), influencing large arterial structure and inducing a mild hypocontractile state in hyperbilirubinaemic Gunn rats (12).

Furthermore, recent reports demonstrate that Gunn rat myocardium is resistant to ischaemia-reperfusion insult, reducing cell death, dysfunction and oxidative injury (12). These changes are accompanied by increased glutathione peroxidise (*Gpx*) and reduced superoxide dismutase (*Sod1*) and phospholamban (*Pln*) gene expression (12). Increased expression of antioxidant genes (*Gpx* and *Sod1*) further support a role for an antioxidant mechanism, in addition to the effect of elevated bilirubin, while reduced *Pln* expression suggests modulation of additional pathways with the potential to affect cardiac function in Gunn myocardium. Although acute administration of bilirubin analogues protects the myocardium from ischaemia-reperfusion from oxidative insult (Chapter 5), it remains unknown to what extent hyperbilirubinaemia effects gene transcription, which could be important in explaining additional effects of bilirubin on cardiac function and stress resistance.

The purpose of the current study was to investigate impact of mild hyperbilirubinaemia in the middle aged female Gunn rat on the myocardial transcriptome as determined via microarray and RT-qPCR analysis.

6.3 Methods

Microarray and RT-qPCR analyses were performed on left ventricular myocardial tissue from 12-13 month old (middle-aged) female Gunn (*n*=6) and litter-mate controls (*n*=6) rats. All 'control' animals were heterozygotes (do not express a hyperbilirubinaemic phenotype), while all Gunn rats were homozygous and visibly jaundiced at birth (and were ear-tagged). Prior to tissue harvest, all animals were housed at the Griffith University Animal Facility and provided with standard laboratory rodent pellets (Specialty Feeds, WA, Australia) and fresh water daily. Animals were sacrificed at 12 months of age with ethical approval from the Griffith University Animal Ethics Committee (MSC/06/12).

6.3.1 RNA extraction and microarray analysis

Myocardial RNA was extracted from 50 mg of ventricular tissue stored at -80ºC in RNAlater (Qiagen, Melbourne, Australia). Tissue was carefully disrupted in TRIzol (Life Technologies, Melbourne, Australia) using a 1 ml syringe and 18G, 21G 23G and 25G needles, and RNA isolated using RNeasy spin columns (Qiagen, Melbourne, Australia) as per manufacturer's instructions. The RNA yield and purity were determined using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) and diluted to a final concentration of 50 ng/µL. RNA integrity was assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Microarray analysis was subsequently performed using GeneChip® Rat Gene 2.0 ST arrays

(Affymetrix, Santa Clara, CA, USA). These whole-transcript microarrays assess >13,900 protein coding transcripts and are designed with a median of 22 unique (25bp) probes per transcript, thus providing superior sequence coverage than other platforms by measuring a median of 550 bases per transcript. Microarray hybridisations were carried out at the Ramaciotti Centre for Genomics (University of New South Wales) according to standard manufacturer's protocols.

6.3.2 Affymetrix microarray data analysis

The individual CEL files generated at the end of microarray scanning were imported into the Affymetrix Expression Console software (Affymetrix, Santa Clara, CA, USA) and gene level RMA normalisation applied. Following data processing all microarray controls (hybridisation, labelling and housekeeping) were within accepted levels. Affymetrix Transcriptome Analysis Console software (Affymetrix, Santa Clara, CA, USA) was used for statistical analysis to enable the identification of differentially expressed genes between the two groups. Functional annotation and pathway analysis was carried out using the DAVID Bioinformatics Database (62). The Benjamini false discovery rate was used to determine enrichment of genes within pathway analysis, with a *P* value of less than 0.1 considered significant. A more conservative *P* value was adopted to determine significance specifically in pathway analysis, to assist in generating hypotheses for further exploration, as is commonly adopted in exploratory microarray studies.

6.3.3 Validation of microarray via RT-qPCR

RT-qPCR was performed on seven transcripts, identified to be significantly differentially expressed by microarray analysis. Briefly, cDNA was synthesised from

