Nitrosative Stress and the Pathogenesis of Takotsubo Syndrome: Insights from a Novel Female Rat Model.

By

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Contents

Abstract

Introduction: Takotsubo Syndrome (TS), also known as Takotsubo Cardiomyopathy or Broken Heart Syndrome, is a poorly recognised condition presenting with similar symptoms to an acute coronary syndrome. Characterised by what appears to be transient dysfunction and inflammation of the left ventricle, TS was initially thought to be relatively rare and benign, however as more is understood about the condition this has found to be untrue. It has now been established that TS shares a similar in-hospital mortality to acute myocardial infarction and there is development of a heart failure phenotype that persists after the index admission. The pathogenesis remains uncertain, although substantial evidence now suggests that catecholamines and associated β_2-adrenoceptor activation play a critical role. Furthermore, the biochemical pathways that result in myocardial inflammation and energetic impairment in this condition are still yet to be elucidated.

Methods: In four experimental chapters, this thesis examines the evidence for, and role of, nitrosative stress in the pathogenesis of TS. Initial investigations were conducted in myocardial biopsy tissue recovered from patients dying of TS, with immunohistochemistry utilised to assess myocardial content of 3-NT, TXNIP and PAR. Subsequent experiments revolved around the creation and use of a rodent model of TS, using echocardiography to assess functional parameters, and immunohistochemistry and Western blot to assess myocardial content of 3-NT, TXNIP, PAR, CD68, CD45, PARP-1, iNOS, eNOS and NFκB.

Results: Initial experiments from a pilot human post-mortem study revealed the presence of nitrosative stress within the myocardium of patients dying from TS, which was in contrast to investigations in patient plasma during the acute phase of TS.

A female rodent model of TS was developed using a bolus intra-peritoneal (IP) administration of isoprenaline, in Sprague-Dawley rats aged 4-5 months, mimicking the characteristics of human TS. There was significant apical left ventricular dysfunction, infiltration of inflammatory cells and evidence

of nitrosative stress. Mortality was consistent with that seen in other catecholamine rodent models of TS.

The role of activation of PARP-1 in TS, the “energetic sink” enzyme, was investigated in our female rodent model, using bolus IP administration of the PARP-1 inhibitor 3-aminobenzamide. Results showed that inhibition of PARP-1 was successful in reducing the apical dysfunction associated with isoprenaline administration. Intriguingly, mortality and left ventricular function were not linked, with mortality in this group comparable to isoprenaline alone.

As β_2-adrenoceptor activation is linked to nitric oxide synthases, the role of NOS was investigated, using the NOS inhibitor L-NAME. Inhibition of NOS was unsuccessful in reversing isoprenaline-associated LV dysfunction, but reduced mortality in this model, providing further evidence that function and mortality are not intimately related.

Conclusions: In summary, nitrosative stress has been found to play a significant role in the pathogenesis of TS. Downstream activation of PARP-1 and associated energetic impairment appears to be related to left ventricular dysfunction, while mortality is linked to NOS activation. Further investigations involving peroxynitrite formation/decomposition or suppression of inflammation may provide incremental therapeutic targets, in a condition which currently has no definitive therapeutic strategy.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Signed:

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Abbreviations

αAR	Alpha adrenergic receptor
α_2AR	Alpha-2 adrenergic receptor
βAR	Beta adrenergic receptor
β_1AR	Beta-1 adrenergic receptor
β_2AR	Beta-2 adrenergic receptor
β_3AR	Beta-3 adrenergic receptor
ADMA	Asymmetric dimethylarginine
ADP	Adenosine diphosphate
ACE	Angiotensin converting enzyme
ACh	Acetylcholine
ACS	Acute coronary syndromes
AMI	Acute myocardial infarction
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AR	Adrenergic receptor
ARB	Angiotensin receptor blocker
ATP	Adenosine triphosphate
BNP	B-type natriuretic peptide
BH4	Tetrahydrobiopterin
Ca^{2+}	Calcium
CABG	Coronary artery bypass graft
CAD	Coronary artery disease
CAG	Coronary angiography
cAMP	Cyclic adenosine monophosphate
CCTA	Coronary computed tomography angiography
CFR	Coronary flow reserve
CK	Creatine kinase
CK-MB	Creatine kinase muscle/brain
CMR	Cardiac magnetic resonance imaging

CRP	C-reactive protein
DDAH	Dimethylarginine dimethylaminohydrolase
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
ECG	Electrocardiogram
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
Gi	G-inhibitory
Gs	G-stimulatory
GLS	Global longitudinal strain
GRK	G-protein coupled receptor kinase
GRK5	G-protein coupled receptor kinase 5
GSH	Glutathione
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
Hs-CRP	High sensitivity C-reactive protein
HUVEC	Human umbilical vein endothelial cell
iNOS	Inducible nitric oxide synthase
IL-1	Interleukin 1
IL-1α	Interleukin 1-alpha
IL-1β	Interleukin 1-beta
IL-1RA	Interleukin 1 receptor antagonist
IP	Intraperitoneal
IV	Intravenous
IVUS	Intravascular ultrasound
K_{ATP}	ATP-sensitive potassium channel
kDa	Kilodalton
KPSS	Potassium physiological saline solution
L-NAME	L-NG-Nitroarginine methyl ester
LBBB	Left bundle branch block
LDL	Low density lipoprotein

LGE	Late gadolinium enhancement
LV	Left ventricle
LVEF	Left ventricular ejection fraction
LVOT	Left ventricular outflow tract
LVOTO	Left ventricular outflow tract obstruction
MACCE	Major adverse cardiac and cerebrovascular events
MAO	Monoamine oxidase
MCP-1	Monocyte chemo-attractant protein-1
MDA	Malondialdehyde
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MI	Myocardial infarction
MINOCA	Myocardial Infarction with Nonobstructive Coronary Arteries
mPTP	Mitochondrial permeability transition pore
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
NaHS	Sodium hydrosulfide
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOS	Nitric oxide synthase
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NSTEMI	Non-ST-elevation myocardial infarction
NT-proBNP	N-terminal pro b-type natriuretic peptide
$O2^-$	Superoxide
OH^-	Hydroxyl radical
$ONOO^-$	Peroxynitrite
OVX	Ovariectomised
OVX-E	Ovariectomised with estrogen therapy
PAR	Poly (ADP-ribose)
PARP-1	Poly (ADP-ribose) polymerase-1
PCI	Percutaneous coronary intervention
PCr	Phosphocreatine

PET	Positron emission tomography
PKA	Protein kinase A
PKC	Protein kinase C
PTX	Pertussis toxin
QTc	Corrected QT interval
RIRR	ROS-induced ROS release
ROS	Reactive oxygen species
RV	Right ventricle
RWMA	Regional wall motion abnormalities
SCAD	Spontaneous coronary artery dissection
SD-1	Syndecan-1
SEM	Standard error of the mean
SERCA	Sarco-endoplasmic reticulum calcium ATPase
SOD	Superoxide dismutase
SPECT	Single photon emission computed tomography
STEMI	ST-elevation myocardial infarction
TIMI	Thrombolysis in myocardial infarction
TNF-α	tumour necrosis factor-α
TS	Takotsubo syndrome
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UA	Unstable angina
VF	Ventricular fibrillation
VT	Ventricular tachycardia

Publication list

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1 Introduction

1.1 Acute coronary syndromes and myocardial cell death

To better understand Takotsubo Syndrome in the current clinical and research landscape, it is important to examine acute coronary syndromes (ACS) in some detail. Cardiac emergencies diagnosed as ACS represent a substantial burden on the healthcare system in many countries. In Australia approximately 75,000 hospitalisations and 10,000 deaths per year are attributed to ACS, with this number expected to increase to 13,675 deaths by 2020[1]. The term ACS refers to any group of clinical symptoms compatible with acute myocardial ischemia, and includes unstable angina (UA), non-ST-segment elevation myocardial infarction (NSTEMI), and ST-segment elevation myocardial infarction (STEMI). The condition of acute coronary artery spasm (variant/Prinzmetal's angina) also presents as ACS.

1.1.1 Clinical Presentation

Typical presentation of a patient with ACS is usually qualitatively similar to that of one with stable angina pectoris, though quantitatively different: symptoms are usually "random" in occurrence, more severe, more prolonged and more alarming. Usually, widely distributed, but often mainly central chest pain is described as squeezing, aching or burning, radiating to the left arm, jaw or neck and is prolonged in duration (>20 min). Other common symptoms include dyspnoea, nausea, a sense of heightened anxiety and unprovoked fatigue[2]. Along with *a priori* risk, history and physical examination, ECG and laboratory tests expedite rapid diagnosis.

1.1.1.1 The ECG

An ECG is the single most important diagnostic test in a patient with suspected ACS and should be administered immediately upon presentation (Class I recommendation)[3]. ST-segment elevation and possibly relevant left bundle branch block (LBBB) indicate the need for emergency reperfusion. Transient ST-segment elevation, ST-segment depression and/or T-wave inversion are all highly suggestive of ACS and warrant early coronary angiography in the vast majority of cases. The fact that the posterior and lateral walls of the left ventricle are not well represented on a 12-lead ECG means that although patients with a normal ECG are less likely to have severe ischemia, this cannot be excluded on the basis of ECG alone.

1.1.1.2 Biomarkers

1.1.1.2.1 Cell death

The presence of elevated cardiac biomarkers such as cardiac troponins in patients with suspected ACS signifies the presence of myocardial injury and associated cell death. This most commonly reflects ACS. However myocardial injury/cell death can occur unrelated to ischemia, for example in heart failure, myopericarditis, pulmonary embolism, trauma and atrial fibrillation, amongst many others, and therefore must be viewed in clinical context.

Cardiac troponins are now the most sensitive, specific and rapidly accessible biomarkers available, significantly improving the speed and threshold for detection of cardiac injury. Cardiac troponins become elevated in the blood stream one to six hours after an acute myocardial infarction (AMI), and can remain elevated for up to two weeks thereafter. As troponin levels may be chronically elevated in other conditions, the key issues are fluctuation and the need to be matched with ischemic symptoms[4]. Other potential biomarkers include creatine kinase and its myocardial isoform (CK and CK-MB) and myoglobin. However, these are not recommended for the initial diagnosis where cardiac troponin is available[5].

1.1.1.2.2 Other biomarkers

Occurrence of acute cardiac injury, whether associated with infarction or otherwise, is virtually always characterised by the release of a number of nonspecific markers of disturbance of homeostasis and of inflammatory response.

Notably, B-type natriuretic peptide (BNP) is released into plasma, particularly in the presence of impaired cardiac function, while concentrations of non-specific inflammatory markers such as C-reactive protein (CRP) rise rapidly[6]. Recently it has been shown that release of syndecan-1 (SD-1), a component of the vascular endothelial glycocalyx, is markedly increased in plasma, especially after reperfusion of infarcts[7].

1.1.1.3 Cardiac catheterisation findings

As mentioned previously, ACS presentations with ST-segment elevation are now routinely treated with cardiac catheterisation and coronary stenting as a first-line intervention. In the setting of STEMI, there is more likely to be an occlusive thrombus caused by plaque rupture/erosion, whereas in NSTEMI it more common to find non-occlusive thrombus. The extent and duration of coronary thrombosis is determined by several factors, including: extent of exposed plaque, degree of stenosis/surface irregularities (disruption to laminar flow) and the balance of a thrombotic/thrombolytic environment[8].

The Thrombolysis in Myocardial Infarction (TIMI) grade flow scoring system[9] can then be used to assess the impact of coronary obstruction on regional myocardial perfusion. TIMI 0 flow (no perfusion) refers to the absence of any antegrade flow beyond a coronary occlusion. TIMI 1 flow (penetration without perfusion) is only minor anterograde flow, with incomplete perfusion distal to the occlusion. TIMI 2 flow (partial reperfusion) is anterograde flow that is delayed or slowed. TIMI 3 is normal coronary flow.

1.1.1.4 The role of magnetic resonance imaging (MRI): The gold standard?

Progressive increases in sophistication of cardiac MRI make a strong case (outlined below) for its place as the new "gold standard" for the precise diagnosis of suspected ACS. However, there are major technical limitations to its utility, and performance of MRI should not delay coronary intervention in patients in whom STEMI is a probable diagnosis.

Although the ECG and other imaging modalities, such as echocardiography and computed tomography are highly useful in the diagnosis of ACS, they may not be able to definitively distinguish ACS from other conditions. Cardiac MRI has several benefits, including most importantly the ability to produce accurate and highly reproducible imaging with both tissue characterisation and prognostic abilities which outperform traditional clinical assessment[10]. There is also further utility to be found in the relatively new imaging techniques such as T2-weighted sequences in the detection of oedema, particularly useful in differentiating culprit lesion/s in the acute-on-chronic setting in established CAD patients or late gadolinium enhancement (LGE), which allows differentiation between irreversibly damaged myocardium and only stunned, ischemic myocardium that is still viable.

Aside from feasibility in emergency situations such as STEMI, there are of course other limitations associated with cardiac MRI, mainly related to cost and potential patient incompatibility (any metallic implants, claustrophobia etc.), however in the majority of cases these are greatly outweighed by the diagnostic and prognostic value that can be provided.

1.1.2 Definitions

In 2000, the first universal definition of AMI was provided by the Joint European Society of Cardiology (ESC)/American College of Cardiology (ACC) Committee. Since that time, there have been two redefinitions with the ESC/ACC Committee expanded to form the "Task Force". The third and current definition was published in October 2012. This definition includes not only AMI per se but also several proposed subtypes of AMI (Type 1 – 5)[2].

Type 1 AMI, which equates most closely to "classical" AMI, is caused by an acute spontaneous atherothromboembolic coronary event. This event can be related to plaque rupture, fissuring, ulceration, erosion or dissection with the resultant intraluminal thrombus causing reduced myocardial blood flow. Thus, Type 1 AMI reflects the classical clinical presentation.

Type 2 AMI, also known as secondary AMI, is proposed to be a more heterogeneous entity, where a condition other than CAD is presumed to contribute to an acute imbalance between oxygen supply and demand. Type 2 AMI might occur in a patient with or without significant CAD. However, there is no actual evidence that this proposed pathophysiology ever occurs.

Type 3 AMI is reserved for the rare cases where death occurs in patients with symptoms suggestive of myocardial ischemia, and with presumed new ischaemic ECG changes before blood samples could be obtained or before cardiac biomarkers could rise.

Types 4 and 5 AMI are iatrogenic infarctions associated with percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG), respectively.

There are several advantages to classifying AMI in this way: for example, Types 3 and 4 reflect at least presumptively the same net loss of myocardial cells due to coronary occlusion; only the circumstances vary. This also carries medicolegal advantages. However, there are potential disadvantages, especially as regards Types 2 and 5, which are inherently controversial.

As regards Type 2 infarcts, in general there is evidence of myocardial cell death without structural evidence of an underlying localised area of infarction/s. The suggestion that these cases are "infarcts" due to excessive myocardial oxygen demand is largely presumptive. Similarly, the nature of cardiac cell death during coronary bypass surgery (Type 5 infarcts) is sometimes, but not always, due to graft occlusion.

1.1.3 Treatment

Emergency reperfusion therapy to re-establish blood flow to the artery/arteries affected in the STEMI form of ACS has been shown to reduce infarct size[11] and mortality[12], and this can be achieved by either using mechanical (PCI) or pharmacological (thrombolytic) means. The most crucial element in reperfusion therapy is limitation of the duration of ischemia, irrespective of which method is chosen. Current guidelines state that patients selected for thrombolytic therapy should receive treatment within 30 minutes of presentation (door to needle time) and that in patients undergoing PCI, balloon inflation in the infarct related artery should occur at most within 90 minutes of presentation (first medical contact to balloon time)[13].

In general, primary PCI is more effective in restoring coronary blood flow than thrombolysis[14], with consequently greater benefits in reducing mortality, recurrent MI and stroke risk. PCI is also associated with a lower risk of bleeding. Currently, the main residual indication for thrombolysis is occurrence of STEMI remote from an angioplasty facility.

1.1.4 Pathophysiology of various forms of ACS: current views

ACS is most commonly caused by the rupture of an atherosclerotic plaque (usually with a thin fibrous "cap"), with resultant partial or complete occlusion of a coronary artery[15], accounting for approximately 70% of fatal events in acute myocardial infarctions. Plaque progression can develop over many years, but is significantly accelerated in the setting of cardiac risk factors such as hypertension, smoking, hyperlipidaemia, diabetes and a familial history of coronary artery disease (CAD)[16]. However, up to 30% of cases exhibit plaque erosion rather than rupture[17]. In the process of plaque rupture, there is activation of platelets and the coagulation cascade due to exposure of the (pro-aggregatory) subendothelial collagen, leading to thrombus formation. Once a thrombus has formed, it can either partially or completely occlude the lumen of the artery, at the site of formation or as a distal embolisation. Occlusion of the artery results in evolving infarction of the distal myocardium, with associated ECG patterns (STEMI/NSTEMI with T wave inversion, ST-segment

depression etc.). Importantly, it has been known for many years that the probability of plaque rupture is independent of severity of the associated coronary stenosis. Rather, the presence of anatomic features including a thin fibrous "cap", a large lipid core populated by numerous inflammatory cells, abundant production of inflammatory matrix metalloproteinases, and a relative lack of smooth muscle cells are known to predispose rupture of a "vulnerable plaque"[15].

In most cases, prolonged myocardial ischemia leads to the development of major or minor degrees of cell death in the ischemic zone. The extent of cell death varies with the size of ischemic zone, duration of ischemia, and presence/absence of preconditioning (that is, protective) stimuli[18].

1.1.5 Plaque status: ruptured, eroded or absent?

Although there are difficulties associated with the concepts of Types 2 and 5 infarcts (discussed previously), verifiable myocardial injury may occur in a number of other circumstances: emboli, coronary vasospasm and inflammation. Type 2 AMI patients are likely to be older, more likely to be female, have lower blood troponin values, and have more comorbidities[19].

As mentioned in Chapter 1.1.4, the majority of infarcts can be attributed to rupture of "vulnerable plaques": these are cholesterol-laden, with thin, inflamed fibrous caps. The contributions of systemic inflammatory activation, as against the role of inflammatory stimulus via plaque contents such as oxidised LDL, are subjects of intense current interest (for review see Hansson *et al* (2015)[20]).

However as previously mentioned, up to 30% of patients with clinical evidence of ACS have eroded, rather than frankly ruptured, plaques[17]. The implications of plaque erosion are currently poorly studied. On one hand, it is possible that the pathophysiology is identical to that of plaque rupture, with thrombosis following platelet activation and aggregation. On the other hand, plaque erosion might result from inflammatory shedding of the protective endothelial glycocalyx layer (for review see Lipowsky[21]). If this were the case, there would be a component of endothelial activation, platelet activation and propensity towards vasoconstriction/spasm around such plaques[22].

Finally, a small number of patients with definite, MRI-verifiable acute infarcts appear to have "normal" coronary arteries. This association has recently been termed MINOCA[23]. Of course, some of these infarcts might reflect coronary emboli or eroded plaques, the latter not being seen on routine coronary angiography. Then again, investigations in this area are in their infancy, and perhaps other causes will emerge.

1.1.5.1 Infarcts independent of plaque rupture

1.1.5.1.1 Emboli

A rare cause of AMI, coronary artery embolism can be caused by various mechanisms and sources of thrombus formation, including hypercoagulable states, atrial fibrillation, aortic sinus thrombosis, valve prostheses, infective and noninfective endocarditis, intracardiac thrombi, patent foramen ovale with paradoxical embolism, and even benign and malignant tumours[24]. In these cases, not only coronary angiography but also echocardiography (transthoracic or transoesophageal), and intravascular ultrasound (IVUS) are potentially useful in diagnosing the source of the emboli. Depending on the cause, treatment may involve angioplasty +/- thrombus aspiration or simply supportive medical therapy for distal emboli. For hypercoagulable states and atrial fibrillation, anticoagulant therapy is required.

1.1.5.1.2 Spontaneous coronary artery dissection

Spontaneous coronary artery dissection (SCAD) is a relatively rare condition involving an intramural haematoma (false lumen) which blocks anterograde flow beyond the dissection due to the false lumen blocking the true lumen. SCAD is defined by the underlying pathophysiology and classified as a "primary" dissection, where if a traumatic, iatrogenic or non-coronary process is involved, the term "secondary" artery dissection in used[25].

1.1.5.2 Coronary vasospasm

Coronary vasospasm is defined as sudden and severe vasoconstriction of an epicardial coronary artery, leading to severe, often prolonged, and sometimes total interruption of antegrade flow[24]. Prolonged transmural myocardial ischemia caused by completely occlusive vasospasm may occasionally lead to AMI and in severe cases sudden cardiac death. At elective coronary angiography, coronary arteries may appear completely normal, or in the case of predominantly arteriolar spasm, the "slow coronary flow phenomenon" may be seen[26]. Low-dose intra-coronary acetylcholine (ACh) can be used to demonstrate a propensity towards spasm. Although this is frequently a recurrent and debilitating condition, diagnosis is difficult because in many cases release of conventional markers of myocardial injury does not occur. While coronary spasm is ameliorated by both organic nitrates and calcium antagonists, it is a notoriously recurrent problem, affecting quality of life rather than survival.

1.1.5.3 The evolving concept of a "Type 2 infarct"

Over the past 18 years, a great deal of effort has been directed to the reclassification of AMI[2, 27, 28]. In particular, a series of suggested subtypes of AMI have been proposed, including the "standard" clinical form (Type 1).

Type 2 infarcts remain a subject of considerable controversy in 2018. They are currently defined as being due to "instances of myocardial injury with necrosis, where a condition other than CAD contributes to an imbalance between myocardial oxygen supply and/or demand"[2]. However, while this definition is meant to exclude TS[29], there is no suggested diagnostic sequence to establish the diagnosis. For example, cardiac MRI scanning is not compulsory. Furthermore, there is no suggested means for demonstrating the presence of a regional myocardial supply-demand imbalance (in the absence of fixed local coronary artery disease).

In practice, the combination of release of troponin and the absence of fixed coronary disease may lead to a diagnosis of Type 2 AMI: clearly this would be reached on the basis of limited evidence.

1.1.5.4 Inflammation and ACS: what is known?

Inflammation is indisputably involved in the pathogenesis of ACS and is known to be a major contributor to atherothrombosis (for recent review, see Moreira *et al*[30]). However, prior to 2013 there had been no studies to test the associated Koch's postulates by evaluating effects of inflammatory limitation on cardiovascular outcomes such as AMI, stroke or cardiovascular death. Between 30 – 40% of patients with ACS have diabetes or metabolic syndrome, with these patients having a high prevalence of subclinical inflammation, as determined by increased levels of circulating biomarkers such as (pentameric) CRP[31]. In the PROSPECT trial, where CRP levels were correlated to instability of untreated vulnerable coronary plaques, circulating CRP concentrations strongly predicted the rate of major adverse coronary events[32]. This association between the instability of plaques and circulating CRP levels supports the theory that leukocytes that infiltrate and destabilize plaques release cytokines that upregulate hepatic synthesis of CRP[33]. Furthermore, CRP may induce expression of adhesion molecules, tissue factors, MCP-1 and other inflammatory cytokines, iNOS and superoxide production[34].

CRP is also a mediator of plaque inflammation, via conversion of the circulating pentamer to the monomeric form, which is deposited on unstable plaques[35]. The effects of statins in reducing risks of ACS have increasingly been attributed in part to suppression of inflammation, since the JUPITER trial[36] showed concordance between rosuvastatin-induced suppression of inflammatory markers and improved outcomes. For many years it was uncertain whether the prognostic advantages of cholesterol lowering statins might in part reflect suppression of inflammation[37]. However, two further pieces of evidence strengthening the case of a primary role of inflammation have emerged:- (i) the establishment of rheumatoid arthritis, a non-atheromatous but chronic inflammatory condition, as a coronary risk factor[38], and (ii) very recently, the results of the CANTOS study demonstrated that the "pure" anti-inflammatory agent canakinumab reduced risk of cardiac events in a high risk population, establishing inflammation as an independent coronary risk factor[39].

Other trials of this type are now underway. For example, the Cardiovascular Inflammation Reduction Trial (CIRT)[40] is currently assessing the use of low-dose methotrexate in patients with subclinical

vascular inflammation, while the Effects of Methotrexate Therapy on ST Segment Elevation Myocardial Infarctions trial (TETHYS)[41] will evaluate low-dose methotrexate in STEMI. These trials aim to use an anti-inflammatory treatment to reduce cardiovascular outcomes such as AMI, stroke and cardiovascular death.

1.1.5.4.1 Inflammation and plaque rupture

The mechanisms of plaque rupture have been extensively studied, leading to a now well established correlation between inflammation and plaque vulnerability, with components of both the innate and adaptive immune systems involved[42]. Interactions between lipids and multiple facets of immune function serve to promote premature atherogenesis and accelerate plaque rupture[43]. Furthermore, the association between biomechanics and plaque rupture has also been well studied, with maximum stress on the thin fibrous cap or plaque shoulders, where rupture is most likely to occur. This may be due to focal inflammation caused by mechanical forces within the vessel wall leading to heterogeneity in strain, and subsequent weakening and eventual rupture of the fibrous cap[44].

Currently, the aforementioned CANTOS trial is focused on the inflammatory cytokine interleukin-1 (IL-1) and its role in the progression of atherothrombosis, with a primary goal of reducing cardiovascular events with IL-1β inhibition. IL-1β production is linked to activation of the NLPR3 inflammasome, a complex group of intra-cellular proteins, by crystalline compounds such as silica, asbestos and uric acid crystals, as well as cholesterol crystals and minimally modified LDL cholesterol[45]. Furthermore, human studies have shown that atherosclerotic arteries, when compared to normal coronary arteries, have higher levels of IL-1β present, and that key cells in atherosclerotic plaque biology, monocytes and macrophages, produce the bulk of IL-1β[46].

The IL-1 gene family encodes three major proteins: the proinflammatory IL-1α (a predominantly plasma membrane associated protein, with local effects) and IL-1β (secreted and circulated systemically), and the anti-inflammatory IL-1 receptor antagonist (IL-1RA). It has been shown that IL1-RA is also elevated in ACS patients and that in STEMI patients, elevation of IL-1RA levels precede increases in the level of markers of myocardial necrosis, such as troponin and creatine kinase[43].

1.1.5.4.2 Inflammation, plaque erosion and potential glycocalyx shedding

As compared to plaque rupture, plaque erosion involves a process of thrombus formation without any communication with the necrotic core of an atherosclerotic plaque[44]. Although not yet well understood, it has been suggested that the pathological basis for plaque erosion is based around exposure of the thrombogenic extracellular matrix, and is associated with less inflammation than plaque rupture. Preliminary evidence shows that plaque erosion may be caused by degradation of the endothelial glycocalyx layer, allowing endothelial cells to adopt atherosclerotic behaviour, with increased uptake of oxidised LDL and subsequent activation of inflammatory CD40/CD40L signalling pathways[47].

1.1.5.4.3 Inflammation and coronary artery spasm

As previously mentioned, coronary vasospasm is a notoriously recurrent problem which severely inhibits patient quality of life. The precise mechanisms behind coronary vasospasm are yet to be delineated, however there is a growing body of evidence to suggest that inflammation also plays a contributing role in the pathogenesis of this condition. Early work in an in vivo pig model showed that chronic treatment with a major inflammatory cytokine (IL-1β) resulted in vasospastic responses in the porcine coronary arteries[48].

In a study of 199 patients using intracoronary injection of ACh to provoke vasospasm, levels of hs-CRP were significantly elevated in patients in the coronary spasm group as compared with the non-spasm group[49]. Similarly, a study of 933 patients using a different provocation agent, methylergonovine, showed similar increases in hs-CRP levels in coronary vasospasm patients compared to non-spasm patients[50]. Another study showed that patients with a positive ACh test had not only raised hs-CRP levels, but also significantly elevated concentrations of soluble CD40 ligand, a marker and mediator of inflammation[51].

1.1.5.5 Is it possible for inflammation to primarily involve the myocardium?

Myocarditis is a term which encompasses a large group of conditions involving a broad range of pathological immune processes within the heart, caused by infection, auto-immune disease or cardiotoxic agents[52]. This can be associated with both structural and functional changes in cardiomyocytes, with a range of effects including, but not limited to, chest pain, dyspnoea or palpitations. In turn, inflammation induces global or regional functional impairment, diastolic dysfunction and/or electrical system abnormalities and consequences range from sudden cardiac death to the eventual development of dilated cardiomyopathy[53]. Despite the fact that myocarditis results in myocardial injury with a variable amount of necrosis, it is suggested in the current universal definition of MI that it should not be classified as an MI, but rather as a myocardial injury[2]. There is however continued uncertainty in this area and a strong case has been expressed for the inclusion of myocarditis and associated conditions under the heading of "Type 2 AMI", or alternatively for abandoning the "Type 2" label and placing all such conditions under a broader "acute myocardial injury" term. This would have the added benefit of narrowing the definition of an MI[54].

1.1.5.5.1 Infective

The causes of infective myocarditis are diverse, including viral, bacterial, spirochaetal, fungal, protozoal, parasitic, and rickettsial. Most commonly infective myocarditis is caused by an infection from a cardiotropic virus, followed by active inflammatory destruction of the myocardium[55]. Historically, diphtheritic myocarditis represented a major problem throughout the world, but this is now an extreme rarity[56]. On the other hand, South American trypanosomiasis, or Chagasic myocarditis, remains at epidemic proportions in many South American countries[57]. Although cardiac magnetic resonance is becoming more useful as a diagnostic tool, the endomyocardial biopsy remains the gold standard in cases such as these.

1.1.5.5.2 Auto-immune/auto-inflammatory

On rare occasions, auto-immune or auto-inflammatory diseases such as sarcoidosis, Behcet's disease, eosinophilic granulomatosis with polyangiitis, myositis or systemic lupus erythematosus may have severe cardiac involvement, in the form of myocarditis. The pericardium, myocardium, endocardium, valvular tissue and coronary arteries can all be involved, with non-specific clinical presentations incorporating dyspnoea, fever, chest pain and/or palpitations[58]. Cardiac MRI has shown promise as a non-invasive diagnostic technique, however endomyocardial biopsy continues to be the gold standard here. Currently glucocorticosteroids and immunosuppressive agents are common treatments, along with standard heart failure treatments. Persistent autoimmune responses are thought to underlie the progression of myocarditis to dilated cardiomyopathy, with immune suppression/modulation having shown positive results in these patients, allowing for tailoring of treatments to underlying condition and phase of disease.

1.1.5.5.3 Chemical

Various classes of drugs have been associated with myocarditis. Prescription medicine may cause myocarditis for example, with approximately 3% of patients taking Clozapine (an antipsychotic) developing the condition, usually within the first 2 months of therapy[59]. Similar to this, chemotherapy medication Doxorubicin is known to commonly have myocarditis as a side effect[60]. Chronic use of the recreational drug cocaine is known to cause myocarditis, along with other cardiac consequences such as hypertrophy, dilated cardiomyopathy and heart failure[61]. Hypersensitivity to drugs is another cause of myocarditis, although very rare. Fortunately, the prognosis in both prescription/recreational drug induced and hypersensitivity myocarditis is usually excellent and requires only the removal of the causative drug for improvement to be seen[62]. Aside from prescription and recreational drugs, other pathological stimuli for myocarditis include alcohol, radiation and chemicals such as hydrocarbons and arsenic[53].

1.2 Takotsubo Syndrome

1.2.1 Background

Takotsubo Syndrome (TS), also known as Takotsubo Cardiomyopathy, Broken Heart Syndrome, Stress Cardiomyopathy or Apical Ballooning Syndrome, was first reported in Japanese literature by Sato and colleagues in 1990[63]. The name for the condition was coined as the characteristic shape of the typical TS left ventricle, with narrow hyperactive basal segments and apical ballooning during systole, resembled a Japanese octopus pot, a ”takotsubo” (Figure 1.1). It was thought to be a condition only affecting the Japanese population until the late 1990’s, where two publications appeared, initially from France[64] and then later from the United States[65]. The term Takotsubo was not used with Caucasian patients until 2003 when a small series of 13 patients in Belgium was described[66], followed later the same year by a description of a similar sized cohort in the United States[67]. Since that time, both diagnosis and understanding of TS have improved rapidly, although there is still much we do not know about this condition. Typically, patients present with sudden onset chest pain, dyspnoea or syncope, which makes it difficult for both paramedics and emergency doctors to exclude acute MI.

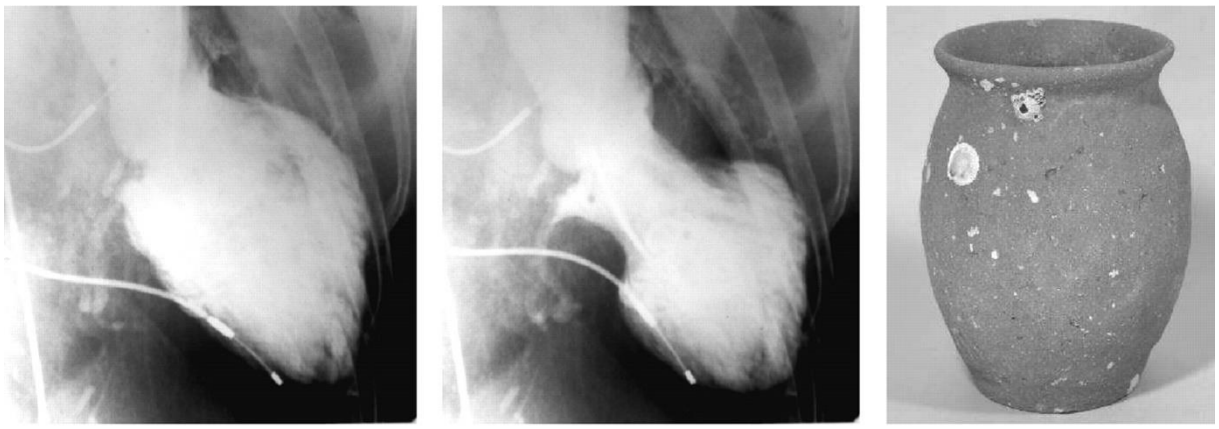


Figure 1.1 Left ventriculogram (A, end-diastolic phase; B, end-systolic phase) in the right anterior oblique projection. The extensive area around the apex shows akinesis, and the basal segments display hypercontraction. C, A picture of a takotsubo, which has a round bottom and narrow neck. Reprinted with permission from Akashi *et al*, 2008[68]

1.2.2 Diagnosis and clinical features

Due to the similarities of TS to AMI in regard to symptoms and presentation, including biomarkers and ECG abnormalities, there are inherent difficulties in diagnosis. The first diagnostic criteria for TS was introduced in 2003 by Abe *et al*[69], followed a year later by a publication from the Mayo Clinic[70]. Four years later the Mayo Clinic criteria were revised[71], notably including neurogenic stunned myocardium and highlighting that there may be concomitant coronary disease, a factor which significantly increases difficulty of diagnosis and has now been shown to be present in 10-29% of cases[72]. The latest criteria for the diagnosis of TS are presented below in Table 1.1, from the International Expert Consensus Document on Takotsubo Syndrome[72].

The position statement from the Taskforce on Takotsubo Syndrome (2016) first suggested an algorithm to assist with the diagnosis of TS[73], which was updated in the international expert consensus on TS, released recently (see Figure 1.2)[74]. Presentations with a spontaneous or physical/emotional stress trigger constitute "primary TS", while its development concomitant with medical/surgical/psychiatric emergencies are considered to represent "secondary TS"[75]. Physical triggers are in fact more common than emotional[76], and in "primary TS" being male and presenting with a physical trigger are independent risk factors of in-hospital mortality[77]. It has been suggested by some authors that acute stressor is not the sole cause of TS, rather that prolonged perceived stress predisposes, or creates vulnerability, to the precipitation of TS[78]. Along with the aforementioned chest pain and shortness of breath, patients must have ECG changes (discussed in more detail below).

