

Imperial College London

Mechanosensitivity of the Failing Myocardium: Role of Mechanical Unloading

Sean Bello

MB BS, BSc (Hons), MRCS (Eng)

IMPERIAL CENTRE FOR TRANSLATIONAL AND EXPERIMENTAL MEDICINE
NATIONAL HEART AND LUNG INSTITUTE
HAMMERSMITH HOSPITAL
LONDON

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Abstract

Left ventricular assist devices (LVADs) were initially developed primarily to serve as a means of bridging heart failure patients to transplantation. However, reports show that significant myocardial functional recovery occurs in some LVAD patients to the extent that device explantation can be achieved without the need for transplantation. This phenomenon, described as reverse remodelling only occurs in about 4-10% of patients treated with LVADs and the basis for this is unknown.

Myocardial fibrosis worsens with mechanical unloading but the mechanism remains poorly understood. Unloading is however associated with a reduction in microvascular luminal diameter suggesting a negative autoregulatory effect of unloading on the coronary collateral circulation. Coronary reperfusion after myocardial injury is associated with heightened regenerative responses in the zebrafish heart. However, the effect coronary reperfusion coupled with unloading has on cardiomyocyte proliferation is unknown. It is known that cardiomyocyte excitation-contraction coupling is improved by unloading but the effect of unloading on whole-heart contractility and on proteins that facilitate efficient excitation-contraction coupling such as JPH2, BIN1, and Tcap, is unknown.

In this thesis, we hypothesised that mechanical unloading of the reperfused ischaemic heart ameliorates unloading-induced increases in fibrosis and stimulates an upregulation of cardiomyocyte proliferation. We also hypothesised that these positive effects of mechanical unloading are associated with improvements in the expression and function of JPH2, BIN1, and Tcap, and in whole-heart contractility.

Hearts of animal models of ischaemic cardiomyopathy, achieved following coronary artery ligation, were mechanically unloaded via heterotopic abdominal heart and lung transplantation after either being revascularised or left permanently ligated. We found that following acute myocardial infarction, ischaemia-reperfusion coupled with mechanical unloading was associated with a remarkable amelioration of the deleterious effect of unloading on myocardial fibrosis and a significant upregulation of cardiomyocyte proliferation. Two major findings that could have significant clinical implications for LVAD-induced myocardial recovery.

Declaration of Originality

All the work in this thesis is my own work and all other externally utilised material has been referenced.

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Poster Presentations

Role of Cardiomyocyte Regeneration in Mechanical Unloading Induced Reverse Cardiac Remodelling

American Heart Association: Scientific Sessions - New Orleans, LA, USA; November 2016

Coronary reperfusion ameliorates the deleterious effect of mechanical unloading on infarct size and interstitial fibrosis after acute myocardial infarction

American Heart Association: Scientific Sessions - Anaheim, California, USA; November 2017

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To my precious daughter Chelsy

Glossary of Abbreviations

AMI	acute myocardial infarction
ANOVA	analysis of variance
bpm	beats per minute
BIN-1	bridging integrator-1
BTR	bridge to recovery
BTT	bridge to transplantation
BW	body weight
BP	blood pressure
Ca²⁺	calcium
CFR	coronary flow reserve
CHF	congestive heart failure
CICR	calcium induced calcium release

CMI	chronic myocardial infarction
DCM	dilated cardiomyopathy
DDR	DNA damage response
DT	destination therapy
EC coupling	excitation-contraction coupling
ECG	electrocardiography
ECM	extracellular matrix
eCSC	endogenous cardiac stem cell
EDPVR	end diastolic pressure volume relationship
EF	ejection fraction
ESPVR	end systolic pressure volume relationship
FS	fractional shortening
HAHT	heterotopic abdominal heart transplant
HAHLT	heterotopic abdominal heart and lung transplant
HFPEF	heart failure with preserved ejection fraction
HR	heart rate

HFREF	heart failure with reduced ejection fraction
HW	heart weight
ICM	ischaemic cardiomyopathy
IF	interstitial fibrosis
IR	infarct ratio
I/R	ischaemia-reperfusion
IS	infarct size
IVC	inferior vena cava
JPH2	Junctophylin-2
LA	left atrium
LAD	left anterior descending
LCA	left coronary artery
LTCC	L-type Ca^{2+} channels
LVAD	left ventricular assist device
LVEF	left ventricular ejection fraction
LVFS	left ventricular fractional shortening

LVEDD	left ventricular end diastolic diameter
LVEDP	left ventricular end diastolic pressure
LVESD	left ventricular end systolic diameter
LVESP	left ventricular end systolic pressure
LVP	left ventricular pressure
MBF	myocardial blood flow
MCS	mechanical circulatory support
MEIS1	myeloid ecotropic viral integration site 1
MI	myocardial infarction
MMP	matrix metalloproteinase
MU	mechanical unloading
MV0₂	myocardial volume oxygen
NCX	sodium-calcium exchanger
OMT	optimum medical therapy
PA	pulmonary artery
PRSW	preload recruitable stroke work

PV	pressure volume
PVA	pressure volume area
RA	right atrium
RAAS	renin angiotensin aldosterone system
ROS	reactive oxidant species
RyR	ryanodine receptor
S.E.M	standard error of mean
SERCA2a	sarcoplasmic & endoplasmic reticulum calcium ATPase
SMA	smooth muscle actin
SR	sarcoplasmic reticulum
SVC	superior vena cava
TAZ	transcription co-activator with PDZ-binding motif
TBS	Tris-buffered saline
Tcap	telethonin
TGF-β1	transforming growth factor-beta 1
TIMP	tissue inhibitor of metalloproteinase

Tn-T	Troponin-T
TTE	transthoracic echocardiography
YAP	yes associated protein

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1 INTRODUCTION

In the last century remarkable progress has been made in the treatment of cardiovascular diseases, in particular, coronary artery disease.(van der Wall, 2013) Most of the cardiovascular risk factors, which were unravelled by observations in large cohorts such as the Framingham study, served as a substrate for pharmacological interventions.(Mahmooda *et al.*, 2014) Besides these milestones in preventive cardiology, the last three decades were marked by several major breakthroughs in the treatment for acute myocardial infarction (AMI), such as the introduction of the coronary care unit, pharmacological reperfusion (i.e., thrombolysis), pharmacological interventions (e.g., beta blockade, ACE inhibitors, antiplatelet drugs, and statins), and improvements in interventional cardiology (i.e., primary percutaneous coronary intervention).(van der Wall, 2013) (Mahmooda *et al.*, 2014) As a result, our ability to provide some patients with a viable less invasive alternative to surgical revascularisation (the gold standard for definitive treatment of coronary artery disease) has developed significantly. This achievement has however come at the expense of a vast increase in patients with congestive heart failure (CHF) a chronic consequence of AMI and the current major clinical challenge to cardiovascular medicine.(Koudstaal *et al.*, 2013) (Cahill and Karbanda, 2017)

1.1 Heart Failure

Heart failure is a clinical syndrome brought about by the inability of the heart to generate adequate cardiac output to meet the body's demand for tissue oxygenation. It is a result of a complex blend of structural, functional, and biological alterations that the myocardium undergoes in response to injury and increased wall stress which culminates in pathological

remodelling with global impairment in systolic (heart failure with reduced ejection fraction – HFREF) or diastolic (Heart failure with preserved ejection fraction – HFPEF) function.(Birks, 2013) (Braunwald, 2013) Heart failure poses a significant healthcare burden occurring in 1-2% of the adult UK population, rising to about 16% in men over the age of 75.(Petersen, Rayner and Wolstenholme, 2002) It is one of the most common reasons for emergency medical admission, readmission and hospital bed-days occupancy accounting for 1 million hospital bed days per year.(Heart and Audit, 2016) Managing patients with heart failure costs the UK National Health Service (NHS) an estimated £625 million per year.(National Institute for Cardiovascular Outcomes Research (NICOR), 2011) Globally, an estimated 38 million people have a current diagnosis of heart failure with a projected 46% exponential rise in prevalence by 2030.(Lawson *et al.*, 2019) In the US, heart failure contributes to over 250,000 deaths per year with an estimated cost to the US economy estimated at over \$39.2 billion annually.(Roger *et al.*, 2012) (Ziaeeian *et al.*, 2017) Globally, an estimated US\$108 billion is spent on heart failure each year.(Lawson *et al.*, 2019)

The underlying pathophysiology of heart failure is multifaceted and includes the sequelae of increased haemodynamic overload, excessive neurohumoral stimulation, ischaemia and arrhythmia related dysfunction, ventricular remodelling, abnormal cardiomyocyte calcium cycling, excessive or inadequate deposition of extracellular matrix proteins, accelerated cell death, and genetic mutations.(Braunwald, 2013) (Ziaeeian *et al.*, 2017) Left ventricular remodelling occurs in several clinical conditions which are broadly classified as idiopathic, non-ischaemic, or ischaemic cardiomyopathy. Idiopathic dilated cardiomyopathy (DCM) is a form of non-ischaemic cardiomyopathy prevalent in relatively young patients aged 20-60 years where the exact cause is initially unknown and often multifactorial, potentially including occult viral myocarditis, abnormalities of the immune system, and toxic, metabolic, or genetic factors. (Hazebroek, Dennert and Heymans, 2012), (Wu, 2007) Other forms of non-ischaemic

cardiomyopathy include those secondary to valvular heart disease, hypertension and tachycardia-induced cardiomyopathy. (Wu, 2007)

Ischaemic cardiomyopathy, a result of chronic lack of oxygen to the heart muscle because of coronary artery disease, is by far the most common aetiology, accounting for approximately 70% of cases. (Birks, 2013) (Moran *et al.*, 2014)

After a myocardial infarction, macrophages, monocytes, and neutrophils migrate into the infarct zone; this initiates intracellular signalling and neurohumoral activation, which localizes the inflammatory response. Changes in circulatory haemodynamics are determined primarily by the magnitude of myocyte loss, the stimulation of the sympathetic nervous system and renin-angiotensin-aldosterone system, and the release of natriuretic peptides.(Birks, 2013) Post-infarction remodelling has been arbitrarily divided into an early phase (within 72 hours) and a late phase (beyond 72 hours). The early phase involves expansion of the infarct zone, which may result in early ventricular rupture or aneurysm formation. Late remodelling involves the left ventricle globally and is associated with time-dependent dilatation, the distortion of ventricular shape rendering it more spherical, establishment of a definitive scar, and mural hypertrophy. The failure to normalize increased wall stresses results in progressive dilatation, recruitment of border zone myocardium into the scar, and deterioration in contractile function.(Jessup and Brozena, 2003)

Heart failure is classified into 4 stages (A-D). This staged approach to its classification is designed to underscore the progressive nature of the disease and the fact that established risk factors in addition to structural abnormalities are necessary for its development. Patients with stage A heart failure are at high risk for the development of heart failure but have no apparent structural abnormality of the heart. Patients with stage B heart failure have a structural abnormality of the heart but have never had symptoms of heart failure. Patients with stage C

heart failure have a structural abnormality of the heart and current or previous symptoms of heart failure. Patients with stage D heart failure have end-stage symptoms of heart failure that are refractory to standard treatment. Clinical manifestations of stage C & D heart failure patients described using the New York Heart Association (NYHA) classification system as follows:

Class I: No limitation of physical activity. Ordinary physical activity does not cause undue fatigue, palpitation, or dyspnoea (shortness of breath).

Class II: Slight limitation of physical activity. Comfortable at rest. Ordinary physical activity results in fatigue, palpitation, or dyspnoea.

Class III: Marked limitation of physical activity. Comfortable at rest. Less than ordinary activity causes fatigue, palpitation, or dyspnoea.

Class IV: Incapable of carrying out any physical activity without discomfort. Symptoms of heart failure at rest.

Despite major advances in the medical management of heart failure, particularly the use of β -adrenoreceptor blockers, inhibitors of the renin-angiotensin-aldosterone system,(McMurray *et al.*, 2013) and more recently the angiotensin receptor-neprilysin inhibitor,(McMurray *et al.*, 2014) in conjunction with devices such as pacemakers, which enhance cardiac synchronisation, and implanted cardiac defibrillators,(Yancy *et al.*, 2013) mortality from heart failure remains very high at an average of 60% at five years after diagnosis(Rose *et al.*, 2001) with one year mortality estimated to be as high as 80% in advanced forms of the disease.(Boothroyd *et al.*, 2013)

Heart transplantation remains the gold standard treatment for end-stage heart failure but the well documented shortage of donor hearts precludes this as a realistic option for a large proportion of patients. It is estimated that just over 5000 donor hearts are available yearly

worldwide with over 150,000 potential recipients.(Lund *et al.*, 2017) (Roger *et al.*, 2012) In addition, about 10% of patients die annually on the heart transplant waiting list.(Roger *et al.*, 2012) In fact, the 2010 report from the International Society of Heart and Lung Transplantation (ISHLT) showed that 52.4% of the patients listed for transplant between the years 2000 and 2005 died within six months without heart transplantation. Thus there is a pressing need for a viable alternative to cardiac transplantation in the management of a large proportion of heart failure patients.

The recent development and widespread use of mechanical circulatory support (MCS) in patients with advanced heart failure refractory to medical therapy has helped bridge the gap to cardiac transplantation to a significant extent in many of these patients.(Holley, Harvey and John, 2014) Short term, extra-corporeal assist devices were initially used to provide MCS but as technology improved throughout the 1990s, durable and implantable assist devices such as Left Ventricular Assist Devices (LVADs) were introduced thus revolutionising the surgical treatment of heart failure refractory to medical intervention.(Holley, Harvey and John, 2014) The viability of LVADs as a Bridge To cardiac Transplantation (BTT) was first demonstrated in a non-randomised study conducted by Frazier *et al* in 1995 which showed that implantation of an LVAD vs Optimum Medical Therapy (OMT) resulted in a 55% reduction in pre-transplant mortality and a significant improvement in 1 year post-transplant survival.(Holley, Harvey and John, 2014) (Frazier *et al.*, 1995) Subsequently, the landmark Randomised Evaluation of Mechanical Assistance in the Treatment of Congestive Heart failure (REMATCH)(Rose *et al.*, 2001) trial established the superiority of LVADs over OMT in the management of end-stage heart failure and this device is now rapidly taking central stage as a viable alternative to heart transplantation for both transplant eligible and ineligible heart failure patients.(Boothroyd *et al.*, 2013) (Interventional *et al.*, 2015)

1.2 Left Ventricular Assist Devices (LVADs)

The conceptualisation, design, and refinement of ventricular assist devices (VADs) over the past 5 decades as therapy for end-stage heart failure has been extraordinary. Since the original VAD design by Michael DeBakey in the early 1960s,(Lee *et al.*, 2013) and the subsequent implantation of the first LVAD as a BTT in 1978,(Cooley *et al.*, 1981) numerous devices for MCS have been engineered.(Lee *et al.*, 2013)

LVADs were originally developed for use as a BTT providing haemodynamic support to patients with decompensated end-stage heart failure whilst awaiting a donor heart.(Holley, Harvey and John, 2014) They brought about significant improvements in end-organ function, haemodynamics, overall functional status and quality of life of these critically ill patients.(Lee *et al.*, 2013) The landmark REMATCH trial which randomised end-stage heart failure patients who were ineligible for heart transplantation to medical management or implantation of the first generation LVAD (HeartMate vented electric LVAD) demonstrated marked reduction (48%) in the risk of death from any cause with device use.(Rose *et al.*, 2001) In addition, 1-year survival rate was 52% in the device group compared to 25% in the medical therapy group.(Rose *et al.*, 2001) These outcomes have led to LVADs currently taking centre stage in the management of advanced heart failure with their application now spreading beyond BTT to Destination Therapy (DT), and even Bridge To Recovery (BTR).(Topkara *et al.*, 2016) LVADs are currently licensed for use in patients who have worsening New York Heart Association (NYHA) Class IV heart failure despite inotropic plus intra-aortic balloon pump support and an estimated 1-year mortality of over 50% with medical therapy.(Osaki *et al.*, 2009) (Hunt *et al.*, 2005)

First generation LVADs such as the pneumatically driven (1994) and subsequently the self-contained vented electric (1998) LVADs were the first group of VADs to gain FDA approval

for use as BTT.(Lee *et al.*, 2013) The basic design of these pulsatile LVADs is illustrated in figure 1 and includes an inflow cannula which is inserted into the left ventricular apex and draws blood out of the heart via the activity of a pumping unit (LVAD). Blood is then pumped into the systemic circulation through the outflow cannula anastomosed to the ascending aorta. A driveline cable from the pump exits the skin and is attached to an external controller that is powered by portable batteries (Fig. 1.1).(Lee *et al.*, 2013) (Rose, Gelijns and Moskowitz, 2001)

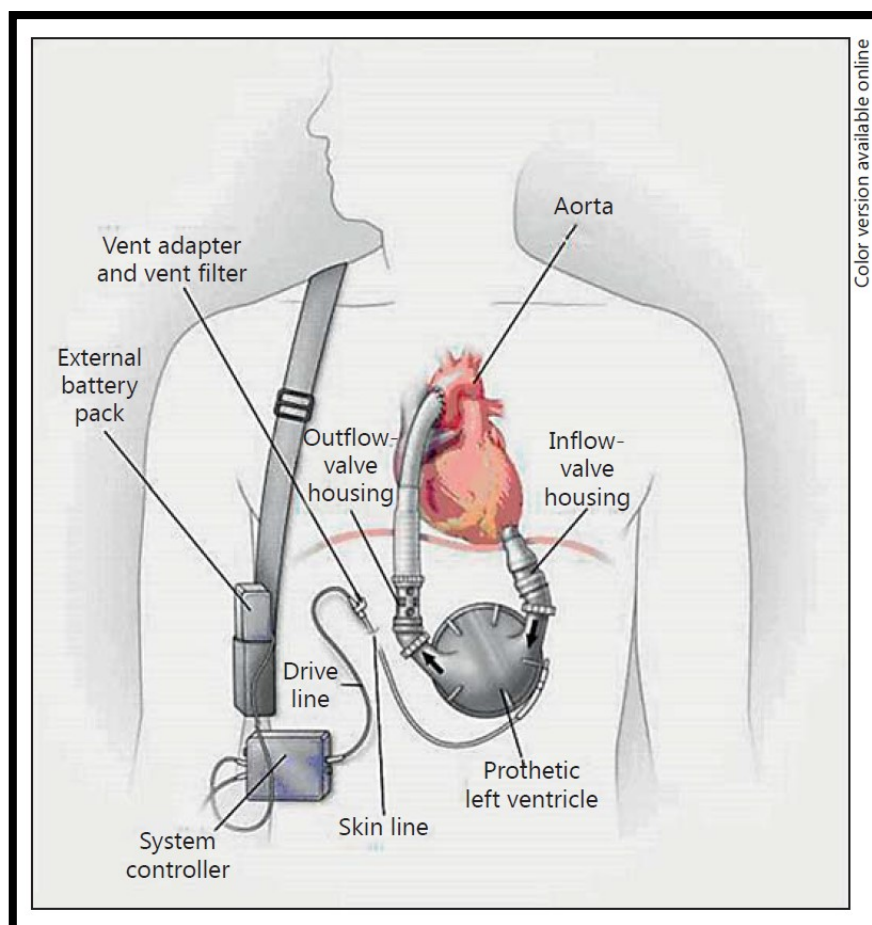


Figure 1.1: The HeartMate XVE, a first generation pulsatile LVAD. The device is implanted below the diaphragm within the abdominal wall or peritoneal cavity with the inflow and outflow conduits anastomosed to the LV apex and ascending aorta respectively. Image reproduced from <http://www.heartfailure.org>.

Despite positive outcomes with these devices however, resistance to widespread use of LVADs persisted for an extended period of time due to limited durability of the device and the high

incidence of adverse events such as bleeding, stroke, and infections related to the large percutaneous driveline.(Lee *et al.*, 2013) This drove the development of newer, more refined devices with much smaller drivelines and the capability of full intra-pericardial implantation. These newer devices which are continuous flow as opposed to the first generation pulsatile flow pumps, are lighter, less noisy and have much better durability compared to the first generation LVADs.(Slaughter *et al.*, 2009)

The two main continuous-flow LVADs currently approved for use by the FDA are illustrated in figure 1.2 and include the second generation axial-flow pump (HeartMate II) and the third generation centrifugal-flow pump (HeartWare).(Rogers *et al.*, 2017)

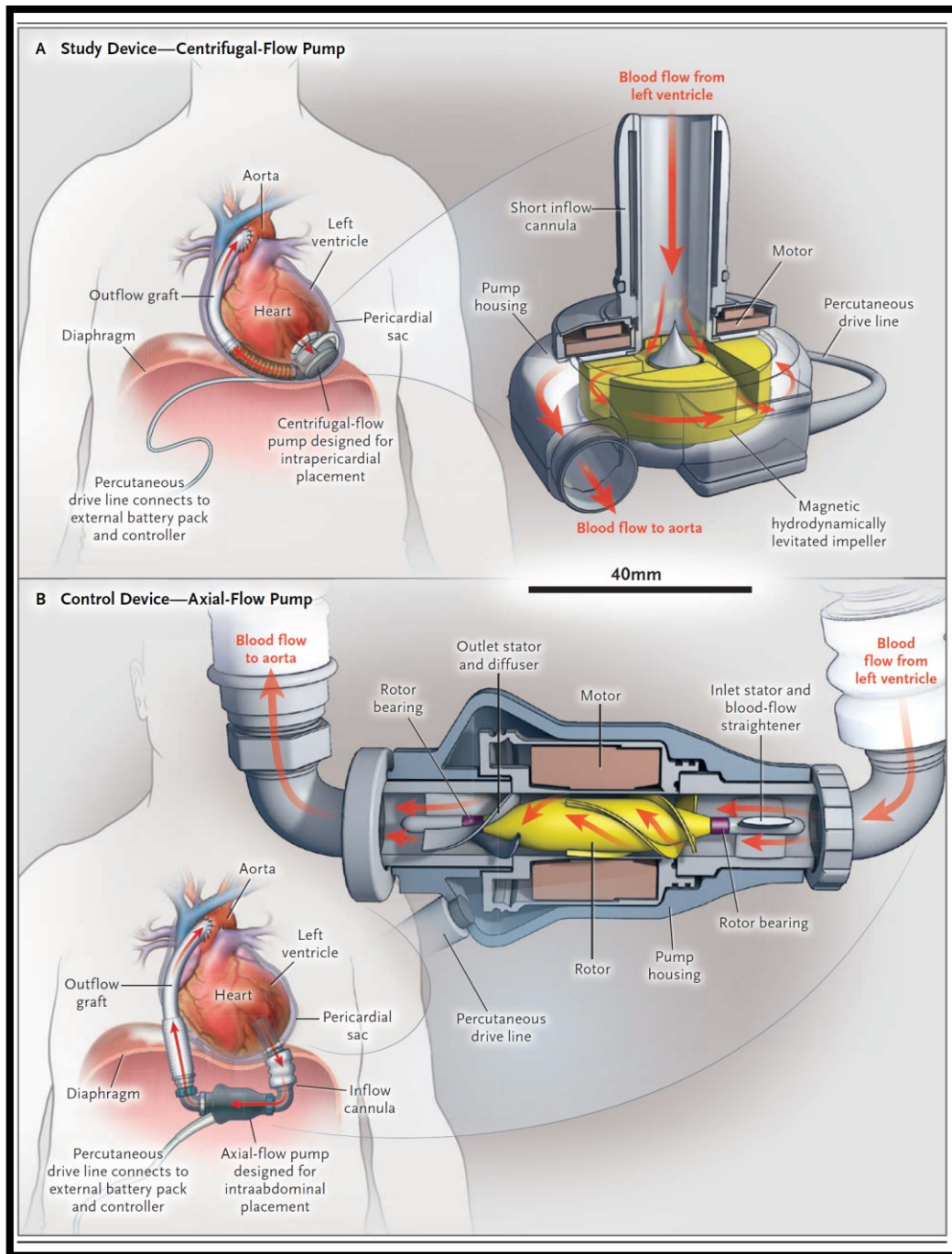


Figure 1.2: (A) The centrifugal flow LVAD (HeartWare) and (B) the axial flow pumps, HeartMate II showing the key differences in technology and implantation. The HeartWare LVAD has magnetic and hydrodynamic levitation of the internal rotor making it more compact and durable facilitating intrapericardial implantation. Image reproduced from Rogers et al. N Engl J Med 2017

The superiority of the newer continuous-flow LVADs over the older devices was shown in the HeartMate II trial, a RCT which compared the continuous-flow LVADs to the pulsatile devices and OMT, and demonstrated that actuarial survival at 2yrs was 8% with OMT, 24% with

HeartMate I vs 58% with HeartMate II.(Slaughter *et al.*, 2009) Most recently, the ADVANCE trial, involving the third-generation HeartWare device reported survival with device use of 92% at 6 months.(Aaronson *et al.*, 2012) In the current era, over 95% of LVAD implants are continuous flow devices and overall survival continues to remain above 80% at 1 year and 70% at 2 years.(Kirklin *et al.*, 2017)

There is thus overwhelming evidence of the superiority of LVADs over OMT in the management of end-stage heart failure patients and the technology continues to improve with much smaller devices being designed. The Impella 2.5 and 5.0/LD MCS devices (Fig. 1.3) for instance are capable of percutaneous insertion through the femoral artery and passage into the left ventricle across the aortic valve under fluoroscopic guidance.(Lemaire *et al.*, 2014)

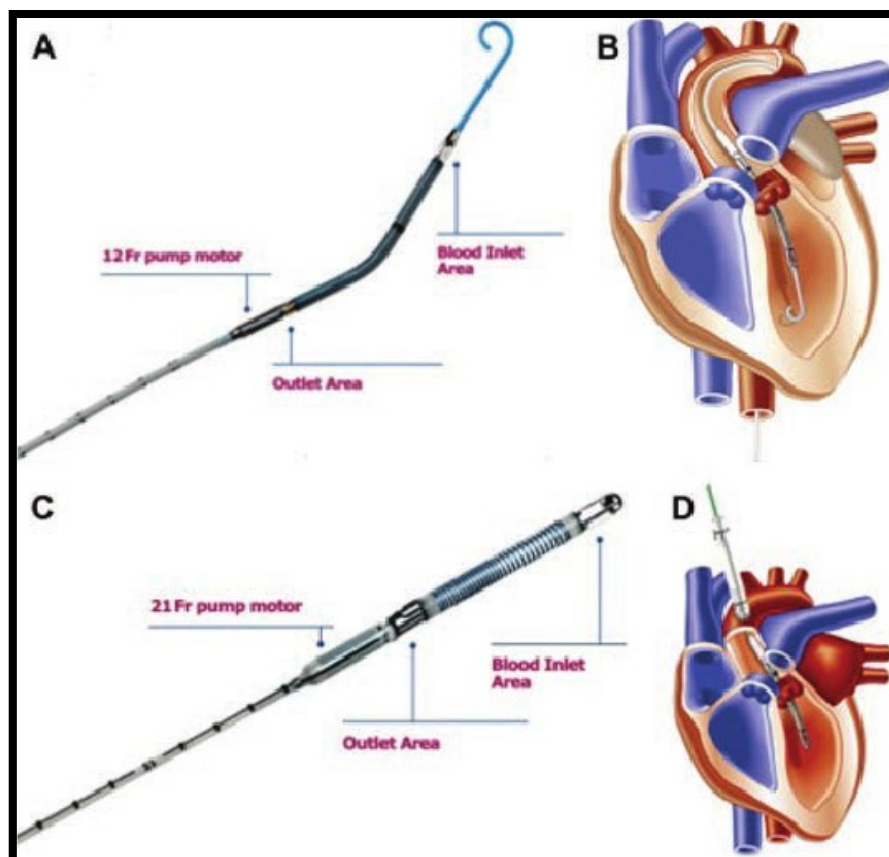


Figure 1.3: (A and B) Impella LP 2.5 and Impella LD pumps respectively, both designed to aid percutaneous insertion through the femoral artery or ascending aorta as shown in C and D respectively. These devices are capable of achieving cardiac outputs ranging from 2.5 to 5L/min. Courtesy of Abiomed, Inc., Danvers, MA

They are able to adequately unload the left ventricle albeit for shorter duration (about 5-18 days compared to conventional LVADs), generating forward flows as high as 2.5 L/min (Impella 2.5) and 5 L/min (Impella 5.0/LD) and stimulating significant improvement in myocardial function.(Lemaire *et al.*, 2014) (Antończyk *et al.*, 2018)

Thus as described earlier, improvements in LVAD design and outcomes has led to a broader use of the device. An area of particular scientific interest presently is with regards the potential for LVADs to be looked upon as not simply a supportive device but as a therapeutic tool capable of stimulating sustained myocardial functional recovery, significant enough to permit device explantation with no further requirement for cardiac transplantation. In essence a BTR device.

There is a growing body of evidence that shows that LVAD use in some heart failure patients is associated with the stemming or even reversal of the pathological processes that culminates in heart failure with significant recovery of myocardial function.(Jakovljevic *et al.*, 2017) (O H Frazier *et al.*, 1996) (Maybaum *et al.*, 2007) This novel phenomenon, described as *reverse remodelling* of the failing heart is thought to encompass the effects of mechanical unloading on the plasticity of cardiac remodelling, the rejuvenation of cardiomyocyte calcium cycling and excitation-contraction coupling, the expression profile of the extracellular matrix (ECM) proteins and cardiac fibrosis, and the possible activation of the endogenous regenerative capacity of the failing adult heart, particularly in the setting of ischaemic cardiomyopathy.(Ibrahim, Navaratnarajah, *et al.*, 2012b) (Drakos *et al.*, 2010) (Canseco *et al.*, 2015)

The concept of reverse remodelling provides a very exciting prospect for the future management of heart failure and is an area currently stimulating much research interest worldwide. This subject forms the cornerstone of this PhD thesis.

1.3 Bridge to Recovery

The ultimate goal of studies investigating the underlying mechanisms of BTR is aimed at arriving at a time when short-term therapeutic use of MCS for heart failure reversal in its early stage, negating the need for future heart transplantation will be possible.(Dandel and Hetzer, 2015) The development of LVADs as a therapeutic tool will also increase the indication for the device. This will result in a large pool of transplant ineligible heart failure patients being provided with a viable alternative to cardiac transplantation, with significant improvements in their long-term outcome and quality of life. These patients are currently left in a debilitated state requiring multiple hospital admissions for inotropic support.(National Institute for Cardiovascular Outcomes Research (NICOR), 2011)

Since the first report of LVAD-induced myocardial recovery by Frazier et al in 1996,(O H Frazier *et al.*, 1996) several clinical and experimental studies showing significant recovery of myocardial function following mechanical unloading of the failing heart have been reported.(Dandel and Hetzer, 2015) (Ibrahim and Terracciano, 2013) In a recent study which investigated the long-term outcome of patients BTR compared to those BTT, the actuarial survival rate at 5 years after LVAD explantation in the BTR group was 73.9%, whereas in the BTT group, where all patients finally received a heart transplant, the actuarial post-transplant survival rate at 5 years was 78.3%.(Birks *et al.*, 2012) Hence, this suggests that patients weaned from LVADs were not at a particular disadvantage when compared to those who underwent cardiac transplantation. This outcome was found to be so even where the indication for LVAD implantation was chronic cardiomyopathy, as opposed to more reversible causes of cardiomyopathy, such as acute myocarditis, post-cardiotomy heart failure or peripartum cardiomyopathy.(Dandel and Hetzer, 2015)

These remarkable findings notwithstanding, it is important to note that LVAD-induced myocardial functional recovery significant enough to permit LVAD removal with sustained cardiac recovery only occurs in a small proportion of heart failure patients.(Mancini *et al.*, 1998) (Jakovljevic *et al.*, 2017) (Potapov *et al.*, 2019) Most multicentre studies report rates of recovery of only 8-10%.(Maybaum *et al.*, 2007) (Farrar *et al.*, 2002) Whilst higher rates of recovery have been reported in some centres, these are yet to be reproduced.(Dandel *et al.*, 2008) (Birks *et al.*, 2006) (Birks *et al.*, 2011) The basis for this low rate of recovery is not fully understood. It is known that the incidence of recovery is disproportionately higher in patients with dilated as opposed to ischaemic cardiomyopathy,(Müller *et al.*, 1997) (Potapov *et al.*, 2019) but the implication of this is yet to be fully characterised. The occurrence of myocardial atrophy after LVAD support in some patients has been noted as a possible contributory factor.(Ito *et al.*, 2003)

Whilst some studies show that rates of recovery can be substantially increased by combining LVAD treatment with pharmacologic therapy,(Birks *et al.*, 2011) (Soppa *et al.*, 2008) and by identifying predictors of potential recovery to facilitate targeted LVAD implantations.(Wever-Pinzon *et al.*, 2016) More work is needed to better understand the clinical pattern of BTR and the biological mechanisms involved in this potentially valuable therapeutic approach to end-stage heart failure.

1.4 LVAD-induced Reverse Remodelling

Over the past decade, a number of studies have shed light into the natural history of end-stage heart failure patients managed with LVADs.(Maybaum *et al.*, 2007) (Terracciano *et al.*, 2004) (Simon *et al.*, 2005) Evidence suggests that by stimulating significant improvement in

myocardial function, LVADs may in fact play a more pivotal role in the management of end-stage heart failure.(O.H. Frazier *et al.*, 1996) (Birks *et al.*, 2006)

By mechanically unloading the left ventricle and taking over a substantial part of the hearts haemodynamic role, LVADs essentially afford the heart a rare period of rest similar to that achieved by the “bed rest” management approach that was originally the mainstay of treatment for congestive heart failure in the 1960s.(Burch and DePasquale, 1966) This period of reduced myocardial work, wall stress, and oxygen consumption that occurs during mechanical unloading with LVADs has been shown to be associated with profound broad-based effects on fundamental properties of cardiac myocytes of end-stage failing human hearts.(Klotz *et al.*, 2007) Myocardial cell size, left ventricular size and mass approach normal, calcium cycling properties, and isolated myocyte contractility are improved, gene expression patterns and signalling pathways are normalized.(Wohlschlaeger *et al.*, 2005) (Ibrahim, Navaratnarajah, *et al.*, 2012a) (Ibrahim, Rao, *et al.*, 2012) These wide ranging structural, functional, and molecular effects that occur after mechanical unloading with LVADs are thought to bring about reversal of pathological remodelling and improvement in myocardial contractility.(Drakos *et al.*, 2007)

In the quest to better understand the underlying mechanism of LVAD-induced reverse remodelling, several studies have thus far identified key elements in the process. These include: the effects of mechanical unloading of the failing heart on cardiomyocyte excitation contraction coupling and, in particular (transverse) t-tubule ultrastructure and calcium homeostasis; (Ibrahim and Terracciano, 2013) (Ibrahim, Navaratnarajah, *et al.*, 2012b) (Chaudhary *et al.*, 2004) (Terracciano *et al.*, 2003) effects on the homeostasis of the extracellular matrix, myocardial fibrosis, and infarct scar; (Drakos *et al.*, 2007) (Klotz *et al.*, 2005a) (Bruggink *et al.*, 2006) (Cave, Kingma and Hearse, 1992) (Kapur *et al.*, 2015) and finally, probable effects on the endogenous regenerative capacity of the adult heart.(Canseco *et al.*, 2015) (Bergmann *et al.*, 2009) (Kurazumi *et al.*, 2014) (Suzuki *et al.*, 2007) (Wohlschlaeger *et al.*, 2010)

1.4.1 Excitation-contraction coupling

Pathological remodelling of the heart as a result of hypertension, ischaemia, or cardiomyopathy ultimately results in impairment of the force of myocardial contraction and an inability of the heart to generate adequate cardiac output to meet the body's needs.(Zhang *et al.*, 2013) Myocardial contraction is governed by the cardiac excitation-contraction (EC) coupling process (Fig. 1.4), whereby modest release of Ca^{2+} into the cytosol from voltage-gated L-type Ca^{2+} channels (LTCCs) lining deep myocyte membrane invaginations called t-tubules stimulate a much larger Ca^{2+} release from adjacent ryanodine receptors (RyRs) in the sarcoplasmic reticulum (SR).(López-López JR(1), Shacklock PS, Balke CW, 1995) (Ather *et al.*, 2013) This process of calcium-induced calcium release (CICR) leads to the production of large Ca^{2+} transients that bathe the contractile machinery within cardiac myocytes and thus stimulate myocardial contraction. (Ibrahim *et al.*, 2010) (Eisner *et al.*, 2017)

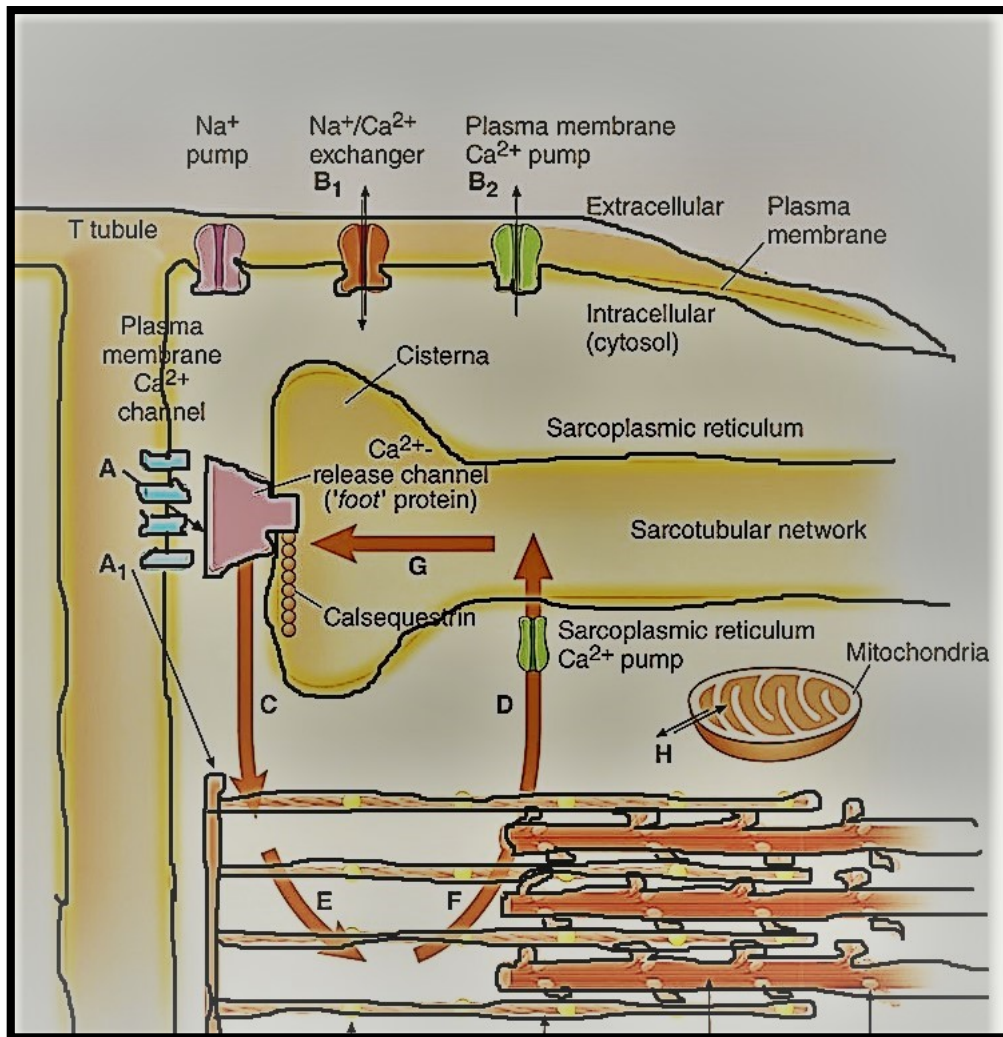


Figure 1.4: Schematic representation of the EC coupling process illustrating the close dyad between the t-tubule and the RyRs in the terminal cistern of the sarcoplasmic reticulum. The arrows denote the direction of Ca²⁺ fluxes with the thick arrows showing the movement of a larger magnitude of the Ca²⁺. Letters A-H illustrates the various Ca²⁺ fluxes during the CICR process from passive entry of Ca²⁺ into the cell from the extracellular fluid (arrow A) following action potential stimulation of the LTCC with only a small portion of the Ca²⁺ (arrow A₁) directly activating the contractile proteins. Activation of the RYRs facilitates the larger efflux of Ca²⁺ from the SR in a cyclical fashion (C-G) from the SR to the contractile elements to aid contraction and back into the SR via the activity of the Sarcoplasmic reticulum ATPase during relaxation. Image adapted from AM Katz: Physiology of the Heart, 4th ed. Philadelphia, Lippincott, Williams & Wilkins, 2005.

Maintenance of the structural integrity of t-tubules and the dyad they form (across a 10-25nm cleft) with RyRs has been shown to be pivotal to efficient calcium transient synchronicity and the force of myocardial contraction.(Ibrahim, Navaratnarajah, *et al.*, 2012a) (Ibrahim *et al.*, 2011a) (Jiang *et al.*, 2019) Studies have shown that this t-tubular structural integrity is load

dependent with chronic mechanical overload, a feature of the failing heart, bringing about disruption of the t-tubule network, derangement in the spatial proximity of LTCCs to RyRs and loss of synchronous cardiomyocyte calcium release.(Ibrahim *et al.*, 2010) (Wei *et al.*, 2010) (Jones, MacQuaide and Louch, 2018) However, reduction of chronic cardiac overload via mechanical unloading has been shown to induce t-tubule reverse remodelling with consequent improvement in calcium transient synchronicity and EC coupling.(Ibrahim and Terracciano, 2013) This outcome is dependent on an optimum degree and duration of unloading as excessive unloading has also been shown to lead to impairment of t-tubule ultrastructure.(Ibrahim *et al.*, 2010) (Ibrahim and Terracciano, 2013) (Oriyanhan *et al.*, 2007)

Thus the maintenance of t-tubular architecture and efficient CICR mechanism is crucial to successful reversal of pathological remodelling in the failing heart.(Ibrahim, Navaratnarajah, *et al.*, 2012a) However, the exact mechanism governing t-tubule structural disruption and molecular mechanisms involved in t-tubule regulation and restoration of its structural integrity are not fully described. A number of proteins associated with t-tubule synthesis and function such as: Junctophilin-2 (JPH-2), BIN-1, and telethonin (Tcap), have been identified as important molecular elements involved in the maintenance of t-tubule structure but the exact role they play in mechanical unloading-induced reverse remodelling is not well described.(Ibrahim *et al.*, 2011a)

1.4.1.1 Cardiac T-tubules and Reverse Remodelling

T-tubules are a well organised network of sarcolemma invaginations (occurring at every 1.8 to 2- μ m intervals) that penetrate deep into the cardiac myocyte interior lying parallel to the Z-line regions in predominantly adult mammalian ventricular myocytes.(Wei *et al.*, 2010) (Hong and Shaw, 2017) They were first illustrated as transversely oriented continuous tubules in

mammalian heart muscle from light microscopy studies by Nyström in 1897.(Hong and Shaw, 2017) More detailed analysis of their ultrastructure in mammalian ventricular myocyte was however first described 60 years later by Lindner in 1956 with the aid of electron microscopy.(Lindner, 1957) This was followed in the 1970s by the discovery of the axial components of t-tubules which travel along the longitudinal axis of cardiac myocytes forming roughly perpendicular junctions with the transversely oriented tubules.(Sperelakis and Rubio, 1971)

T-tubules are rich in ion channels important for action potential generation, regulation, and signalling transduction. Through the junctional contact (dyad) they make with RyRs present in neighbouring SR, t-tubules are able to carry out their main function which is the regulation of cardiac EC coupling by facilitating efficient CICR and myocardial contractility.(Hong and Shaw, 2017) Furthermore, in addition to being an important site for Ca^{2+} entry, t-tubules are also involved in facilitating Ca^{2+} extrusion from the cell via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX).(Smith *et al.*, 2018)

T-tubules possess a phospholipid-rich bilayer that is maintained by a number of membrane scaffolding proteins, intracellular cytoskeleton and the ECM.(Smith *et al.*, 2018) as illustrated in figure 1.5.(Smith *et al.*, 2018)

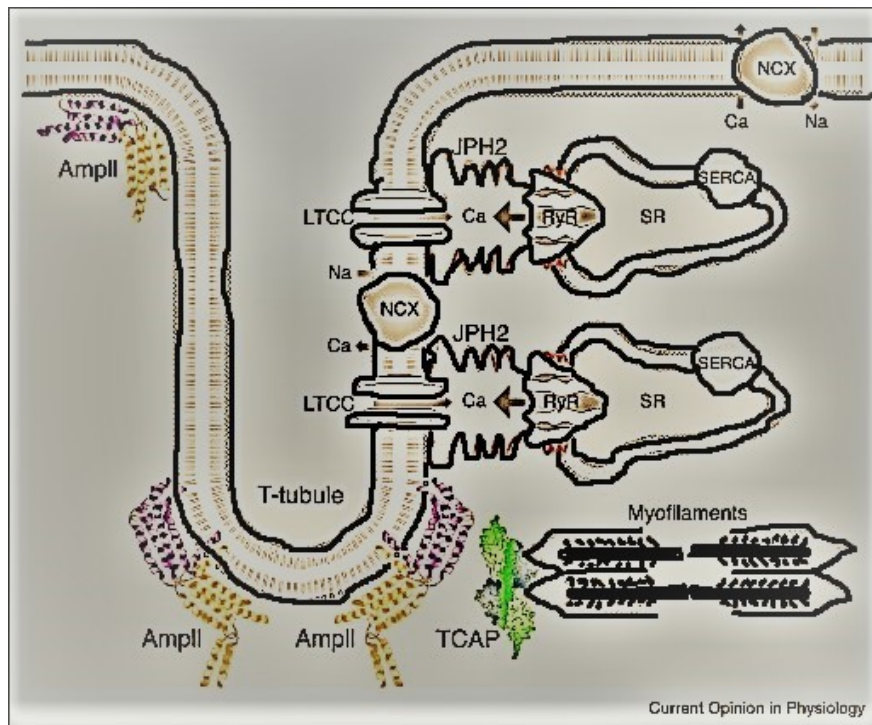


Figure 1.5: Schematic representation of the t-tubule system illustrating the three important proteins AmpII (BIN1), JPH2, and Tcap that are involved in its synthesis and function. Image reproduced from Smith et al. Current opinion in physiology 2018

The role these proteins play in the development and function of t-tubules have been extensively studied and are discussed in detail below, however the effect that mechanically unloading a failing heart has on their expression profile and activity is not well known.(Hong and Shaw, 2017) (Smith *et al.*, 2018) (Manfra, Frisk and Louch, 2017) (Knoll *et al.*, 2002)

Several studies now provide collaborative evidence to show that increased volume loading present in the failing heart plays a key role in t-tubular disruption and the impairment of efficient CICR and myocardial contraction.(Ibrahim and Terracciano, 2013) (Ibrahim *et al.*, 2015) It's been shown that the normal tightly co-localised dyad between LTCCs and RyRs present in the healthy heart is separated in the overloaded failing heart but that this colocalization is restored after mechanical unloading.(Ibrahim, Siedlecka, *et al.*, 2013) The precise molecular mechanisms by which this reversal of pathological remodelling occurs is however still keenly investigated.

1.4.1.2 Junctophilin-2

Junctophilin-2 (JPH2) is a structural membrane protein that has been associated with t-tubule-SR tethering and thus maintaining the tight junctional membrane complexes (JMCs) between LTCCs present in t-tubules and RyRs of the junctional SR.(Smith *et al.*, 2018) As previously described, this tight JMCs or dyads are essential for efficient EC coupling and JPH2 has been shown to be critical to their formation and maintenance by acting as a spacer molecule within the dyadic space as illustrated in figure 1.5.(Reynolds *et al.*, 2013) (Reynolds et al, 2017) Its importance in t-tubule development has been described in studies showing that JPH2 knockdown predisposes to impaired t-tubule maturation, leading to heart failure in developing mice.(Chen *et al.*, 2013)

Furthermore, progressive decrease in contractile function seen in heart failure has been shown to be paralleled by a progressive decrease in cardiac JPH2 levels and disruption of t-tubule structural integrity.(Wei *et al.*, 2010)(Reynolds et al, 2017) (Lyon *et al.*, 2009) In contrast however, overexpression of JPH2 in the failing heart results in the stabilisation of t-tubule structure, a reduction in Ca^{2+} spark frequency and an increase in Ca^{2+} transient amplitude consistent with JPH2-mediated normalization of SR Ca^{2+} handling.(Reynolds et al, 2017) (Van Oort *et al.*, 2012)

The precise mechanisms by which JPH2 downregulation occurs during heart failure is not well described. Elevated ventricular wall stress has been suggested as a possible trigger, with a number of studies showing decrease in JPH2 expression with increasing cardiac load (Wei *et al.*, 2010) (Frisk *et al.*, 2016) and recovery of expression with reduction in mechanical load.(Xie *et al.*, 2012) (Chen *et al.*, 2012) These studies highlight the effect pressure loading and unloading has on the expression profile of JPH2 and t-tubule architecture.

Previous studies in this laboratory have investigated the effects volume load has on t-tubule structural integrity and Ca^{2+} handling showing that mechanical unloading of the failing heart via heterotopic abdominal heart transplantation (HAHLT) was capable of stimulating recovery of t-tubule structure and function in a degree and duration of unloading dependent manner.(Ibrahim and Terracciano, 2013) (Ibrahim, Kukadia, *et al.*, 2012) However, whilst the expression profile of JPH2 was noted to be reduced in the loaded failing heart, recovery of expression was not observed with mechanical unloading despite improvement in t-tubule structure.(Ibrahim, Navaratnarajah, *et al.*, 2012a) The study did not assess the effect of mechanical unloading on the regularity of JPH2 expression. As JPH2 is important in t-tubule-SR tethering, the regularity of its expression along t-tubules as opposed to absolute numbers might be of more relevance. In this study the efficacy of JPH2 activity indexed by the regularity of its expression along t-tubules is evaluated with the aid of the Fourier transform.

1.4.1.3 Bridging Integrator-1 (BIN1)

BIN1, also referred to as amphiphysin II, is a scaffolding protein that plays an important role in dyadic assembly and maintenance.(Fig 1.5) (Hong *et al.*, 2010) It is specifically associated with the induction of membrane invaginations, t-tubule biogenesis and the trafficking of LTCCs.(Hong and Shaw, 2017) (Hong *et al.*, 2012) With this significant role in establishing key components of the contractile unit of the heart it is not surprising that BIN1 downregulation has been reported in human and animal models of heart failure with associated disruptions in t-tubule architecture.(Caldwell *et al.*, 2014) BIN1 genetic knockout has also been shown to be embryologically lethal and to predispose to dilated cardiomyopathy in the adult heart.(Manfra, Frisk and Louch, 2017) (Laury-Kleintop *et al.*, 2015)

The mechanism by which BIN1 loss in the failing heart results in t-tubule disruption is not well described.(Manfra, Frisk and Louch, 2017) Whilst cardiomyocyte retubulation in the failing heart secondary to SR Ca²⁺ ATPase (SERCA) gene delivery has been shown to correlate with significant upregulation of BIN1 protein expression, little is known of the effect mechanical unloading has on BIN1 expression profile.(Caldwell *et al.*, 2014) (Lyon *et al.*, 2012)

1.4.1.4 Telethonin (Tcap)

Tcap is a 19-KDa protein that is part of the stretch-sensitive complex of the z-disc (fig 1.4). It links this stretch-sensitive complex with other proteins in the t-tubule membrane and it is thought to be a load-dependent regulator of t-tubule structure.(Knoll *et al.*, 2002) Tcap knockout mice have been shown to exhibit progressive disruption of the t-tubule network during development(Manfra, Frisk and Louch, 2017) whilst Tcap downregulation in the adult heart is associated with the development of heart failure.(Bryant *et al.*, 2014) In keeping with its stretch-sensitive effects, the deleterious manifestation of Tcap loss is pronounced mainly in the presence of increased ventricular load.(Ibrahim, Siedlecka, *et al.*, 2013)

Similar to BIN1, SERCA2a gene therapy-induced recovery of t-tubular architecture was associated with an augmented expression of Tcap in the failing heart.(Ibrahim, Siedlecka, *et al.*, 2013) However, the precise effects of mechanical unloading on Tcap expression profile and its activity as indexed by the regularity of its expression along t-tubules in the mechanically unloaded failing heart is not known.(Lyon *et al.*, 2012)

In summary, the evidence presented here show that the expression of these three key proteins, involved in facilitating efficient t-tubule function, is downregulated by the loaded failing heart. The hypothesis in this part of the thesis is that mechanical unloading of the failing heart

stimulates an upregulation in the expression and activity of JPH2, BIN1, and Tcap, with consequent improvement in EC-coupling and myocardial contractility.

1.4.2 The Extracellular Matrix

The cardiac extracellular matrix (ECM) is a living network of proteins, particularly fibrillar type I collagen which helps to maintain the structural integrity of the myocardium.(Weber, 1989) Fibrillar collagen type I is the principal constituent of the ECM and accounts for about 85% of all collagen in the myocardium with fibrillar collagen type III and type V accounting for about 10-15% and 5% respectively.(Weber *et al.*, 1988) (Nagaraju *et al.*, 2019) Other major constituents of the ECM include: fibronectin, elastin, laminin, fibrillin, proteoglycans and glycoproteins.(Segura, Frazier and Buja, 2014) Together these proteins help to provide the protective scaffolding in the myocardium and promote adequate myocardial function by facilitating the transmission of electrical and mechanical forces between myocytes.(Segura, Frazier and Buja, 2014)

ECM proteins are secreted by flat spindle shaped cells of mesenchymal origin known as cardiac fibroblasts. These cells, the third most populous in the adult heart, remain largely quiescent following embryonic development.(Zhou and Pu, 2016) In response to appropriate stimuli, most commonly myocardial injury, cardiac fibroblasts become activated by the cytokine Transforming Growth Factor beta 1 (TGF β -1) and differentiate into a more mobile and contractile phenotype termed myofibroblasts. Myofibroblasts typically express smooth muscle cell markers such as alpha smooth muscle actin (α SMA), smooth muscle myosin heavy chain, vinculin, paxillin, and tensin that are absent in quiescent cardiac fibroblasts.(Weber *et al.*, 2013) (Nagaraju *et al.*, 2019) They have a greater synthetic ability to produce ECM proteins

and thus their presence leads to increased synthesis and accumulation of fibrotic deposits, myocardial stiffness and disruption of myocyte-myocyte interactions with consequent impairment of cardiac function.(Weber *et al.*, 2013) The persistence of myofibroblasts in the heart long after scar formation has been identified as a key pathophysiological process that leads to pathological remodelling after myocardial infarction.(Weber *et al.*, 2013)

In addition to the secretion of ECM proteins, cardiac fibroblasts are also responsible for maintaining the homeostasis of the ECM. They secrete the ECM-regulatory proteins Matrix Metalloproteinases (MMPs) which are a family of functionally related enzymes involved in fibrillar collagen denaturation and degradation.(Klotz *et al.*, 2005a) There are a number of MMPs involved in myocardial remodelling after injury such as: MMPs 1-14.(Felkin *et al.*, 2009) MMP-1 breaks down collagen type I, II, and III, and increase in its activity has been shown to correlate with reduced expression of type I collagen, a reduction in myocardial contractile force, muscle fibre slippage, and muscle realignment predisposing to thinning of the myocardium with consequent chamber dilatation.(Klotz *et al.*, 2005a) (Gunja-Smith *et al.*, 1996)

Cardiac fibroblasts also secrete Tissue Inhibitors of Metalloproteinases (TIMPs) which inhibit MMPs. There are four main TIMPs. TIMPs 2-4 are predominantly expressed in healthy myocardium whilst TIMP-1 levels are very low in healthy hearts but increase significantly in disease. TIMP-1 is the better characterized TIMP and has been shown to bind to and inactivate a range of MMPs including MMP-1.(Weber *et al.*, 2013)

1.4.2.1 ECM changes following myocardial injury

Myocardial infarction leads to loss of large numbers of cardiomyocytes as a result of necrotic cell death and this compromises the structural integrity of the myocardium predisposing the

heart to excessive dilatation and possible rupture. However, immediate adaptive changes in the constituents of the cardiac ECM play a vital role in maintaining the hearts structural integrity after injury.(Weber *et al.*, 2013)

Shortly after MI, inflammatory cells including macrophages accumulate in the infarct territory and express renin which activates the renin-angiotensin-aldosterone system (RAAS) thus generating angiotensin II at the site of injury.(Sun *et al.*, 2001) (Sun *et al.*, 1994) (Sun and Weber, 1996) Angiotensin II binds to the angiotensin II type 1 (AT1) receptor resulting in the upregulation of the expression and activation of TGF- β 1.(Sun *et al.*, 1998) Myofibroblast generation then occurs through TGF- β 1 mediated activation of resident cardiac fibroblasts, bone marrow-derived and epithelial cell-derived fibroblasts (the latter two via the Smad pathway), and together with the accumulated inflammatory cells, mount a healing response that involves collagen fibrillogenesis with consequent formation of an infarct scar.(Towbin, 2007) (Iwano *et al.*, 2002) (Weber *et al.*, 2013) This adaptive process of ‘reparative fibrosis’ helps to replace lost cardiomyocytes and preserve the structural integrity of the heart albeit with an increase in myocardial stiffness from the deposition of crossed-linked fibrillar collagen type 1.(Weber *et al.*, 2013)

After the initial scar formation, persistence of myofibroblasts at the site of injury as opposed to their progression towards apoptotic cell death (as occurs in most other organs) leads to excessive ECM protein deposition, abnormal ECM degradation, impaired systolic and diastolic function as well as progressive ventricular dilatation and wall thinning.(Willems *et al.*, 1994) (Spinale, 2007) In addition to effects at the site of injury, excessive myofibroblast activity also leads to the generation of soluble signals that are capable of traversing the interstitial space to distant sites where they activate the development of interstitial and perivascular adventitial fibrosis in regions remote from the area of myocardial injury.(Weber *et al.*, 2013) This maladaptive process of ‘reactive fibrosis’ perpetuates the pathological remodelling of the

myocardium that culminates in the development of heart failure.(Brower *et al.*, 2006) (Felkin *et al.*, 2009) (Weber *et al.*, 2013)

1.4.2.2 The cardiac ECM and reverse remodelling after mechanical unloading

Several recent studies have identified the cardiac ECM as possibly having a significant influence on the degree of reverse remodelling that occurs during mechanical unloading with LVADs.(Schaefer *et al.*, 2019) The ECM is at present the only part of the unloaded failing heart that appears to portray features that are not in keeping with the recovery process.(Klotz *et al.*, 2007) (Klotz *et al.*, 2005b) It is now widely accepted in human and animal studies that myocardial fibrosis worsens after unloading the failing heart.(Schaefer *et al.*, 2019) (Lok *et al.*, 2015) (Navaratnarajah *et al.*, 2013) (Drakos *et al.*, 2010) The mechanism by which this occurs is however not fully understood.