500 ng of total RNA using QuantiTect Reverse Transcription Kits (Qiagen, Melbourne, Australia) according to manufacturer's guidelines. Two-step RT-qPCR was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). PCR primers (Table 2.1) were designed with PerlPrimer software (151) according to standard guidelines (8). Briefly, primer pairs for each transcript were designed to have a target annealing temperature of 60°C, span an intron/exon boundary and generate an amplicon between 75-150bp in size. Briefly, cDNA was diluted 1:20, 4 µL of which was combined with 5 µL 1X RT^2 SYBR Green qPCR mastermix (Qiagen, Melbourne, Australia) and 100 nM of each primer (Intergrated DNA Technologies, Baulkham Hills, Australia) in a final reaction volume of 10 µL. Seven genes (*Col3a1*, *Fmod, Igf1, Mmp2, Nrep, Rxfp1, Sparc*) were selected to validate array data, based upon high abundance and positive and negative regulation based on microarray data. The phosphoglycerate kinase 1 (*Pgk1*) gene was evaluated among several potential reference genes and validated as the most stably expressed (stability M-value = 0.26) in all analyzed samples. All quantitative PCR data is expressed as a fold change relative to wild-type controls, calculated using the ΔCq method.

6.3.4 Statistical analysis.

Data are mean±SD. For group comparisons (Gunn rat *vs*. wild type control), unpaired *t*-tests were used to assess statistical significance of differences. A *P* value of <0.05 was considered statistically significant for comparison of gene expression between the groups. The Benjamini-Hochberg procedure for multiple comparisons was used to control for false rate discovery in the pathway analysis. A *P* value of <0.1 was considered significant due to the conservative nature of this method.

Table 6.1: *Primer sequences of target genes used within RT-qPCR analysis.*

Symbol	Gene name	Forward Sequence	Reverse Sequence
Col3a1	collagen, type III, alpha 1	TCAAGAGCGGAGAATACTGG	TCAGCACCAGCATCTGTC
Fmod	fibromodulin	AGGATCAATGAGTTCTCCATCAG	CGCTTGATCTCGTTCCCA
Igf1	insulin-like growth factor 1	TTTACTTCAACAAGCCCACAG	CAGCCTCCTCAGATCACAG
Mmp2	matrix metallopeptidase 2	GTAGACGCTGCCTTTAACTG	TCATTGTATCTCCAGAACTTGTCC
Nrep	neuronal regeneration related protein	GGAACTCTTGGTCTGGGTC	GAAGTCTTCCCTTAGTAAGACCTC
Rxfp1	relaxin/insulin-like family peptide receptor 1	AAACAATAAGATTCGCTCCGTC	GTCTTCAATTATCAGCCATTCCAG
Sparc	secreted protein, acidic, cysteine-rich (osteonectin)	GGACCATGCAAATACATTGCC	GAGGTTGTTGCCCTCATCTC
Pgk1	phosphoglycerate kinase 1	AGCTCCTGGAAGGTAAAGTC	CTGCACTAACACCAAATGGA

6.4 Results

General details of Gunn and wild-type rats are provided in Table 6.2. As observed previously, body weight was significantly reduced in Gunn rats. Left ventricular mass was significantly reduced, however, when normalised to body weight, no significant difference existed.

Table 6.2: General body and cardiovascular parameters of Gunn and wild-type rats (*n*=8)

Parameters	Control	Gunn	P-Value
Body weight, g	259±30	200±32*	<0.01
Blood [UCB], µmol/L	1.4±0.6	46.4±11.4*	<0.01
HR, bpm	329±29	319±35	0.62
LV mass, g	0.70±0.05	0.58±0.11*	0.03
LV mass/body weight	0.0028±0.0003	0.0029±0.0007	0.86

*P<0.05 vs. control. HR, heart rate; LV, left ventricular. Data are mean±SD.

Microarray analysis revealed significant differences in the expression of 304 transcripts (81 up-regulated, 223 down-regulated), 97 (49 up-regulated, 48 down-regulated) of which were protein encoding genes. Initial statistical analysis using the Benjamini-Hochberg false discovery (FDR) correction for multiple comparisons yielded no statistically significant differential expression between the animal groups. Therefore, unadjusted *P* values were used to identify target genes for validation. Whilst this relaxed statistical threshold may potentially introduce numerous false positives, greater confidence in the data is achieved due to the whole-transcriptome nature of the microarrays (i.e. median of 22 unique probes per gene is assayed on these microarrays) and was followed by RT-qPCR validation of gene expression. A volcano plot (Figure 6.1) of the microarray data shows the 304 differentially expressed genes which met both

fold-change [>1.3] and P-value thresholds [P<0.05]; Figure 6.1). Hierarchical clustering of the 304 significantly expressed genes demonstrates that the Gunn and control groups did not clearly partition into two distinct clusters (Figure 6.2). Two of the Gunn samples clustered within the control group, which most likely indicates heterogeneous biological variability within some of the genes identified.