Table 1.1. International Takotsubo Diagnostic Criteria (InterTAK Diagnostic Criteria). Reprinted with permission from Ghadri *et al*, 2018[72]

1.	Patients show transient[a] left ventricular dysfunction (hypokinesia, akinesia, or dyskinesia) presenting as apical ballooning or midventricular, basal, or focal wall motion abnormalities. Right ventricular involvement can be present. Besides these regional wall motion patterns, transitions between all types can exist. The regional wall motion abnormality usually extends beyond a single epicardial vascular distribution; however, rare cases can exist where the regional wall motion abnormality is present in the subtended myocardial territory of a single coronary artery (focal TTS).[b]
2.	An emotional, physical, or combined trigger can precede the takotsubo syndrome event, but this is not obligatory.
3.	Neurologic disorders (e.g. subarachnoid haemorrhage, stroke/transient ischaemic attack, or seizures) as well as pheochromocytoma may serve as triggers for takotsubo syndrome.
4.	New ECG abnormalities are present (ST-segment elevation, ST-segment depression, T-wave inversion, and QTc prolongation); however, rare cases exist without any ECG changes.
5.	Levels of cardiac biomarkers (troponin and creatine kinase) are moderately elevated in most cases; significant elevation of brain natriuretic peptide is common.
6.	Significant coronary artery disease is not a contradiction in takotsubo syndrome.
7.	Patients have no evidence of infectious myocarditis.[b]
8.	Postmenopausal women are predominantly affected.

(a) Wall motion abnormalities may remain for a prolonged period of time or documentation of recovery may not be possible. For example, death before evidence of recovery is captured. (b) Cardiac magnetic resonance imaging is recommended to exclude infectious myocarditis and confirm diagnosis of takotsubo syndrome.

Hypotension and/or shock are often described at presentation, seen in at least 10-15% of patients, although the physiological basis for this is unclear with LV function often near normal. The hypotension/shock is more likely to be multifactorial in origin[79]. Pericardial effusions are common, seen in approximately 43% of patients on cardiac MRI early after admission. However pericardial tamponade is a relatively rare complication, with an incidence of <1%[73]. The most common in-hospital complications of TS are acute heart failure (12-45%) and cardiogenic shock (6-20%). Severe LV dysfunction, with associated apical ballooning, increases risk for LV thrombus and subsequent systemic embolism[74].

1.2.2.1 Diagnostic algorithm: overall approach

The diagnostic algorithm suggests urgent coronary angiography to investigate the possibility of culprit coronary disease if ST-elevation is noted on ECG, and if found, then the diagnosis of acute coronary syndrome can be made. If, however, there is no culprit coronary disease present, further imaging with cardiac MRI and echocardiography, investigation of cardiac biomarkers and serial ECGs is recommended to elucidate possible causes for symptoms, such as hypertrophic cardiomyopathy, pericarditis or viral myocarditis. This construct therefore places (fundamentally negative) coronary angiography as the pivotal investigation, used essentially as a means of diagnosing TS via exclusion of plaque rupture. This position is increasingly under challenge.

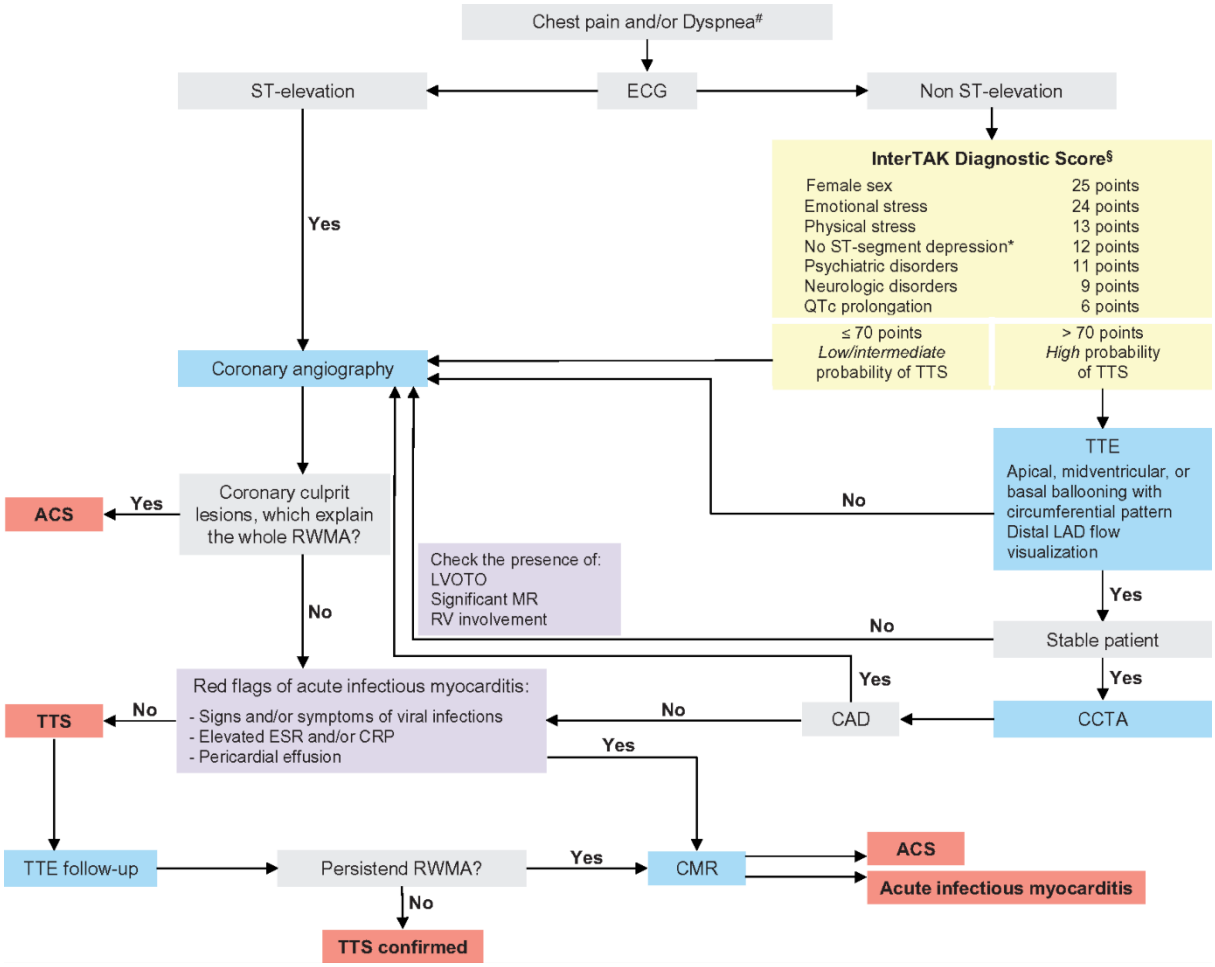


Figure 1.2. Diagnostic algorithm of takotsubo syndrome. #Applied to patients who are seeking medical emergency departments with e.g. chest pain and/or dyspnoea. §The InterTAK Diagnostic Score did not include patients with pheochromocytoma induced takotsubo syndrome in which atypical pattern are more frequently noted. *Except in lead aVR. ACS, acute coronary syndrome; CAD, coronary artery disease; CCTA, coronary computed tomography angiography; CMR, cardiac magnetic resonance; CRP, c-reactive protein; ECG, electrocardiogram; ESR, erythrocyte sedimentation rate; InterTAK, International Takotsubo Registry; LAD, left anterior descending coronary artery; LVOTO, left ventricular outflow tract obstruction; MR, mitral regurgitation; QTc, QT-time corrected for heart rate; RV, right ventricle; RWMA, regional wall motion abnormality; TTE, transthoracic echocardiography; TTS, takotsubo syndrome. Reprinted with permission from Ghadri *et al*, 2018[74].

Approximately 12 months after the release of the aforementioned Takotsubo Taskforce position statement, and as a result of analysis of the International Takotsubo Registry, a clinical score to estimate the probability of TS and distinguish TS from ACS was proposed – the InterTAK Diagnostic Score[80]. Seven variables are combined to form the Score, each weighted differently with individual scoring values - female sex 25, emotional trigger 24, physical trigger 13, absence of ST-segment depression (except in lead aVR) 12, psychiatric disorders 11, neurologic disorders 9, and QTc prolongation 6 points. When using a score value of ≥50 nearly 95% of TS patients were diagnosed correctly (sensitivity 94.7%). The InterTAK score is used in the latest diagnostic algorithm in cases where patients have a non ST-elevation presentation, a score of >70 suggests a high probability of TS.

1.2.2.2 ECG

As ECG changes are quite heterogenous in TS patients, varying with race, patient characteristics, wall motion abnormalities and triggers involved, it is beneficial to individually assess each relevant segment of the ECG[81]. These ECG changes are also time dependent and some are not only seen in the acute phase, but also months later. Life-threatening ventricular arrhythmias, such as torsades de pointes, VT, or VF occur in 3.0–8.6% of cases and are a frequent cause of death, occurring mainly in the subacute phase (2-4 days post index event)[74].

1.2.2.2.1 The PR segment

Usually associated with pericarditis, PR segment depression was present in nearly two-thirds of TS patients, significantly more than that seen in STEMI patients (62% vs 3%)[82]. Of the patients who had cardiac magnetic resonance, only 19% showed any signs of pericarditis, such as pericardial effusion or late gadolinium enhancement.

1.2.2.2.2 The QRS complex

Multiples studies have suggested that low QRS voltage and amplitude attenuation of QRS could be potential markers of TS[83, 84]. Initially, a specific distribution of amplitude attenuation was noted in inferior, anterior and lateral leads in 93.5% of TS cases. Follow-up studies confirmed this, with

observation of an acute decrease in QRS amplitude in both TS and ACS patients, followed by a progressive recovery of QRS amplitude in TS patients only. This recovery was also associated with improvement of LV function and reduction in cardiac damage markers.

1.2.2.2.3 The J wave

The J wave has been postulated as a hyperacute marker of TS, fading away after the first few hours[81]. Seen in approximately one third of all cases, the J wave appears related to ventricular vulnerability to arrhythmias. Due to its transient nature and limited studies investigating presence of the J wave, it currently holds only minor clinical value.

1.2.2.2.4 The ST segment

Early studies suggested a very high prevalence of ST segment elevation in TS patients. It was for this reason that traditionally TS has been compared with STEMI, and more specifically anterior STEMI (given that anterior S-T elevation occurs most frequently). A recent sub-analysis of the InterTAK registry compared ECG data from ST-elevation and non-ST-elevation TS patients, with those from STEMI and NSTEMI patients. The results showed that in the setting of ST elevation, there was a 100% positive predictive value with 100% specificity for TS versus STEMI when there was ST elevation in -aVR as well as in the anteroseptal leads. This had a sensitivity of 12% in STEMI-like TS patients. With regards to non ST-elevation, T wave inversion in I, aVL, V5 and V6 or ST-elevation in -aVR with T wave inversion in any lead was >95% specific for TS versus NSTEMI[85].

1.2.2.2.4 The T wave

The T wave is the most common ECG segment to be affected in TS, although it appears to be more common in Caucasian patients, with race playing a large role in ECG presentation[86]. T wave inversion usually involves a large number of leads, frequently in V2-V6, and present with greater magnitude than those seen in ACS (also known as “giant inverted T waves”). Inverted T wave morphology in TS resembles the traditional Wellens’ ECG pattern, correlating with the TS-associated myocardial oedema that is visualised by magnetic resonance imaging[87]. The Wellens’ ECG pattern persists after the

apparent recovery of left ventricular function, consistent with the presence of oedema post acute attack. It has also been noted that atypical forms of TS are associated with less T wave inversion.

1.2.2.2.4 The QT interval

Widespread T wave inversion and acquired QT prolongation has been noted in conditions such as pheochromocytoma and intracranial bleeding, consistent with a role for the sympathetic nervous system in repolarisation patterns, as ventricular repolarisation time is significantly increased in TS patients. QT interval prolongation can persist for up to six months after recovery of systolic function and cardiac biomarkers in patients with TS[81]. Typically, QTc prolongation peaks at 2-4 days post onset of symptoms, while occasional patients develop torsades de pointes, typically in the first 48 hours.

1.2.2.3 Plasma biomarkers

There currently is no single biomarker that can completely differentiate TS from AMI, especially in an emergency clinical setting. There are, however, several helpful biomarkers that can be used to markedly expedite the diagnosis of TS.

There is substantial evidence that catecholamine release (or administration of exogenous catecholamines) initiates many, if not most, TS attacks[88-90]. During admission, elevated plasma catecholamine levels have been documented in TS patients, and Wittstein *et al*[91] established that in the presence of ST-segment elevation, catecholamine levels in plasma are considerably higher for TS than for STEMI. Furthermore, it has been shown that plasma normetanephrine levels correlate directly with severity of the attack[92, 93]. However, as neither the prospect for diagnostic utility nor practicality is appealing for plasma catecholamine levels, this remains essentially a research tool.

B type natriuretic peptide (BNP) and N-terminal proBNP (NT-proBNP) are both substantially elevated not only in acute TS but remain above normal levels at three months post-attack[93], and are also elevated to higher levels than those seen in admissions for ACS, even in TS patients with no clinical evidence of acute heart failure. Elevation of BNP and NT-proBNP levels can be (and is usually regarded

as) indicative of increased wall stress, which in theory corresponds with the typical regional wall dysfunction associated with TS. However, the sheer extent of elevation remains disproportionate from this point of view: ultimately speaking, it is very rare for patients with TS to develop acute pulmonary oedema, despite the presence of severe hypotension. Therefore it has been suggested that BNP/NT-proBNP release is related to inflammatory activation, as time course of release parallels that of persistent myocardial oedema[94]. On the other hand, cardiac troponin is elevated to a lesser extent in TS when compared with ACS. Looking at these two simple biomarkers together could play a role in the early clinical recognition of TS, and more specifically as a differential diagnosis for ACS. Furthermore, creatine kinase and myoglobin are not usually markedly raised in TS patients[95], presenting another possible point of difference between ACS and TS admissions.

A recent publication has shown that TS is associated with glycocalyx shedding, a process which might theoretically contribute to the oedema associated with the condition[96]. In this study levels of syndecan-1 (SD-1), a component of the glycocalyx, were elevated in the plasma of patients during the acute phase of TS. However, patients admitted with AMI also showed significant elevation of SD-1[97], suggesting lack of utility of this measure for differentiation between these conditions.

Overall, these biochemical tests suggest that TS is associated with only minimal myocardial necrosis, and instead with considerable inflammatory activation, accounting for the often-remarkable elevation of NT-proBNP concentrations in plasma, and for the (relatively non-specific) release of SD-1.

1.2.2.4 Myocardial changes

1.2.2.4.1 Macroscopic structural changes

As mentioned earlier, there is significant oedema associated with TS. Although much of the previous literature showed that oedema was present, a study in 2012 was the first to show that the oedema was essentially global and slowly resolving in nature, measured using T2-weighted cardiac magnetic resonance[92]. Added to this, the severity of oedema seen in these patients correlated with plasma normetanephrine levels. Given that this oedema has no conventional haemodynamic basis,

incremental vascular permeability due to glycocalyx "shedding"[96] may represent a substantial component of its genesis.

1.2.2.4.2 Molecular changes

In biopsy data obtained by Nef *et al* (2008) from three patients with acute TS and also after function recovery, there was an increase in Nrf2-induced genes in the acute phase, with these genes known to be activated in the presence of reactive oxygen species (ROS). Catecholamines are also known to stimulate ROS production and indeed in these biopsies, patients with TS were shown to have elevated levels of superoxide. Oxidative stress has been shown to have not only direct damaging effects on myocardial and vascular cells, but also indirect effects[98]. In a previous set of patients studied in the same laboratory, biopsies showed the absence of oncotic and apoptotic cell death as evidenced via TUNEL, C9 staining and electron microscopy[99].

Intracellular lipid accumulation has also been shown to be present during the acute phase of TS, with biopsy data from 4 patients showing severe accumulation in the akinetic myocardium. This accumulation was not present in biopsy data from TS patients post-functional recovery, suggesting that the impairment of lipid metabolism is transient and specific to the affected myocardium[100].

Post-mortem microscopy of a patient who died of TS showed contraction bands, myocardial fibrosis and diffuse infiltration of leukocytes and macrophages throughout the LV. The inflammatory changes and contraction bands that were noted differentiated TS from AMI[101].

In a larger cohort of patients recruited in the acute phase, studied by cardiac MRI/MRS, and followed up four months later, there was significant decrease noted in resting cardiac energetic status when compared to healthy controls[102], measured using the phosphocreatine/adenosine triphosphate ratio (PCr/ATP). Again, this had improved from the acute phase by the 4-month follow up, but still remained substantially below healthy control levels.

1.2.2.5 Imaging

1.2.2.5.1 Echocardiography

Early echocardiography plays a crucial role in the diagnosis of TS by virtue of its versatility and accessibility, with the standard result showing wall motion disturbances that go beyond the region of a single coronary artery. The typical wall motion pattern seen on echocardiography is one of hypokinesis in the apical and/or mid-ventricular segments, with hyperkinesis of the basal segments[103, 104]. The reduction in ejection fraction that is evident on admission typically improves rapidly over approximately seven days. This typical form of TS with hallmark circumferential wall motion abnormality pattern is easily distinguished from variant forms such as midventricular dysfunction and apical sparing (discussed further below).

Associated with TS may be potentially severe complications which can be detected using echocardiography, such as mitral regurgitation, LV outflow tract obstruction, left ventricular thrombus formation or ventricular rupture[105].

The role of echocardiography is also vital in the assessment of recovery post acute TS. The use of more accurate measures of LV function such as global longitudinal strain (GLS), as compared with the gross assessment of wall motion using an ejection fraction, has shown that subtle LV dysfunction persists at least three months post-TS admission[94]. The study found that this impairment was also clinically significant, with reduced GLS at three months correlating with a reduction in patients' quality of life, as assessed by the SF-36 questionnaire. Not only was systolic LV dysfunction shown to persist, despite the normalisation of LV ejection fraction and volumes, diastolic dysfunction was also present at four months post acute attack[106].

Along with GLS there are other non-conventional echocardiography techniques that could prove to be useful in the assessment of TS, such as tissue Doppler, circumferential and radial strain, 3D and 4D imaging/strain and myocardial contrast echocardiography. These techniques are more accurate and sensitive to changes in LV, RV and coronary microcirculatory function, and are becoming increasingly accessible as technology rapidly improves in this area.

1.2.2.5.2 Nuclear medicine: cardiac innervation and perfusion

^{123}I-metaiodobenzylguanidine (^{123}I-MIBG) is a norepinephrine analogue and scintigraphy with this radiotracer can be used to evaluate some aspects of sympathetic nervous system function in conditions such as dilated cardiomyopathy. This technique has been used to correlate reduced uptake of norepinephrine at the synaptic cleft with the regional wall motion abnormalities which characterise TS[107].

The intriguing aspect of a finding of decreased catecholamine reuptake into sympathetic nerve endings in TS (which could in theory be replicated by measurement of catecholamine "spillover" into the coronary sinus) is that it theoretically provides a basis for ongoing myocardial exposure to high local catecholamine concentrations beyond the initially postulated endogenous or exogenous "surge". It is also consistent with precipitation of TS by inhibitors of catecholamine reuptake such as venlafaxine[108].

A number of studies, particularly in the early or acute phase, have used radionucleotide markers of myocardial perfusion to demonstrate impairment of perfusion to the LV apex in the days following onset of TS[109, 110]. These findings beg the question of whether such changes are "primary" (e.g. associated with coronary spasm[111]) or "secondary" to compression of intramyocardial arterioles. Inflammation and decreased ATP are known to cause a reduction in myocardial relaxation[112], and a subsequent increase in intramural pressure, resulting in compression of these arterioles.

1.2.2.5.3 Cardiac magnetic resonance imaging

Cardiac MRI is paramount in the diagnosis of TS, with different imaging techniques as part of the modality allowing for differentiation of TS from AMI. In the classical variant of TS (atypical variants are discussed later) cine cardiac MRI accurately demonstrates the regional wall motion disturbances that give TS its name, specifically, mid-apical segment akinesis/hypokinesis with basal segment sparing/hyperkinesis[113]. These images also provide an accurate measurement of LV systolic function and volumes, which can then be used in serial follow up examinations to track functional recovery. Representative MRI images are shown in Figure 1.3.

As mentioned in the previous section, RV involvement can occur in some cases of TS, with approximately one third of patients exhibiting biventricular involvement[114]. Imaging of the RV can be often difficult using echocardiography due to limited echo windows, which is where cardiac MRI displays another advantage, allowing for high resolution imaging of the RV in motion and in 3D.

The assessment and characterisation of oedema in TS is achieved by using sequences in which image contrast is weighted by intrinsic magnetic relaxation times. Using an inversion technique to remove signals from fat and blood, cardiac MRI can be used to accurately image myocardial oedema. Short T1 inversion recovery images are used in TS, with specific localisation of oedema noted to be in the myocardial segments which have been stunned, commonly with high signal intensity and a diffuse or transmural distribution[105]. TS can be distinguished from AMI using this method, as the localisation of oedema in AMI always follows vascular distribution, whereas TS does not[114]. Differentiating TS from acute myocarditis is also theoretically possible, as the T1 signal in acute myocarditis is more heterogeneous and not distributed transmurally. T2-weighted cardiac MRI has also shown to be useful in assessing myocardial oedema, as Neil et al (2012) used to assess TS patients in the acute phase compared to matched controls[92]. In this study, not only was there evidence of oedema in patients with TS, but also the extent of the oedema correlated with the regional contractile disturbance. To date the pathophysiologic mechanism resulting in oedema has not been delineated, although increased wall stress and inflammation have been suggested as possible causes.

Late gadolinium enhancement (LGE) sequences are an important tool that can be used in the differentiation of TS from AMI. Roughly 10-15 minutes after gadolinium contrast agents are injected, LGE images can be taken. AMI is often characterised by LGE, with high signal intensity, whereas only 10-40% of TS patients exhibit LGE, which is low-intensity[113]. This technique therefore represents a means to make the diagnosis of TS from a positive standpoint, rather than as an exclusion process. However, cardiac MRI is rarely available on an acute basis.

Finally, cardiac MRI can be used as a sensitive tool for the assessment of intracardiac thrombi, a known complication of TS[115]. While echocardiography is useful in this regard, it appears that there are a

number of false positive diagnoses. Incidence of intracardiac thrombi in echocardiography reports is approximately 8%, whereas this number is significantly lower in cardiac MRI reports, at around 2%[114].

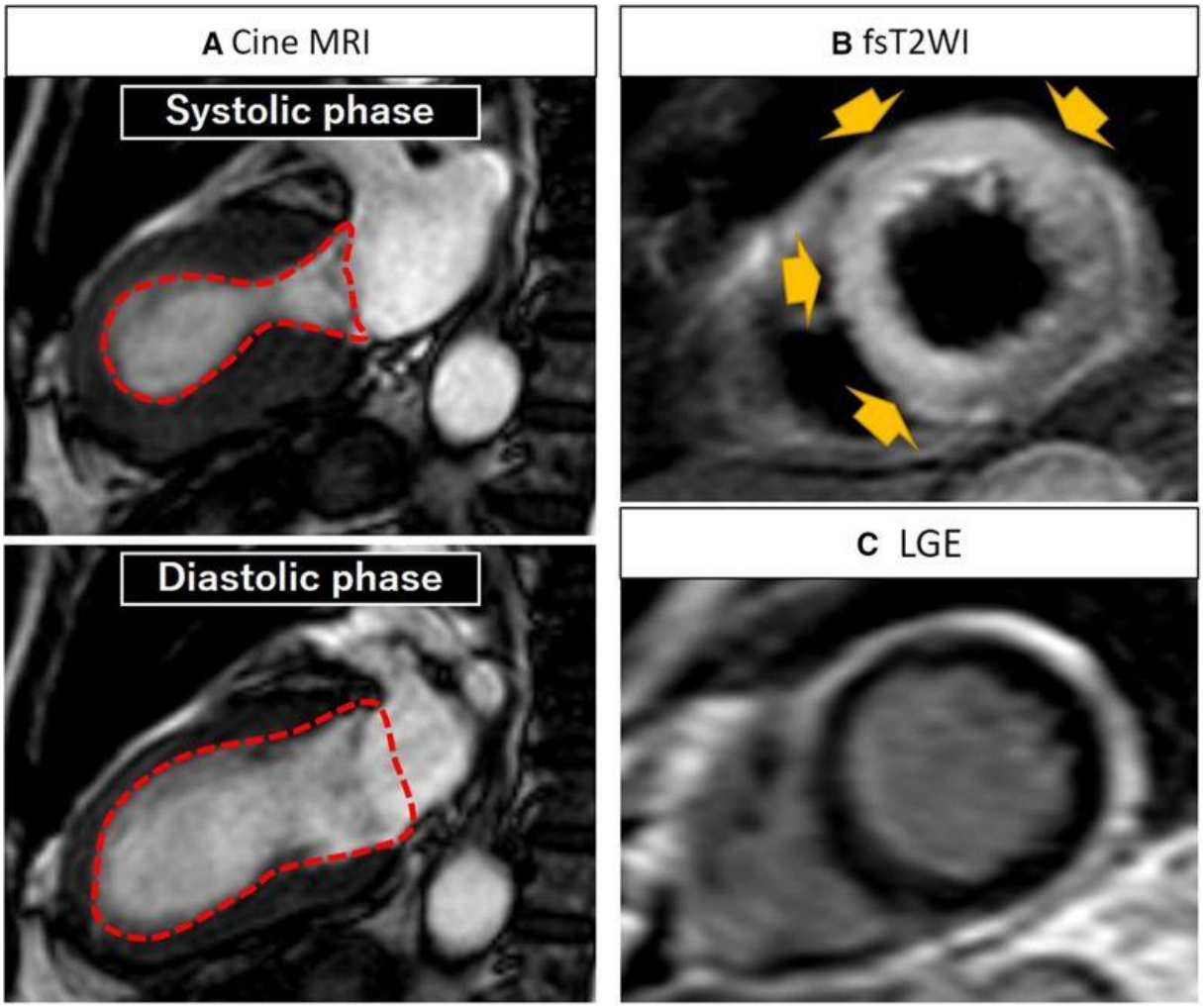


Figure 1.3. CMR imaging of Takotsubo Syndrome. (A) Wall motion abnormalities at the mid-ventricular to apical anterior segments with cine imaging as apical ballooning. High signal intensity with fat-saturated T2-weighted images (fsT2WI) (yellow arrows) due to the myocardial oedema (B), but the absence of the late gadolinium enhancement (LGE) (C) are shown. Reprinted with permission from Manabe *et al*, 2018[116]

1.2.2.5.4 Angiography/left ventriculography

TS is most commonly diagnosed during coronary angiography (CAG), a diagnosis of exclusion, where either "clean" coronary arteries or those without relevant stenoses (although these may be haemodynamically significant, they do not explain the sites of LV dysfunction i.e. "bystander CAD") are often found[117]. Frequently during CAG there is an associated finding of microvascular dysfunction, as evidenced by increased TIMI frame count[105]. Left ventriculography is then used to determine regional wall motion abnormalities that are associated with TS, while also allowing for detection and quantitation of mitral regurgitation.

1.2.2.5.5 Cardiac computed tomography angiography

In cases where CAG +/- left ventriculography are contraindicated, such as terminal malignancy and intracranial bleeding, cardiac computed tomography angiography (CCTA) may be an appropriate non-invasive alternative[74]. Although radiation exposure may be significant due to image acquisition throughout the cardiac cycle, CCTA can provide information on both coronary artery anatomy and regional LV function.

1.2.2.6 Atypical variants

While the classic presentation of TS involves apparent stunning of the apical myocardium (with/without mid ventricular involvement), it is not uncommon for there to be involvement of other segments of the LV, a biventricular variant of TS or indeed for TS to be isolated to the RV. The four main phenotypes of TS are displayed in Figure 1.4.

The typical form of TS is seen in ~50-82% of cases, with variability between reported series of patients differing significantly[73]. The two next most common variants are "inverted TS", where the basal segments are akinetic and apical segments spared, and the mid-ventricular variant, where only the mid-LV wall segments are affected. Intriguingly, there have also been reports of patients who suffer a recurrence of TS presenting with a different anatomical variant than seen in the index admission[118, 119].

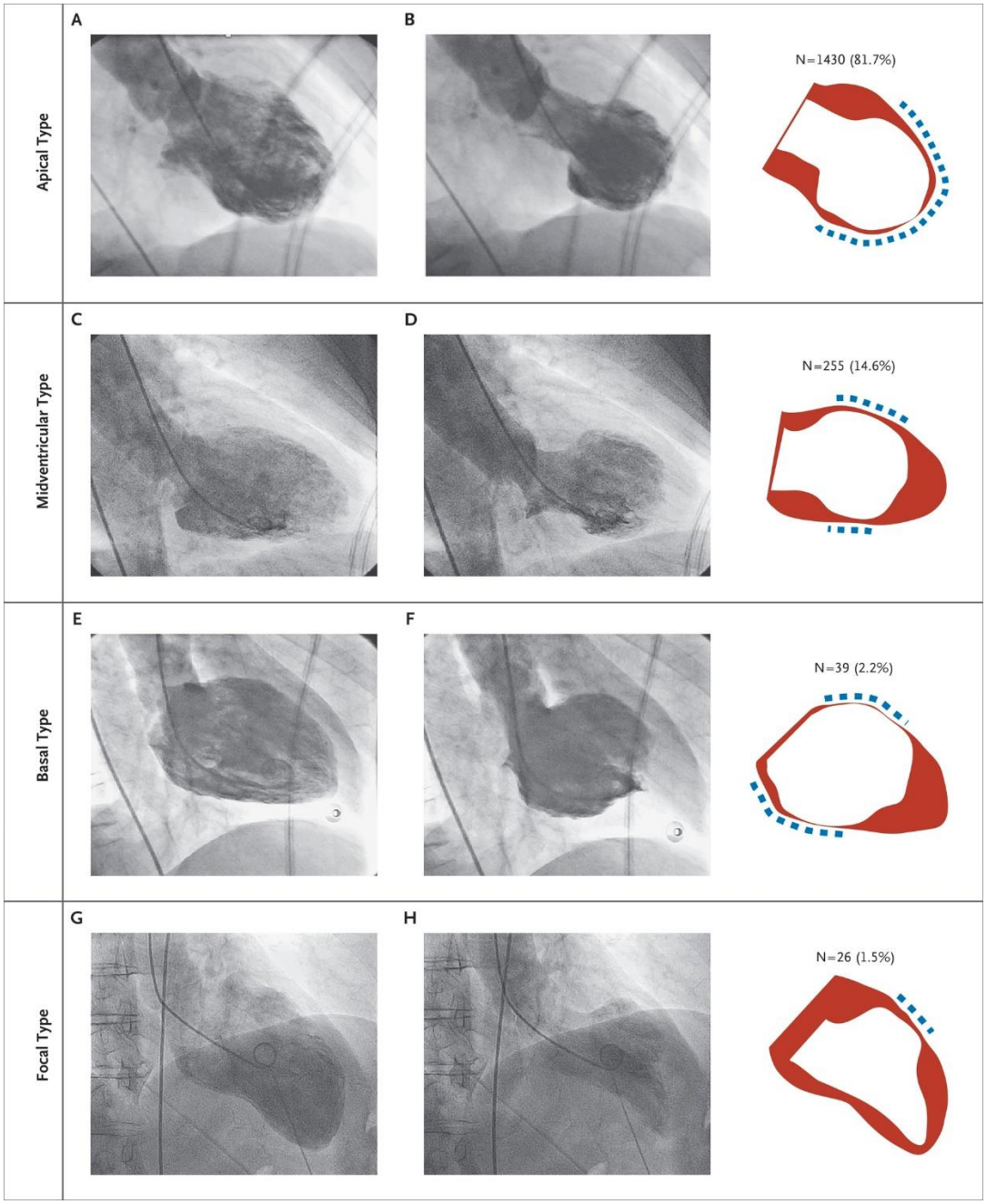


Figure 1.4. Among 1750 study patients, the most common type of Takotsubo Syndrome was the apical type (in 81.7% of patients) (Panels A and B), followed by the midventricular type (in 14.6% of patients) (Panels C and D), the basal type (in 2.2% of patients) (Panels E and F), and the focal type (in 1.5% of patients) (Panels G and H). Reproduced with permission from Templin *et al*, 2015[76].

1.2.3 Epidemiology

Approximately 90% of all TS patients are women, with a mean age of 67-70 years, and around 80% are over 50 years of age. There have however been reports of young children with TS[120, 121], and even in a premature neonate born in the 28th gestational week[122]. While around 2% of patients presenting to hospital with ACS are "traditionally" identified as having TS[123], this number rises to ~7% of all patients with presumed MI, who are in fact TS[124]. Based on discharge data in the United States, TS accounted for around 0.02% of hospitalisations in 2008[72]. This does not account for misdiagnosed or undetected cases of TS, which is more likely to occur in centres without access to PCI[125]. Recurrence rates have now been established as approximately 1-2% annually[76, 126]; this may be decreased by therapy with ACE inhibitors. Overall recurrence rate is approximately 5%, most commonly occurring between three weeks and 3.8 years after the index event[127]. One of the most important findings to emerge from the International Takotsubo Registry (currently the largest registry of TS patients) is that TS is not as benign as first thought, with long term morbidity and mortality comparable with that of MI[76].

1.2.3.1 Psychiatric/neurologic disorders: the heart-brain connection?

The International Takotsubo Registry results also showed that 42.3% of TS patients were found to have an acute, former, or chronic psychiatric disorder (three times higher than age- and sex-matched patients with ACS) - this was a considerable surprise, as the association had previously escaped detection. Indeed, in a prospective study the prevalence of anxiety or depression was 78% in TS patients, significantly higher than that in ACS patients[128]. Neurologic disorders such as seizures, cerebral haemorrhage or migraine followed a similar pattern, almost twice as prevalent in TS patients compared to matched ACS patients. Recent research has also investigated the possibility of a heart-brain interaction in patients with TS. A study of 22 patients with TS and 39 matched healthy controls showed substantial anatomical differences in the limbic network of the two groups. The insula, amygdala, cingulate cortex and hippocampus which are involved in control of emotional processing, cognition and the autonomic nervous system were all found to be anatomically different, with the

authors postulating that these differences resulted in less efficient affective processing[129]. The authors did note however, that causation cannot be implied from the results of a cross-sectional study, and future longitudinal studies would be necessary.

1.2.3.2 Genetic predisposition

Currently there has only been limited investigation of genetic predisposition to TS, with conflicting results[73]. There are case reports of familial TS cases, recorded in two sisters[130, 131] and in two mother-daughter pairs[132, 133]. However, studies regarding functional polymorphisms in relevant genes such as oestrogen receptor-α, β1- and β2-adrenergic receptors and GRK5 have not provided clear results, and only had relatively small sample sizes[134]. Further work is required to assess the possibility that TS has genetic risk factors.

1.2.3.3 The role of gender and oestrogen

Considering the prominence of TS in post-menopausal women, there is a obvious suggestion of hormonal involvement, with the risk of TS increasing with age. This striking disparity in the distribution of TS between genders is not unique, with other cardiac conditions showing imbalances also, such left ventricular hypertrophy, where women are more protected than men[135] or indeed the opposite in conditions such as torsades de pointes arrythmias, where the female disposition towards longer QT interval puts them at greater risk[136]. It should be noted though that each condition is affected differently by the sets of sex hormones, despite the comparatively greater level of cardioprotection that females have over males.

Oestrogen is known to change the normal response to catecholamines, reducing inotropic and chronotropic responses after chronic exposure, as well as altering vascular reactivity and acting in a cardioprotective manner[123]. In this regard, there has been a suggestion by some authors that declining rates of hormone replacement may be exposing post-menopausal females to increased risk for TS[137].

A review of TS reports by Kuo *et al* (2010) did not find a single female receiving oestrogen replacement therapy, though this finding is certainly limited by the lack of medication reporting in these cases[138]. As yet there is no systematic evidence to connect oestrogen levels with the development of TS.

There has been much research regarding catecholamine effects/toxicity in the vasculature and myocardium, and whether there is a potential for a different level of susceptibility in females (see review by Hinojosa-Laborde *et al*, 1999[139]). Some points relevant to TS are as follows: - i) stress mediated vasoconstriction is enhanced in post-menopausal women[140], ii) ovariectomised rats have been shown to exhibit increased β_1AR expression versus sham operated rats[141], and iii) rat models have displayed reduced sarcoendoplasmic reticulum Ca^{2+} transport ATPase (SERCA) activity in post-menopausal animals[142]. If increased catecholamine exposure in hearts of females were coupled with what is seen experimentally in regard to increased β_1AR responsiveness/impaired calcium handling, this could potentially explain some of the predominance of TS in females.