The traditional concept is that increased load leads to increased wall stress and stretch, activation of the RAAS pathway and thus activation of TGF- β 1 leading to increases in myofibroblast activity with consequent increase in collagen deposition, myocardial stiffness, diastolic and systolic dysfunction.(Jugdutt and Butler, 2007) Conversely, decreased mechanical load would be expected to decrease angiotensin II levels, collagen deposition and myocardial stiffness, and thus improve diastolic and systolic function. However, many contemporary reports show this not to be the case. (Drakos *et al.*, 2010) (Wang *et al.*, 2013) (Lok *et al.*, 2015) It has been suggested that whilst cardiomyocyte hypertrophy is primarily load-dependent, with mechanical unloading bringing about a decrease in hypertrophy with near normalisation of diastolic properties of hearts in end-stage heart failure as evidenced by an improved LV passive end-diastolic pressure-volume relation and a trend toward improved

cardiomyocyte function. Calcium cycling, gene expression, fibroblast activation and myofibroblast propagation are primarily load-independent and as such ECM remodelling is not reversed by mechanical unloading alone.(Jugdutt and Butler, 2007) Fibroblast activation has been shown to be predominantly a result of increase in the activity of the RAAS pathway.(Jugdutt, 2003) (Peng *et al.*, 2005) (Ferrario *et al.*, 2005) The increased angiotensin II protein expression seen after mechanical unloading has thus been suggested as a possible cause of the worsening fibrosis seen after LVAD use. (Klotz *et al.*, 2005c) However, the use of ACE-inhibitors in conjunction with mechanical unloading with LVADs has not been shown to bring about significant reduction in myocardial fibrosis.(Farris *et al.*, 2017)

Thus the exact underlying mechanism by which mechanical unloading of the failing heart brings about increases in myocardial fibrosis with potential deleterious consequences on LVAD-induced myocardial recovery remains largely unknown. What is now clear from several contemporary studies is that mechanical unloading of the failing heart leads to a significant increase in the absolute content of type I and III collagens and the ratio of collagen type I to III in the ECM.(Madigan *et al.*, 2001) (McGowan *et al.*, 2003) (McCarthy *et al.*, 1995) (Li *et al.*, 2001) Furthermore, mechanical unloading has also been associated with a decrease in MMP-1 levels and activity whilst TIMP-1 levels increase tremendously after unloading thus generating MMP-1:TIMP-1 protein expression ratios that predispose to increases in the total amounts of type I and III collagen deposition.(Klotz *et al.*, 2005c)

1.4.2.3 The role of the collateral circulation

An important yet largely unexplored element that could shed more light into the underlying mechanism by which fibrosis worsens with LVADs is the impact, if any, that mechanical unloading has on the collateral circulation after myocardial infarction.

The heart is an obligate aerobic organ that requires a steady supply of oxygen to meet its demand for energy. With elevated metabolic demand, myocardial blood flow (MBF) has the potential to rise up to 5-fold through auto-regulatory effects on the microcirculation.(Xydas *et al.*, 2005) Studies have demonstrated that the heart has a congenitally developed microcirculation that is predominantly redundant at rest but in times of increased metabolic need become highly effective intercoronary collateral channels.(BAROLDI, MANTERO and SCOMAZZONI, 1956) (Rentrop *et al.*, 1985) (Fulton, 1964b) (Fulton, 1964c) It's been shown that following an acute significant obstruction (~80%) or occlusion of a major epicardial coronary artery, these collateral vessels become stimulated to undergo a process of arteriogenesis (figure 1.6 A) where they dilate, remodel and thus provide an alternative blood source to the infarct territory and neighbouring myocardium helping to limit the extent of necrotic cell death and infarct size.(Fulton, 1964b) (Yoon *et al.*, 2009) (van Royen *et al.*, 2009)

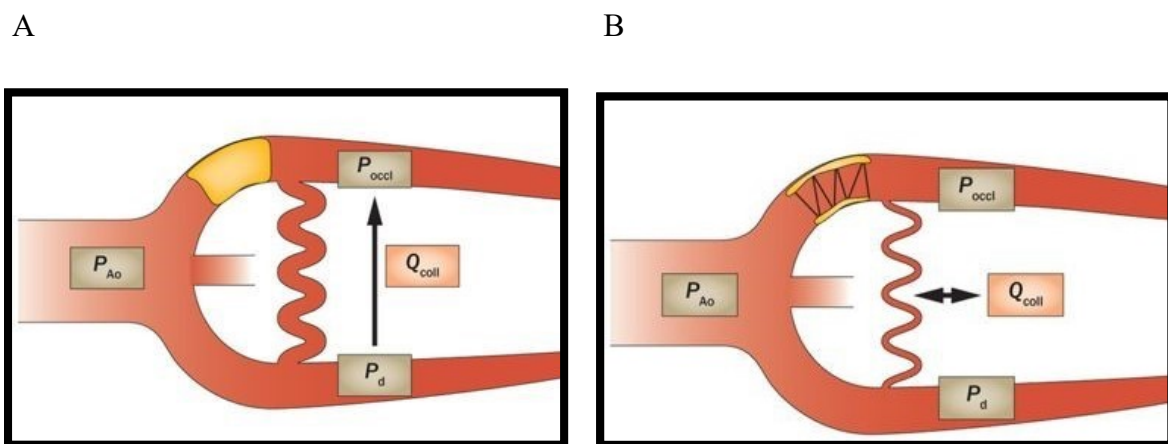


Figure 1.6: Autoregulation of the collateral circulation. Beyond a coronary occlusion (A), P_{occl} is sustained by Q_{coll} , provided by a healthy donor artery in which $P_d = P_{Ao}$. Removal of the occlusion (B) restores normal antegrade flow and promotes a prompt normalization of P_{occl} , with subsequent derecruitment of Q_{coll} . Abbreviations: P_{Ao} , aortic pressure; P_d , driving pressure; P_{occl} , pressure beyond the coronary occlusion; Q_{coll} , collateral flow. Adapted from Nature Reviews Cardiology, 2014.

Several reports have shown that patients with a robust collateral coronary circulation have a significantly lower mortality from myocardial infarction.(Fulton, 1964a) (Antoniucci *et al.*,

2002) (Seiler, 2009) (Kim *et al.*, 2016) (Meier *et al.*, 2012) (Nakai *et al.*, 1995) These highly autoregulated microvasculatures help to match MBF to changes in myocardial oxygen consumption ($MV\dot{O}_2$) thus preserving cardiomyocyte function.(Xydas *et al.*, 2005) Once blood flow in the parent (non-autoregulated) vessel is re-established (figure 1.6 B) these collateral channels rapidly become non-functional and return to their collapsed redundant state.(Rentrop *et al.*, 1985)

It has been reported that coronary flow reserve (CFR), the contribution of the microvasculature to increased $MV\dot{O}_2$, is diminished in heart failure patients treated with LVADs.(Tansley *et al.*, 2004) This is thought to be a result of the markedly decreased LV workload during mechanical unloading which reduces $MV\dot{O}_2$ and diminishes the autoregulatory stimulus on the microcirculation.(Tansley *et al.*, 2004) Furthermore, it has also been shown that mechanical unloading with LVADs is associated with a reduction in microvascular luminal diameter in the failing heart, further suggesting the presence of a link between LVAD use and negative autoregulatory effects on the collateral circulation.(Drakos *et al.*, 2010)

In summary, the reported deleterious effect mechanical unloading has on the ECM, with worsening of myocardial fibrosis and chamber stiffness, poses a clear limitation on the degree of LVAD-induced myocardial recovery achievable. The observation that mechanical unloading with LVADs predisposes to a decline in CFR and is associated with a reduction in microvascular luminal diameter in the failing heart raises important yet unanswered questions as to the effect alterations in blood flow to the infarct territory will have on LVAD-induced changes in the ECM.

Thus the hypothesis in this section is that in a failing ischaemic heart, coronary reperfusion aimed at returning blood flow through the parent, non-autoregulated coronary vessel supplying

the infarcted myocardial territory, ameliorates the deleterious effect mechanical unloading has on the ECM.

1.4.3 Endogenous Proliferative Capacity of the Adult Heart

The previously well held paradigm that the heart was a post-mitotic organ and all cardiac myocytes were terminally differentiated and thus incapable of re-entering the cell cycle has recently been challenged.(He and Zhou, 2017) This century long dogma was driven by the observation that the adult mammalian heart showed no meaningful regenerative response to myocardial injury. Instead, the vast majority of cardiomyocytes lost after MI is substituted by scar tissue contributing to the contractile demise of the heart and subsequent development of heart failure. (Kajstura *et al.*, 2010)

Discoveries of significant cardiomyocyte regenerative capacity in some lower vertebrates such as the zebrafish and neonatal mouse hearts helped stimulate intensive research into the adult mammalian heart's potential for self-renewal leading to the widely reached consensus now that cardiomyocytes are indeed formed throughout life albeit at a disputed frequency.(Vadakke-Madathil and Chaudhry, 2018) (Malliaras *et al.*, 2013) (Nadal-Ginard, Ellison and Torella, 2014) (Hsieh *et al.*, 2007) (Jopling *et al.*, 2010) Studies have shown that the adult mammalian heart does possess an intrinsic form of cellular homeostasis that permits regeneration and formation of new cardiac myocytes and vasculature. (Kajstura *et al.*, 2010) (Bergmann *et al.*, 2009)

The frequency at which new myocytes are formed remains controversial with studies reporting regenerative rates ranging from less than 1% to 4% (Fleischmann *et al.*, 2010) In humans, a

retrospective study using ^{14}C birth dating of cells established the presence of a modest degree of cardiomyocyte renewal of about 1-2%, but also noted that this regenerative capacity continued to decline with age.(Vadakke-Madathil and Chaudhry, 2018) (Kajstura *et al.*, 2010) They showed that throughout life, the heart is capable of achieving a myocyte turnover of about 50% with most of this renewal occurring in early years. (Kajstura *et al.*, 2010) This degree of cell turnover is however insufficient to replace the large number of cardiomyocytes (up to 1 billion in humans) that are lost following a large infarct, meaning there remains a need for the development of novel strategies aimed at augmenting the heart's regenerative capacity. (Porrello and Olson, 2014) Furthermore, the exact mechanism of cardiomyocyte renewal in the adult heart is not well defined. It is still unknown whether the adult mammalian heart generates new cardiomyocytes through myogenic differentiation of endogenous cardiac stem cells (eCSC) or via the dedifferentiation and proliferation of pre-existing resident cardiomyocytes that re-enter the cell cycle.(He and Zhou, 2017) (Hsieh *et al.*, 2007) (Porrello *et al.*, 2013) It has been suggested that both of these mechanisms might be at work in the mammalian heart. (Malliaras *et al.*, 2013)

The exact regenerative potential of the exogenous administration of eCSCs either via intracoronary infusion or intramyocardial injection remains a matter marred in controversy.(Vadakke-Madathil and Chaudhry, 2018) Despite investigations with a wide variety of cell types such as; cardiac-derived cKit⁺ cells, cardiac side population cells, bone marrow side population cells, and cardiac Sca-1⁺ cells, results of stem cell transplantation has remained ambiguous. Most believe the reported functional improvements observed are mediated through paracrine mechanisms rather than true transdifferentiation into working myocardium.(Lee, 2018) (Vadakke-Madathil and Chaudhry, 2018)

Notwithstanding these controversies, there remains an ongoing drive towards developing therapeutic strategies aimed at activating the endogenous regenerative capacity of the heart to a level whereby meaningful repair of the injured heart can be achieved. (Koudstaal *et al.*, 2013)

1.4.3.1 MI and cardiomyocyte regenerative potential

A study designed to determine the absolute rates and relative magnitudes of induced secondary cardiomyocyte proliferation and cardiomyogenic differentiation of recruited endogenous stem cells in the normal and infarcted mammalian heart found that in the normal adult mouse heart, cardiomyocyte turnover occurs mainly through the proliferation of resident myocytes, without any significant contribution by endogenous progenitors. (Malliaras *et al.*, 2013) However, after myocardial injury, cardiomyocyte proliferative rate increased significantly and this increase was due to the activation of the heart's endogenous progenitor pool. (Malliaras *et al.*, 2013) These findings are in line with several other reports in animal studies which show that most eCSC are quiescent, following injury, they get rapidly activated, multiply, and differentiate, taking on myocardial cell lineages to replace lost cardiomyocytes. (Ellison *et al.*, 2013)

Myocardial injury appears to stimulate cardiomyocyte proliferation by upregulating genes associated with cell-cycle progression, such as those associated with the G0/G1 transition (Cyclin D1, Cyclin-Dependent Kinase 4), the G1/S transition (Cyclin E, Cyclin-Dependent Kinase 2) and the G2/M transition (CyclinA1-2, E2F1). These genes are also upregulated in embryonic and neonatal hearts known to possess robust regenerative potential. (Malliaras *et al.*, 2013) (Fleischmann *et al.*, 2010) (Porrello *et al.*, 2011) However, this increase in regenerative capacity associated with myocardial injury has been shown to be limited only to the first few days after birth. (Porrello and Olson, 2014) (Mahmoud *et al.*, 2013) Cardiomyocyte cell cycle arrest and a significantly dampened regenerative response to myocardial injury

occurs at about 7 days after birth but the basis for this has not been well described.(Mahmoud *et al.*, 2013) (A. Uygur, 2017)

1.4.3.2 Effect of mechanical load on cardiac regeneration

Recent work has identified a correlation between the postnatal (P) cardiomyocyte cell cycle arrest that occurs at day 7 after birth (P7) and the upregulation of myeloid ecotropic viral integration site 1 (MEIS1). (Mahmoud *et al.*, 2013) Meis1 belongs to the TALE (three amino acid loop extension) family of homeodomain transcription factors that are central regulators of cardiac differentiation during embryonic development. (Paige *et al.*, 2012) (Wamstad *et al.*, 2012) The study showed that MEIS1 knockout in adult mice was associated with a 10 fold increase in the number of mitotic cardiomyocytes. By contrast, forced overexpression of MEIS1 inhibited neonatal heart regeneration following induction of myocardial injury at P1, whilst wild-type hearts regenerated normally. (Mahmoud *et al.*, 2013) Furthermore, induction of MI in a neonatal mouse at P7 was associated with a significant rise in MEIS1 expression and a decline in myocyte proliferation, an observation that was absent when MI was induced at P1. (Mahmoud *et al.*, 2013) With the aid of cell cycle PCR array, the group showed that MEIS1 deletion resulted in the downregulation of cyclin-dependent kinase inhibitors e.g. CDKN2B, CDKN3, & CDKN1A in isolated cardiomyocytes, as well as the stimulation of a number of positive regulators of the cell cycle such as cdk4, MCM3 and CHEK1. (Mahmoud *et al.*, 2013) This report suggests that MEIS1 might play a significant role in the development of cardiomyocyte cell cycle arrest that occurs at P7 and the growth transition the heart undergoes shortly after birth from hyperplastic to hypertrophic growth. As this transition also coincides with the increased hypertrophic stimuli from volume loading of the heart, it is

reasonable to postulate that the removal of mechanical load from the heart using LVADs could have an effect on MEIS1 expression and thus the regenerative response of the heart to injury.

Mechanical load also affects the activity of cell-intrinsic signalling pathways that regulate cardiomyocyte proliferation. The Hippo pathway is an evolutionarily conserved pathway that regulates organ size and growth during development in a range of animals from *Drosophila* to humans by restraining cellular proliferation, inducing apoptosis and regulating cell fate decisions. Mechanical wall stress, amongst a number of other stimuli, is capable of activating the Hippo pathway which leads to phosphorylation of transcription co-activators YAP and TAZ in mammals and their extrusion from the nucleus. Deactivation of the Hippo pathway results in YAP/TAZ protein dephosphorylation and their translocation back to the nucleus where they can then induce cell proliferation.(A. Uygur, 2017) (Heallen *et al.*, 2012) (Xin *et al.*, 2013) The effect mechanical unloading has on the expression of phosphorylated YAP (pYAP) has not been described.

Furthermore, studies have shown that the postnatal heart's exposure to increase in mechanical load also stimulates cardiac mitochondrial biogenesis resulting in a build-up of mitochondrial mass. (Piquereau *et al.*, 2013) This leads to increases in reactive oxygen species (ROS) production, oxidative DNA damage, and activation of DNA damage response (DDR), an important mechanism of cell cycle arrest in postnatal mammalian cardiomyocytes. (Puente *et al.*, 2014) This study showed that mechanical unloading using LVADs was able to bring about a reduction in mitochondrial mass and DDR, and thus reverse the metabolic cascade that results in cardiomyocyte cell cycle arrest, ultimately reverting the heart back to its hyperplastic growth phenotype. (Canseco *et al.*, 2015)

This possible role of mechanical unloading on cardiomyocyte proliferation is further supported by reports showing that cells at the periphery of myocardial infarction undergo endomitosis

and polyploidization instead of conventional mitosis. In fact, the majority of cardiomyocytes in heart failure harbour a polyploid as opposed to the normal diploid genome. (Wohlschlaeger *et al.*, 2010) Interestingly, upon relief of wall stress via mechanical unloading there was a more than 2 fold increase in diploid cardiomyocytes with a significant decrease in the number of polyploid cardiomyocytes. (Wohlschlaeger *et al.*, 2010) This finding suggests that mechanical unloading might facilitate conventional mitosis instead of endomitosis. It supports the notion that the vast polyploidy of cardiomyocytes in the failing heart is a result of repeated rounds of DNA synthesis with an incomplete attempt at mitosis that is blocked after completion of DNA replication. When hemodynamic stress is relieved by LVAD, this blockade is released, thus promoting karyokinesis, cytokinesis, and real cell duplication. The decrease in mean DNA content per cardiomyocyte noted suggests not only that karyokinesis with a decreased DNA content per nucleus is taking place but also that cell division or progenitor cell proliferation occurs after unloading, resulting in a reduction in total DNA per cardiomyocyte. (Wohlschlaeger *et al.*, 2010)

1.4.3.3 Coronary reperfusion and cardiac regeneration

In addition to the potential positive effects of mechanical unloading on the regenerative capacity of the heart it has also been noted that coronary reperfusion after myocardial injury plays an important role in the regenerative response shown by the zebrafish heart. (Marín-juez *et al.*, 2016) In the zebrafish, strong early angiogenic revascularisation occurs after injury with coronary vessel sprouting into the injured area developing within 15hr of cryoinjury and with an established coronary plexus forming within 2 days. Furthermore, blocking early vascular sprouting into the damaged territory was significant enough to inhibit cardiomyocyte proliferation. (Marín-juez *et al.*, 2016) In this research, the effect mechanical unloading of the

heart (accomplished via HAHLT in a rat model of MI) with or without ischaemia-reperfusion has on the regenerative response of the adult heart to injury is investigated. The effects on the expression profiles of MEIS1 and pYAP are also described.

The hypothesis in this section is that mechanical unloading of the heart following myocardial infarction potentiates the proliferation of new cardiomyocytes and facilitates the downregulation of cardiomyocyte cell cycle arrest.

1.4.4 Whole-Heart Contractility after Mechanical Unloading

The ultimate goal of research into the effects mechanical unloading has on the regression of pathological remodelling is to develop interventions that will culminate in reproducible improvements in myocardial function. Thus developing effective techniques for the accurate physiological assessment of myocardial function after mechanical unloading is of paramount importance to the study of reverse remodelling. To date, research showing improvements in cardiac contractility secondary to interventions aimed at achieving reverse remodelling in the mechanically unloaded failing rat heart have relied predominantly on data from isolated cells and cardiac tissue slices.(Xie *et al.*, 2012) (Ibrahim, Kukadia, *et al.*, 2012) (Ibrahim, Navaratnarajah, *et al.*, 2012b) In this laboratory, mechanical unloading of the failing rat heart achieved via heterotopic abdominal heart and lung transplantation (HAHLT, discussed in chapter 2) has been extensively shown to bring about improvement in cardiomyocyte contractility by: the restoration of t-tubular structural integrity, decrease in action potential duration, increase in Ca^{2+} transient amplitude, reduction in Ca^{2+} spark frequency, and improvement in Ca^{2+} transient synchrony.(Ibrahim and Terracciano, 2013) (Ibrahim, Kukadia, *et al.*, 2012) (Ibrahim, Rao, *et al.*, 2012) (Navaratnarajah *et al.*, 2014) These reports and many

others have helped to shed light on the possible underlying mechanisms responsible for mechanical unloading-induced reverse remodelling. However, the assessment of functional improvement in myocytes or cardiac tissue slices does not provide an adequate representation of the functional status of the heart as a whole.

In vivo pressure-volume (PV) loop conductance technology has taken centre stage in the functional assessment of intra-thoracic rodent hearts after experimentation and provides a vast amount of haemodynamic parameters under varying physiological loading conditions. In addition, by altering preload, the PV loop conductance catheter is able to obtain load-independent haemodynamic indices that specifically reflect the contractile state of the LV. In vivo PV loop studies can't be employed to assess LV functional improvement after HAHLT because the transplanted rodent heart is mechanically unloaded in the recipient's abdomen. The ex vivo working/ejecting heart system facilitates the reloading of the isolated rodent whole-heart physiologically through the cannulation of the left atrium. This technique allows the functional assessment of the mechanically unloaded rodent whole-heart to be possible and is thus of particular value in this study.

This ex vivo approach to the evaluation of myocardial contractility in a failing rat heart at the whole-heart level using the working/ejecting heart system in conjunction with the PV loop conductance catheter has however not been previously undertaken. To the best of our knowledge this will be the first report of the ex vivo assessment of LV function of a mechanically unloaded rodent heart after HAHLT.

The isolated working/ejecting heart preparation is a modification of the well-known and widely employed Langendorff isolated heart perfusion system used to study the effect of pharmacological interventions on myocardial function, myocardial ischaemia studies, electrical conduction, vascular reactivity, endothelial and smooth muscle function.(Liao,

Podesser and Lim, 2012) (BROADLEY, 1970) In the Langendorff or non-ejecting heart preparation the isolated heart is perfused in a retrograde fashion through the aorta. Isovolumic intraventricular balloon method of LV pressure assessment is then employed for measuring LV performance.(Liao, Podesser and Lim, 2012) (Soppa *et al.*, 2008) (Liu *et al.*, 2015) Whilst less technically challenging to set up compared to the working/ejecting heart system, the Langendorff system does not put the heart through physiological relevant work nor does it provide the opportunity to assess myocardial function at varied preload and afterload conditions as achieved during in vivo pressure-volume loop studies.(Pacher *et al.*, 2008) (Paterek and Mackiewicz, 2016)

In the working/ejecting heart preparation, antegrade perfusion of the isolated heart occurs via the flow through the left atrium, loading the left ventricle and culminating in ejection through the aorta. This preparation has the advantage of facilitating assessment of the heart ex vivo whilst it is being put through physiologically relevant mechanical work.(Liao, Podesser and Lim, 2012) Furthermore, as opposed to the isovolumic preparation, the ejecting heart system allows measurement of LV performance under various loading conditions such as left ventricular filling at a constant preload, ventricular ejection against an afterload, assessment of load-independent indices of LV contractility by varying preload etc.(Liao, Podesser and Lim, 2012) Utilising the ejecting heart system, a wide range of haemodynamic data is obtainable from PV loop studies providing useful information on myocardial contractility.(Paterek and Mackiewicz, 2016)

This technique allows the physiological evaluation of contractile function in infarcted hearts that had been mechanically unloaded via HAHLT. The ability to obtain, in both loaded and unloaded rat hearts, load-independent indices of myocardial contractility and chamber stiffness, such as the end-systolic pressure volume relationship (ESPVR), and end-diastolic pressure volume relationship (EDPVR) respectively, the pressure volume area (PVA) which

reflects total myocardial oxygen consumption (MV_{O_2}) per cardiac cycle, and preload recruitable stroke work (PRSW) which is a highly sensitive marker of myocardial contractility that is independent of load, heart size and heart rate are of significant value. Other parameters noted in this study include the diastolic constant tau and the ejection fraction (EF) both load-dependent markers of diastolic function and contractility respectively.(Grieve *et al.*, 2004) (Kass *et al.*, 1986) (Glower *et al.*, 1985)

1.4.4.1 Pressure-Volume Loop Studies in the Rat Heart

The in vivo assessment of LV systolic and diastolic function, and myocardial contractility in the small rodent heart with the aid of miniaturised PV loop catheters is now well established following its first application over two decades ago.(Ito *et al.*, 1996) (Pacher, 2004) (Radovits *et al.*, 2013) (Lapointe *et al.*, 2003) PV loops provide a characteristic representation of the cyclical steps occurring during a cardiac cycle with multiple heart beats during steady state becoming superimposed on each other producing the typical loop pattern as illustrated in figure 1.7.

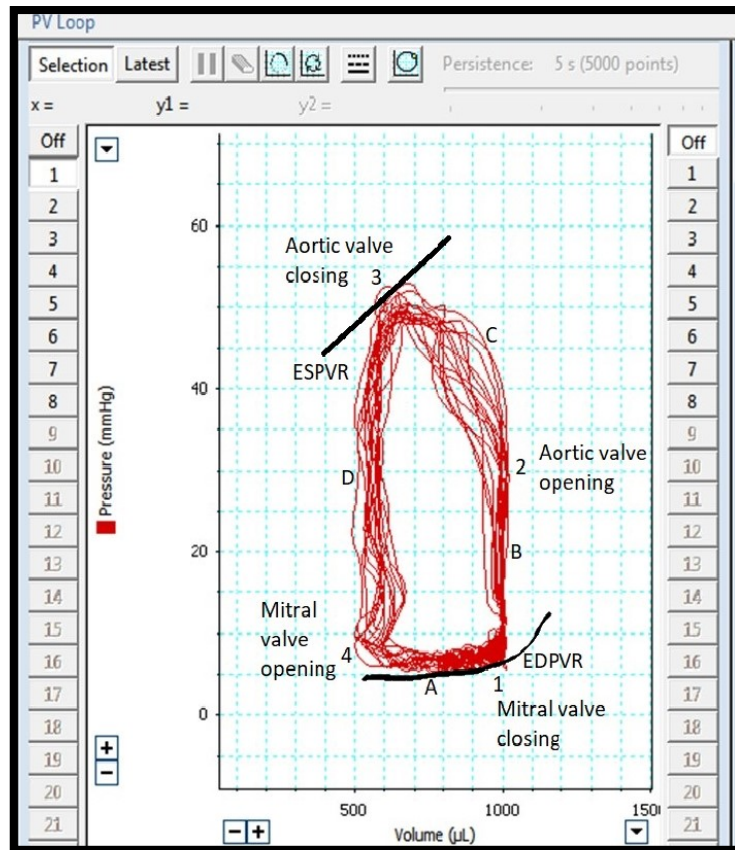


Figure 1.7: Ex vivo PV loop study obtained from a healthy rat heart showing the various stages of the cardiac cycle.

Phase (A) in figure 1.7 represents the period of diastolic filling starting from the opening of the mitral valve (4) to its closure (1) once left ventricular end-diastolic pressure (LVEDP) or preload exceeds the left atrial (LA) pressure. Phase (B) represents isovolumetric ventricular contraction which ends once the left ventricular pressure (LVP) exceeds the diastolic pressure in the aorta (afterload). The aortic valve opens (2) and ventricular ejection phase (C) resumes. During this phase the LV volume decreases as LVP increases to a peak value (peak systolic pressure) and then decreases as the ventricle begins to relax leading to the closure of the aortic valve (3). Isovolumetric ventricular relaxation phase (D) then resumes ending once left ventricular end-systolic pressure (LVESP) drops below the LA pressure resulting in the opening of the mitral valve (4) and thus completing the loop.(Physiology, 2011) The straight

black line depicts the ESPVR, a load-independent index of myocardial contractility, whilst the curved black line depicts the EDPVR, a load independent index of ventricular chamber stiffness. The PV loop parameters noted in this study are described below.

1.4.4.2 ESPVR

The ESPVR represents the inotropic state of the left ventricle and is the maximal pressure that can be developed by the ventricle at any given left ventricular volume.(Physiology, 2011) (Baan, van der Velde and Steendijk, 1992) It is a linear relationship and intercepts the volume or x-axis at V_0 (maximum volume at which pressure is still zero mmHg) just beyond the zero mark showing that a certain amount of volume in the ventricle is necessary for the generation of pressure.(Physiology, 2011) The ESPVR is generated experimentally by occluding the inferior vena cava (in vivo models) or the LA cannula (ex vivo models) thus reducing the flow to the left ventricle.(Pacher *et al.*, 2008) This decreases ventricular preload and causes the PV loop to get smaller over several heart beats with the upper left hand corner of each loop falling on the EDPVR as illustrated in figure 1.8.

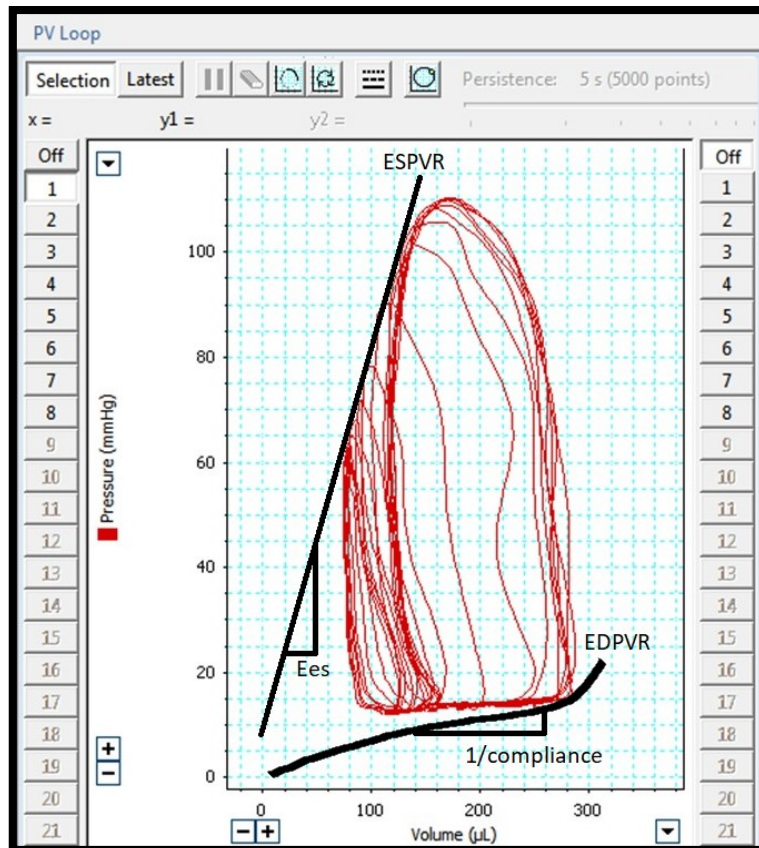


Figure 1.8: Ex vivo PV loop study obtained from a healthy rat heart after preload occlusion illustrating the load-independent markers of ventricular systolic (ESPVR) and diastolic (EDPVR) function.

The slope of the ESPVR is the end-systolic elastance (Ees fig. 1.8) and it represents the efficiency of interactions of the intrinsic contractile elements of the myocardium (actin and myosin). The elastance is increased by positive inotropic agents and decreased in the presence of negative inotropes.(Physiology, 2011)

1.4.4.3 EDPVR

The EDPVR represents the PV relationship at a state of maximum ventricular relaxation and is an indicator of the hearts diastolic function.(Physiology, 2011) The relationship between the EDV and LVP is non-linear and its slope represents the stiffness or compliance (Fig. 1.8) of

the left ventricle with reduction in compliance which occurs in left ventricular hypertrophy, scarring after MI, and dilated cardiomyopathy being associated with high left ventricular filling pressures and diastolic dysfunction.(Grieve *et al.*, 2004) (Mirsky, 1984)

1.4.4.4 PVA

The PVA represents the total energy consumption during a cardiac cycle and it encompasses the energy consumption of the myocardium during non-contractile metabolism also known as the elastic potential energy (PE) and that utilised during mechanical contraction or stroke work (SW) as illustrated in figure 1.9.(Suga *et al.*, 1983)

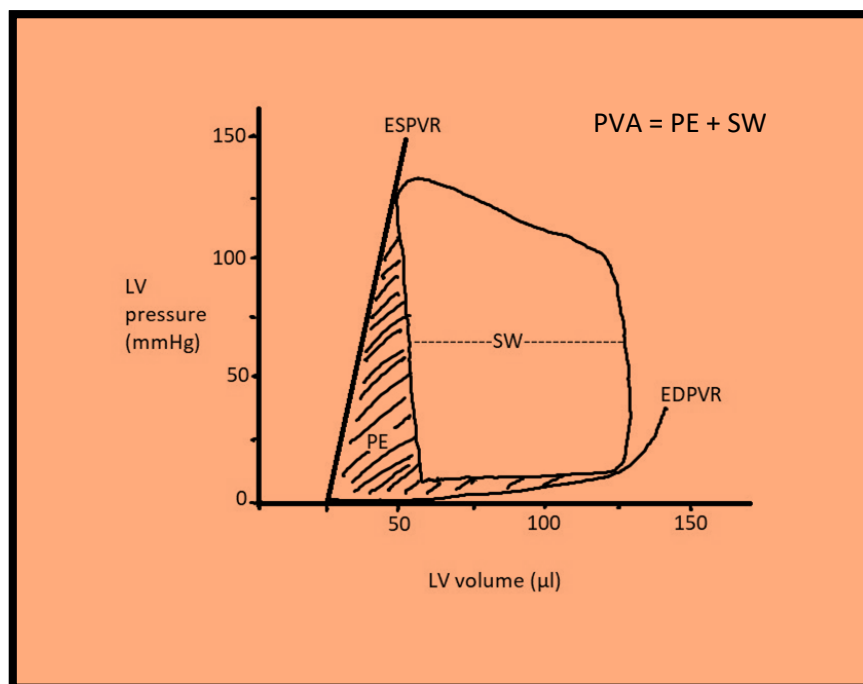


Figure 1.9: Schematic representation of the PV loop showing PE (shaded area) and SW (PV loop area)

1.4.4.5 PRSW

The PRSW is the slope of the linear relationship between the SW and EDV, and is a load-independent marker of myocardial contractility which is most sensitive to changes in systolic function. It is not affected by changes in heart rate or ventricular size making it of particular value in this study. (Ruetten *et al.*, 2005) (Berry *et al.*, 2004)

1.4.4.6 Tau

Tau is the isovolumetric diastolic time constant utilised to quantify the rate of ventricular relaxation and thus provides a measure of left ventricular diastolic function.(Asfour *et al.*, 1999) It is sensitive to changes in afterload and heart rate.

1.4.4.7 EF

The EF is a widely employed parameter in animal and human studies that is accepted as an easily reproducible index of ventricular contractility.(Physiology, 2011) It is obtained by echocardiography and is defined as the ratio between the stroke volume (EDV – ESV) and EDV. The EF represents the percentage of the EDV that is ejected with each heartbeat. Normal values in healthy hearts range from 55% to 65%.(Physiology, 2011) In this study in vivo assessment of cardiac function in the loaded rat hearts was achieved via echocardiographic determination of fractional shortening (FS) which was then converted to EF with the aid of a standardised formula.

1.5 AIMS and HYPOTHESES

The evidence presented here shows that increased mechanical load seen in heart failure has a wide ranging effect on the homeostasis of the heart that culminates in pathological remodelling. Evidence is also presented showing that mechanical unloading with LVADs is capable of bringing about regression of this remodelling with in some cases substantial improvement in myocardial function.

The effect mechanical unloading has on improving excitation-contraction coupling in the failing heart, particularly its effects on t-tubule ultrastructure and function, and Ca^{2+} homeostasis has been widely studied in this laboratory but more still needs to be understood. For instance, little is known of the effect mechanical unloading has on the activity of key molecular elements known to be pivotal for maintaining t-tubular structural integrity and function.

The deleterious effect mechanical unloading has on the extracellular matrix is widely reported in many contemporary studies but none have provided an explanation as to why myocardial fibrosis worsens with unloading. Evidence provided in this thesis highlights a well-documented inhibitory effect mechanical unloading has on myocardial microvasculature and CFR, however, no studies have directly compared the presence or absence of reperfusion of the parent coronary artery supplying an infarct territory on the long-term effects of mechanical unloading on the ECM.

Evidence presented in this thesis show that increase in mechanical load in the heart might be associated with the cardiomyocyte cell cycle arrest noted to occur in the first few days after birth. Shortly after birth, the heart undergoes transformation from hyperplastic growth to hypertrophic growth due in part to increases in haemodynamic wall stress with upregulation of molecular inhibitors of cardiomyocyte proliferation such as MEIS1 and pYAP. Furthermore,

it has also been shown that adequate coronary blood flow to the infarct territory facilitates cardiomyocyte proliferation. To date, no studies have investigated the effect ischaemia-reperfusion coupled with mechanical unloading has on the regenerative capacity of the adult heart.

There is thus sufficient evidence to suggest that the these three broad areas discussed above i.e. the excitation-contraction coupling, the extracellular matrix, and the endogenous proliferative capacity of the heart might contribute significantly to the recovery of myocardial function seen after mechanical unloading.

This thesis thus addresses the hypothesis that mechanical unloading of the failing heart brings about modifications of the myocardium that induce regression of pathological remodelling through modulation of excitation-contraction coupling, extracellular matrix homeostasis and alteration in the heart's endogenous proliferative capacity, and these effects culminate in improvement in whole-heart contractile function.

Specifically, the hypotheses addresses the concept that mechanical unloading of the failing heart:

1. Stimulates improvement in the expression and function of JPH2, BIN1 and Tcap.

This hypothesis was tested by completing cell immunohistochemistry on isolated cardiomyocytes from failing loaded and unloaded rat hearts to determine the expression profiles of JPH2, BIN1, and Tcap, and the regularity of their alignment along t-tubules with the aid of the Fourier transform. (Chapter 3)

2. Predisposes to an increase in myocardial fibrosis and infarct size but this deleterious effect of unloading is ameliorated in the presence of coronary reperfusion.

This hypothesis was tested by completing histological analysis on 15µm frozen tissue cryosections of loaded and unloaded, permanently ligated and ischaemia-reperfused rat hearts for the quantification of myocardial fibrosis and infarct size using Sirius red/Fast green collagen staining. Western blotting was also employed on these samples for the determination of the dynamic expression profiles of the ECM proteins TGF-β1, αSMA, MMP and TIMP. (Chapter 4)

3. Potentiates the proliferation of new cardiomyocytes by downregulating the expression of MEIS1 and pYAP

This hypothesis was tested by completing immunohistochemistry staining using the cellular marker of proliferation Ki67, cardiomyocyte marker troponin (cTnT), fibroblast marker vimentin, and the membrane marker wheat germ agglutinin (WGA). Immunohistochemistry was carried out on 15µm frozen tissue cryosections of loaded and unloaded, permanently ligated and ischaemia-reperfused rat hearts. Western blotting was also employed on these samples for the determination of the dynamic expression profiles of MEIS1 and pYAP. (Chapter 5)

4. Generates improvement in whole-heart contractility

This hypothesis was tested by determining whole-heart contractile function in a rat heart model of mechanical unloading using the ex vivo working/ejecting heart system to reload the heart in order to obtain physiological PV-loop haemodynamic parameters. (Chapter 6)

2 MATERIALS & METHODS

2.1 Animal Models

All animal procedures were performed in accordance with the Guide for the care and Use of Laboratory Animals (National Research Council 2011). In brief, animals were observed in the operating theatre for an hour following recovery from general anaesthesia at which point signs of pain or distress were acted upon in accordance with Home Office regulations. Animals were checked twice a day for the first 3 post-operative days during which analgesia and antibiotics were given. Daily weights were obtained for the first week after surgery to pick up subtle signs of failure to thrive. Once daily health checks continued thereafter.

Male syngeneic Lewis rats weighing 250-300g (purchased from Charles River, UK) were utilised to create models of myocardial infarction (MI), ischaemia-reperfusion (I/R), and mechanical unloading (U) via heterotopic abdominal heart & lung transplantation (HAHLT). Lewis rats are inbred and as such were chosen to avoid the need for immunosuppression after transplantation. They have also been shown to develop large infarct sizes, LV chamber dilatation, and progress to LV dysfunction with much lower mortalities more consistently following coronary artery ligation compared to several other strains of rat. (Liu *et al.*, 1997) These animals have also been extensively used in our laboratory thus facilitating standardisation. (Ibrahim, Navaratnarajah, *et al.*, 2012a) (Navaratnarajah *et al.*, 2013)

2.1.1 Myocardial Infarction Surgery & Ischaemia-Reperfusion

2.1.1.1 Permanent Coronary Artery Ligation

Permanent ligation of the Left Anterior Descending artery (LAD) in rats is a well-established technique employed to create animal models of myocardial infarction (MI) and subsequent heart failure secondary to ischaemic cardiomyopathy. (Goldman and Raya, 1995) (Liu *et al.*, 1997) The technique was first described in 1954 (Gross and Beck, 1954) and whilst other methods have been developed to create animal models of cardiac hypertrophy and heart failure, for clinical relevance, permanent coronary artery ligation in rats remains a very valuable tool as it closely mimics the pathological processes encountered in the human setting. The structural, functional, and biochemical changes that occur after coronary artery ligation in rats are very similar to those in humans. (Goldman and Raya, 1995) It has been observed that the pathophysiology of heart failure after significant myocardial infarction in both rats and humans occur in a similar fashion, with un-infarcted regions of the myocardium progressively deteriorating in function without the need for new or repeated ischaemic events. (Goldman and Raya, 1995) Rats are also much more affordable to manage and to study in large numbers compared to larger animals such as pigs.

In this study, the aim of carrying out permanent coronary artery ligation in Lewis rats is the development of severe heart failure to mimic the syndrome of chronic ischaemic cardiomyopathy observed in humans. Previous studies have shown that permanent coronary ligation to a left ventricular territory large enough to generate an infarct size of above 46% is required to facilitate the development of congestive heart failure. (Pfeffer *et al.*, 1979) Severe heart failure i.e. ejection fraction (EF) <40% develops in Lewis rats by 12 weeks post permanent LAD ligation. (Ibrahim, Navaratnarajah, *et al.*, 2012a) Echocardiography was

completed 1 week after coronary ligation and animals with an EF less than 50% were selected. Previous studies in the department have shown that animals with EF less than 50% 1 week after MI were more likely to develop severe heart failure at 12 weeks. (Navaratnarajah *et al.*, 2013) This technique whilst effective meant a large number of animals whose EF fell above the 50% mark at 1wk had to be culled. I observed that the reason for the significant disparity in the severity of myocardial infarction in the animals was mainly due to the distribution of blood flow to the interventricular septum. Whilst in humans the right coronary artery (RCA) only supplies blood to 33% of the septum, in rats the RCA supplies blood to 50% of the septum. (Ahmed *et al.*, 1978) This means only rats with a left dominant system (49.1%) where the septum was wholly supplied by the left coronary artery (LCA) developed significant heart failure after coronary ligation. I modified the current technique for MI surgery by including an additional ligature to the posterior descending artery (PDA). The PDA is the main branch of the RCA (in a right dominant system) or LCA (in a left dominant system) that provides blood supply to the septum. This modification led to 100% of animals developing significant heart failure at 12wks post procedure compared to LAD ligation alone. (Fig. 2.1)

detrimental effects on the injured heart that starts with the development of reversible functional impairment (myocardial stunning) to death of cardiac myocytes that were viable immediately before myocardial reperfusion (lethal reperfusion injury). (Hausenloy and Yellon, 2013a) (Wu, 2018)

Lethal reperfusion injury (LRI) has been shown to culminate in cardiomyocyte death and bring about an increase in infarct size. (Hausenloy and Yellon, 2013a) It is thought to account for the high rate of death of about 10% that occurs despite early intervention after an acute MI and the subsequent progression to heart failure seen in almost 25% of cases. (Keeley, Boura and Grines, 2003) The exact mechanism by which LRI culminates in cardiomyocyte cell death is not however fully understood. It remains unclear if LRI is an independent mediator of cardiomyocyte death, distinct from the preceding ischaemic injury, or simply worsens the ongoing cellular injury that occurs after an acute MI. (Kloner, 1993) None-the-less, several experimental studies have shown that interventions instituted early during myocardial reperfusion can significantly reduce myocardial infarct size and improve outcomes (Yellon and Baxter, 2000) although this has not been successfully reproduced in clinical trials. (Davidson *et al.*, 2019)

The operative steps were implemented as previously described. (Gross and Beck, 1954) In brief, syngeneic male Lewis rats were anaesthetised using the inhalation anaesthetic agent isoflurane at 3-5% for induction & 1.5-2.5% for maintenance. Oxygen flow was kept at 3L/min for induction and 1.5-2L/min for maintenance. The anaesthetic circuit used was the Bain's co-axial facemask. The animals were shaved from the neck to the groin once adequate anaesthesia was confirmed by loss of pedal reflexes. They were then placed supine and upright on a board. The tongue was retracted and epiglottis straightened using a rodent laryngoscope (Pioneer veterinary products, L4694) to aid access to the vocal cords. (Fig. 2.2)



Figure 2.2: Rat intubation

Intubation was achieved using a 16 G vascular cannula which is mounted on a blunt-tipped trocar needle. The trocar provides rigidity and is withdrawn following successful passage of the cannula through the vocal cords and into the trachea. The cannula was secured and connected to a volume controlled ventilator (Model 683 Rodent ventilator, Harvard 101Apparatus Ltd, UK) through a Y-tube thus permitting the delivery of inspiratory, and the removal of expiratory gasses. Synchronous chest wall movements with the ventilator provided confirmation of successful intubation and maintenance of anaesthesia was then achieved with isoflurane at 1.5-2% and oxygen flows of 1.5-2L/min. The tidal volume was kept at about 6-8ml/kg with a ventilatory rate of about 40breaths/min. The animal was placed supine on a draped heat mat with its outstretched limbs taped and its body temperature maintained at about 37⁰ C.

Heart rate and oxygen saturation were monitored throughout the procedure using a rodent SATs monitor connected to its foot. Once adequacy of anaesthesia was confirmed by loss of pedal reflexes, a left anterolateral incision was made over the fourth rib and a thoracotomy achieved by cutting through the intercostal muscles. The ribs were spread with a self-retaining retractor to expose the beating heart. The parietal pericardium was excised and the proximal LAD was ligated using 6/0 prolene sutures. Assessment of the apex of the heart was carried out to determine onset of the infarct. The heart was then lifted cephalad to gain access to the PDA which was also ligated using 6/0 prolene sutures. The lungs were inflated and the chest wall temporarily closed. Animals were then left on the ventilator for 90 mins. This was taken as sufficient time to allow transmural myocardial infarction to occur. In the permanently ligated group, the animal was recovered after 90 mins whilst in the ischaemia/reperfusion (I/R) subgroup, the chest wall was reopened after 90 mins and the ligatures to the proximal LAD and PDA were released to return blood flow to the infarct territory. The ribs and muscle layers were then re-approximated using 5/0 vicryl rapid sutures and skin closed using a subcuticular approach with 4/0 monocryl. Isoflurane is turned off and the animal allowed to wake whilst gradually lowering the minute ventilation to stimulate intrinsic ventilatory effort. The animal was then placed in the recovery chamber until fully recovered.

2.1.2 Echocardiography

Transthoracic echocardiography (TTE) was performed in the permanently ligated Lewis rats at 1 week and 12 weeks after MI surgery to quantify the extent of myocardial dysfunction. Only rats with significant LV dysfunction i.e. EF<40% at 12 weeks post MI and echocardiographic evidence of significant LV chamber dilatation were added to the study group.

TTE was performed under general anaesthesia with the rat lying supine with its limbs outstretched to gain access to the left chest wall. Induction of anaesthesia was achieved using isoflurane (5% induction and 1.0% maintenance), mixed with oxygen (1-2 L/min). Maintenance anaesthesia was generally set at 1.5% isoflurane and 1 L/min oxygen flow. A pulse oximeter was used to monitor heart rate and oxygen saturations during anaesthesia and these were maintained at about 200bpm and 98-100% respectively. Hair over the left axilla and anterior chest wall was shaved and ultrasound transmission gel (Sonogel®, AnaWiz Ltd, UK) was applied on the chest wall to optimise the acoustic signal. A 14 MHz phased-array, transducer (15L8-S, Siemens® Medical Solutions, USA) was then used to obtain 2D parasternal short axis views of the LV at the mid-papillary level. On M-mode view, measurements of LV internal diameter in diastole (LVIDd, Fig. 2.3b), and systole (LVIDs, Fig. 2.3c) were obtained and the difference between them used to determine chamber size.

LV fractional shortening and ejection fraction were obtained using the following formulas $(LVIDd-LVIDs/LVIDd)$ and $(LVIDd^3-LVIDs^3/LVIDd^3)$ respectively.

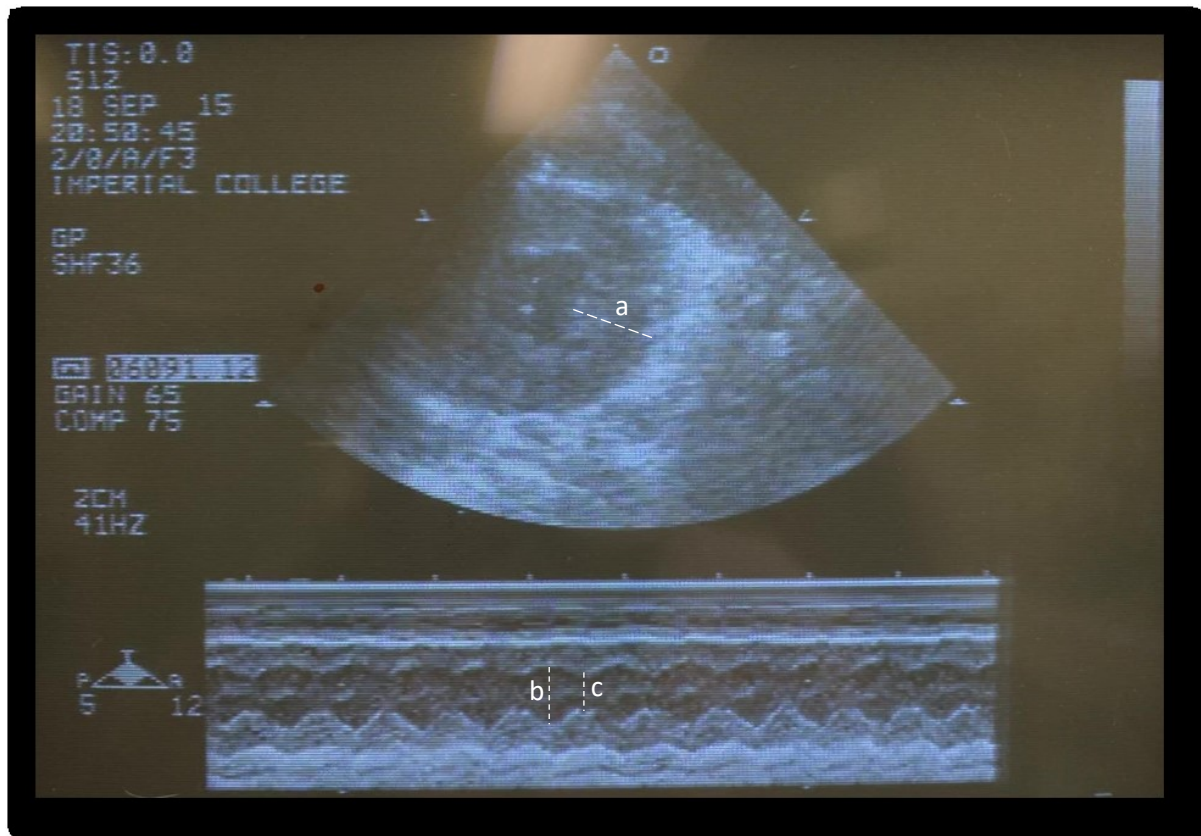


Figure 2.3: M-mode (short-axis view) image of a healthy rat heart (CTR) showing normal myocardial wall thickness (a) and fractional shortening (b/c).

The parasternal short axis view obtained at papillary muscle level highlights normal LV myocardial wall thickness (Fig. 2.3a) in the control heart but this is significantly reduced in the infarcted heart leading to chamber cavity enlargement and heart failure (Fig. 2.4).

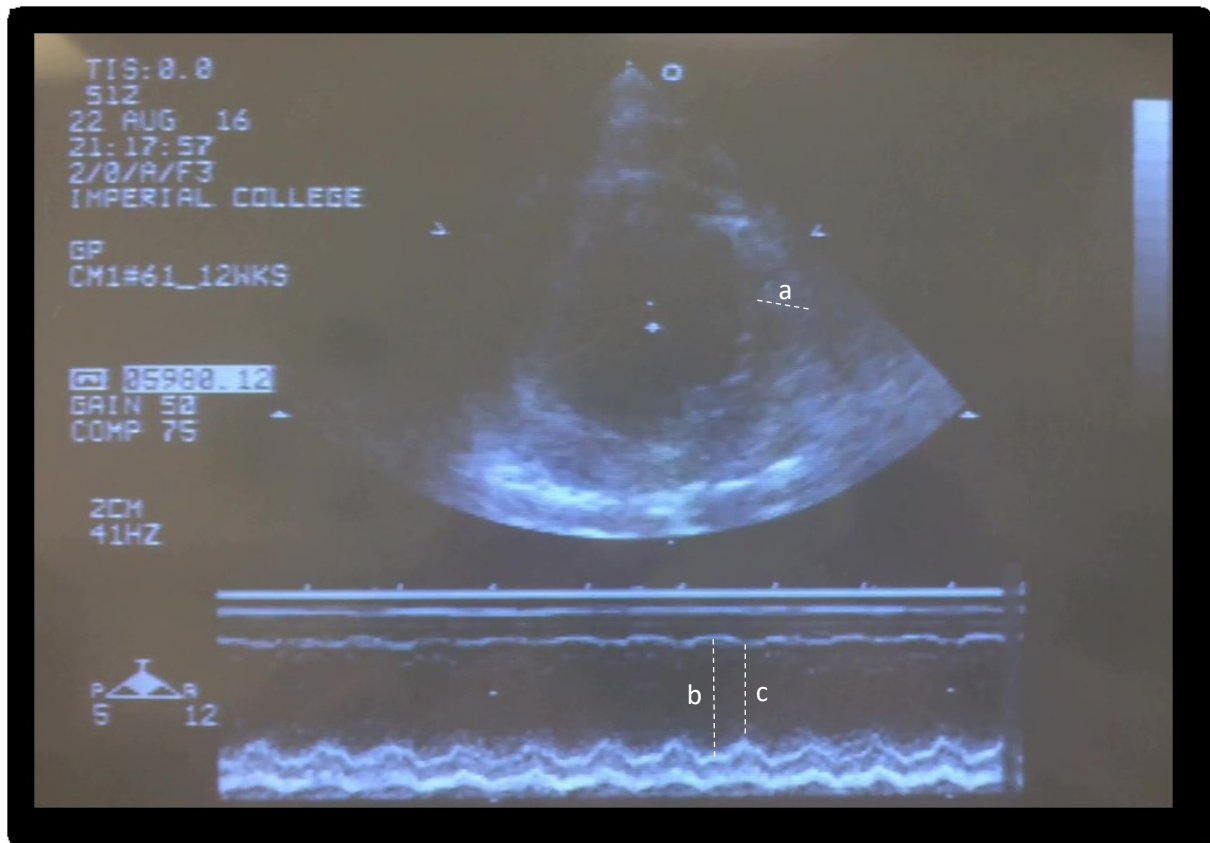


Figure 2.4: M-mode (short-axis view) image of a failing rat heart at 12 weeks showing reduced wall thickness (a), enlarged LV chamber size (b-c) and impaired fractional shortening (b/c).