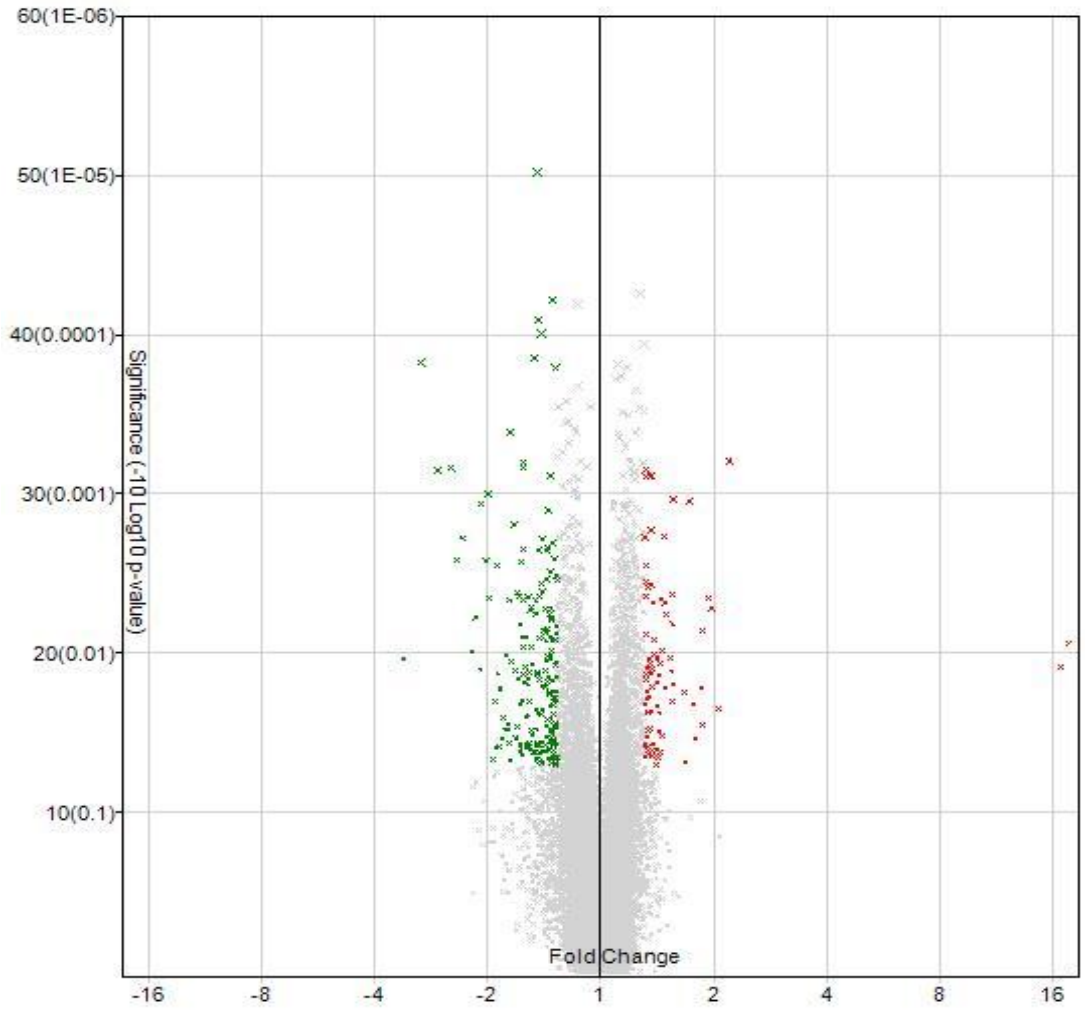

Figure 6.1: *Individual gene expression changes between groups.* Volcano plot representing fold changes of individual gene expression between the groups (x-axis) and *P* values (y-axis) in Gunn vs. controls. Red crosses indicate up-regulated transcripts and green crosses indicate down-regulated transcripts in Gunn *vs.* control animals (*n*=6/group).