1.2.4 Treatment and outcomes

Thus far, treatment of TS in both the short- and long-term has been entirely empirical. There is no reported randomised study in the current literature. Table 1.2 summarises the various common agents and modalities which have been widely reported to be used as therapy in the acute and post-discharge phases of TS.

Most reports of acute therapy relate to the relatively common problem of shock, and therefore deal with very small numbers of patients. The issue of delay in onset of treatment may be important, given current difficulties in rapid diagnosis of TS.

Table 1.2 Agents/modalities of treatment used in TS

Phase	Agent/modality	Example
Acute	Catecholamines*	Volman *et al*, 2011[143]
	Heparin/oral anticoagulant	Herath *et al*, 2017[144]
	Levosimendan*	Santoro *et al*, 2013[145]
	Verapamil	Abu-Fanne *et al*, 2007[146]
	Intra-aortic balloon pump*	Lisi *et al*, 2014[147]
Post-discharge	Aspirin	Bertaina *et al*, 2017[148]
	β-blockers	Templin *et al*, 2015[76]
	ACE inhibitors	Cacciotti *et al*, 2012[149]
	Angiotensin receptor blocker (ARB)	Abanador-Kamper *et al*, 2017[150]
	Statins	Santoro *et al*, 2014[151]

*Proposed in hypotensive/shocked patients

Larger series are now available re post-discharge therapy. In the registry-based report of Templin *et al* (2015), β-blockers were used in 78.1% of patients, while 79.3% were prescribed ACE inhibitors or ARBs. It was noted here that discharge treatment with β-blockers did not significantly affect outcomes, while ACE inhibitors and ARBs improved survival at one year[76]. A meta-analysis by Singh *et al* (2014) indicated that the risk of recurrence of TS was lower in patients discharged on ACE inhibitors or ARBs. These appear to be the only "positive" therapeutic data on TS in the current literature[126].

Although TS is still considered by many to be a generally benign disease, recent studies have shown that rates of cardiogenic shock and death are comparable to that of ACS patients[76, 152-154]. The currently accepted early in-patient mortality is between 3 and 5%, as a result of ventricular arrhythmias, cardiac rupture, thromboembolic stroke or persistent shock[124]. There is no accurate method to estimate rates of out of hospital deaths that are likely to have been caused by TS but are attributed to MI. LVEF <45%, atrial fibrillation, neurologic disorders but not TS phenotype were independent predictors of death after adjustment for cofounders[155]. Furthermore, despite the prevalence of TS being significantly greater in females, male patients have an up to three-fold increased rate of death and major adverse cardiac and cerebrovascular events (MACCE)[156].

Templin *et al* (2015) published data from the largest TS registry to date, which showed that rates of death and MACCE are approximately 5.6% and 9.9% per-patient year respectively. Two other studies showed that these rates are comparable to those in ACS patients, and significantly worse than patients without CAD[152, 153]. It has been noted that diabetes mellitus (DM) is present in a lower proportion of patients with TS than in age/gender-matched controls[157]. In this report, the author observes that for DM to exert a "protective effect" for the occurrence of TS, there must be autonomic neuropathy and/or catecholamine hyposecretion, known complications of severe DM. While it is theoretically possible that diabetes is directly responsible for a "protective effect" against the precipitation of TS, as suggested by some authors, this discrepancy could also reflect the necessity for intact NO signalling to generate $ONOO^-$ (as a pivotal aspect of TS pathogenesis: see Chapter 1.2.8).

1.2.5 Pathogenesis/proposed mechanisms

Since its initial description at the beginning of the 1990s, the pathogenesis of TS has remained an area of much debate, with several hypotheses being put forward over this time. The suggested mechanisms can broadly be categorised as either vascular or myocardial in nature and are not necessarily mutually exclusive.

1.2.5.1 Ruptured plaque/aborted MI

As previously discussed, the rupture of vulnerable atherosclerotic plaques can result in an MI, with occlusion of the coronary vessel. It has been suggested that an occlusion of this kind could be the precipitating event in TS, with thrombolysis and recanalisation occurring before angiography[158]. Although this hypothesis cannot be tested with angiography, there has been evidence to suggest that disrupted eccentric plaques of the left anterior descending artery have been visualised using intravascular ultrasound[159].

There are however several problems with this hypothesis, which make it an unlikely pathogenic mechanism; firstly, the typical area of hypo/akinesia seen with TS is greater than that supplied by a single coronary artery. Secondly, ECG changes remain during angiography, which should not be the case if thrombolysis had resolved the coronary occlusion. Finally, histopathology in TS shows characteristic contraction band necrosis, whereas coagulation necrosis is characteristic in AMI. Data from animal experiments (see Chapter 1.2.6) also provide evidence of non-involvement of plaque rupture.

Although there is significant evidence to suggest that aborted MI is unlikely to be the cause of TS, it by no means suggests that there cannot be concurrent coronary artery disease in TS patients, as the severe chest pain experienced in ACS may be a significant stress factor which could trigger TS[160-162].

1.2.5.2 Multi-vessel coronary artery spasm

When authors Sato and Dote first described TS in 1990[63], their hypothesis for the pathogenesis of TS was that the left ventricular dysfunction characteristic of TS was caused by myocardial stunning due to multi-vessel coronary artery spasm. In this small series of five patients, the authors observed spontaneous multi-vessel spasm in two patients, with ergonovine administration causing spasm in two others. The same authors then reported that approximately 70% of TS patients had coronary spasm during a provocation test[163].

Since then, there have been inconsistent reports of spasm in TS patients, with one review stating that only 5-10% of cases reported spontaneous vasospasm[123], while another suggested that this figure could be as low as 1.4% in the 212 patients investigated[164]. Provocation-induced vasospasm showed a higher incidence, however this was still only 28% of the 84 patients studied and the results varied widely in different series, from 0 to 100%[164].

In addition to the inconsistent reports of spasm, reverse or mid-ventricular variants of TS cannot be explained by the coronary spasm hypothesis[165]. Furthermore, catecholamines and beta-receptor

agonists such as epinephrine and dobutamine, which have significant vasodilator effects, have both been shown to induce TS[166]. Overall, coronary artery spasm is not common in TS patients, which suggests that although it may be causative in a small subset of patients, it may also be a concurrent process in some patients, similar to coronary artery disease.

1.2.5.3 LVOT obstruction

The hypothesis that LVOT obstruction is the mechanism behind TS revolves around patients with susceptible hearts (generally older women with smaller ventricles and prominent septal bulges) developing a dynamic mid-ventricular obstruction under catecholamine stress. Theoretically this large pressure gradient between the apex and the base of the LV might result in subendothelial ischemia in the apical region and the characteristic apical ballooning[167]. Only a minority of TS patients have any evidence of LVOT obstruction however (~25%), and it also does not account for atypical variants and RV involvement[165]. Furthermore, patients with obstructive hypertrophic cardiomyopathy do not develop apical a/hypokinesis. This then may more likely represent a complication of TS rather than the cause.

1.2.5.4 Microvascular dysfunction

Of the "vascular" hypotheses re pathogenesis of TS, microvascular dysfunction presents the strongest case. The coronary microcirculation involves the pre-arterioles and arterioles (<500μm), responsible for modulating the coronary blood flow in response to neural, mechanical or metabolic factors[168].

Using TIMI frame-count as a semiquantitative invasive technique, microvascular resistance has been suggested to be increased in patients with TS on angiography[169, 170]. Khalid *et al*, using a "corrected TIMI frame-count" technique found that there was microvascular dysfunction in TS patients compared to controls, however only in the left anterior descending artery, with no difference seen between

controls and TS patients in other vessels[171]. Conversely, other authors have found that there are no significant differences in TIMI frame-counts between TS patients and controls[172].

Another technique of investigating microvascular dysfunction is coronary flow reserve (CFR). Using a Doppler guidewire technique in eight consecutive TS patients, it was observed that CFR was decreased in all 3 coronary arteries in the acute setting[173]. Concurrent to improvement of LV wall motion abnormalities, CFR also improved in these patients. These findings raise the possibility that microvascular dysfunction may reflect increased extramural pressure during diastole due to incomplete LV relaxation.

Non-invasive techniques to assess myocardial dysfunction have also been studied, such as Doppler transthoracic echocardiography and myocardial contrast echocardiography. These techniques provide an analysis of coronary flow and any RWMA, with results showing that compared with ventricular segments that do not have any motion abnormalities, the "stunned" parts of the LV had reduced myocardial blood flow in the acute setting[168, 174]. A study in 20 consecutive patients concluded there was a transient impairment of CFR during the acute phase of TS, due to a reduced vasodilating capacity of the microvasculature. The impairment was correlated to parameters of systolic function, but not diastolic function[175]. Single photon emission computed tomography (SPECT) ^{99m}Tc-sestamibi or tetrafosmin perfusion studies are another method of assessing myocardial perfusion, and unsurprisingly tracer uptake was shown to be reduced with systolic dysfunction in the acute phase of TS, suggesting microvascular dysfunction[105, 125, 176].

In addition to this substantial evidence, microvascular dysfunction has been associated with other conditions that predominate in post-menopausal women, such as Syndrome X. In a recent review however, it has been suggested that microvascular dysfunction may be the result, rather than the cause, of apical "stunning" and its precipitants[167]. This is supported by evidence that catecholamines, both directly and indirectly, have the ability to trigger coronary microvascular dysfunction and LV contractility impairment in predisposed subjects[168]. Furthermore, other conditions such as dilated

cardiomyopathy also show decreased microvascular function resulting from reduced muscle function, rather than a precipitant to reduced function.

1.2.5.5 Catecholamines and catecholamine-induced signalling

These hypotheses represent the most promising starting point in the search for the pathophysiological mechanism behind TS, with both animal and human studies confirming the significant role of catecholamines (presumably acutely released) in the pathogenesis of TS[165].

The pharmacological induction of TS through various agents across different drug classes represents an area of significant controversy regarding the pathogenesis of TS. There have been many case reports of the inotropic β-agonist dobutamine inducing TS, commonly after dobutamine stress echocardiography[88, 89, 177, 178]. Similarly, the use of classes of antidepressants which prevent tissue reuptake of catecholamines (such as tricyclics and serotonin norepinephrine reuptake inhibitors) in some patients has triggered TS, both in therapeutic and over dosage scenarios[108, 179, 180]. At least five cases of cocaine use triggering TS have been published in recent years, with many such cases likely misdiagnosed as patients are unlikely to receive an echocardiogram under these circumstances[181, 182]. Furthermore, there are also reports that administration of adrenaline via the use of an Epipen©, or even the venom of the Irukandji jellyfish (acting via increased sympathetic activation) has precipitated TS[183, 184]. While it seems that pharmacological induction of TS is quite common, it has also been noted that the withdrawal of opioids has been able to induce TS, which raises the question of whether this represents only the release of catecholamines as a component of the withdrawal syndrome[185, 186].

1.2.5.5.1 Direct myocardial stunning

Direct myocardial stunning, as a result of blood-borne catecholamine toxicity, has been suggested as a component of the pathogenesis of TS after an association with emotional or physical stress immediately preceding the onset of symptoms, as stress is known to release large quantities of catecholamines[187]. This hypothesis is supported by i) initial reports of high plasma catecholamine levels seen in patients during the acute phase of TS[188], ii) numerous reports of TS being induced by exogenous

administration of catecholamines[180, 189, 190], and iii) the association between TS and patients with pheochromocytoma (in fact, even before the first reports of TS)[191-193].

The proposed mechanism suggests that acutely elevated catecholamine levels tend to decrease the viability of myocytes through cAMP-mediated calcium overload, resulting in the characteristic contraction band necrosis seen in TS[91]. Excess circulating catecholamines are also a source of oxygen-derived free radicals, which are known to cause direct myocyte injury, in conjunction with interfering with sodium and calcium transporter function[194, 195].

As with other proposed hypotheses, despite strong supporting evidence, there is a counter argument which challenges the direct catecholamine myocyte toxicity hypothesis. Firstly, high levels of circulating catecholamines are not consistently reproduced in all studies of TS[125, 196]. Secondly, the majority of patients do not present with a history of an acute emotional or physical stressor[164]. Finally, animal models have shown an apical-to-basal cardiac gradient of β_2-adrenoceptors (β_2AR), which are acted upon by circulating catecholamines[197]. This only fits with the typical apical variant of TS, and is unable to explain the inverted or mid-ventricular variants. It has the overall effect, however, of making it unnecessary to postulate hyper-responsiveness to β-stimulation.

1.2.5.5.2 Hyperactivation of the sympathetic nervous system

Death subsequent to activation of the sympathetic nervous system due to an acute stressor is not a new concept, with the well-known paper “Voodoo death” published in 1942 reporting on anecdotal experiences of death from fright[198]. TS itself is preceded by a recognised emotional or physical stressor around 70% of the time[199]. It is also well known that various acute intracranial diseases and injuries can induce TS, irrespective of lesion localisation, with an increase in intracranial pressure and hyperactivation of the cardiac sympathetic nervous system being the hypothesised cause[200].

As previously mentioned, TS (excluding cases with ST elevation on initial ECG) is associated with characteristic T-wave inversion and prolongation of the corrected QT-interval on ECG. A recent review article has provided evidence to link the disruption of cardiac sympathetic nerve terminals to reversible

T-wave inversions, where multiple acute cardiac and non-cardiac conditions induced transient ECG changes consistent with those seen in TS[199].

Evidence in animal studies suggests that brain stimulation, induction of experimental intracranial haemorrhage, and stellate ganglion stimulation may induce ECG and myocardial changes which resemble changes seen in TS. Researchers were then able to prevent or reverse these changes with surgical (spinal cord transection or severing) or pharmacological (reserpine, propranolol) sympathectomy[201]. Similarly in human studies, intracranial diseases/injuries have been noted to induce TS[202, 203]. Norepinephrine spillover from the cardiac sympathetic nerve terminals can decrease myocyte viability through cAMP-mediated calcium overload, manifesting as contraction band necrosis, which is known to be a hallmark histological finding in TS[201].

In a study on patients with subarachnoid haemorrhage myocardial scintigraphy was used to assess myocardial perfusion (technetium sestamibi) and sympathetic innervation (Iodine 123 meta-iodobenzylguanidine (^{123}I-MIBG)), with correlation to LV function assessed by echocardiography. The authors concluded that "*LV systolic dysfunction in humans with subarachnoid haemorrhage is associated with normal myocardial perfusion and abnormal sympathetic innervation*", with the excessive release of norepinephrine from myocardial sympathetic nerve terminals possibly responsible for the damage to both myocytes and nerve terminals[204]. Intriguingly, a recent review of 959 TS studies showed that the prevalence of diabetes mellitus was significantly rarer in TS patients (10.2-17%) when compared to the average population (26.9% [National Health and Nutrition Examination Survey])[205]. The authors concluded that diabetes mellitus-induced autonomic neuropathy may actually be cardioprotective in the setting of TS, limiting the local and blood-borne catecholamine surge that results from intense sympathetic stimulation.

In addition, hyperactivation of the sympathetic nervous system may be able to explain the different regional wall motion abnormalities and atypical variants of TS, where many of the other proposed mechanisms cannot. There is dense sympathetic innervation within the myocardium that is distributed

on a regional basis, with this distribution appearing to follow the wall motion abnormalities that are associated with TS[201, 206].

As previously mentioned, there is evidence to show that TS is associated with significant myocardial oedema in the acute phase, likely of inflammatory origin[92] (although it is certainly possible, given recent findings regarding glycocalyx shedding[96] that increased vascular permeability is also relevant). These findings are consistent with the sympathetic hyperactivity hypothesis, where it has been suggested that excessive catecholamine levels result in a chemical myocarditis[165], but also may trigger glycocalyx shedding[207].

1.2.5.5.3 Post-receptor variability in β_2AR-based signalling

There is a longstanding and now substantial literature related to the physiological role of myocardial β_2AR stimulation in heart failure, a circumstance under which β_1ARs are partially internalised[208, 209]. In summary, it has been suggested by many studies that: -

(i) Myocardial β_2ARs are coupled both to Gs (G-stimulatory) and Gi (G-inhibitory) proteins, and that the former are in turn coupled both to activation of adenylate cyclase and to that of nitric oxide synthase[210, 211].

(ii) oxidative stress results in PKA-related feedback and inhibition of β_2AR-Gs coupling, resulting in predominantly Gi-based signalling under these circumstances[212].

This biased signalling therefore results in predominantly negative inotropic effects of β_2 stimulation, as summarised in Figure 1.5.

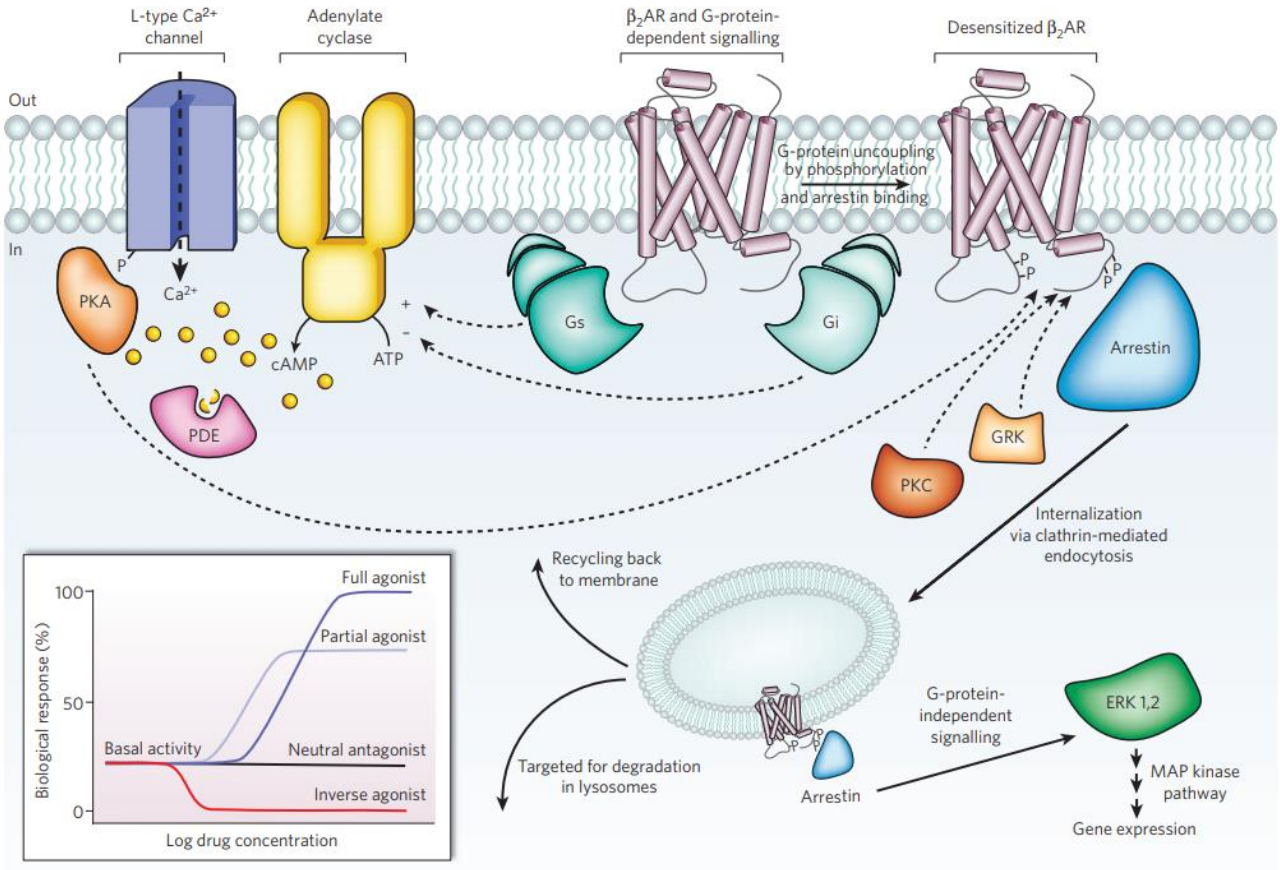


Figure 1.5. Diverse signalling pathways regulated by the type 2 beta adrenergic receptor (β_2AR). The β_2AR can activate two G proteins, Gαs and Gαi (part of the Gs and Gi heterotrimers, respectively), which differentially regulate adenylate cyclase. Adenylate cyclase generates cyclic AMP (cAMP), which activates protein kinase A (PKA), a kinase that regulates the activity of several cellular proteins including the L-type Ca2+ channel and the β_2AR. cAMP second messenger levels are downregulated by specific phosphodiesterase proteins (PDEs). Activation of the β_2AR also leads to phosphorylation by a G-protein-coupled receptor kinase (GRK) and subsequent coupling to arrestin. Arrestin is a signalling and regulatory protein that promotes the activation of extracellular signal-regulated kinases (ERK), prevents the activation of G proteins and promotes the internalization of the receptor through clathrin-coated pits. PKC, protein kinase C. The inset shows classification of ligand efficacy for GPCRs. Many GPCRs exhibit basal, agonist-independent activity. Inverse agonists inhibit this activity, and neutral antagonists have no effect. Agonists and partial agonists stimulate biological responses above the basal activity. Efficacy is not directly related to affinity; for example, a partial agonist can have a higher affinity for a GPCR than a full agonist. Reprinted with permission from Rosenbaum *et al* (2009)[210]. Note: This schematic does not recognise the potential importance of biased β_2AR-targeted post-receptor signalling, which was poorly understood in 2009.

Lyon *et al*[197] proposed some additional details which might apply in the circumstance of TS, labelling the relevant process as "stimulus trafficking". Under normal physiological conditions epinephrine binds to both β_1- and β_2ARs, however with higher affinity for β_2ARs, which results in activation of the Gs-protein–adenyl cyclase–PKA pathway and subsequent positive inotropic response. At the higher epinephrine concentrations ("supraphysiological") that are present after an acute stressor, there is a switch in signalling, a process known as stimulus trafficking, whereby specifically β_2ARs instead couple to Gi-proteins with subsequent negative inotropic effects. The Gi-protein coupled β_2ARs either switch back to Gs-protein coupling or are internalised and degraded once the epinephrine surge has been cleared from circulation, allowing return of myocardial function. This is thought to be a cardioprotective mechanism (although negatively inotropic) to prevent βAR-coupled Gs protein pathway overactivation which induces proapoptotic pathways in the cardiomyocyte. With a higher βAR density in the apical myocardium as compared with the basal myocardium, and if it is assumed that myocardial perfusion is balanced, the typical TS phenotype can be explained by the (potentially) increased responsiveness of the apical myocardium relative to the base. This however does not account for atypical forms of TS.

Although there has been much progress in recent years and broad investigation into the many potential mechanisms behind TS, there is still currently no definitive answer. From the available evidence, it appears that there is significant involvement of the sympathetic nervous system, whether through blood-borne catecholamines from the adrenal medulla or via drug therapy, or primarily via sympathetic innervation of the heart[72]. It remains quite likely, as there is convincing evidence for multiple hypotheses, that there is not a single mechanism at play, rather that multiple mechanisms are working in concert.

1.2.6 Animal models of TS

Despite the wealth of knowledge that can be gained from observations and studies in human patients, animal models remain a key means by which to both better understand a condition and also to test potential therapeutic strategies. There are obvious limitations attached to animal models, as they can never be a true substitute for what occurs in the human body, but they remain an important part of understanding disease pathology. In TS there are essentially two groups of animal models, which are defined by the method in which stress is induced in the animal: i) psychogenic stress models, whereby the animal is placed in a stressful situation and, ii) exogenous pharmacological stress models, where administration of exogenous compounds (i.e. catecholamines) are used to induce TS.

1.2.6.1 Psychogenic stress

By far the lesser investigated of the two categories of animal models, the psychogenic stress models offer a more "real life" situation. The advantage of this is that they represent a more translatable platform to test potential therapies, but this is also their main disadvantage, with any model (strictly by nature) being non-specific for a particular cause.

It has been well established that handling and forced immobilisation of animals activates an acute stress response, with increases in plasma levels of epinephrine and norepinephrine[213]. One of the first animal models of TS, and certainly the first major psychogenic model, was created by Ueyama and colleagues in Wakayama[214]. The researchers had previously used an immobilisation technique involving 30 minutes of dorsal recumbency restraint to induce a stress response in rats, with the goal of examining "*glucocorticoid effects on indices of CA (catecholamine) release, metabolism, and synthesis, and on CA biosynthetic enzyme activities and gene expression*"[187]. In the 2002 study however, the researchers used this immobilisation technique and assessed LV function with left ventriculography, showing that there was reversible LV apical ballooning. Pre-treatment with amosulalol hydrochloride (a non-specific α/β-adrenoceptor antagonist) normalised LV function under stress, while the use of a calcium channel blocker or nitroglycerine had no significant effect. Further

experiments from the same laboratory suggest a protective effect when rats were premedicated with the long acting calcium channel blocker azelnidipine, preventing the cardiac dysfunction seen in the acute stress model[215].

The same team of researchers continued down this pathway, by investigating the role of oestrogen supplementation in ovariectomised rats subjected to similar immobilisation stress[216]. The results of this study showed that oestrogen supplementation was able to prevent LV dysfunction in these rats, suggesting a cardioprotective effect of oestrogen and perhaps (part of) the reason that TS is seen mainly in post-menopausal women. In further experiments by this group, oestrogen supplementation in ovariectomised rats appeared to reduce sympathetic outflow from the brain and increased heat shock protein 70 and ANP levels, further indicating cardioprotective effects of oestrogen[217, 218].

A separate group of Japanese researchers used immobilisation stress and the addition of an α_2-adrenoceptor agonist to assess involvement of α_2AR-Gi-protein coupling in the pathophysiology of TS[219]. Ejection fraction and specifically anterior LV wall motion were supressed post injection of xylazine, while posterior wall motion was preserved, and serum epinephrine levels were elevated. Pre-treatment with the Gi-protein inhibitor pertussis toxin (PTX) reversed the LV dysfunction, but had an associated mortality rate of ~30%. The researchers were able to show that by enhancing Gi-protein coupling in rats exposed to immobilisation stress, they were able to induce transient and regional LV dysfunction, suggesting that this was a pivotal mechanism of induction of negative inotropic changes. α_2AR-agonists are known to inhibit stimulation-evoked release of epinephrine and norepinephrine, however the combination of immobilisation stress and xylazine resulted in an increase of serum epinephrine compared to animals subjected to immobilisation stress alone. Researchers hypothesised that increased calcium influx, particularly within the adrenal medulla, as well as within the myocardium, was responsible for the raised epinephrine levels seen in these rats.

A different model of psychogenic stress was used by Wideman *et al*, whereby the social defeat paradigm was used to model "stress induced cardiomyopathy" (TS) in rats[220]. This particular technique involves placing an intruder rat into the cage of a "resident" rat to illicit a stress response, while

monitoring ECG data from a biotelemetry transmitter that had previously been implanted into the rats. After a set period of time the experiment was completed, and the intruder rat was humanely killed, after which blood and the heart tissue were taken for analysis. Heart weight/body weight ratio, left ventricle/body weight ratio, heart length, plasma corticosterone levels, and plasma troponin I levels of intruder rats were significantly higher as compared to control rats. The increased heart weight and LV weight/body ratio were hypothesised to represent a result of LV hypertrophy, although this postulate seems obscure in light of the acuity of the changes. On the other hand, plasma norepinephrine levels were not significantly greater in the intruder rats when compared with controls.

The advances made by these initial animal models are significant, in that they concur with what is seen clinically in TS. The findings of raised catecholamine levels and strong evidence of adrenoceptor signalling pathways are important, with the evidence of a cardioprotective effect of Gi-protein signalling, though the origin of the catecholamines is uncertain in these models. The role of sex hormones is also yet to be fully elucidated, though there appears to be some cardioprotection offered by oestrogen in this manner.

1.2.6.2 Exogenous catecholamines

Although the use of catecholamines to cause heart failure in animal models is far from a new concept, the earliest (to our knowledge) animal model of TS using exogenous catecholamines was in 2009[221]. In comparison to the psychogenic stress models previously discussed (see 2.6.1), exogenous catecholamine models are a significantly more robust way to investigate TS. Added to this, psychogenic stress models and their use in rodents is still ethically controversial, due to the extreme nature of the experimental protocol.

Appendix 1 summarises the available literature concerning the utilisation of animal models to investigate acute cardiotoxicity of catecholamines. Over the period 2009-2018, a total of 16 publications have appeared, all but one using a rat or mouse model[100, 221-235]. Only two studies used female animals. All studies used agents which stimulate cardiac βAR, and isoprenaline was the most

commonly utilised agent. In all cases, regional left ventricular hypokinesis was induced, although not always in a predominantly apical location.

Of these studies, nine evaluated some aspects of myocardial structure and seven extended evaluation beyond 24 hours. Mortality was specified by most authors, and varied greatly, with some authors finding no acute (<2hrs) mortality and others finding high mortality rates. Appendix 1 provides a summary of the models and the main results for each. There are however some findings that warrant further discussion: -

In two of the early studies, it was noted by both Paur *et al* (2012)[222] and Shao *et al* (2013)[100] that β_2AR blockade increased mortality while ameliorating hypokinesis. This finding suggests that β_2ARs play a significant role in the pathogenesis of TS, with over-stimulation resulting in negative inotropy which, somewhat paradoxically, is also cardioprotective. These findings also support clinical observations with regard to β-blocker therapy: - this appears to be ineffective in the treatment of TS[76]. This was contradicted, however, in the study by Sachdeva *et al* (2014)[229], where pre-treatment with β-blockers actually increased survival, though it had no effect on any structural alterations that were caused by the high dose of isoprenaline. Similarly, Cao *et al* (2015)[231] were able to return all haemodynamic parameters to baseline by pre-treating with specific β_2AR antagonist. There has been no analysis to date as to whether patients pre-treated with β-blockers prior to onset of TS have abnormally good or bad outcomes.

One of the most significant findings, and the most consistent finding between different models other than the initiation of TS with catecholamines, is the presence of inflammatory infiltration into the myocardium. In comparison to an acute MI, where inflammation is limited and there is comparatively more necrosis, animal models of TS are characterised by inflammatory cell infiltration (specifically monocytes/macrophages) and limited necrosis. Again, these findings in catecholamine TS models correlate to clinical findings of significantly raised NT-proBNP levels and NT-proBNP/BNP ratios, which is known to occur in inflammatory states.

One of the more intriguing results came from the Redfors *et al* (2014)[225] study, which showed that different catecholamines cause different TS phenotypes. The administration of isoprenaline resulted in apical dysfunction, resembling the classic TS phenotype, whereas all other catecholamines resulted in basal dysfunction or "reverse TS". This may be due to adrenoceptor density gradients within the myocardium, with isoprenaline being a specific β_2AR, while other catecholamines act on other βAR and/or αAR.

Arguably the most promising therapeutic results are from Zhang *et al* (2017)[234], where the Chinese group showed that the H_2S donor NaHS reversed functional damage and ameliorated mortality rate in their rat model. Added to this, the use of NaHS was associated with diminution of oxidative stress. In a condition where there is currently no definitive therapeutic strategy, results such as this are of extreme interest, warranting significant further investigation. H_2S is now recognised as the third member of the gasotransmitter family, along with nitric oxide (NO) and carbon monoxide. Previous studies of H_2S interactions with myocardial inflammatory states have been limited, though there is evidence that H_2S inhibits expression of adhesion molecules in human umbilical vein endothelial cells (HUVECs), induced by inflammatory cytokines such as tumour necrosis factor-α (TNF-α)[236]. In addition, H_2S hampers TNF-α-induced monocyte-endothelial interactions by downregulating the expression of the monocyte chemo-attractant protein-1 (MCP-1). Particularly in the vasculature, H_2S is largely regarded as a vasodilator acting in similar fashion to NO[236], but the relationship with NO level was not explored in the study by Zhang *et al*.

Since there is a substantial body of evidence to suggest that some of the cytoprotective effects of H_2S are mediated by incremental formation and/or effect of NO[237-239], this finding is superficially counter-intuitive. However, H_2S also inhibits O_2^- production[240], scavenges $ONOO^-$[241] and reduces oxidative stress[236]. Thus, the reported protective role of H_2S/H_2S donors (such as NaHS) in TS is theoretically feasible and merits further, primarily mechanistic, investigation.

The only study which has so far investigated the potential effects of oestrogen as a cardioprotective agent was by Cao *et al* (2015)[231]. In this study the authors showed that in an ovariectomised group of

animals, initiation of TS with catecholamines resulted in more severe cardiac dysfunction and injury. Subsequent administration of oestrogen improved haemodynamic parameters. These results echo those from Ueyama *et al* (2003)[216], where oestrogen was again found to have a cardioprotective effect in a psychogenic stress model, as compared with the exogenous catecholamine model used by Cao *et al* (2015)[231].

1.2.7 Cellular models of TS

Although the majority of experimental research into TS has been conducted solely in vivo, there have been some small studies utilising cellular models, which require brief discussion. Firstly, Paur and colleagues published a manuscript in 2012 which had significant implications in understanding the pathogenesis of TS, specifically in regard to biased βAR signalling, through the use of a rodent model[222]. This study contained a component of cellular experimentation using isolated rat cardiomyocytes, which showed that epinephrine-induced negative inotropy could be reversed with G_i blockade (using PTX), without a significant change in cAMP levels. The group also showed that β_1AR blockade or overexpression of β_2AR or G_i protected against isoprenaline-induced cell death. Shao *et al* (2013) showed that stressing HL-1 cardiomyocytes with isoprenaline resulted in significant lipid accumulation, corresponding to results from their in vivo work[224]. Depression in cellular electrical activity was also noted in these cells.

Willis and colleagues (2015) isolated ventricular myocytes and mitochondria from isoprenaline-treated rats, where they found altered mitochondrial oxidative metabolic state, increased mitochondrial fragility, and oxidative stress, as well as dysfunctional Ca^{2+} handling[230]. The authors hypothesised that impaired energy synthesis and exacerbated ROS production were responsible for these changes. Finally, Borchert *et al* (2017) used pluripotent stem cells derived from patients with TS, describing what appears to be the first completely in vitro model of TS[242]. They found that sudden stress could be achieved using short-term catecholamine exposure, and that this resulted in both β_1- and β_2AR activation, negative inotropy, depressed electrical activity and lipid accumulation within these cells.

1.2.8 Oxidative and nitrosative stress

Reactive oxygen (ROS) and reactive nitrogen species (RNS), including superoxide (O_2^-), nitric oxide (NO), peroxynitrite ($ONOO^-$) and hydrogen peroxide (H_2O_2), are unstable molecules generated normally during cellular metabolism, balanced by endogenous antioxidants, such as superoxide dismutase (SOD) and catalases[243]. When there is an imbalance between ROS/RNS generation and antioxidant capacity, a state of oxidative and/or nitrosative stress occurs. As previously discussed in sections 1.2.5.5 and 1.2.6.2, there is substantial evidence from both human and animal studies for catecholamines to play a central role in the pathogenesis of TS, though as yet there has been relatively little progress in regard to the exact effects within the myocardium. High levels of catecholamines, both directly and through the activation of α- and βAR, are known to induce both oxidative and nitrosative stress through various biochemical pathways, which requires examination in the context of TS.

1.2.8.1 Oxidative stress

There is now substantial evidence to implicate catecholamines in the induction of oxidative stress within the myocardium. Firstly, αAR activation by catecholamines (e.g. noradrenaline) leads to increased activity of NADPH oxidase and subsequent generation of the O_2^- anion within cardiomyocytes[244]. Secondly, MAO-dependent oxidative deamination of catecholamines stimulates formation of hydrogen peroxide (H_2O_2), which may be converted to the highly reactive hydroxyl radical ($OH^\cdot$) through metal catalysis[245]. Thirdly, catecholamines are readily oxidized into toxic compounds termed “aminochromes”, a spontaneous but low rate process (autooxidation). This process is, however, markedly accelerated in the presence of oxidants and free radicals such as O_2^-, redox metals (especially iron and copper) and by enzymatic catalysis. Aminochromes result in direct cardiotoxicity via inhibition of oxidative phosphorylation and depression of calcium binding, while indirectly increasing oxidative stress through the formation of large amounts of ROS[246].