Loss of wall thickness was most pronounced in the anterolateral aspect of the left ventricle (Fig. 2.4a), the territory supplied by the ligated coronary artery.

2.1.3 Heterotopic Abdominal Heart & Lung Transplantation

Heterotopic Abdominal Heart & Lung Transplantation (HAHLT) in rats is a well-established technique that had been initially employed to facilitate studies in cardiac transplant immunology. (Abbott *et al.*, 1964) (Ono and Lindsey, 1969) (Minanov *et al.*, 1997) It involves anastomosing the heart and lung en bloc from a donor onto the abdominal aorta of a healthy recipient. This model allows a much less profound or partial unloading of the LV to be

achieved. Only a single anastomosis of the ascending aorta of the donor heart to the abdominal aorta of the recipient is completed. As the inferior vena cava (IVC) and superior vena cava (SVC) are ligated and the pulmonary circulation remains intact, the LV receives additional blood from the right ventricle (RV), via the pulmonary veins after passing through the lungs as illustrated in figure 2.5.

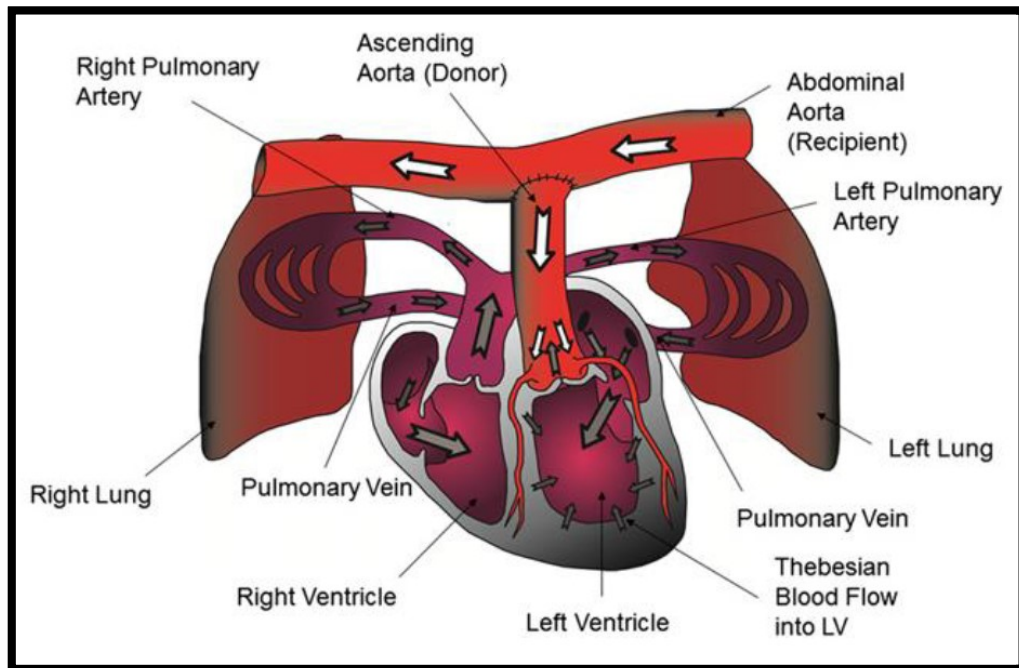


Figure 2.5: Schematic representation of HAHLT. In this preparation the donor heart and lung en bloc is transplanted by anastomosing the ascending aorta of the donor to the abdominal aorta of the recipient. The LV is only partially unloaded here because the venous return from the coronary sinus travels through the lungs and empties into the left atrium and eventually the LV. Figure reproduced from Ibrahim 2012.

The HAHLT model contrasts to the fully unloaded rodent heart model, the Heterotopic Abdominal Heart Transplant (HAHT), where both the ascending aorta and the pulmonary artery of the donor are anastomosed to the abdominal aorta and IVC of the recipient respectively (figure 2.6). Partial unloading of the heart is more in line with current LVAD use and is less likely to bring about significant myocardial atrophy. (Estep et al. 2003; Tang-Quan et al. 2010; Wang et al. 2008).

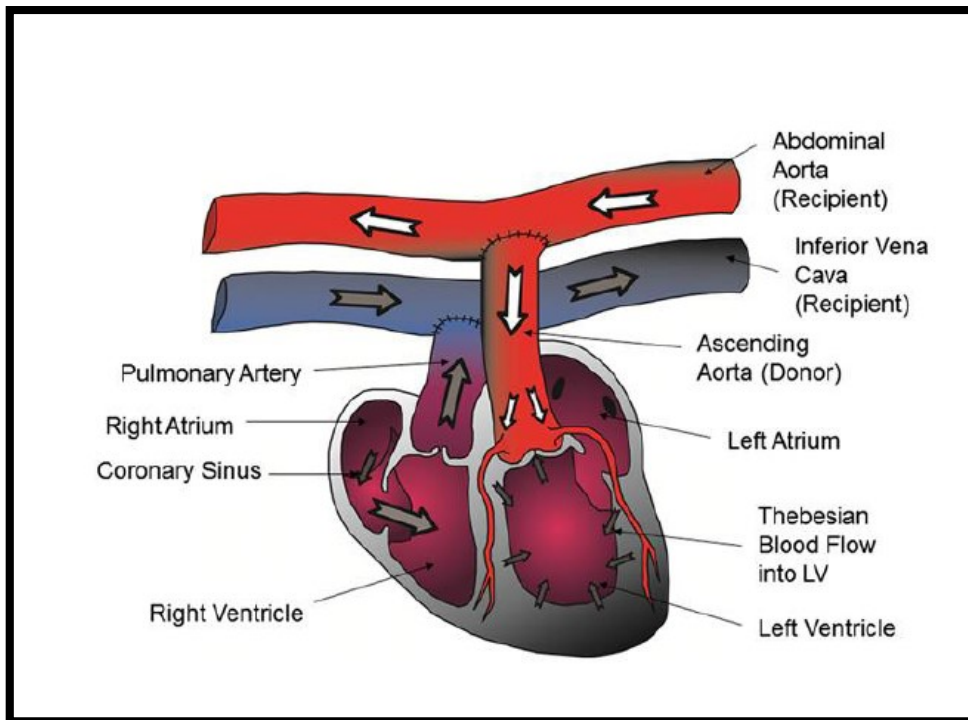


Figure 2.6: Schematic representation of HAHT. In this preparation the donor heart alone is transplanted. The lungs are excised and the pulmonary veins ligated. Transplantation is completed by anastomosing the ascending aorta of the donor to the abdominal aorta of the recipient. The pulmonary artery is then anastomosed to the IVC. The LV is fully unloaded here because the venous return from the coronary sinus empties into the IVC. Figure reproduced from Ibrahim 2012.

The use of the HAHLT model as a tool to provide mechanical unloading of hearts from rat models of heart failure has allowed significant strides to be made in the understanding of the pathophysiological basis of heart failure and reverse remodelling. (Ibrahim, Terracciano and Yacoub, 2012) This technique has been used in this laboratory for several years. (Ibrahim, Navaratnarajah, *et al.*, 2013)

In this study, HAHLT was employed to facilitate the investigation of the underlying mechanism responsible for mechanical unloading-induced reverse remodelling with particular emphasis on the effect of unloading on the molecular elements known to be key to efficient EC coupling, the extracellular matrix and the adult heart's endogenous proliferative capacity after MI. Mechanical unloading takes away a substantial workload from the heart and as a result brings about a reduction in myocardial oxygen consumption ($MV\dot{O}_2$). Since the sequelae of

acute coronary obstruction is a consequence of the mismatch between myocardial oxygen supply and demand it is unsurprising that a number of studies have shown an association between mechanical unloading after MI and a reduction in infarct size and interstitial fibrosis. (Bruckner *et al.*, 2001) (Maybaum *et al.*, 2007) (Thohan *et al.*, 2005) However, this finding remains controversial as other studies have reported no improvement or in fact a worsening of myocardial fibrosis after mechanical unloading. (Klotz *et al.*, 2005c) (Patel *et al.*, 2011) (Drakos *et al.*, 2010) The reason for this dichotomy remains unknown. Mechanical unloading has however been associated with a reduction in microvascular luminal diameter in the failing heart, thus suggesting a link with negative auto-regulatory effects on the collateral circulation. (Drakos *et al.*, 2010) This finding implies that impairment in coronary blood flow to the infarct region might contribute to the worsening fibrosis that occurs after mechanical unloading. The potential cardioprotective effect of mechanically unloading on the injured heart early during myocardial reperfusion has been explored although most of these studies mainly investigated the short term effects (mins to hours) of mechanical unloading after I/R on infarct size. (Cave, Kingma and Hearse, 1992) (Smalling *et al.*, 1992) (Achour *et al.*, 2005) (Kapur *et al.*, 2013) (Kapur *et al.*, 2015) (Saku *et al.*, 2016) To the best of our knowledge this is the first study to investigate long-term effects (days to weeks) of mechanical unloading after I/R on infarct size.

The operative steps are as previously described. (Ibrahim, Navaratnarajah, *et al.*, 2013) Two syngeneic male Lewis rats were utilised to avoid graft rejection. Anaesthesia was achieved using the inhalation anaesthetic agent isoflurane at 3-5% for induction & 1.5-2.5% for maintenance. Oxygen flow was kept at 3L/min for induction and 1.5-2L/min for maintenance. The anaesthetic circuit used was the Bain's co-axial facemask. The animals were shaved from the neck to the groin once adequate anaesthesia was confirmed by assessing pedal responses. They were then placed supine on a draped heat mat with outstretched limbs taped and body temperature maintained at about 37⁰ C. The animals were prepped with povidone iodine and

draped. Intraoperative pain relief consisted of a single subcutaneous dose of vetergesic (buprenorphine) at 0.12mg/kg. Adequate hydration was achieved by giving 1ml bolus of warm saline subcutaneously every hour. (Fig 2.7)

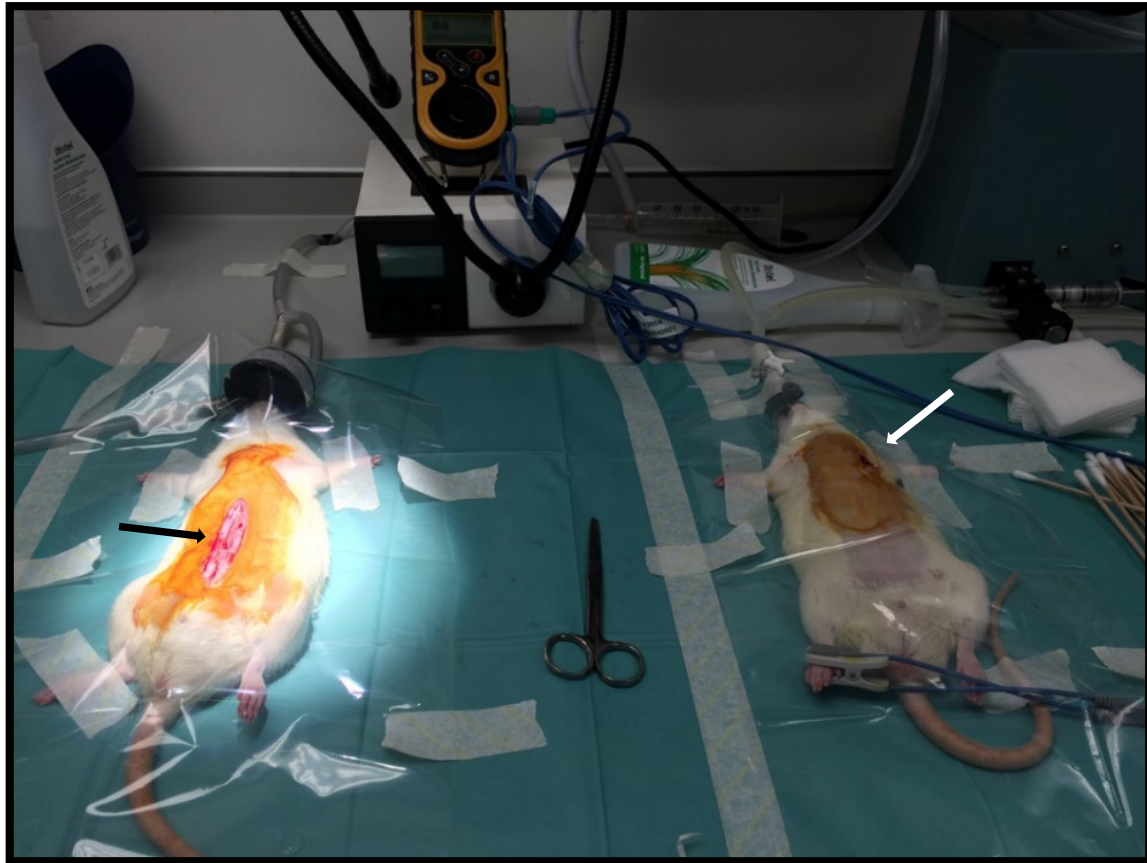


Figure 2.7: Two Lewis rats being prepared for HALHT. The rat on the right has had 90 mins of LAD and PDA ligation via a left mini thoracotomy (white arrow). The rat on the left was the recipient with a midline laparotomy (black arrow) being initiated.

The recipients' abdominal aorta was accessed via a midline laparotomy incision using size 22 blade and its intestine was placed in a saline soaked gauze swab. A sufficient length for anastomosis was exposed on the abdominal aorta using blunt dissection and blood flow was blocked above and below this region using two 3cm titanium atraumatic bulldog artery Clamps (Harvard Apparatus, 81314230). The heat mat was switched off to reduce the risk of spinal cord ischemic. With the aid of a microscope, a 0.5cm midline longitudinal aortotomy was carried out with a size 11 blade. The donor abdominal aorta was then accessed and blood flow

blocked as above. Adequate heparinisation was achieved by injecting heparin at 10000 IU/Kg into the IVC using a 30G needle and pausing for 2 minutes. The donor abdominal aorta was then cannulated with a 20-gauge arterial line (Leadercath arterial, Vygon UK Ltd, FSQ049) using a catheter-over-wire technique. The donor heart was arrested in diastole by injecting 50mls of cold St Thomas II cardioplegia solution. The IVC was simultaneously transected to prevent overload. The sternum was cut open with a large scissors and the heart explanted after ligating the IVC and SVC using 4/0 Mersilk sutures. Figures 2.8 & 2.9 shows the permanently ligated heart and the I/R hearts at the point of harvest for transplantation. Note that after infusing cardioplegia the infarct territory can be seen clearly in the permanently ligated heart which is absent in the I/R hearts.

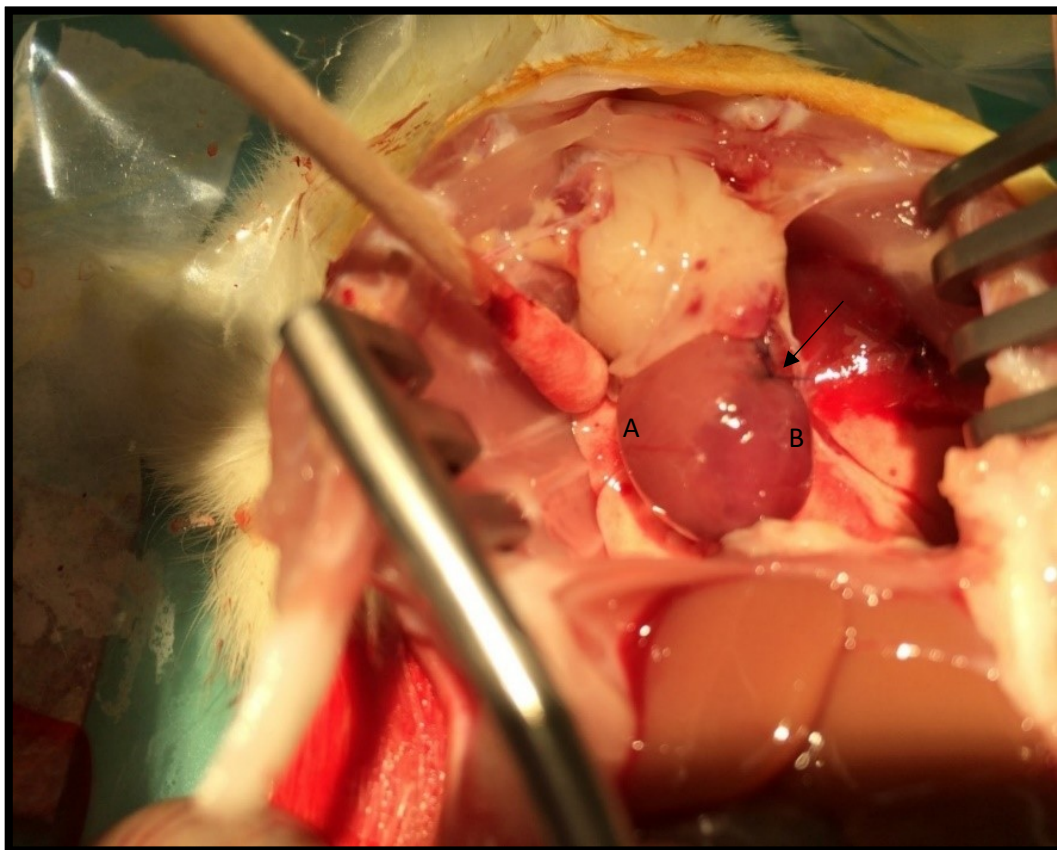


Figure 2.8: Thoracic cavity of the rat opened revealing the permanently ligated heart after infusion of St Thomas II cardioplegia solution showing blockage of flow to the infarct region (B). The arrow is pointing at the LAD ligature. A swab stick is used to expose areas of the heart blanching by the infused cardioplegia (A) thus providing a clear indication of the region with an occluded coronary circulation (B).

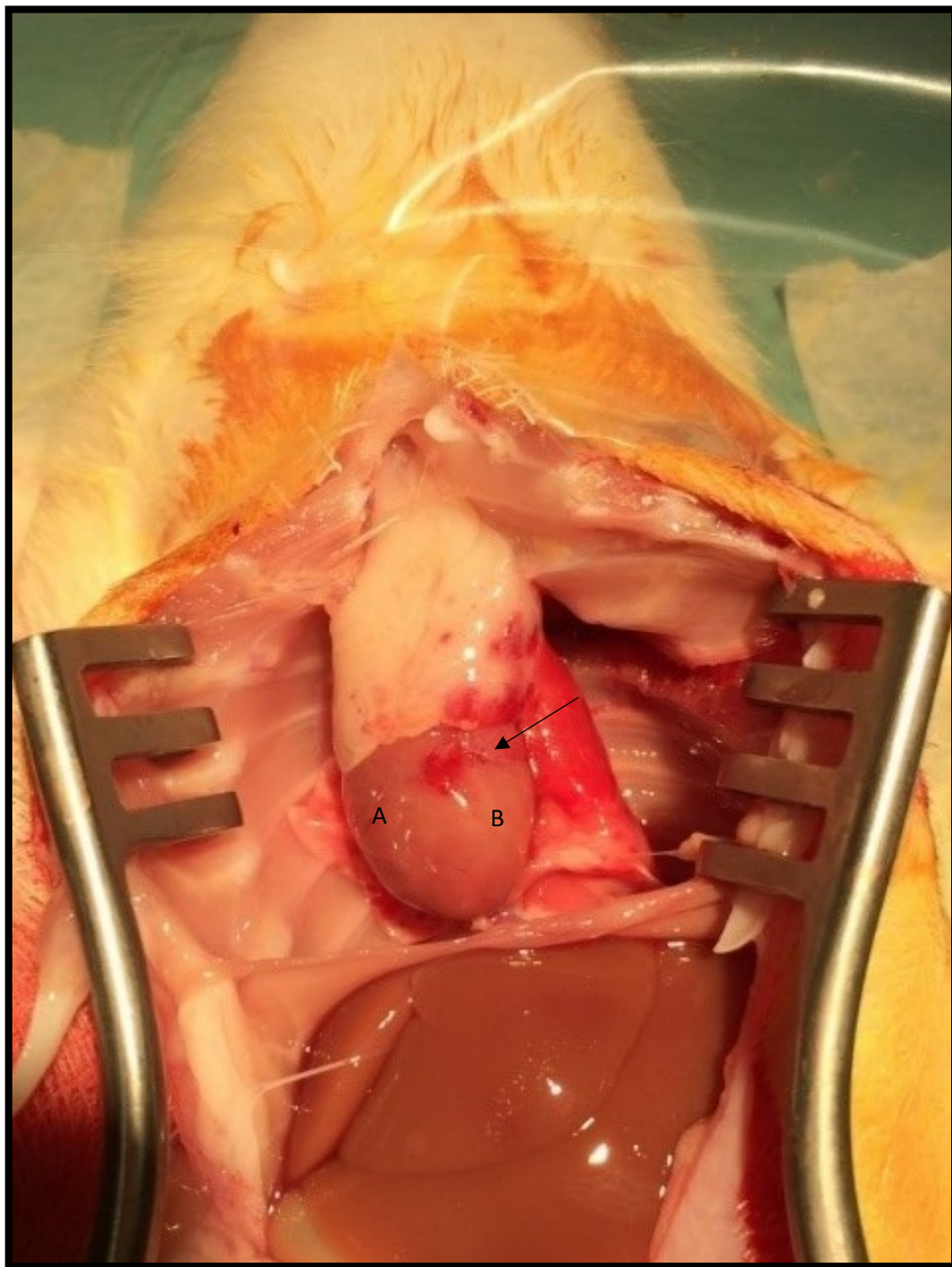


Figure 2.9: Ischaemia/Reperfusion heart after infusion of St Thomas II cardioplegia solution showing return of flow to the infarct region. There is now no clear demarcation between regions (A) and (B) indicating return of coronary flow through the LAD after removing the ligature (arrow).

The donor aorta was cut into shape with microscissors and anastomosed to the recipient's abdominal aorta using 8/0 prolene sutures. The bulldog clamps were removed at completion and haemostasis achieved. The donor heart beat returned after a few seconds. The heat mat was switched back on, the abdominal contents returned and the anterior abdominal wall muscle was closed with 5/0 vicryl sutures. A subcuticular skin closure was achieved using 4/0 monocryl sutures. Post-operative analgesia was achieved by injecting carprofen (rimadyl) subcutaneously at 0.5mg/kg at the end of the operation and once daily for two days. Antibiotic cover consisted of enrofloxacin given subcutaneously at 1mg/kg at the end of the operation and daily for two days. The animal was then placed in the recovery chamber until fully recovered after which it was returned to its housing unit.

2.1.4 Experimental groups and Operative Mortality

In this study, healthy control (CTR) Lewis rat hearts were either loaded (CTR-L) or mechanically unloaded (CTR-U). Animal models of MI were divided into 8 groups:

- a. Acute MI Loaded (AMI-L) – here permanently ligated hearts were explanted for analysis 2 or 7 days after coronary ligation.
- b. Acute MI Reperfusion Loaded (AMI/R-L) – here I/R hearts were explanted for analysis 2 or 7 days after coronary ligation.
- c. Acute MI Unloaded (AMI-U) – here permanently ligated hearts were explanted 90 minutes after coronary ligation and mechanically unloaded via HAHLT. These hearts were then explanted for analysis 2 or 7 days after transplantation.
- d. Acute MI Reperfusion Unloaded (AMI/R-U) – here I/R hearts were explanted 90 minutes after coronary ligation and mechanically unloaded via HAHLT. These hearts were then explanted for analysis 2 or 7 days after transplantation.

- e. Chronic MI Loaded (CMI-L) – here permanently ligated hearts were explanted for analysis 16 or 20 weeks after coronary ligation provided they had an EF less than 40% at 12 weeks.
- f. Chronic MI Reperfusion Loaded (CMI/R-L) – here I/R hearts were explanted for analysis 16 weeks after coronary ligation. An EF less than 40% was not a requirement in the I/R group.
- g. Chronic MI Unloaded (CMI-U) – here permanently ligated hearts were explanted at 12 weeks if their EF was less than 40%, and mechanically unloaded via HAHLT. These hearts were then explanted for analysis 4 or 8 weeks after transplantation.
- h. Chronic MI Reperfusion Unloaded (CMI/R-U) – here I/R hearts were explanted at 12 weeks and mechanically unloaded via HAHLT. These hearts were then explanted for analysis 4 weeks after transplantation.

In total, 128 coronary artery ligations were completed with an operative mortality of 18.75%. 95 HAHLT were carried out with a mortality of 21%. The operative mortality in the transplant group was significantly affected by a viral outbreak in the laboratory.

2.1.5 Heart harvest and preparation

At the respective time points (days 2 and 7, & 16 and 20 weeks) animals were weighed and adequately anaesthetised. For the loaded infarcted heart harvest, the animal was sacrificed using schedule 1 cervical dislocation. The chest wall was cut open rapidly and the heart explanted and inserted into cold heparinised St Thomas II cardioplegia solution. For the unloaded heart harvest, the recipient's abdominal aorta was clamped above and below the point of anastomosis to the donor aorta using bulldog clamps. The unloaded heart was then removed

and rapidly inserted into cold St Thomas II cardioplegia solution. The recipient's sternum was then cut open and its heart removed and also rapidly arrested in cardioplegia solution.

For most preparations, apart from the cell isolation and working heart experiments (both described below), the lungs and atria were excised after heart explantation and the ventricle weighed. The ventricular weight (VW) to body weight (BW) ratio (VW:BW) for each heart was recorded.

The ventricle was placed in an acrylic zivic rat heart slicer matrix with 2mm coronal section slice intervals (Zivic instruments, HSRA002-1) and sliced into 5 coronal segments A-E. (Fig. 2.10)

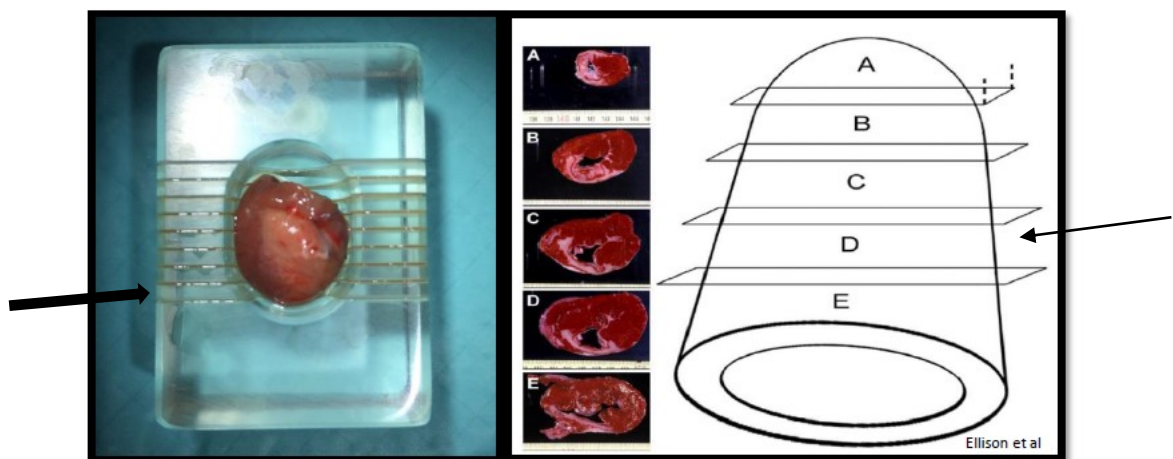


Figure 2.10: Zivic slicer (thick arrow) provides 2mm slices of heart tissue A-E (thin arrow). The atria of the explanted heart was resected at the atrioventricular septum, ventricular weight determined, and 2mm ventricular slices were obtained for analysis.

Whilst still submerged in cold St Thomas II cardioplegia solution, the left ventricular aspect of segments C & E were each further divided into their respective chambers: anterior, posterior, and septal to facilitate regional assessment of protein expression. The tissues were then snap frozen in liquid nitrogen and stored in -80°C for protein analysis. Segments A, B & D were

frozen in the Optimal Cutting Temperature compound (OCT) and also stored in -80°C for histological analysis and immunohistochemistry.

2.2 Laboratory techniques & Experiments

2.2.1 Frozen tissue & Cryosections

Each heart segment A, B, and D was cleaned in cardioplegia solution and submerged flat in the base of a 5ml sterilin tube (ThermoFisher scientific, Z5PS) containing 1ml of OCT compound. The tissue was then snap frozen in isopentane mixed with dry ice and stored in -80°C ready for sectioning.

15 μm cryosections were obtained using a cryostat at proper operating temperature of -20°C to -30°C . The frozen OCT embedded tissue was mounted onto a cryostat object disk. Care was taken to make sure the disk was at room temperature and smeared with some OCT before the specimen was mounted. The disk was then placed on the cryobar in the cryostat and rapidly began to freeze. Once adequately frozen the disk was inserted into the microtome object disk holder and held firmly in place by a screw. By turning the flywheel on the cryostat, the specimen was gradually brought towards the mounted microtome knife to obtain 15 μm thin sections. The first few sections were discarded until a full cross-section of the sample was obtained. These were then mounted onto glass slides (SuperFrost Plus, Thermo Scientific, 630-0954), 3 sections per slide and stored in -80°C ready for immunohistochemistry or histological analysis. To facilitate consistent, good quality cryosections, this aspect was carried out by an experienced member of the team (Dr Charanjit Singh).

2.2.2 Immunohistochemistry of frozen tissue sections for the analysis of cardiomyocyte proliferation

The immunohistochemistry staining technique was applied on frozen tissue sections to facilitate regional assessment of cardiomyocyte proliferation. Ki67, a cellular marker for proliferation that is strictly associated with cell proliferation was used in addition to the cardiomyocyte marker cTnT, the fibroblast marker vimentin, and the membrane marker WGA. Primary antibody specificity was determined using secondary antibody only control. The procedure was completed using a standardised protocol. In brief, glass slides containing 15µm tissue sections were submerged into pre-cooled methanol (-20°C) for 5 mins. Afterwards, 1ml of 1.5% triton in 10mM phosphate buffered saline (PBS) was spread over each glass slide to permeabilise the tissue for 20 mins. During this time, blocking solution made up of 1% bovine serum albumin (BSA) and 0.1% triton both in PBS was freshly prepared. After 20 mins of permeabilization the tissue section was submerged in blocking solution, covered in foil and kept in the fridge at 4°C for 1 hour. The slides were then drained of fluid and a circular hydrophobic barrier was drawn around the tissue using a PAP pen (Sigma-Aldrich, Z377821-1EA). The tissue sections were kept overnight at 4°C after introducing the following primary antibodies: rabbit anti-Ki67 (1:300, Abcam, ab16667), mouse anti-cardiac troponin (1:300, ThermoFisher, MA512960), and chicken anti-vimentin (1:3000, ThermoScientific, PA1-10003).

After a minimum of 12 hours the tissues were washed with PBS 2x for 15 mins each and the following secondary antibodies from ThermoFisher were introduced at a dilution of 1:1000: Alexa 488 donkey anti-rabbit (A21206), Alexa 546 donkey anti-mouse (A10036), Alexa 568 goat anti-chicken (A11041) and Alexa Fluor 555 conjugate (wheat germ agglutinin) (1:100 ThermoFisher, W32464). The sections were kept at 4°C covered in foil for 1 hour. This was

followed by 3 washes in PBS for 15 mins each. Finally, the tissue sections were covered with the nuclei stain Hoechst 33342 (1:1000, Thermoscientific, H3570) for 10 mins at 4⁰C, washed 2x with PBS for 15 mins each, and with the aid of 50% glycerol in PBS as mounting media, borosilicate rectangular glass coverslips (22mm x 40mm, VWR, 631-0138) were placed over the tissue and secured around the edges with nail varnish. Prepared samples were then kept at 4⁰C ready for imaging.

2.2.3 Histological analysis of frozen tissue sections for the quantification of myocardial fibrosis and infarct size

The 15µm cryosections prepared as described above were also processed with histological stains to assess the degree of fibrosis and infarct size in the rat models of MI. Sirius red/Fast green collagen staining was employed using a standardised protocol. In brief, the glass slides containing tissue sections are taken from -80⁰C storage and hydrated in ethanol gradations i.e. 100% ethanol, 70% ethanol, and distilled water each for 20s duration. The Sirius red/Fast green solution was filtered to remove debris and the slides were submerged in solution for 35 mins, agitating 2-3x every 10 mins. The stained slides were then washed in 100% ethanol and again agitated 2x every 20s. Under the fume cupboard, the slides were immersed in two separate xylene containing troughs, each immersion lasting about 5 mins to 10 mins to eliminate glass-like appearance on the slides. Using a DPX mountant (Sigma-Aldrich, 06522-100ML), coverslips were carefully applied to avoid air bubbles. Slides were allowed to dry overnight at room temperature in preparation for imaging.

2.2.4 Cardiomyocyte isolation and fixation

Heart-lung blocs were collected and cardiomyocytes isolated by an experienced cell isolator in our departmental (Peter O’Gara) using a well-established protocol. (Cardiology and Republic, 1983) Cells were plated and fixed using a standardised protocol. In brief, 13mm round borosilicate glass coverslips (VWR, 631-0150) were dipped in 100% ethanol, heat dried and placed in Falcon polystyrene 12 well plates (VWR, 10062-894). To facilitate cell binding about 25ml/well of the extracellular matrix protein laminin (2µg/ml, Cultrex, 3446-005-01) was spread evenly over the coverslips and allowed to dry for 20 mins. 200µl of the isolated cell solution was then placed on each coverslip and stored in the culture cupboard for 1 hour. Fixation was completed by draining excess cell solution and adding freshly prepared 1ml of 4% paraformaldehyde (Honeywell, F1635-500ML) to each well and left for 10 mins. This was followed by 3 washes with PBS 2 mins apart. The cells were then stored at 4⁰C submerged in PBS and ready for immunohistochemistry.

2.2.5 Cell immunohistochemistry for the analysis of t-tubular structural proteins & MEIS1

Immunohistochemistry was carried out on isolated cardiomyocytes to determine the expression profiles of specific proteins identified as key molecular elements necessary for maintaining the structural integrity of t-tubules. These proteins include: BIN-1, Junctophilin 2 (JPH-2), and Tcap. The specific expression profile of MEIS1 in loaded and unloaded cardiomyocytes was also investigated. Primary antibody specificity was determined using secondary antibody only control and immunoblot primary antibody control. Immunostaining was completed using a standardised protocol. In brief, fixed cells were permeabilised with 0.5% triton X-100 in PBS

for 15 mins at room temperature. Following 1x wash with PBS, the cells were blocked by adding freshly prepared 10% fetal calf serum (FCS) diluted 10x in PBS into the 12 well plate and leaving for 30 mins at room temperature. The coverslips were then taken out of the wells and the cell side placed in contact with 50µl of each respective primary antibody and stored at 4°C overnight. The following primary antibodies from Santa Cruz were used: monoclonal mouse anti-BIN1 (sc-30099), rabbit anti-JPH2 (sc-51313), mouse anti-Tcap (sc-8726), and goat anti-Meis1 (sc-10599). The cells were washed in PBS the next day and the following secondary antibodies (ThermoFisher, 1:500) were applied for 1 hour: Alexa 488 donkey anti mouse (A21202), Alexa 546 donkey anti rabbit (A10040), Alexa 488 donkey anti goat (A11055). The coverslips are then washed in PBS and distilled water and mounted onto glass slides with the aid of the mounting medium vectashield hardset (Vector labs, H-1500). The stained cells are stored at 4°C ready for imaging. Cell analysis was completed using ImageJ and MATLAB software.

2.2.6 Western Blotting

Western blotting technique was used to determine the expression profiles of a number of key proteins such as: proteins that play significant roles in the regulation of cardiomyocyte regeneration e.g. MEIS1 and YAP, and proteins involved in modulating the extracellular matrix e.g. TGF-β1, αSMA, MMP and TIMP.

2.2.6.1 Rat heart harvest, tissue homogenisation & protein quantification

Hearts were collected as described in 2.1.3 (heart harvest & preparation). Tissue from the anterior and posterior wall of the left ventricle (snap frozen in liquid nitrogen) were ground

into fine particles, weighed and homogenised in 2x lysis buffer (Cell Signaling Technology, 9803S) at 1ml per 100mg of tissue. The composition of 1ml of 2x CST lysis buffer was as follows: 200µl 10x lysis buffer, 664µl DNase/RNase free distilled water (Gibco by life technologies, 10977-035), 100µl 10% Sodium Dodecyl Sulfate (SDS) (ThermoFisher, 24730020), 10µl Phosphatase inhibitor 2 (Sigma-Aldrich, P5726), 10µl Phosphatase inhibitor 3 (Sigma-Aldrich, P0044), 10µl Protease inhibitor cocktail (Cell Signaling Technology, cat no. 5871S), 5µl Phenylmethylsulfonyl Fluoride (PMSF) (Cell Signaling Technology, 5871S), and 1µl Dithiothreitol (DTT) (Sigma-Aldrich, 43816-10ML).

The tissue homogenate was centrifuged at 14000rpm for 10mins at 4⁰C and the supernatant stored in -80⁰C. With the aid of bicinchoninic acid assay (BCA assay) (ThermoFisher, cat no. 23227), protein quantification from the supernatant was determined against bovine albumin via a standard curve.

2.2.6.2 Gel preparation, electrophoresis, transfer, & primary antibody application

Two 17-well, 1.0mm, NuPAGE 4-12% Bis-Tris Protein Gels (ThermoFisher, NP0329BOX) were mounted onto the electrophoresis holder and immersed in the Mini Gel Tank (ThermoFisher, A25977) containing 500ml of running buffer. The running buffer is made up of 25ml 20x NuPAGE MOPS SDS running buffer (ThermoFisher, B0001) and 475ml Milli-Q water. Protein samples (respective concentration determined using a standard curve as described in 2.2.6.1) were loaded into 15 inner wells at 10µl per well whilst 10µl color prestained protein standard (11-245kDa, BioLabs, P7712S) was added to the first and last wells. The gel tank was covered and connected to a power source (Bio-Rad, PowerPac300, 164-5050) with an electric current at 200V passed for 30 min. Once the proteins had travelled

down the gel, the gel was then transferred onto an Immun-blot polyvinylidene (PVDF) membrane (Bio-Rad, 162-0174) and sandwiched in a Mini Blot Module (ThermoFisher, B1000). This is then immersed in the Mini Gel Tank after adding about 500ml of transfer buffer. The transfer buffer is composed of 25ml 20x NuPAGE transfer buffer (ThermoFisher, BT00061) and 475ml Milli-Q water. Transfer was completed by connecting to a power source and passing electric current through the tank at 20V for 2hr.

The membrane was blocked in 5% milk (Marvel original dried skimmed milk) for 1hr and immersed in primary antibody overnight at 4°C. Primary antibodies used are as follows: goat anti-Meis1 (1:100, Santa Cruz, sc-10599), rabbit anti-phospho-YAP (1:1000, Cell Signaling Technology, 13008s), rabbit anti-total-YAP(1:1000, Cell signalling technology, 4912s), rabbit anti-TGF-β1 (1:200, Santa Cruz, sc-31609), mouse anti-αSMA (1:1000, Dako, M0851), rabbit anti-TIMP1 (1:1000, Santa, Cruz, sc-5538), and rabbit anti-MMP1 (1:1000, Proteintech, 10371-1-AP).

2.2.6.3 Secondary antibody application, washes and development

The next day, the membrane was washed in TBS-Tween 3x for 10 mins each and immersed in secondary antibody for 1hr at room temperature. Secondary antibodies (Santa Cruz) were as follows: rabbit anti-goat IgG-HRP for Meis1 (1:500, sc-2768), goat anti-rabbit IgG-HRP for YAP, TIMP1 & MMP1 (1:2000, sc-2030), mouse anti-rabbit IgG-HRP for TGF-β1 (1:500, sc-2357), goat anti-mouse IgG-HRP for αSMA (1:2000, sc-2005).

Membranes were then washed in TBS-Tween for 30 mins (3x for 10 mins each) and a further three washes this time in TBS for 10 mins each was completed. Membrane development was carried out using electrochemiluminescence (ECL) (gelifesciences, RPN2235) and Syngene

G:BOX chemiluminescence imaging device. The data was then analysed using imageJ software.

2.2.7 The Ex vivo Rodent Working Heart and PV Loop System

The working heart model, a modification of the Langendorff isolated perfused heart system is designed to permit physiological evaluation of the functional status of the heart ex vivo. (Neely *et al.*, 1967) (Manuscript and Transfer, 2012) (Grieve *et al.*, 2004) The technique has been employed in several large animal models but to the best of our knowledge this is the first report of the working heart being used to investigate changes in myocardial function in a rat model of myocardial infarction and mechanical unloading.

2.2.7.1 Working heart setup

The working heart setup is illustrated in figure 2.11. It includes a 2L capacity reservoir (A) with an oxygen delivery pole in situ which allows vigorous gassing of the perfusate i.e. Krebs-Henseleit (KH) solution with combined 95% O₂ and 5% CO₂ to obtain a PO₂ of about 66 kPa. KH solution consists of (in mM) 119.1 NaCl, 4.7 KCl, 25 NaHCO₃, 0.94 MgSO₄, 1.2 KH₂PO₄, and 11.5 Glucose. The oxygenated perfusate was then circulated, with the aid of a peristaltic pump (B, Radnoti Masterflex, 170100) through a warm jacketed circuit (Radnoti Thermal Circulator pump, 170051G) to maintain the temperature of the perfusate at 37-38.5⁰C. The perfusate was pumped through a filter and two bubble-traps (C) before reaching the aortic

cannula (D) in Langendorff mode or the left atrial cannula (E) in working heart mode. (Fig. 2.11)

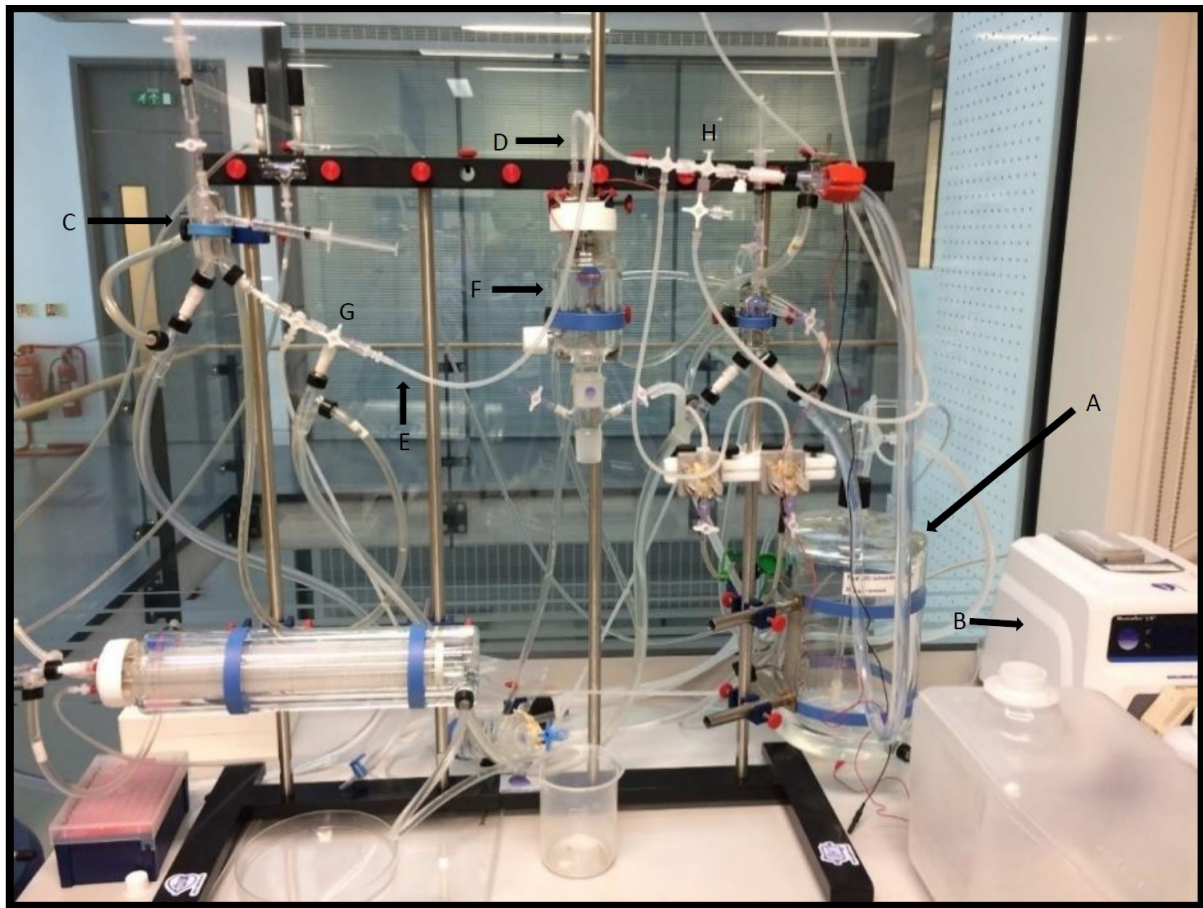


Figure 2.11: Rodent ex vivo Working heart System. The rat heart with the lungs excised and the pulmonary veins, SVC and IVC ligated was suspended in the glass chamber (F). The ascending aorta and left atrium were cannulated, with cannulas D & E respectively. In the working heart mode, perfusate loads the left ventricle via the left atrium and the heart ejects this out through the ascending aortic cannula (D).

The heart was suspended in a glass chamber (F) and its electrical activity was monitored using spring clip electrodes attached to the heart (ADinstruments, MLA1210). Data was acquired with the Animal Bio Amp hardware (ADinstruments, FE136). Pressure transducers connected to the aortic and left atrial lines were de-aired and used to provide information on aortic perfusion pressure in Langendorff mode and on preload and afterload in working heart mode. A flow meter (Transonic, T402-TB) was connected to the left atrial line via a flow probe

(Transonic, 2PXN-probe, 1.8mm ID) and these provided real-time data on perfusate flow into the left atrium. The afterload chamber was initially set at a height of about 50 cmH₂O. PowerLab 8/35 hardware and Labchart pro software (ADInstruments) were used for data acquisition. With the setup completed, an acid wash was carried out by passing a solution containing 40mls of 100% HCL in 2L of Milli-Q water through the system. The system was then primed with KH solution. (Fig. 2.11)

2.2.7.2 Rat heart harvest, cannulation and attaining baseline parameters

Loaded, unloaded and control hearts were explanted as illustrated in 2.1.3. The heart(s) were transferred to the lab where left atrial cannulation through one of the pulmonary veins was carried out whilst the heart remained in cold cardioplegia solution. The other pulmonary veins, the IVC and SVC were ligated and an opening was created in the pulmonary artery trunk. The peristaltic pump was started at a rate of 4rpm during aortic cannulation to avoid introducing air into the coronary circulation. Heart contraction resumed within a few seconds and the perfusate flow rate was increased slowly up to 8mls/min achieving an aortic perfusion pressure in Langendorff mode of about 80-90mmHg. The heart was enclosed in a warm glass chamber and its temperature maintained at about 38⁰ C. The hearts temperature was monitored with a T-type implantable thermocouple probe (ADInstruments, MLT1401). Once stable at 38⁰ C, the heart was left in Langendorff mode for 15-20mins. After 20mins the heart was switched into working heart mode by turning off flow into the aortic cannula whilst simultaneously opening its outflow line to the afterload chamber. The flow to the left atrium was opened and left atrial pressure (preload) maintained at 3-5mmHg. The heart's performance was now assessed against predetermined parameters before a decision was made to proceed with the experiments. These parameters include: Sinus rhythm, absent or only infrequent premature ventricular contractions

(PVC), and adequate heart rate of about 200bpm. Figure 2.12 shows a picture of the heart in working mode.

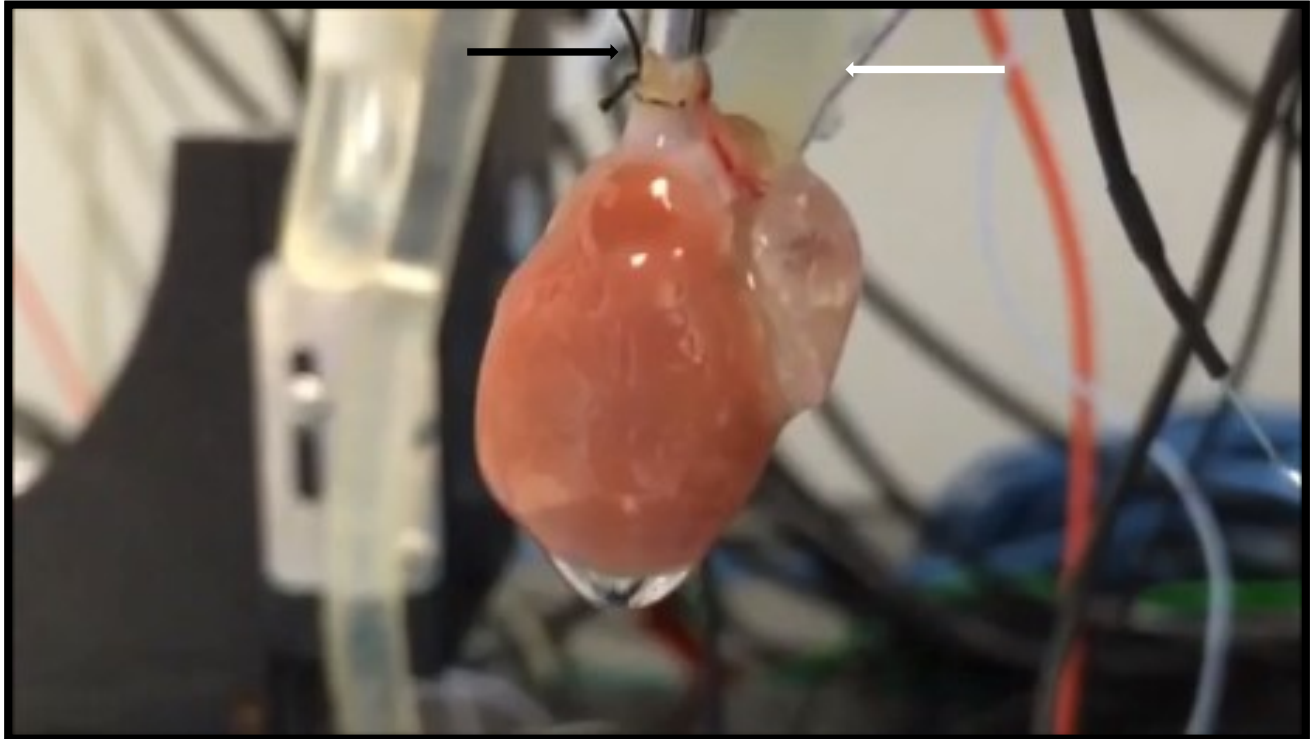


Figure 2.12: Control rat heart in working mode. The left atrium and left ventricle are in view. Note the aortic cannula black arrow, left atrial cannula white arrow & coronary effluent dripping beneath.

2.2.7.3 Assessment of LV function & myocardial contractility using PV loop catheter

With the heart in working mode, myocardial systolic and diastolic function, and load-independent indices of myocardial contractility were determined using a 1.9F Scisense micro-conductance catheter (Transonic, FTE-1912B-6018) inserted into the left ventricular cavity through the apex of the heart with the aid of a 25G needle. (Fig. 2.13)

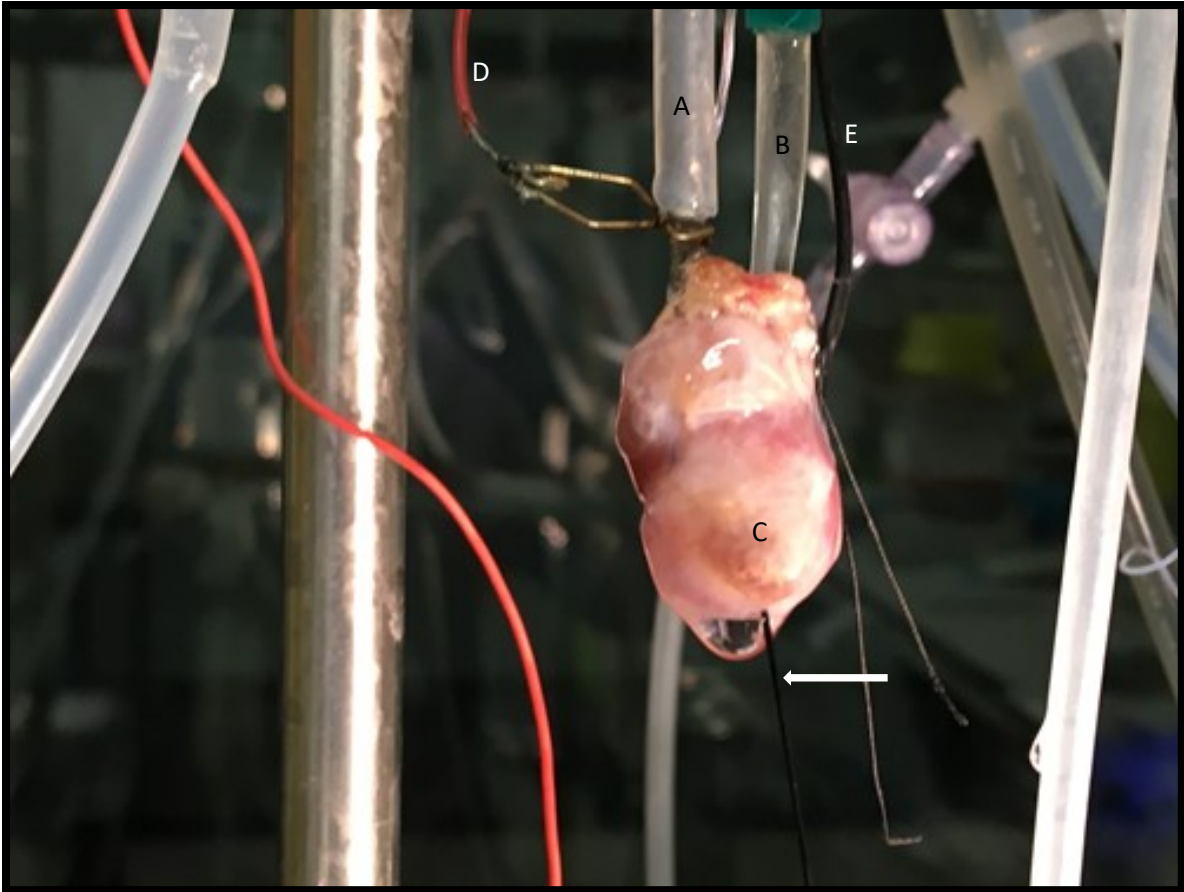


Figure 2.13: Infarcted heart with a scisense catheter inserted apically into the left ventricle (white arrow) and connected to the pressure-volume data acquisition hardware. A and B are the aortic and left atrial cannulas respectively. The left ventricular infarct scar (C). D and E are spring clip electrodes used for monitoring the hearts electrical activity.

The catheter was advanced until the proximal conductance coils are positioned in the apical cavity. It was secured in place to the apex with a purse string stitch using 8/0 prolene sutures. The catheter was connected to the Scisense ADV500 Pressure-Volume data acquisition hardware (Scisense Inc, FY097B) and optimal catheter position and pressure-volume analysis was determined by the shape of the PV loops obtained using LabChart software. (Fig. 2.14)

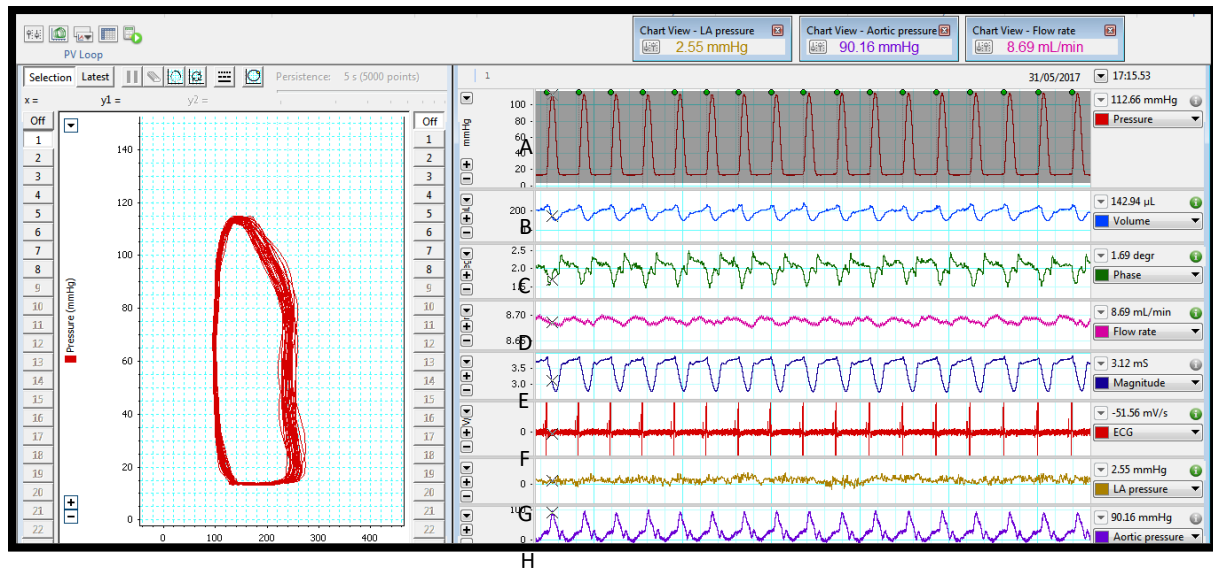


Figure 2.14: PV loop trace of the cardiac cycle obtained following appropriate ex vivo catheter placement into the rats heart. The stages of the cardiac cycle represented by the PV loop was described in section 1.4.4.1. The systolic and diastolic pressure attained during the cardiac cycle is shown on the arterial pressure trace (A) and is also shown on the y-axis of the PV loop trace. The x-axis of the PV loop represents the change in volume in the left ventricle with each beat and represented by the waveform (B). The phase (C) or muscle measurement trace and magnitude (E) are utilised by the software for accurate volume measurements. Perfusate flow to the heart was measured (D). F, G and H represent the electrical activity of the heart, left atrial pressure and aortic pressure traces respectively.