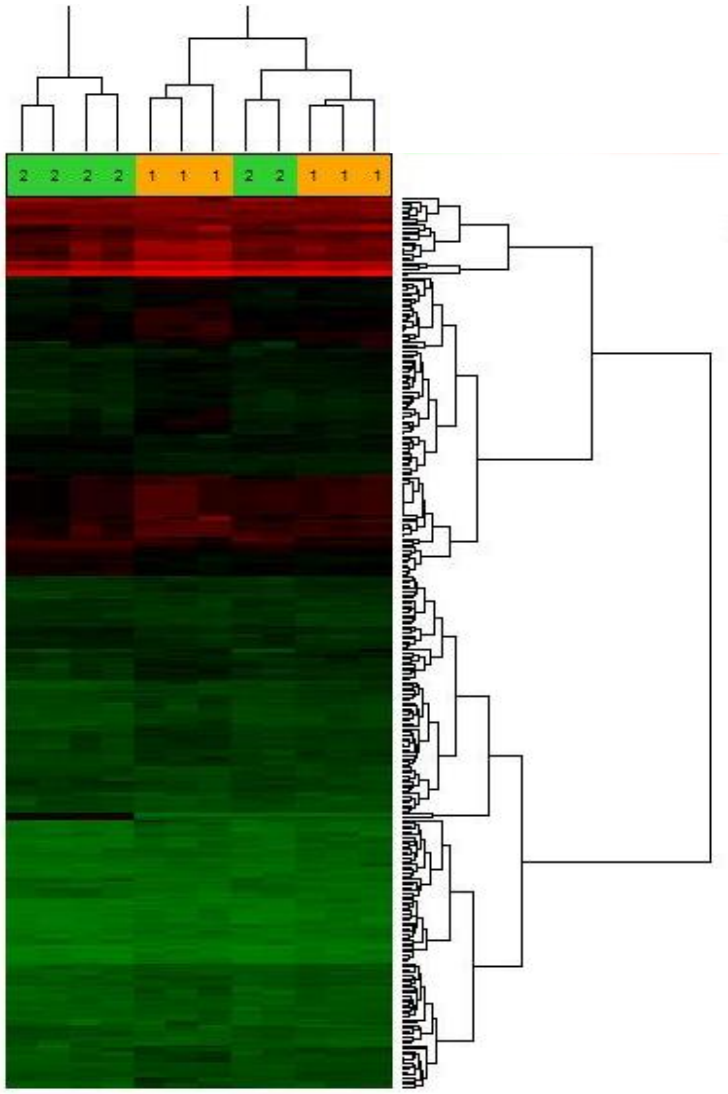

Figure 6.2: *Gene clustering differences between groups.* Heat map representing clustering of genes in Gunn (green boxes) vs. controls (orange boxes; *n*=6/group)

Functional annotation and canonical pathway analysis using the DAVID bioinformatics database indicated differential enrichment of genes in twelve annotated clusters (summarised in Tables 6.3-6.6). In particular, extracellular matrix composition and organisation were differentially regulated between the groups, in addition to pathways associated with vascular development and response to extracellular stimulus.

Table 6.3: Enrichment of genes coding for cellular components in left ventricular Gunn *vs.* control myocardium

Cellular component	**Count**	**ES**	**P value**	**Benjamini *P* value**
*Proteinaceous extracellular matrix**	9	1.79	<0.01	<0.01
*Extracellular matrix**	9	1.79	<0.01	<0.01
*Fibrillar collagen**	3	1.79	<0.01	0.03
*Extracellular region part**	11	1.79	<0.01	0.03
*Collagen**	3	1.79	<0.01	0.07
*Extracellular region**	13	1.79	0.02	0.2
*Extracellular space**	7	1.79	0.03	0.3

*Benjamini *P*-value<0.1. ES, enrichment score

Table 6.4: Enrichment of genes coding for biological processes in left ventricular Gunn *vs.* control myocardium

Biological process	**Count**	**ES**	**P value**	**Benjamini *P* value**
*Extracellular matrix organization**	5	1.79	<0.01	<0.01
Skeletal system development	7	1.79	<0.01	0.18
Response to extracellular stimulus	7	1.79	<0.01	0.18
Response to cAMP	4	1.79	<0.01	0.23
Collagen metabolic process	3	1.79	<0.01	0.25
Collagen fibril organization	3	1.79	<0.01	0.24
Skin development	3	1.79	0.01	0.26
Response to wounding	7	1.79	0.01	0.29
Response to cytokine stimulus	4	1.79	0.01	0.35
Regeneration	4	1.79	0.01	0.37
Bone development	4	1.79	0.02	0.40
Would healing	4	1.79	0.04	0.52
Response to oxidative stress	4	1.79	0.04	0.53
Epidermis development	3	1.79	0.07	0.67
Blood vessel development	4	1.79	0.07	0.68
Vasculature development	4	1.79	0.08	0.68
Skeletal system morphogenesis	3	1.79	0.08	0.69
Regulation of cell size	3	1.79	0.18	0.91
Regulation of cellular component size	3	1.79	0.25	0.95
Enzyme linked receptor protein signaling pathway	3	1.79	0.33	0.98
Regulation of apoptosis	3	1.79	0.79	1.0
Regulation of programmed cell death	3	1.79	0.79	1.0
Tissue morphogenesis	7	1.53	0.00	0.23
Morphogenesis of an epithelium	5	1.53	0.00	0.24