Finally, intracellular Ca^{2+} overload, both in the cytosol and mitochondria, triggers oxidative stress. This process is described as the "mitochondriocentric signal transducer–effector (MSTE) pathway", where myocardial Ca^{2+} build-up results in downstream phosphorylation of multiple Ca^{2+}-cycling proteins. βAR activation-linked progressive increase in mitochondrial Ca^{2+} results in a rapid change in the permeability of the inner mitochondrial membrane, followed by swelling of the mitochondrial matrix, loss of respiratory control and generation of ROS, further exacerbating Ca^{2+}-induced mitochondrial dysfunction[246]. Antioxidants have proven to be beneficial in other catecholamine induced cardiotoxicity models, including the use of melatonin[247], quercetin[248], ascorbic acid[249] or *N*-acetylcysteine[250]. In this setting of oxidative stress and Ca^{2+} overload, a process known as "ROS-induced ROS release" (RIRR) occurs, feeding further oxidative stress and stimulating efflux of pro-apoptotic molecules[251]. This process is the result of opening of the mitochondrial permeability transition pore (mPTP), which causes mitochondrial depolarisation and cessation of oxygen transport[252]. The degree to which the mPTP opens, and hence the level of RIRR with subsequent pro-apoptotic molecule release, determines whether cells are able to recover (minimal opening), or if they die by apoptosis (moderate opening), if necrosis results (substantial, irreversible opening)[246, 253].

1.2.8.2 Nitrosative stress

Cardiomyocytes, endocardial endothelium, coronary endothelium and cardiac nerves are all sources of NO produced by Ca^{2+}-dependent NOS, with NO necessary for normal physiological cardiac function, modulated in part by decrease of intracellular Ca^{2+}. NO is formed from L-arginine by three isoforms of nitric oxide synthase (NOS): neuronal (nNOS), endothelial (eNOS) and inducible (iNOS)[254]. Biological actions of NO include regulation of vascular function and platelet aggregation, inhibition of vascular smooth muscle cell proliferation and LDL oxidation[255]. These, and other, functions of NO are mainly executed through NO-cGMP signalling, but also through protein modification by S-nitrosylation. The regulation of NO production is physiologically maintained in part by inhibitory actions of asymmetric dimethylarginine (ADMA), a potent endogenous non-selective NOS substrate, activity of which is itself

modulated through NO concentration-dependent negative feedback regulation of dimethylarginine dimethylaminohydrolase (DDAH) activity[256]. Production of NO via iNOS has been studied most extensively in sepsis, where inflammatory stimuli have been shown to cause increased iNOS expression and subsequent vascular dysfunction[254].

In the myocardium, nNOS and eNOS play a significant role in the modulation of cardiac contractility and relaxation[257, 258], for example in chronic heart failure, where the use of a NOS inhibitor led to potentiation of the βAR inotropic response[259]. Activation of βAR by catecholamines results in the increased production of NO by the Ca^{2+}-dependent isoforms of NOS, nNOS and eNOS[257]. Intriguingly, eNOS can produce both NO (via its oxygenase function) and O_2^- (via its reductase function). This occurs after eNOS "uncoupling" where due to reduction in tetrahydrobiopterin (BH4), the eNOS monomer produces O_2^-, while in the presence of abundant BH4 the dimer produces NO[260] (Figure 1.6). There is evidence that in aging, there is phenotypic upregulation of the third NOS isoform, iNOS, which has been shown to be coupled to βAR in the setting of ischemia[261].

Neither NO or $O2^-$ are particularly toxic on their own, due to the highly efficient clearance mechanisms for each, SOD readily scavenges $O2^-$ while NO rapidly diffuses through tissues into red blood cells, where it is converted into nitrate. The problem arises, however, if the two come into contact with each other, they will always spontaneously form $ONOO^-$ in a diffusion limited reaction, as NO is the only known biological molecule to react faster with $O2^-$ than SOD[255].

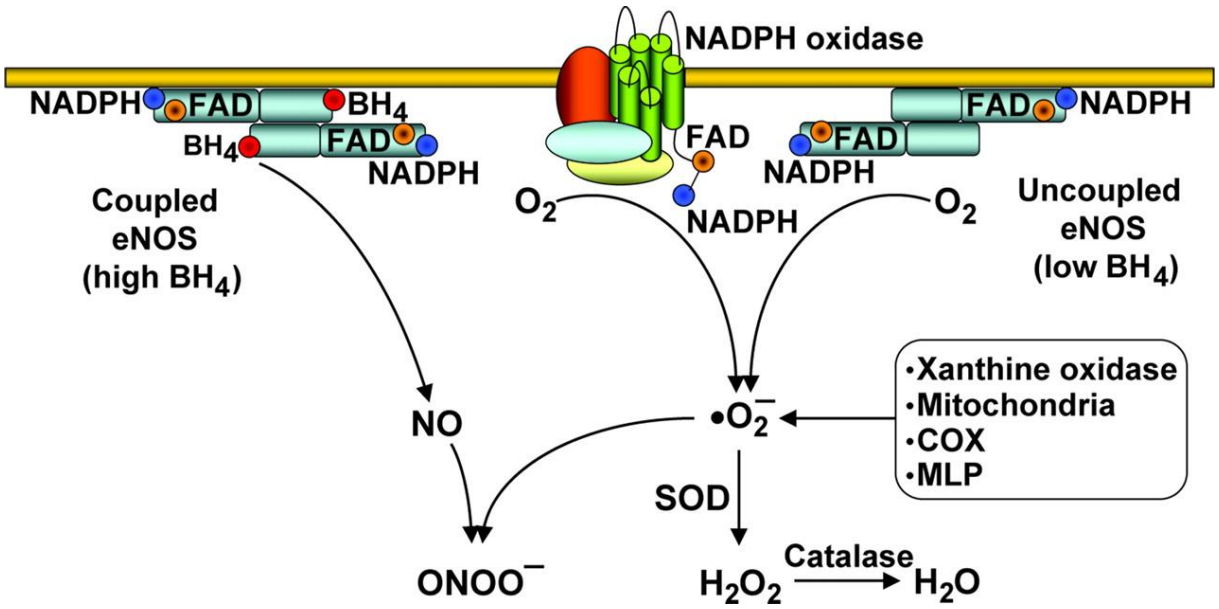


Figure 1.6. Schematic of the balance between NO and ROS that modulates NO bioavailability. When ROS production is increased, tetrahydrobiopterin (BH4) generation is reduced, and eNOS produces superoxide ($O2^{-}$). Excess generation of $O2^{-}$ by different sources (NADPH oxidase, uncoupled eNOS, xanthine oxidase, myeloperoxidase, cyclooxygenase, mitochondria) will reduce NO bioavailability and convert NO into peroxynitrite ($ONOO^{-}$), which has deleterious effects. Reduced activity of SOD will also result in enhanced ROS accumulation in the vascular wall. COX indicates cyclooxygenase; FAD, flavin adenine dinucleotide; MLP, myeloperoxidase; NADPH, reduced nicotinamide adenine dinucleotide; XO, xanthine oxidase. Reprinted with permission from Schiffrin, 2008[260]. Note: This schematic omits the role of asymmetric dimethylarginine (ADMA) as a competitive inhibitor of NOS.

$ONOO^-$ formation results in a myriad of downstream consequences, with a comprehensive review of these published by Pacher *et al* (2007)[255]. There are four consequences with distinct relevance to TS, the first being tyrosine nitration of proteins within cardiomyocytes, such as desmin, myosin heavy chain and α-actin, which could result in deleterious effects on cardiac contractility. Secondly, $ONOO^-$ triggers lipid peroxidation and subsequent formation of products such as lipid hydroperoxyradicals, conjugated dienes, and aldehydes, ultimately resulting in membrane permeability and fluidity changes. Thirdly, $ONOO^-$ has been shown to disrupt the endothelial glycocalyx, something that has been recently associated with TS[96]. Finally, and possibly most importantly with regard to this thesis, $ONOO^-$ can cause single-strand DNA breakage, the trigger for activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP)[262].

In short, the normal function of PARP-1 is, upon activation through detection of DNA damage, to allow for DNA repair and cell recovery using cellular NAD^+ to build poly(ADP-ribose) polymers (PAR)[263]. In circumstances of significant DNA damage there is hyperactivation of PARP-1, resulting in significant loss of NAD^+ and a marked decrease in cellular ATP, establishing essentially an "energetic sink"[264]. An additional role of PARP-1 is in the upregulation of inflammatory processes, with experimental models devoid of functional PARP-1 alleviating expression of a host of proinflammatory mediators, including cytokines, chemokines, adhesion molecules and enzymes (such as iNOS). It also reduced tissue infiltration of activated phagocytes in experimental models of inflammation, circulatory shock, and ischemia-reperfusion[255, 265].

These findings suggest that there may be a significant role for oxidative and nitrosative stress in the pathogenesis of TS, potentially via direct action on myocardial cells, through disruption of mitochondrial function and/or through activation and upregulation of inflammatory pathways.

1.2.9 Thioredoxin interacting protein (TXNIP)

TXNIP was initially characterised as a protein which variably bound to both thioredoxin-1 and -2, thus neutralising the antioxidant effects of these proteins. However, it has more recently emerged that TXNIP exerts thioredoxin-independent effects, including activation of inflammatory processes (via the NLPR3 inflammasome) and limiting insulin release (via a glucose-sensing component of the gene)[266, 267]. Although TXNIP expression is modulated by blood glucose levels, it may also be released in response to other stimuli, including disordered shear stress and hypoxia (for summary, see Chong *et al*, 2014[268]). It has also been shown that TXNIP and NO exert mutually reciprocally suppressive effects[269, 270].

Because glycocalyx shedding occurs in the acute phase of TS[96], with resultant development of disordered responses to shear stress[271], it would be expected that TXNIP expression might be increased in blood vessels and myocardium. However, this has not been previously evaluated. While there are no specific treatments to supress TXNIP expression, metformin and the L-type calcium channel blockers verapamil and diltiazem exert that effect.

1.2.9 Important residual issues

1.2.9.1 Precise molecular cause of medium-term disability and long-term mortality rates

In a recent cardiac MRI/MRS-based study which followed up patients who had previously suffered an acute attack of TS at least one year prior, there was found to be a persistent impairment of cardiac function[272]. Despite the fact that TS was previously thought to be relatively benign, it is becoming increasingly clear that this is not the case; patients in this study displayed impaired cardiac energetic status and reduced maximal oxygen consumption on exercise, as a result of significant cardiac limitation. The authors in fact concluded that TS resulted in patients developing a persistent, long-term heart failure phenotype. Another study has shown that GLS is impaired at three months post acute attack, and this is associated with not only raised NT-proBNP levels, but also an impairment of quality of life (as assessed by SF-36 questionnaire)[94].

Longer-term follow-up in a retrospective study of 56 patients admitted with TS showed that 10 patients died after discharge with a mean survival of 4.47 years, and these deaths were all due to non-cardiac causes[273]. Indeed, a number of authors have commented on a high prevalence of cancer in TS[274], an association which cannot purely reflect precipitation of TS by some anti-neoplastic drugs[275]. There is then an apparent dichotomy between the causes of medium-term disability (i.e. energetic impairment and reduced quality of life) and the long-term mortality associated with TS.

1.2.9.2 Mitochondrial function

Little is currently known about cardiac mitochondrial function in patients with TS, although there is evidence from both patient biopsies and animal models to suggest dysfunction. Biopsy results from TS patients in one study noted evidence for increased production of O_2^{-}[98], while other studies noted decreased myocardial glucose uptake[276, 277]. The finding of intracellular lipid accumulation in both human TS and animal models[224] is also relevant, as this can lead to superoxide formation and subsequent mitochondrial dysfunction[278, 279]. Other animal models have also noted mitochondrial damage and dysfunction[235], which could be the result of direct actions by high levels of catecholamines to stimulate mitochondrial respiration. In a study by Willis *et al* (2015), mitochondria isolated from rats 2-weeks post-isoprenaline displayed decreased oxidative metabolism, membrane fragility, and enhanced oxidative stress[230]. Superoxide is also known to be released in the degradation of unstable oxidised catecholamines[252]. In addition to oxidative stress, in this setting, there is potential for interactions between NO and O_2^{-} to form $ONOO^{-}$, resulting in nitrosative stress.

There is then potential for energetic impairment to be critically involved in both the initial and ongoing myocardial dysfunction associated with TS. Added to findings of energetic impairment and mitochondrial dysfunction discussed above, it has been shown that intense catecholamine stimulation results in depletion of high energy phosphates after ~5 minutes[280]. Furthermore, energetic impairment is implicated in the pathogenesis of several other forms of heart failure, where it has been proposed

to play a central role[281]. Further experiments are required to elucidate the biochemical pathways leading to, and the clinical significance of, energetic impairment in the pathogenesis of TS.

1.3 Scope of the present study

After consideration of the literature and identification of gaps therein, the major objectives of the experiments described in this thesis were:

1) To obtain immunohistological data to test the hypothesis that TS in humans is associated with the development of nitrosative stress, thus implying increased formation of $ONOO^-$ anion via interaction between tissue catecholamines and NO. This component of the study involved immunohistological investigation of a small group of patients dying early post-onset of TS.
2) To develop an animal model for the acute stages of TS, via injection of isoprenaline in female rats. The model was to be used to study the relationships between short-term impairment of left ventricular systolic function and inflammatory/pro-inflammatory changes in the myocardium.
3) Using this female rat model, to test the hypothesis that formation of the "energy sink" enzyme poly(ADP-Ribose) (PAR), via activation of PAR polymerase-1 (PARP-1) within myocardium contributes to development of contractile impairment.
4) To evaluate the contribution of (potentially supra-normal) intrinsic generation of nitric oxide, catalysed by nitric oxide synthases, to the development of TS in this model.

2 Methods

2.1 Rat model

The basis for our experiments was to develop and use an animal model that was able to closely mimic the demographics of human TS. To that end we eventually chose to study the effects of isoprenaline on the myocardium of ageing female rats. For all three sequential experiments ageing (4-5month old) female Sprague-Dawley rats (200-300g) were used. Initially, preliminary experiments were conducted with isoprenaline alone to assess the impact of age and dose of isoprenaline on tolerability, with results showing that in rats aged >8 months the mortality rates following injection of 5mg/kg isoprenaline were unacceptably high (>50%). Therefore, the age range of 4-5 months and a single dose of isoprenaline at 5mg/kg, given intraperitoneally, were eventually selected. Under these conditions, the short-term mortality (manifest generally between 0.5-2 hours) was approximately 35%, which is similar to that reported by a number of other investigators (see Appendix 1 for a review of other animal models of TS). Immediately post ISO administration the rats began to display a state of torpor, most prominent in the animals that perished.

The initial experimental protocol was as follows: Rats in treatment groups (non-control) received baseline echocardiography under isoflurane anaesthesia, followed by a single intraperitoneal injection of isoprenaline, with follow-up echocardiography performed 24 hours later. Animals were then sacrificed, and myocardium was harvested for immunohistological and immunoblotting analyses. Control rats were also subjected to serial echocardiography to maintain blinding, and were sacrificed after 24 hours, without exposure to isoprenaline, in order to provide immunohistochemical and immunoblotting controls.

In two subsequent series of experiments, we evaluated the impact of pre-treatment with either a) PARP-1 inhibitor 3-aminobenzamide (3AB: 50mg/kg intraperitoneal injection) administered 30 minutes prior to isoprenaline (ISO/3AB) or b) NOS inhibitor L-NG-Nitroarginine methyl ester (L-NAME: 50mg/kg intraperitoneal injection) administered 30 minutes prior to isoprenaline (ISO/L-NAME).

Comparisons of each of these treatment groups were made with rats treated with isoprenaline alone to assess any beneficial effects of these compounds. Subsets of rats were also treated with 3AB or L-NAME alone to assess any deleterious effects on cardiac function caused by the potential therapeutics without isoprenaline involvement.

Sample size calculations for the study had to await completion of echocardiography evaluations at baseline and are discussed in the statistics section (see section 5. Statistical methodology).

2.1.1 Echocardiography

2.1.1.1 Methods

Although control rat echocardiograms were not analysed, all animals underwent echocardiography (pre-treatment echoes were used as paired controls for 24hr follow-up in all of the treated animals). Prior to baseline echocardiography all animals were weighed and placed into individual cages. The common echocardiography procedure was as follows: Rats were anaesthetised using a nose cone at a dose of 2% isoflurane initially, reduced to 0.5% once asleep. In preparation for echocardiography animals were placed on their backs on a warming pad, their chests shaved, and three limb-leads attached for electrocardiogram recording. The echocardiography was performed using a Vivid 7 ultrasound system with a special small animal 10s 2298589 sector array probe (GE Ultrasound, Horten, Norway), with a frequency of 4.0 – 10.5MHz. The left ventricle was imaged in parasternal long axis view and three levels of the parasternal short axis view, allowing for complete imaging of ventricle and regional analysis. Animals were returned to individual cages post baseline echocardiography +/- treatment and monitored closely for the first 2 hours thereafter, then at 1-hour intervals for the next four hours.

2.1.1.2 Views acquired

Three pre-defined levels were focused on with imaging in the parasternal long axis view: (i) basal – measurements were performed at 3mm below the mitral annulus, (ii) mid-ventricle – measurements were recorded at the level of the papillary muscle and (iii) apical – measurements were performed 3mm from the left ventricular apex. Short axis views at the level of the apex with image depth set at approximately 2-2.5cm with a frame rate of 240-260 frames per second were recorded for radial strain measurement.

2.1.1.3 Analysis of images

2.1.1.3.1 Radial strain calculation

Echocardiographic images were transferred to an EchoPAC PC workstation with Q analysis software enabled (GE version 11.1.1), where data was analysed using a speckle-tracking algorithm. This validated software recognises the seemingly random "speckle" pattern of each individual section of myocardium, and as this pattern is relatively stable between individual frames, can track the change in size or shape of these sections over time. Peak radial strain was measured at the apex of the left ventricle, by selecting tracking regions and assessing for endocardial tracking quality. The apical radial strain value is an average of the six segments into which the left ventricular apex is divided when performing Q analysis. Blinded analysis to treatment status and phase of treatment was used at all times. Inter-observation coefficient of variability for measurement of apical strain was 13%. Figure 2.1 shows a representative image of an apical image being analysed using this technique, along with the associated results. Apical radial strain measurements were made in triplicate, with care taken to ensure quality tracking of the endocardium across all segments.

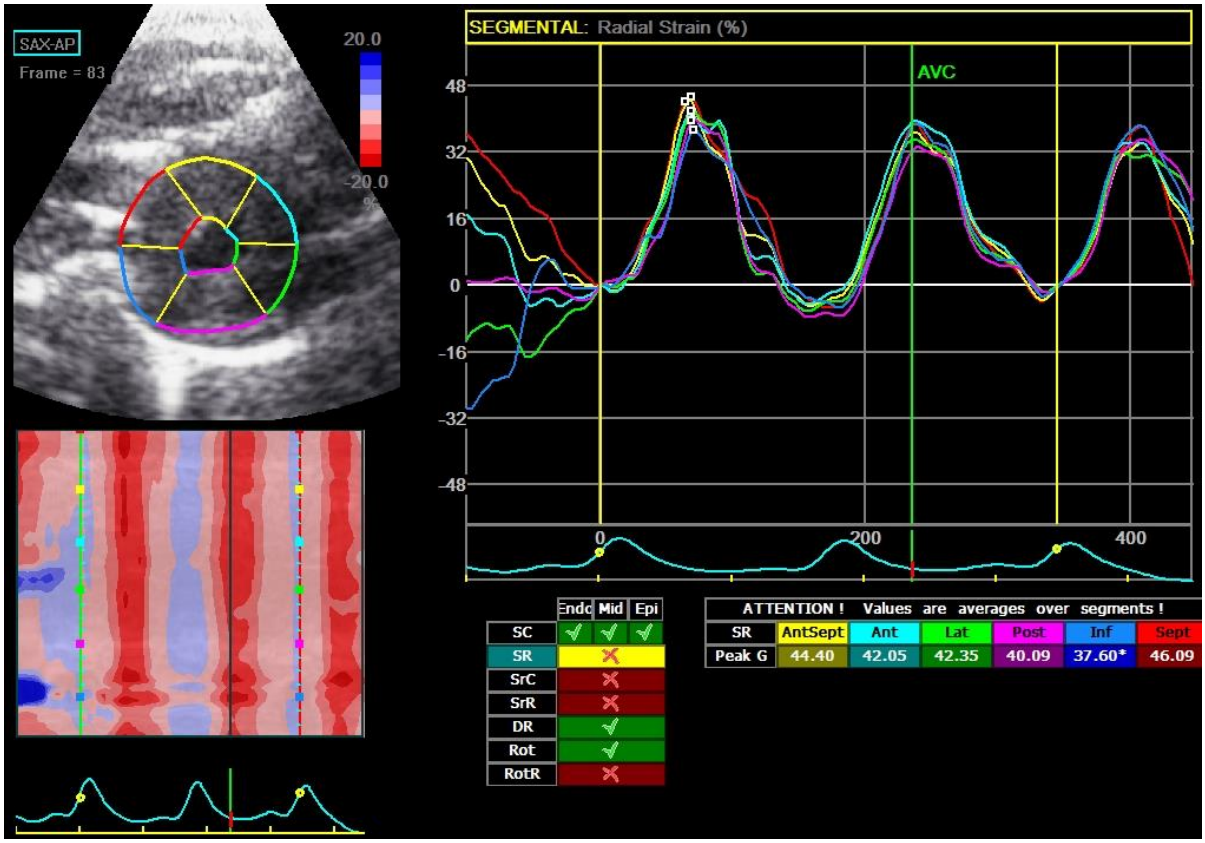


Figure 2.1. Representative image of apical radial strain analysis using the Q analysis software.

2.1.1.3.2 Fractional area shortening (FAS)

2D images of the LV in the parasternal view were measured at three predefined locations: - basal LV measurements were taken 3mm from the mitral annulus, mid-ventricular measurements were taken at the level of the papillary muscle and apical measurements were taken 3mm from the LV apex. FAS is calculated as the percentage change in LV cavity dimension, measured from end-diastole to end-systole. LV wall thickness at apical and basal level was also determined from these parasternal 2D images (both septal and posterior wall thickness were recorded, with mean apical wall thickness used in statistical analysis).

2.1.2 Preparation of rat myocardium

Following 24hr follow-up echocardiography all rats were sacrificed under anaesthesia, with care taken to ensure non-responsiveness to tail pinch, after which hearts and aorta were excised. Hearts were

initially divided into basal and apical left ventricular sections, followed by small tissue sections being removed from each side of the heart (apical/basal). One small section was embedded in optimal cutting temperature compound (OCT) to be frozen, while a second section was snap frozen in liquid nitrogen to be used for immunoblotting. The remaining heart tissue was fixed in 4% formaldehyde for paraffin embedding, to be used for immunohistochemical analyses. Thoracic aortic sections were excised and immediately placed into the organ bath for assessment of vascular reactivity.

2.2 Immunohistological methods

Myocardial content of the following was determined by immunohistochemistry:

a) 3- Nitrotyrosine (3-NT), a marker of nitrosative stress
b) Poly-ADP-ribosylated proteins (PAR), the principal product of PARP-1 activation
c) Thioredoxin interacting protein (TXNIP), a pro-inflammatory intracellular protein which is synthesized in response to disordered regional wall stress. Previous studies from our group have suggested that expression of TXNIP and of NO are reciprocally related[269].
d) CD68, as a marker of monocyte/macrophage infiltration
e) CD45, as a marker of leukocyte infiltration

2.2.1 Protocol

In all cases, the methodology was analogous, with variation only in the primary and secondary antibodies and dilution utilized. Thus, the sequence of quantitation involved the following steps:

4µm sections of tissue were cut and mounted onto Superfrost Ultra Plus slides (Menzel, Germany), then allowed to dry overnight in a 37˚C oven. Slides were deparaffinised, beginning with xylene, followed by 100% ethanol and then through decreasing concentrations of ethanol to finish in distilled water. The deparaffinising steps were xylene: 3 x 5min, 100% ethanol: 3 x 3min, 90% ethanol: 1 x 3min, 70% ethanol: 1 x 3min, 50% ethanol: 1 x 3min, 1x Phosphate buffered saline (PBS): 1 x 3min.

The next step involved antigen retrieval in citrate buffer. The buffer was preheated in a microwave for 9min before the slides were immersed and heated for 20min at 100˚C. Following antigen retrieval, slides were first placed in dH_2O, followed by PBS 2 x 5min. Excess PBS was removed and a wax circle was drawn around each section. Then slides were blocked for endogenous peroxidises using 3% H_2O_2 for 30min, then washed in PBS 3 x 5min. After again removing excess PBS sections underwent protein block using DAKO serum free protein block (DAKO, Agilent Technologies, USA) for 30min.

To measure the myocardial concentrations of different compounds, different primary antibodies were used for each stain, as follows – TXNIP (MBL Anti-TXNIP (VDUP1) mAb K0205-3): 1:500 dilution, 3-NT (Upstate #06-284): 1:500 dilution, PAR (Trevigen 4335-MC-100-AC): 1:100 dilution, CD68 (ED1, Santa Cruz sc-59103): 1:50 dilution and CD45 (OX30, Santa Cruz sc-53047): 1:25 dilution. All primary antibodies were diluted in DAKO antibody dilulent solution (DAKO, Agilent Technologies, USA). Protein block was removed but slides were not washed, then 100µL of primary antibody was applied, then sections were left to incubate overnight at 4˚C.

The following day, sections were washed four times in DAKO wash buffer (1x) for 5min each, followed by one 5min wash in PBS, before secondary antibody application. As for primary antibodies, the secondary antibodies differed slightly for different stains, as follows – for 3NT the premade DAKO EnVision+ System- HRP Labelled Polymer Anti-Rabbit (k4003) (DAKO, Agilent Technologies, USA), for TXNIP, PAR, CD68 and CD45 Santa Cruz goat anti-mouse IgG-HRP (sc-2005): 1:100 dilution in PBS (Santa Cruz Biotechnology, USA). 100µL was applied to sections, which were then left to incubate for 40min at room temperature. Sections were then washed four times in DAKO wash buffer (1x) for 5min each, followed by one 5min wash in PBS before visualisation.

For visualisation, liquid DAB+ Substrate Chromogen System (DAKO k3467, Agilent Technologies, USA) was applied to each section before being placed in distilled water to finish reaction. Different visualisation times were employed for different stains, from 10sec for 3NT, TXNIP and PAR, to 1min for CD45 and CD68, depending on rates of stain development. Slides were incubated in Harris haematoxylin for 10 seconds and then washed in dH_2O for 1 min, followed by three dips in Scott's tap water for counterstaining and again washed in dH_2O for 1 min. Slides were then dehydrated, following

a reverse order of the deparaffinising steps – 90% ethanol: 1 x 3 min, 100% ethanol: 2 x 3 min, xylene: 2 x 5min. Coverslips were applied, with care taken to remove any air bubbles trapped between the glass, followed by air drying and subsequent imaging.

2.2.2 Imaging

For imaging, the Nanozoomer (Hamamatsu Photonics K.K., Japan) system was used, with all slides required to be cleaned of excess adhesive prior to imaging. The imaging process is analogous for all slides, with an overview image initially taken of each slide, which allows selection of desired imaging area and focus points/positioning. The process of Z-stacking was also used to take multiple images at slightly varying depths (0.5micron intervals), which provided a more uniformly in-focus image. Between 8 and 12 focus points are manually selected for each section, depending on the size of the section and the area of interest. The Nanozoomer system takes the information provided during the set-up process based on the overview image and creates high-resolution images of each section, allowing for accurate analysis.

2.2.3 Analysis

All analyses were performed by investigators blinded as to rat treatment group. Reproducibility of estimates was evaluated via determination of coefficient of variability. The images generated by Nanozoomer were processed utilising Aperio ImageScope v12.3 (Leica Biosystems, Germany). Before using the software to determine the proportion of positive stain, anything that should not be part of the analysis (artefact/damaged tissue) was removed from the area of interest. The removal of these parts of the image creates a more accurate assessment of the amount of stain per total area. The algorithm provided with the ImageScope software (Positive Pixel Count v9) generates a measure of the proportion of the area of interest occupied by the stain and presents it as a percentage of the total number of pixels in the area of interest.

2.3 Immunoblotting studies

Given the central importance of the 3-NT data to the experiment, a supplementary assay was performed utilising immunoblotting (with blots in triplicate). Apical and basal sections of rat hearts which had been snap frozen were ground to a fine powder under liquid nitrogen and homogenized in 1x lysis buffer that contained protease and phosphatase inhibitor cocktails. The protein concentrations of supernatant were quantified in triplicate using the DC protein assay (BioRad), based on modifications of the Lowry method. Samples were then prepared in Laemmli sample buffer for separation at 30 µg protein/sample. These proteins were separated by using a 7.6% SDS polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Sigma).

Blots were blocked with 5% nonfat milk in TBS-0.01% Tween at room temperature for two hours and then probed overnight at 4^0C using primary monoclonal antibodies 3NT (Abcam, Cambridge, UK, ab61392 1:4000). The same blot was washed and re-probed for βactin (Abcam, Cambridge, UK, ab8226 1:5000) to determine equal sample loading and then incubated with a secondary HRP-conjugated anti-mouse antibody (Santa Cruz Biotechnology, 1:5000). The bands were visualised utilising ImageQuant LAS 4000 (GE Healthcare Life Science). Band intensities were quantified using Multi Gauge Software. Effects on expression of two bands of 3-NT relative to β-actin were quantified for all experiments, with Figure 2.2 illustrating the multiple bands of 3-NT noted.

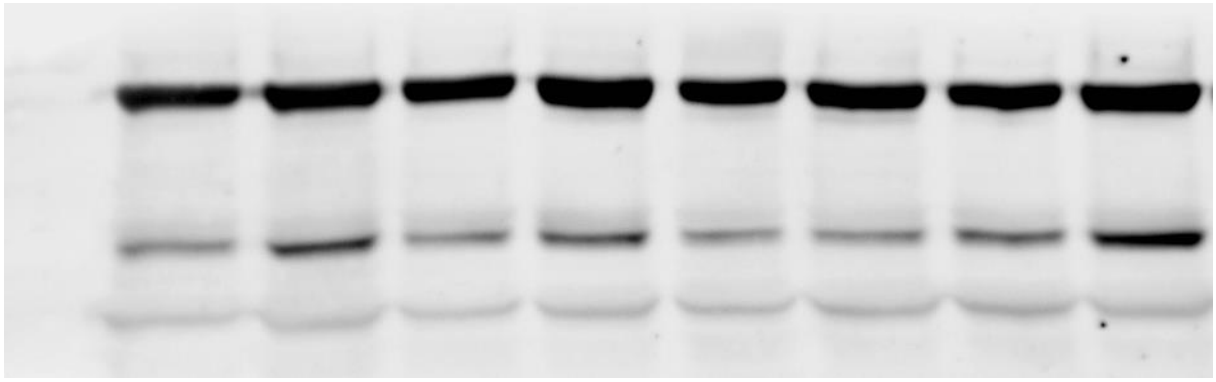


Figure 2.2. Sample Western blot for 3-NT with two distinct molecular weights, compared with β-actin.

For subsequent experiments evaluating pre-treatment with 3AB or L-NAME, immunoblotting was also performed to compare expression of PARP-1 and of its product PAR in hearts 24 hours post ISO treatment. The methodology was as for 3-NT procedure, but utilising the primary antibodies PARP (Cell Signaling Technologies, #9542: 1:1000 dilution) and PAR (Trevigen #4335-MC-100-AC: 1:800 dilution). The ratio of PAR generation to PARP-1 expression was utilised as a measure of PARP-1 activity.

To further evaluate inflammatory activation in this model, Phosphorylated and Total NFκB were also investigated. The methodology was as for 3-NT procedure, but utilising the primary antibodies (Phosphorylated NFκB – Cell Signalling Technology, #3033 1:2000, Total NFκB – Cell Signalling Technology, #8242 1:1000), a dilution of 1:2000 for β-actin and 1:5000 for the secondary antibody (Santa Cruz Biotechnology, anti-rabbit). For a more accurate assessment, the ratio of phosphorylated NFκB to total NFκB was used as a marker of NFκB activation.

Finally, immunoblotting was also used to assess expression of two NOS isoforms. Methodology was analogous to the other experiments above, with different antibodies: - eNOS (BD610297; 1:2500 dilution) , secondary anti-mouse antibody (Santa Cruz Biotechnology, 1:5000), iNOS (Abcam, Cambridge, UK, ab95441 1:500); secondary anti-rabbit antibody (Santa Cruz Biotechnology, 1:5000).

2.4 Organ bath

Aortic rings (3mm) were suspended (under two grams tension) in organ baths (15ml) containing Krebs solution at 37 degrees Celsius & gassed with carbogen (95% oxygen 5% carbon dioxide). Isometric tension was recorded via two wires through the lumen, one of which was connected to a force transducer and the other fixed. Arteries were allowed to equilibrate for 60 minutes & then contracted with potassium physiological saline solution (KPSS) initially, to assess viability, and then twice more (at 30-minute intervals) to establish a stable response. Cumulative application of noradrenaline (NA; 0.001-30 μMolar) to aortic rings was used to determine contractility, followed by washout (90 minute). This dose-response to NA was used to set up submaximal constriction in the aortic rings following

washout, after which vasorelaxant effects of cumulative application of acetylcholine (ACH; 0.001-10 μMolar) were determined.

2.5 Statistical methodology

Echocardiographic parameters were assessed via paired comparison of baseline and 24-hour echocardiograms, with evaluation being performed while blinded as to experimental sequence. For the initial isoprenaline vs control experiment comparisons of echocardiographic parameters were undertaken using paired t-tests for normally distributed parameters (assessment of echocardiographic changes in ISO rats were compared between baseline (pre-isoprenaline) and 24hrs post-isoprenaline administration). D'Agostino & Pearson omnibus normality tests were performed to ensure normality of the data.

Evaluation of differences in content and regional distribution (apical vs. basal myocardium) of 3-NT, PAR and TXNIP from immunoblotting studies was performed utilising 2-way ANOVA. Correlations between left ventricular apical radial strain and extent of accumulation of apical 3-NT and TXNIP within myocardium were sought utilising Spearman's correlation, as was correlation between apical 3-NT and TXNIP, and 3-NT and PAR content.

For evaluation in subsequent experiments of the impact of 3AB and/or L-NAME on ISO-induced changes, all statistical comparisons (unpaired) were between subsequently treated ISO and ISO/3AB or ISO and ISO/L-NAME treated rats. The effects of 3AB or L-NAME were evaluated utilising 2-way ANOVA.

Differences in expression of 3-NT, PAR, TXNIP, PARP-1, iNOS and eNOS were assessed using immunoblotting studies and these data were analysed via 2-way ANOVA.

Analysis of mortality between ISO and other treatment groups was performed using Fisher's exact test.

Sigmoidal dose response curves were created for vascular reactivity with both NA-mediated contraction and ACh-mediated relaxation.

All data are expressed as mean ± SEM unless otherwise stated.

2.6 Human post-mortem study

TS patients were selected from those admitted to two Adelaide tertiary referred hospitals over a 3-year period (2012-2014). The clinical diagnosis of TS in these cases was based on typical regional wall motion abnormalities at ventriculography, with exclusion of relevant coronary disease in the cases studied. Following patients' demise, permission was sought from their relatives for performance of a limited autopsy involving the heart. The protocol was approved by the relevant Ethics of Human Research Committee.

At autopsy, apart from myocardial biopsies of apical and basal left ventricle being taken for further histological and immunohistological study, there was exclusion of coronary artery occlusion and histological evidence of acute myocardial infarction. Control patients were age-matched individuals who had died of non-cardiac causes over the preceding 3 years. Biopsies of control patients were processed identically to those for TS patients.