Multiple PV loops were generated by altering preload and afterload. The slope of these loops provided volume-independent indices of myocardial contraction such as: end systolic pressure-volume relationship (ESPVR), end diastolic pressure-volume relationship (EDPVR), and preload recruitable stroke work (PRSW). (Fig. 2.15) Several other haemodynamic parameters such as stroke volume (SV), cardiac output (CO), Stroke work (SW), dP/dt max, dP/dt min, and the relaxation constant tau were also obtained.

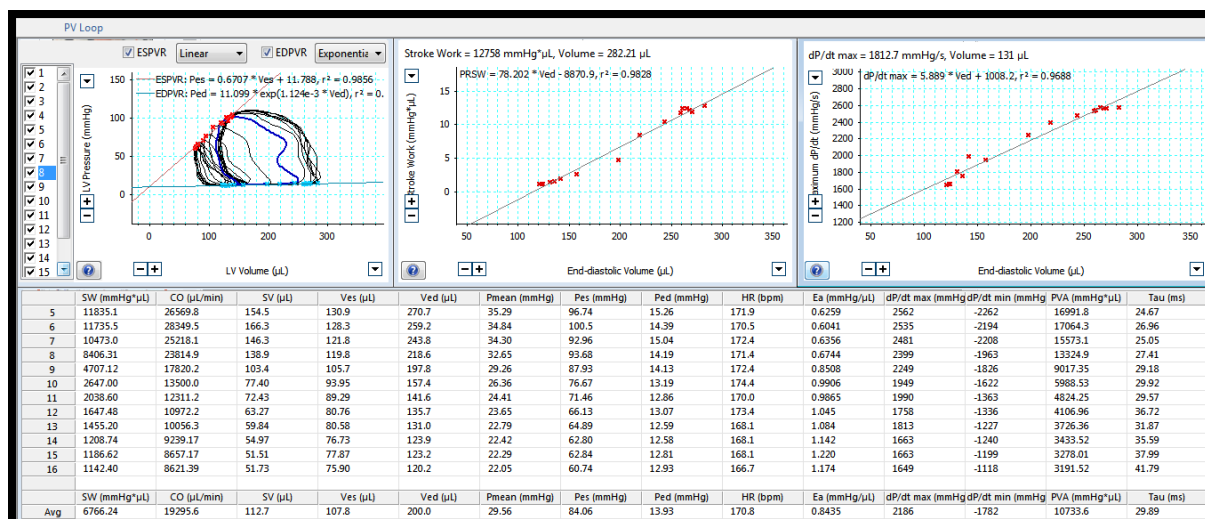


Figure 2.15: PV loop analysis table showing volume-dependent and independent haemodynamic data from an ex vivo working rat heart.

2.3 Statistical analysis

A two-tailed t-test was used for the comparison between independent groups of normally distributed data as determined by Kolmogorov-Smirnov testing. One-way analysis of variance (ANOVA) followed by Bonferroni post hoc test for individual significant differences was used. All statistical analyses were done using Prism 7 software (GraphPad Software, Inc.). The results are presented as means $\pm$ SD. The significance threshold was set at $p < 0.05$ for all data. A minimum of 3 rats were used in each experimental group and n numbers represented the number of rats studied. * denotes $P < 0.05$, ** denotes $P < 0.01$ and *** denotes $P < 0.001$.

3 Effects of mechanical unloading of the failing heart on the expression profiles of JPH2, BIN1, and Tcap, and their regularity along t-tubules

3.1 INTRODUCTION

Pathological remodelling during heart failure accounts for significant depression of calcium transients – an intrinsic component of the EC coupling process which directly affects myocardial contractility.(Ibrahim, Navaratnarajah, *et al.*, 2012a) Chronic mechanical overload-induced disruption of the transverse tubule (T-tubule) network has been shown to account for this loss of calcium transient synchrony seen in heart failure.(Ibrahim *et al.*, 2011b) (Ibrahim, Navaratnarajah, *et al.*, 2012a) Several studies have provided evidence to show that the structural integrity of T-tubules is load dependent and it appears that reduction of chronic cardiac overload through mechanical unloading can stimulate T-tubule reverse remodelling with concomitant decrease in action potential duration, increase in Ca^{2+} transient amplitude, reduction in Ca^{2+} spark frequency, and improvements in Ca^{2+} transient synchrony.(Ibrahim and Terracciano, 2013) (Ibrahim, Kukadia, *et al.*, 2012) (Ibrahim, Rao, *et al.*, 2012) (Navaratnarajah *et al.*, 2014) However, the effects mechanical unloading in this setting has on the effective expression (indexed by their regularity along t-tubules) of key molecular elements known to be involved in T-tubule regulation and the restoration of its structural integrity are not fully described. Junctophilin-2 (JPH-2), BIN-1, and telethonin (Tcap) have been identified

as important proteins involved in the maintenance of T-tubule structure but the exact role they play in reverse remodelling is unknown.(Ibrahim *et al.*, 2011a)

In this section of the thesis, the changes in regularity along t-tubules and thus effective expression of JPH2, Tcap, and BIN1 in a mechanically unloaded failing rat heart is investigated.

3.2 METHOD

3.2.1 Chronic myocardial infarction and Mechanical unloading

Lewis rat models of chronic MI that had developed severe heart failure, defined as an EF<40% on echocardiography at 12 weeks post permanent coronary artery ligation were studied as detailed in section 2.1.1.1. Rats with adequate heart failure were then either observed for a further 8 weeks (loaded group) or had their hearts explanted at 12 weeks and transplanted into the abdomen of healthy age-matched recipients where they were mechanically unloaded for a period of 8 weeks as detailed in section 2.1.2(e & g). The recipient's heart was used as the loaded control. Unloaded control hearts were healthy age-matched hearts mechanically unloaded for a duration of 8 weeks.

3.2.2 Cardiomyocyte fixation and cell immunohistochemistry

Heart-lung blocs were collected and cardiomyocytes isolated, plated and fixed using a well-established protocol as detailed in section 2.2.4. Cell immunohistochemistry was then completed to determine the expression profiles of t-tubular structural proteins, BIN-1, Juncophilin 2 (JPH-2), and Tcap as described in section 2.2.5.

Imaging of the stained cells was completed with the aid of confocal microscopy and data acquisition was done using Matlab.

3.2.3 Protein density and regularity analyses using Fourier transformation in MATLAB

High-resolution images of the cells stained with the respective proteins were taken and analysed on ImageJ. The high-resolution images were adapted into binary images in ImageJ, and plot profiles of a central portion of these images were then loaded onto MATLAB (MATLAB 2018a, the MathWorks Inc., MA, USA) where Fourier analysis was performed to obtain their regularity and density.

3.2.4 Western blotting

Western blotting was used to determine the effect of mechanical unloading on the dynamic expression profiles of JPH2, BIN1, and Tcap as described in section 2.2.6. Primary antibody control was determined via the immunoblot (Western blot) method where the antibody specificity to the molecular weight of the protein of interest was determined.

3.2.5 Statistics

The expression profiles of JPH-2, BIN-1, and Tcap were each compared between the loaded and unloaded permanently ligated failing hearts and age-matched loaded and unloaded healthy hearts. The filled bars in each figure represents loaded hearts and the empty bars unloaded hearts.

A two-tailed t-test was used for the comparison between independent groups of normally distributed data as determined by Kolmogorov-Smirnov testing. One-way analysis of variance (ANOVA) followed by Bonferroni post hoc test for individual significant differences was used. All statistical analyses were done using Prism 7 software (GraphPad Software, Inc.). Five animals (n=5) were studied in each subgroup. The results are presented as means $\pm$ SD. The significance threshold was set at $p < 0.05$ for all data. * denotes $P < 0.05$, ** denotes $P < 0.01$ and *** denotes $P < 0.001$.

3.3 RESULTS I: Echocardiographic Assay of Cardiac Function

The development of cardiac enlargement after myocardial infarction was indexed by a rise in ventricular weight/body weight ratio, dilatation of the LV chamber, and significant deterioration in LV function ($EF < 40\%$). These factors were taken as indicators of successful progression to heart failure in the Lewis rats. Figure 3.1 shows the development of significant cardiac enlargement by 12 weeks after coronary artery ligation.

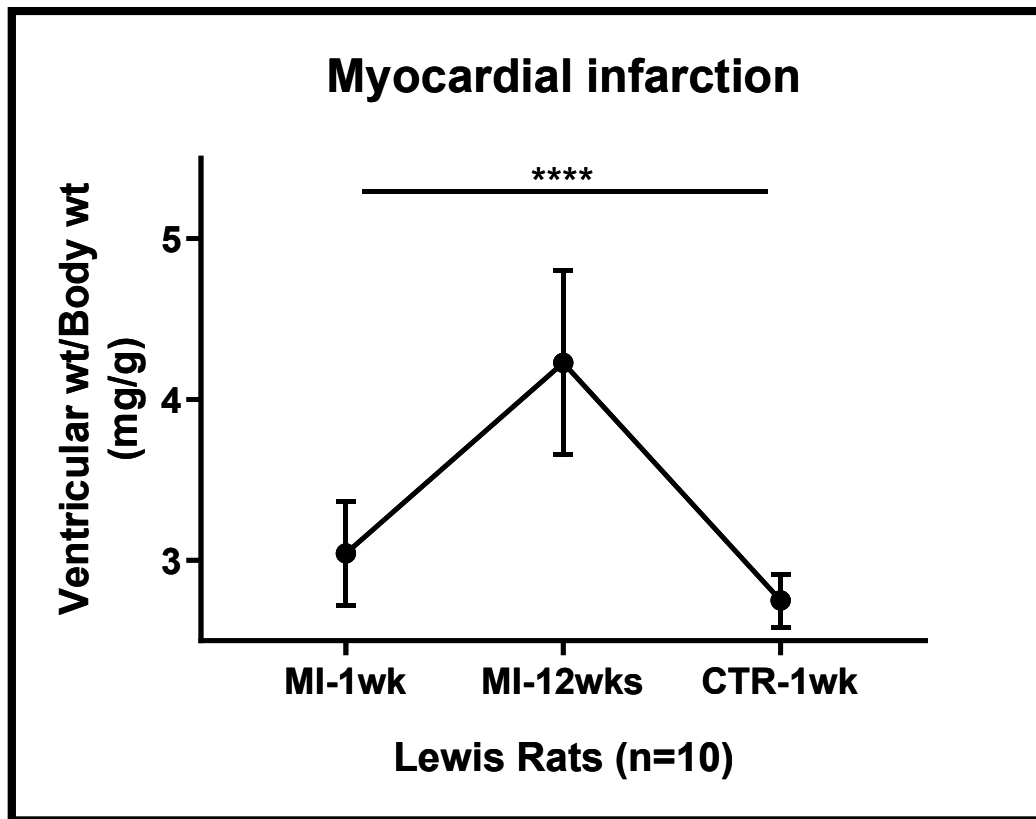


Figure 3.1: Significant increase in ventricular weight/body weight ratio in the loaded chronically infarcted hearts by 12 weeks following coronary artery ligation compared to that in the control hearts and infarcted hearts at 1 week. There were 10 rats in each group.

Transthoracic echocardiography of the rats was obtained at 1 week and 12 weeks following coronary artery ligation to assess LV chamber geometry and function as described in section 2.1.2. Figures 3.2 to 3.3 illustrate the changes in LV chamber size and function following myocardial infarction.

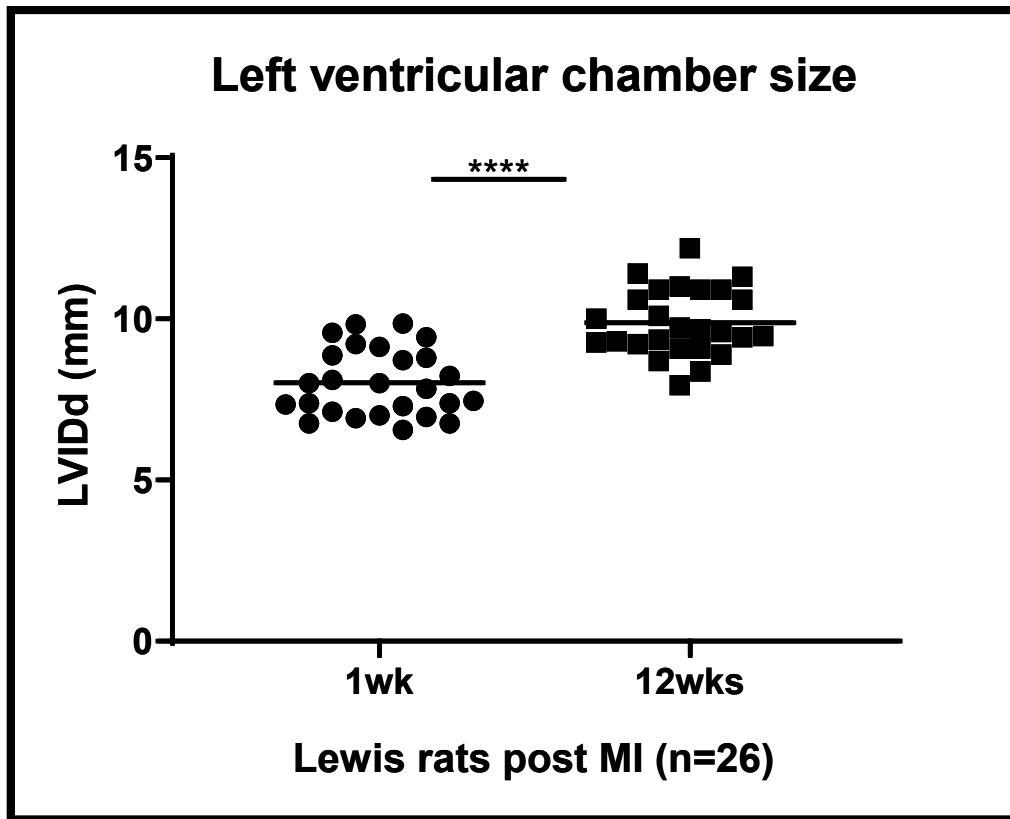


Figure 3.2: Significant increase in left ventricular chamber size in rat models of myocardial infarction 1 week compared to 12 weeks following coronary artery ligation indicating successful development of heart failure.

Significant increase in LV chamber size occurred by 12 weeks following coronary artery ligation (CMI-1wk vs CMI-12wks $p < 0.0001$) as shown in figure 3.2, and this correlated with a significant reduction in ejection fraction (CMI-1wk vs CMI-12wks $p < 0.0001$) as illustrated in figure 3.3.

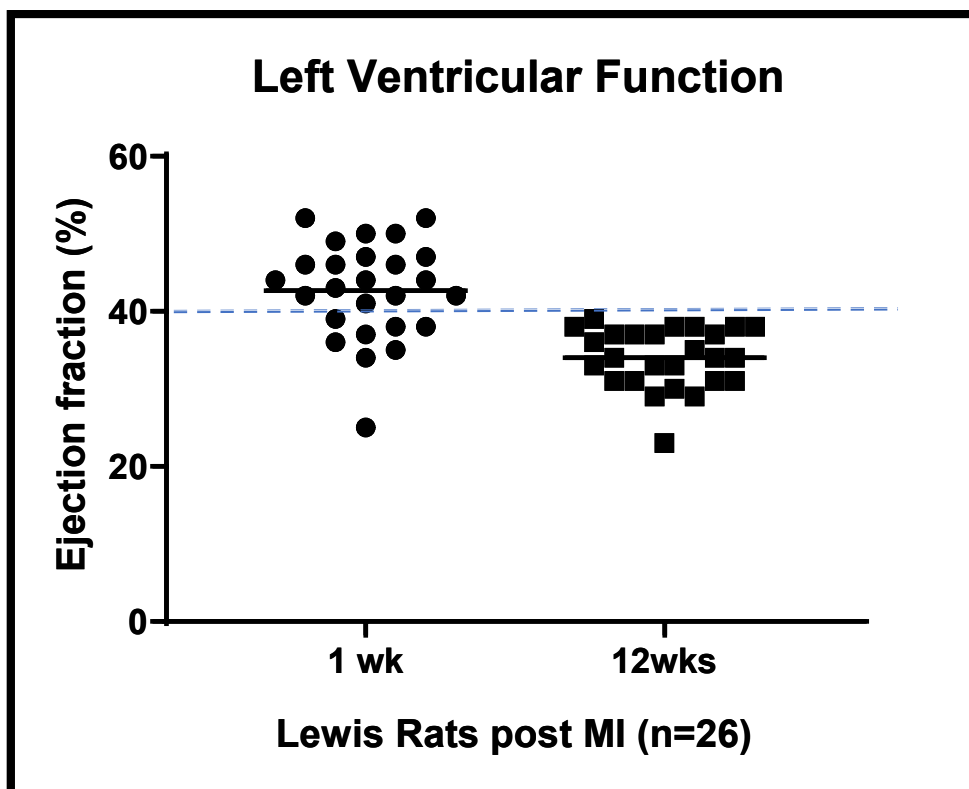


Figure 3.3: Echocardiography of infarcted Lewis rats showing evidence of significant heart failure ($EF < 40\%$) at 12wks after LAD + PDA coronary ligation. The data at 12wks is the same as that illustrated in fig 2.1.

3.4 RESULTS II: Dynamic Expression Profile, Regularity and Density of JPH2, Tcap and BIN1 in the Failing Mechanically Unloaded Heart

3.4.1 Effect of mechanical unloading on JPH2 regularity, density and expression

The data showed that mechanical unloading of the healthy rat heart (CTR-U) was associated with a significant decrease in the regularity of junctophilin compared to control (CTR-L 12.18 ± 0.7259 , vs CTR-U 3.124 ± 0.4758 , $p < 0.0001$) as illustrated in figure 3.4. This impaired regularity of junctophilin was similar to that seen in the loaded chronically infarcted heart compared to control (CTR-L 12.18 , vs CMI-L 2.378 $p < 0.0001$). However, mechanical unloading of the failing heart was associated with a significant improvement in junctophilin regularity (CMI-L 2.378 ± 0.6377 , vs CMI-U 6.903 ± 0.8297 , $p = 0.0024$) as shown in figure 3.4.

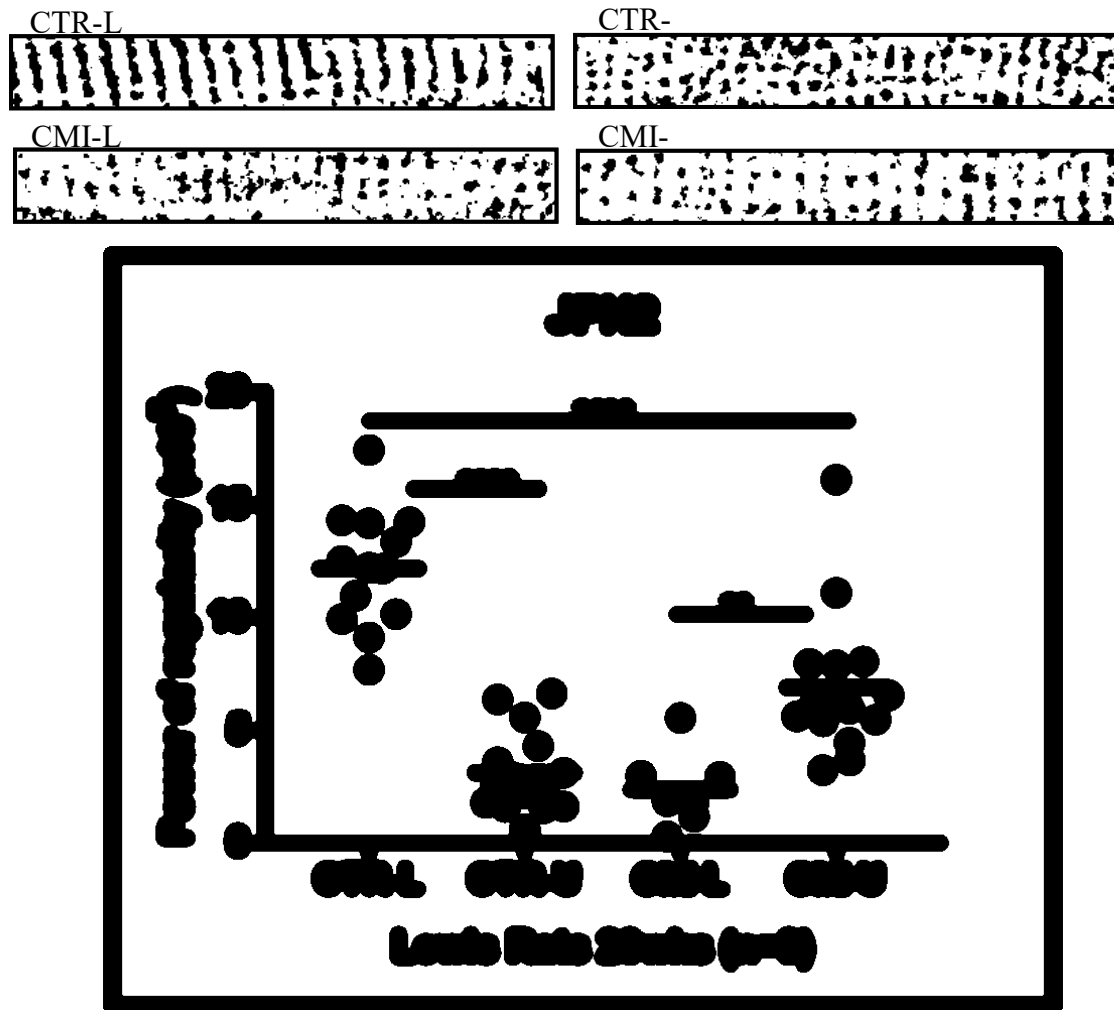


Figure 3.4: Cell immunochemistry data showing the effect of mechanical unloading on the power of regularity of juncctophilin in healthy and chronically infarcted hearts compared to control. Representative computed results of immunofluorescence images obtained using imageJ and MATLab software are shown above. Three rats were used for each experiment and the dots represents number of cells.

In contrast to the effect of mechanical unloading on the regularity of juncctophilin, there was no statistically significant difference in juncctophilin density after mechanical unloading of the chronically infarcted heart or control. (Fig. 3.5)

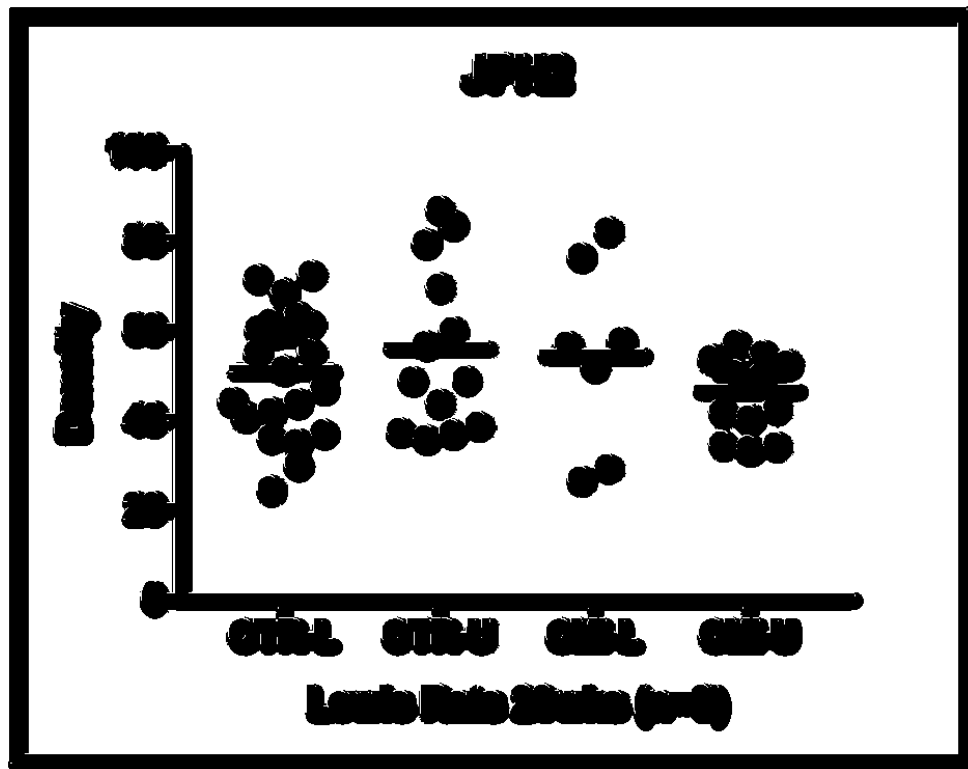


Figure 3.5: Cell immunofluorescence data showing the effect of mechanical unloading on the density of junctophilin in healthy and chronically infarcted hearts compared to control. Representative computed results of immunofluorescence images used for analysis are shown in figure 3.4. Three rats were used in each experiment and the dots represent number of cells.

Similarly, analysis of junctophilin protein expression by western blotting also showed that mechanical unloading of the healthy heart was not associated with a significant change in protein expression as shown in figure 3.6.

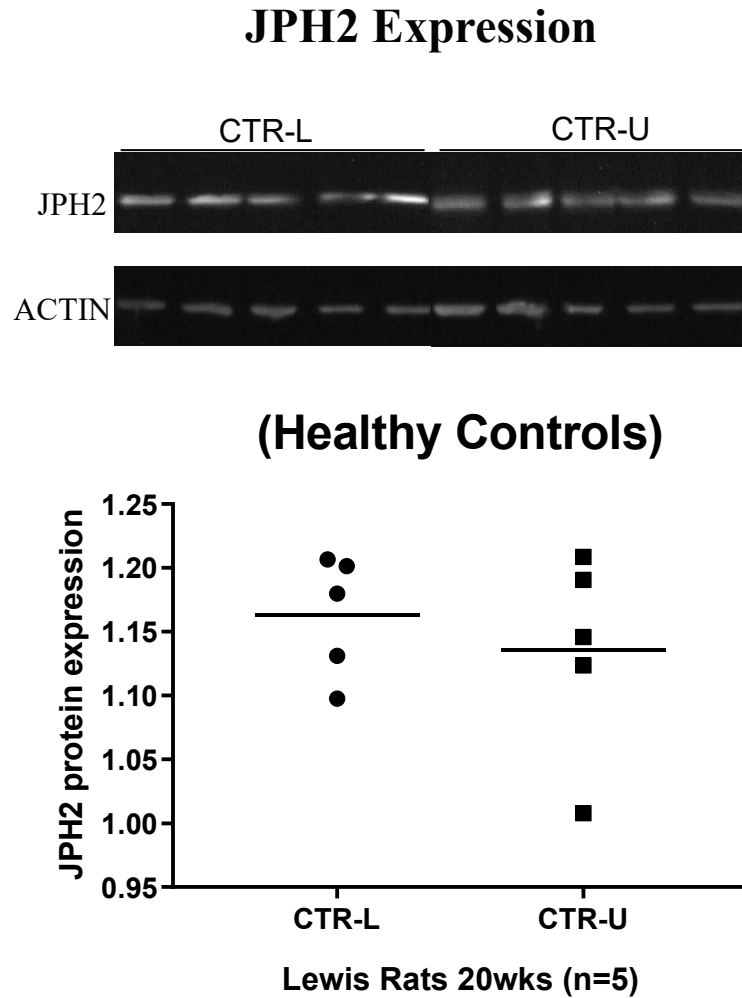


Figure 3.6: Western blot data showing the effect of mechanical unloading on the protein expression profile of junctophilin in healthy control hearts. The dots represents number of rats.

3.4.2 Effect of mechanical unloading on Tcap regularity, density and expression

Cell immunohistochemistry data showed that mechanical unloading of the healthy and chronically infarcted heart was associated with a significant impairment of Tcap regularity (CTR-L 18.81

± 1.958 , vs CTR-U 4.139 ± 0.6827 , $p < 0.0001$ and CMI-L 22.20 , vs CMI-U 12.95 , $p = 0.0337$ respectively) as illustrated in figure 3.7.

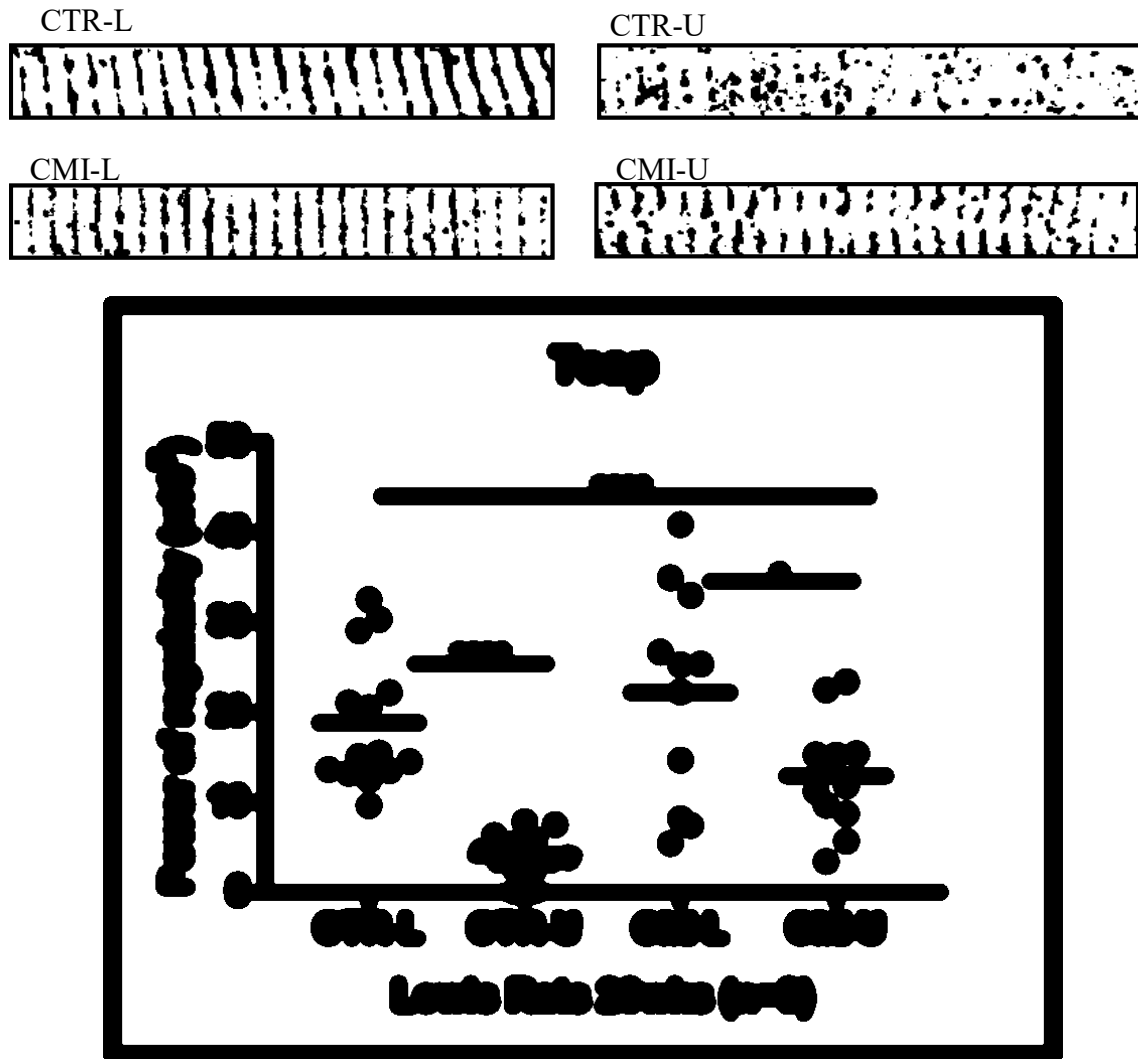


Figure 3.7: Cell immunochemistry data showing the effect of mechanical unloading on the power of regularity of Tcap in healthy and chronically infarcted hearts compared to control. Representative computed results of immunofluorescence images obtained using imageJ and MATLAB software are shown above. Three rats were used in each experiment and the dots represents number of cells.

There was however no statistically significant difference in Tcap density after mechanical unloading in both the control and infarcted hearts (Fig. 3.8).

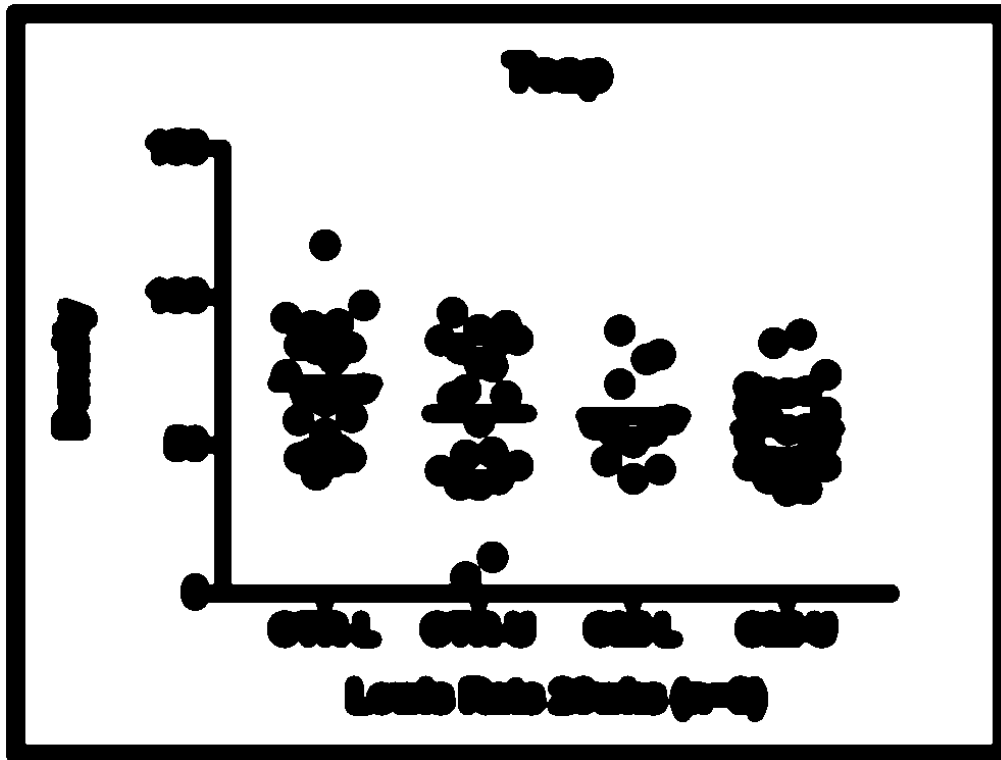


Figure 3.8: Cell immunochemistry data showing the effect of mechanical unloading on the density of Tcap in healthy and chronically infarcted hearts compared to control. Representative computed results of immunofluorescence images used for analysis are shown in figure 3.7. Three rats were used in each experiment and the dots represents number of cells.

Analysis of Tcap protein expression via western blotting showed a statistically significant downregulation of Tcap expression after mechanical unloading in healthy hearts (CTR-L 3.035 ± 0.419 , vs CTR-U 0.9121 ± 0.1274 , $p=0.0013$) as shown in figure 3.9.

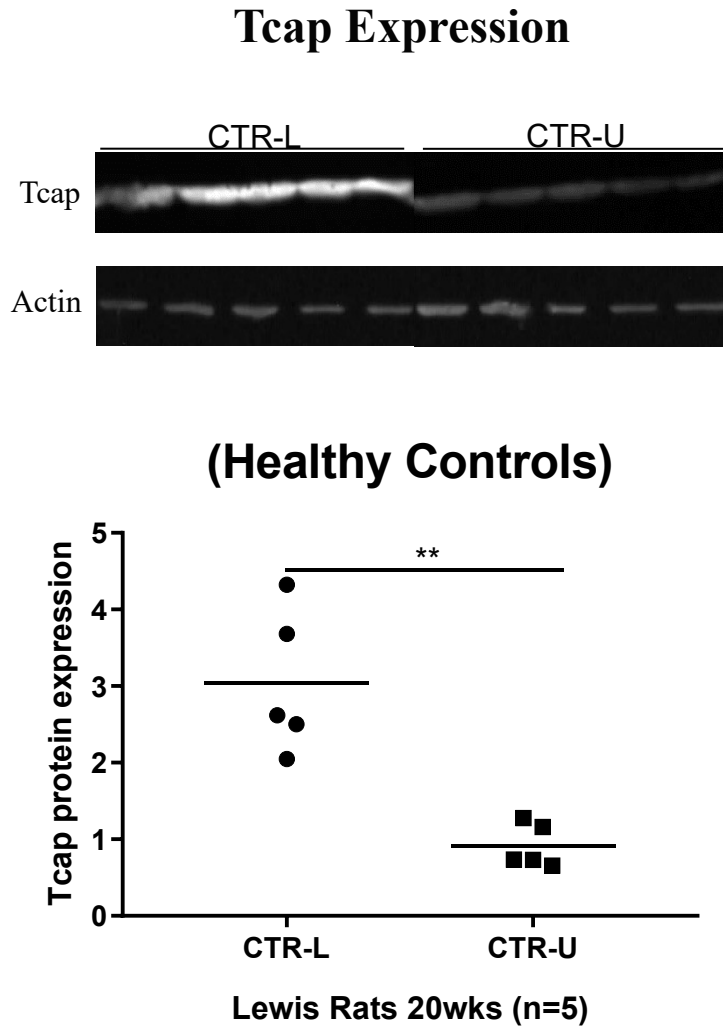


Figure 3.9: Western blot data showing the effect of mechanical unloading on the protein expression profile of Tcap in healthy control hearts. The dots represents number of rats.

3.4.3 Effect of mechanical unloading on BIN1 regularity, density and expression

Cell immunochemistry data showed no statistically significant difference in BIN1 regularity or density after mechanically unloading healthy or chronically infarcted rat hearts (Fig. 3.10 and 3.11).

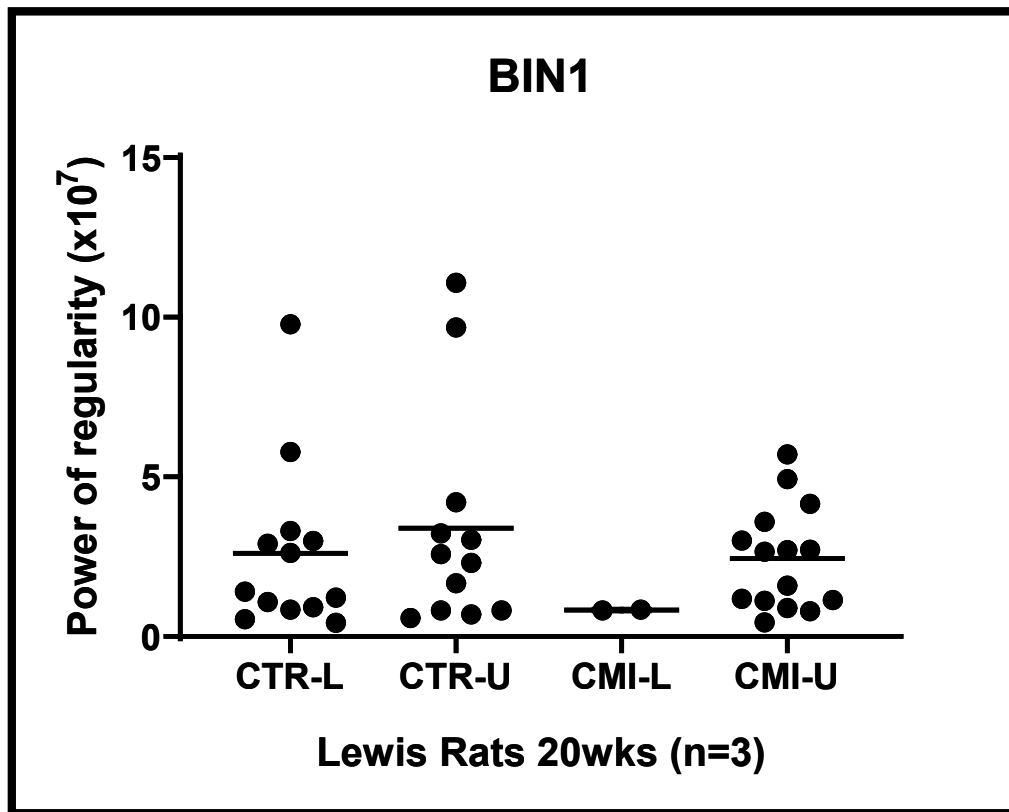
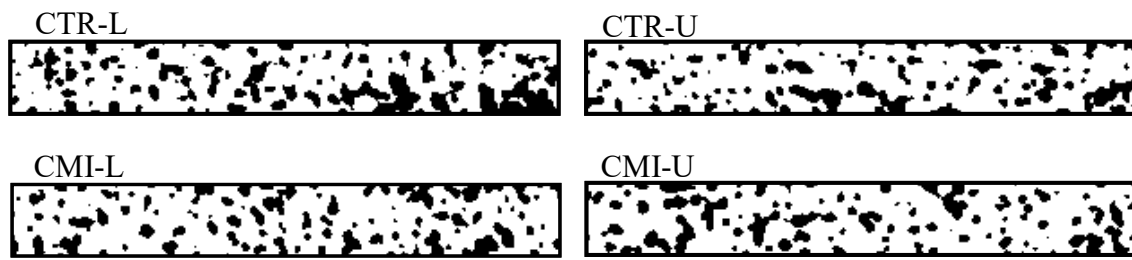


Figure 3.10: Cell immunochemistry data showing the effect of mechanical unloading on the power of regularity of BIN1 in healthy and chronically infarcted hearts compared to control. Representative computed results of immunofluorescence images obtained using imageJ and MATLab software are shown above. Three rats were used for each experiment and the dots represents number of cells. Unfortunately due to the severity of failure in the CMI-L hearts only 2 cells were adequate for BIN1 analysis of power of regularity.

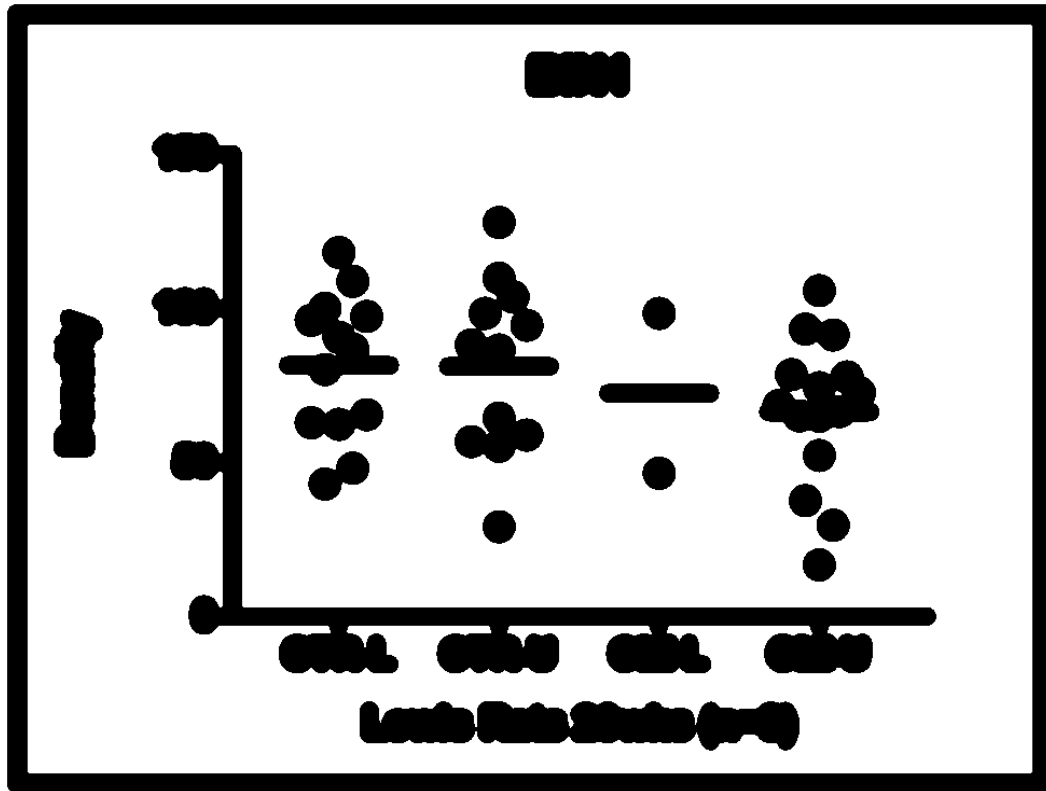
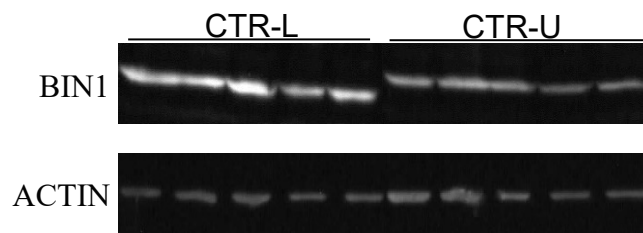


Figure 3.11: Cell immunochemistry data showing the effect of mechanical unloading on the density of BIN1 in healthy and chronically infarcted hearts compared to control. Representative computed results of immunofluorescence images used for analysis are shown in figure 3.10. Three rats were used for each experiment and the dots represents number of cells.

Similarly, analysis of BIN1 protein expression via western blotting also showed no statistically significant difference in BIN1 expression following mechanical unloading as shown in figure 3.12.

BIN1 Expression



(Healthy Controls)

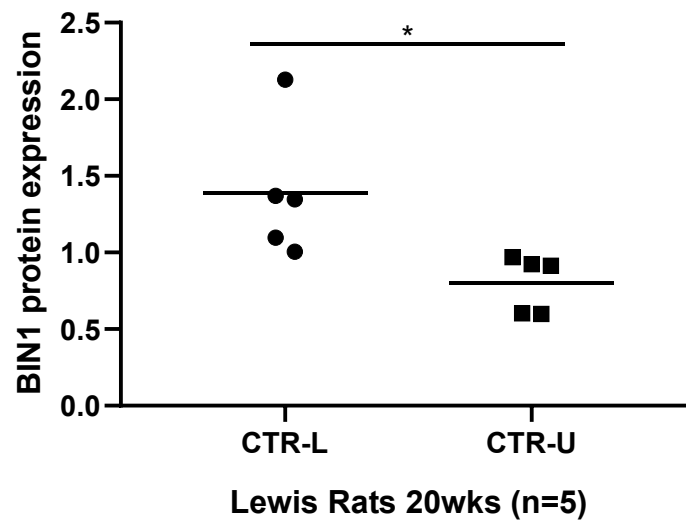


Figure 3.12: Western blot data showing the effect of mechanical unloading on the protein expression profile of BIN1 in healthy control hearts. The dots represents number of rats.

3.5 DISCUSSION

3.5.1 Load variation and the regularity of JPH2, Tcap & BIN1 after MI

These data show that alterations in mechanical load after the development of severe heart failure secondary to ischaemic cardiomyopathy in the Lewis rat heart is associated with significant changes in the regularity of alignment to t-tubules of JPH2 and Tcap but not BIN1. This finding is consistent with the expected effect of mechanical load variations on the regularity of JPH2 and Tcap due to their individual roles as anchoring proteins (maintaining the tight junctional membrane complexes between LTCCs in t-tubules and RyRs of the junctional SR) and stretch-sensitive proteins (linking the stretch-sensitive complex of the z-disk with other proteins in the t-tubule membrane) respectively. This is not the case with BIN1 because it is ubiquitously expressed, its role in the biogenesis of t-tubules and the trafficking of LTCCs is not reliant on its spatial alignment with t-tubules.(Caldwell *et al.*, 2014)

Previous published data showed that the protein expression profile of JPH2 is significantly downregulated in the loaded failing heart and that mechanical unloading via HAHLT did not bring about significant upregulation of JPH2 protein expression despite the presence of improvements in t-tubule structure and function following unloading in this setting.(Ibrahim, Navaratnarajah, *et al.*, 2012a) (Caldwell *et al.*, 2014) Data from this study however shows that whilst the density and protein expression profile of JPH2 appear not to be significantly altered by mechanical unloading, the regularity of JPH2 is significantly increased when the loaded failing heart undergoes a period of load reduction via HAHLT. This emphasises the concept

that the efficient spatial orientation as opposed to quantity of JPH2 is of more significance to mechanical unloading induced reverse remodelling.

Interestingly, these data also show that mechanical unloading of the healthy (control) heart brings about a significant reduction in JPH2 regularity. The decreased regularity seen in the mechanically unloaded control heart is to a similar degree to the disruption in regularity of JPH2 observed in the overloaded chronically infarcted heart. This indicates a bimodal response of the heart's contractile apparatus to variations in load. Load reduction in the presence of a physiological load status appears to have a significant detrimental effect on the activity of JPH2. This finding is in line with previous reports showing that the structural integrity of t-tubules is load-dependent with excessive overload or excessive mechanical unloading bringing about disruption in t-tubule structure and function.(Ibrahim and Terracciano, 2013)

Tcap has been shown to be a load-dependent regulator of t-tubule structure and function.(Ibrahim, Siedlecka, *et al.*, 2013) Tcap activity is enhanced with increased mechanical load and its absence is associated with the development of more pronounced overload-induced t-tubule dysfunction.(Ibrahim, Siedlecka, *et al.*, 2013) (Chu, Koshman and Samarel, 2012) Data from this study show the presence of enhanced Tcap regularity in the loaded control and infarcted hearts with mechanical unloading in both these hearts resulting in significant reduction in Tcap regularity. These data suggests a possible contribution of disruption in Tcap activity in the detrimental effects on the heart sometimes observed during mechanical unloading and warrants further investigating.

3.6 SUMMARY

Data from this experiment provides new insight into the possible underlying mechanisms involved in the recovery of contractile function noted in isolated cardiomyocytes after the failing heart is mechanically unloaded. Significant improvement in JPH2 regularity was observed after unloading indicating a greater importance of JPH2 regularity to improved t-tubule function. This might help to explain the previously published observation that the reduced expression of JPH2 seen in heart failure is not recovered after mechanical unloading despite improvements in cell function.(Ibrahim, Navaratnarajah, *et al.*, 2012a) Furthermore, the significant impairment in Tcap regularity with unloading also highlights a possible role of Tcap in some effects seen with mechanical unloading that are not in keeping with the recovery process.

4 Effect of mechanical unloading of the ischaemia-reperfused heart on infarct size and interstitial fibrosis

4.1 INTRODUCTION

Several contemporary studies have identified the cardiac ECM as possibly having a significant influence on the degree of reverse remodelling that occurs during mechanical unloading.(Schaefer *et al.*, 2019) (Lok *et al.*, 2015) (Drakos *et al.*, 2010) The ECM is at present the only part of the unloaded failing heart that appears to portray features that are not in keeping with the recovery process.(Klotz *et al.*, 2007) It is now widely accepted in human and animal studies that myocardial fibrosis increases after unloading the failing heart.(Schaefer *et al.*, 2019) (Navaratnarajah *et al.*, 2013) (Drakos *et al.*, 2010) The mechanism by which this negative effect of unloading occurs is however not fully understood. Reversal of fibrosis remains a challenge and much work is ongoing towards the identification of the main elements in the fibrotic process that will serve as viable therapeutic targets. Several studies have shown that angiotensin II, transforming growth factor beta 1 (TGF- β 1), matrix metalloproteinases (MMPs) particularly MMP1 which degrades collagen type 1, and tissue inhibitor of metalloproteinases (TIMPs), mainly TIMP1 (the better characterized TIMP that binds to and inactivates a range of MMPs including MMP1) play pivotal roles in the pathological process that leads to fibrosis.(Kinoshita *et al.*, 2014) (Barton *et al.*, 2003) Furthermore, drugs such as ivabradine and ramipril have been shown in animal and human studies to ameliorate cardiac

fibrosis yet increased fibrosis remains a feature of LVAD unloaded failing human hearts. (Navaratnarajah *et al.*, 2013) (Milting *et al.*, 2006) (Klotz *et al.*, 2005c) Thus the exact underlying mechanism by which mechanical unloading of the failing heart brings about increases in myocardial fibrosis with potential deleterious consequences on LVAD-induced myocardial recovery remains largely unknown.

An interesting, yet largely unexplored element in the process of worsening myocardial fibrosis during LVAD use is the effect mechanical unloading has on coronary blood flow after myocardial infarction. The heart is an obligate aerobic organ that requires a steady supply of oxygen to meet its demand for energy, in the absence of which cell death and fibrosis develops. A set of major non-autoregulated coronary blood vessels are the predominant source of blood flow around the heart. In addition however, the heart is also enriched with a network of smaller autoregulated collateral blood vessels.(van Royen *et al.*, 2009) (BAROLDI, MANTERO and SCOMAZZONI, 1956) These latter coronary vessels form the hearts microcirculation and have been shown to be predominantly redundant at rest but in times of increased metabolic need become highly effective intercoronary collateral channels.(BAROLDI, MANTERO and SCOMAZZONI, 1956) (Rentrop *et al.*, 1985) (Fulton, 1964b) (Fulton, 1964c) With elevated metabolic demand, myocardial blood flow (MBF) has the potential to rise up to 5-fold through autoregulatory effects on the microcirculation.(Xydas *et al.*, 2005) Conversely, in the absence of metabolic need, i.e. after reperfusion of the parent coronary vessel, these collateral channels rapidly become non-functional and return to their collapsed redundant state at rest.(Rentrop *et al.*, 1985) Thus these highly autoregulated microvasculatures help to match MBF to changes in myocardial oxygen consumption (MVO_2) in an attempt at preserving cardiomyocyte function.(Xydas *et al.*, 2005)

Studies have reported that coronary flow reserve (CFR), the contribution of the microvasculature to increased MVO_2 is diminished in heart failure patients treated with

LVADs.(Tansley *et al.*, 2004) This is thought to be a result of the markedly decreased LV workload during mechanical unloading which reduces $MV\dot{O}_2$ and diminishes the autoregulatory stimulus on the microcirculation.(Tansley *et al.*, 2004) Furthermore, it has also been shown that mechanical unloading with LVADs is associated with a reduction in microvascular lumenal diameter in the failing heart with associated worsening of interstitial fibrosis.(Drakos *et al.*, 2010) These reports highlight a potential link between LVAD use and negative autoregulatory effects on the collateral circulation. No studies have however evaluated the extent of interstitial fibrosis in the failing heart after mechanical unloading in the presence or absence of reperfusion of the non-autoregulated parent coronary vessel.

In this section, the effect ischaemia-reperfusion has on the development of interstitial fibrosis and propagation of an infarct after acute and chronic myocardial infarction coupled with mechanical unloading is investigated. The dynamic changes in the protein expression profiles of TGF- β 1, α SMA, MMP1, and TIMP1 is also reviewed.

4.2 METHODS

4.2.1 Myocardial infarction and Ischaemia-reperfusion model

Rodent models of acute and chronic MI with or without ischaemia-reperfusion were utilised. Details are described in section 2.1.1. In the chronic MI group, echocardiography was carried out at 12 weeks post coronary artery ligation to determine the development of heart failure. Failing hearts were then explanted and transplanted into the abdomen of healthy recipients

where they were mechanically unloaded for a period of 4 weeks as detailed in section 2.1.1.1. In the permanently ligated chronic MI group a minimum EF less than 40% was a requirement for inclusion into the study as this was taken as an indication of adequate occlusion of the LAD and PDA.

4.2.2 Mechanical unloading model

The heterotopic abdominal heart transplantation technique was used as the model of mechanical unloading. Details are described in section 2.1.2. Figure 5.1 shows a schematic representation of the experimental protocol used for developing the animal models. Twelve groups were studied, 8 acute MI groups and 4 chronic MI groups as described in 2.1.2.

4.2.3 Histological analysis of frozen tissue sections

Sirius red/Fast green stain was used for histological analysis of frozen tissue sections from the study groups as described in section 2.2.3.