*Benjamini *P*-value<0.05. ES, enrichment score

Table 6.5: Enrichment of genes coding for molecular functions in left ventricular Gunn *vs.* control myocardium

Molecular function	**Count**	**ES**	**P value**	**Benjamini *P* value**
Extracellular matrix structural constituent	3	1.79	<0.01	0.70
Structural molecule activity	7	1.79	0.03	0.91
Identical protein binding	4	1.79	0.46	1.0
Olfactory receptor activity	11	1.53	0.04	0.85

ES, enrichment score

Table 6.6: Enrichment of KEGG pathway in left ventricular Gunn *vs.* control myocardium

Canonical Pathway	**Count**	**ES**	**P value**	**Benjamini *P* value**
ECM-receptor interaction	4	1.79	<0.01	0.41
Focal adhesion	5	1.79	0.02	0.45
Olfactory transduction	8	1.35	0.30	1.0

ES, enrichment score

RT-qPCR analysis of seven selected transcripts confirmed a linear correlation between microarray and PCR determined gene expression. Seven of these genes were determined to be significantly differentially expressed, including *Col3a1, Igf1, Mmp2, Nppb, Nrep, Rxfp1, Sparc* (Figure 6.3; Table 6.7). Pearson's correlation of fold change results obtained using the two different methods indicated preliminary validation of the microarray data (R^2=0.77).

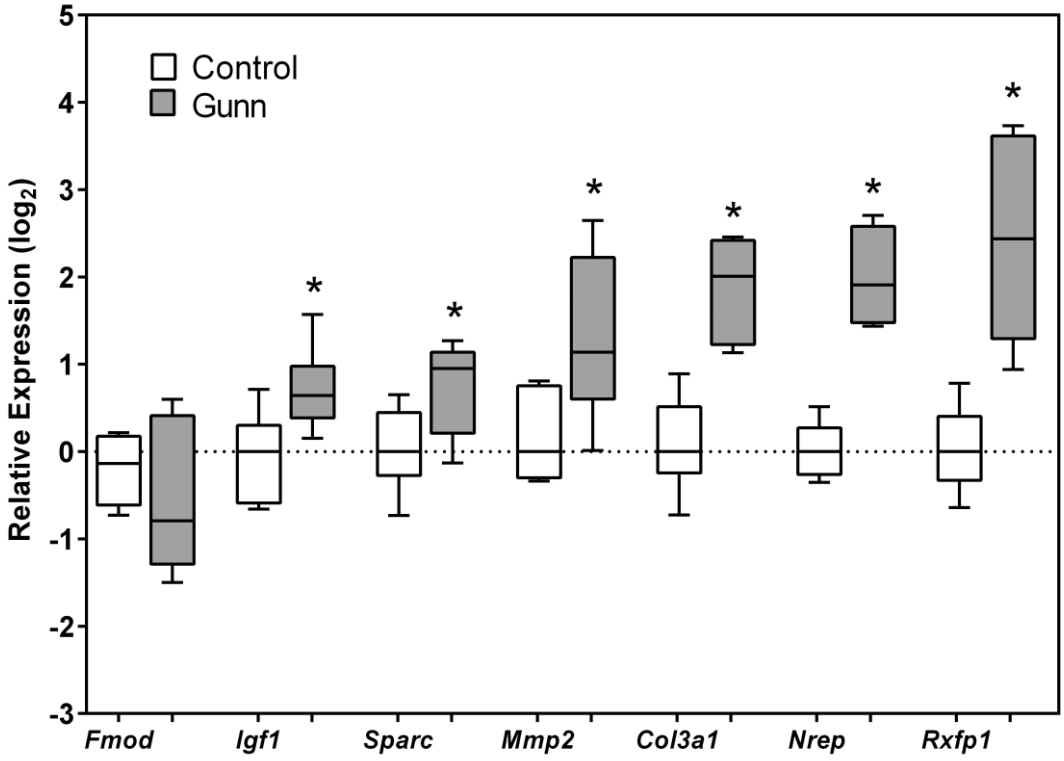

Figure 6.3: *RT-qPCR validation of genes.* RT-qPCR fold changes of seven selected genes, in Gunn and control left ventricular myocardium, **P*<0.05 *vs.* control. (n=6/group).