Myocardial content of the following was determined by immunohistochemistry:

a) 3-NT, as a marker of nitrosative stress
b) PAR, representing the principal product of PARP-1 action
c) TXNIP, a pro-inflammatory intracellular protein

Methods for immunohistological assessment were analogous to those used with the rat myocardial tissue, as stated earlier in this chapter (see Chapter 2.1), differing only in the primary antibody used: - TXNIP primary antibody (MBL Anti-TXNIP (VDUP1) mAb K0205-3) at a dilution of 1:50 in PBS, 3-NT primary antibody (ABCAM #AB61392) at a dilution of 1:100 in PBS and PAR primary antibody (Trevigen 4335-MC-100-AC) at a dilution of 1:100 in PBS.

3 Inflammatory Activation and Nitrosative Stress in TS: A Human Post-Mortem Pilot Study

Abstract

Introduction: Takotsubo Syndrome (TS) remains a poorly understood, and likely underdiagnosed, clinical entity, despite increased recognition and research interest in recent years. TS is associated with myocardial inflammation, prolonged LV dysfunction and severe clinical complications, including hypotensive shock and death. Catecholamine release has been implicated in the pathogenesis of TS, along with alterations in nitric oxide signalling and myocardial energetics. In this short chapter we compared post-mortem myocardial samples of patients dying from TS with patients dying of non-cardiac conditions, with the aim of better understanding the biochemical pathways underlying the condition.

Methods: Over a five-year period, 237 patients presented with TS and were followed up. 5 of these died, and post-mortem myocardial tissue was salvaged for immunohistological analyses, utilising markers of nitrosative stress (3-nitrotyrosine (3-NT)), PARP-1 activation (poly(ADP-ribose) (PAR)), and inflammatory activation (thioredoxin interacting protein (TXNIP)). TS patient myocardial samples were compared to those from four control patients, who had died of non-cardiac causes. Apical and basal sections were compared to assess for regioselectivity.

Results: There was a significant increase in myocardial 3-NT content in patients dying of TS, compared to controls. There was no significant difference between these groups in PAR or TXNIP content.

Conclusions: This is the first evidence of increased nitrosative stress in the heart of patients dying from TS. Although this does not implicitly suggest a direct role for nitrosative stress in the pathogenesis of TS, it does correlate with clinical findings, such as inflammatory activation and disturbed nitric oxide signalling, which warrants further investigation.

3.1 Introduction

In general, while the pre-hospital mortality associated with TS is unknown, that which occurs during index admission after initial diagnosis is approximately 4%[124]. Most of these deaths are due to the development of shock, for which there is no universally agreed treatment. Occasionally, in patients with "secondary" TS, death reflects the concomitant extracardiac crisis, such as subarachnoid haemorrhage[282].

In our laboratory, we have recently documented that TS patients exhibit "supra-normal" nitric oxide (NO) signalling compared to controls, including unusually low plasma concentrations of the eNOS inhibitor asymmetric dimethylarginine (ADMA)[283]. Combined with evidence that β_2ARs are coupled to NOS[284], we sought to evaluate whether peroxynitrite ($ONOO^-$) generation might be a central component of inflammatory activation in TS. In particular, we were interested in the potential for $ONOO^-$ release to activate poly(ADP-ribose) polymerase 1 (PARP-1), with consequent energetic impairment[263]. PARP-1 activation, largely initiated as a response to $ONOO^-$-induced DNA damage, results in depletion of NAD, and therefore may represent a form of "energy sink" and a possible basis for these observations.

In order to evaluate changes in myocardial biochemistry associated with the acute phase of TS, most previous investigators have utilised myocardial biopsy[98, 100]. However, the safety of this technique in TS is incompletely established. We therefore decided to evaluate, wherever possible, the changes in the hearts of patients dying in the acute in-hospital phase of TS, and to utilise non-cardiac death in an ageing population of evaluation in "control" hearts. It was recognised that only a limited number of hearts from TS patients would be available, precluding the use of formal statistical comparison.

Our aims for this pilot study were as follows: -

1) To determine whether there was any evidence for activation of the peroxynitrite/PARP-1 cascade in severe TS.
2) To determine whether expression of TXNIP, a pro-inflammatory protein which is synthesised in response to hypoxia and non-laminar flow[285], is increased within the TS myocardium.

3.2 Methods

Over a 5-year period, 237 patients presented to our institution with TS, and were followed up, with an annual mortality amongst patients of approximately 2%. Thirteen patients died within 72 hours after the establishment of the diagnosis of TS; of these, five underwent autopsy and evaluation of myocardium with consent from the family. Amongst these patients, the majority presented as an apparent ACS and one presented as an out of hospital cardiac arrest. All patients underwent coronary angiography on presentation: - none had clinically relevant fixed coronary disease. Demographic data from the five TS patients, and of four control patients (three female, one male) of similar ages, who had died of non-cardiac causes, are listed in Table 3.1. There were no marked differences between patients and controls other than the occurrence of TS. In all cases, left ventricular biopsies were taken at the levels of apex, mid-ventricle and base, and were embedded in paraffin as for the rat ventricular biopsies for subsequent immunohistological study.

3.3 Results

Increased release of NO potentially induces formation of peroxynitrite ($ONOO^-$) anion, with associated redox stress, protein nitration[286] and downstream activation of the α-arrestin thioredoxin-interacting protein (TXNIP)[287].

Table 3.1. Patient characteristics

	TS (5)	Control (4)
Age (mean ± SD)	66 ± 14	72 ± 13
Female	5(100%)	3(75%)
Hypertension	3(60%)	2(50%)
Diabetes	1(20%)	0(0%)
ACE inhibitors	3(60%)	2(50%)
Beta blockers	0(0%)	1(25%)
GFR >60	2(40%)	3(75%)
Apical Variant TS	3(60%)	N/A

*Because of small numbers, no formal statistical analyses were performed

Conventional histology on patients with TS revealed the presence of neutrophil and macrophage infiltration in the myocardium as evidenced by the presence of positive staining for CD15 and CD168.

Three of the four TS patients in whom 3-NT was able to be assayed demonstrated myocardial 3-NT content greater than the range for controls, and in two cases, far greater (Figure 3.1). Elevated 3-NT content also tended to correspond to increased PAR levels, although here overall differences between patients and controls were marginal (Figure 3.2). There was no significant difference between apical and basal myocardium.

Myocardial TXNIP content was similar for TS patients and controls (32.1 ± 17.8% and 22.4 ± 7.4%). TXNIP content also did not differ between the apical and basal segments of patients with TS (Figure 3.3).

The results of this pilot human study therefore revealed an approximately 6-fold increase in 3-NT level in patients dying of TS compared to age-matched controls. However, analogous findings were not seen in results from assessment of plasma 3-NT concentrations in a separate study of 22 patients with TS and nine healthy controls, suggesting that there are no substantial systemic manifestations of nitrosative stress (see Figure 3.4).

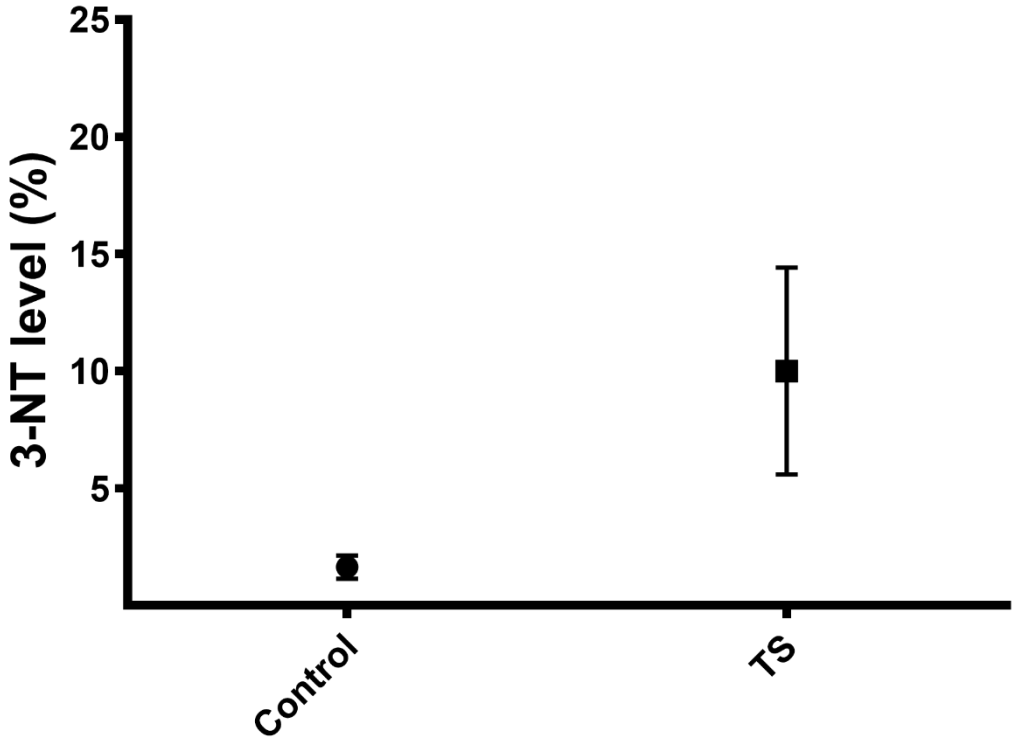


Figure 3.1. 3-NT level measured in the myocardium of five patients dying from Takotsubo Syndrome (TS) and four controls (Control). Error bars represent SEM.

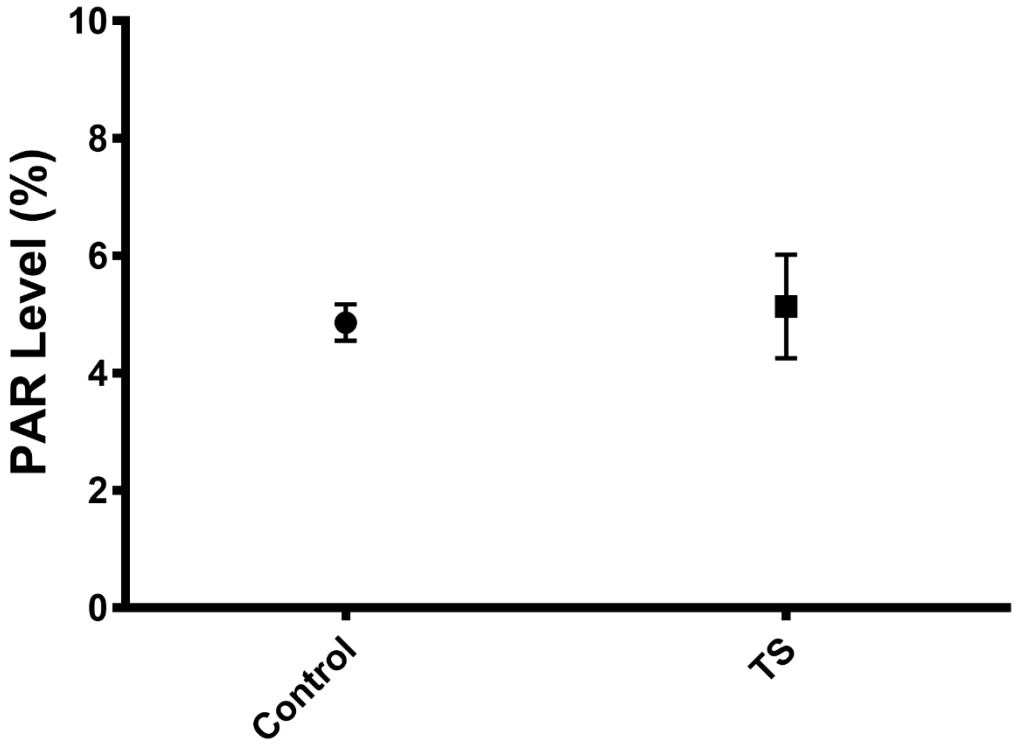


Figure 3.2. PAR level measured in the myocardium of five patients dying from Takotsubo Syndrome (TS) and four controls (Control). Error bars represent SEM.

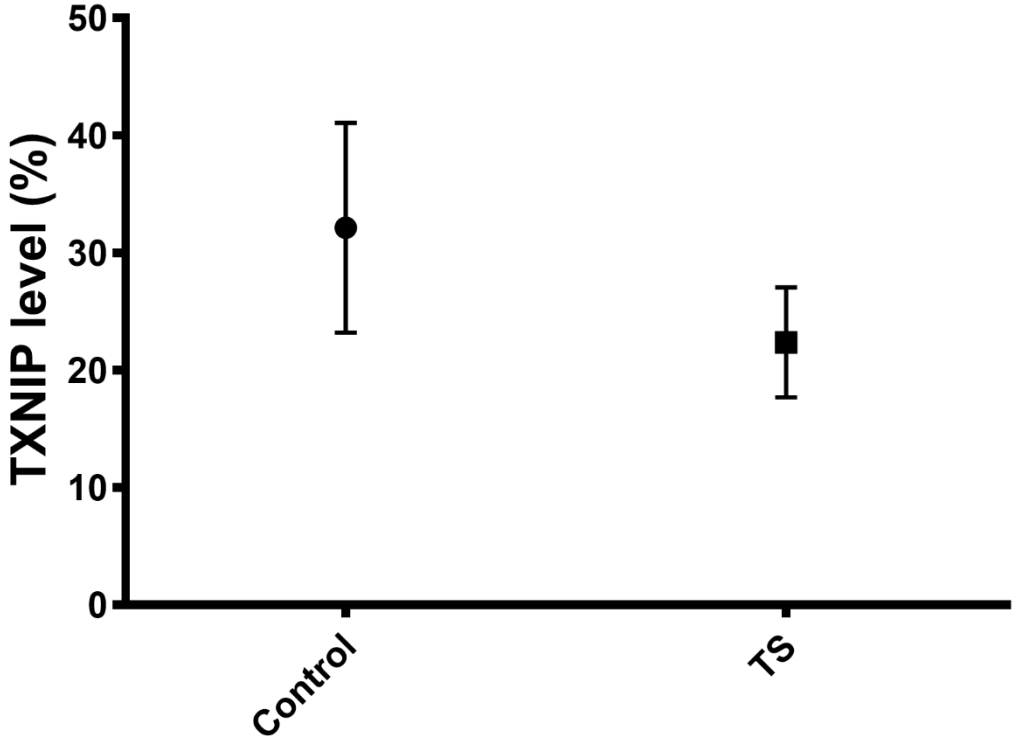


Figure 3.3. TXNIP level measured in the myocardium of five patients dying from Takotsubo Syndrome (TS) and four controls (Control). Error bars represent SEM.

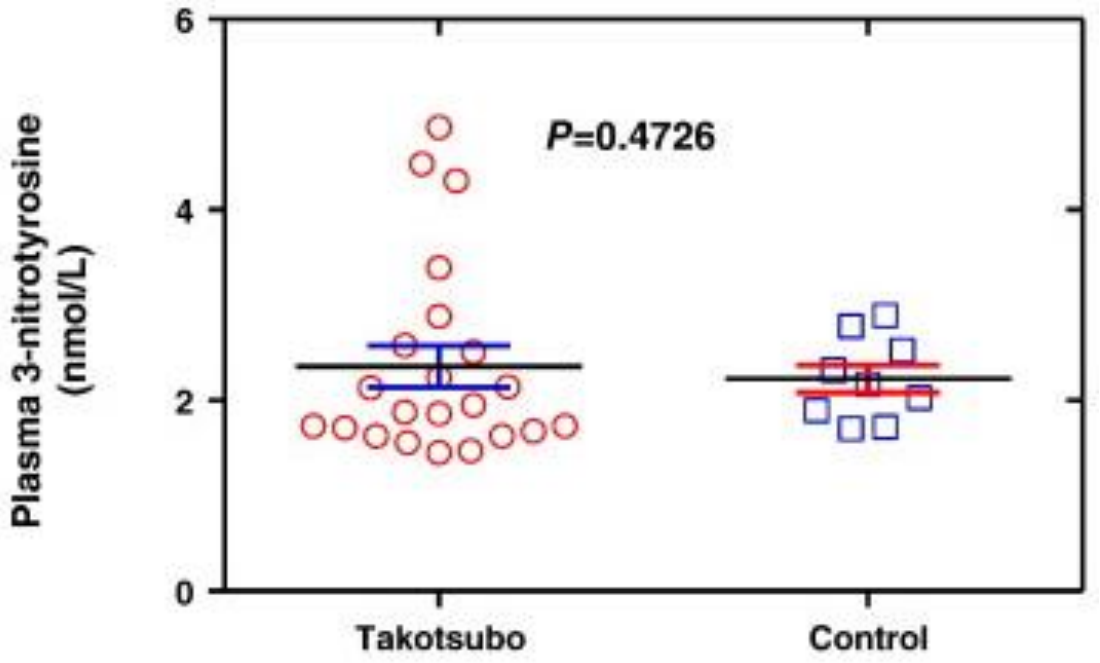


Figure 3.4. Concentration of 3-NT in plasma of 22 Takotsubo Syndrome patients (Takotsubo) and nine healthy subjects (Control). Modified and reprinted with permission from Kayacelebi *et al* (2013).

3.4 Discussion

This brief chapter summarises efforts from our laboratory to glean, in the patient context, evidence that TS is associated with increased nitrosative stress. We were therefore also interested in the possibility that $ONOO^-$-activated PARP-1 might be relevant to the genesis of negative inotropic changes.

The first phase of our experiments involved determination of 3-NT concentrations in plasma during the acute phase of TS, as published in collaboration with Prof D Tsikas' group[288]. The results of these experiments showed that there was virtually no evidence of excessive 3-NT formation (see Figure 3.4). We therefore initiated the current post-mortem histological study, in which we showed substantial elevation of tissue 3-NT content in patients dying of TS, as compared to those dying of non-cardiac causes.

These results need to be qualified: the patients studied were theoretically among the most severely affected within the spectrum of TS, and post-mortem study, not being immediate, may have been affected by biochemical changes after patient death.

Furthermore, myocardial PAR content was not elevated. This disconcerting finding is not totally unexpected, given that activation of PARP-1 tends to be transient[265]: there seems to be no easy/practical way to evaluate this in humans.

Finally, we evaluated TXNIP expression. TXNIP would offer a potential avenue for exerting a "multiplier effect" regarding inflammatory activation[289] and its expression is likely to increase with hypoxia and/or non-laminar flow[268]. The current findings suggest, however, that myocardial TXNIP generation is unlikely to be central in the pathogenesis of TS.

4 Establishment of a Novel TS Rat Model

Abstract

Introduction: Takotsubo Syndrome (TS) was first reported approximately 28 years ago and was thought to be a relatively rare and benign condition. In fact, as the condition is more widely recognised and reported, evidence has now shown that TS is associated with inflammatory activation and myocardial oedema, and persistent LV dysfunction for at least three months. Despite the growing interest in TS in recent years, there has only been minor progress in delineation of a pathogenic mechanism. To date, the most important advance has implicated a catecholamine "surge", activating β_2AR with biased post-receptor signalling, resulting in negative inotropy. With this in mind, we aimed to design a catecholamine-induced female rat model of TS that mimicked the characteristics seen in patients.

Methods: Female Sprague-Dawley rats, aged 4-5 months, received either no treatment (control, n=12) or 5mg/kg isoprenaline (ISO, n=20). Echocardiography was performed at baseline pre-treatment and 24 hours post treatment. Hearts and thoracic aortae were salvaged after 24-hour timepoint, for immunohistochemistry, immunoblotting and vascular reactivity studies. Immunohistochemical analyses were used to investigate myocardial accumulation of cellular inflammatory markers (CD45 and CD68), 3-nitrotyrosine (3-NT), poly(ADP-ribose) (PAR) and thioredoxin interacting protein (TXNIP). Immunoblotting analyses involved expression of 3-NT, PAR, TXNIP, NFκB, iNOS and eNOS. Thoracic aortae were assessed for reactivity to noradrenaline (contractility) and acetylcholine (relaxation).

Results: In this model, administration of a bolus dose of ISO was associated with an acute mortality rate of 35%. Among surviving rats, ISO resulted in significant impairment of apical LV function, as assessed by apical radial strain or fractional area shortening ($p < 0.0001$ for both, paired t-test). ISO was associated with a substantial increase in wall thickness ($p < 0.0001$, paired t-test), and intramyocardial accumulation of the monocyte/macrophage marker CD68 ($p = 0.023$, 2-way ANOVA).

There was also increased myocardial content of 3-NT ($p = 0.0003$), PAR ($p = 0.002$) and TXNIP ($p = 0.0001$) compared to control animals. One of the two bands noted during immunoblotting for 3-NT also displayed a significant increase in ISO-treated animals compared to controls ($p = 0.034$). ISO significantly impaired the relaxation response ACh in isolated aorta ($p = 0.001$).

Discussion: In this novel female catecholamine-induced rat model, administration of ISO is associated with apical LV dysfunction, inflammatory infiltration and nitrosative stress, correlating with and extending our previous findings from clinical and post-mortem investigations. These results suggest that this is a robust and reproducible animal model of TS, with scope for use in future therapeutic investigations.

4.1 Introduction

The results of our pilot human post-mortem studies suggested a link between nitrosative stress and TS, which warranted further investigation. TS is now known to be a condition associated with intense inflammatory activation and impairment of LV function, lasting significantly longer than originally suspected[272]. The most significant advance in delineation of the pathogenesis of TS came from Paur *et al* (2012), where researchers were able to induce TS in rats, and show that biased βAR signalling resulted in negative inotropy[222]. The overdose of epinephrine given to rats in that study resulted in a switch from βAR-Gs to -Gi-mediated signalling, in what is thought to be a cardioprotective mechanism. Other rat models of TS have also used different catecholamine exposure to initiate regional LV dysfunction, the hallmark characteristic of TS, such as isoprenaline[100, 225, 229].

In view of the predominant occurrence of TS in ageing women, we sought to develop an animal model specifically in female rats older than those used in most other rat models. This was achieved using single intraperitoneal (IP) injections of isoprenaline (ISO). The study was approved by the institutional Ethics Committee, and subsequently performed in female Sprague-Dawley rats.

Preliminary experiments were conducted to evaluate tolerability of ageing/aged female rats to a single dose of ISO: - it was found that in rats aged nine months mortality rates following intraperitoneal injection of 50mg/kg ISO (a dose previously used elsewhere[100, 225]) were unacceptably high (~80%). Therefore, all subsequent experiments were performed in animals aged four–five months, weighing between 230 and 280 grams, utilising a dose of 5mg/kg ISO. Under these conditions, short-term mortality (manifest generally between 0.5-2 hours) was approximately 35%.

4.2 Methods

4.2.1 Rat Model

As detailed in the Methods chapter (see Chapter 2), baseline echocardiography was followed by a single intraperitoneal injection of ISO for treated rats, with follow-up echocardiography performed 24hrs later. Animals were then sacrificed (see Methods), and myocardium was harvested for immunohistological and immunoblotting analyses. Control rats (n=12) were sacrificed after 24 hours, without exposure to ISO, in order to provide immunohistochemical and immunoblotting controls.

4.2.2 Statistics

All experiments were undertaken with measurements performed while blinded as to treatment allocation. The main hypothesis tested (in null form) was that ISO-treated rats would not differ from control animals.

4.3 Results

4.3.1 Mortality

Following injection of ISO alone (5mg/kg, IP), 11 out 31 rats died suddenly within two hours: data for the surviving 20 rats were analysed.

4.3.2 Echocardiographic findings

Overall appearance of the LV included a variable degree of apical hypokinesis and thickening of the ventricular walls at the apex. Echocardiographic changes 24 hours post ISO injection are summarised in Table 4.1. Specifically, there was approximately a 10% increase in heart rate, mean apical LV wall thickness increased (consistent with the presence of myocardial oedema) and apical radial strain

decreased markedly, with a smaller, yet significant, decrease in apical fractional shortening and ejection fraction.

Table 4.1: Echocardiographic parameters for ISO alone - Echocardiographic changes associated with IP administration of ISO (5mg/kg, n=20).

Parameter	**Baseline**	**24hrs**	**p value**
Heart Rate (bpm)	339.3 ± 6.1	376.1 ± 7.6	0.0008
Mean Apical Wall Thickness (mm)	15.8 ± 0.4	19.3 ± 0.5	<0.0001
Apical Radial Strain (%)	-39.5 ± 1.3	-25.3 ± 1.4	<0.0001
Apical Fractional Area Shortening (%)	49.6 ± 1.4	35.5 ± 1.6	<0.0001
Ejection Fraction (%)	86.2 ± 0.9	81.2 ± 1.5	0.001
All values displayed as mean ± SEM. Note that comparison is between pre-treatment status and 24 hours post ISO.			

4.3.3 Histology/immunohistochemistry

LV myocardium in ISO-treated rats was infiltrated predominantly with macrophages/monocytes (CD68+) rather than leukocytes (CD45+), consistent with activation of a cellular component of an inflammatory process (Figure 4.1). There was a significant increase in CD68+ cell staining in ISO-treated versus control rats ($p = 0.023$, 2-way ANOVA interaction), with a significant apex to base gradient in ISO-treated rats ($p = 0.0017$, Bonferroni's post-hoc test).

3-NT accumulated significantly, especially in apical myocardium ($p = 0.0003$ for treatment), as summarised in Figure 4.2A, visualised in Figure 4.3A. 3-NT content was found to be approximately 2.5-fold higher (18.7 ± 3.7% compared to 7.5 ± 1.3%) in the apical myocardium of ISO-treated animals when compared to controls. There was a significant apex to base gradient of 3-NT concentrations in ISO-treated rats (Bonferroni's post-hoc test, $p = 0.041$).

Given that protein nitration is often regarded as a "fingerprint" of peroxynitrite[290], we sought correlations between the extent of residual apical radial strain in ISO-treated rats and apical myocardial 3-NT content. The results, shown in Figure 4.4A, showed there was no significant correlation ($r = -0.368$, $p = 0.195$), however the trend observed is actually counterintuitive.

PAR content increased significantly ($p = 0.002$, summarised in Figure 4.2B and visualised in Figure 4.3B), although less markedly than 3-NT. For example, apical PAR content was 2.0 ± 0.1% in controls and 2.4 ± 0.1% in ISO-treated animals. PAR concentrations were significantly greater at apex than base in both ISO-treated and control rats (Bonferroni's post-hoc test, $p = 0.02$ and $p = 0.024$ respectively).

TXNIP content of myocardium in ISO rats increased approximately 2.5-fold relative to control ($p < 0.0001$, summarised in Figure 4.2C and visualised in Figure 4.3C), without a significant apex to base gradient. Apical content of TXNIP was 4.4 ± 1.0% in control, compared with 24.6 ± 4.0% in ISO-treated animals. Although this increase was not dissimilar to that seen with 3-NT, there was no significant correlation between 3-NT and TXNIP content in individual rats (Figure 4.4B, $r = 0.244$, $p = 0.45$). As with 3-NT, there was no significant correlation between apical radial strain at 24hrs post-ISO and apical TXNIP content (Figure 4.4C, $r = -0.116$, $p = 0.72$).

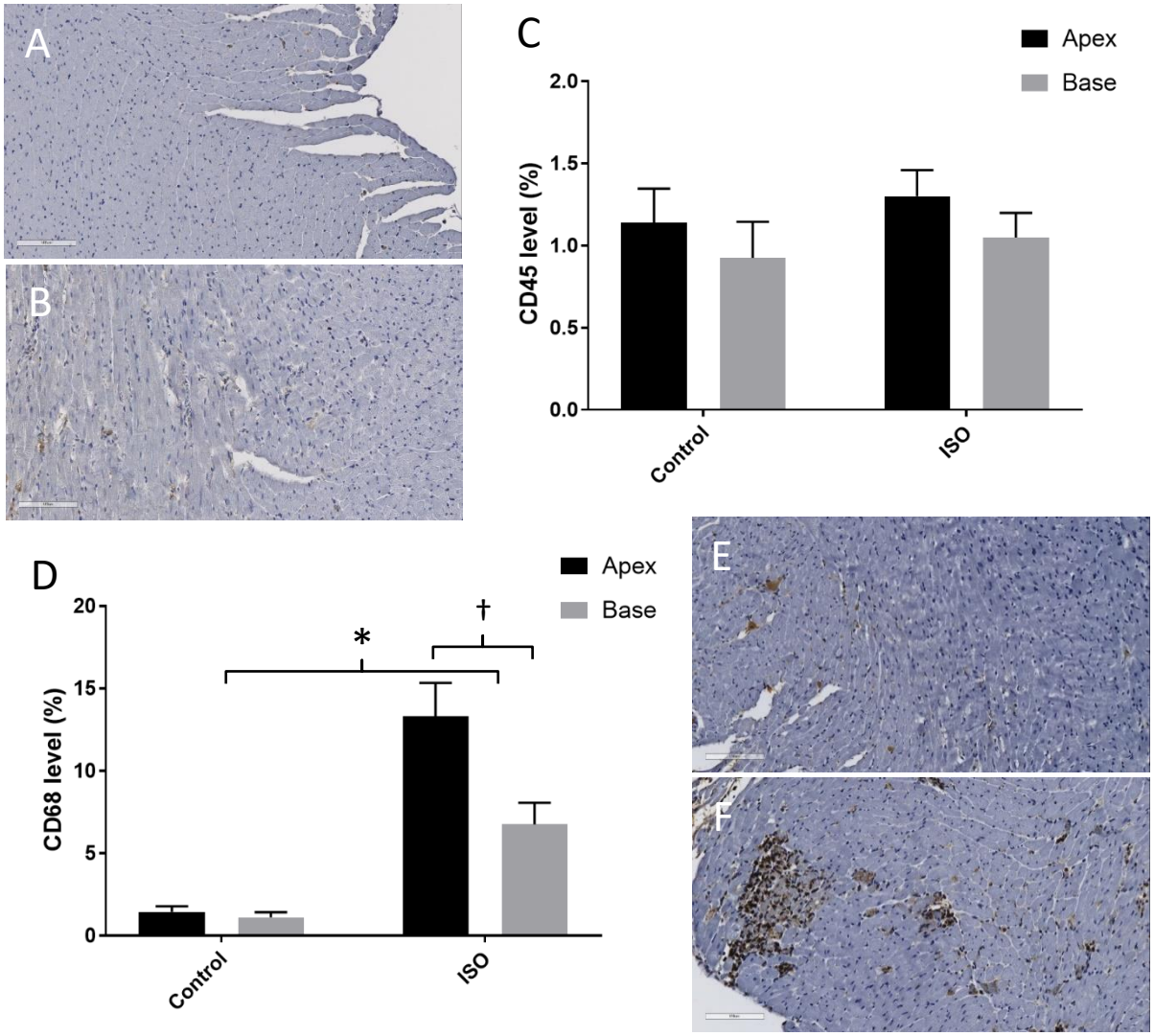


Figure 4.1. Effects of ISO on inflammatory markers - Leukocyte infiltration, as measured by CD45+ expression, is shown in control rat myocardium (A) and ISO-treated myocardium (B), with summary data (C). Macrophage infiltration, measured with CD68+ expression, is shown in control rat myocardium (E) and ISO-treated myocardium (F), with summary data (D). ISO n=10 and control n=9 were analysed, with apex and base for each, black/dark brown nuclei represents positive staining. * indicates $p < 0.05$, 2-way ANOVA. † indicates $p < 0.05$, Bonferroni's post-hoc test.

ANOVA data:		**F**	**p**
C:	ISO	1.53	0.23
	Apex / base	0.59	0.45
	Interaction	0.01	0..92
D:	ISO	6.96	0..013
	Apex / base	44.99	<0.0001
	Interaction	5.68	0..023

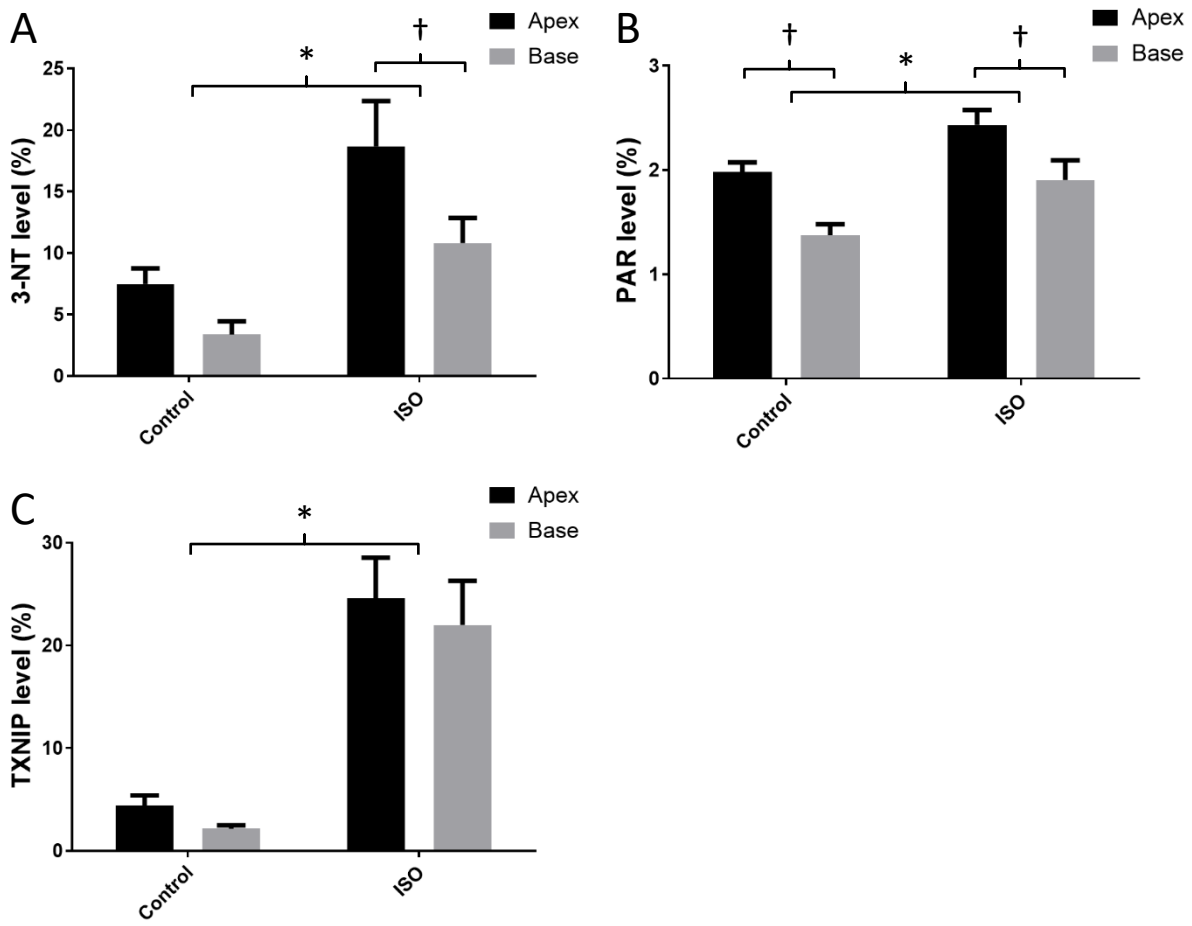


Figure 4.2. Immunohistochemistry data for (A) 3-NT, (B) PAR and (C) TXNIP concentrations in the heart of ISO-treated rats compared with control animals. * indicates $p < 0.05$, 2-Way ANOVA (treatment), † indicates $p < 0.05$, Bonferroni's post-hoc test.