4.2.4 Widefield microscopy and planimetry for the quantification of myocardial fibrosis and infarct size

Images of the stained sections were obtained with the Widefield microscope as described in 2.2.3. The samples were given random codes and blinded to the investigator performing analysis. With the aid of imageJ software, infarct size (IS) and area at risk (AAR) were

determined using planimetry. The IS was the area stained red (Fig. 4.1) whilst the AAR was the whole left ventricle (Fig. 4.1B)

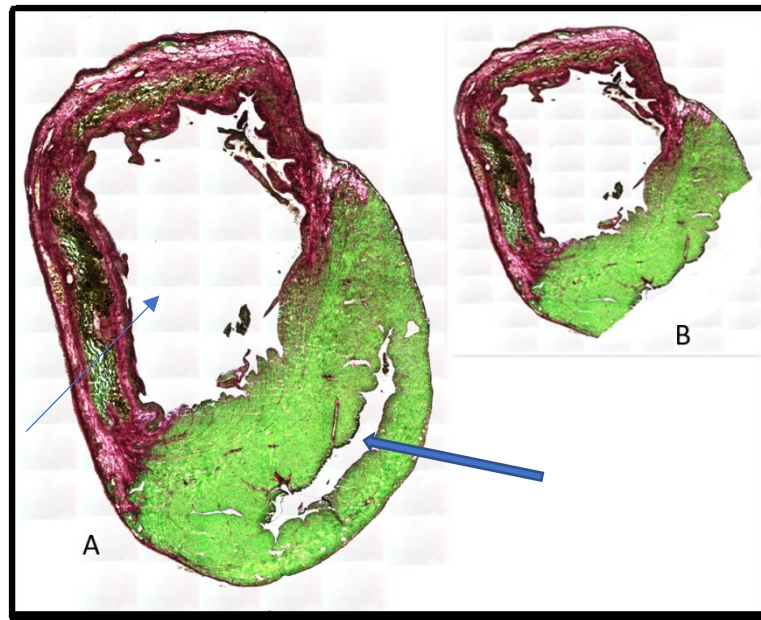


Figure 4.1: Sirius red/Fast green histological stain of a cross-section of the infarcted rat heart showing the extent of necrotic cell death and collagen deposition (red stain) and areas of viable myocardium (green stain). Image B (LV free wall and septum) illustrates the area at risk of infarction following coronary artery ligation. Left ventricle (thin arrow), Right ventricle (thick arrow)

Infarct ratio (IR), calculated as the proportion in percentage (%) of the AAR stained red (i.e. IS/AAR), was used to compare the extent of necrotic cell death and scarring between the study groups. The thresholding tool on imageJ was used to accentuate the contrast between the areas of interstitial collagen deposition and healthy myocardium and hence quantify the extent of interstitial fibrosis (IF).

4.2.5 Western blotting

Western blotting was used to determine the dynamic expression profiles of TGF- β 1, α SMA, MMP-1 and TIMP-1 as described in section 2.2.6.

4.2.6 Statistics

The IR and IF was compared between the loaded and unloaded permanently ligated and ischaemia-reperfused hearts. The filled bars in each figure represents loaded hearts and the empty bars unloaded hearts. The permanently ligated coronary artery group are in blue and the ischaemia-reperfusion group in red.

A two-tailed t-test was used for the comparison between independent groups of normally distributed data as determined by Kolmogorov-Smirnov testing. One-way analysis of variance (ANOVA) followed by Bonferroni post hoc test for individual significant differences was used. All statistical analyses were done using Prism 7 software (GraphPad Software, Inc.). Five animals (n=5) were studied in each subgroup. The results are presented as means $\pm$ SD. The significance threshold was set at $p < 0.05$ for all data. * denotes $P < 0.05$, ** denotes $P < 0.01$ and *** denotes $P < 0.001$.

4.3 RESULTS I: Effect of Mechanical Unloading on Infarct Size after Acute and Chronic Myocardial Infarction

The data showed that in the permanently ligated group, mechanical unloading after acute MI was associated with a statistically significant increase in infarct ratio at both day 2 (AMI-L 36.41 ± 2.951 , vs AMI-U 52.85 ± 3.537 , $p=0.0073$) and day 7 (AMI-L 31.69 ± 3.675 , vs AMI-U 46.21 ± 3.104 , $p=0.0166$).

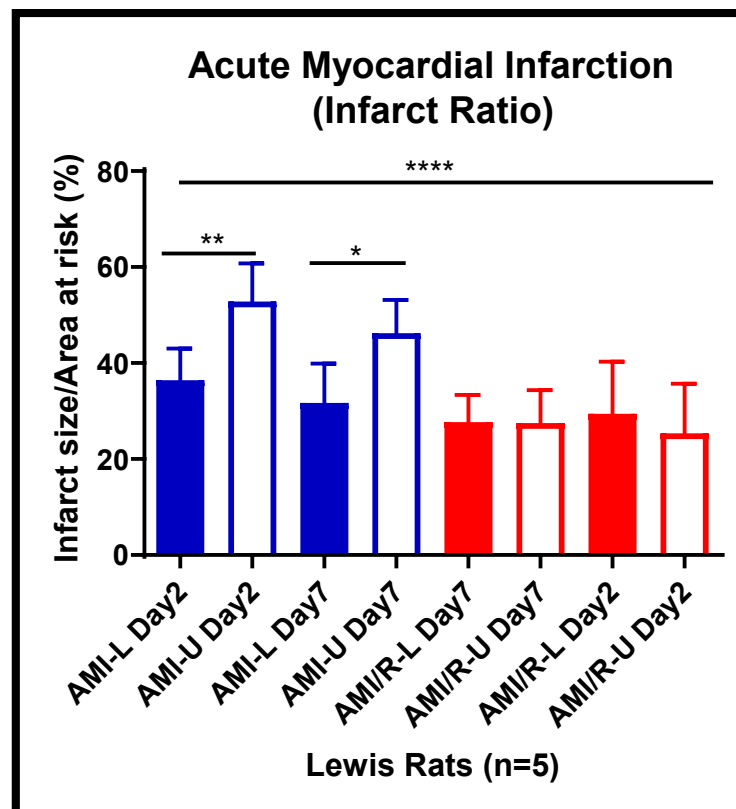


Figure 4.2: Difference in infarct ratio between loaded and unloaded Lewis rat hearts at day 2 and day 7 after permanent coronary artery ligation (blue bars) and ischaemia-reperfusion (red bars). The empty bars represent the unloaded hearts and the loaded hearts are represented by full bars. The infarct ratio is the infarct size/area at risk.

In the ischaemia-reperfusion group however, mechanical unloading after acute MI was not associated with worsening of infarct ratio at day 2 (AMI/R-L 29.44, vs AMI/R-U 25.36, $p=0.5602$) and day 7 (AMI/R-L 27.68, vs AMI/R-U 27.50, $p=0.9651$) as illustrated in figure 4.2.

The average infarct ratio after permanent coronary artery ligation was 34% in the loaded hearts but this rose significantly to 49.5% after mechanical unloading. In the ischaemia-reperfusion group the infarct ratio remained about the same at 29% in the loaded hearts and 27% after mechanical unloading.

In the permanently ligated chronic MI group (Fig. 4.3), there was a statistically significant rise in infarct ratio after mechanical unloading compared to the loaded hearts (CMI-L 24.46, vs CMI-U 41.34, $p<0.0001$). In the ischaemia-reperfusion chronic MI group, the observed increase in infarct ratio associated with mechanical unloading was retained (CMI/R-L 31.69 ± 3.675 , vs CMI/R-U 27.81 ± 2.364 , $p=0.0297$). The infarct ratio rose from 24.5% in the permanently ligated loaded hearts to 41% in the unloaded hearts. In the ischaemia-reperfusion group, infarct ration also rose, from 17.8% to 26%.

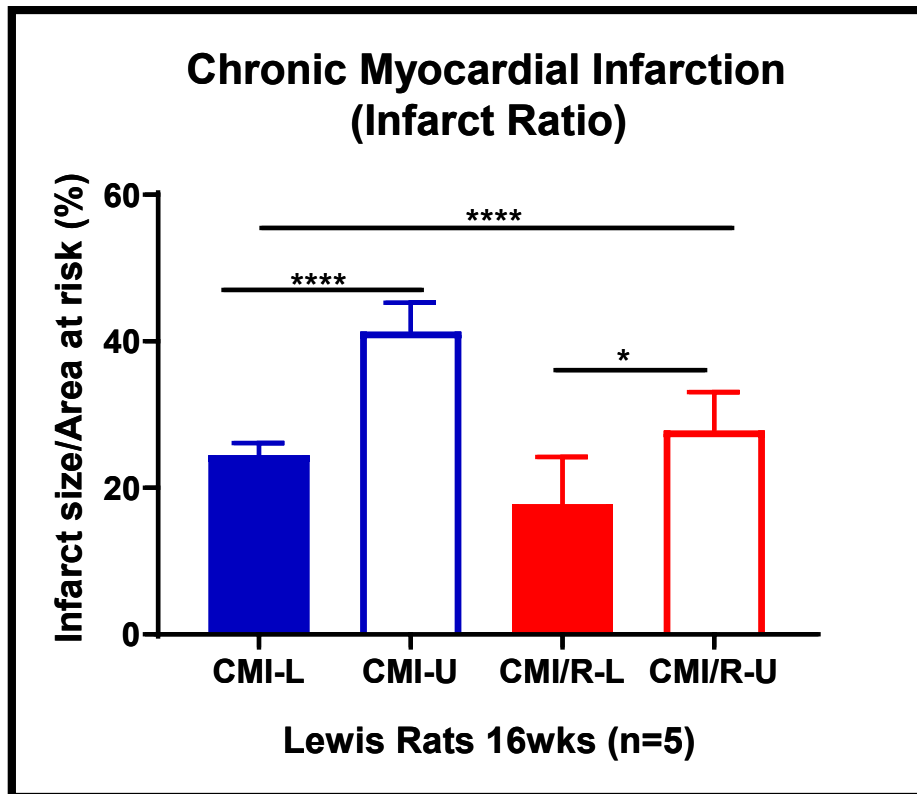


Figure 4.3: Infarct ratio in the chronic permanently ligated and ischaemia-reperfused hearts showing the effects of 4 weeks of mechanical unloading in failing hearts from ischaemic cardiomyopathy. These hearts underwent HAHLT 12 weeks after coronary artery ligation.

Figures 4.4 to 4.7 provide an illustration of the values for IS and AAR for the loaded and unloaded permanently ligated and ischemia-reperfused hearts. These show that in the permanently ligated hearts whilst there was a decrease in AAR with unloading, the actual IS in the unloaded heart increased thus resulting in the observed increase in IR. (Fig. 4.5)

By contrast, there was a decrease in actual IS in the unloaded ischaemia-reperfused hearts hence despite the presence of a reduction in AAR (which was similar in degree to that seen in the unloaded permanently ligated heart), the observed IR was significantly less after ischaemia-reperfusion. (Fig. 4.7)

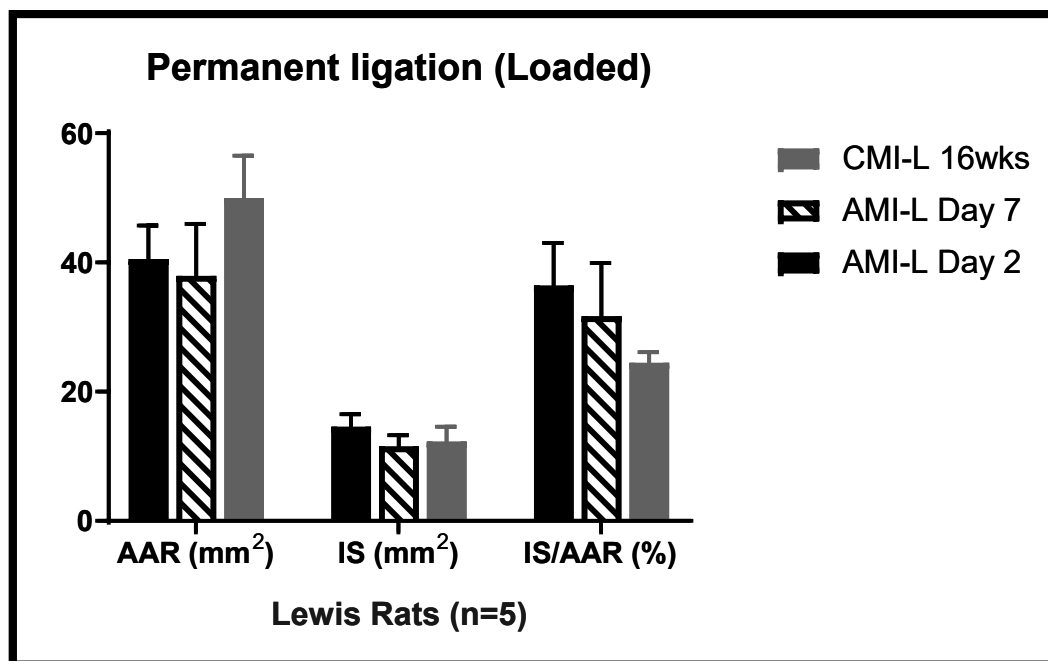


Figure 4.4: Illustration of the AAR, IS, and IR (IS/AAR) in the loaded permanently ligated hearts at days 2, 7 and 16 weeks.

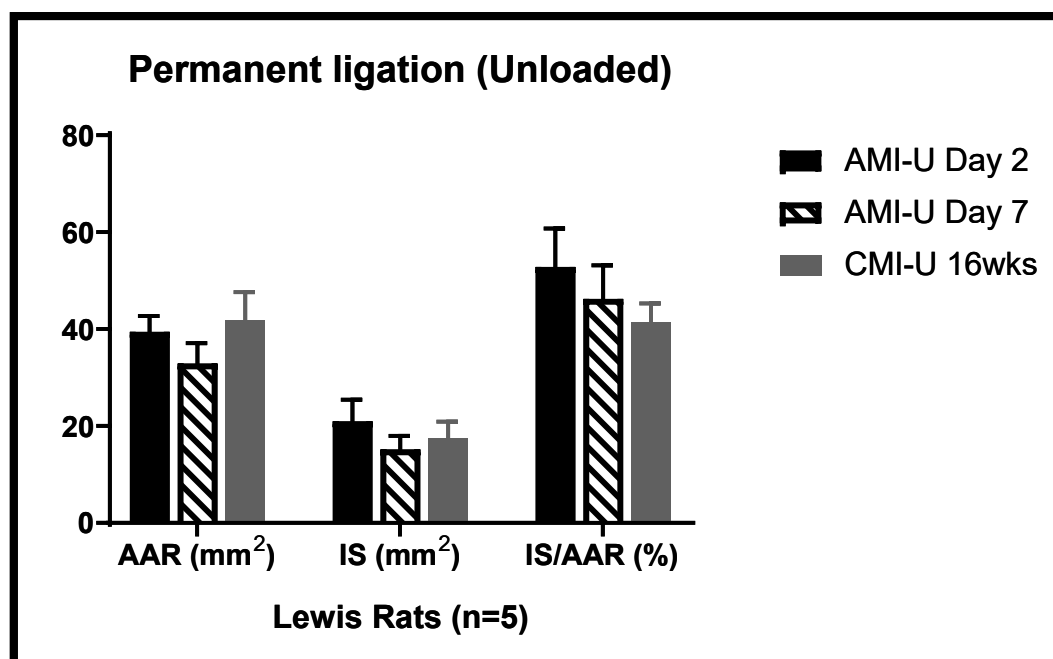


Figure 4.5: Illustration of the AAR, IS, and IR (IS/AAR) in the unloaded permanently ligated hearts at days 2, 7 and 16 weeks. Note that whilst the AAR decreased with mechanical unloading, the IS increased thus accounting for the increase in IR following mechanical unloading in the permanently ligated hearts.

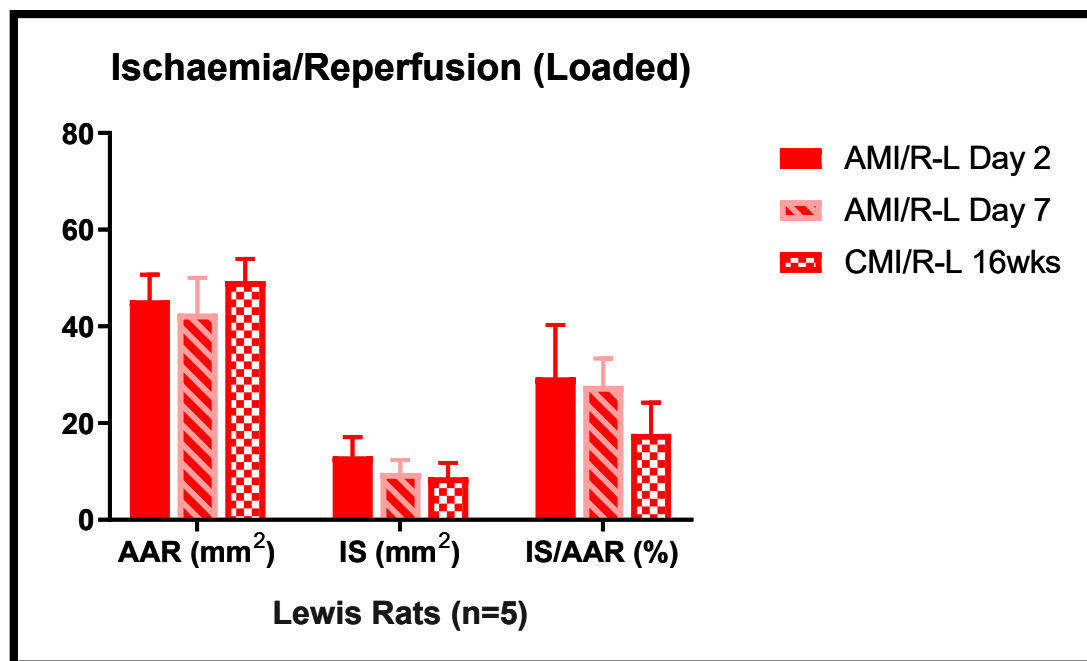


Figure 4.6: Illustration of the AAR, IS, and IR (IS/AAR) in the loaded ischaemia-reperfused hearts at days 2, 7 and 16 weeks

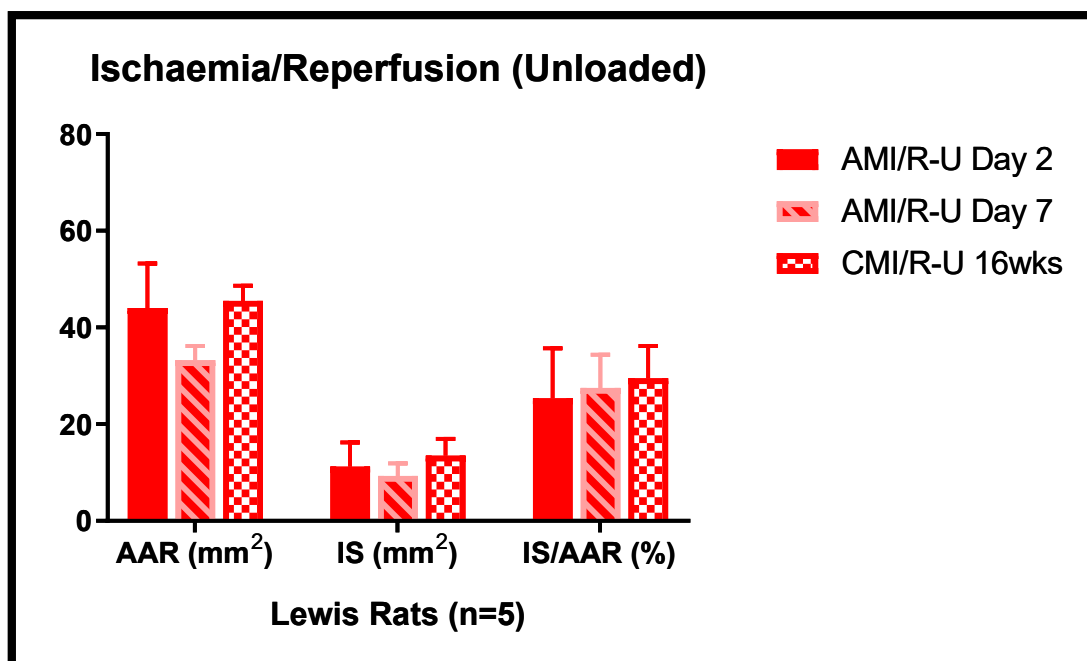


Figure 4.7: Illustration of the AAR, IS, and IR (IS/AAR) in the unloaded ischaemia-reperfused hearts at days 2, 7 and 16 weeks showing no significant change in IS despite a decrease in AAR (particularly at day7) with mechanical unloading. Thus decrease in AAR after mechanical unloading was not the cause of the lack of increase in IR observed in the group.

Furthermore, in an attempt to eliminate the influence of atrophic changes on the observed IR, an average AAR (aAAR) of all the loaded and unloaded hearts (combined) in each group (day 2, day 7 and 16 weeks) was obtained. By using the aAAR to obtain the IR, we believe that a normalisation of the atrophic changes that occur after unloading was achieved and thus the differences in IR obtained between the permanently ligated and ischaemia-reperfused hearts were more a reflection of actual differences in IS in both groups as shown in figures 4.8 and 4.9.

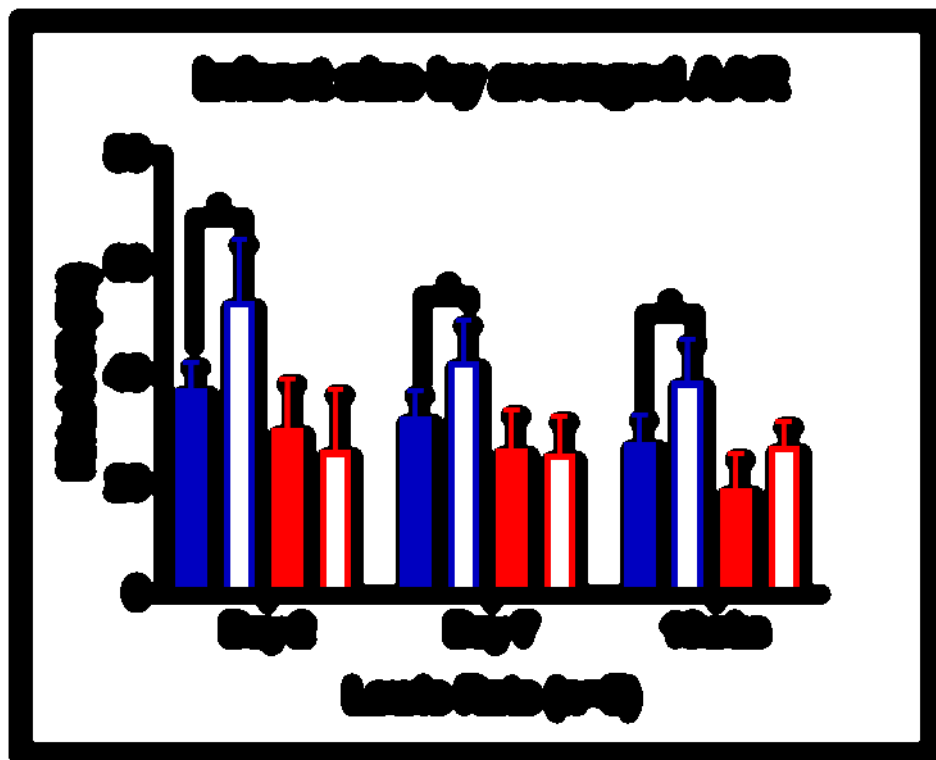


Figure 4.8: Effect of mechanical unloading on IR obtained using the average AAR of the loaded and unloaded hearts in each group. Note the worsening of IR is retained in the mechanically unloaded permanently ligated hearts at day 2, day 7 and 16 weeks.

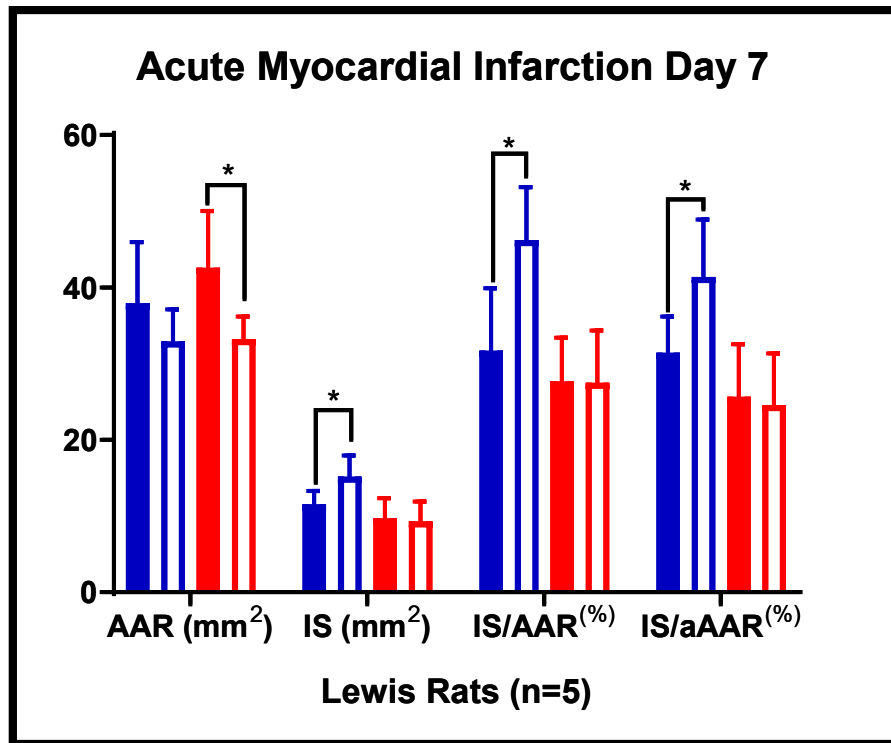


Figure 4.9: Detailed illustration of the AAR, actual IS and IR at day 7. Note here that when the average AAR of the loaded and unloaded hearts was used to determine IR (IS/aAAR) there was no significant change in findings. Mechanical unloading in the permanently ligated hearts still resulted in an increase in IR despite the normalisation of AAR between the loaded and unloaded hearts.

Data from figures 4.8 and 4.9 show that in this case, the myocardial atrophy seen with mechanical unloading was not the cause of the significant increases in IR observed in the unloaded permanently ligated heart, as elimination of the variability in AAR between the two groups did not have an impact on the changes in IR observed.

4.4 RESULTS II: Effect of Mechanical Unloading on Interstitial Fibrosis after Acute and Chronic Myocardial Infarction

Mechanical unloading in the permanently ligated hearts led to a statistically significant worsening of IF compared to loaded hearts at day 2 (AMI-L 8.146 ± 0.4943 , vs AMI-U 10.91 ± 0.2783 , $p=0.0012$) and day 7 (AMI-L 8.600 , vs AMI-U 10.97 , $p=0.0363$). In the ischaemia-reperfusion group however, mechanical unloading after acute MI was not associated with worsening of IF at day 2 (AMI/R-L 8.788 ± 0.2613 , vs AMI/R-U 8.286 ± 0.7162 , $p=0.5287$) and day 7 (AMI/R-L 9.696 ± 1.071 , vs AMI/R-U 8.998 ± 0.4993 , $p=0.5711$) as illustrated in figure 4.10.

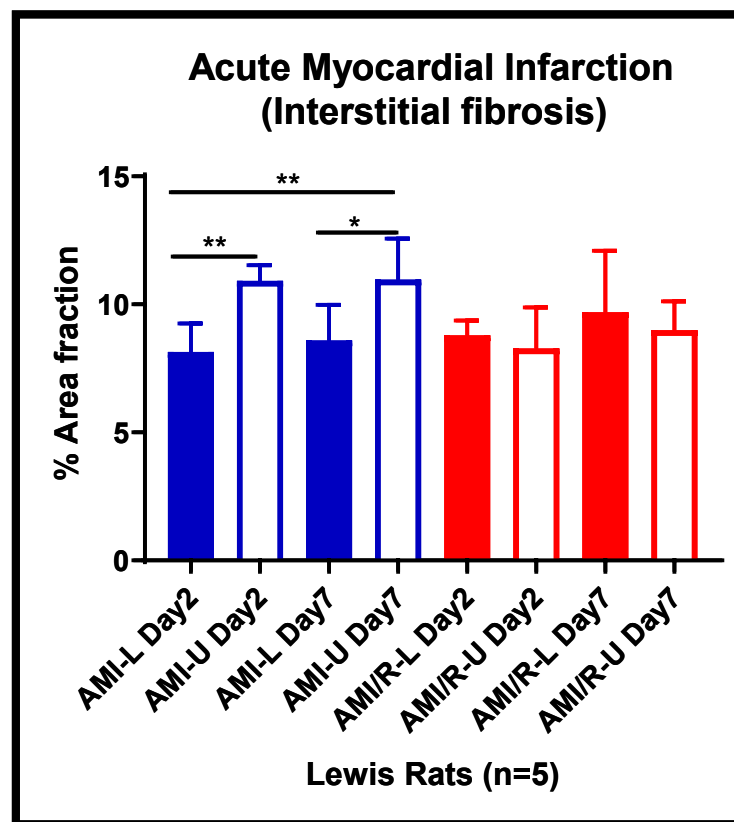


Figure 4.10: Difference in interstitial fibrosis between loaded and unloaded Lewis rat hearts after permanent coronary artery ligation and ischaemia-reperfusion.

The worsening of IF with mechanical unloading observed in the permanently ligated hearts as opposed to the re-perfused hearts was readily visible as shown in figures 4.11 to 4.14. The red stain in the images represent collagen deposition.

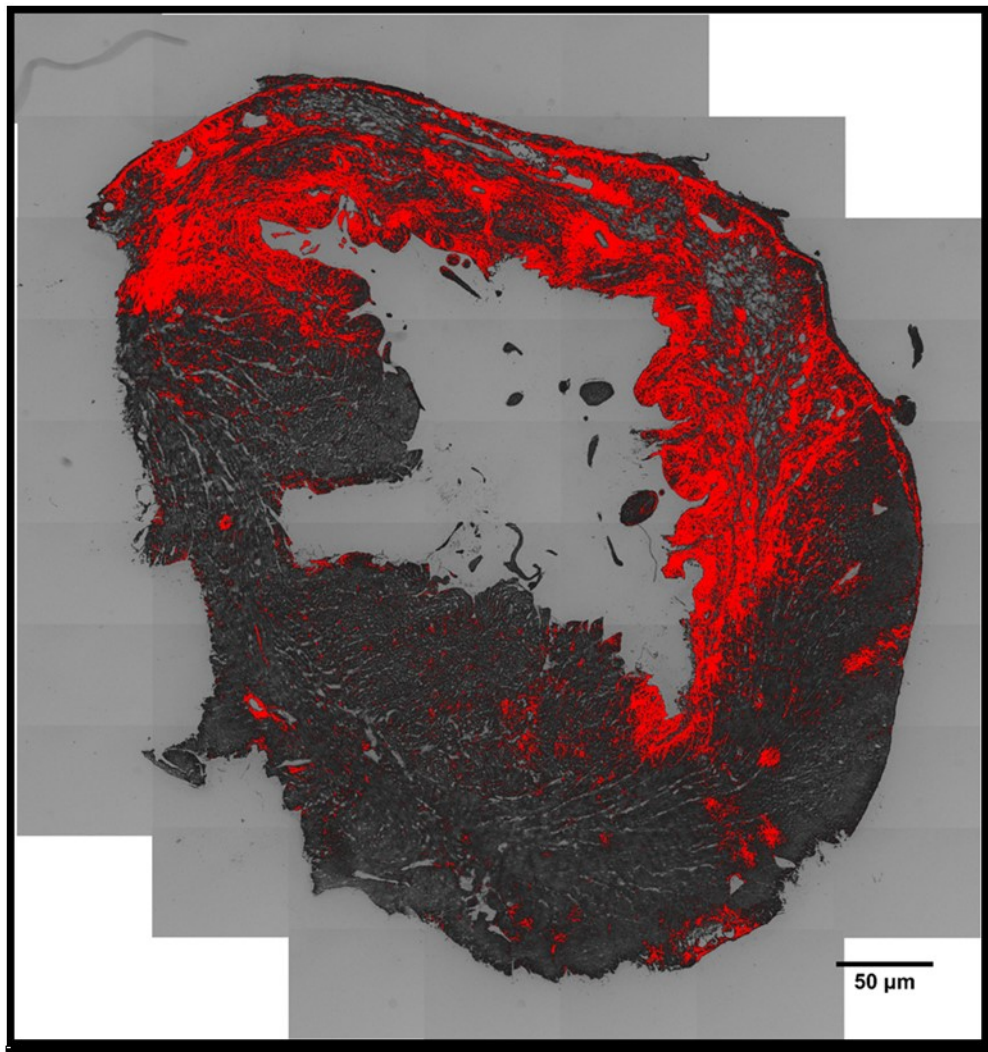


Figure 4.11: IF in an AMI-L sample at day 7. Sirius red staining was used to determine areas of collagen deposition and thus fibrosis (stained red). Thresholding tool on imageJ was used for analysis. Here there is increased deposition of collagen in the infarcted territory with only mild collagen expression in the un-infarcted areas.

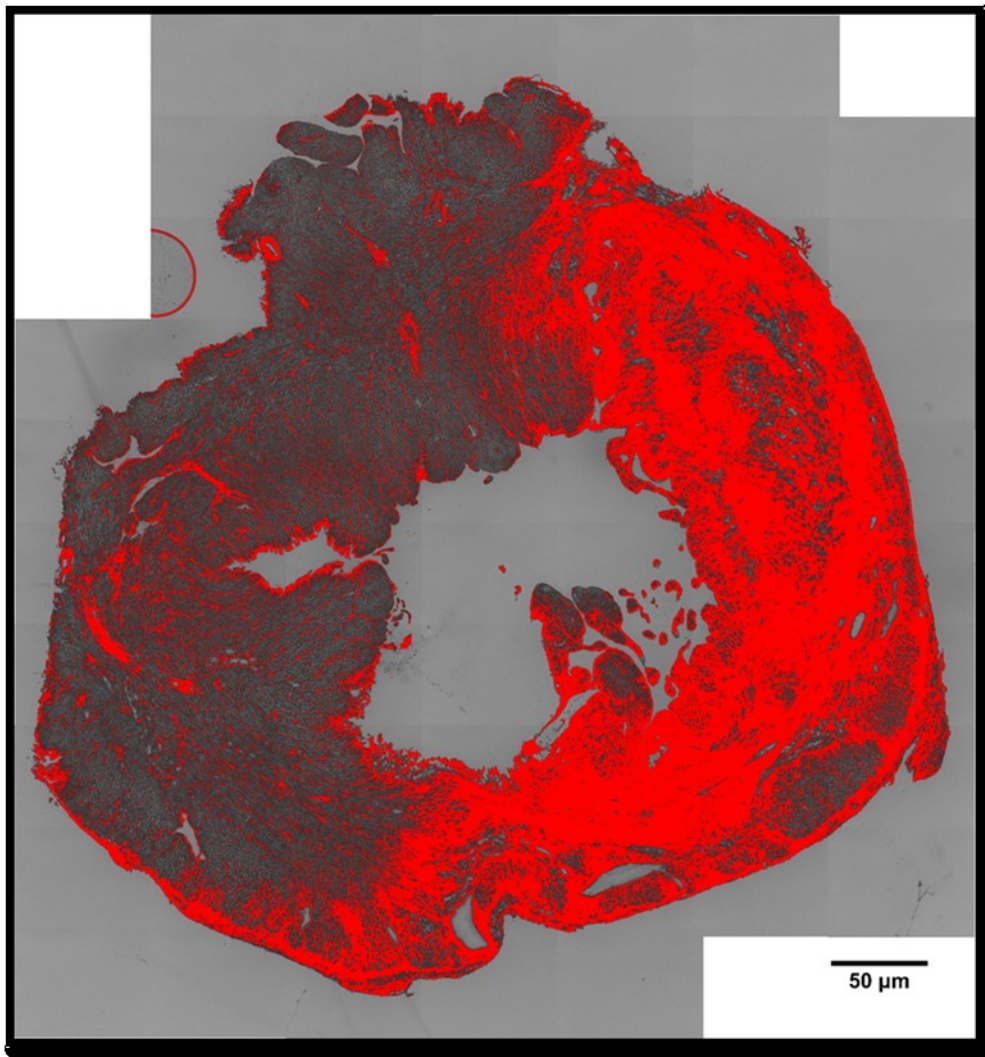


Figure 4.12: IF in an AMI-U sample at day 7 showing increased expression of collagen in the interstitium after mechanical unloading.

Figure 4.12 shows significantly worse IF after mechanical unloading. In the ischaemia-reperfusion group however, mechanical unloading did not bring about significant worsening of IF as shown in Figures 4.13 and 4.14.

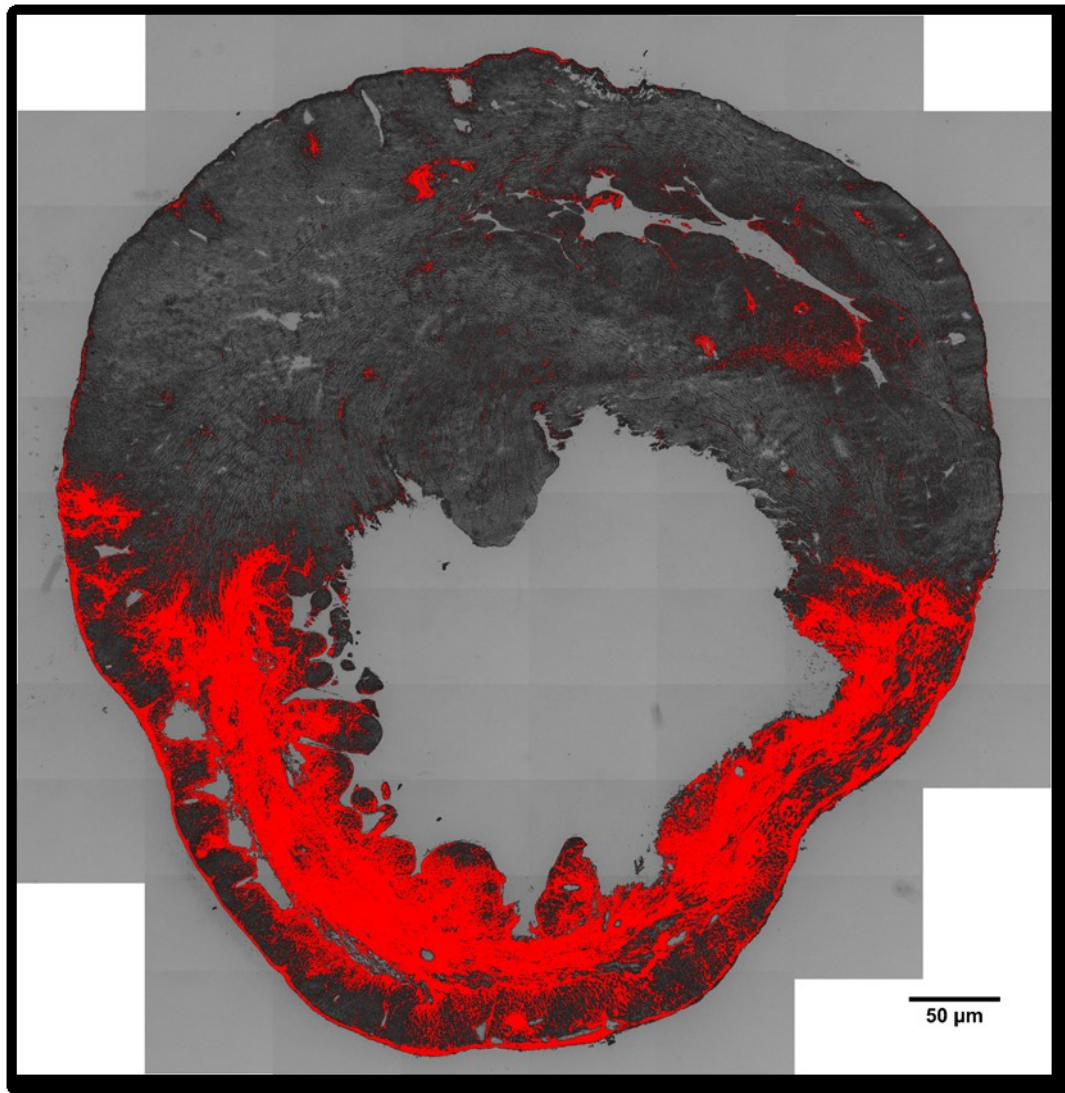


Figure 4.13: IF in an AMI/R-L sample at day 7. This also shows that in the loaded heart collagen expression was predominantly localised to the infarct territory with only mild expression in the interstitium.

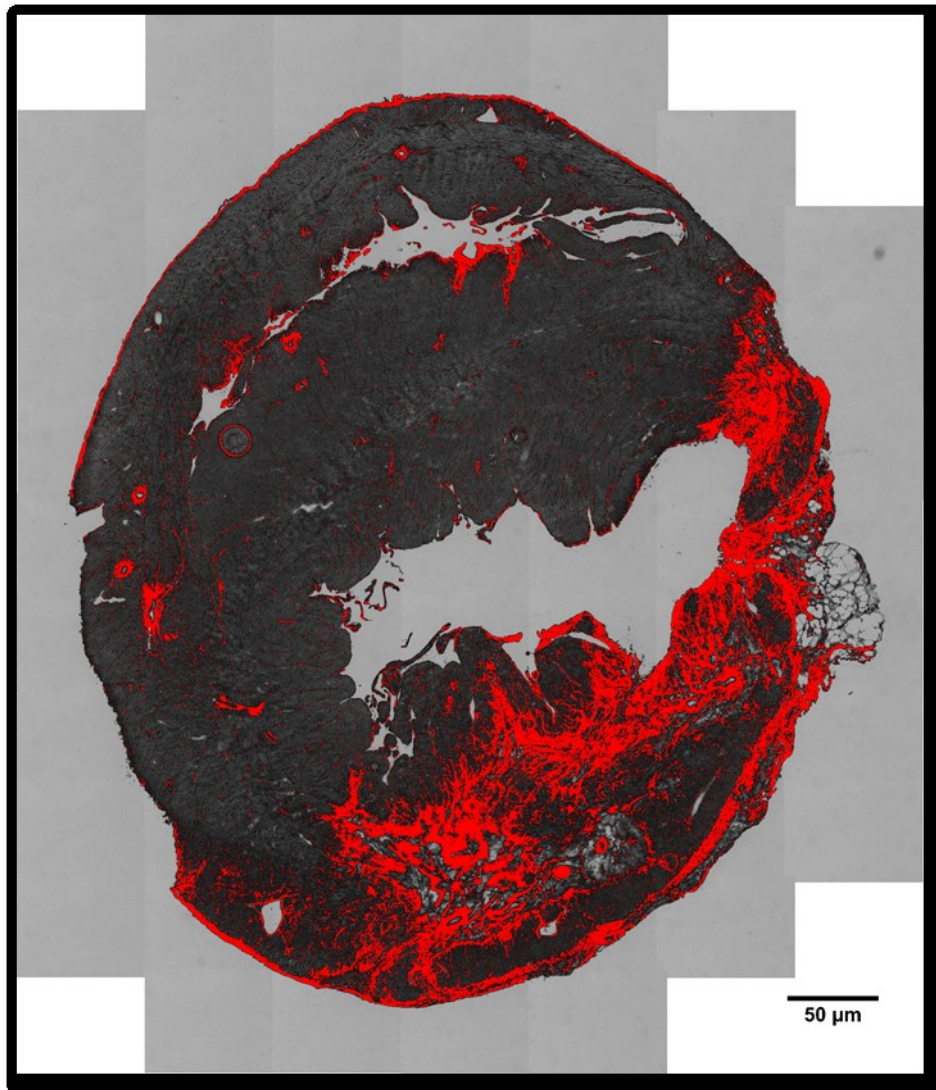


Figure 4.14: IF in an AMI/R-U sample at day 7. Remarkably, as the image shows, after ischaemia-reperfusion, mechanical unloading did not bring about a significant increase in interstitial fibrosis as opposed to the permanently ligated hearts where unloading was associated with higher levels of IF.

Figure 4.14 shows much less collagen deposition in the interstitium after mechanical unloading compared to that observed in the unloaded permanently ligated heart.

In the chronic MI group, mechanical unloading of the permanently ligated hearts also led to a statistically significant increase in interstitial fibrosis compared to the loaded hearts (CMI-L 8.684, vs CMI-U 17.23, $p < 0.0001$). However, in the ischaemia-reperfusion group, the observed

worsening of IF with mechanical unloading was retained (CMI/R-L 8.38 ± 0.4765 , vs CMI/R-U 15.57 ± 0.9938 , $p= 0.0002$). (Fig. 4.15)

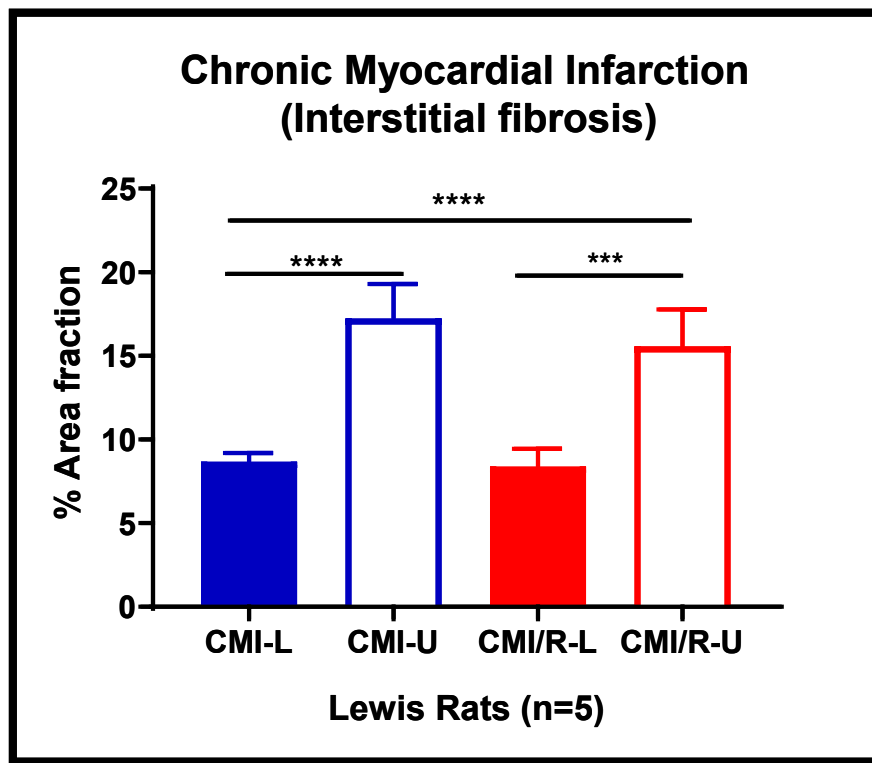


Figure 4.15: Interstitial fibrosis in the chronic permanently ligated and ischaemia-reperfused hearts showing the effects of 4 weeks of mechanical unloading in failing hearts. These hearts underwent HAHLT 12 weeks after coronary artery ligation and were explanted for analysis at 16 weeks having undergone 4 weeks of unloading.

Figures 4.16 to 4.19 are representative samples from each group that help illustrate the changes in IF in the chronically infarcted heart after mechanical unloading.

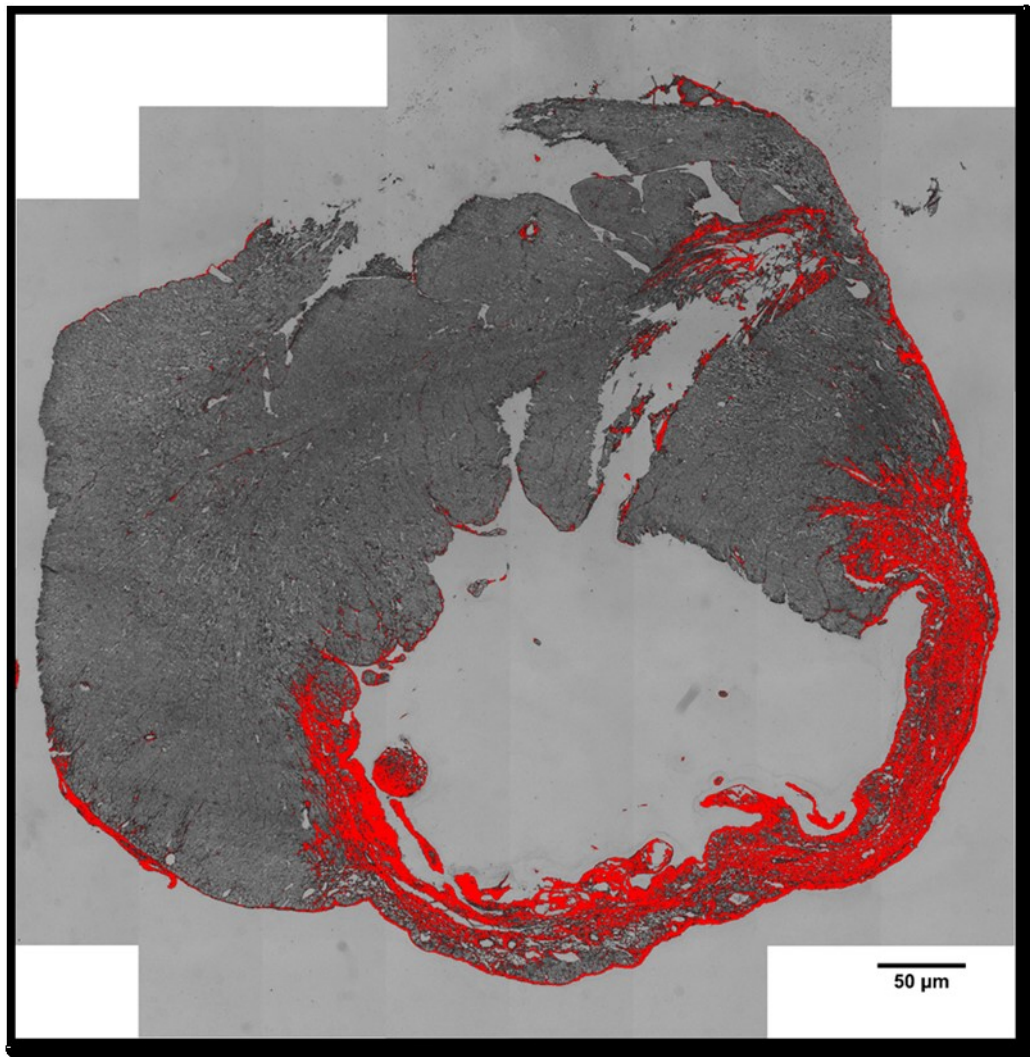


Figure 4.16: IF in a CMI-L rat heart sample showing only mild diffuse collagen deposition in the interstitium.

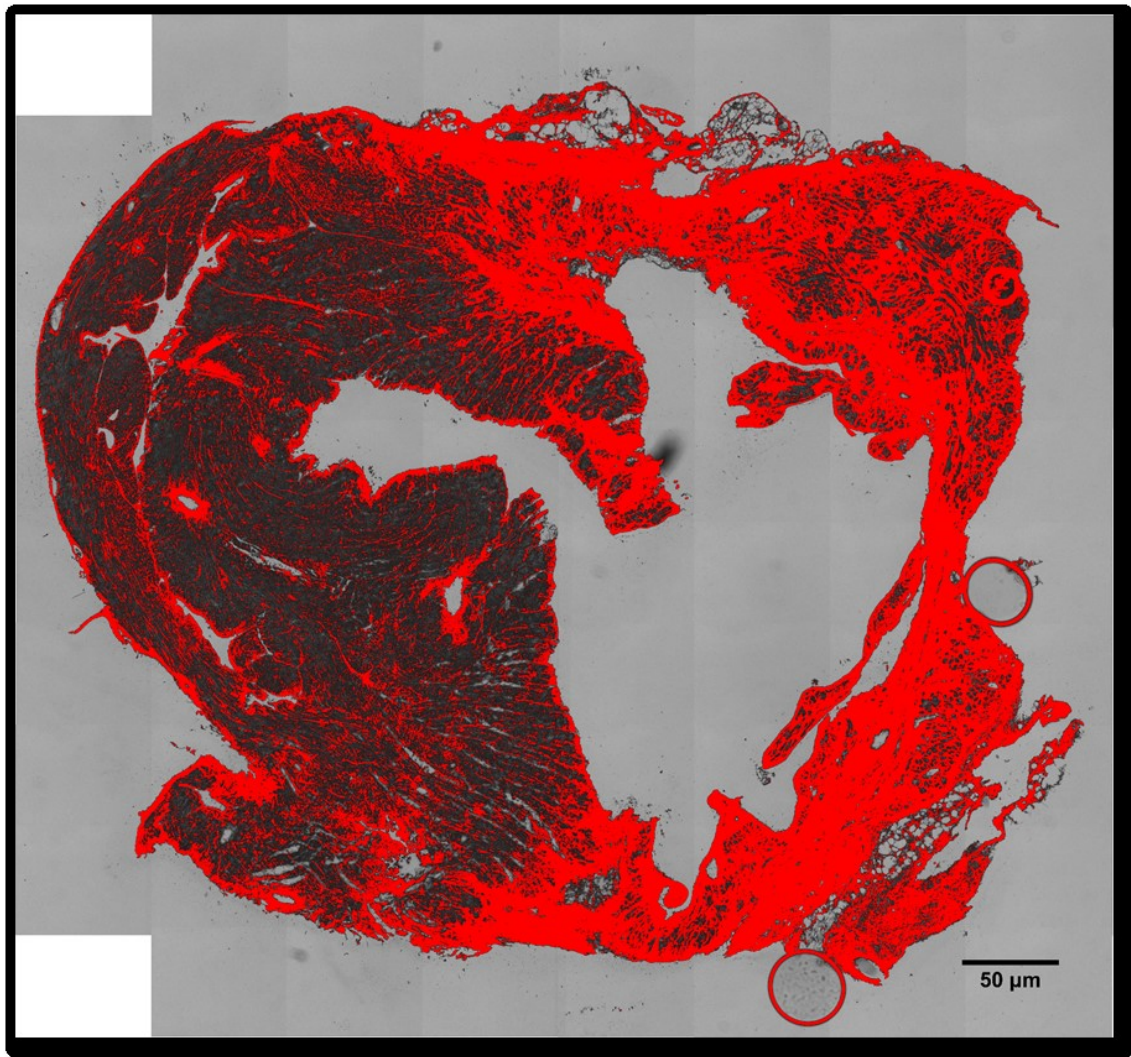


Figure 4.17: IF in a CMI-U sample showing significant upregulation of collagen expression in the interstitium after mechanical unloading.

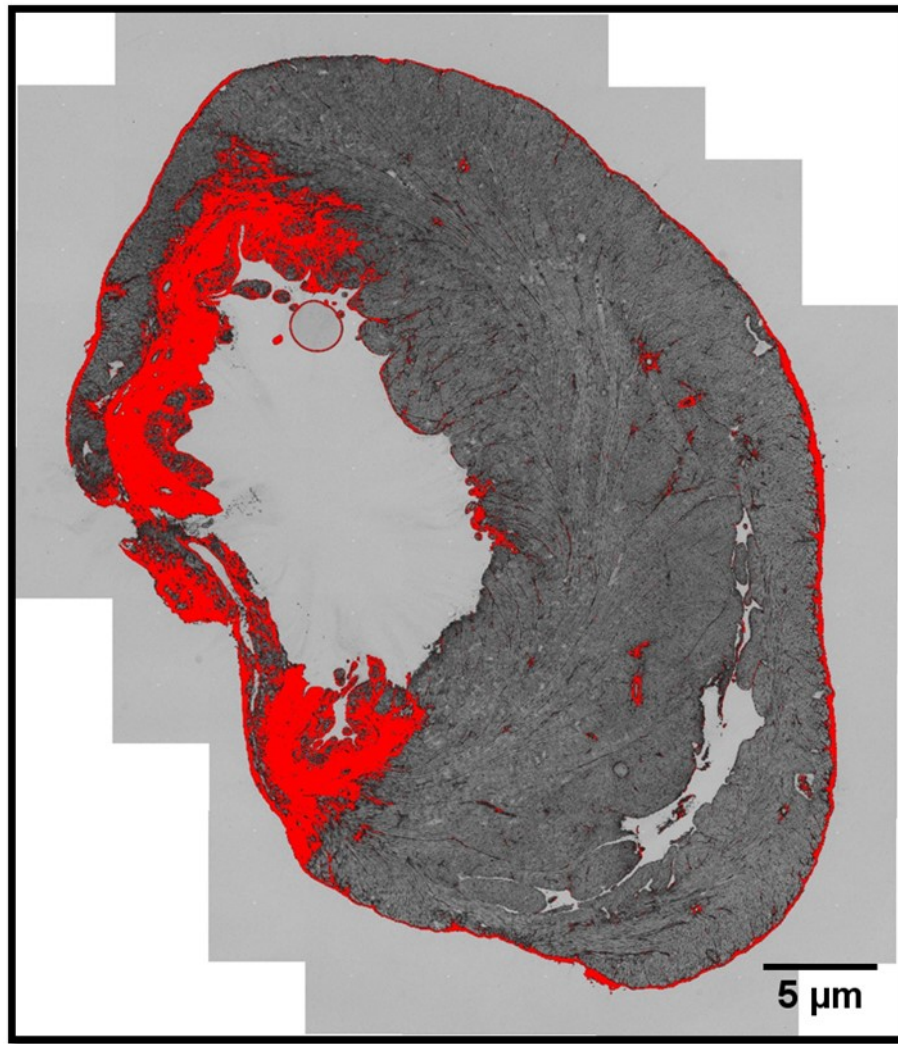


Figure 4.18: IF in a CMI/R-L rat heart sample showing a similar degree of collagen deposition in the interstitium to that of the loaded permanently ligated heart.