Table 6.7: Comparison of gene expression results (fold-change) between microarray and RT-qPCR approaches. Fold change indicated relative to control animals

Gene	Microarray (Fold Change)	RT-qPCR (Fold Change)	Microarray *P* Value	RT-qPCR *P* value
Col3a1	2.19	4.02	<0.01	<0.01
Fmod	-2.02	-1.73	<0.01	0.20
Igf1	1.45	1.56	<0.01	0.02
Mmp2	1.48	2.20	<0.0	0.02
Nrep	2.06	3.05	0.02	0.04
Rxfp1	1.93	5.41	<0.01	<0.01
Sparc	1.49	1.93	0.02	0.03

6.5 Discussion

These microarray data are the first to support the notion of broad transcriptomic modulation in left ventricular tissue from mutant hyperbilirubinaemic Gunn rats versus litter-mate controls. These differences may reflect novel physiological effects of bilirubin within myocardial tissue. Differences in gene expression were focused on transcription of growth factors and their receptors, elements of the ECM and response to olfactory signaling. These data are interesting and important given the stress resistant phenotype of hyperbilirubinaemic Gunn hearts, and implicate the effects of bilirubin exist well beyond its commonly accepted antioxidant properties. We expect that these data will open a new avenue of research investigation, focused upon understanding the transcriptional regulatory effects of bilirubin, which will importantly contribute to revealing new physiological roles for unconjugated bilirubin in influencing cardiac and vascular structure and function. These data are also suggestive of effects on cardiac structure and function, which may have important implications for understanding the resistance of Gunn animals to hypertension (218, 240) and ischaemic stress resistance.

Pathway analysis revealed differential enrichment of genes in pathways regulating extracellular matrix organisation in Gunn rats. In support of these findings, previous reports suggest that increased serum bilirubin improves would healing. Interestingly however, these studies attribute this effect to bilirubin's anti-inflammatory and antioxidant properties, with little justification (4, 190). The current data suggest that modulation in gene expression may also contribute to improved would healing. Caution, however, should be applied this this speculation, given that effects on wound healing were investigated in deep skin excision models, whereas these data were generated in myocardial tissue, which clearly has a different cellular composition and function to

skin.

Interestingly, olfactory receptor activity/transduction was also modulated in Gunn myocardium (Table 6.5 and 6.6). These data coincide with recent reports demonstrating that genes regulating taste and smell sensors exist in the heart and suggest that these genes might play important roles in detecting external signal. Given the novelty of this area of research, characterisation of these gene products and their function in the heart remains in their infancy. However, in the context of mild hyperbilirubinaemia, it is interesting to speculate that such sensors might be responding to elevated bilirubin within these animals.

As previously reported by Bakrania *et al.*, despite evidence of a hypocontractile state in isolated myocardium; ejection fraction, fractional shortening, stroke volume and cardiac output are maintained in Gunn *vs.* wild-type rats (12). These data support the idea of an adequate compensation/maintenance of function *in vivo*. Shifts in ECM makeup evidenced here could influence intrinsic cardiac contractility. For example, cardiac remodeling is an adaptive response to mechanical stress, induced by changes in pre- and afterload on the heart. Exercise and heart failure provide two divergent, however, highly relevant models to investigate the effects of favourable and detrimental mechanical stress on the heart. In both conditions cardiac muscle hypertrophies, and both are usually associated with increased heart mass (248). However, heart failure is accompanied by reduced cardiac function (ie. reduced ejection fraction) while, exercise induced hypertrophy is not (248). Interestingly, a critical differentiating factor of these forms of hypertrophy is the involvement of signaling molecules that stimulate growth pathways. The Akt and ERK 1/2 signaling pathways are responsible for proliferation,

growth and cardiomyocyte survival. In pathological circumstances, hypertrophy is a result of activation of G-coupled protein receptors (GCPR) by molecules such as endothelin-1 (ET-1), which a potent vasoconstrictor and increases afterload on the heart. Incidentally, expression of ET-1 was significantly down-regulated in Gunn hearts (from micro-array dataset, data not shown in results section; fold change -1.22, P=0.02). However, physiological hypertrophy is stimulated by the activation of insulin-like growth factor-1 receptors (IGF1R), and consequent activation of phosphoinositide 3-kinase (PI3K) and the Akt pathway (248). Serneri *et al*. compared insulin-like growth factor 1 (IGF-1) concentrations in healthy males who engage in competitive exercise, to males who did not (171), and showed IGF-1 was increased *and* appeared to be the only growth factor responsible for physiological hypertrophy in these subjects. Furthermore, McMullen *et al*. reported physiologically hypertrophied myocardium in mice overexpressing IGF1R (159).