ANOVA	**data:**	**F**	**p**
A:	ISO	14.95	0.0003
	Apex / base	6.16	0.17
	Interaction	0.61	0.44
B:	ISO	10.27	0.002
	Apex / base	13.77	0.0005
	Interaction	0.072	0.79
C:	ISO	45.5	<0.0001
	Apex / base	0.66	0.42
	Interaction	0.003	0.96

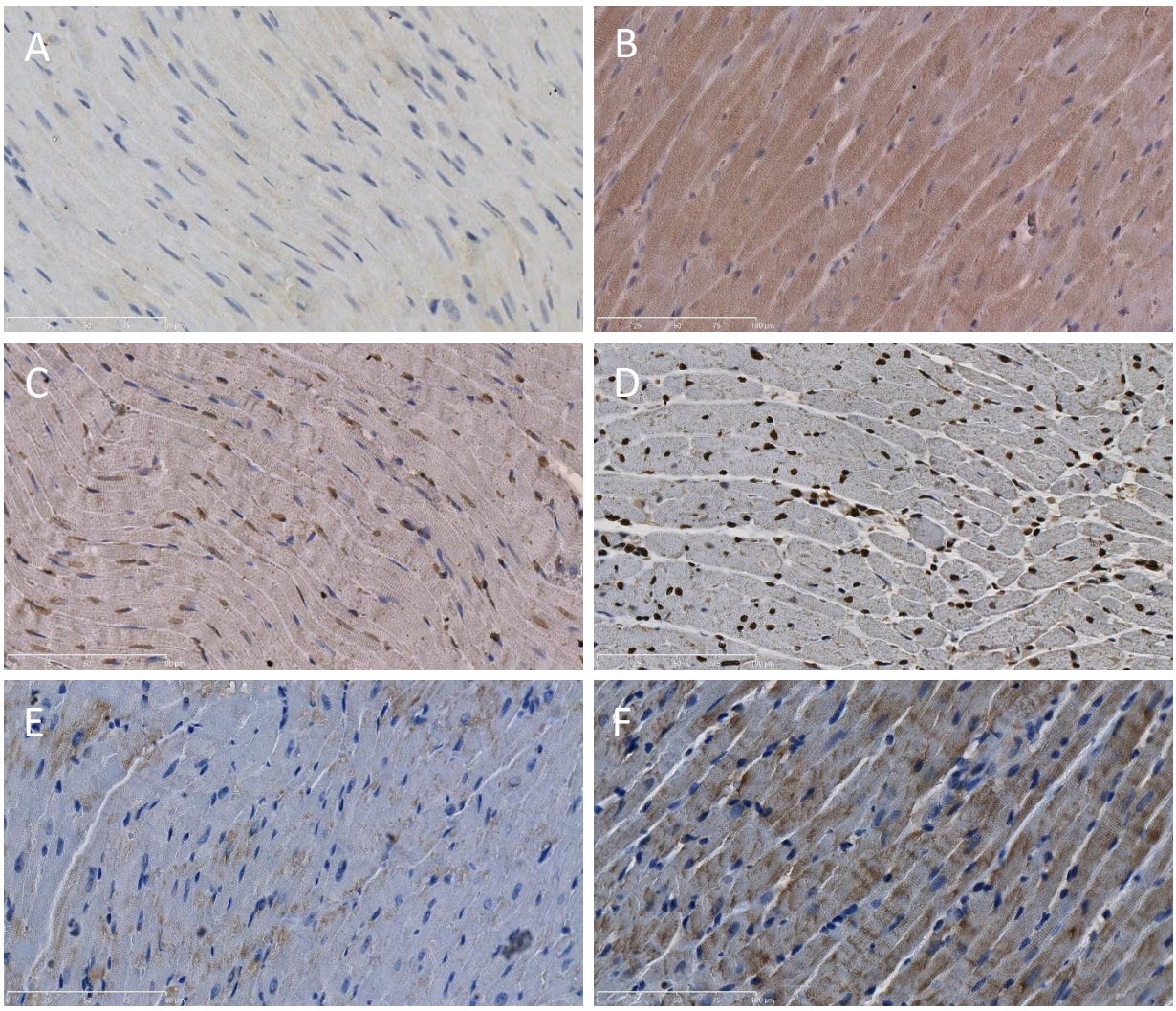


Figure 4.3. Effects of ISO on 3-NT, PAR and TXNIP expression in the myocardium. 3-NT accumulation is shown by brown staining in (A) control rat myocardium and (B) ISO-treated myocardium. PAR accumulation is shown by darkly stained nuclei in (C) control rat myocardium and (D) ISO-treated myocardium. TXNIP accumulation is shown by brown staining in (E) control rat myocardium and (F) ISO-treated myocardium. All images are from apical samples.

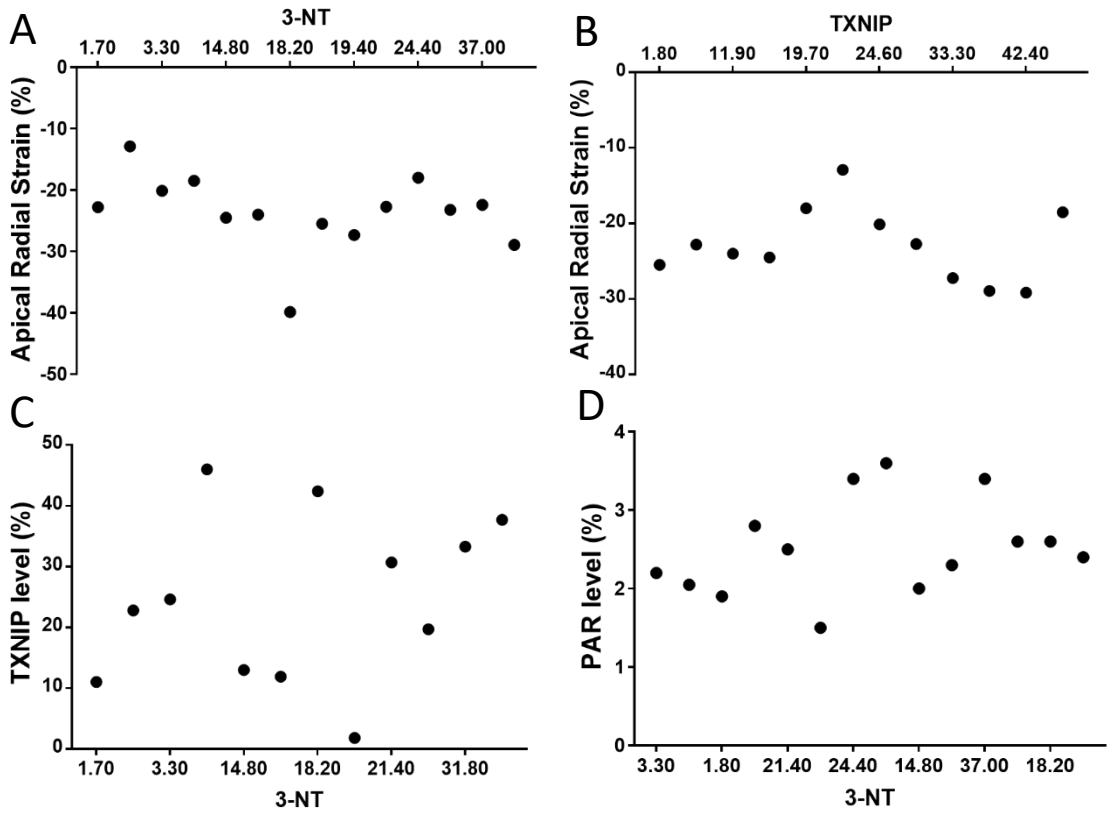


Figure 4.4. Correlations between apical radial strain at 24 hours post-ISO and (A) 3-NT and (B) TXNIP. Correlation between apical 3-NT and apical TXNIP content and apical PAR content are shown in (C) and (D) respectively. There were no statistically significant correlations for any of these data sets.

4.3.4 Immunoblotting

3-NT immunoblotting consistently generated two bands of molecular weight 47 and 70kDa respectively (see Figure 4.5), as shown in previous studies[291]. The differences between ISO and control were quantitated regarding each of the individual bands, rather than averaging data. These revealed (via 2-way ANOVA) a significant increase in the 47 kDa band ($p = 0.034$) (Figure 4.5A) but no significant increase in the 70 kDa band ($p > 0.05$) (Figure 4.5B) with ISO treatment, without obvious regional variability.

There were no significant differences noted in any of the other parameters investigated using the Western blot technique, which included expression of: - PAR, TXNIP, NFκB (ratio of phosphorylated: total NFκB was used a measure of NFκB activity), iNOS, eNOS and PARP.

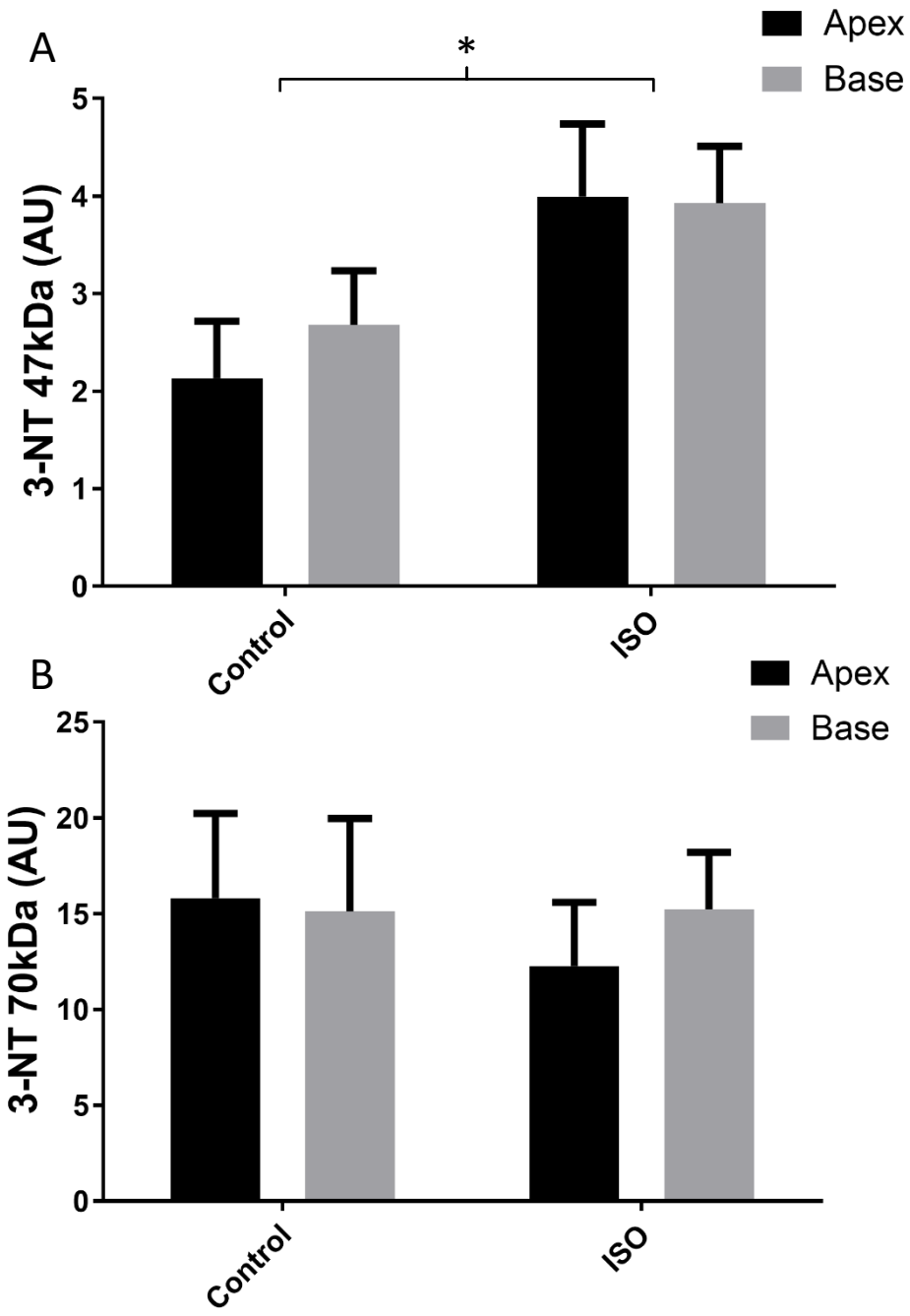


Figure 4.5. Graphical representation of Western Blot data for ISO vs control rats, at two distinct bands (A) 47kDa and (B) 70kDa. * = $p<0.05$, via 2-way ANOVA.

4.3.5 Vascular reactivity

Evaluation of contractile responses to noradrenaline 24 hours post ISO revealed no significant changes in either maximal response (Emax) or in concentration associated with 50% of maximal effect (EC50) [see Figure 4.6].

However, post ISO there was substantial and significant increase in the EC50 (logM) for acetylcholine (ACh) ($p = 0.001$, Figure 4.7), consistent with attenuation of the NO-mediated vasodilator effects of ACh.

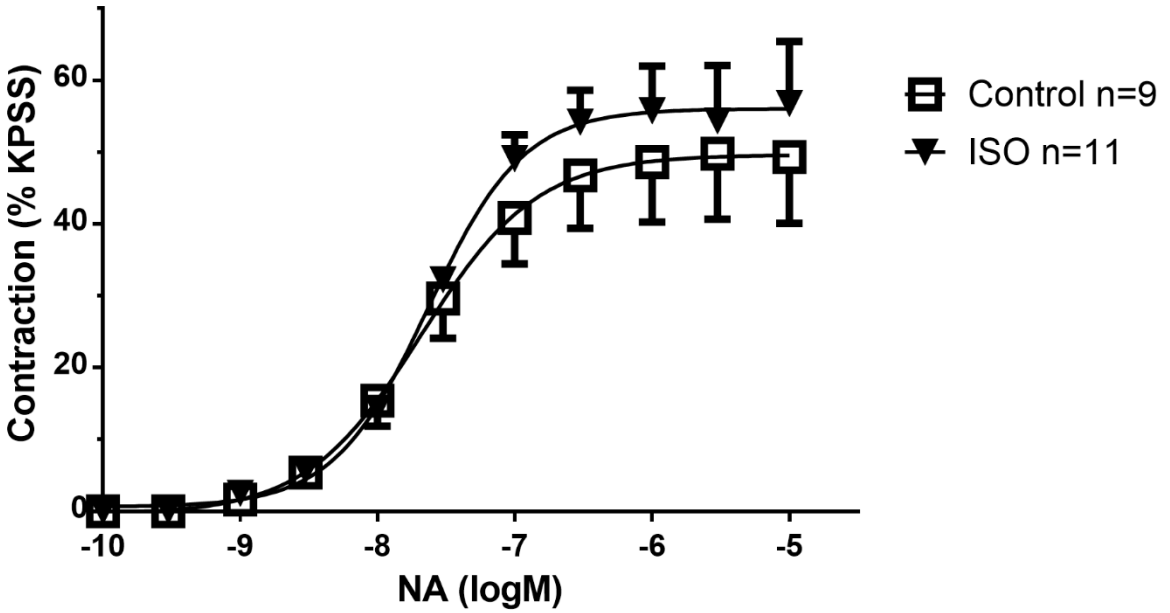


Figure 4.6. Contractile concentration-response curves for isolated abdominal aortic rings from ISO and control rats in response to noradrenaline (NA). There were no significant differences between treatment groups in this respect.

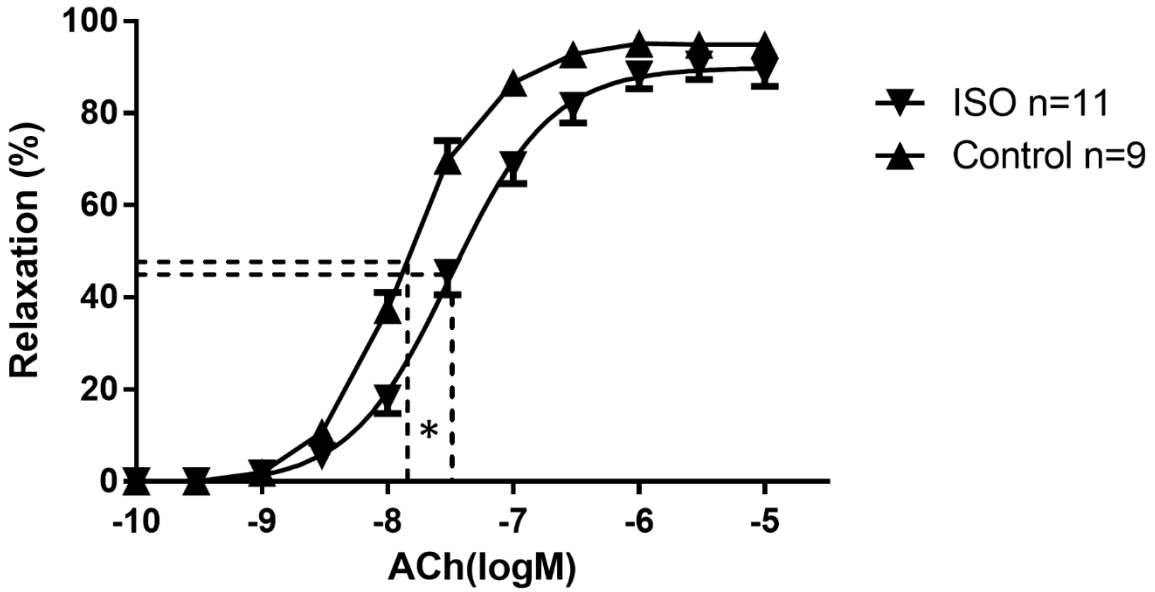


Figure 4.7. Relaxation concentration-response curves for isolated abdominal aortic rings from ISO-treated and control rats. Vessels were contracted with NA and relaxation was induced in response to acetylcholine (ACh). *EC50 (logM) for control = -7.85 ± 0.05, EC50 (logM) for ISO = -7.48 ± 0.07.

4.4 Discussion

The current experiments were conducted to establish a new model of TS in female rats, with the purpose of evaluating the possible occurrence, and potential pathophysiological impact of catecholamine-stimulated nitrosative stress in an animal model of TS. We found that in female rats, 24 hours post injection with ISO, there was significant evidence of TS-like reductions in LV function[225] as measured by echocardiography, along with an increase in LV wall thickness. Furthermore, these functional alterations were associated with intramyocardial accumulation of 3-NT, a conventional marker of nitrosative stress[286], mirroring results from our pilot post-mortem study (discussed in Chapter 3). There was also myocardial accumulation of PAR, a product of PARP-1 activation and thus a marker and mediator of myocardial energetic impairment[292], through its actions as an "energy sink"[263]. Myocardial TXNIP content was also significantly increased in the hearts of ISO treated rats, consistent with increased stimuli for its expression, such as withdrawal of NO effect[270] and/or disruption of laminar flow and associated shear stress[268], a process known to be precipitated by glycocalyx shedding, a recently delineated component of TS[96].

This model also demonstrated the presence of inflammatory infiltrate, with a substantial increase in CD68+ cells and no significant difference in CD45+ cell content, and thus representing predominantly macrophage/monocyte infiltration. This is relevant to the association between TS and glycocalyx shedding, as an atherosclerotic mouse model[293] has shown that macrophage/monocyte infiltration may result primarily from glycocalyx shedding, which itself is engendered largely by matrix metalloproteinase (MMP) activation. The pathophysiological role of glycocalyx shedding and the basis for inflammatory infiltration of the myocardium, along with their respective contributions to myocardial cell damage are yet to be defined, although these findings suggest a significant role in the pathogenesis of TS in this model. An important issue arising from these findings is the potential time of onset of macrophage/monocyte infiltration in TS, and the role which this infiltration plays in the pathogenesis of the condition.

As regards nitrosative stress in this model, previous work in our laboratory utilising venesection from patients, shown TS to be associated with reduced levels of ADMA and increased platelet responsiveness to NO[283]. These findings, taken together with the fact that catecholamines activate myocardial β_2AR, which are linked to NOS[294], suggests that there is increased production/availability of NO. It has previously been shown that β_2AR density relative to that of β_1ARs is greatest in the LV apical myocardium[222] and in the rat ventricle, β-adrenoceptor-stimulated release of NO increases with senescence[295]. Indeed, in the current experiments to establish this model, even in control rat hearts there was a trend for apical 3-NT content to exceed that of basal. In the presence of O_2^-, the current results imply increased formation of $ONOO^-$, and associated development of nitrosative stress. This propensity for increased NO generation to induce nitrosative stress in the presence of oxidative stress has previously been shown, in both models of central neuronal injury[296] and anthracycline-induced cardiac injury[297]. These results thus establish the occurrence of nitrosative stress as a potentially central mechanism for apical hypocontractility in TS. On the other hand, there was no tendency for myocardial 3-NT content to predict the extent of contractile impairment (see Figure 4.4). These results could have two possible explanations: (i) peroxynitrite, while activating PARP-1, also exerts other physiological effects on the heart, some of these with positive inotropic implications, or, (ii) there are additional, non-$ONOO^-$-mediated, mechanisms of negative inotropy.

Evaluation of changes in vascular reactivity of isolated abdominal aortic rings 24 hours post ISO revealed attenuation of vasodilator responsiveness to acetylcholine (ACh) without any changes in constrictor responses to noradrenaline. These results imply the development of "endothelial dysfunction" post ISO, despite previous evaluations in patients showing enhanced anti-aggregatory responses to the NO donor SNP[283].

These results are consistent with our recent finding that TS is associated with glycocalyx shedding[96], a process which denudes the vascular endothelium and is associated with disturbances of physiological responses to changes in wall stress[22]. Such changes may include decreased release of NO from the endothelium, as well as increased TXNIP generation[268], which was observed within the myocardium in

these investigations. In turn, these findings might explain the occasional reports (indeed, in the very first report of TS) of the presence of multivessel coronary artery spasm in association with TS[63, 298].

Finally, the presence of concomitant myocardial inflammation with associated nitrosative stress raises the issue of what is the "primary target" for the cardiac damage in TS. The presence of nitrosative stress raises the possibility that both myocardium and glycocalyx may be directly affected: peroxynitrite is a known precipitant of glycocalyx shedding[299]. As peroxynitrite is extensively diffusible[255], it is also possible that its generation within the myocardium (via biased β_2-AR/G_i protein-based signalling) may be the "primary event", with the glycocalyx affected via peroxynitrite diffusion.

Overall, this model of TS has been shown to be reproducible and to correlate well with what is seen clinically in patients with TS: - there is significant reduction in apical LV function expressed in multiple echocardiographic parameters, there is infiltration of inflammatory cells; and there is increased LV wall thickness, which may be associated with myocardial oedema, now known to be characteristic in TS. Significantly, there is also evidence of nitrosative stress, which we had previously discovered in our pilot human post-mortem study[300]. It must also be mentioned that previous analyses[288] had failed to show significant elevation of 3-NT concentrations in the plasma of TS patients. It is possible however that 3-NT is not markedly released from myocardium into peripheral blood. Furthermore, the evidence of elevated myocardial concentrations of PAR correlate with clinical findings of impaired energetic status in patients with TS[102]. The results of this study suggest that this model can be useful in not only the investigation into the pathogenesis of TS, but also in the search for effective treatment options, an area which is currently unexplored in the clinical context.

5 Investigating the Role of PARP-1 Activation in TS

Abstract

Introduction: Takotsubo Syndrome (TS) is catecholamine-induced, prolonged myocardial inflammatory disorder, occurring particularly in ageing females. TS is known to be associated with ONOO- formation and DNA damage, resulting in poly(ADP-ribose)-1 (PARP-1) activation and subsequent PAR production. This cascade potentially results in an "energy sink" through the usage of ADP in the production of PAR. Therefore, in this rat model of TS, we investigated the role PARP-1 inhibitor 3-aminobenzamide (3AB) in reducing catecholamine-induced LV dysfunction.

Methods: Methods were analogous to those used in previous studies with this female Sprague-Dawley rat model. Briefly:

(i) rats were divided into treatment groups – ISO alone (5mg/kg IP), ISO/3AB (5mg/kg ISO + 50mg/kg 3AB IP), 3AB alone (50mg/kg IP) and control.

(ii) Echocardiography was performed at baseline pre-treatment and 24hrs post treatment.

(iii) Hearts and thoracic aortae were salvaged after 24-hour timepoint, for immunohistochemistry, immunoblotting and vascular reactivity studies.

Results: Addition of 3AB resulted in a significant improvement in LV function, as measured by apical radial strain, at 24 hours post-ISO ($p = 0.0002$, 2-way ANOVA). Immunohistological analyses displayed decreased myocardial content of CD68+ cells (monocytes/macrophages, $p = 0.006$, 2-way ANOVA (treatment)) and increased accumulation of 3-NT, PAR and TXNIP (respectively $p = 0.047$, 0.002 and 0.007, 2-way ANOVA (treatment)). There was no effect on noradrenaline-induced vascular contractility, but 3AB did in part restore acetylcholine-induced vascular relaxation compared to ISO alone ($p = 0.027$).

Discussion: Myocardial inflammation in this model of TS was associated with nitrosative stress and PARP-1 activation. Inhibition of PARP-1 limited LV dysfunction post-ISO, without significant effect on mortality, suggesting a dichotomy in this model between inotropic status and survival.

5.1 Introduction

Takotsubo syndrome (TS) is a condition of disordered cardiac energetics[102] and persistent LV dysfunction, with associated impairment of quality of life for at least three months after initial attack[94]. Although inflammation and oedema have been associated with TS[92], there have as yet not been any investigations into the pathogenic mechanisms by which the inflammatory activation could arise.

We have shown in Chapter 4 that our novel female rat model of TS is able to reproduce some of the key clinical characteristics, namely regional LV dysfunction and inflammatory infiltration. The finding of significant $ONOO^-$ accumulation within the myocardium of ISO-treated rats provides evidence of nitrosative stress. As $ONOO^-$ is known to activated PARP-1[262], the increased accumulation of PAR found in these rats was not a surprising finding. The process of PAR production however, the primary product of PARP-1 activation, is one which can result in high energy phosphate depletion, potentially creating an "energy sink"[264]. To further investigate this, and any potential effects on cardiac function, a PARP-1 inhibitor was used in this next set of experiments.

5.2 Methods

5.2.1 Rat model

The ISO rat model described in the previous chapter, developed for use with female Sprague Dawley rats, was used in this subsequent series of experiments to compare and contrast the effects of ISO alone and ISO plus the PARP-1 inhibitor 3-aminobenzamide (3AB) in an animal model of TS. Rats were either administered 3AB (50mg/kg, IP) 30 minutes before ISO (5mg/kg, IP) (ISO/3AB group) or ISO alone (5mg/kg, IP) (ISO). All techniques and methodology were analogous to those detailed in previous chapters. The dosage regimen for 3AB was chosen on the basis of previous experiments in Sprague-Dawley rats by Miki *et al*, 2007[301].

Four rats were treated with 3AB (50mg/kg, IP) alone.

5.2.2 Statistics

All experiments were undertaken with measurements performed while blinded as to treatment allocation. The main hypothesis tested (in null form) was that ISO/3AB would not differ from ISO alone.

5.3 Results

5.3.1 Mortality

Mortality with ISO/3AB was ~35% (all within two hours of ISO/3AB injections), not significantly different from ISO alone ($p > 0.999$, Fisher's exact test). Data are presented on the 19 surviving rats.

5.3.2 Echocardiographic changes

Apical radial strain and fractional shortening remained preserved in the ISO/3AB group at 24 hours compared with the ISO alone group (time-treatment interaction values $p = 0.0002$ and $p = 0.0028$ respectively). Results are summarised in Table 5.1. Other echocardiographic measurements, such as apical wall thickness or heart rate, were not significantly different between ISO alone and ISO/3AB groups.

3AB alone induced no changes in wall motion (data not shown).

Table 5.1. Echocardiographic parameters for ISO vs ISO/3AB - Echocardiographic changes associated with IP administration of ISO alone (ISO: 5mg/kg, n=20) and ISO + 3AB (ISO/3AB: 50mg/kg, n=19). Analysis is via 2-way ANOVA, but only p values are for time/treatment interaction are given.

	ISO		**ISO/3AB**		
Parameter	**Baseline**	**24hrs**	**Baseline**	**24hrs**	**p value**
Heart Rate (bpm)	339.3 ± 6.1	376.1 ± 7.6	346.8 ± 5.9	372.7 ± 5.1	0.38
Mean Apical Wall Thickness (mm)	15.8 ± 0.4	19.3 ± 0.5	15.3 ± 0.2	18.3 ± 0.3	0.45
Apical Radial Strain (%)	-39.5 ± 1.3	-25.3 ± 1.4	-43.3 ± 1.0	-37.2 ± 1.0	0.0011
Apical Fractional Area Shortening (%)	49.6 ± 1.4	35.5 ± 1.6	46.8 ± 1.1	41.6 ± 1.1	0.0013
Ejection Fraction (%)	86.2 ± 0.9	81.2 ± 1.5	83.7 ± 1.0	82.0 ± 1.0	0.15

All values displayed as mean ± SEM. Note that comparison is between pre-treatment status (Baseline) and 24 hours post ISO (24hrs).

5.3.3 Histology/immunohistochemistry

CD68 content decreased in ISO/3AB compared to ISO alone ($p = 0.006$, 2-way ANOVA) (Figure 5.1). It should also be noted that CD68 distribution was predominantly periapical in ISO-treated rats ($p = 0.016$, 2-way ANOVA), with a similar trend in the ISO/3AB group ($p = 0.2$).

3-NT content increased significantly in 3AB-treated compared to ISO-alone treated animals ($p = 0.047$ for treatment), as summarised in Figure 5.2A, visualised in Figure 5.3A. There were no regioselective differences noted in 3-NT accumulation.

PAR content was increased in the ISO/3AB group compared to the ISO-alone group (treatment $p = 0.002$) as summarised in Figure 5.2B, visualised in Figure 5.3B. There was a trend toward regioselectivity, for example apical PAR content was 3.2 ± 0.3% and basal 2.5 ± 0.2% in ISO/3AB treated rats, however this was not significant after a Bonferroni's post-hoc test was performed ($p > 0.05$).

Overall, TXNIP content was further elevated in the myocardium of ISO/3AB rats compared to ISO alone (treatment $p = 0.007$), as summarised in Figure 5.2C, visualised in Figure 5.3C. There were no regioselective differences noted in TXNIP accumulation.

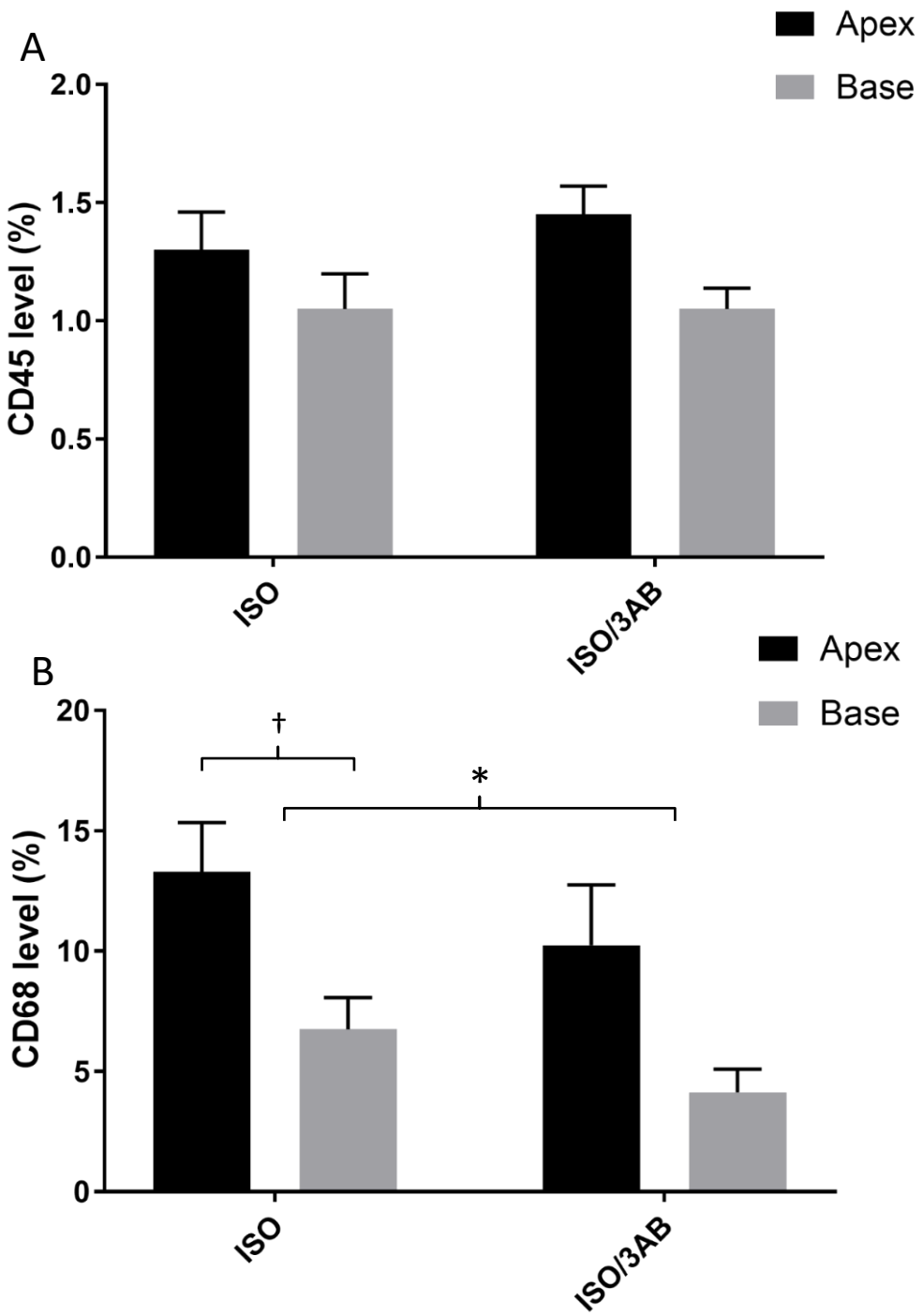


Figure 5.1. Effect of 3AB on inflammatory markers in ISO (n=10) and ISO/3AB (n=4) groups. CD45+ staining (leukocytes) is shown in (A) and CD68+ staining (monocytes/macrophages) is shown in (B). * indicates $p < 0.05$, 2-way ANOVA), † indicates $p < 0.05$, Bonferroni's post-hoc test.

ANOVA data:		**F**	**p**
A:	Treatment	3.86	0.06
	Apex / base	0.21	0.66
	Interaction	0.21	0.66
B:	Treatment	8.9	0.006
	Apex / base	1.79	0.019
	Interaction	0.01	0.92

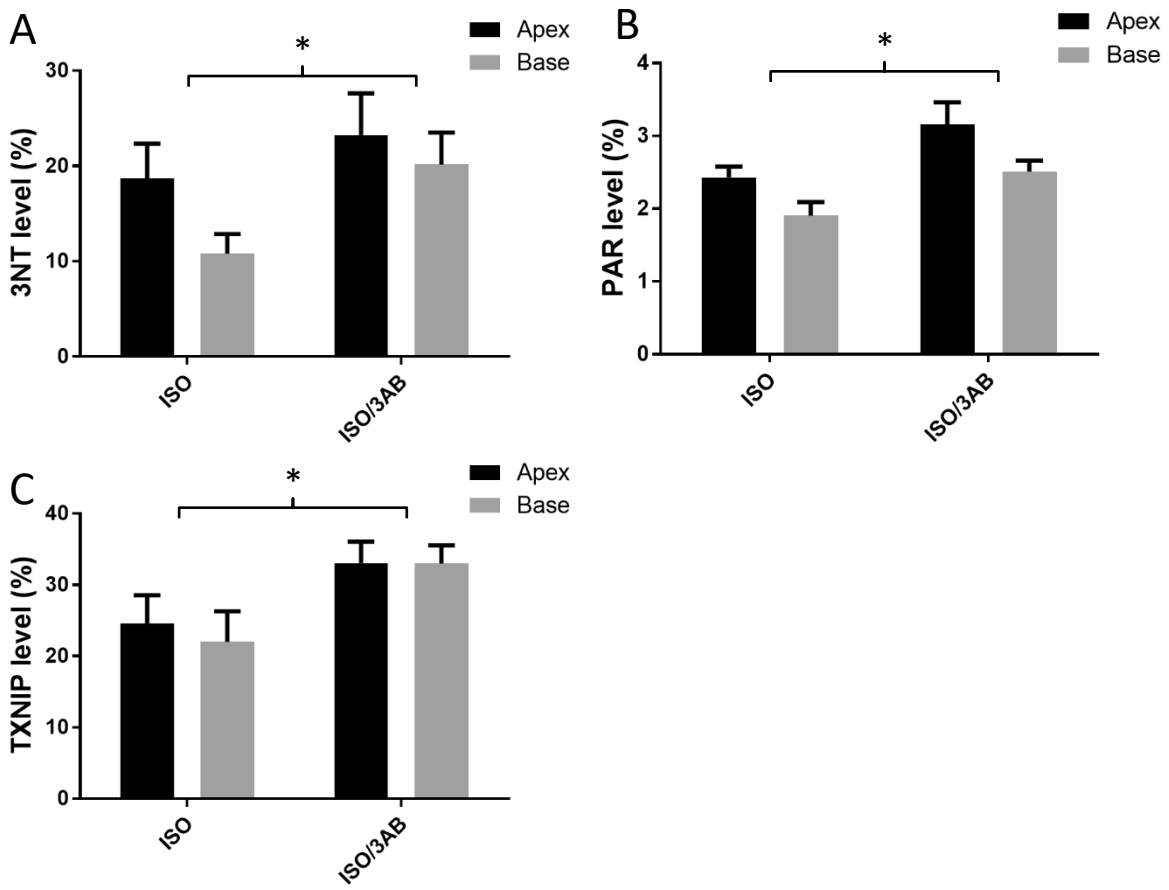


Figure 5.2. Immunohistochemistry data for (A) 3-NT, (B) PAR and (C) TXNIP concentrations in the heart of ISO-treated rats compared with ISO/3AB animals. * indicates $p < 0.05$, 2-Way ANOVA (treatment).