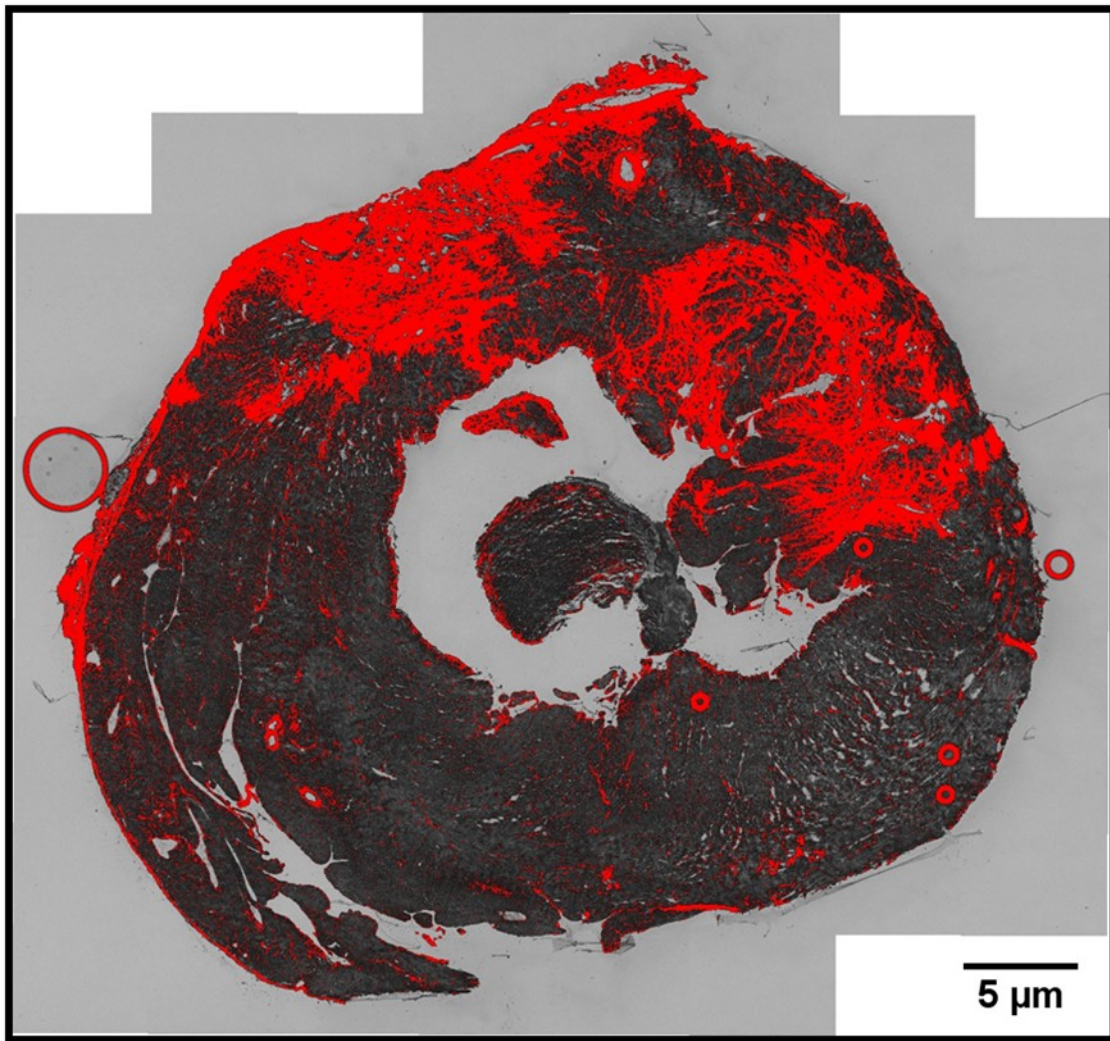


Figure 4.19: IF in a CMI/R-U rat heart sample, again showing significant upregulation of collagen deposition in the interstitium after mechanical unloading. It is interesting to note here that fibrosis increased despite reperfusion in the chronic hearts whilst this did not occur in the reperfused acute hearts.

Figures 4.10 to 4.19 thus show that interstitial fibrosis worsens after mechanical unloading in permanently ligated hearts. In the acute ischaemia-reperfused hearts, mechanical unloading was found not to lead to worsening fibrosis. However, this apparent protective effect was lost in the chronic ischaemia-reperfused hearts where unloading was found to also lead to an increase in interstitial fibrosis.

4.5 RESULTS III: Effect of Mechanical Unloading on the Dynamic Expression Profiles of α SMA, TGF- β 1, MMP-1, and TIMP-1, after Acute and Chronic Myocardial Infarction

4.5.1 Effect of mechanical unloading on α SMA expression after acute & chronic myocardial infarction

In the loaded permanently ligated heart, AMI resulted in a statistically significant rise in α SMA protein expression compared to control at both day 2 (CTR 0.2854 ± 0.08482 , vs AMI-L 1.33 ± 0.01442 , $p=0.0005$) and day 7 (CTR 0.2854 ± 0.08482 , vs AMI-L 1.842 ± 0.3022 , $p=0.0291$) as shown in figures 4.20 & 4.21. Mechanical unloading was associated with a further rise in α SMA protein expression at both day 2 and day 7 but these were not statistically significant.

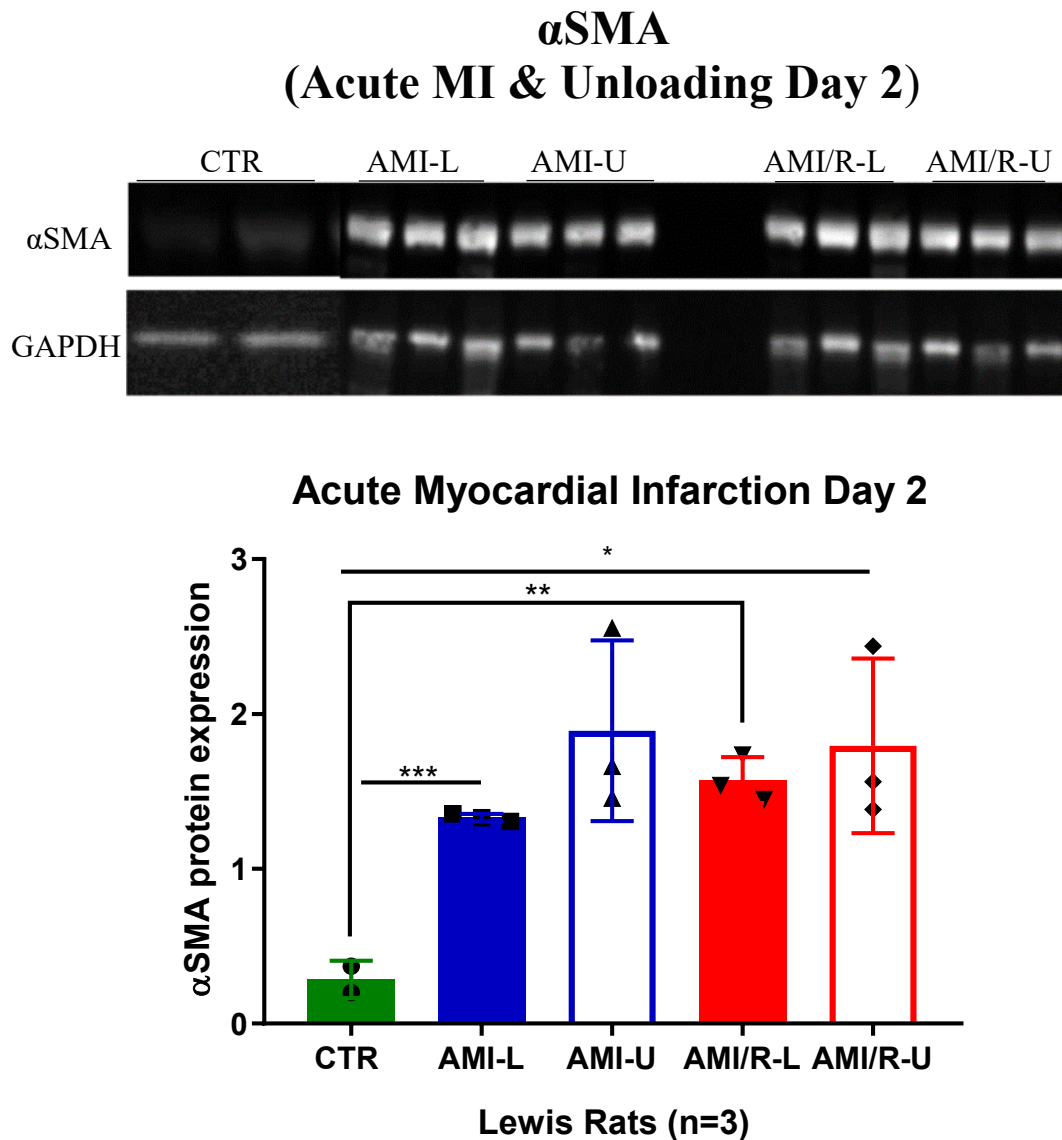


Figure 4.20: α SMA expression profile after permanent coronary artery ligation and ischaemia-reperfusion showing the effect of mechanical unloading at day 2. The CTR samples were run on the same membranes as AMI but repositioned in the figure for visual clarity.

In the ischemia-reperfusion group, α SMA protein expression also rose significantly in the loaded hearts at day 2 compared to control (CTR 0.2854 ± 0.08482 , vs AMI/R-L 1.573 ± 0.08503 , $p = 0.0020$).

After mechanical unloading there was no statistically significant difference in α SMA protein expression at day 2. However, at day 7, mechanical unloading after ischaemia-reperfusion led

to a statistically significant decrease in α SMA protein expression (AMI/R-L 1.9 ± 0.1324 , vs AMI/R-U 1.185 ± 0.2172 , $p = 0.0483$) as shown in figure 4.21.

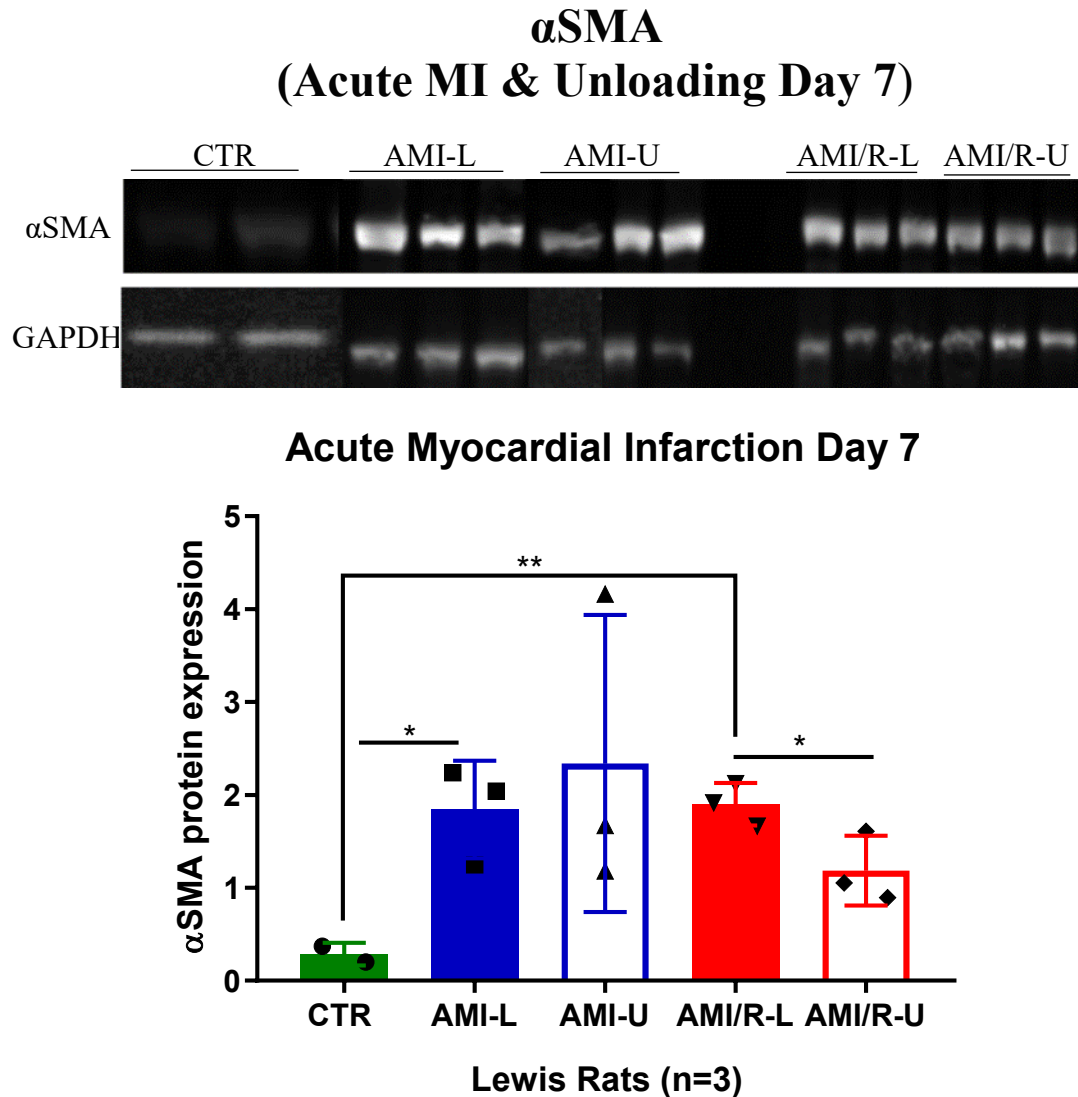


Figure 4.21: α SMA expression profile after permanent coronary artery ligation and ischaemia-reperfusion showing the effect of mechanical unloading at day 7. The CTR samples were run on the same membranes as AMI but repositioned in the figure for visual clarity.

After chronic myocardial infarction (16 weeks), α SMA protein expression in the loaded permanently ligated hearts remained significantly higher than control (CTR 1.343, vs CMI-L 2.613, $p = 0.0072$). Mechanical unloading in the permanently ligated group led to a statistically significant increase in α SMA protein expression (CMI-L 2.613 ± 0.1016 , vs CMI-U $5.032 \pm$

0.254, $p= 0.0009$). In the ischaemia-reperfusion group, there was also a significant rise in α SMA protein expression compared to control (CTR 1.343 ± 0.1916 , vs CMI/R-L 2.89 ± 0.1523 , $p= 0.0078$). Mechanical unloading in this group also resulted in a statistically significant increase in α SMA protein expression (CMI/R-L 2.890, vs CMI/R-U 3.855, $P= 0.0306$). (Fig. 4.22)

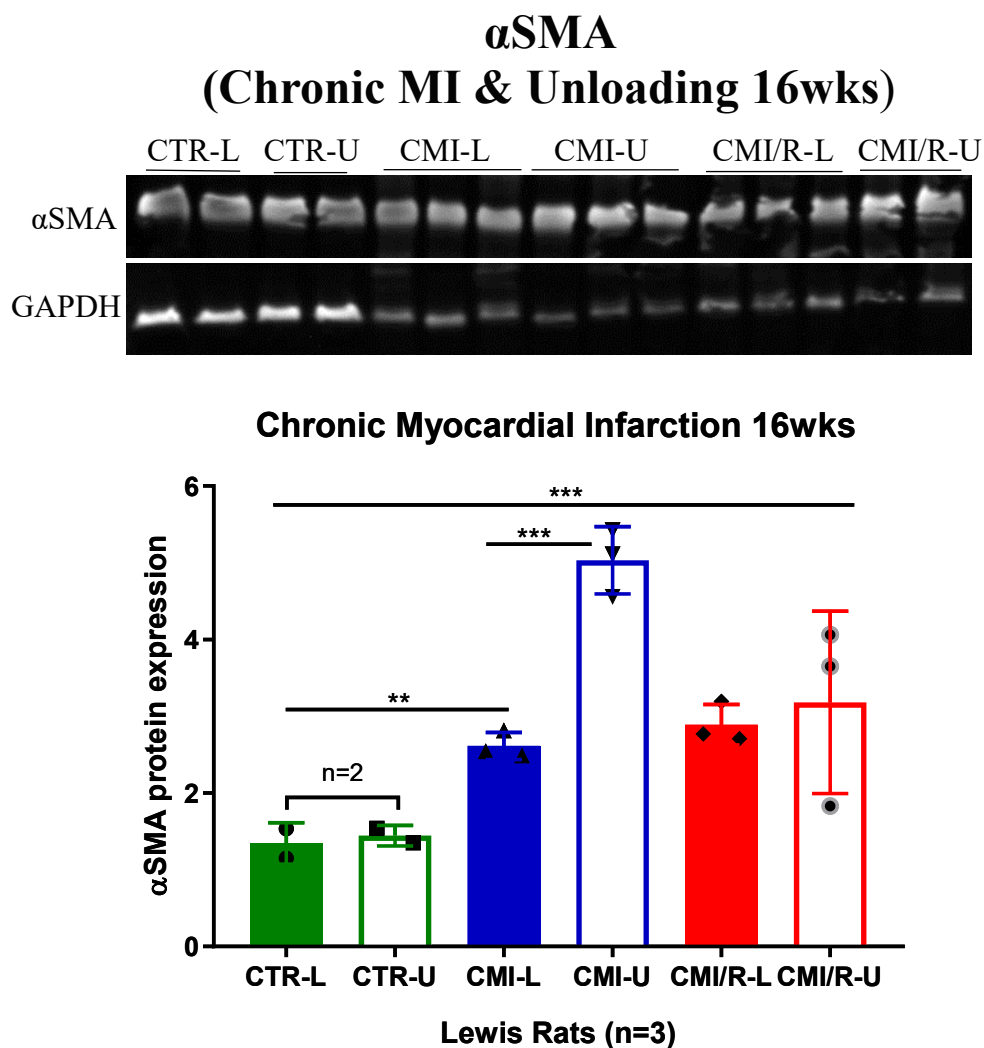


Figure 4.22: α SMA protein expression in the failing heart at 16 weeks following permanent coronary artery ligation and ischaemia-reperfusion showing the effect of 4 weeks of mechanical unloading in the chronically infarcted heart. Three rats were tested in each group except where indicated.

4.5.2 Effect of mechanical unloading on TGF- β 1 expression after acute & chronic MI

Compared to the loaded hearts, mechanical unloading after permanent coronary artery ligation and ischemia-reperfusion did not bring about a statistically significant difference in TGF- β 1 protein expression at day 2 or day 7 as shown in figures 4.23 and 4.24.

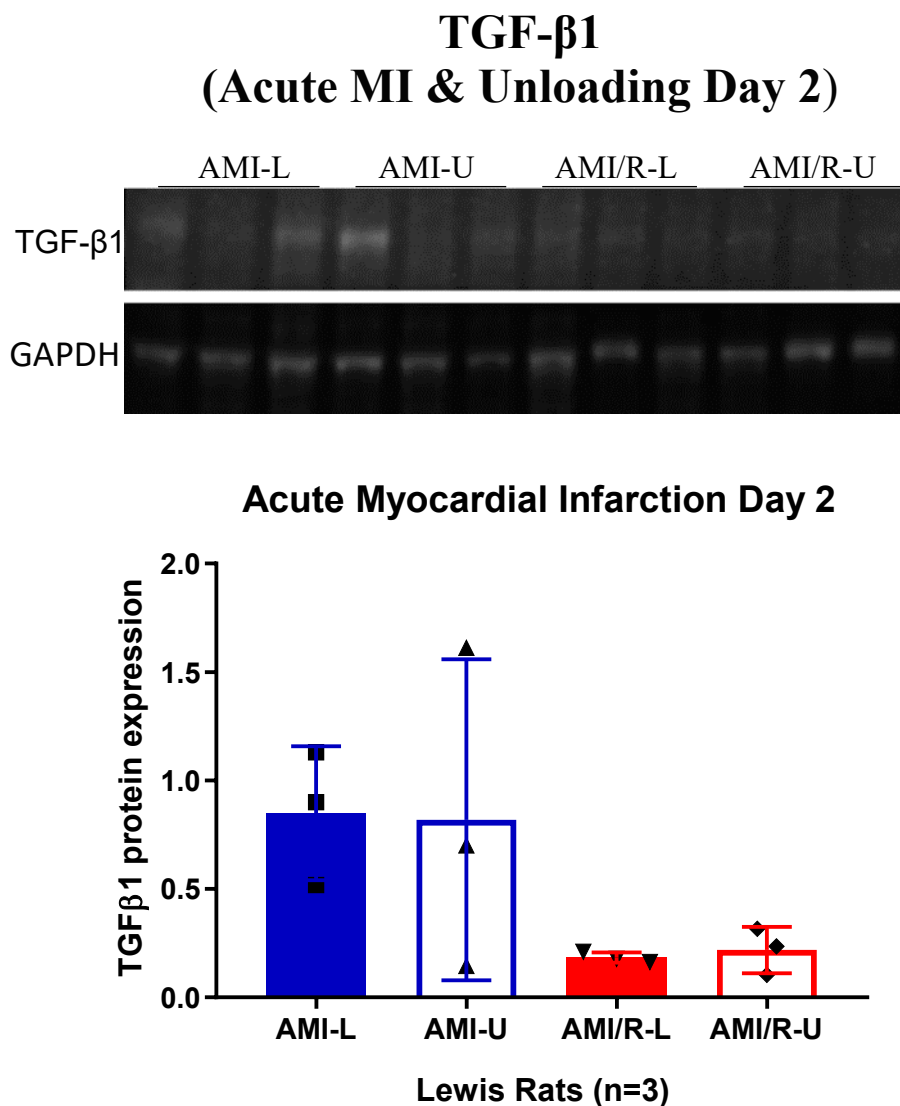


Figure 4.23: TGF- β 1 expression profile after permanent coronary artery ligation and ischaemia-reperfusion showing the effect of mechanical unloading at day 2.

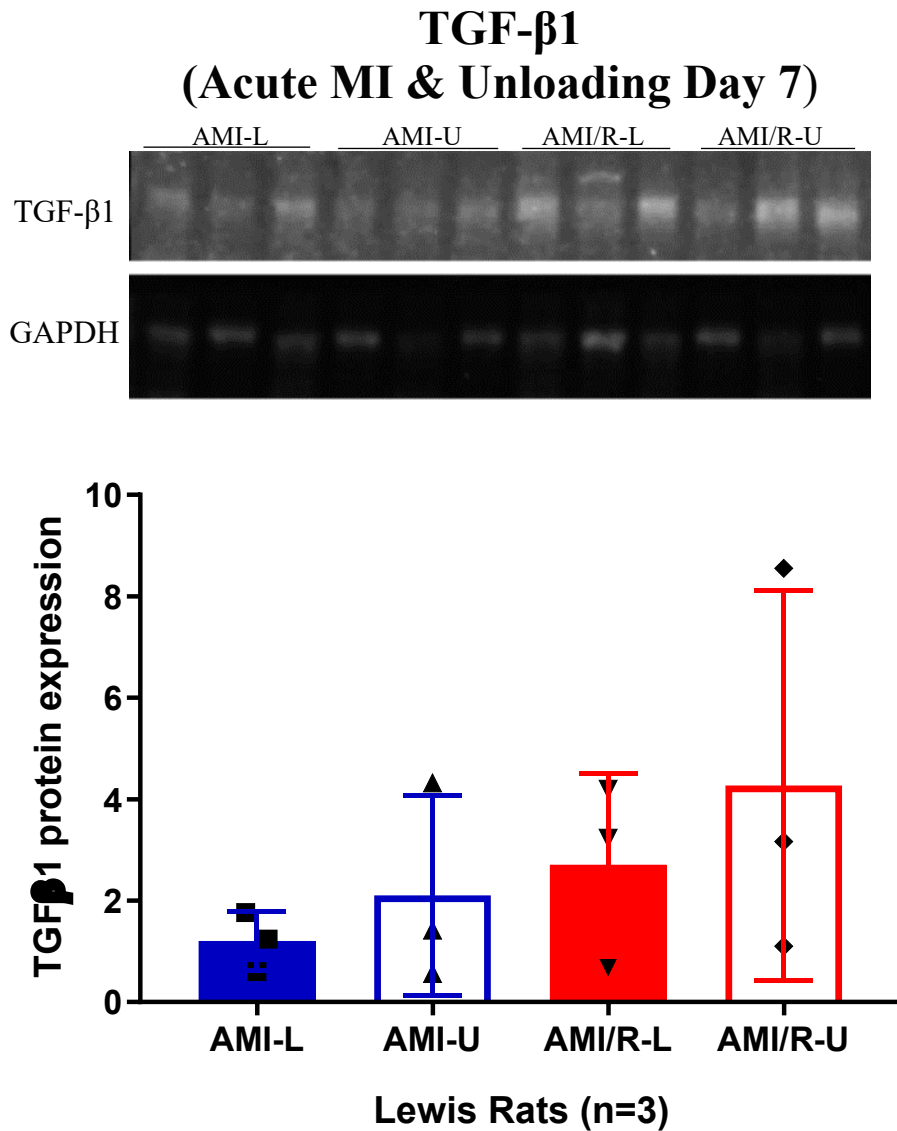


Figure 4.24: TGF- β 1 expression profile after permanent coronary artery ligation and ischaemia-reperfusion showing the effect of mechanical unloading at day 7.

After 16 weeks, mechanical unloading led to a statistically significant increase in TGF- β 1 protein expression after permanent coronary ligation (CMI-L 1.930, vs CMI-U 4.922, $p=0.0146$) and ischaemia-reperfusion (CMI/R-L 1.890, vs CMI/R-U 3.540, $P=0.0429$). (Fig. 4.25)

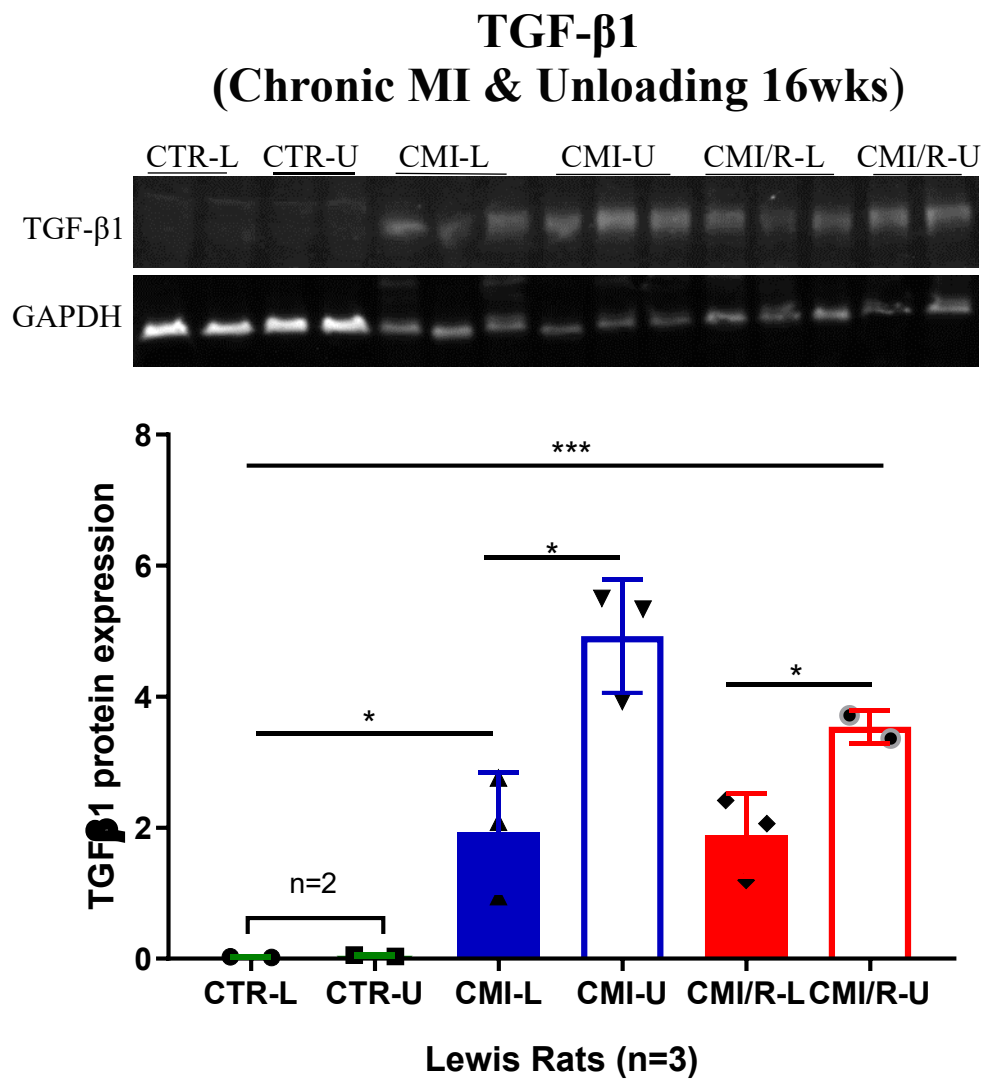


Figure 4.25: TGF- β 1 protein expression in the failing heart at 16 weeks following permanent coronary artery ligation and ischaemia-reperfusion showing the effect of 4 weeks of mechanical unloading in the chronically infarcted heart.

The expression levels of TGF- β 1 in the chronic ischaemia-reperfused hearts were significantly higher than in the acute (day 2) hearts (AMI-L 0.1833, vs CMI-L 1.890, $P=0.0094$) and (AMI/R-U 0.2182, vs CMI/R-U 3.115, $P=0.0028$). (Fig. 4.26)

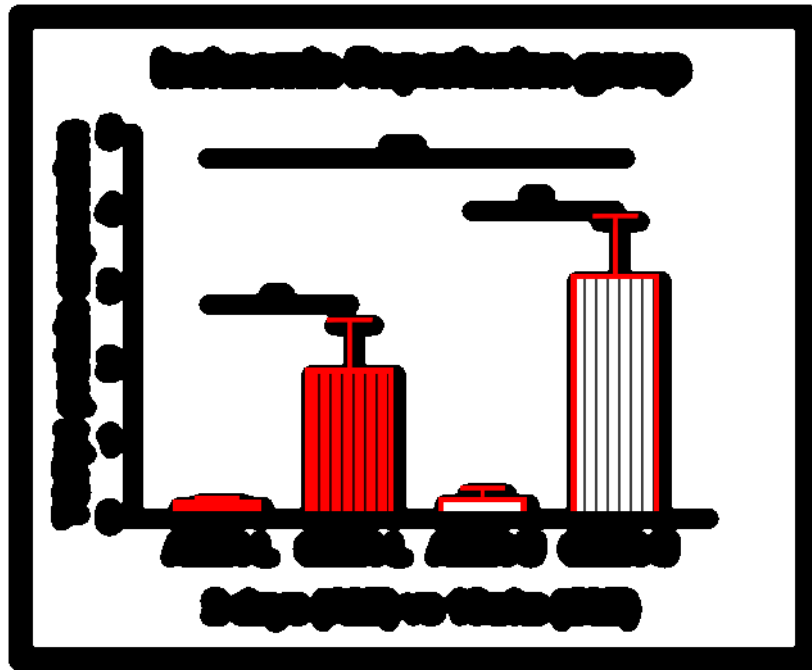


Figure 4.26: TGF- β 1 protein expression after acute and chronic myocardial infarction in the ischaemia-reperfusion group showing significant upregulation of expression in the chronic hearts.

4.5.3 Effect of mechanical unloading on MMP-1 and TIMP-1 expression after acute & chronic MI

In the acute group at day 7, mechanical unloading after permanent coronary artery ligation led to a statistically significant decrease in the expression of MMP-1 (AMI-L 0.4570, vs AMI-U 0.2251, $p = 0.0198$). There was an equally significant decrease in MMP-1 expression after mechanical unloading in the ischaemia-reperfusion group (AMI/R-L 0.3798, vs AMI/R-U 0.1962, $p = 0.0374$). Ischaemia-reperfusion alone did not generate a significant difference in MMP-1 protein expression as shown in figure 4.27.

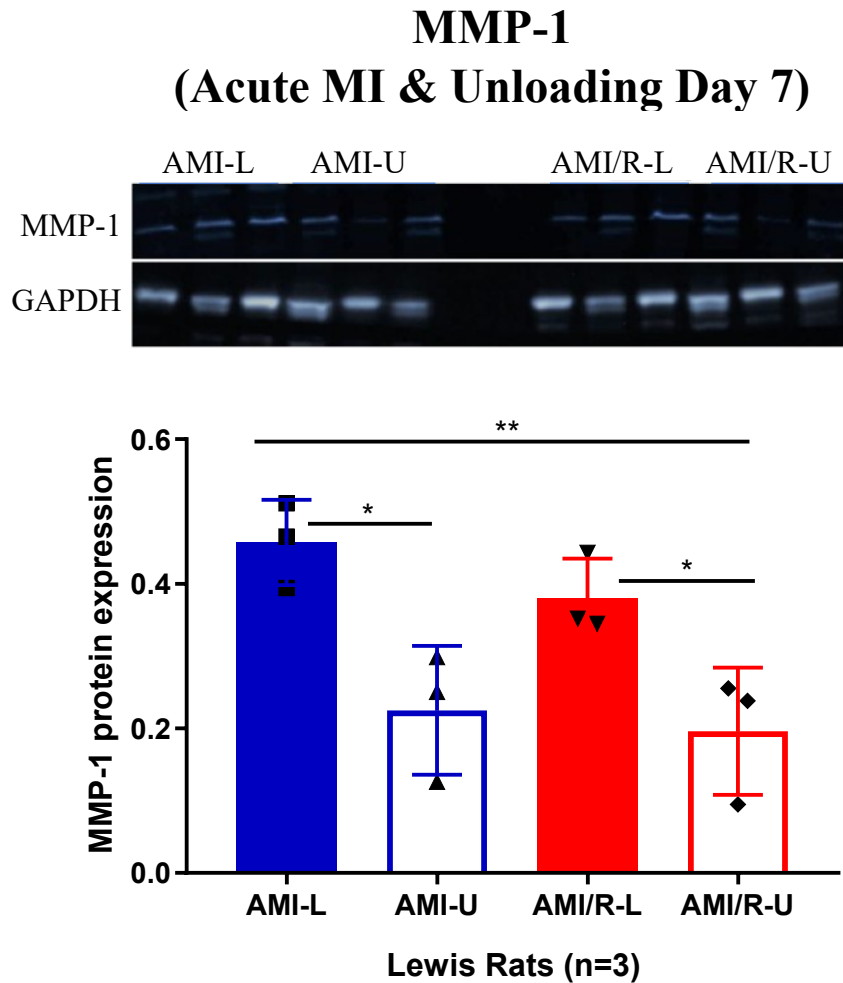


Figure 4.27: MMP-1 expression profile after permanent coronary artery ligation and ischaemia-reperfusion showing the effect of mechanical unloading.

Mechanical unloading did not bring about a significant change in TIMP-1 protein expression in the permanently ligated or ischaemia-reperfusion hearts at day 7 (AMI-L 0.2525, vs AMI-U 0.3537, $p=0.1356$) or (AMI/R-L 0.4474, vs AMI/R-U 0.2517, $p=0.1585$) respectively. (Fig. 4.28) There was however a statistically significant increase in TIMP-1 protein expression in the loaded re-perfused compared to the loaded permanently ligated hearts (AMI-L 0.2525 ± 0.04175 , vs AMI/R-L 0.4474 ± 0.05626 , $p=0.0498$) as shown in figure 4.28.

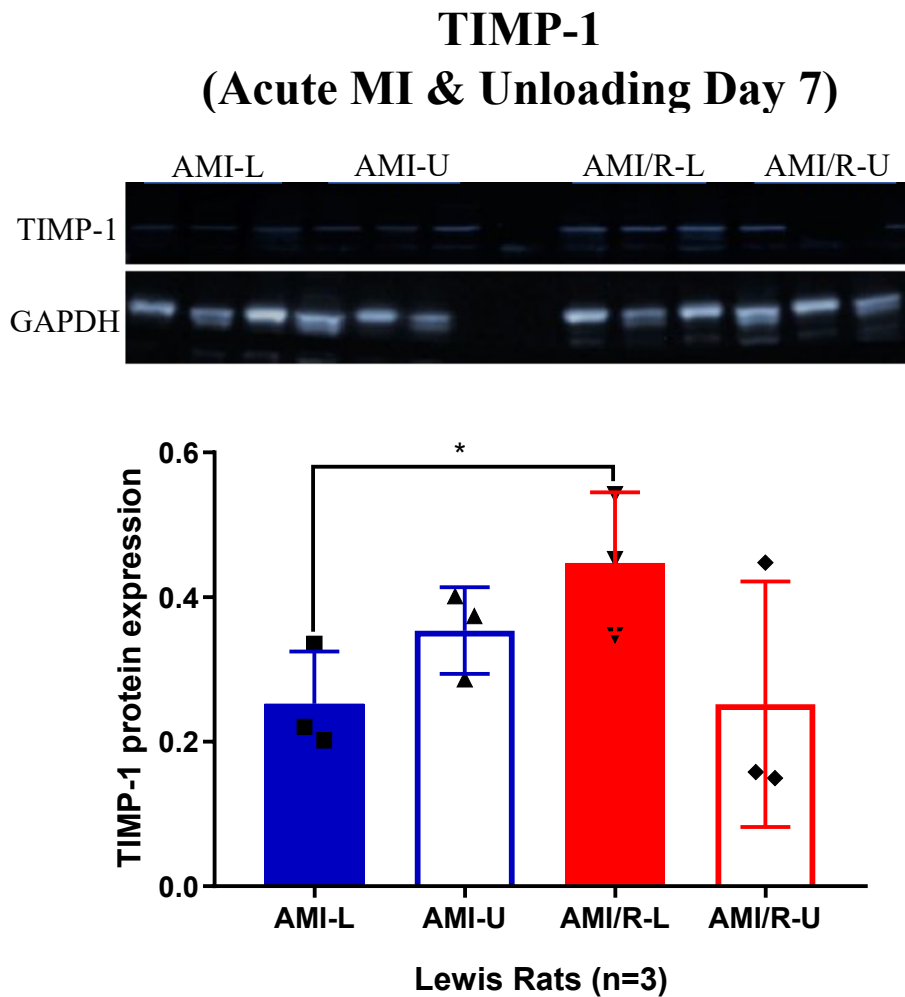


Figure 4.28: TIMP-1 expression profile after permanent coronary artery ligation and ischaemia-reperfusion showing the effect of mechanical unloading.

There was a statistically significant decrease in the ratio of MMP-1 : TIMP-1 after mechanical unloading in the permanently ligated hearts at day 7 (AMI-L 1.934, vs AMI-U 0.6759, $p=0.0440$). Ischemia-reperfusion was associated with a statistically significant downregulation of MMP-1 : TIMP-1 protein expression ratio compared to the permanently ligated hearts (AMI-L 1.934, vs AMI/R-L 0.8475, $p=0.0473$). There was no statistically significant difference in MMP-1 : TIMP-1 ratio between the loaded and unloaded hearts after ischemia-reperfusion as shown in figure 4.29.

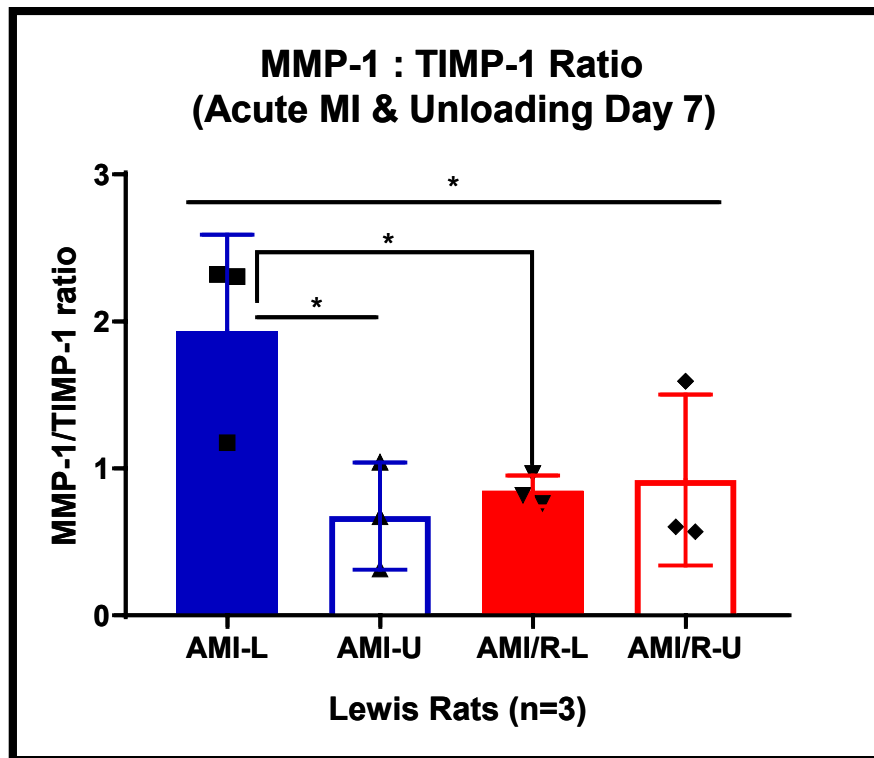


Figure 4.29: MMP-1 : TIMP-1 ratio after permanent coronary artery ligation and ischaemia-reperfusion showing the effect of mechanical unloading.

In the chronic setting, after 16 weeks MMP-1 protein expression was significantly lower compared to control in the permanently ligated loaded (CTR-L 0.6564 ± 0.2276 , vs CMI-L 0.08924 ± 0.03266 , $p= 0.0477$) and unloaded hearts (CTR-U 0.5717 ± 0.06769 , vs CMI-U 0.06796 ± 0.04516 , $p= 0.0073$). After chronic MI, mechanical unloading did not bring about a statistically significant difference in MMP-1 expression in the permanently ligated or ischaemia-reperfusion hearts as shown in figure 4.30.

MMP-1 (Chronic MI & Unloading 16wks)

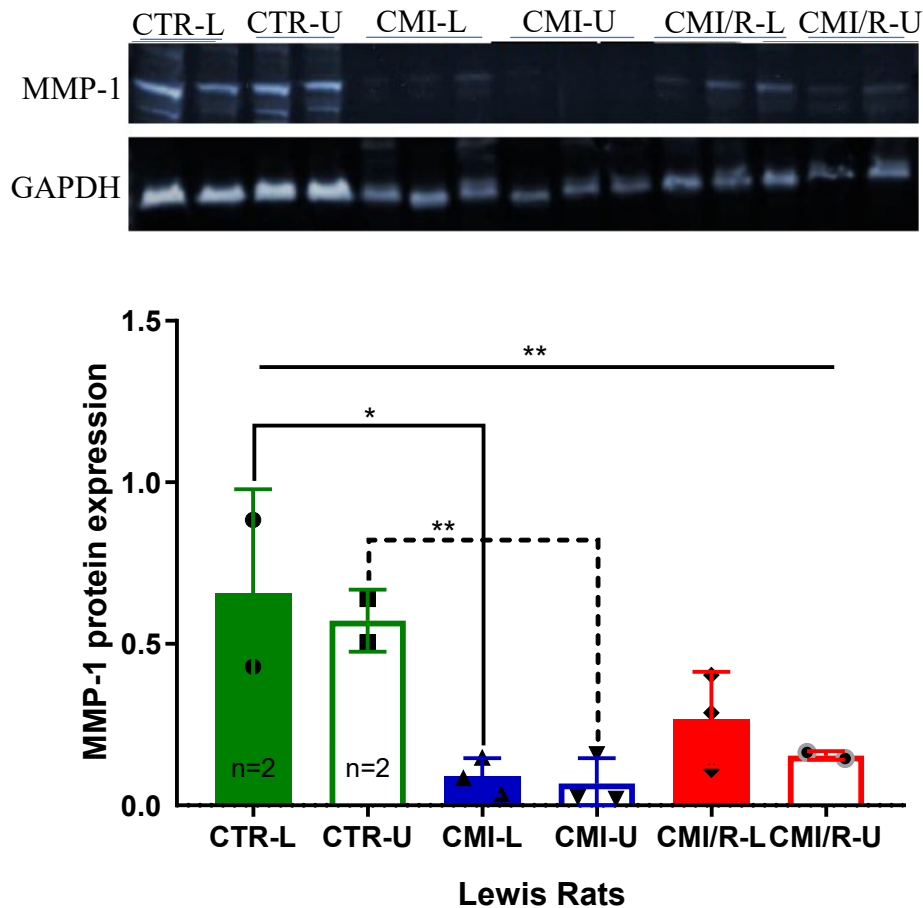


Figure 4.30: MMP-1 expression profile after permanent coronary artery ligation and ischaemia-reperfusion showing the effect of mechanical unloading in the chronically infarcted heart. Mechanical unloading of the permanently ligated heart resulted in a significant reduction in MMP-1 protein expression compared to control (dotted line).

TIMP-1 protein expression in the chronically infarcted heart was also significantly lower than control in the permanently ligated loaded (CTR-L 0.6101 ± 0.02437 , vs CMI-L 0.2548 ± 0.03716 , $p = 0.0062$) and unloaded heart (CTR-U 0.7318 ± 0.002767 , vs CMI-U 0.1342 ± 0.004414 , $p = 0.0001$). (Fig. 4.31) Mechanical unloading in the permanently ligated hearts led to a statistically significant reduction in TIMP-1 protein expression (CMI-L 0.2548 ± 0.03716 ,

vs CMI-U 0.1342 ± 0.004414 , $p= 0.0322$). There was also a reduction in TIMP-1 expression after mechanical unloading in the ischemia-reperfusion group although this did not reach statistical significance. (Fig. 4.31)

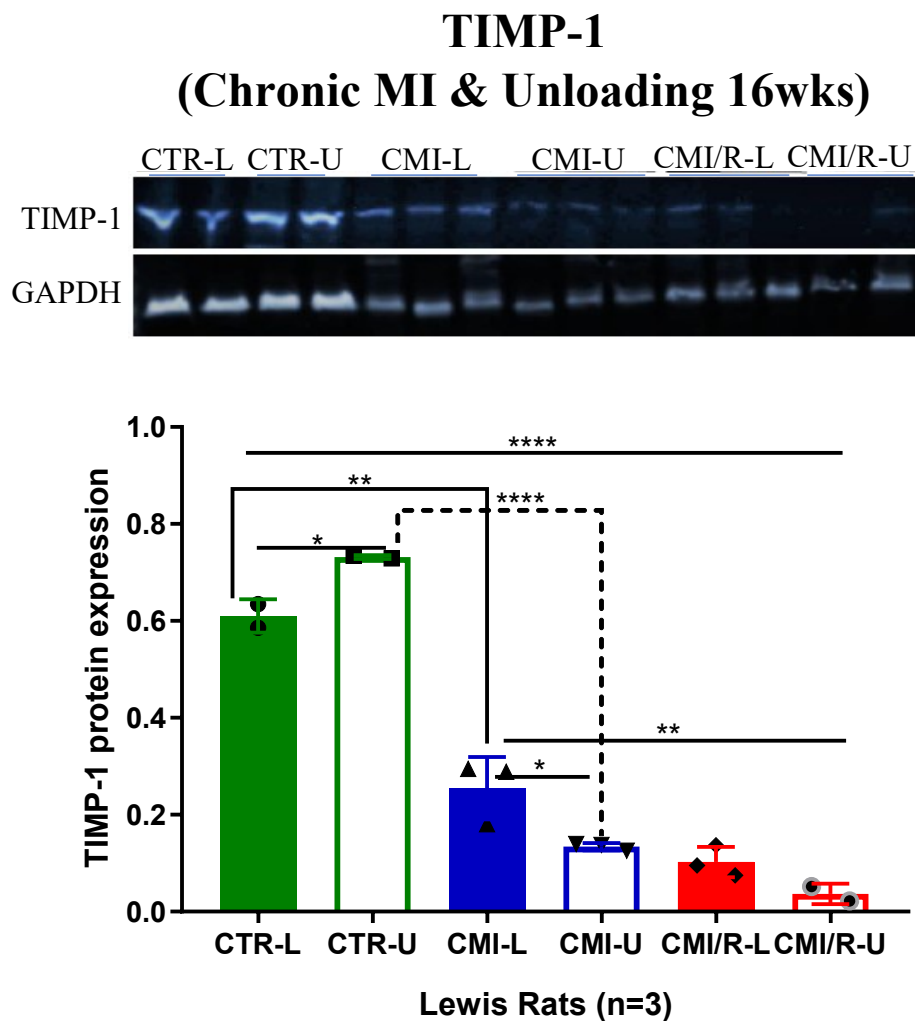


Figure 4.31: TIMP-1 expression profile after permanent coronary artery ligation and ischaemia-reperfusion showing the effect of mechanical unloading in the chronically infarcted heart.

MMP-1 : TIMP-1 ratio after chronic MI was not significantly altered by mechanical unloading in the control, permanently ligated or ischemic-reperfusion groups. The trend in MMP-1 : TIMP-1 ratio was higher in the ischemia-reperfusion group compared to the permanently

ligated and control groups but this was not statistically significant due to large variability in the data. (Fig. 4.32)

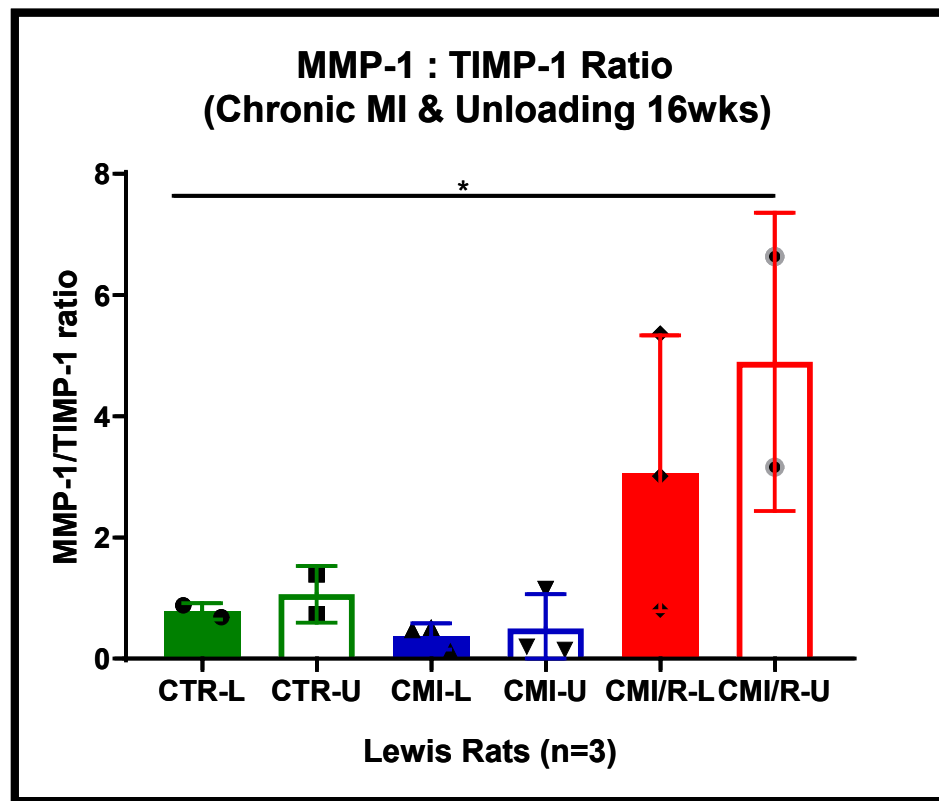


Figure 4.32: MMP-1 : TIMP-1 ratio after permanent coronary artery ligation and ischaemia-reperfusion in chronic hearts showing the impact of mechanical unloading.

SUMMARY OF EFFECTS OF MECHANICAL UNLOADING

Protein	<u>AMI-L Vs AMI-U</u>	<u>AMI/R-L Vs AMI/R-U</u>	<u>CMI-L Vs CMI-U</u>	<u>CMI/R-L Vs CMI/R-U</u>
TGF-β1	NO CHANGE	NO CHANGE	INCREASED	INCREASED
αSMA	NO CHANGE	DECREASED	INCREASED	INCREASED
MMP-1	DECREASED	DECREASED	NO CHANGE	NO CHANGE
TIMP-1	NO CHANGE	NO CHANGE	DECREASED	NO CHANGE
MMP-1 : TIMP-1	DECREASED	NO CHANGE	NO CHANGE	NO CHANGE

Table 4.1: Outline of effects of mechanical unloading on the dynamic expression profiles of TGF- β 1, α SMA, MMP-1, TIMP-1, MMP-1:TIMP-1 ratio. No change means no statistically significant difference between the loaded and unloaded hearts. Increased or Decreased pertains to the effect of mechanical unloading on the respective protein expressions.

4.6 DISCUSSION

Data from this study show that mechanical unloading after acute and chronic myocardial infarction predisposes the heart to an increase in infarct ratio and interstitial fibrosis. Infarct ratio rose by 16% and 15% in the acute permanently ligated heart after 2 days and 7 days of mechanical unloading respectively. Similarly, there was a significant rise in interstitial fibrosis after 2 days (33%) and 7 days (21%) of mechanical unloading in the permanently ligated hearts. Remarkably however, there was no significant difference in infarct ratio or interstitial fibrosis in the ischaemia-reperfusion group after the same duration of mechanical unloading.

These findings suggest that the deleterious effect of mechanical unloading on infarct size and interstitial fibrosis could be ameliorated by re-establishing coronary circulation to the ischaemic territory. To the best of our knowledge this is the first study to report these findings and it has very significant potential clinical implications for LVAD-induced myocardial recovery in failing hearts.

To date the ECM remains the only part of the unloaded failing heart that appears to portray features that are not in keeping with the recovery process.(Klotz *et al.*, 2007) Several other studies have also shown a worsening of fibrosis with mechanical unloading but the underlying mechanism of this deleterious effect has remained poorly understood.(Schaefer *et al.*, 2019)

The findings from this study suggests a link between the re-establishment of blood flow in a large less autoregulated vessel and the amelioration of on-going ischemic injury to the heart during mechanical unloading. As described in section 4.1, mechanical unloading has been associated with a reduction in CFR. A finding that is believed to be a result of the markedly decreased LV workload during mechanical unloading which reduces MV02 and diminishes the autoregulatory stimulus on the microcirculation designed to match MBF to changes in MV02.(Tansley *et al.*, 2004) In addition, mechanical unloading with LVADs leads to a reduction in microvascular lumenal diameter in the failing heart, providing further evidence of a link between LVAD use and negative auto-regulatory effects on the collateral circulation.(Drakos *et al.*, 2010) The role of the microcirculation in limiting the extent of myocardial injury following obstruction to a large coronary vessel cannot be overemphasized. The presence of a robust collateral circulation and better outcomes after myocardial infarction is established.(Fulton, 1964a) (Antoniucci *et al.*, 2002) (Seiler, 2009) (Kim *et al.*, 2016) (Meier *et al.*, 2012) (Nakai *et al.*, 1995)

Whilst this study does not provide specific evidence of the effects of mechanical unloading on the collateral circulation after myocardial injury, the main difference between the two groups studied (permanent coronary artery ligation and ischemia-reperfusion groups) is the re-establishment of blood flow in the parent coronary vessels and thus elimination of the need for collateral blood flow. In fact it has been previously shown that once blood flow in the parent vessel is re-established, collateral channels rapidly become non-functional and return to their collapsed redundant state.(Rentrop *et al.*, 1985) It is thus reasonable to ascribe the predominant basis of the differences seen between the two groups studied to effects on the collateral circulation.

This finding which was only possible due to the unique design of this study has significant implications for LVAD-induced recovery because it makes the case for heart failure patients to have myocardial viability studies done prior to LVAD implantation. The findings here suggest that in patients with ischemic cardiomyopathy, myocardial function might be impaired further during mechanical unloading due to a negative auto-regulatory consequence of unloading on the collateral circulation. It is reasonable to suggest that this might play a major role in the significantly lower incidence of LVAD-induced myocardial functional recovery seen in ischemic cardiomyopathy patients.