Bilirubin protects against ischaemia-reperfusion injury in several organs including the heart (12, 17, 45, 54, 88), and elevated serum bilirubin is consistently correlated with reduced CVD risk (173, 207, 243). Interestingly, antioxidants can also inhibit the harmful effects of chronic inflammation (140), which is a hallmark of CVD. Apoptosis is partly responsible for cell death arising from ischaemia-reperfusion injury. Many reports suggest up-regulation of anti-apoptotic genes significantly reduces ischaemic tissue injury (34, 95, 258). Interestingly, *Igf1* and transcript for the non-specific *Igf1* receptor, *Rxfp1*, are considered pro-survival genes and were both significantly up-regulated in Gunn hearts (Table 6.7). Insulin-like growth factor-1 (IGF-1) triggers Akt and ERK 1/2 signaling pathways via activation of PI3K (81, 95, 181, 258). Several studies also report beneficial effects of IGF-1 in ischaemia-reperfusion

(34, 63, 175). For example, post-ischaemic IGF-1 treatment improves wall stiffness, infarct size and post-ischaemic function (175), and stimulates angiogenesis (63). Conversely, expression of *Igf1* and other growth factors is repressed with aging (200), which may contribute to increased susceptibility of the heart to ischaemic stress. If follow-up studies demonstrate that the *Igf-1*/*Rxfp1* pathway is activated and results in phospho-activation of survival kinase pathways in middle-aged Gunn rats, this may indeed demonstrate an important additional cardioprotective mechanism in this model (200).

The expression of *Rxfp1*, a non-specific IGF-1 receptor was confirmed to be upregulated in Gunn hearts using qRT-PCR, and promotes ERK pathway activation and hypertrophy/stress resistance via IGF-1 signaling, suggestive of a dynamic state of remodeling. Additionally, genes involved in angiogenesis were differentially expressed, including up-regulation of *Mmp2* (involved in the breakdown of ECM) *vs*. down-regulation of *Fmod* (which inhibits angiogenesis *in vitro* and *in vivo* (115, 268)). Gunn rat hearts undergo dramatic changes in function through development (ie. between 3 and 12 months of age), which may involve remodeling of the ECM. From 3 to 12 months of age, the velocity of exiting the left ventricle steadily increases, however, this increase is clearly attenuated in Gunn rats *vs.* controls. Furthermore, ejection time is significantly prolonged in older Gunn hearts. These data suggest the presence of altered cardiac/vascular remodeling and hypocontractility, in response to life-long exposure to elevated bilirubin. Given the enrichment of genes within pathways (cellular components, biological processes, molecular functions) relating to extracellular matrix, it is tempting to speculate that bilirubin may affect fibroblast function and vascular development, resulting in dilated large arteries and altered mechanical function in

myocardium. To better characterise such effects, imaging of ECM components including collagen and elastin, might complement these data and support evidence of transcriptomic modulation documented here.

6.6 Conclusion

This study provides preliminary evidence of transcriptomic modulation of important cell signalling pathways in myocardium from hyperbilirubinaemic Gunn rats. In particular, transcriptomic changes relevant to cardiac and ECM remodelling, together with inhibition of apoptotic processes, were identified. These findings are consistent with previously reported shifts in contractile function, stress-resistance and cardiovascular structure/function in Gunn rats. Confirmation of parallel proteomic/post-translational modification is warranted, together with interrogation of the specific functional relevance of these molecular changes. Whether they arise directly as a result of bilirubin, or indirectly as a result of other impacts (eg. reduced oxidative stress) also awaits analysis.

CHAPTER 7

CONCLUSION

7.1 Project summaries

This thesis explored the effect of hyperbilirubinaemia on cardiac function, structure and stress resistance. Furthermore, these studies provide preliminary evidence of modulated cardiac gene transcription associated with hyperbilirubinaemia. The first study (Chapter 3) provided an initial characterisation of cardiac phenotype in female hyperbilirubinaemic Gunn rats, which showed significantly reduced myocardial contractility *ex vivo* that is effectively compensated for *in vivo*. Aortic ejection velocities and pressure gradient are reduced *in vivo,* likely as a result of significant aortic dilatation. These vascular changes may beneficially influence afterloading, cardiac function and remodeling processes. Preliminary evidence in this study suggested endogenous hyperbilirubinaemia had the potential to improve mechanical recovery from ischaemia-reperfusion injury in the female Gunn heart. These promising data stimulated further inquiry to determine whether changes in cardiac phenotype and stress resistance were sex-dependant.