ANOVA	**data:**	**F**	**p**
A:	ISO	14.95	0.0003
	Apex / base	6.16	0.17
	Interaction	0.61	0.44
B:	ISO	10.27	0.002
	Apex / base	13.77	0.0005
	Interaction	0.072	0.79
C:	ISO	45.5	<0.0001
	Apex / base	0.66	0.42
	Interaction	0.003	0.96

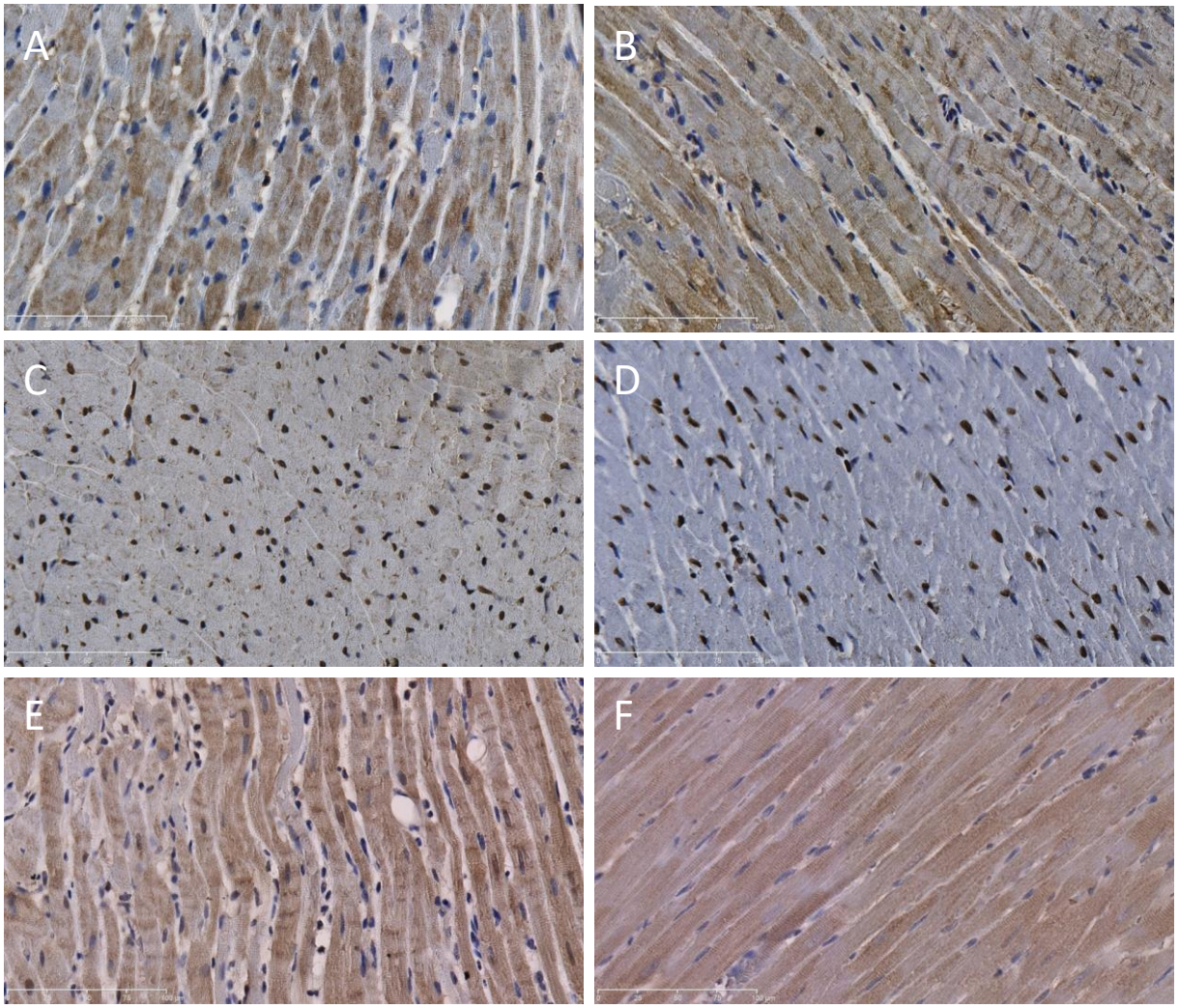


Figure 5.3. Effects of 3AB on 3-NT, PAR and TXNIP expression in the myocardium. 3-NT accumulation is shown by brown staining in (A) ISO-treated rat myocardium and (B) ISO/3AB-treated myocardium. PAR accumulation is shown by darkly stained nuclei in (C) ISO-treated rat myocardium and (D) ISO/3AB-treated myocardium. TXNIP accumulation is shown by brown staining in (E) ISO-treated rat myocardium and (F) ISO/3AB-treated myocardium. All images are from apical samples.

5.3.4. Immunoblotting

PARP-1 immunoblotting (116kD fragment) demonstrated that apical PARP-1 expression did not significantly differ at 24hrs between ISO and ISO/3AB treated rats (6.9 ± 2.7 vs 2.1 ± 0.8: ANOVA: $p = 0.16$).

PAR content also did not vary significantly (37.9 ± 10.7 for ISO and 32.8 ± 8 for ISO/3AB: $p = 0.9$).

Phosphorylated:total NFκB ratios were not significantly different between groups ($p = 0.43$). The immunoblotting data is not graphically displayed.

5.3.5. Vascular reactivity

Evaluation of contractile responses to noradrenaline 24hrs post ISO in ISO alone and ISO/3AB groups revealed no significant changes in either maximal response (Emax) or in concentration associated with 50% of maximal effect (EC50) [see Figure 5.4].

However, post-ISO in the ISO/3AB group there was substantial and significant decrease in the EC50 (logM) for acetylcholine (ACh) ($p = 0.027$, Figure 5.5), consistent with restoration of the NO-mediated vasodilator effects of ACh.

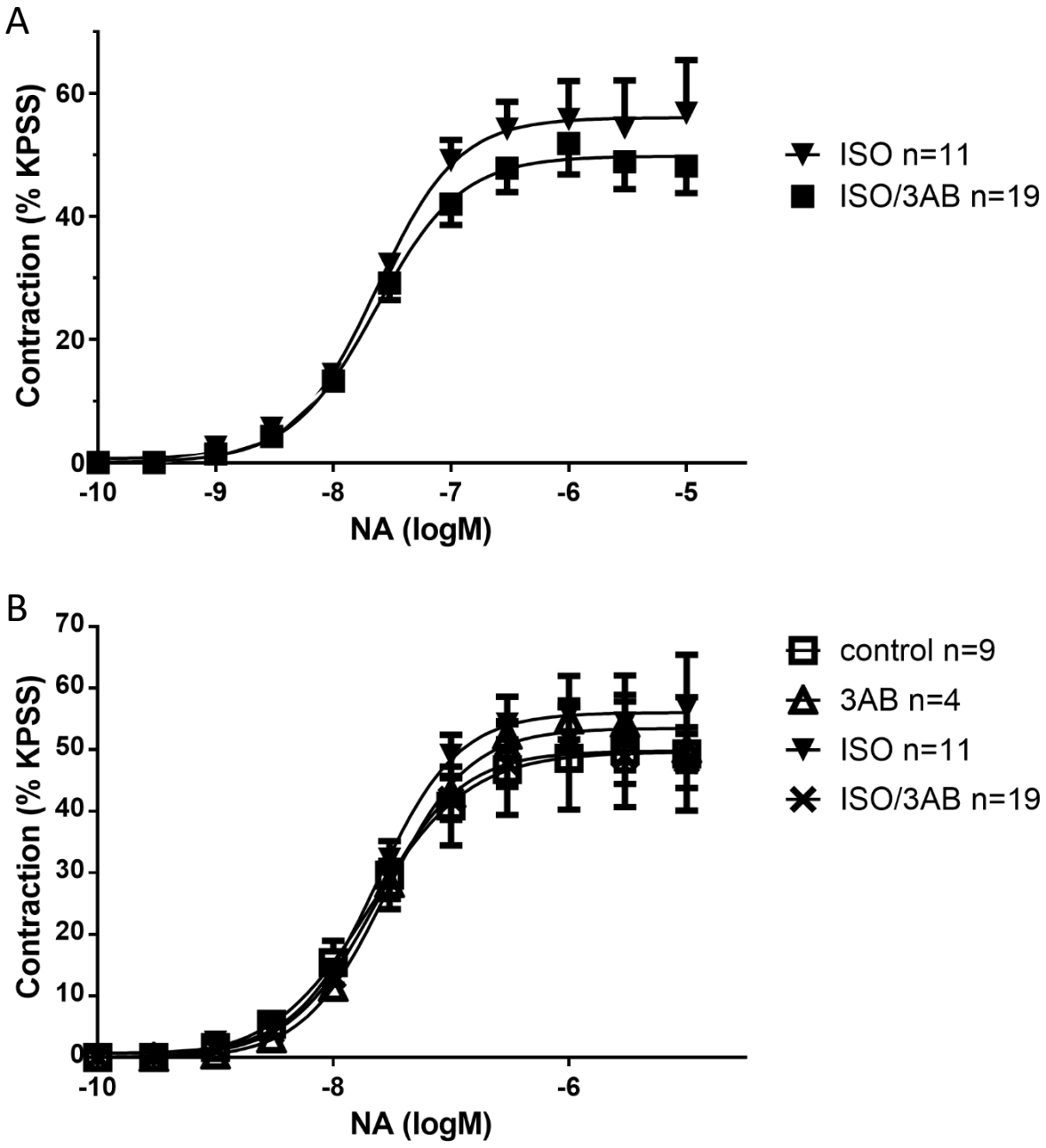


Figure 5.4. A) Contractile concentration-response curves for isolated thoracic aortic rings from ISO and ISO/3AB treated rats in response to noradrenaline (NA). B) All treatment groups involving 3AB, in relation to control and ISO-alone treated animals. There were no significant differences between treatment groups in this respect.

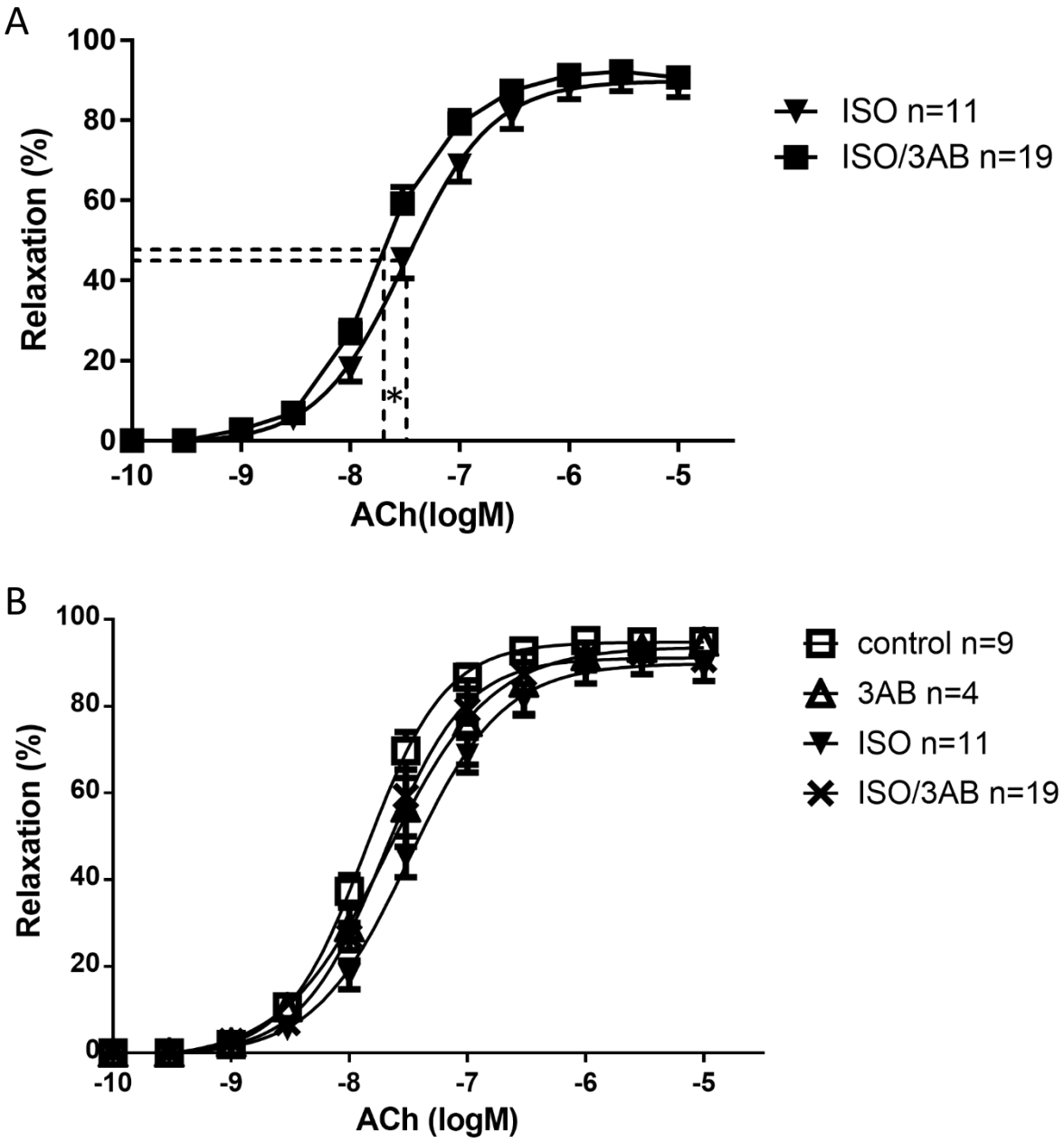


Figure 5.5. A) Relaxation concentration-response curves for isolated thoracic aortic rings from ISO and ISO/3AB treated rats. Vessels were contracted with NA and relaxation was induced in response to acetylcholine (ACh). *EC50 (logM) for ISO = -7.48 ± 0.07, EC50 (logM) for ISO/3AB = -7.70 ± 0.06.). B) All treatment groups involving 3AB, in relation to control and ISO-alone treated animals.

5.4 Discussion

The major findings of the current study were that the PARP-1 inhibitor 3AB attenuated the negative inotropic effects and associated monocyte accumulation in the myocardium of ISO in the rat model, without significantly modifying acute mortality. At 24hrs, there was no residual biochemical evidence of PARP-1 inhibition, as characterised by myocardial PAR content: indeed, that was increased in comparison with data in untreated rats. Finally, 3AB had no effect on 3-NT or TXNIP accumulation within the myocardium, but potentiated ACh-induced relaxation of rat aortae. Many of these findings have major implications regarding understanding of the potential role of PARP-1 activation in TS.

First, the current experiments represented a logical next-step following the findings of evidence that TS is associated with nitrosative stress within the myocardium. For example, our pilot data in hearts of patients dying from TS[300] showed evidence of increased myocardial content of 3NT, a "fingerprint" of peroxynitrite ($ONOO^-$) effect[290]. In turn, $ONOO^-$ functions, among other things, as a PARP activator[262]. Substantial activation of PARP-1 results in development of energetic impairment, to the extent that the enzyme, despite its importance in DNA repair, is often regards as an "energy sink"[264]. Clinically, three recent studies from the Aberdeen group[102, 124, 272] have demonstrated that impairment of myocardial energetics occurs for at least four months after onset of TS, and the current findings, in association with prolonged presence of inflammatory oedema within the myocardium, suggest that PARP-1 activation may be prolonged.

That being the case, the question arises: why was myocardial PAR content elevated 24hrs post 3AB administration? In spite of the large dose of 3AB utilised in the current study, the duration of PARP-1 inhibition by 3AB is short, and previous studies have suggested that "rebound" activation of PARP-1 occurs as myocardial 3AB concentrations fall[302]. Therefore, if the strategy of PARP-1 inhibition were to be translated to clinical use, it would be advisable to ensure that administration of the inhibitor were sufficiently frequent to ensure that continuous effect was achieved.

The assumption from the current data is that negative inotropic changes reflect, at least in part, PARP-1 mediated energetic impairment. While this is sound, it must be noted that myocardial redox stress

and energetic state were not measured in those experiments. Furthermore, improvement in energetic state does not necessarily imply need for reversal of PARP-1 inhibition:- several agents acting on other systems, such as inhibition of the mitochondrial membrane enzyme CPT-1, or of cardiac fatty acid oxidisation, may also improve energetics[303, 304].

The finding that acute mortality did not decrease significantly despite partial salvage of contractile impairment was quite surprising, but the results were quite robust. All deaths occurred within 90 minutes of ISO(±3AB) administration, and were often preceded by signs of torpor in the affected rats. It is currently uncertain whether these deaths were primarily due to the development of shock, or to tachyarrhythmias, as no blood pressure or ECG recording was undertaken. There is therefore a considerable need to evaluate in greater detail what precisely occurs in the first 90 minutes post initiation of TS. Such information would potentially enrich knowledge of pathophysiology in patients:- while both shock and tachyarrhythmias occur in substantial proportions of patients[74], very little information is available about the earliest stages of TS.

The fact that contractile impairment was ameliorated without reduction in mortality has at least one major parallel in the literature. Paur *et al*[222] utilised a rat model of TS and found that biased activation of G_i-based post-receptor signalling from myocardial β_2AR mediated negative inotropic changes. However, while specific β_2AR blockade attenuated negative inotropic effects, it markedly increased mortality rates. Again, the mechanism(s) underlying this paradox was never fully elucidated, although it was proposed that β_2AR activation in TS was, at least in the short term, cardioprotective. Apart from the fact that this was not investigated further, it is important in the context of the current study to speculate as to whether such "protection" might have occurred by limitation of $ONOO^-$-mediated effects other that PARP-1 activation[255] or whether NOS activation via β_2AR stimulation[257] might play a critical role. This area of uncertainty has been addressed in part in Chapter 4.

There has been considerable controversy in the literature as to whether TS might be, primarily, a coronary vascular, rather than myocardial, disease, a sort of acute coronary syndrome (see [305, 306]). For example, coronary spasm has occasionally been reported in TS cases[307]. In the current study,

evaluation of vascular reactivity was performed, utilising thoracic aortic rings, perfused in vitro. The results showed that 3AB did not significantly affect contractile responses to noradrenaline (Figure 5.4), but potentiated relaxation responses to ACh (Figure 5.5), which are mediated by NOS activation within vascular endothelium[308]. Therefore, loosely speaking, 3AB may be considered to have "improved endothelial function" in these vessels. The question therefore arises: was endothelial function ever impaired? Patients with TS display supranormal NO-based signalling in platelets[283]. However, we have recently demonstrated that the acute stages of TS are characterised by substantial activation of glycocalyx shedding[96], an inflammatory process which can be initiated by catecholamines[207], and which results, not only in loss of the role of the glycocalyx in maintaining laminar flow, but also in attenuation of endothelial "barrier" function, so that development of tissue oedema and of white blood cell infiltration of tissues are augmented[22]. As to the impact on vascular reactivity, studies to date are limited, but it is certainly conceivable that NO-mediated relaxation may be impaired.

Importantly, in the current study, 3AB reduced myocardial CD68 content, a finding which could be explained by limitation of damage to the endothelial glycocalyx, with resultant limitation of monocyte infiltration from the blood. On the other hand, 3AB did not significantly attenuate ISO effects on thickening of the apical LV wall, probably representing development of oedema.

These results therefore raise an additional perspective about the pathogenesis of TS, suggesting that coronary vascular dysfunction (and increased permeability) may play a role in the initiation and/or perpetuation of inflammatory changes within the myocardium.

There are several potentially clinically relevant issues which arise from these experiments, apart from the potential utility of inhibitors of $ONOO^-$ effect (e.g. "scavengers") or of NOS. For example, doxycycline, a non-specific metalloproteinase inhibitor, protects glycocalyx integrity[309] and therefore might limit inflammatory infiltration, myocardial oedema and perhaps even the development of hypotension/shock. Chapter 6 contains data related to the issue of NOS inhibition.

6 Impact of NOS Inhibition on the Evolution of TS in a Rat Model

Abstract

Introduction: Takotsubo Syndrome (TS) is, at least predominantly, an acute inflammatory condition of the myocardium, triggered by a catecholamine "surge". Although catecholamines are known to activate β-adrenoceptors, some of which are coupled to NO (nitric oxide) synthases, as yet there have been no studies specifically to assess the role of NO in TS. Therefore, the effects of the non-specific NOS inhibitor L-NAME were assessed in this rat model of TS

Methods: Methods were analogous to those used in previous studies with this female Sprague-Dawley rat model. Briefly:

(i) rats were divided into treatment groups – ISO alone (5mg/kg IP), ISO/L-NAME (5mg/kg ISO + 50mg/kg L-NAME IP), L-NAME alone (50mg/kg IP) and control.

(ii) Echocardiography was performed at baseline pre-treatment and 24hrs post treatment.

(iii) Hearts and thoracic aortae were salvaged after the 24-hour timepoint, for immunohistochemistry, immunoblotting and vascular reactivity studies.

Results: The most striking result in this series of experiments was that the addition of L-NAME to ISO resulted in amelioration of ISO-induced mortality ($p = 0.0043$, Fisher's exact test versus ISO alone). There was no significant effect on the main functional endpoint of apical radial strain in the ISO/L-NAME group, although when assessed by fractional area shortening, these rats had less impaired systolic function compared to rats treated with ISO alone ($p = 0.026$). Immunohistochemical analyses in ISO/L-NAME rats displayed a substantial decrease in monocyte/macrophage accumulation ($p = 0.002$) and a marked increase in myocardial TXNIP accumulation ($p < 0.0001$), but no significant changes in 3-NT or PAR content. Western blot results, however, showed a decrease in one of the two bands of 3-NT (47kDa) ($p = 0.0096$) and increases in eNOS and TXNIP expression ($p = 0.0027$ and $p =$

0.0015 respectively). Vascular reactivity was impaired in ISO/L-NAME rats, in both contractile- and relaxation-response in isolated thoracic aortae.

Discussion: While the addition of L-NAME was not clearly successful in reducing ISO-induced LV dysfunction in this experiment, there was a significant reduction in mortality and monocyte/macrophage accumulation within the myocardium. Therefore, the impact of incremental NO release in this model of TS includes modulation of both cellular inflammatory infiltration and mortality risk, while exerting at most minor effects on negative inotropic changes.

6.1 Introduction

The experiments described in Chapters 4 and 5 have demonstrated that:

(i) Single dose intraperitoneal injection of ISO into female Sprague-Dawley rats can induce inflammatory changes within the myocardium, with evidence of nitrosative stress, and LV wall motion changes mimicking TS.

(ii) There is associated impairment of ACh-induced NO-mediated vasodilatation in systemic arteries.

(iii) The PARP-1 inhibitor 3AB ameliorates negative inotropic changes in this model, suggesting that they arise from $ONOO^-$-mediated activation of PARP-1. Notably, early mortality was similar among rats treated with ISO alone or ISO/3AB, at around 35%.

At this stage therefore, a schematic for the pathogenesis of TS within the myocardium would be that shown in Figure 6.1.

However, as detailed in this schematic, an essential component of the cascade culminating in PARP-1 activation (and presumptive energetic impairment) is the activation of NOS, coupled to β_2AR stimulation[211]. This would ensure release of NO and increase the likelihood of peroxynitrite formation. We already have epidemiological evidence that patients with TS demonstrate "paradoxical" increases in NO signalling[283].

The principal objective of the current study was to evaluate the effects on myocardium of the addition of the non-specific NOS inhibitor L-NG-Nitroarginine methyl ester (L-NAME) to ISO. We considered that in theory, L-NAME might exert similar overall effects to those of 3AB, with the additional action of limiting 3-NT formation.

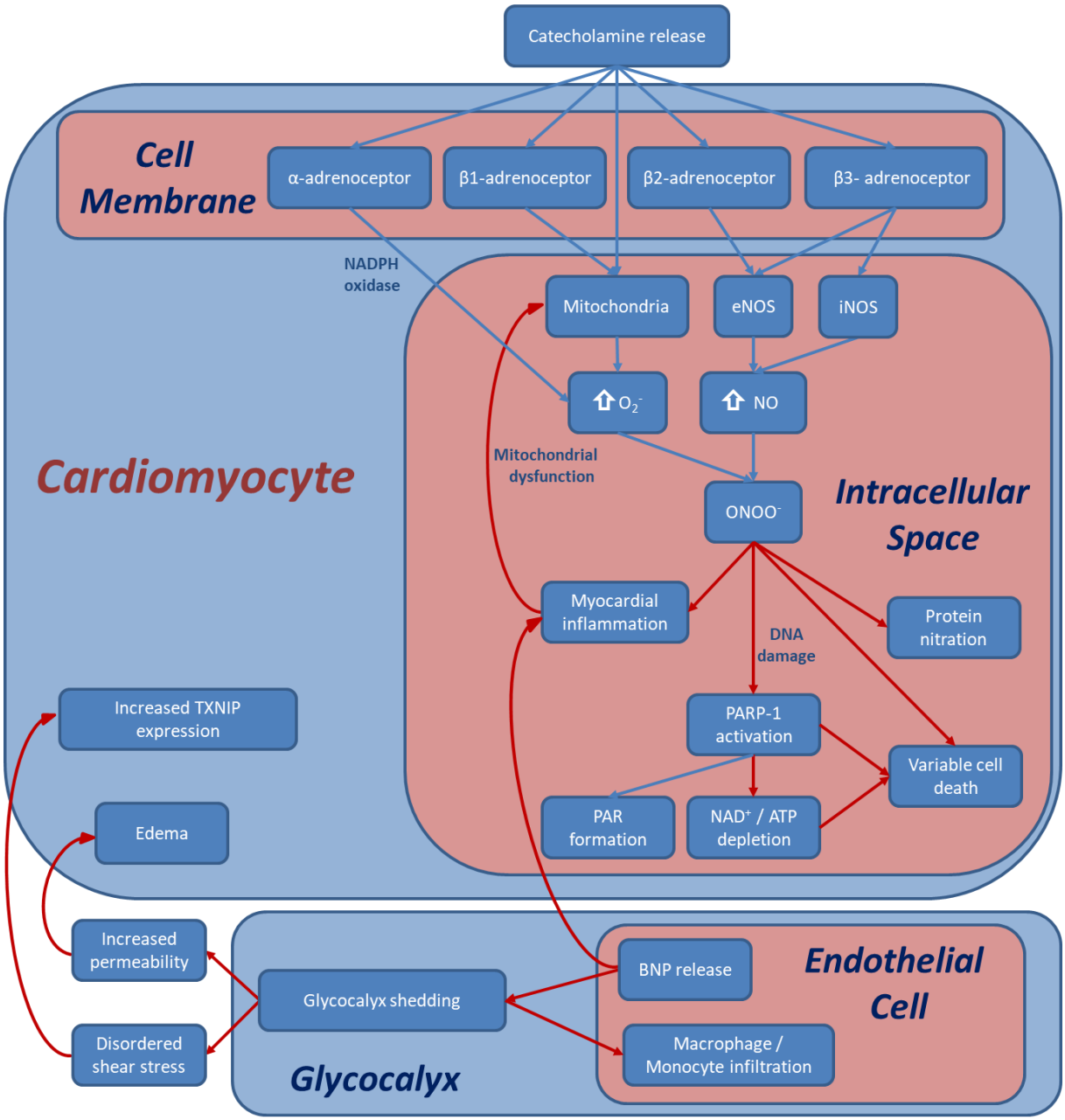


Figure 6.1. Proposed schematic for the pathogenesis of TS: status as at end of experiments outlined in previous Chapters 4 and 5.

6.2 Methods

6.2.1 Rat model

As described in Chapters 2, 4 and 5, the female Sprague Dawley rat model was again used in this series of experiments, with the aim being to investigate the role of NOS in TS, by using the non-selective NOS inhibitor L-NG-Nitroarginine methyl ester (L-NAME). Rats were either administered L-NAME (50mg/kg, IP) 30 minutes before ISO (5mg/kg, IP) (ISO/L-NAME group) or ISO alone (5mg/kg, IP) (ISO). All techniques and methodology were analogous to those detailed in previous chapters. Five rats were treated with L-NAME (50mg/kg, IP) alone.

6.2.2 Statistics

All experiments were undertaken with measurements performed while blinded as to treatment allocation. The main hypothesis tested (in null form) was that ISO/L-NAME would not differ from ISO alone.

6.3 Results

6.3.1 Mortality

While this was not the primary purpose of the experiments, the results were of great interest and are displayed in Figure 6.2.

Rats treated with either ISO or ISO/L-NAME often became unwell about 5-10 minutes after initial injection. However, none of the rats treated with ISO/L-NAME died ($p = 0.0043$, Fisher's exact test). In this chapter, data are presented on the 17 ISO/L-NAME rats and rats treated with ISO alone.

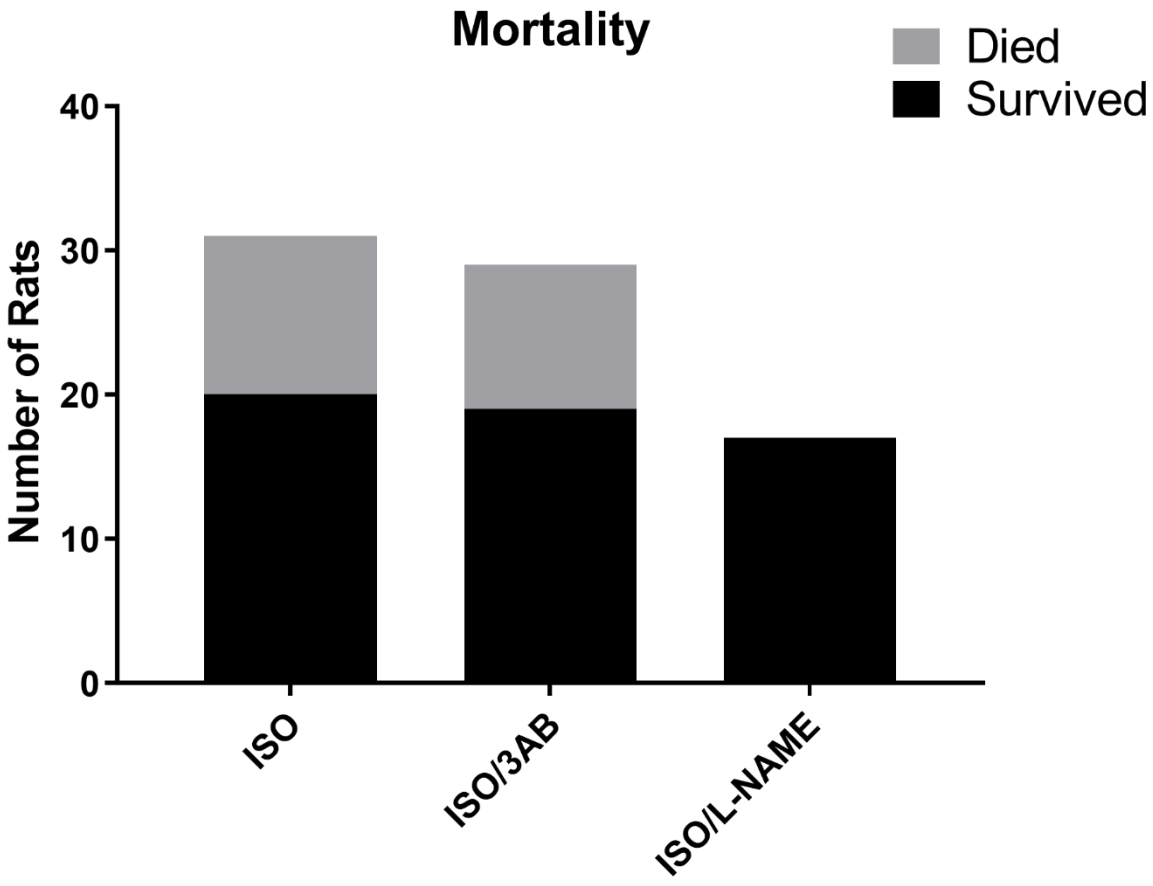


Figure 6.2. Reduction in mortality in ISO/L-NAME rats vs ISO alone (p = 0.0043, Fisher's exact test).

6.3.2 Echocardiographic findings

These data, summarised in Table 6.1, proved to be somewhat complex.

First, the induction of tachycardia post ISO tended to be diminished ($p = 0.055$, 2-way ANOVA: time/treatment interaction). Furthermore, while there was a trend towards detrimental impact on apical radial strain with ISO/L-NAME ($p = 0.16$) there was actually a significant decrease in impact on apical fractional area shortening ($p = 0.026$). Ejection fraction and apical wall thickness were not significantly different between treatment groups. These confusing results are discussed further below.

L-NAME alone induced no changes in wall motion (data not shown).

Table 6.1. Echocardiographic parameters for ISO vs ISO/L-NAME - Echocardiographic changes associated with IP administration of ISO alone (ISO: 5mg/kg, n=20) and ISO + L-NAME (ISO/L-NAME: 50mg/kg, n=17). Analysis is via 2-way ANOVA, but only p values are for time/treatment interaction are given.

	ISO		**ISO/L-NAME**		
Parameter	**Baseline**	**24hrs**	**Baseline**	**24hrs**	**p value**
Heart Rate (bpm)	339.3 ± 6.1	376.1 ± 7.6	353.8 ± 5.7	365.4 ± 5.9	0.055
Mean Apical Wall Thickness (mm)	15.8 ± 0.4	19.3 ± 0.5	16.1 ± 0.4	19.3 ± 0.6	0.72
Apical Radial Strain (%)	-39.5 ± 1.3	-25.3 ± 1.4	-41.3 ± 1.5	-23.0 ± 1.5	0.16
Apical Fractional Area Shortening (%)	49.6 ± 1.4	35.5 ± 1.6	45.4 ± 1.2	38.0 ± 1.7	0.026
Ejection Fraction (%)	86.2 ± 0.9	81.2 ± 1.5	83.6 ± 1.1	77.7 ± 1.5	0.72

All values displayed as mean ± SEM. Note that comparison is between pre-treatment status (Baseline) and 24 hours post ISO (24hrs).

6.3.3 Histology/immunohistochemistry

Similar to ISO/3AB, CD68 content decreased in ISO/L-NAME rats compared to ISO alone ($p = 0.002$, 2-way ANOVA) (Figure 6.3A). This reduction was significant in apical samples ($p = 0.002$) after Bonferroni's post-hoc testing. No significant changes occurred in CD45 accumulation (Figure 6.3B).

Distribution of 3-NT was periapical in both ISO/L-NAME and ISO alone treated animals ($p = 0.001$ for region), as summarised in Figure 6.4A, visualised in Figure 6.5A. There were, however, no differences noted in 3-NT accumulation between treatment groups.

PAR content was not significantly changed in the ISO/L-NAME group compared to the ISO alone group (treatment $p > 0.05$) as summarised in Figure 6.4B, visualised in Figure 6.5B. There was regioselectivity of PAR accumulation ($p = 0.034$ for region, 2-Way ANOVA), however after a Bonferroni's post-hoc test this was only significant ($p < 0.05$) in animals treated with ISO alone.