In the chronic group, mechanical unloading after permanent coronary artery ligation was also associated with a statistically significant rise in infarct ratio and an almost doubling of interstitial fibrosis. Intriguingly however, in the chronic ischaemia-reperfusion group, the amelioration of the deleterious effect of mechanical unloading that was observed in the acute ischaemia-reperfused hearts was absent. Mechanical unloading in this group was also associated with a large and statistically significant rise in infarct ratio and interstitial fibrosis. Thus whilst in the acute MI hearts the release of coronary artery ligation after 90 minutes of occlusion led to an amelioration of the deleterious effect of mechanical unloading on infarct

size and interstitial fibrosis at days 2 and 7, these data show that 16 weeks after the release of the ligature this protective effect of reperfusion is lost. No evidence has been obtained in this study to explain this difference; however, as the only difference between the permanently ligated and ischaemia-reperfused hearts studied is the presence or absence of a patent coronary artery, a possible explanation could be that coronary artery re-occlusion occurs in the chronic hearts. It is well known that within 2 weeks of injury to the intima of a coronary artery, fibrin, platelets, and acute inflammatory cells aggregate at the site of injury and predispose to thrombosis, and by 4 weeks, neointimal growth from smooth muscle cell deposition in the injured media occurs leading to vessel occlusion.(Farb *et al.*, 1999) This process, described as intimal hyperplasia has been strongly linked to the overexpression of TGF- β 1 in vascular smooth muscle cells that leads to increased extracellular matrix protein production and artery narrowing.(Khan and Sheppard, 2006)

In an attempt to better understand the acute and chronic effects of mechanical unloading on the heart after permanent coronary ligation and ischemia-reperfusion, the expression profiles of α SMA, TGF- β 1, MMP-1, and TIMP-1 in each group was determined via western blotting.

At day 2 after MI, α SMA protein expression was significantly higher than control in all groups as expected but there was no statistically significant difference between the loaded and the unloaded groups (table 2), although this could possibly be due to the presence of variability in the data from the unloaded hearts. At day 7, a similar α SMA protein expression profile to day 2 was obtained in the permanently ligated hearts, however, there was a statistically significant decrease in α SMA levels after mechanical unloading in the ischemia-reperfusion group. This might indicate that the activity of myofibroblasts is downregulated in the unloaded ischemia-reperfusion group with consequent decrease in collagen deposition and hence the amelioration of the worsening of interstitial fibrosis seen in the permanently ligated group after unloading.

In the chronic hearts, α SMA protein expression was significantly higher in the unloaded permanently ligated hearts compared to the loaded hearts (table 4.1), possibly accounting for the 98.5% increase in interstitial fibrosis seen in this group. Furthermore, in contrast to the finding in the acute hearts, ischaemia-reperfusion was still associated with a statistically significant increase in α SMA protein expression after mechanical unloading compared to the loaded hearts. This might help to explain the absence of the amelioration of worsening fibrosis after unloading that is shown to occur in the acute ischaemia-reperfused hearts.

The expression profile of TGF- β 1 after acute MI did not correlate with α SMA protein expression at similar time points. There was no statistically significant differences in TGF- β 1 expression between the groups at day 2 and day 7. In the chronic hearts however, TGF- β 1 protein expression was significantly higher in the mechanically unloaded permanently ligated hearts compared to the loaded heart and this correlates well with the expression profile of α SMA in this group. Furthermore, there was also a statistically significant increase in TGF- β 1 expression after mechanical unloading in the ischemia-reperfusion group. This increased expression level of TGF- β 1 in the unloaded chronic ischemia-reperfusion group also correlates with α SMA expression and the 85% increase in interstitial fibrosis in the mechanically unloaded chronically infarcted hearts despite ischaemia-reperfusion. It is worth noting that the expression levels of TGF- β 1 in the chronic ischaemia-reperfused hearts were also significantly higher than in the acute hearts (Fig. 4.2). This might be a consequence of the development of intimal hyperplasia in the chronic hearts as previously described.

Following ECM protein deposition from increased α SMA activity, MMPs and TIMPs play significant roles in regulating the ECM. The expression profiles of MMP-1, TIMP-1, and the ratio of MMP-1 : TIMP-1 in each group was investigated as these proteins are known to regulate collagen deposition.

At day 7, mechanical unloading led to a statistically significant decrease in MMP-1 protein expression in both the permanently ligated and ischemia-reperfused hearts (table 4.1). Furthermore, ischemia-reperfusion alone did not appear to impact on the expression profile of MMP-1. This downregulation of MMP-1 with mechanical unloading at day 7 might partly explain the increased fibrosis observed in the mechanically unloaded permanently ligated hearts. The reduced level of fibrosis seen in the unloaded ischemia-reperfusion group in the presence of a downregulated MMP-1 protein expression could be indicative of decreased collagen production in this group as the low levels of TGF- β 1 and α SMA noted would suggest. MMP-1 : TIMP-1 ratio was significantly downregulated after mechanical unloading in the permanently ligated hearts at day 7 providing further supportive evidence of decreased collagen degradation in this group. There was no statistically significant difference between the loaded and unloaded hearts after ischemia-reperfusion, thus suggesting that mechanical unloading did not lead to a decrease in collagen degradation in this group.

In the chronic hearts, MMP-1 and TIMP-1 expression in the permanently ligated hearts was significantly lower than control correlating with the increased fibrosis in these hearts. The ratio of MMP-1 : TIMP-1 was however not significantly different between the loaded and unloaded hearts. No statistically significant difference in MMP-1 : TIMP-1 ratio was noted between the loaded and unloaded hearts in the ischemia-reperfusion group although there were very large variations in the data.

4.7 SUMMARY

Understanding the underlying mechanisms that regulate the changes in the ECM of a failing heart mechanically unloaded with an LVAD remains a challenge. Data from this study supports

most contemporary reports that show that fibrosis worsens with mechanical unloading. Interestingly however, this is the first report to show that after 90 minutes of ischaemia, returning blood flow through the ligated parent coronary vessels (LAD & PDA) ameliorates the deleterious effect of mechanical unloading on interstitial fibrosis and infarct size.

There was a significant upregulation of TGF- β 1 and α SMA following mechanical unloading especially in the chronic permanently ligated hearts indicating increased ECM protein deposition. Furthermore, in line with several reports, this study also confirms the downregulation of matrix metalloproteinase activity with mechanical unloading as shown by a significant decrease in the MMP-1 : TIMP-1 ratio. These findings might explain the reduction in collagen degradation in failing human hearts supported with left ventricular assist devices.

Finally, contrary to some reports, infarct size was seen to worsen with mechanical unloading in this study. This might be due to differences in study design as studies showing improvement in infarct size with mechanical unloading were carried out over very short periods of unloading lasting only a few hours. The findings of this study suggests that mechanical unloading might have negative autoregulatory effects on the collateral circulation. Effects that could be responsible for predisposing mechanically unloaded permanently ligated hearts to a worsening of infarct size. These effects develop over days (van Royen *et al.*, 2009) and might have been missed in the studies showing improvement in infarct size after only a few hours of unloading.

The unique design of this study which over a period of days and weeks directly compared the changes in the ECM of mechanically unloaded permanently ligated hearts to those where blood flow was re-established in the parent vessel has raised an intriguing previously unknown finding which should stimulate further research into developing a better understanding of LVAD effects on infarct size and interstitial fibrosis after myocardial infarction.

5 Effect of mechanical unloading on the endogenous proliferative capacity of the heart

5.1 INTRODUCTION

Intensive research into the adult mammalian heart's capacity for self-renewal has led to the consensus that cardiomyocytes are formed throughout adult mammalian life albeit at a disputed frequency.(He and Zhou, 2017) (Malliaras *et al.*, 2013) (Nadal-Ginard, Ellison and Torella, 2014) (Hsieh *et al.*, 2007) It has also been shown that after myocardial injury, cardiomyocyte proliferative rate increased significantly and that this increase was due to the activation of the heart's endogenous progenitor pool. (Malliaras *et al.*, 2013) In addition, emerging reports suggests that cardiomyocyte regeneration might in fact be involved in the observed LVAD-induced myocardial functional recovery described in some end-stage heart failure patients.(Malliaras *et al.*, 2013) (Koudstaal *et al.*, 2013) (Ellison, Nadal-Ginard and Torella, 2012) (Wohlschlaeger *et al.*, 2010) (Suzuki *et al.*, 2007) These studies and others have provided evidence to suggest that haemodynamic load might play an important role in facilitating cardiomyocyte proliferation.

Furthermore, recent work has identified a correlation between the postnatal (P) cardiomyocyte cell cycle arrest that occurs at P7 and the upregulation of myeloid ecotropic viral integration site 1 (MEIS1).(Mahmoud *et al.*, 2013) MEIS1 belongs to the TALE (three amino acid loop extension) family of homeodomain transcription factors that are central regulators of cardiac differentiation during embryonic development.(Paige *et al.*, 2012) (Wamstad *et al.*, 2012) The

study showed that MEIS1 knockout in adult mice was associated with a 10 fold increase in the number of mitotic cardiomyocytes. By contrast, forced overexpression of MEIS1 inhibited neonatal heart regeneration following induction of myocardial injury at P1, whilst wild-type hearts regenerated normally. The effect mechanically unloading the injured heart has on the expression profile of MEIS1 has not been investigated and is evaluated in this research.

Mechanical wall stress, amongst a number of other stimuli, is also known to be capable of activating the Hippo pathway, an evolutionarily conserved pathway that regulates organ size and growth during development.(A. Uygur, 2017) Increased mechanical wall stress leads to phosphorylation of transcription co-activators YAP and TAZ in mammals and their extrusion from the nucleus with consequent inhibition of cell proliferation. Deactivation of the Hippo pathway results in YAP/TAZ protein dephosphorylation and their translocation back to the nucleus where they can then induce cell proliferation. (A. Uygur, 2017) The effect mechanical unloading has on the expression of YAP has not been described and is investigated.

Finally, the effect coronary reperfusion (after acute myocardial infarction) has on the regenerative capacity of the heart after 2 days and 7 days of mechanical unloading has not been investigated. There is evidence that shows that in the loaded heart, improvement in coronary blood flow to the ischaemic territory after myocardial injury facilitates cardiomyocyte proliferation.(Marín-juez *et al.*, 2016)

In this study, the effect mechanical unloading of the heart (accomplished via HAHLT in a rat model of MI) with or without ischaemia-reperfusion has on the regenerative response of the adult heart to injury is investigated. The effects on the expression profiles of MEIS1 and YAP are also described.

5.2 METHODS

5.2.1 Acute MI and Ischaemia-reperfusion model

Rodent models of acute MI with or without ischaemia-reperfusion were utilised. Details are described in section 2.1.1.

5.2.2 Mechanical unloading model

The heterotopic abdominal heart transplantation technique was used as the model of mechanical unloading. Details are described in section 2.1.2.

5.2.3 Mode of detection of proliferating cells

Immunohistochemistry staining of frozen tissue sections using the antibody Ki67 was the technique employed in this study to determine cell proliferation. Ki67 is a protein known to be strictly associated with cell proliferation and has been used as the marker of choice in several studies as it is exclusively expressed during the active phases of the cell cycle i.e. G1, S, G2, and mitosis, but is absent in the resting phase (G0) and in differentiated cells. (Tanaka *et al.*, 2011) Ki67 has been shown to be a more sensitive and specific marker of cell proliferation compared to other markers such as the Proliferating Cell Nuclear Antigen (PCNA) or the thymidine analogue Bromodeoxyuridine (BrdU). The expression of the latter two have been shown to be significantly affected by DNA damage and repair.(Bologna-Molina et al, 2013) Ki67 immunohistochemistry is however limited in that it only provides information about the

proliferative rate of cells at the point of heart harvest. As such it does not provide an accurate representation of the rate at which cells are proliferating. It is thus important to note that the data provided here represents an underestimation of the actual proliferative capacity of the adult heart. Immunohistochemistry was the preferred approach as opposed to flow cytometry as immunohistochemistry preparations afford regional assessment of proliferation, and deals with the confounding issue of multinucleation as tissues are cut in transverse section meaning only one nucleus or none was visible. As the study was carried out on frozen tissue cross sections, only one Ki67 positive nucleus per cell was counted. In fact, cells with missing nucleus due to the level at which the tissue was cut were not counted further underestimating the degree of proliferation.

5.2.4 Confocal & Widefield microscopy

The permanently ligated, ischaemia-reperfused, loaded or unloaded groups (2.1.2 a-d) were observed for a period of 2 and 7 days after surgery. Hearts were then explanted (see section 2.1.3) for analysis on day 2 or day 7. Cell proliferation was only tested in acute hearts. The chronic hearts were not tested as the evidence for the hearts regenerative response to injury is mainly in the first few days after injury.

Following immunochemical staining with cTnT, Ki67, WGA, vimentin, and Hoechst or DAPI (as described in sections 2.2.1 and 2.2.2) samples were imaged under confocal microscopy with z-stacking tool utilised to verify cardiomyocyte nuclei. Widefield microscopy was used to aid regional analysis of cell proliferation. Proliferating cells were counted manually from three distinct regions of the stained left ventricle as illustrated in figure 5.2, these include: Anterior infarct borderzone (between the anterior wall of the right ventricle and septum, and the anterior aspect of the left ventricular free wall), Posterior infarct borderzone (between the posterior wall

of the right ventricle and septum, and the posterior aspect of the left ventricular free wall), and Distant myocardium (midpoint of the interventricular septum). Each region counted was about 1.4mm^2 and contained approximately 2000 cardiomyocytes. The total number of proliferating cardiomyocytes in each 1.4mm^2 region was noted as the proliferative capacity whilst the total number of proliferating cardiomyocytes in each 1.4mm^2 zone divided by the total number of cardiomyocytes (approximately 2000 cells) in that zone was noted as the proliferative rate at the point of harvest. The proliferative capacity provides a global index of the proliferative activity of each heart whilst the proliferative rate was employed for comparative analysis.

Strict criteria were followed to avoid counting non-cardiomyocyte nuclei or counting more than one nucleus per cell. These criteria were as follows:

- a. Thresholding tool on imageJ analysis software was used to eliminate autofluorescence.
- b. Only regions with cardiomyocytes cut in transverse section were included in the count and only one positive nucleus per cell was counted.
- c. Only cardiomyocytes with positive nuclei positioned centrally in the cell or well clear of the cell membrane (stained with WGA) were counted.

5.2.5 Western blotting

The expression profiles of MEIS1 and YAP in free wall of the left ventricle of the loaded and unloaded subgroups were determined via Western blotting and compared to control (see section 2.2.6). Data were obtained using imageJ.

5.2.6 Statistical analysis

Proliferating cells were counted after using the strict criteria in 5.2.3 a-c to identify cardiomyocyte nuclei labelled by Ki67. A two-tailed t-test was used for the comparison between independent groups of normally distributed data as determined by Kolmogorov-Smirnov testing. One-way analysis of variance (ANOVA) followed by Bonferroni post hoc test for individual significant differences was used. All statistical analyses were done using Prism 7 software (GraphPad Software, Inc.). Five animals (n=5) were studied in each subgroup. The results are presented as means $\pm$ SD. The significance threshold was set at $p < 0.05$ for all data.

* denotes $P < 0.05$, ** denotes $P < 0.01$ and *** denotes $P < 0.001$.

5.3 RESULTS I - Regional Evaluation of Cardiomyocyte Proliferation in Permanently Ligated and Ischaemia-Reperfused Hearts

Cardiac troponin (cTnT) positive cells (stained red in Fig. 5.1 below) expressing Ki67 (green) in their nuclei (blue) were counted as proliferating cardiomyocytes.

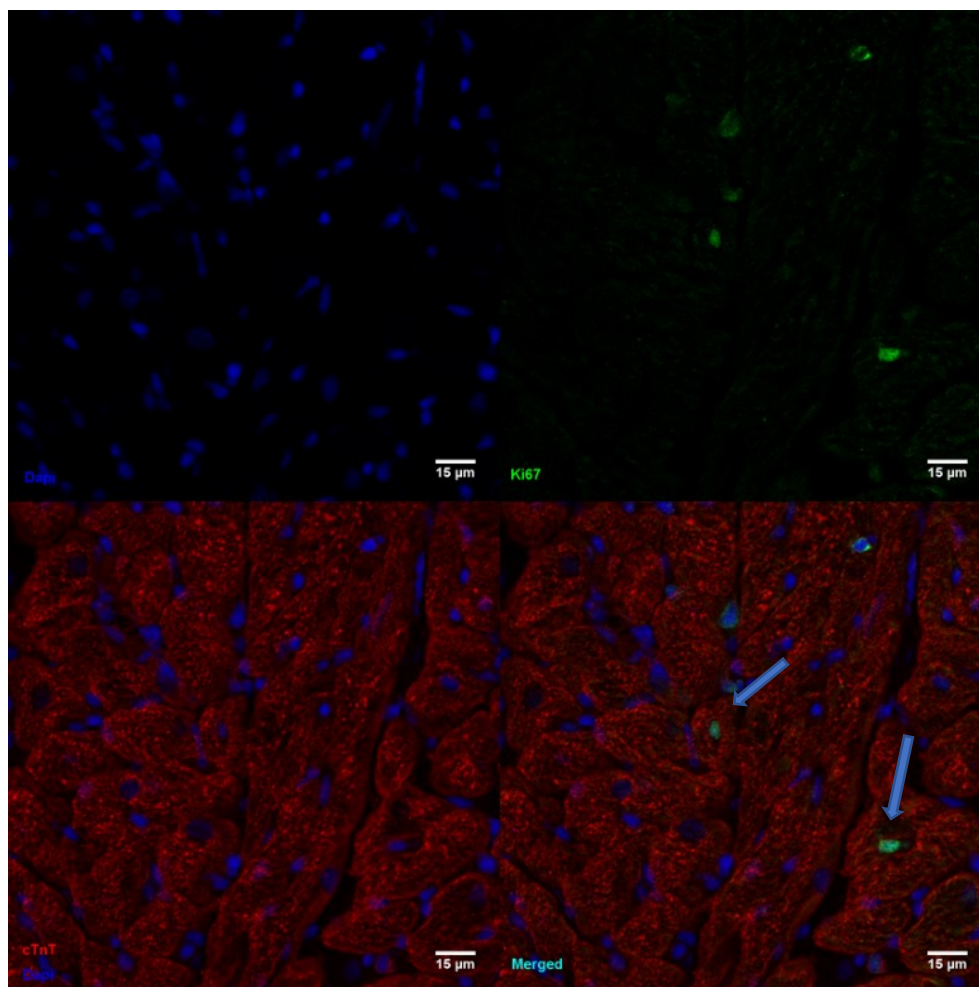


Figure 5.1: Confocal microscopic image of a 15µm frozen cross section of a Lewis rat heart after immunohistochemistry staining using cTnT (red) to identify cardiomyocytes, DAPI (blue) for the nuclei, and Ki67 (green) to detect proliferating cells (blue arrows).

Cross-sectional analysis allowed regional evaluation of cell proliferation as illustrated in figure 5.2.

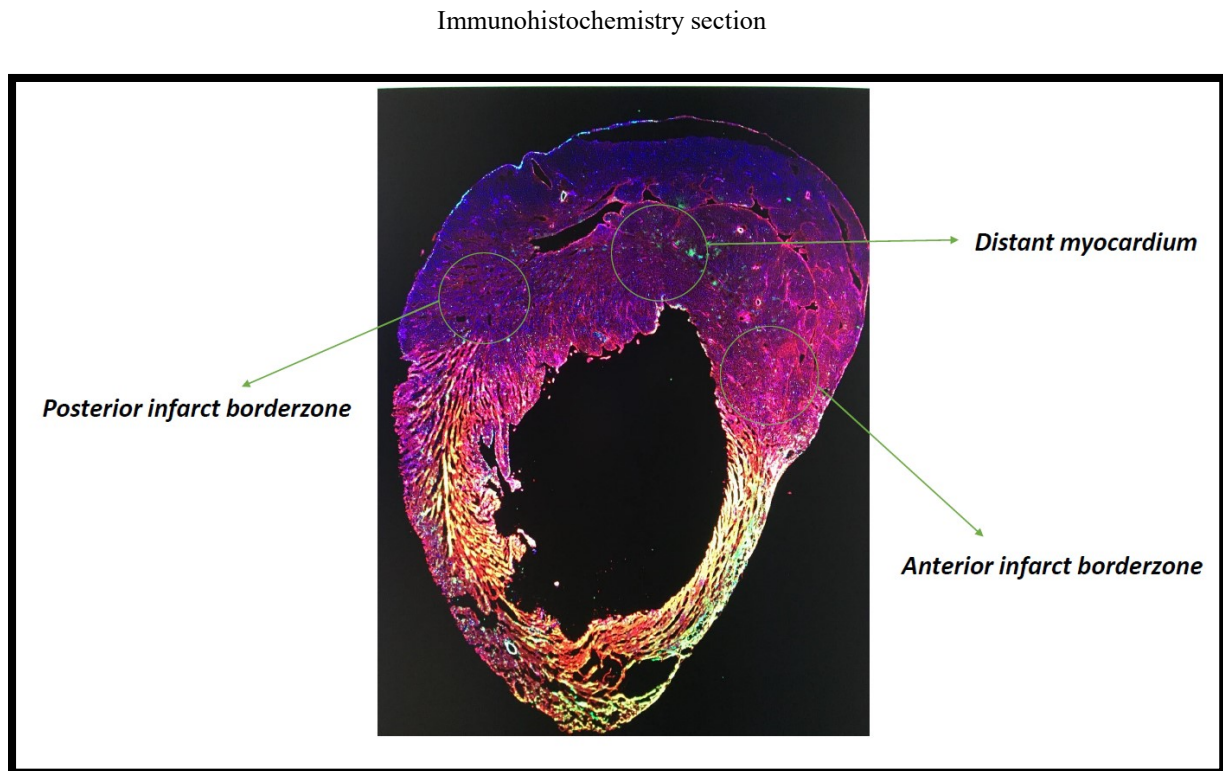


Figure 5.2: Bright field microscopic image of an immunohistochemistry specimen. The frozen rat heart tissue section was stained with Ki67 (green), Hoechst (blue), and WGA (red). The three regions evaluated to determine cardiomyocyte proliferation are shown.

Analysis of cardiomyocyte proliferation was completed on day 7 samples as most reports of significant regenerative responses to myocardial injury were described after this period of injury.(Haubner *et al.*, 2012)(Sturzu *et al.*, 2015) In each figure below, the filled bars represent loaded hearts and the empty bars unloaded hearts. The permanently ligated coronary artery group are in blue and the ischaemia-reperfusion group in red.

5.3.1 Anterior infarct borderzone

In the anterior infarct borderzone at day 7, cardiomyocyte proliferation was 4 times higher in the loaded infarcted hearts compared to control after permanent coronary artery ligation (AMI-L 6.8 ± 1.594 , vs CTR 1.8 ± 0.5831 , $p=0.0185$). After mechanical unloading, there was a 22 times increase in cardiomyocyte proliferation in the unloaded hearts compared to control but this did not reach statistical significance (AMI-U 40.20, vs CTR 1.800, $p=0.515$). (Fig. 5.3) There was no statistically significant difference in proliferation between the loaded and unloaded hearts in the anterior infarct borderzone after permanent coronary artery ligation.

After ischaemia-reperfusion however, there was a statistically significant 5 times increase in cardiomyocyte proliferation in the loaded hearts compared to control (AMI/R-L 9.2 ± 1.685 , vs CTR 1.8 ± 0.3742 , $p= 0.0027$). Mechanical unloading coupled with ischaemia-reperfusion stimulated a significant increase in cardiomyocyte proliferation compared to both the control hearts (AMI/R-U 55.4 ± 13.43 , vs CTR 1.8 ± 0.3742 , $p= 0.004$) and the loaded reperfused hearts (AMI/R-U 55.4 ± 13.43 , vs AMI/R-L 9.2 ± 1.685 , $p=0.0092$) as shown in figure 5.4.

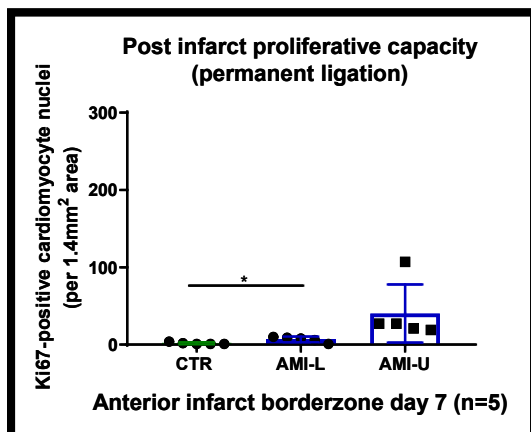


Figure 5.3: Proliferative capacity of Ki67-positive cells in the anterior infarct borderzone 7 days after permanent coronary artery ligation. The dots represent number of rats (n=5) in each group.

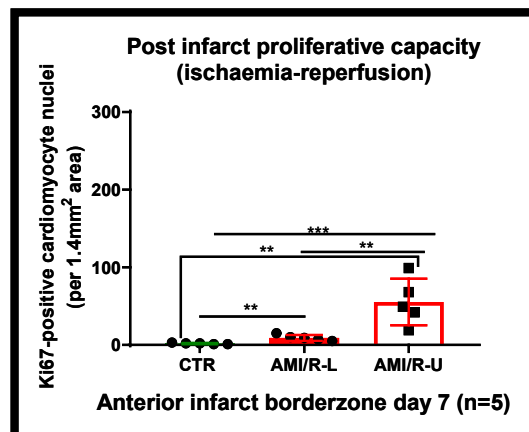


Figure 5.4: Proliferative capacity of Ki67-positive cells in the anterior infarct borderzone 7 days after ischaemia-reperfusion.

The proliferative rate in the control, loaded, and unloaded hearts was 0.1%, 0.3%, and 2% in the permanently ligated hearts, and 0.1%, 0.4%, and 2.6% in the re-perfused hearts respectively. (Fig. 5.5 & 5.6)

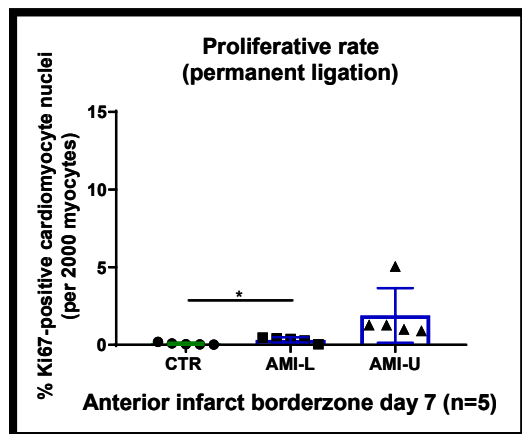


Figure 5.5: Proliferative rate of Ki67-positive cells in the anterior infarct borderzone 7 days after permanent coronary artery ligation.

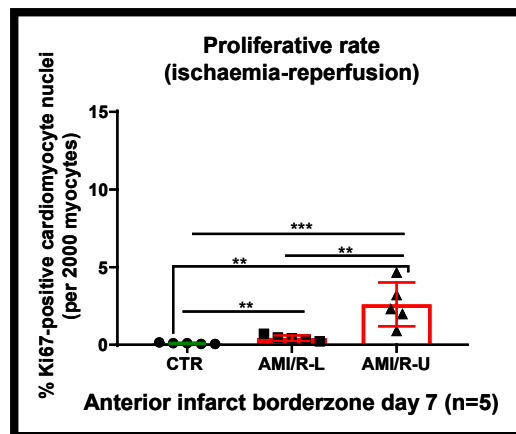


Figure 5.6: Proliferative rate of Ki67-positive cells in the anterior infarct borderzone 7 days after ischaemia-reperfusion.

5.3.2 Posterior infarct borderzone

In the posterior infarct borderzone at day 7, permanent coronary artery ligation was associated with 6 times increase in cardiomyocyte proliferation in the loaded hearts compared to control but this was not statistically significant (AMI-L 41.8 ± 19.79 , vs CTR 1.8 ± 0.5831 , $p=0.0781$). In the unloaded hearts, there was a large statistically significant increase in cardiomyocyte proliferation compared to control (AMI-U 71 ± 22.16 , vs CTR 1.8 ± 0.5831 , $p=0.0142$) after permanent coronary artery ligation. However, there was no statistically significant difference

between the unloaded and loaded permanently ligated hearts although this was possibly due to large variability in the data. (Fig. 5.7)

In the ischaemia-reperfusion group, there was a statistically significant 5 times increase in proliferation in the loaded hearts compared to control (AMI/R-L 9.8 ± 2.437 , vs CTR 1.8 ± 0.3742 , $p=0.0118$). After mechanical unloading, cardiomyocyte proliferation rose quite significantly compared to both the control (AMI/R-U 200.2 ± 21.67 , vs CTR 1.8 ± 0.3742 , $p=0.0001$) and the loaded hearts (AMI/R-U 200.2 ± 21.67 , vs AMI/R-L 9.8 ± 2.437 , $p=0.0001$). (Fig. 5.8)

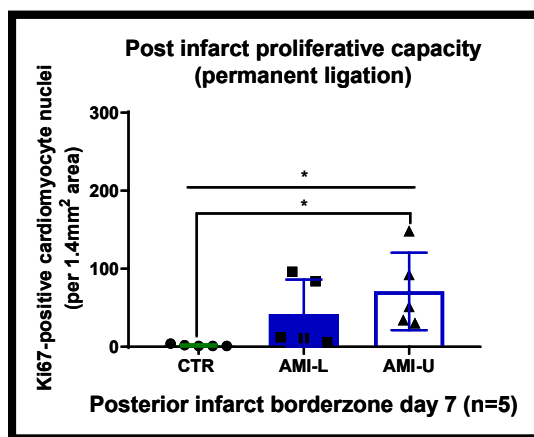


Figure 5.7: Proliferative capacity of Ki67-positive cells in the posterior infarct borderzone 7 days after permanent coronary artery ligation

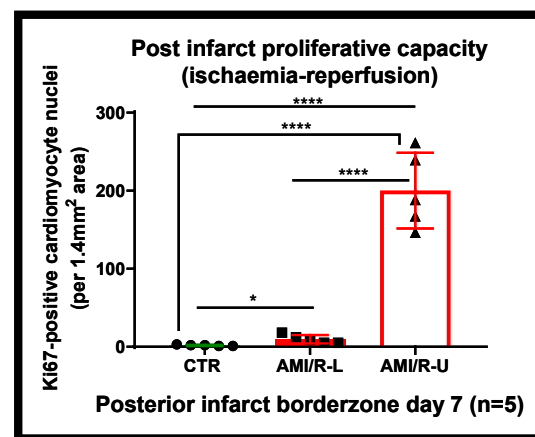


Figure 5.8: Proliferative capacity of Ki67-positive cells in the posterior infarct borderzone 7 days after ischaemia-reperfusion

The proliferative rate in the control, loaded, and unloaded hearts was 0.1%, 0.6%, and 3.7% in the permanently ligated hearts, and 0.1%, 0.5%, and 10.4% in the re-perfused hearts respectively. (Fig. 5.9 & 5.10)

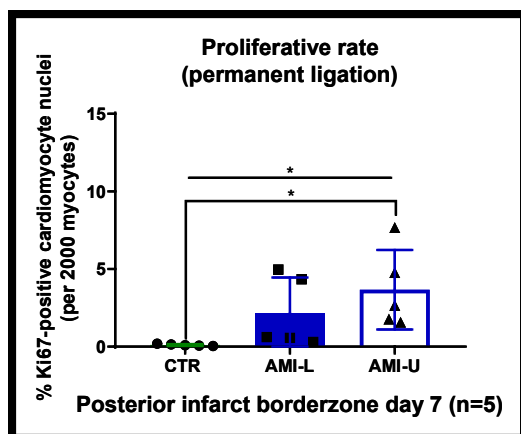


Figure 5.9: Proliferative rate of Ki67-positive cells in the posterior infarct borderzone 7 days after permanent coronary artery ligation

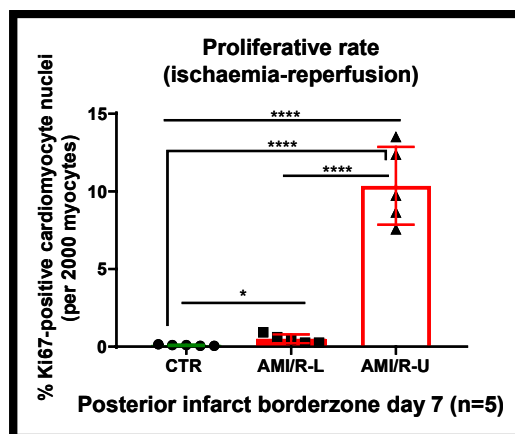


Figure 5.10: Proliferative rate of Ki67-positive cells in the posterior infarct borderzone 7 days after ischaemia-reperfusion

The remarkable increase in cardiomyocyte proliferation noted in the posterior infarct borderzone after ischaemia-reperfusion in the unloaded compared to the loaded heart is quite vivid as illustrated in figure 5.11.

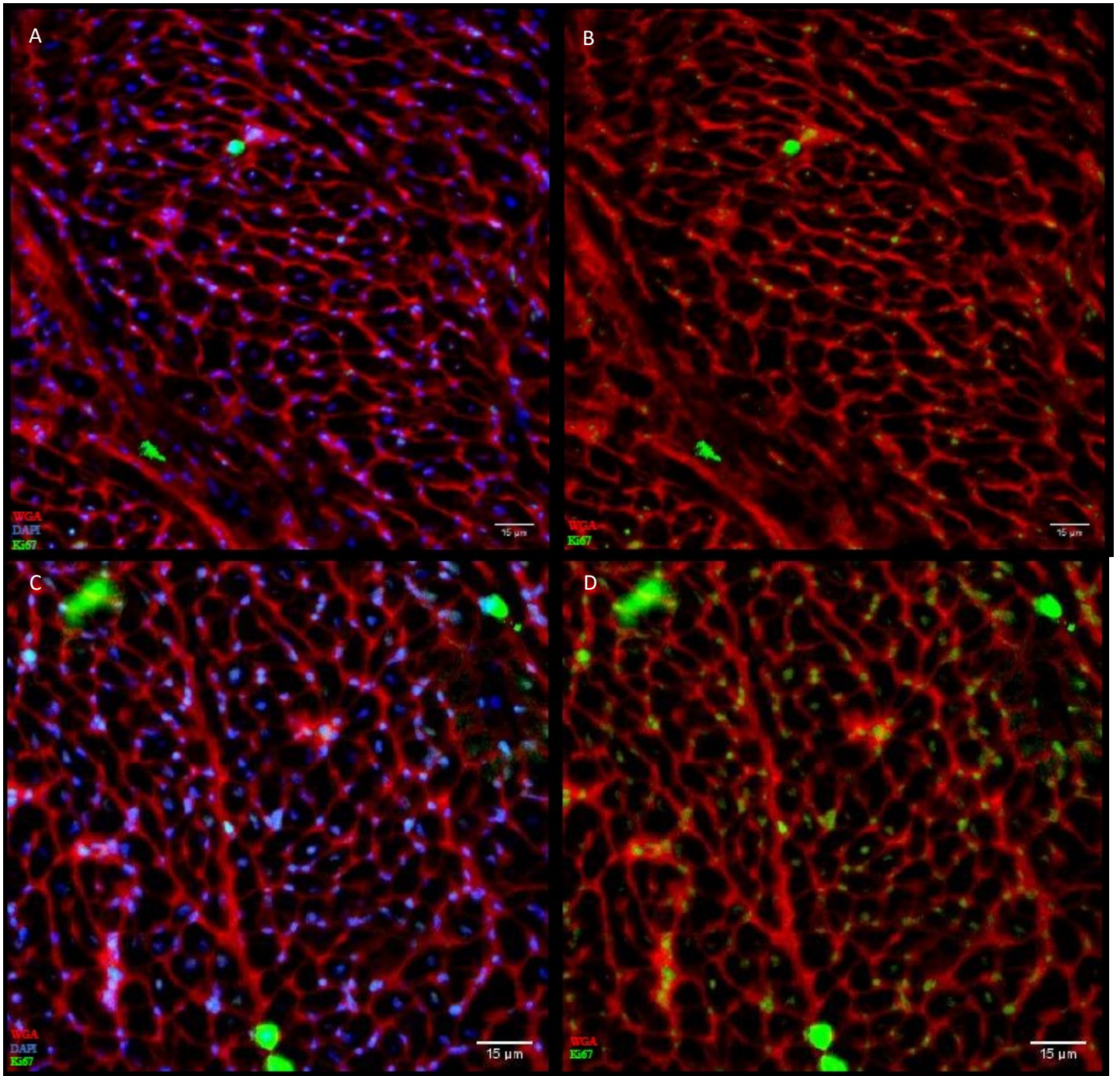


Figure 5.11: Representative samples of an unloaded permanently ligated heart (A&B) and an unloaded ischaemia-reperfused heart (C&D) showing significant cardiomyocyte proliferation in the reperfed heart. The red stain is the membrane marker WGA which marks the cardiomyocyte membrane here. The green stains are Ki67 positive nuclei. The blue nuclei marker (DAPI) was silenced in B & D to illustrate the difference in Ki67 expression.

5.3.3 Distant myocardium

In the distant myocardium at day 7, there was a larger increase in cardiomyocyte proliferation compared to that observed in the posterior infarct borderzone. After permanent coronary artery ligation, cardiomyocyte proliferation was over 20 times higher in the loaded hearts compared to control (AMI-L 44.8 ± 21.18 , vs CTR 1.8 ± 0.3742 , $P= 0.0769$) and there was a 40 times increase after mechanical unloading (AMI-U 76.8 ± 51.59 , vs CTR 1.8 ± 0.3742 , $P= 0.1841$). None of these were however statistically significant due to large variations in the data. (Fig. 5.12) There was no statistically significant difference in proliferation between the loaded and unloaded hearts after permanent coronary artery ligation.

In the ischaemia-reperfusion group, there was a statistically significant increase in proliferation in the loaded hearts compared to control (AMI/R-L 40.2 ± 13.93 , vs CTR 1.8 ± 0.3742 , $p=0.0248$). Mechanical unloading resulted in a very large and statistically significant increase in cardiomyocyte proliferation compared to control (AMI/R-U 258 ± 53.18 , vs CTR 1.8 ± 0.3742 , $p= 0.0013$). Compared to the loaded hearts, mechanical unloading brought about a statistically significant 7 times increase in proliferation (AMI/R-U 258 ± 53.18 , vs AMI/R-L 40.2 ± 13.93 , $p= 0.0042$). (Fig. 5.13)

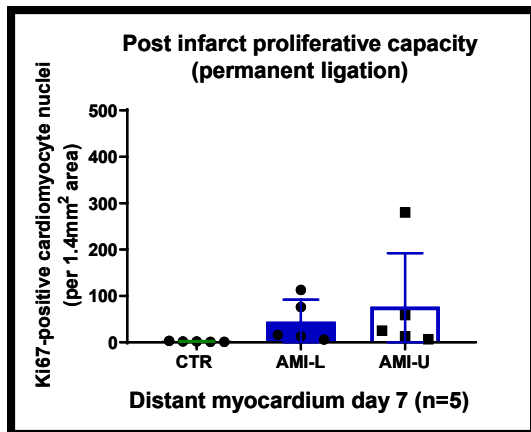


Figure 5.12: Proliferative capacity of Ki67-positive cells in the distant myocardium 7 days after permanent coronary ligation

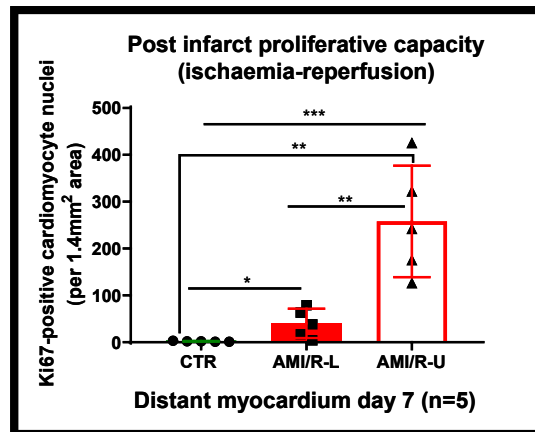


Figure 5.13: Proliferative capacity of Ki67-positive cells in the distant myocardium 7 days after ischaemia-reperfusion

The proliferative rate in the control, loaded, and unloaded hearts was 0.1%, 2.1%, and 3.9% in the permanently ligated hearts, and 0.1%, 1.9%, and 13.4% in the re-perfused hearts respectively. (Fig. 5.14 & 5.15)

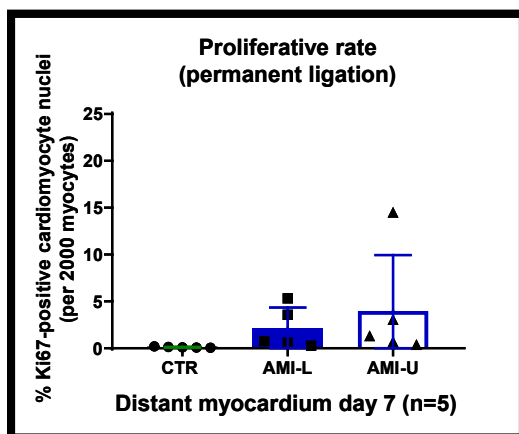


Figure 5.14: Proliferative rate of Ki67-positive cells in the distant myocardium 7 days after permanent coronary artery ligation

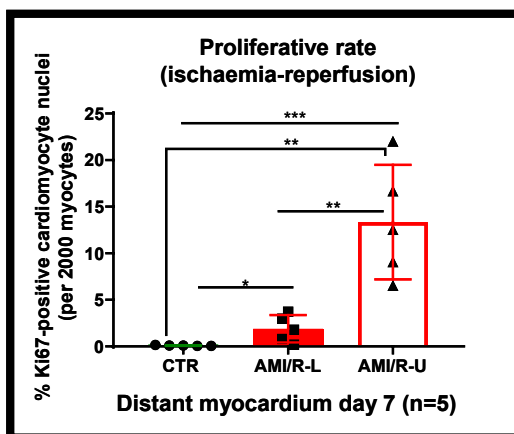


Figure 5.15: Proliferative rate of Ki67-positive cells in the distant myocardium 7 days after ischaemia-reperfusion

5.4 RESULTS II: Dynamic Expression Profiles of MEIS1 and YAP

As described in 5.1 above, significant upregulation of MEIS1 expression has been shown to correlate strongly with the cardiomyocyte cell cycle arrest that occurs from day 7 after birth. In this study, there was no statistically significant difference in MEIS1 protein expression between the loaded and unloaded hearts after permanent coronary artery ligation and ischaemia-reperfusion. There appears to be a trend towards higher levels of MEIS1 protein expression in the ischaemia-reperfusion group compared to the permanently ligated group but this did not reach statistical significance probably due to the very high variability in the data. (Fig. 5.16)

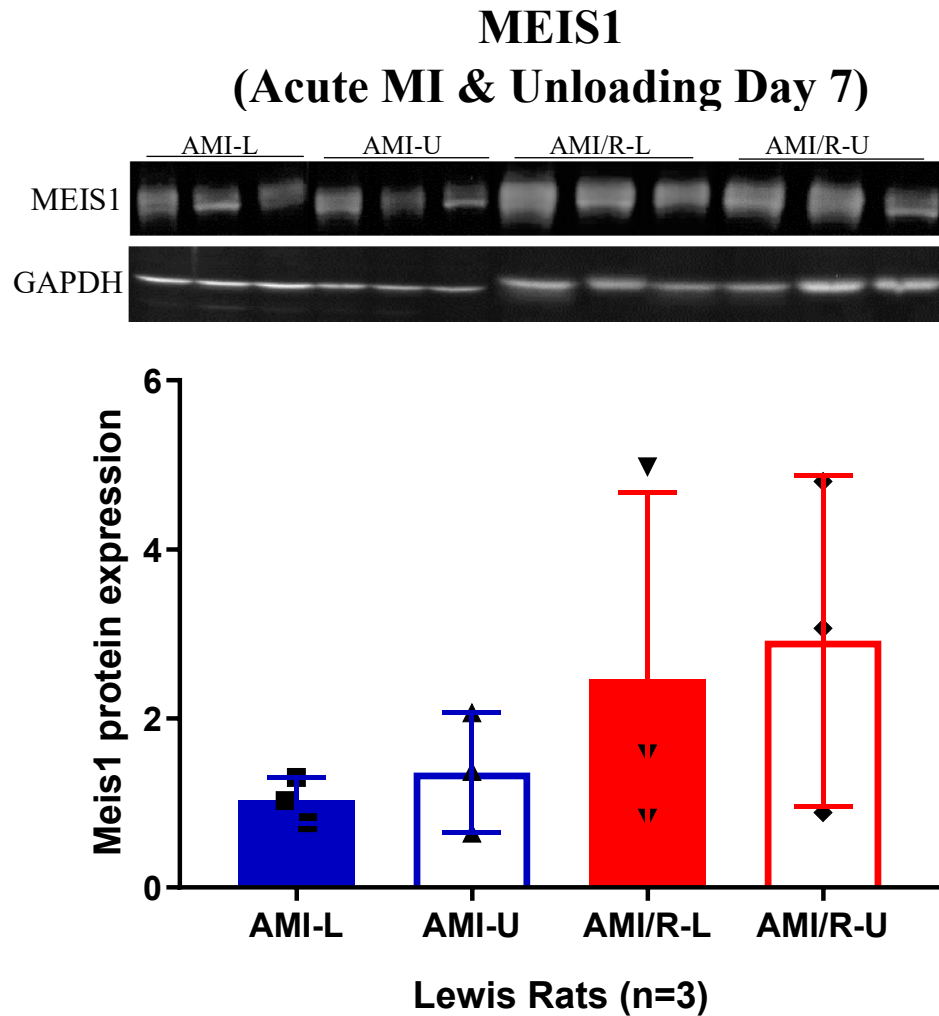


Figure 5.16: Effect of mechanical unloading on MEIS1 protein expression 7 days after permanent coronary artery ligation and ischaemia-reperfusion.

YAP is a protein whose presence in the cell nucleus has been shown to be critical to the induction of cell proliferation (5.1.3).(Bassat *et al.*, 2017) (A. Uygur, 2017) (Heallen *et al.*, 2012) (Xin *et al.*, 2013) Once phosphorylated, YAP is extruded out of the nucleus as phosphorylated YAP (pYAP), thus an increase in the expression of pYAP is synonymous with a decrease in cell proliferation.(Xin *et al.*, 2013)

After permanent coronary artery ligation, there was a statistically significant increase in pYAP expression in the unloaded hearts compared to the loaded hearts (AMI-L 0.5908 ± 0.2313 , vs

AMI-U 1.514 ± 0.2217 , $P=0.0450$). (Fig. 5.17). However, in the ischaemia-reperfusion group, mechanical unloading did not bring about a statistically significant difference in pYAP expression compared to the loaded hearts (AMI/R-L 0.8667, vs AMI/R-U 0.5908, $p=0.5198$), although the trend was towards a decrease in expression. Mechanical unloading after ischaemia-reperfusion was associated with a statistically significant decrease in pYAP protein expression compared to the unloaded permanently ligated hearts (AMI/R-U 0.4991, vs AMI-U 1.514, $p=0.0104$) (Fig. 5.17).

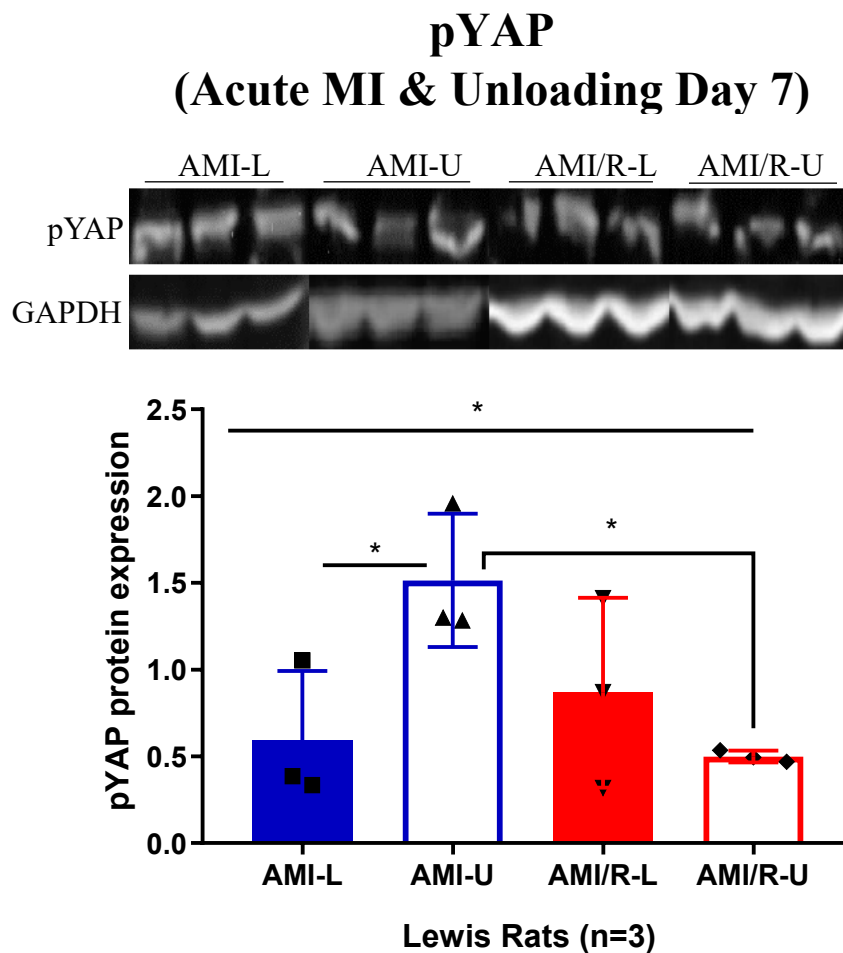


Figure 5.17: Effect of mechanical unloading on pYAP protein expression after permanent coronary artery ligation and ischaemia-reperfusion.

5.5 DISCUSSION

The results of this research show that the adult heart retains an intrinsic proliferative capacity that is stimulated after acute myocardial infarction.(Kajstura *et al.*, 2010) (Bergmann *et al.*, 2009) The data also support the consensus that this intrinsic proliferative response to myocardial injury is modest.(Lee, 2018) (Hsieh *et al.*, 2007) (Jopling *et al.*, 2010) Increases in cardiomyocyte proliferative rate after injury ranged from 0.3% to 2.1% in the loaded permanently ligated and reperfused hearts, a rise that isn't significant enough to bring about meaningful regeneration of lost myocardium after myocardial infarction. This finding thus emphasises the need to develop novel strategies capable of enhancing the adult heart's intrinsic proliferative capacity.

As discussed in sections 5.1.3 and 5.1.4, there is evidence to show that mechanical load and the presence or absence of adequate coronary reperfusion after myocardial infarction play important roles in the ability of the heart to regenerate lost myocardium.(Marín-juez *et al.*, 2016) Previous studies have mainly reviewed the effects of mechanical unloading on the survival and differentiation of cardiac stem cells exogenously implanted into the infarcted heart, (Kurazumi *et al.*, 2014) or the effects of mechanical unloading on the proliferative capacity of chronically infarcted hearts. (Canseco *et al.*, 2015) One study investigated the effect of mechanical unloading on the endogenous proliferative capacity of acutely infarcted hearts; (Suzuki *et al.*, 2007) however, the effect of coronary reperfusion in this setting was not investigated. To the best of our knowledge, this is the first study that investigates the possible synergistic effects of mechanical unloading and coronary reperfusion on the endogenous proliferative capacity of the acutely infarcted adult heart.

The results of this study show that whilst mechanical unloading of the adult heart after acute myocardial infarction brings about an increase in cardiomyocyte proliferation (2%, 3.7%, and

3.9%, in the anterior borderzone, posterior borderzone, and distant myocardium respectively), that was larger than that stimulated by myocardial infarction alone, this effect was not sufficient to generate significant increases in proliferation. Similarly, coronary reperfusion alone did not remarkably alter the rate of cardiomyocyte proliferation in the loaded heart as seen by the regional proliferative rates of 0.4%, 0.5%, and 1.9% in the anterior borderzone, posterior borderzone, and distant myocardium respectively.

When combined however, mechanical unloading and coronary reperfusion were associated with a much larger (2.6%, 10.4%, and 13.4%, in the anterior borderzone, posterior borderzone, and distant myocardium respectively), and statistically significant rise in cardiomyocyte proliferation particularly in the regions of the myocardium furthest away from the primary area of infarct, i.e. the posterior infarct borderzone or distant myocardium. The remarkable upregulation of cardiomyocyte proliferation seen here raises two important points: firstly, that mechanical unloading and coronary reperfusion after acute myocardial infarction have a very strong synergistic effect on the capacity of the adult heart to form new cardiomyocytes. Secondly, that this synergistic effect is most pronounced in the region of the myocardium furthest away from the primary infarct site.

These two findings are not unexpected and have significant clinical implications. It has been previously shown that mechanical unloading after myocardial injury is associated with improvements in myocardial wall stress, cardiomyocyte calcium cycling properties, contractility and normalisation of gene expression patterns and signalling pathways.(Barbone *et al.*, 2001) (Terracciano *et al.*, 2004) (Blaxall *et al.*, 2003) The improvement in overall heart function brought about by unloading improves the neurohumoral milieu around the heart after injury thus providing conditions conducive to cardiomyocyte proliferation. In addition, coronary reperfusion returns blood flow to the infarct territory, providing cells residing particularly in the posterior infarct borderzone and distant myocardium where less damage had

been incurred following the infarct the necessary nourishment to prevent apoptosis and enhance cell growth. Myocytes in the distant regions benefit the most from mechanical unloading and data from this study shows that these conditions culminate in significant upregulation of their proliferative capacity.

To further understand the underlying mechanism by which the synergistic effects of mechanical unloading and coronary reperfusion brings about improvement in the endogenous proliferative capacity of the heart, the expression profiles of MEIS1 and YAP were investigated.

Data from this research shows that mechanical unloading after both permanent coronary ligation and ischaemia-reperfusion was not associated with a statistically significant difference in MEIS1 protein expression, although this might be due to the large variability in the data. Ischaemia-reperfusion and mechanical unloading appears to be associated with higher levels of MEIS1 compared to the unloaded permanently ligated hearts although this did not reach statistical significance (AMI/R-U 2.918, vs AMI-U 1.361, $p=0.2659$).

As described in section 1.4.3.2, MEIS1 upregulation has been strongly linked to the cardiomyocyte cell cycle arrest that occurs at day 7 after birth.(Mahmoud *et al.*, 2013) These data suggests that despite the presence of conditions favourable enough to bring about an upsurge in the proliferative capacity of the heart, there remains an upregulation of MEIS1 expression. This indicates that mechanical unloading and/or coronary reperfusion might have no direct regulatory effects on MEIS1 expression. Furthermore, these results imply that MEIS1 expression might be strongly affected by the proliferative state of cardiomyocytes, rising to dampen any significant attempt at increases in cardiomyocyte proliferation.

To determine the effect mechanical unloading and coronary reperfusion have on the activity of one of the cell-intrinsic signalling pathways that regulate cardiomyocyte proliferation the

expression profiles of YAP were investigated. YAP is a transcription co-activator whose presence in the cell nucleus is vital for cardiomyocyte proliferation.(Bassat *et al.*, 2017) When phosphorylated by the Hippo pathway (which regulates organ size and growth), YAP becomes extruded from the nucleus and is located in the cytosol as pYAP with consequent reduction in cell proliferation.(Bassat *et al.*, 2017) Thus high levels of pYAP are associated with a downregulation of cellular proliferation. (Xin *et al.*, 2013) (Yeo *et al.*, 2012) Data from this study showed that mechanical unloading combined with coronary reperfusion resulted in a statistically significant decrease in the levels of pYAP compared to the unloaded permanently ligated hearts (AMI/R-U 0.4991, vs AMI-U 1.514, $p=0.0104$). This finding correlates well with the significantly high levels of cardiomyocyte proliferation seen after mechanical unloading and coronary reperfusion.

5.6 SUMMARY

These data shows that after acute myocardial infarction, mechanical unloading combined with coronary reperfusion stimulates a significant increase in cardiomyocyte proliferation. This increased proliferation was seen to be more pronounced in the posterior infarct borderzone and distant myocardium. It is reasonable to conclude that this synergistic effect on cardiomyocyte proliferation is achieved at least in part by decreases in the levels of inhibition of YAP. MEIS1 is a strong regulator of cardiomyocyte proliferation and its expression is not inhibited by mechanical unloading and/or coronary reperfusion.

6 Effect of mechanical unloading of the failing heart on whole-heart contractility

6.1 INTRODUCTION

The assessment of myocardial functional improvement in the rat heart after interventions aimed at achieving mechanical unloading-induced reverse remodelling remains suboptimum. The use of isolated cardiomyocytes and cardiac tissue slices to quantify changes in cardiac contractility after mechanical unloading has been of immense value to the field.(Xie *et al.*, 2012) (Ibrahim, Kukadia, *et al.*, 2012) (Ibrahim, Navaratnarajah, *et al.*, 2012b) However, this approach does not give an accurate representation of whole-heart function. Evaluation of myocardial contractility at the whole-heart level after HAHLT takes account of the heterogeneity of the heart's function but more importantly reflects the overall function of both the healthy and the impaired territories of the injured heart.