The second study (Chapter 4) indicated that the unique cardiac phenotype reported in aged female Gunn rats, was recapitulated in aging male Gunn rats. These findings were important because they excluded oestrogen as a likely mediator of protection in female Gunn rats and indicated that an endogenous unconjugated hyperbilirubinaemia is responsible for protection. Furthermore, this study showed that bilirubin likely contributes to cardioprotection by inhibiting myocardial lipid and protein oxidation, which occurred in association with reduced infarct size and improved functional recovery. Importantly, this protection was clear in middle-aged rats, in which defence mechanisms (ie. cellular signalling, antioxidant defences) are usually impaired. These data agree well with *in vitro* and *ex vivo* analysis of bilirubin's antioxidant

properties and suggest that these properties may protect hearts of individuals with benign hyperbilirubinaemia (ie. persons with Gilbert's syndrome) from ischaemic cardiac injury. Based upon the findings of the first two chapters, these data also suggested that targeted manipulation of bilirubin concentrations, particularly before or after ischaemia, might present a viable therapeutic avenue for cardiac protection.

The third study (Chapter 5) tested whether bilirubin treatment of 'normobilirubinaemic' Wistar rat hearts, either before ischaemia or immediately upon reperfusion, would protect it from ischaemic injury. This study demonstrated that the administration of a novel water soluble bilirubin analogue BRT, at concentrations similar to that of unconjugated bilirubin in Gunn rats, protects cardiac tissue from I-R injury. This protection was, like in male Gunn rats, accompanied by reduced markers of oxidative damage. Bilirubin ditaurate administration during *reperfusion* appeared to most greatly improve post-ischaemic function, infarct size, lipid peroxidation and protein oxidation. These data were important because the availability and effectiveness of post-infarct treatments are greatly limited. Therefore, BRT might represent an effective treatment in individuals suffering from myocardial infarction.

The final study (Chapter 6) aimed to provide new insight and avenues for future investigation regarding transcriptomic modulation of important cell signalling pathways in myocardium from hyperbilirubinaemic Gunn rats. In particular, transcriptomic changes relevant to ECM remodelling, inhibition of apoptotic processes and interestingly, olfactory receptor signalling, were significantly affected in Gunn rats. Preliminary validation of the array was performed using quantitative real time polymerase reaction, and furthermore, demonstrated up-regulation of IGF-1 and IGF-1

receptor gene expression in Gunn myocardium. Changes in the expression of cardiac/ECM remodelling pathways and genes regulating apoptosis were consistent with previously reported shifts in contractile function, stress-resistance and cardiovascular structure in Gunn rats, providing novel insight into new physiological effects of bilirubin.

7.2 Future studies

The four studies included in this thesis contribute to a new body of research suggesting that increased serum bilirubin improves stress resistance and post-ischaemic outcomes in rodents. Furthermore, these studies uncover potential mechanisms, in addition to bilirubin's antioxidant capacity, which might protect hearts from injury in individuals with life-long exposure to higher serum bilirubin, via modulation of cardiac function and aortic remodelling. This information not only advances our understanding of the basis of protection from CVD in human Gilbert's syndrome, but also may assist in developing novel means of manipulating myocardial function and I-R resistance. These studies provide a strong foundation for the further testing of bilirubin analogues in small and larger animal *in vivo* studies, including the testing of dosage and duration of treatment, which would precede clinical trials. The National Institute of Health has sponsored a consortium for pre-clinical assessment of cardioprotective therapies (CAESAR), which aims to standardise rigorous experimentation on larger animals prior to clinical trials. This could represent an excellent funding opportunity to realise the translational effects of bilirubin therapy in the context of cardioprotection. Currently, no therapy has demonstrated efficacy as a post ischaemic therapy in CAESAR trials, yet data collected in this thesis strongly suggest that bilirubin could represent a viable candidate for testing. If further trials are successful, bilirubin infusion by first

responders might represent an effective means to treat patients suffering from myocardial infarction. Such a treatment would provide valuable time to allow for the administration of thrombolytic therapy (usually hours later), allowing complete reperfusion before myocardial necrosis is complete. If such a treatment were successful, the use of bilirubin; a molecule previously considered benign at best, and toxic at worst, might realise its full potential as a critical cytoprotective molecule and in the process, save thousands of lives in the future.

CHAPTER 8

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