The in vivo assessment of myocardial contractility using the PV conductance technology is now widely accepted as the optimum approach to investigating changes in intra-thoracic heart function in small animal studies.(Pacher *et al.*, 2008) The application of PV loop studies in the in vivo assessment of contractility in transplanted rat hearts after HAHLT has not been reported. In vivo LV pressure measurements have been described after HAHLT but this was in the absence of volume measurements and did not investigate the function of the reloaded failing heart after unloading.(Mizuno, Weisel and Li, 2005) The use of MRI and 2D speckle tracking echocardiography for the in vivo assessment of contractility is possible but this has only been

done in unloaded transplanted hearts to detect signs of allograft rejection in transplant immunology studies.(Jassal *et al.*, 2009) (Pieper *et al.*, 2010) (Wu *et al.*, 2009)

The optimum assessment of improvements in LV contractile function in the mechanically unloaded failing heart requires that the LV is reloaded and made to work against a physiological afterload similar to that encountered by the healthy loaded heart. MRI and 2D speckle tracking echocardiography could be employed in the in vivo assessment of LV contractile function after a second-stage operation to reload the LV following HAHLT.(Asfour *et al.*, 1999) However, reloading the heart in vivo via a second-stage operation is technically challenging. In addition, MRI and echocardiography have the limitation of being affected by variations in load making specific evaluation of LV function inadequate.

The ideal approach to assessing LV contractile function after HAHLT is via employing the ex vivo isolated working/ejecting heart system in conjunction with the PV conductance catheter technology. By reloading the LV of the mechanically unloaded failing heart ex vivo (described in section 2.2.7.1) and making the heart eject against varying levels of afterload, the heart is essentially put through rigorous physiologically relevant work. Assessment of LV contractile function under these conditions provides a more accurate representation of the degree of myocardial functional recovery after mechanical unloading. However, the ex vivo evaluation of whole-heart contractile function after HAHLT by utilising the working/ejecting heart system to carry out PV loop studies from the mechanically unloaded failing rat heart has not been previously undertaken. To the best of our knowledge, this is the first report of the ex vivo assessment of whole-heart contractility in the mechanically unloaded failing heart and this technique is of particular value in this study.

6.2 METHODS

6.2.1 The Working Heart and PV Loop System

Eight rats were studied, 3 control (healthy loaded) hearts, 3 chronically infarcted loaded hearts, and 2 chronically infarcted unloaded hearts. The working heart and PV loop system was used to obtain ex vivo whole-heart haemodynamic indices of myocardial function from each of these rat hearts as detailed in section 2.2.7.

6.2.2 Statistics

A two-tailed t-test was used for the comparison between independent groups of normally distributed data as determined by Kolmogorov-Smirnov testing. One-way analysis of variance (ANOVA) followed by Bonferroni post hoc test for individual significant differences was used. All statistical analyses were done using Prism 7 software (GraphPad Software, Inc.). Three rats were studied in the CTR and CMI-L groups while 2 rats were studied in the CMI-U group. The results are presented as means $\pm$ SD. The significance threshold was set at $p < 0.05$ for all data.

* denotes $P < 0.05$, ** denotes $P < 0.01$ and *** denotes $P < 0.001$.

6.3 RESULTS

6.3.1 Ex vivo PV Loop Analysis of the Rat Working Heart System

In healthy rat ex vivo working hearts, PV loop data obtained following trans-apical catheter placement showed significant systolic blood pressure development of about 115mmHg and diastolic pressure of about 10mmHg as shown in figure 6.1.

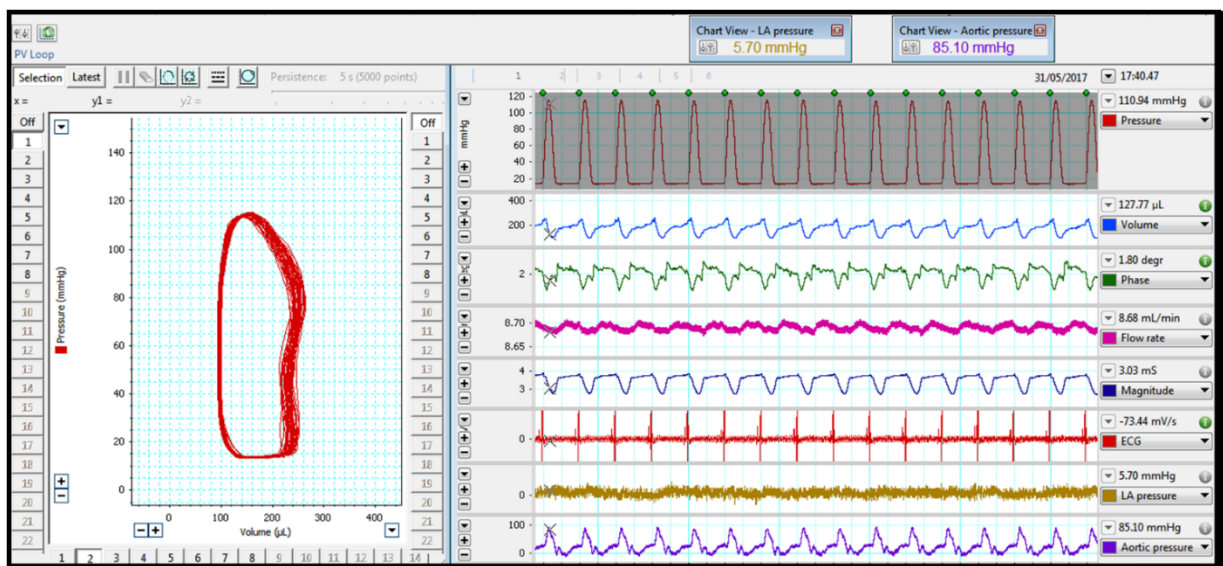


Figure 6.1: Representative PV loop study from a healthy ex vivo working rat heart showing steady state systolic and diastolic pressures during the cardiac circle.

Loaded failing hearts (16wks after myocardial infarction) that were put through physiological work via the ex vivo working heart system showed systolic blood pressure development that was significantly lower (80mmHg) than that seen in healthy aged matched hearts. These hearts also had a higher end-diastolic pressure (25mmHg) compared to the healthy hearts indicative of the presence of diastolic dysfunction (Fig. 6.2).

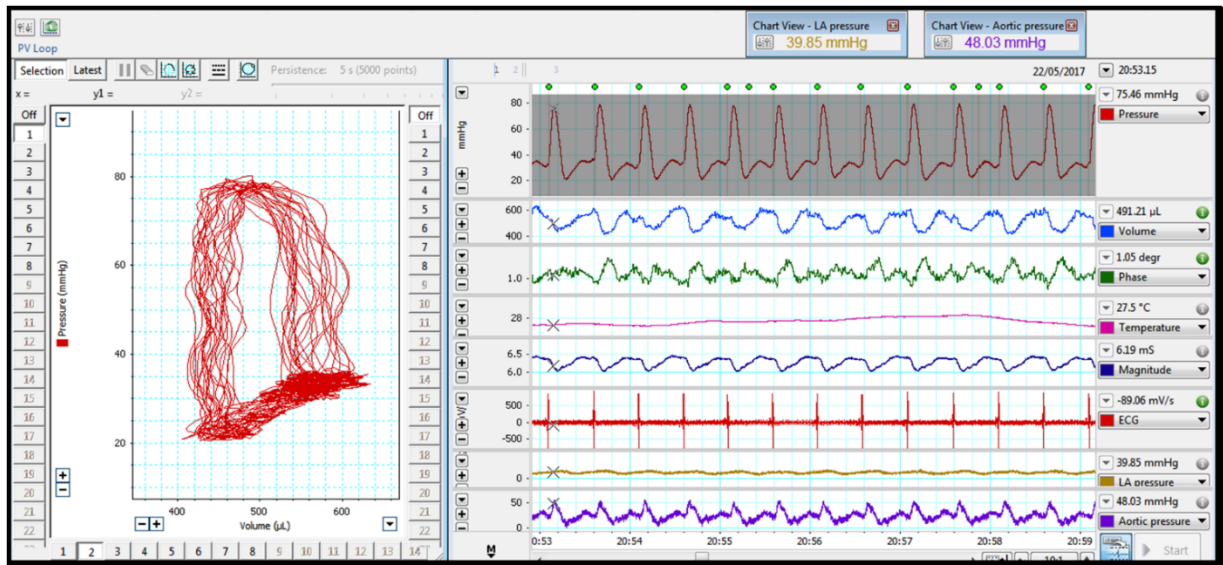


Figure 6.2: Pressure-volume loop from an infarcted ex vivo working rat heart showing low systolic pressure generation and evidence of diastolic dysfunction with higher diastolic filling pressures.

In failing hearts that have been mechanically unloaded via HAHLT, significant myocardial atrophy occurred (CMI-L vs CMI-U $p=0.0001$) as shown in figure 6.3.

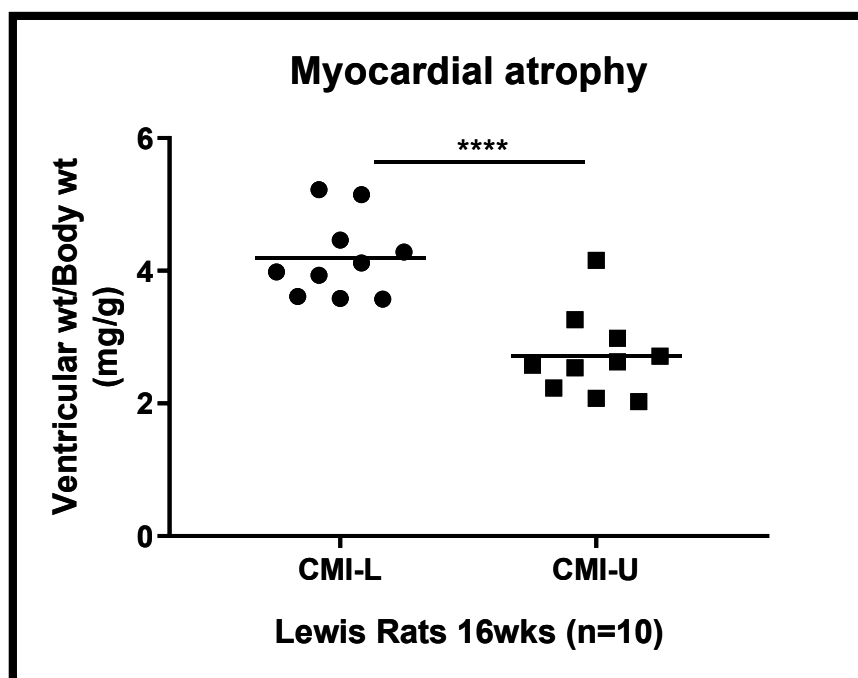


Figure 6.3: Significant myocardial atrophy was noted in the mechanically unloaded failing hearts. These hearts had undergone unloading via HAHLT for 4 weeks after the development of significant heart failure at week 12. The dots represent number of rats.

There was a much smaller LV chamber size which was predominantly made up of scar tissue as depicted in figure 6.4 (arrow).

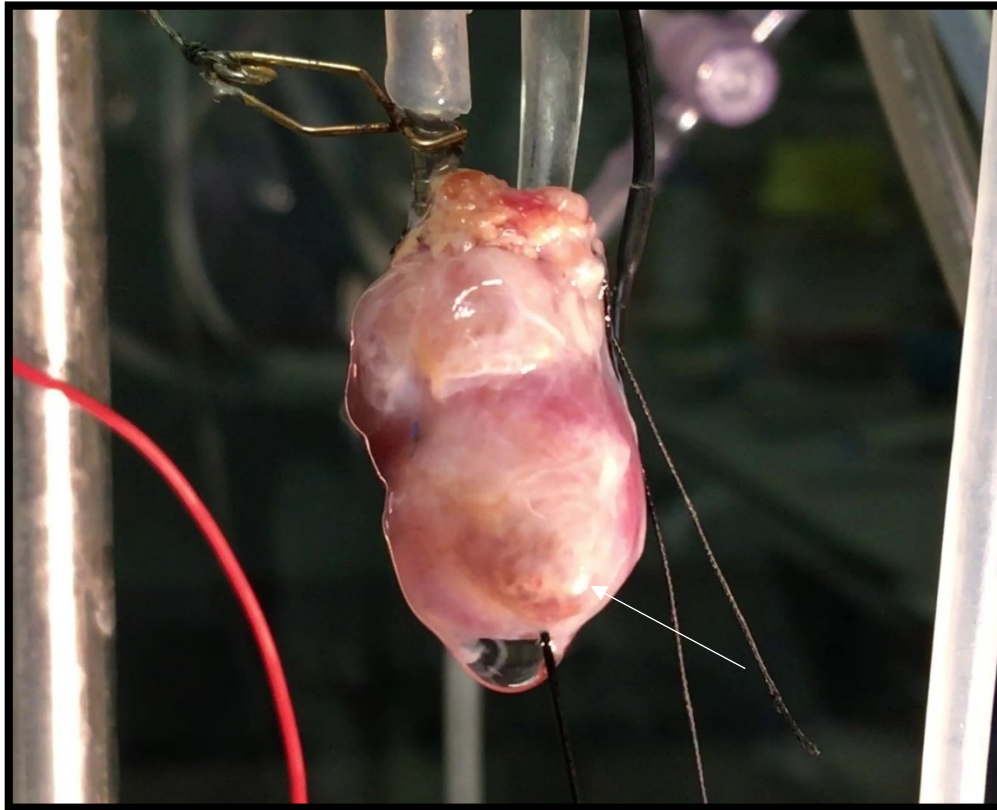


Figure 6.4: Picture of a mechanically unloaded failing rat heart with its atrophied left ventricle being exposed to increased physiological load via the ex vivo working heart system. The PV loop catheter can be seen exiting from the LV apex.

PV-loop studies in the working mechanically unloaded failing hearts showed the presence of a small LVEDV, a low systolic blood pressure generation (64mmHg), and a very low stroke volume indicative of a significantly impaired systolic and diastolic function as shown in figure 6.5.

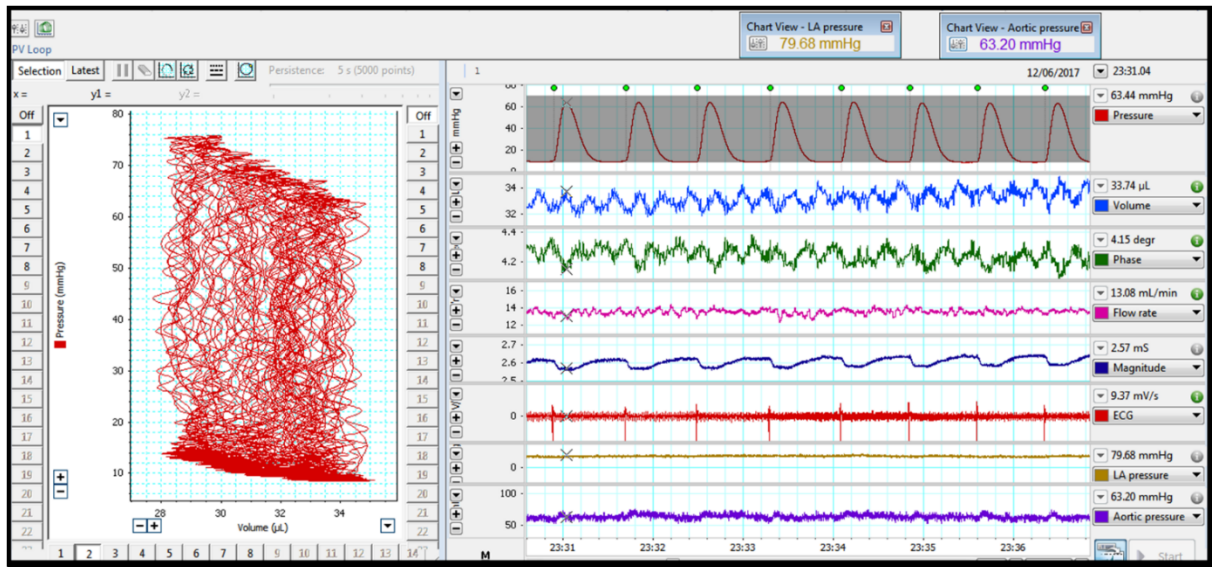


Figure 6.5: Pressure-volume loop from an unloaded infarcted ex vivo working rat heart showing significantly impaired systolic and diastolic function.

At steady state, the following haemodynamic indices of myocardial function were obtained from PV-Loop studies during ex vivo working heart experiments. All n=3 unless where otherwise stated. (Table 6.1)

Cardiac Function

	Control heart (n=3)	Chronic MI Loaded (n=3)	Chronic MI Unloaded (n=2)
SW (mmHg* μ l)	12785 $\pm$ 1184	4750 $\pm$ 2098.5	6.3 $\pm$ 2
CO (ml/min)	23.9 $\pm$ 1.9	24.6 $\pm$ 13.6	0.143 $\pm$ 0.06
SV (μ l)	156.9 $\pm$ 11.6	170.7 $\pm$ 55.8	2.3 $\pm$ 0.5
HR (bpm)	152.2 $\pm$ 9.8	150.7 $\pm$ 10	62 $\pm$ 17.7
LVEDV (μ l)	221.8 $\pm$ 13.8	558.2 $\pm$ 76.3	31.6 $\pm$ 3.1
LVESV (μ l)	110.7 $\pm$ 8	469.5 $\pm$ 58.9	31.2 $\pm$ 2.7
LV Dev P (mmHg)	101.1 $\pm$ 0.75	51 $\pm$ 1.4	58.5 $\pm$ 9
LVEDP (mmHg)	14.8 $\pm$ 0.75	32.43 $\pm$ 6.5	11.2 $\pm$ 2.8
+dP/dt (mmHg/s)	2541 $\pm$ 82	1301 $\pm$ 204.5	742.3 $\pm$ 47.7
-dP/dt (mmHg/s)	2182 $\pm$ 81.5	941 $\pm$ 157	335.8 $\pm$ 47.7
EF (%)	67.2 $\pm$ 6.4	30.8 $\pm$ 8.7	7.4 $\pm$ 1.5
Tau (ms)	25.8 $\pm$ 1.2	97.1 $\pm$ 6	153.1 $\pm$ 36.6

Table 6.1. Cardiac function from ex vivo working rat hearts. SW: stroke work; CO: cardiac output; SV: stroke volume; HR: heart rate; LVEDV: left ventricular end-diastolic volume; LVESV: left ventricular end-systolic volume; LV Dev P: left ventricular developed pressure; LVEDP: left ventricular end-diastolic pressure; +dP/dt: first derivative LV pressure positive; -dP/dt: first derivative LV pressure negative; EF: ejection fraction; Tau: diastolic constant. Data given as mean $\pm$ SD, n represents number of hearts studied.

Preload occlusion in the healthy and chronically infarcted rat hearts was employed as illustrated in figures 6.6 (control heart) and 6.7 (infarcted heart), and this provided load independent

indices of myocardial contractility such as ESPVR, EDPVR and PRSW as described in section 1.4.1.5. Unfortunately, preload occlusion could not be completed in the mechanically unloaded chronically infarcted hearts due to the significant decrease in LVEDV secondary to decreased LV chamber size from unloading-induced myocardial atrophy.

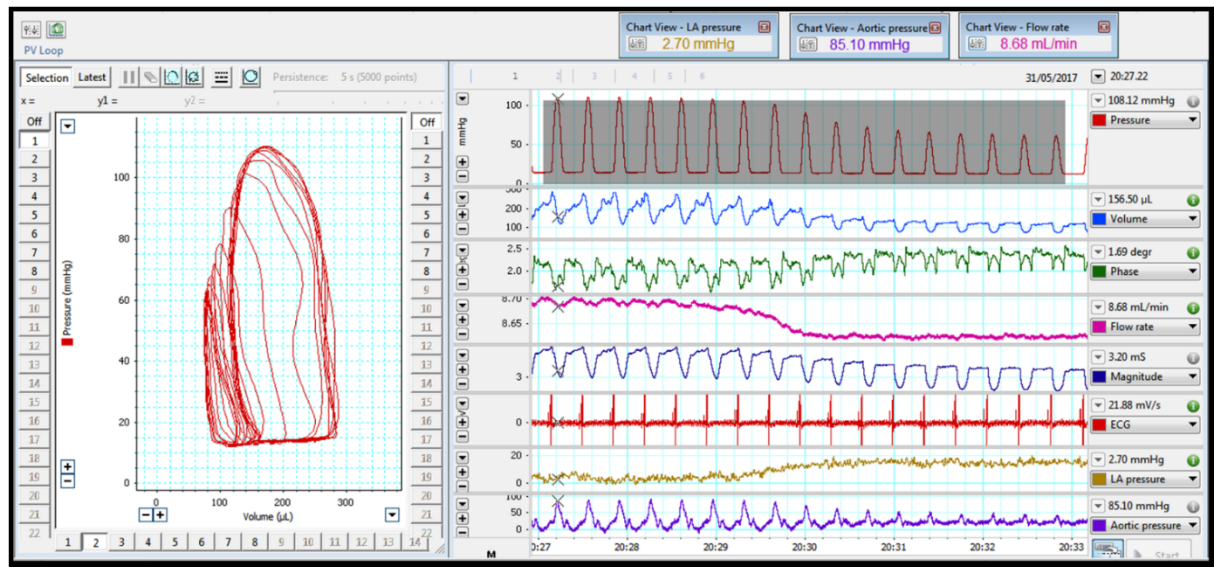


Figure 6.6: Preload occlusion in a healthy ex vivo working rat heart for the assessment of load independent indices of cardiac function.

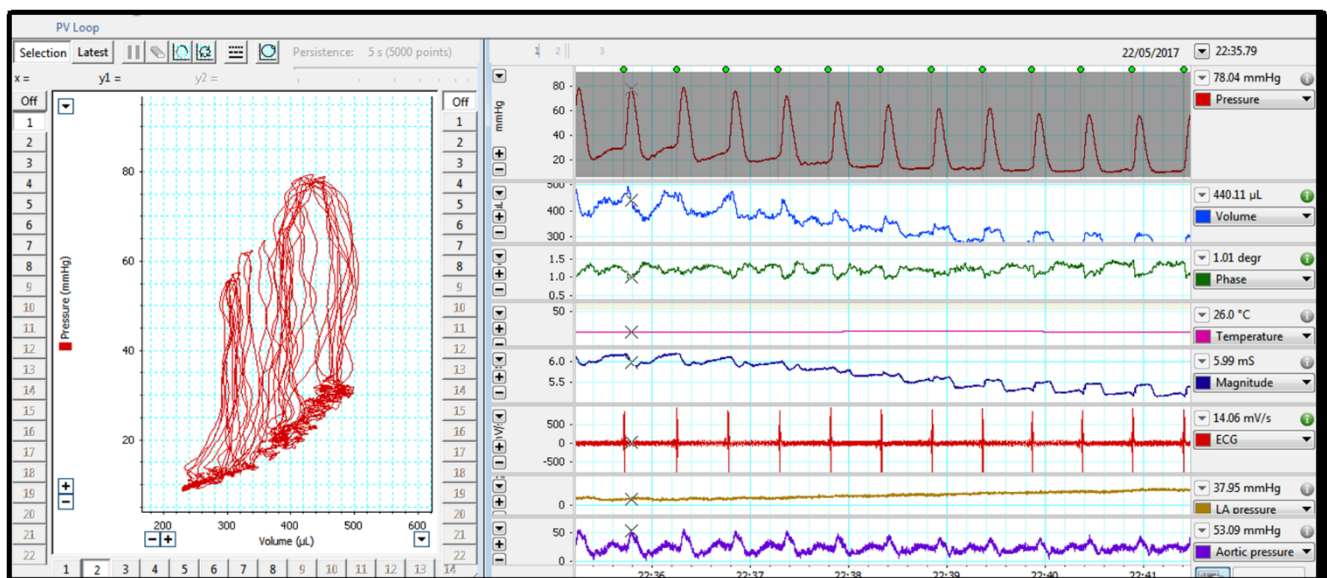


Figure 6.7: Preload occlusion in an infarcted ex vivo working rat heart for the assessment of load independent indices of cardiac function.

The differences in the ESPVR and EDPVR between the healthy loaded (Controls) heart (Fig. 6.8) and the chronically infarcted heart (Fig. 6.9) is shown below.

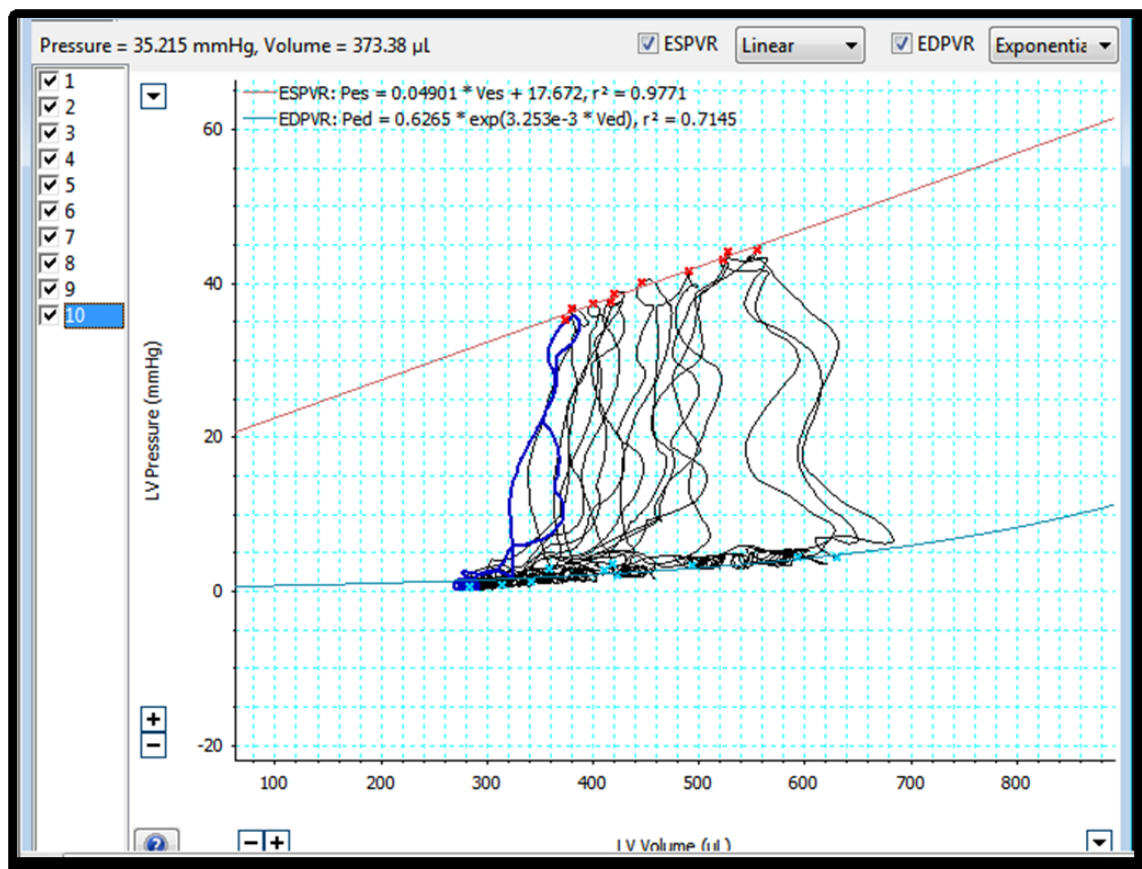


Figure 6.8: ESPVR and EDPVR in a control rat heart after preload occlusion during ex vivo isolated working heart and simultaneous PV loop analysis using a scisense catheter.

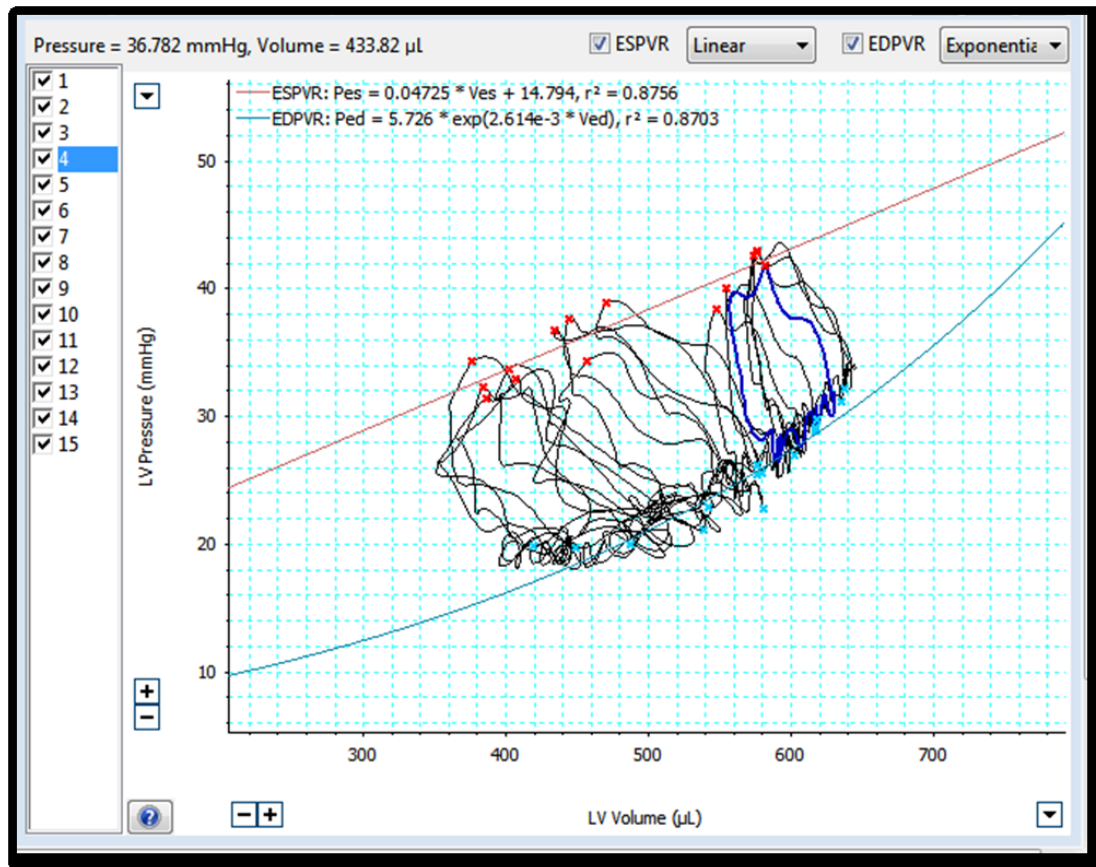


Figure 6.9: ESPVR and EDPVR in a loaded chronically infarcted rat heart after preload occlusion during ex vivo isolated working heart and simultaneous PV loop analysis using a scisense catheter.

Consistent with the development of systolic dysfunction after chronic myocardial infarction, the slope of the ESPVR (mmHg/ μ l) in the control hearts was significantly higher than in the infarcted hearts (CTR-L 0.04906 vs CMI-L 0.04730; $p < 0.0001$). Similarly the development of diastolic dysfunction was observed in the infarcted hearts as confirmed by the presence of a significantly higher EDPVR (mmHg/ μ l) compared to control (CMI-L 5.726 vs CTR-L 0.6262; $p < 0.0001$). (Figures 6.8 and 6.9) The slope of the EDPVR is inversely proportional to the compliance of the left ventricle.

Finally, the PRSW, a highly sensitive load-independent marker of myocardial contractility was also obtained after preload occlusion. PRSW (mmHg) was found to be significantly reduced

after chronic myocardial infarction (CTR-L 78.22 vs CMI-L 21.59; $p < 0.0001$) as illustrated in figures 6.10 and 6.11

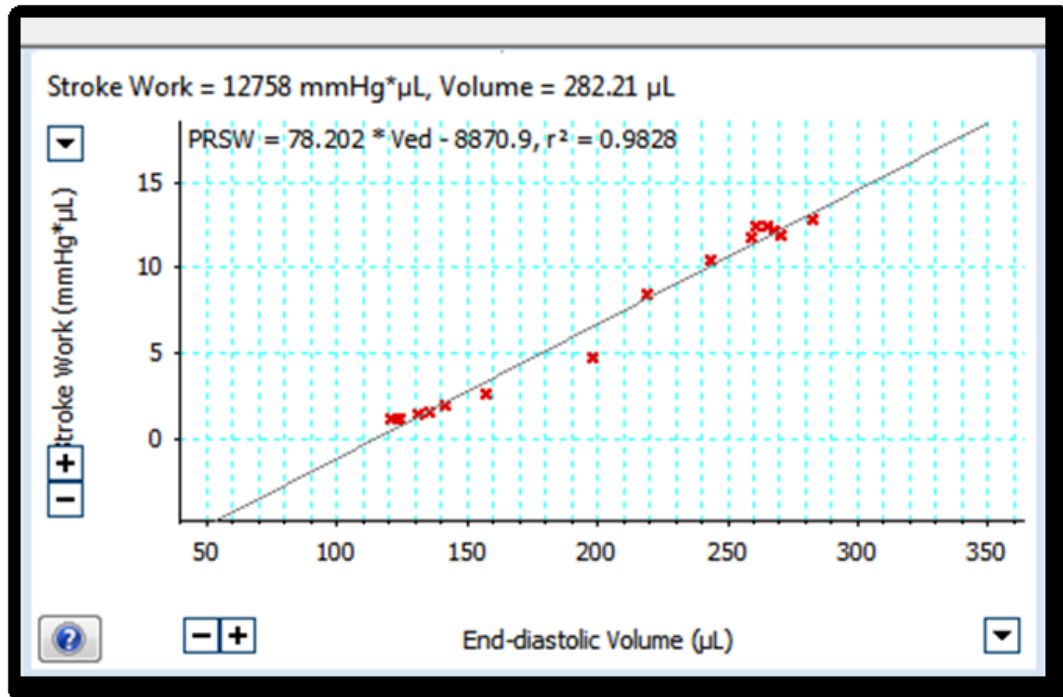


Figure 6.10: PRSW in a loaded healthy rat heart (control) after preload occlusion during ex vivo isolated working heart and simultaneous PV loop analysis using a scisense catheter.

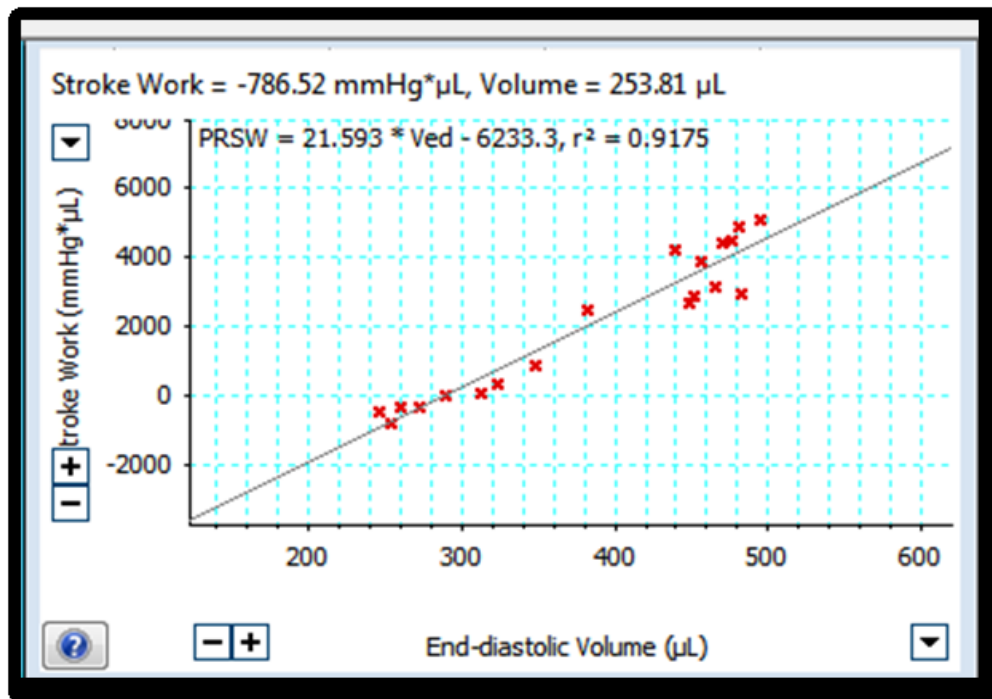


Figure 6.11: PRSW in a loaded chronically infarcted rat heart after preload occlusion during ex vivo isolated working heart and simultaneous PV loop analysis using a scisense catheter.

6.4 DISCUSSION

6.4.1 Ex vivo rodent whole-heart contractility after MI & HAHLT

The re-introduction of physiological load to the infarcted mechanically unloaded rat heart in order to determine the effect of unloading on whole-heart contractility after MI has not been reported. Several studies have shown that after myocardial infarction, mechanical unloading via HAHLT results in improvements in isolated cardiomyocyte contractility.(Ibrahim and Terracciano, 2013) (Ibrahim, Kukadia, *et al.*, 2012) Whether this effect translates into

improvement in whole-heart contractility has not been investigated. By employing the ex vivo rodent ejecting/working heart and PV loop system, indices of myocardial contractility were obtained in the control, failing, and unloaded failing hearts. This novel experiment showed firstly that ex vivo evaluation of a mechanically unloaded rodent heart subjected to physiological load using the working heart system combined with deployment of a miniaturised PV loop catheter is indeed achievable. Ex vivo haemodynamic parameters obtained were similar to those obtained from in vivo rat PV loop experiments.(Connelly *et al.*, 2006)

Compared to the failing heart, the control heart was able to generate significantly higher systolic pressures, SW, LV Dev P, and $+dP/dt$ whilst the failing heart had much higher LVEDV, LVESV, and tau (table 6.1). This is indicative of significantly impaired systolic and diastolic function in the chronically infarcted heart. When compared to the loaded failing hearts, significant myocardial atrophy occurred in the mechanically unloaded failing hearts (Fig. 6.3) and the resultant reduced LV chamber size culminated in much lower LVEDV in the unloaded hearts. However, the systolic pressure obtained from the unloaded failing hearts and LV Dev P (a marker of contractility) were comparable to the loaded hearts. Mechanical unloading of the chronically infarcted heart was however associated with significant worsening of LV diastolic function as indexed by $-dP/dt$ and tau (table 6.1).

6.5 SUMMARY

The findings from this study suggests a significant worsening of whole-heart diastolic function after the failing heart undergoes a period of mechanical unloading and this is consistent with previously described findings in this report and other published reports highlighting a

worsening of fibrosis after mechanical unloading.(Drakos *et al.*, 2010) (Klotz *et al.*, 2007) Due to the presence of significant myocardial atrophy after 16 weeks of mechanical unloading and the consequent reduction in LV chamber size and filling, it was not possible to adequately determine if the previously published improvements in isolated cardiomyocyte contractile function seen with unloading translates to improved whole-heart contractile function.

A methodology for the ex vivo assessment of the rat hearts' myocardial function after HAHLT is now established and this should facilitate future studies into the effects of mechanical unloading on the whole-hearts' contractile function after it is exposed to novel interventions.

7 GENERAL DISCUSSION

7.1 INTRODUCTION

In this final chapter, I will summarise the key findings of this thesis and how results from the three broad subject areas; the excitation-contraction coupling, the extracellular matrix, and the endogenous proliferative capacity of the mechanically unloaded failing heart converge to raise further questions that potentially have immediate translational relevance. The strengths and limitations of this research study will be discussed, and finally, the areas in need of further study in addition to the future directions LVAD-induced reverse remodelling should take will be proposed.

7.2 Mechanosensitivity of the Failing Myocardium: Role of Mechanical Unloading

KEY FINDINGS:

7.2.1 Excitation-Contraction Coupling

The process of calcium-induced calcium release which leads to the production of large synchronous Ca^{2+} transients and efficient myocardial contractility is heavily reliant on the tight junctional membrane complexes or dyads between LTCCs present in t-tubules and

RyRs.(Eisner *et al.*, 2017) The structure of these dyads are maintained by the activity of key anchoring, stretch-sensitive, and synthetic proteins such as JPH2, Tcap, and BIN-1 respectively as described in 1.4.1.(Manfra, Frisk and Louch, 2017) The role these molecular elements play in the reported mechanical unloading induced recovery of the structural integrity of t-tubules and these dyads disrupted in the failing heart are largely unknown.

Data presented in this thesis shows that the co-localisation or efficacy of JPH2 and Tcap activity, indexed by the regularity of their expression along t-tubules is significantly altered by mechanical unloading. Mechanical unloading of the failing heart was associated with a significant increase in JPH2 regularity and by contrast, a statistically significant decrease in Tcap regularity. These effects were not observed with BIN-1, where mechanical unloading of the failing or control hearts was not associated with any significant alterations in its regularity or density. This is possibly due to BIN-1's ubiquitous expression which is different to that of JPH2 and Tcap, two anchoring and stretch-sensitive proteins respectively, whose activities are reliant on their close co-localisation with t-tubules. The observed increase in JPH2 regularity in the mechanically unloaded failing heart is a significant finding as it provides insight into the possible role a key structural membrane protein, important in t-tubule-SR tethering has in the reported recovery of t-tubule structural integrity noted after the failing heart is mechanically unloaded. Notably however, mechanical unloading did not bring about any significant alterations in JPH2 density. This implies that therapeutic strategies aimed at improving the regularity of JPH2 in the failing heart might be of more clinical relevance.

Furthermore, it is interesting to note that the upregulation of JPH2 regularity with mechanical unloading was only observed in the unloaded failing heart. In the healthy (control) heart with normal physiological load, mechanical unloading was associated with a significant decrease in JPH2 regularity to a degree similar to that observed in the loaded failing heart. This indicates a bimodal response of the heart's contractile apparatus to variations in load, with excessive

load reduction, as occurs when a healthy heart is unloaded, also having a significant detrimental effect on the activity of JPH2. This finding is in line with previous reports showing that the structural integrity of t-tubules is load-dependent with excessive overload or excessive mechanical unloading bringing about disruption in t-tubule structure and function.(Ibrahim and Terracciano, 2013) (Jones, MacQuaide and Louch, 2018)

The regularity of Tcap was significantly decreased by mechanical unloading in both the failing and control hearts. By contrast there was a similar degree of Tcap regularity in both the loaded control and chronically overloaded failing hearts, suggesting that Tcap activity is enhanced in the presence of increased mechanical load. This finding further emphasises Tcap's role as a load-dependent regulator of t-tubule structure with the deleterious manifestation of Tcap loss being more pronounced mainly in the presence of increased ventricular load. These data suggests a possible contribution of disruption in Tcap activity in the detrimental effects on the heart sometimes observed during mechanical unloading and warrants further investigating.

7.2.2 Extracellular Matrix

The ECM remains the only known part of the unloaded failing heart that appears to portray features that are not in keeping with the recovery process with many contemporary studies showing that interstitial fibrosis worsens during mechanical unloading with LVADs.(Schaefer *et al.*, 2019) (Lok *et al.*, 2015) (Drakos *et al.*, 2010) The underlying mechanism by which this increase in fibrosis occurs with LVAD use has not been described.

Data from this thesis is to the best of our knowledge the first to report a possible mechanistic link between mechanical unloading and the worsening of fibrosis. We are the first to show that after acute myocardial infarction via permanent coronary artery ligation, mechanical unloading through HAHLT results in a significant worsening of interstitial fibrosis but this increase in

fibrosis is absent in the setting of ischaemia-reperfusion. These data implies that re-establishing blood flow through the previously ligated major coronary vessel has a protective effect on the propagation of interstitial fibrosis during mechanical unloading. We have not shown the exact underlying mechanism by which coronary reperfusion and mechanical unloading brings about this protective effect, however, there is collaborative evidence that suggests that it might be a result of the effect mechanical unloading has on the autoregulation of the collateral circulation.

It has been shown that mechanical unloading is associated with a reduction in microvascular luminal diameter in the failing heart and a significant reduction in coronary flow reserve.(Drakos *et al.*, 2010) This effect of mechanical unloading on the microvasculature in a failing heart is believed to be a result of the markedly decreased LV workload during mechanical unloading which reduces myocardial oxygen consumption in the injured heart and thus diminishes the autoregulatory impulse that should stimulate arteriogenesis in the microcirculation.(Tansley *et al.*, 2004) This autoregulatory stimulus is required to match myocardial blood flow to changes in myocardial oxygen demand. The effect of autoregulation on the collateral circulation has been previously described in studies showing that once blood flow in the parent vessel is re-established following a period of ischaemia, collateral channels rapidly become non-functional and return to their collapsed redundant state.(Rentrop *et al.*, 1985) The impact diminished flow in the microvasculature can have on the propagation of interstitial fibrosis or infarct size after myocardial infarction cannot be overemphasised. It has been shown that the presence of a robust collateral circulation is associated with much better outcomes after myocardial infarction.(Meier *et al.*, 2012)

Interestingly, this protective effect, a result of releasing the obstructing ligature and returning blood flow to the parent coronary vessels, was observed only in the acute group (2 and 7 days post ischaemia-reperfusion and unloading) and not in the chronic hearts (16 weeks post ischaemia-reperfusion but only 4 weeks of unloading). Whilst the coronary ligatures were

released 90 minutes after LAD and PDA occlusion in both the acute and chronic groups, the ischaemia-reperfused heart was immediately mechanically unloaded only in the acute group. In the chronic group, the ischaemia-reperfused heart was harvested 12 weeks after myocardial infarction and then mechanically unloaded for 4 weeks. We believe that the absence of the protective effect of reperfusion in the chronic hearts and consequent increase in interstitial fibrosis with mechanical unloading is probably a result of the gradual re-occlusion of the injured parent coronary vessels as a result of a process called intimal hyperplasia. TGF- β 1 protein expression has been shown to be significantly upregulated during intimal hyperplasia and it is of note that this data shows that the expression of TGF- β 1 was significantly higher in the chronic ischaemia-reperfused hearts compared to the acute group.(Khan and Sheppard, 2006)

Intimal hyperplasia is a common consequence of vascular injury. It is a process whereby the intimal layer of an injured blood vessel becomes thickened due to the proliferation of vascular smooth muscle cells and proteoglycan-rich extracellular matrix located between the endothelium and the internal elastic lamina. It is a gradual process that takes about 4 weeks to progress to re-occlusion of the vessel after reperfusion.

Whilst this does not provide enough evidence to show that alterations in flow through the coronary collateral circulation is responsible for the absence of worsening interstitial fibrosis after mechanical unloading, it is yet another collaborative evidence to that effect. The unique design of this research whereby the only difference between the mechanically unloaded hearts was the presence or absence of coronary reperfusion raises important questions about the role ischaemia-reperfusion plays in the reported worsening of interstitial fibrosis after mechanical unloading with LVADs. Further studies are needed to corroborate this finding.

7.2.3 Endogenous Proliferative Capacity of the Adult Heart

There is now sufficient evidence in support of the paradigm shift away from the previously held concept that the adult heart is incapable of self-regeneration. Despite the major transition the heart undergoes from hyperplastic to a predominantly hypertrophic growth shortly after birth, studies have shown that the adult heart still retains a degree of intrinsic regenerative capacity albeit at a substantially slower rate, incapable on its own of bringing about meaningful replacement of lost cardiomyocytes after injury.(Lee, 2018) (He and Zhou, 2017) Whilst a number of studies have shown that the endogenous proliferative rate of the adult heart is stimulated following myocardial injury, to the best of our knowledge we are the first to show that this proliferative capacity is significantly upregulated by a combination of ischaemia-reperfusion and mechanical unloading.

Data presented in this thesis show that following acute myocardial infarction brought about by 90 minutes of coronary artery ligation, release of this ligature and return of coronary blood flow to the infarct territory combined with mechanical unloading via HAHLT was associated with a remarkable increase in the proliferation of new cardiomyocytes particularly in the areas of the myocardium distant from the site of injury. Acute myocardial infarction, ischaemia-reperfusion, or mechanical unloading on their own did not result in similar outcomes. This remarkable synergistic effect of combining myocardial injury, reperfusion, and mechanical unloading for a duration of 2 to 7 days has never been reported but has major clinical implications. We have previously reported in this thesis that mechanical unloading after acute myocardial infarction brought about by permanent coronary artery ligation was associated with a significant worsening of interstitial fibrosis and infarct size, a deleterious effect of unloading that was absent after reperfusion.

The observation that following ischaemia-reperfusion and mechanical unloading, the reduction in the propagation of interstitial fibrosis coupled with reductions in TGF- β 1 and α SMA protein expression, contribute to the generation of areas of the myocardium where the proliferation of new cardiomyocytes are encouraged raises significant questions for future investigations into the underlying factors contributing to the muted regenerative response of the adult heart to injury. This finding further raises concerns about the impact increase in fibrosis appears to have in hindering the ability of the failing heart to undergo substantial recovery during mechanical unloading with LVADs and warrants further study.

7.2.4 Whole-heart Contractility

Finally, the evaluation of whole-heart contractility by physiologically reloading the left ventricle of a mechanically unloaded failing heart via the ex-vivo ejecting or working heart system showed a significant worsening of diastolic function whilst systolic function was maintained. There was no evidence of significant improvement in whole-heart contractility in the mechanically unloaded failing hearts compared to the loaded infarcted hearts but it is important to note that the mechanically unloaded hearts had undergone significant myocardial atrophy. Most of the healthy myocardium had atrophied leaving predominantly fibrous tissue behind hence the significant increase in diastolic pressure. The remaining viable myocardium in the unloaded failing hearts were able to generate a LV Dev P (marker of contractility) similar to the loaded infarcted hearts. It is possible that this is the basis for reports showing improved contractility in isolated cardiomyocytes or myocardial tissue slices from mechanically unloaded failing hearts which does not however equate to sustained functional recovery in the mechanically unloaded failing human heart. More needs to be done to address LVAD-induced

myocardial atrophy and the propagation of myocardial fibrosis and infarct size seen with mechanical unloading.

To the best of our knowledge this is the first reported case of a mechanically unloaded failing rat heart being functionally assessed *ex vivo* by physiologically reloading its left ventricle. This has significant implications for future studies wishing to assess *ex vivo* whole-heart functional response to therapeutic interventions.

7.3 IMPLICATIONS

The most important translational element that should emanate from work presented in this thesis involves investigating the potential effect coronary revascularisation during LVAD implantation into a chronically infarcted failing heart will have on the propagation of interstitial fibrosis and the incidence of myocardial recovery. As worsening fibrosis during LVAD use currently remains the only aspect of the unloaded failing heart that is not in keeping with the recovery process, its amelioration might lead to significant increase in LVAD-induced myocardial functional recovery.

Furthermore, the observation presented in this thesis that ischaemia-reperfusion after acute myocardial infarction coupled with mechanical unloading is associated with a significant upregulation of cardiomyocyte proliferation has major future research implications. A substantial intrinsic stimulus to the heart to proliferate new cardiomyocytes in an attempt to replace lost myocardium after injury clearly exists. Coronary reperfusion aimed at returning blood flow to the infarct territory coupled with the reduction of myocardial oxygen consumption and elimination of the toxic neurohumoral milieu in the injured heart via

mechanical unloading appears to create a favourable environment for cardiomyocyte proliferation. Future studies in regenerative cardiology need to harness this concept to aid improve outcomes.

Finally, to date small animal studies testing the effects mechanical unloading via HAHLT along with other interventions have on myocardial contractility have relied mainly on changes obtained from tissue slices and isolated cardiomyocytes. The heterogeneity of the hearts contractile function makes this an obvious translational limitation. Work presented in this thesis shows that robust physiological haemodynamic data can be obtained *ex vivo* from explanted mechanically unloaded rat hearts using the ejecting/working heart protocol described. This novel technique means *ex vivo* physiological evaluation of changes in whole-heart contractility can be accomplished in future studies thus providing much more reliable data for translational purposes.

7.4 STRENGTHS AND LIMITATIONS

The unique design of this study permitted the unravelling of potential answers to a number of key challenges currently facing the world of regenerative cardiology and LVAD-induced myocardial recovery. Three major, previously unreported findings discovered in this study have substantial translational and future research implications. These include: firstly, the synergistic role coronary reperfusion coupled with mechanical unloading has in ameliorating the increase in interstitial fibrosis seen with LVAD use; secondly, the discovery that ischaemia-reperfusion when combined with mechanical unloading leads to significant upregulation of cardiomyocyte proliferation; and thirdly, showing that the *ex vivo* evaluation of contractility

in a mechanically unloaded rat heart by using a novel ejecting or working-heart setup to obtain physiological PV-loop haemodynamic parameters is indeed possible.

Cardiomyocyte proliferation was determined by the presence of Ki67 protein expression in cardiomyocyte nuclei. The use of Ki67 as a proliferative marker in this study is limited by the fact that it only identifies cells that had re-entered the cell cycle at the time of heart harvest as opposed to other proliferative markers such as BrdU which identifies all proliferating cells over the experimental period. The proliferative rate thus noted here is an underestimation of the actual regenerative response of the heart to the experimental conditions. Furthermore, whilst Ki67 identifies cells at any of the phases of the cell cycle aside from G0 (G1, S, G2 and M phases), it does not determine the presence of true cell division as opposed to binucleation or multinucleation. Future studies using markers of mitosis such as Aurora B and or phosphorylated histone will be beneficial.

We did not provide strong evidence to explain the underlying mechanism by which coronary reperfusion brought about significant amelioration of the increase in interstitial fibrosis noted with mechanical unloading. The study was however designed such that the only difference between the groups studied was the return of coronary blood flow through the major non-autoregulated coronary vessels hence making the concept of autoregulated collateral circulation a probable underlying mechanism. Other limitations include the lack of strong housekeeping proteins for western blotting, the limited array of proteins screened and the low n numbers in some experiments. Primary antibody specificity was determined at the beginning of the study. This was not repeated in tandem with each experiment which could have introduced some errors to the data.

Inbred Lewis rats were used in this study in order to avoid any requirements for immunosuppression. However the presence of partial immune-reaction cannot be excluded.

Also despite attempts at achieving partial unloading in all experiments, the occurrence of full unloading in some rats cannot be excluded.

In the analysis of JPH2, BIN1 and Tcap, the power of regularity of these proteins along t-tubules was used for qualitative assessment of expression. T-tubules were not directly analysed as these had previously been studied in the laboratory. Furthermore, the severity and duration of heart failure resulted in some cells being too damaged and earlier mechanical unloading might have been of value. In addition, the duration of unloading predisposed to significant myocardial atrophy with reduction in LVEDV. This resulted in an inability to obtain load-independent parameters of contractility in the mechanically unloaded chronically infarcted hearts.

Finally, TGF- β 1 and α SMA are non-specific markers of fibroblast and myofibroblast activity respectively and as such some caution is required in the data interpretation.

7.5 FUTURE DIRECTIONS

Data presented in this thesis highlights the significant role synergistic effects of ischaemia-reperfusion coupled with mechanical unloading after acute myocardial infarction appears to have in preventing the unwanted propagation of interstitial fibrosis after acute myocardial infarction. In addition, the data shows that this synergistic effect also helps to create an environment around the infarcted heart that is conducive for new cell formation. A number of questions however remain unanswered. Firstly, the arrested progression of interstitial fibrosis following coronary reperfusion and mechanical unloading was only observed in the acute group not the chronic group where interstitial fibrosis worsened despite completing coronary

reperfusion 12 weeks prior to mechanical unloading. We reported a significant worsening of TGF- β 1 and α SMA protein expression in these chronic hearts compared to the acute group, which is consistent with the development of intimal hyperplasia. However, future studies should investigate the underlying mechanism responsible for the loss of this protective effect of ischaemia-reperfusion in the chronic hearts.

Secondly, whilst the synergistic effect of ischaemia-reperfusion led to significant upregulation of cardiomyocyte proliferation, better understanding of the underlying mechanism is needed to aid future therapies. MEIS1, a powerful regulator of cardiomyocyte cell cycle arrest was significantly upregulated by permanent coronary artery ligation and remained high even after ischaemia-reperfusion, thus positioning itself as probably a very powerful inhibitor of hyperplasia in the adult heart. Considering significant cardiomyocyte proliferation did occur in the ischaemia-reperfused hearts despite high levels of MEIS1, other pathways would appear to have been facilitated notwithstanding heightened MEIS1 activity. Low levels of pYAP have been shown to be associated with increased cell proliferation. Data presented in this thesis showed that after ischaemia-reperfusion coupled with mechanical unloading, pYAP levels were significantly downregulated compared to the permanently ligated and unloaded hearts. This sheds light onto a probable alternative facilitative molecular pathway that is activated during ischaemia-reperfusion coupled with mechanical unloading despite the inhibitory effects of MEIS1. Future studies aimed at identifying other facilitative pathways activated during ischaemia-reperfusion and mechanical unloading to help better characterise these findings are needed.

7.6 CONCLUSION

Whilst the last 60 years has seen significant strides made in the treatment of cardiovascular diseases, the management of end-stage heart failure remains a challenge. Despite significant improvements in outcome following heart transplantation, the low numbers of donor hearts mean a significant mismatch persists between those in need of transplantation and available donor hearts to go round. Following the first implantation of an LVAD as a bridge to cardiac transplantation over 40 years ago, the last 2 decades have seen this device grow in prominence, providing a large proportion of transplant eligible and ineligible patients with a comparable alternative to cardiac transplantation. Data presented in this thesis concurs with reports from many studies that highlight an even grander role of LVADs in the future management of end-stage heart failure. Particularly novel are our findings that coronary reperfusion after myocardial injury coupled with mechanical unloading stimulates a significant upregulation of cardiomyocyte proliferation and induces a downregulation of interstitial fibrosis, two major findings that have significant implications for LVAD-induced myocardial recovery.

Further studies are required to build upon the reports of this thesis in order to further unravel the true effects of LVADs on the failing heart. There is great hope that the next 20 years will see LVADs develop into much more than a bridging device but grow into an established therapeutic tool, capable of facilitating reversal of pathological remodelling. A sophisticated off-the-shelf cure for heart failure.

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