There was a marked increase in TXNIP content in the myocardium of ISO/L-NAME rats compared to ISO alone (treatment $p < 0.0001$), as summarised in Figure 6.4C, visualised in Figure 6.5C. There were no regioselective differences noted in TXNIP accumulation.

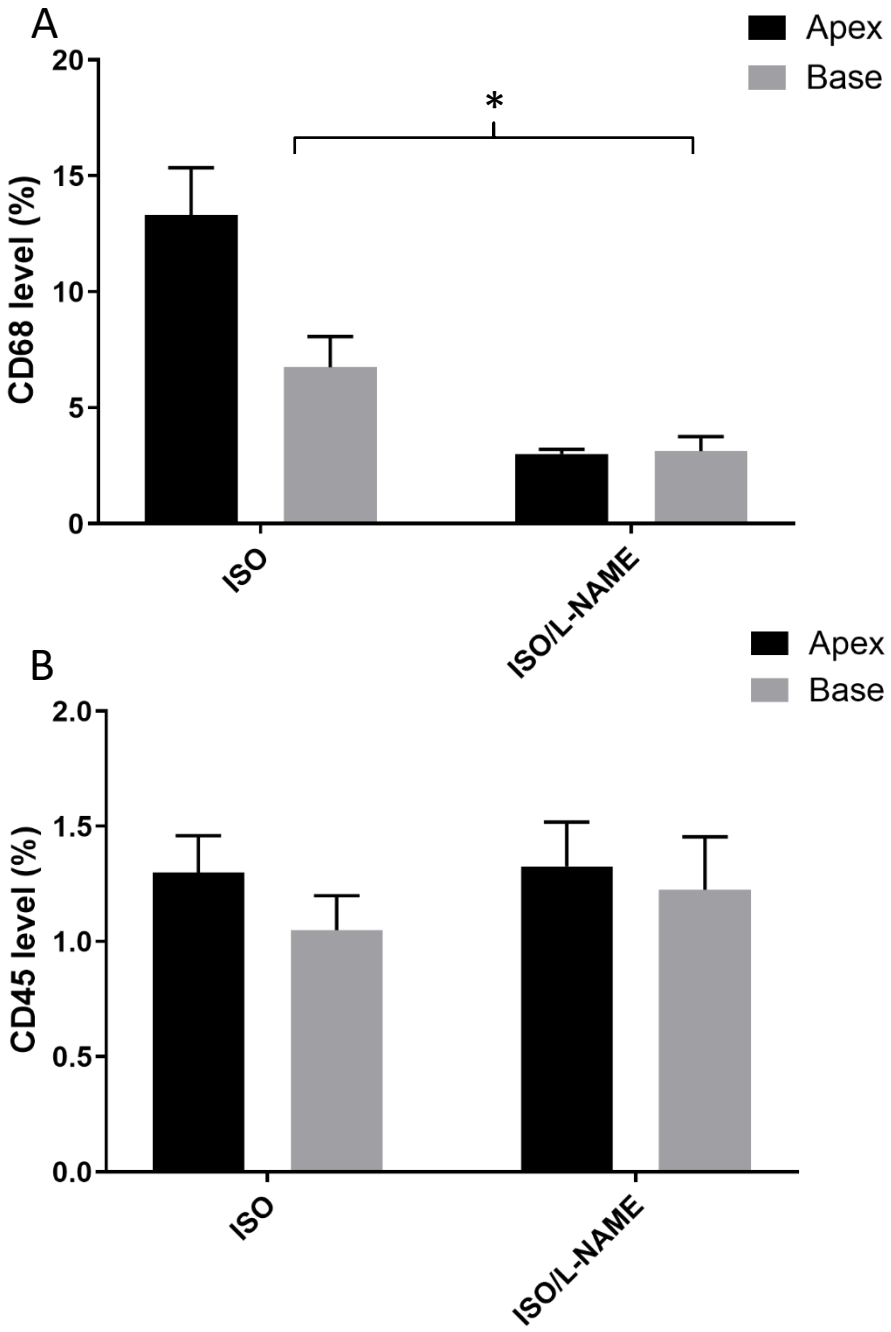


Figure 6.3. Effect of 3AB on inflammatory markers in ISO (n=10) and ISO/L-NAME (n=4) groups. CD68+ staining (monocytes/macrophages) is shown in (A) and CD45+ staining (leukocytes) is shown in (B). * indicates $p < 0.05$, 2-way ANOVA).

ANOVA	**data:**	**F**	**p**
A:	Treatment	2.63	0.12
	Apex / base	12.36	0.002
	Interaction	2.84	0.11
B:	Treatment	0.88	0.36
	Apex / base	0.29	0.6
	Interaction	0.16	0.69

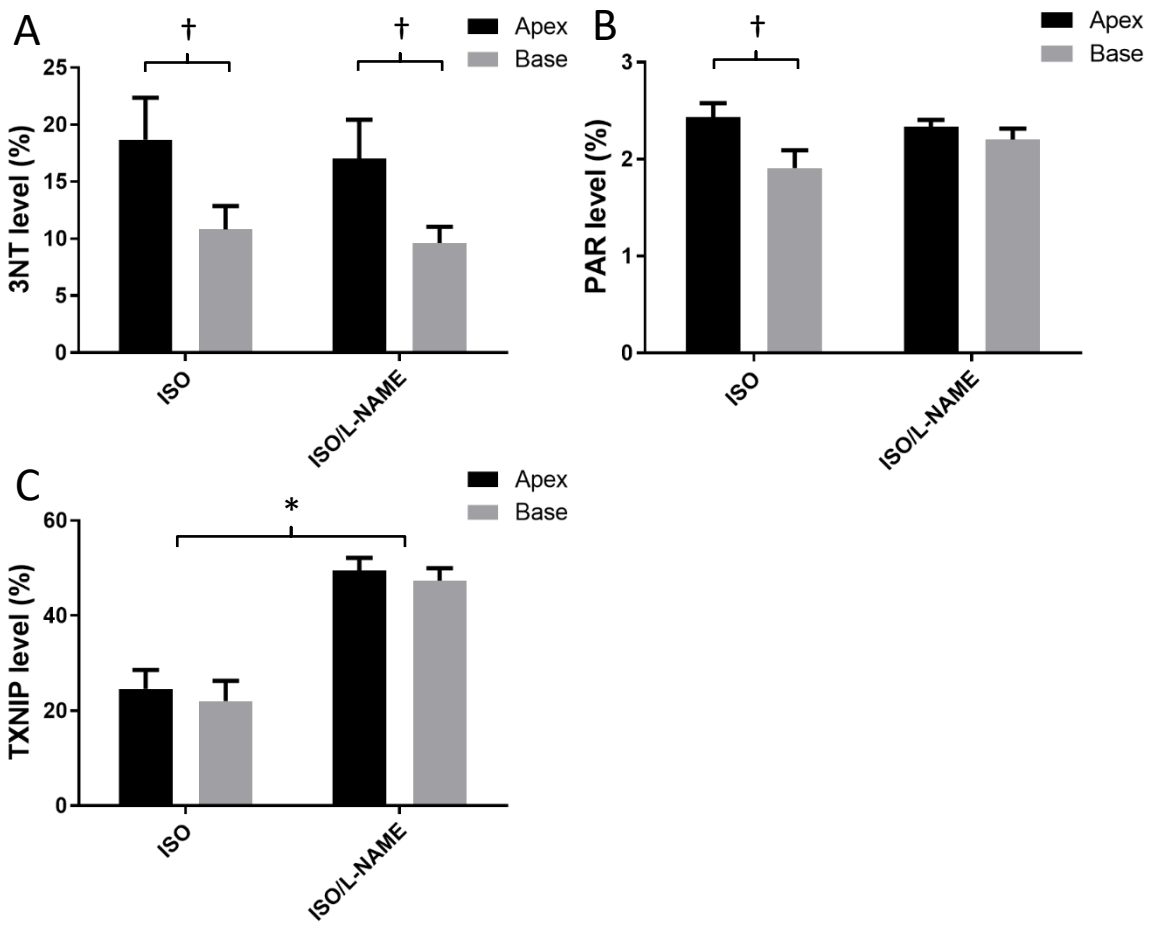


Figure 6.4. Immunohistochemistry data for (A) 3-NT, (B) PAR and (C) TXNIP concentrations in the heart of ISO-treated rats compared with ISO/3AB animals. * indicates $p < 0.05$, 2-way ANOVA (treatment), † indicates $p < 0.05$, Bonferroni's post-hoc test.

ANOVA	**data:**	**F**	**p**
A:	Treatment	0.25	0.62
	Apex / base	7.21	0.0099
	Interaction	0.006	0.94
B:	Treatment	0.41	0.52
	Apex / base	4.71	0.034
	Interaction	1.67	0.2
C:	Treatment	50.31	<0.0001
	Apex / base	0.44	0.51
	Interaction	0.0035	0.95

6.3.4 Immunoblotting

A significant decrease in 3-NT (47kDa), and increases in TXNIP and eNOS accumulation were noted on immunoblotting. These are shown in Figure 6.5 (A, B and C respectively). There were no significant differences noted between ISO/L-NAME rats and those treated with ISO alone in any of the other Western Blot investigations (3-NT (70kDa), PAR, PARP-1 (89kDa and 116kDa) and iNOS).

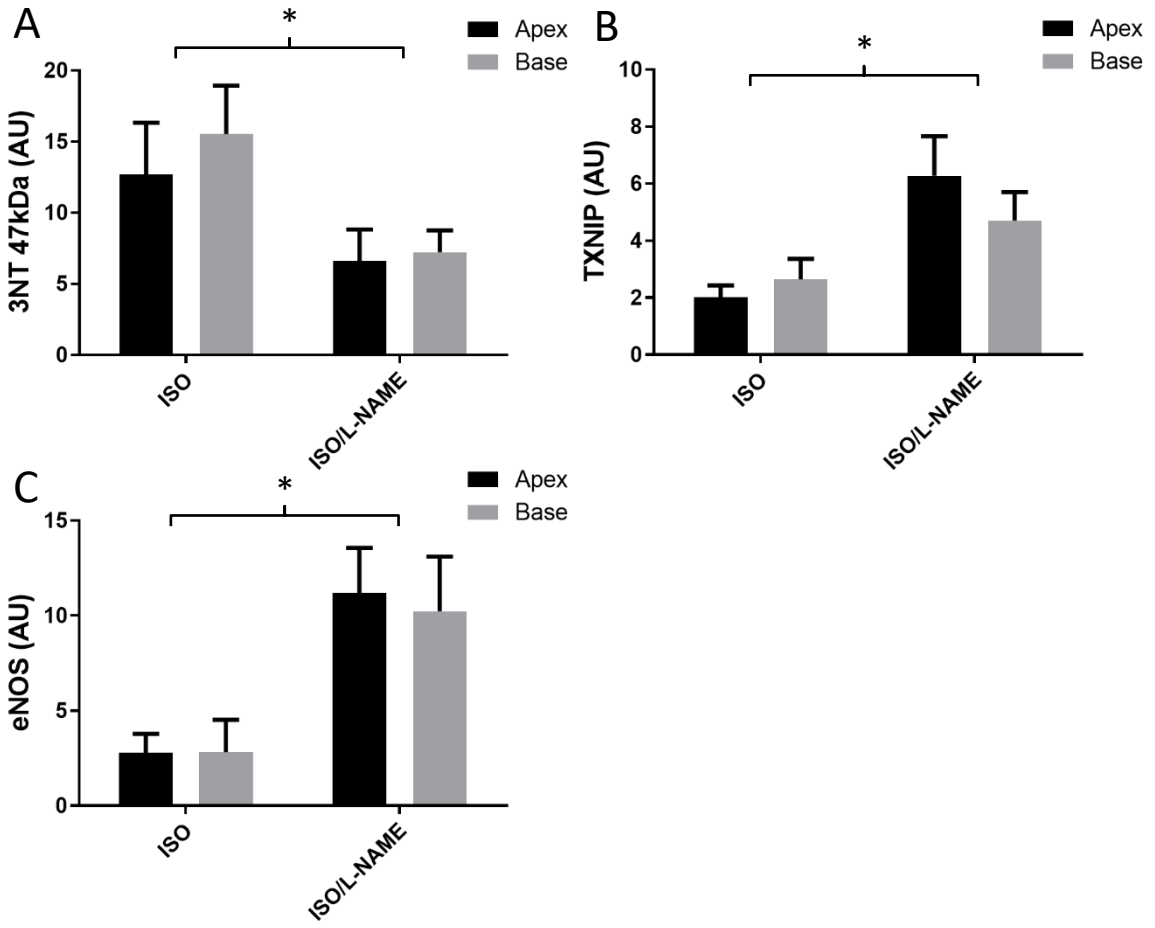


Figure 6.5. Immunoblotting data for (A) 3-NT (47kDa), (B) TXNIP and (C) eNOS concentrations in the heart of ISO-treated rats compared with ISO/L-NAME animals. AU: arbitrary units, * indicates $p < 0.05$, 2-Way ANOVA (treatment).

ANOVA	**data:**	**F**	**p**
A:	Treatment	7.35	0.0096
	Apex / base	0.42	0.52
	Interaction	0.18	0.68
B:	Treatment	10.02	0.0027
	Apex / base	0.23	0.64
	Interaction	1.21	0.28
C:	Treatment	12.43	0.0015
	Apex / base	0.04	0.84
	Interaction	0.05	0.83

6.3.5 Vascular reactivity

Contractile response to noradrenaline was markedly increased in ISO/L-NAME treated rats, compared with those treated with ISO alone, with maximal effect (Emax) significantly elevated ($p < 0.0001$), as shown in Figure 6.6.

ISO/L-NAME treated rats also displayed an impaired relaxation response to ACh, with both log EC50 and Emax significantly reduced in comparison to rats treated with ISO alone ($p = 0.0117$ and $p = 0.0337$ respectively), shown in Figure 6.7.

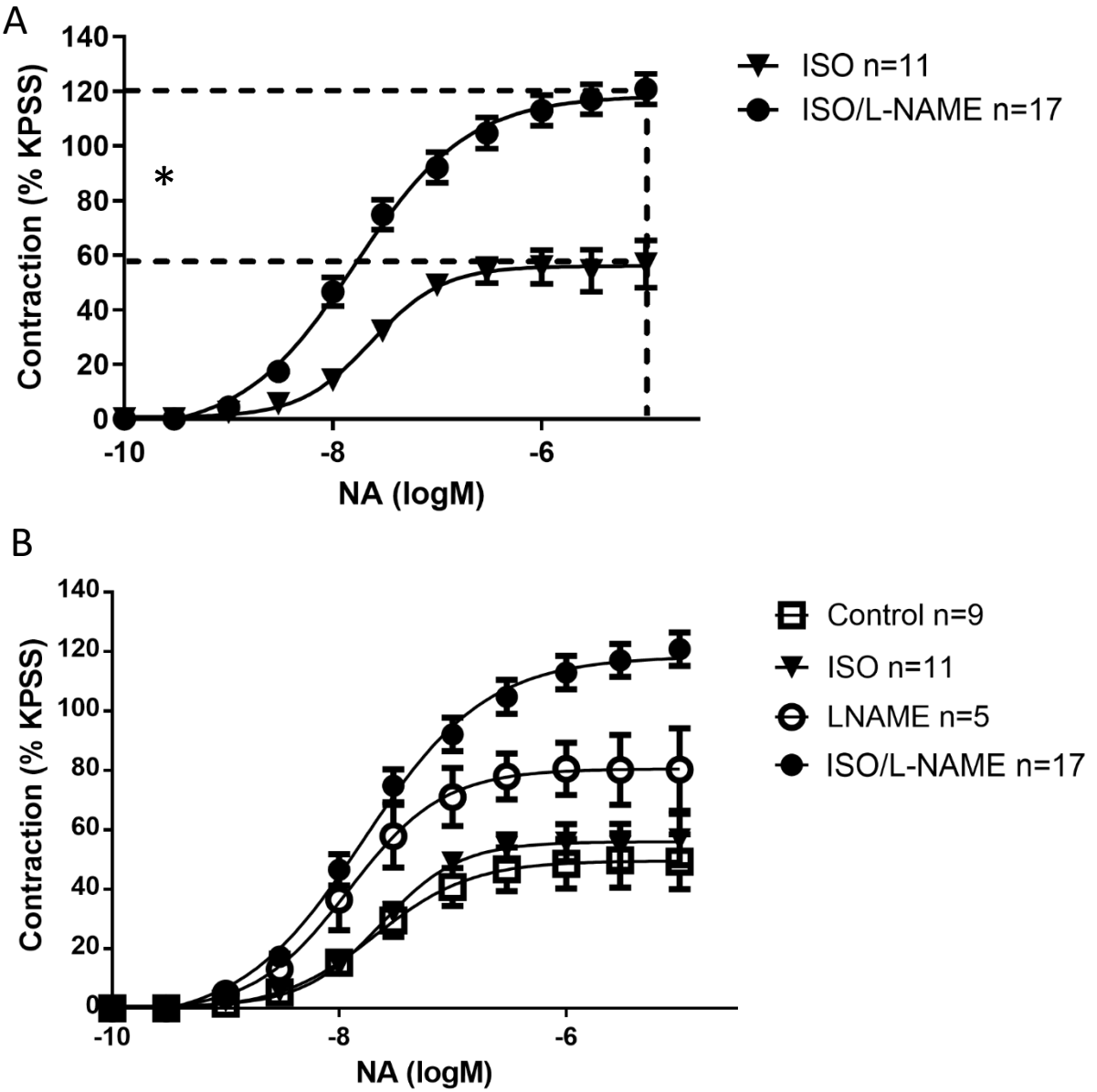


Figure 6.6. A) Contractile concentration-response curves for isolated thoracic aortic rings from ISO and ISO/L-NAME treated rats in response to noradrenaline (NA). There was a significant difference in Emax between treatment groups (*Emax ISO 56.63 ± 6.61, ISO/L-NAME 120.48 ± 5.31). B) All treatment groups involving L-NAME, in relation to control and ISO-alone treated animals.

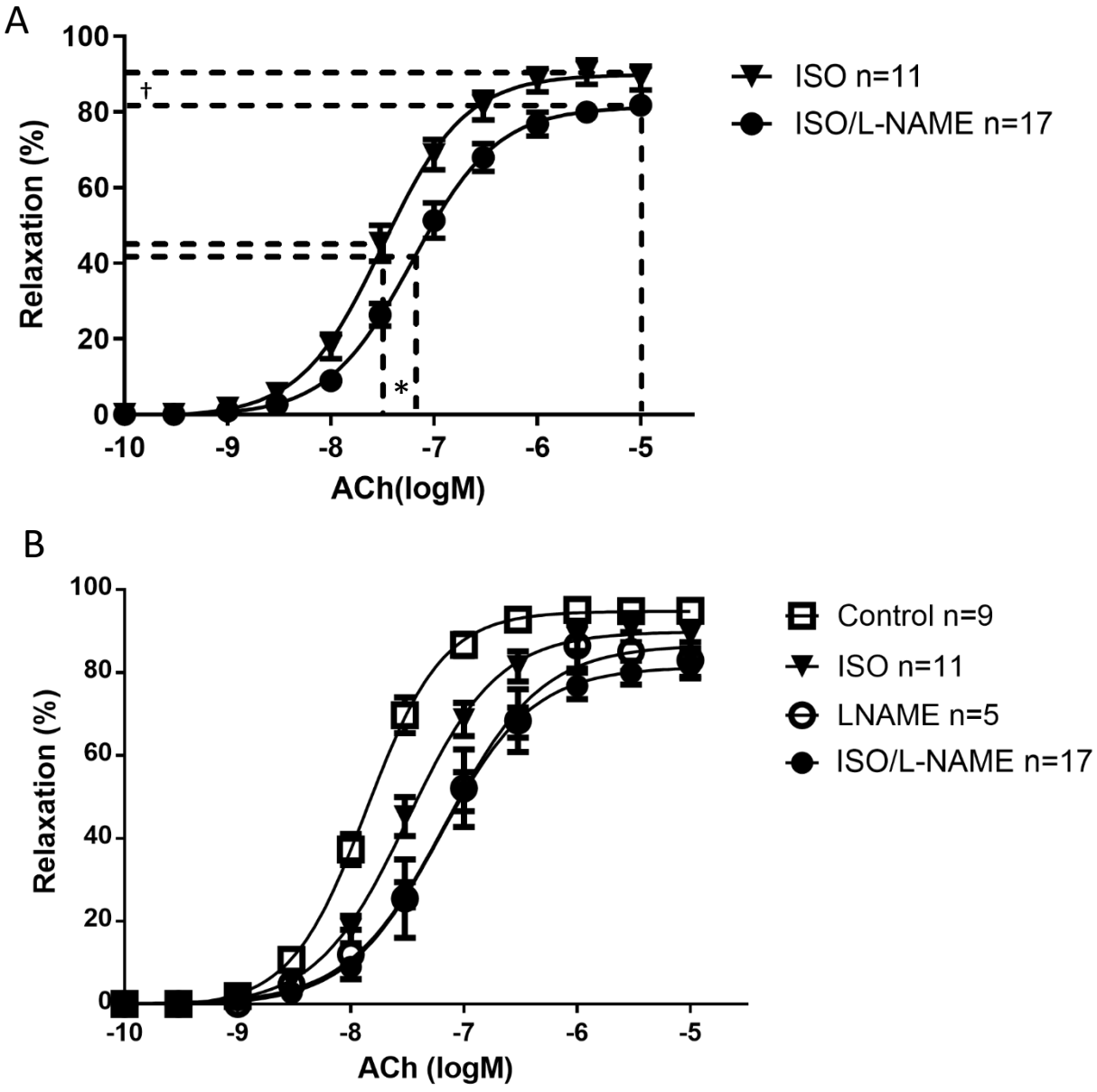


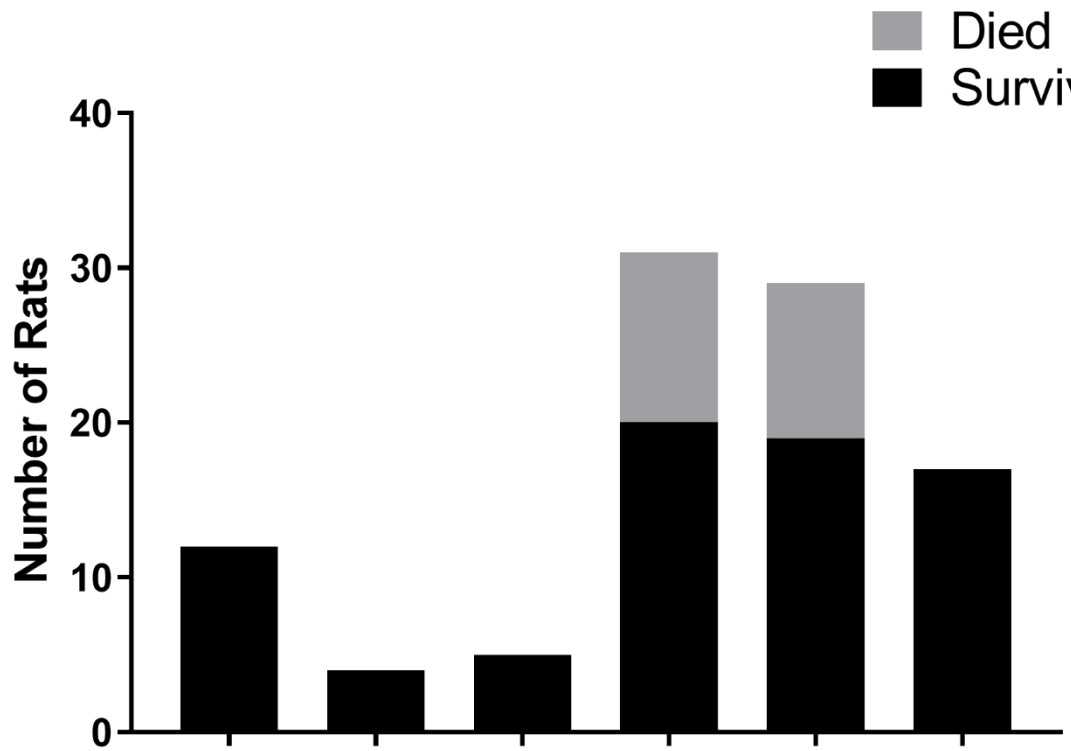
Figure 6.7. A) Relaxation concentration-response curves for isolated thoracic aortic rings from ISO and ISO/L-NAME treated rats. Vessels were contracted with NA and relaxation was induced in response to acetylcholine (ACh). *EC50 (logM) ISO = -7.48 ± 0.07, ISO/L-NAME = -7.17 ± 0.08, †Emax ISO = 90.67 ± 3.11, ISO/L-NAME 81.85 ± 2.44. B) All treatment groups involving L-NAME, in relation to control and ISO-alone treated animals.

6.4 Discussion

The purpose of the experiments in the current study was to utilise the non-specific NOS inhibitor L-NAME to test the hypothesis that both the inotropic changes and intracellular inflammatory activation associated with this model of TS are mediated via NOS activation, and indeed presumably mainly eNOS activation.

L-NAME was well tolerated when administered alone, and did not substantially alter apparent initial malaise of ISO-treated rats. Despite this, no rat treated with ISO/L-NAME died as a result of these injections, which was unexpected. Figure 6.2 displayed mortality data for the ISO and ISO/L-NAME treatment groups, however, a summary of mortality rates for all animals involved in experiments in Chapters 4-6 is shown here in Figure 6.8.

The mortality difference for ISO/L-NAME-treated rats vs ISO alone and ISO/3AB was significant ($p = 0.0167$, Chi-squared test). These mortality differences will be discussed later, because they did not represent the primary objective of these experiments.



ISO	-	-	-	+	+	+
3AB	-	+	-	-	+	-
L-NAME	-	-	+	-	-	+

Figure 6.8. Mortality across all treatment groups: Control, 3AB alone, L-NAME alone, ISO alone, ISO/3AB and ISO/L-NAME.

The next issue are the changes seen on echocardiography. Bearing in mind that 3AB had limited the negative inotropic impact of ISO (see Chapter 5), it would have been expected that L-NAME would exert similar or even greater effects. However, the results were equivocal. Considering the primary measure of LV systolic function (apical radial strain), there was no significant impact of L-NAME treatment; indeed, if anything, there was a trend towards greater impairment than with ISO alone (see Table 6.1). On the other hand, impact of ISO/L-NAME on apical fractional area shortening was significantly less than that of ISO alone. These confusing results may also have been affected by two other factors: -

i) L-NAME tended to limit ISO-induced tachycardia ($p = 0.055$), implying that this tachycardia is partially NOS-mediated. The resultant effects on the force-frequency relationship of the left ventricle[310] might have contributed to alteration in apparent inotropic effects in either direction[311].
ii) L-NAME impaired peripheral vascular NO signalling, as shown by potentiation of noradrenaline-induced vasoconstriction (see Figure 6.7). Although strain rate estimates are relatively afterload- and heart rate-independent[312], radial strain alone may be more sensitive to such changes, and this may have led to the differential impact on apical radial strain measures.

In light of these unclear results with non-specific NOS inhibition, comparison with results previously reported in other circumstances of increased oxidative/nitrosative stress is merited. In a model of experimental MI in rats, selective iNOS inhibition induced a significant reduction in mortality and an improvement in function in rats with MI and congestive heart failure[313]. Plasma nitrite and nitrate levels were elevated at eight weeks post ligation in the MI group, and crucially, only eight weeks of NOS inhibition was successful in limiting this, as compared to only the first two weeks post ligation. There was no significant difference in infarct size between to two NOS inhibition groups (but in both, infarcts were substantially smaller than without treatment). The 8-week NOS inhibition group also showed significantly better cardiac performance than the 2-week group. Similarly, in diabetic rats, selective iNOS inhibition was successful in reducing infarct size and improving LV function after ischemia-reperfusion injury[314]. In an ischemia-reperfusion study also using L-NAME, however, there

was no change in infarct size, only an improvement in mean aortic blood pressure[315]. Another study compared the response to βAR stimulation in young and aging hearts, and examined the role of iNOS in aging related myocardial ischemic injury[261]. The researchers were able to show that iNOS blockade not only improved cardiac function after ISO in isolated rat hearts, but also significantly reduced NO production and subsequent $ONOO^-$ formation. In the treatment of septic shock, for example, selective iNOS inhibition had more beneficial effects than NE on pulmonary artery pressures, gas exchange, mesenteric blood flow, microcirculation, and lactate concentration[316]. The SHOCK II trial, however, showed that non-selective NOS inhibition provided only modest increases in MAP at 15 min compared with placebo, while having no effect on mortality[317].

Of course, the current data do not permit definitive conclusions to be drawn regarding the apparent paradoxical relationship between effects of L-NAME (in the current experiments) and 3AB (in Chapter 5) on inotropic status and acute mortality. One possibility is that rats die of hypotensive shock, and that L-NAME somewhat mitigates this effect. Maximal fall in systolic blood pressure in rats treated with ISO, as reported by Redfors *et al* (2014)[225] or Zhang *et al* (2017)[234], occurs rapidly post ISO injection (within the first few minutes). This is in contrast to other animal models of TS in which epinephrine was used as the catecholamine (Paur *et al*, 2012[222] and Cao *et al*, 2015[231] for example), where hypertension was noted immediately post injection. Therefore, limitation of hypotension might be relevant to mortality reduction: this would require specifically designed experiments for confirmation. Furthermore, it cannot be completely excluded at this stage that 3AB may have induced a minor reduction in mortality.

As regards the relationship with negative inotropy, Paur *et al* (2012) were the first to show that agents which protect against negative inotropy (in their case the β2AR antagonist ICI-118,551) may actually increase mortality[222]. A later study by Shao *et al* (2013) had similar findings, with increased mortality associated with β2AR blockade, though in animals that did survive there was improved cardiac contractility[100]. In this case however, the most obvious explanation is that PARP-1 activation constitutes the main mechanism for negative inotropy.

Turning to cellular inflammatory infiltration of the myocardium, ISO/L-NAME significantly inhibited accumulation of CD68+ cells (monocytes/macrophages). The cause of this reduction may well have been greater maintenance of the diffusion barrier of the glycocalyx[318]. To confirm this, it would have been necessary to measure glycocalyx thickness and/or release into plasma of components of the glycocalyx[96]. This was not done. However, if indeed this is the correct explanation for this finding, it would be expected that an inhibitor of glycocalyx shedding, such as low-dose doxycycline[309] would mimic these findings. It also remains to be determined whether monocyte activation within the myocardium contributed to other biochemical changes observed.

We had postulated that ISO/L-NAME would limit not only NO release, but, consequently, $ONOO^-$ formation. Thus, it would be expected that myocardial content of 3-NT would diminish. In the event, this was significant only when immunoblotting was used, and not with immunohistochemistry (see Figures 6.4A and 6.5A). The most likely cause of this substantial disparity is the presence of two 3-NT-associated bands on Western blotting, as seen in all experiments. It may be that the band with apparent MW 70kDa did not change substantially in intensity, partially masking overall changes when all components of 3-NT are correlated together, as in immunohistochemistry.

Normally, changes in 3-NT formation should have parallel effects on PARP-1 activation and therefore PAR formation[263]. However, PAR content did not change and there is no clear-cut explanation for this finding. The obvious explanation for this is that inhibition of NOS, and in particular of eNOS, acts as a stimulus for increased eNOS expression. Given that the duration of clinical effect of a single dose of L-NAME is only approximately 6 hours[319], increased eNOS expression would thereafter effect rebound increases in nitrosative stress and PARP-1 activation. Certainly, at 24hrs, eNOS expression was markedly increased. Evaluation of changes earlier after NOS inhibition might have clarified this issue.

An intriguing and very exciting finding in this component of the study was a marked increase in myocardial content of TXNIP following ISO/L-NAME treatment (see Figures 6.4C and 6.5B). This carries important mechanistic and consequential implications. First, TXNIP expression is increased by non-laminar flow[268], which would have resulted from glycocalyx erosion. There is also evidence that NO

suppresses TXNIP expression[269]. Irrespective of the cause, TXNIP activates the NLPR3 inflammasome[320], thus potentially contributing to perpetuation of inflammatory changes within the myocardium, and to associated energetic impairment. Finally, TXNIP exerts positive inotropic effects[321], and therefore may have tended to contribute to limitation of negative inotropy. In this regard, it is to be noted that ISO/3AB treatment (see Chapter 5) also increased TXNIP expression. Evaluation of TXNIP contributions in this model would have required use of a specific DNAzyme, as used by Huang *et al* (2016)[322] or Tan *et al* (2015)[323].

Thus, in conclusion, assuming that the results for ISO/L-NAME can be extrapolated to humans, this form of pharmacotherapy would be clinically promising for the early stages of TS. Specifically, increasing blood pressures and decreasing cellular inflammatory infiltration might be pivotal to limiting mortality in the acute stages of the disease. However, further experiments in this rat model are indicated: -

(1) Early, to confirm haemodynamic changes as postulated above

(2) Later, to evaluate effects on recovery of contractility

Finally, the results described in this chapter alter our conception of the cellular pathways involved in TS. First, there is increasing (but incomplete) evidence that hypotension is induced by NOS activation. Second, it appears that negative inotropic changes are most closely related to PARP-1 induced impairment of energetics. Last, the pathophysiology of TS is now increasingly related to vascular dysfunction.

An extension of the pathophysiological concepts involved is detailed in the following chapter.

7 Conclusions and Future Perspectives

7.1 Summary and implications

This thesis was predominantly focused on attempts to delineate (at least to some degree) the biochemical pathways involved in the pathogenesis of TS. The first experimental chapter relates to pilot investigations using post-mortem tissue of patients dying from TS. The following three chapters concern the use of a novel female rat model of TS, and are an extension of results obtained in the initial study.

The results from the initial human post-mortem study (Chapter 3) gave us impetus to pursue nitrosative stress as a key modulator of the characteristic functional impairment and/or inflammatory change associated with this condition. Due to the small numbers of patient samples, limited conclusions can be made from these data beyond this.

Development of the animal model of TS achieved the aim of mimicking the human TS (Chapter 4): - rats treated with a single dose of ISO displayed characteristics of the condition which have been extensively reported clinically, including apical LV dysfunction[72], increased myocardial wall thickness (likely oedematous)[92], inflammatory infiltration[91] and markers of nitrosative stress[300]. Given that nitrosative stress-coupled PARP-1 activation can cause myocardial energetic impairment, we sought to investigate the effect of the PARP-1 inhibitor 3AB in this model (Chapter 5). The addition of 3AB to ISO resulted in significant reductions in LV dysfunction and inflammatory infiltration, without any noticeable effect on mortality. As NO is required for generation of $ONOO^-$, we then utilised the NOS inhibitor L-NAME (Chapter 6), with the aim of reducing both functional impairment and nitrosative stress. In fact, the addition of L-NAME to ISO failed to have substantial effects on LV function compared to ISO alone, despite inducing a stunning amelioration of mortality in this group. Taken together, the data from chapters 4-6 suggest an important role for nitrosative stress in the pathogenesis of TS,

though there may not be a “silver bullet” in addressing the apparent and partial dichotomy between LV dysfunction and survival.

Our results regarding disordered vascular function post-ISO, and various clinical reports (including the first descriptions of TS in the literature[63]) of an association with coronary vasospasm suggest that TS is potentially a combined vascular and myocardial disorder. There is evidence from experiments in other conditions, such as diabetes, that modulation of oxidative stress can be beneficial in restoring vascular function. Low doses of doxycycline, a tetracycline class antibiotic, have been shown to have antioxidant effects in many tissues, including the heart, via inhibition of matrix metalloproteinases. A study in diabetic rats was able to demonstrate that administration of low-dose doxycycline over four weeks ameliorated/prevented vascular endothelial and contractile dysfunction, in conjunction with significantly reducing oxidative stress[324]. The fact that TS has also been associated clinically and experimentally with oxidative stress[98, 230, 234, 325], and now in this thesis implicating nitrosative stress, suggests that some types of antioxidant therapy may be a viable therapeutic pathway. However, it remains to be determined whether such therapy should be targeted specifically at nitrosative stress.

7.1.1 Mechanistic implications

To date, there is no consensus regarding the pathogenesis of TS, other than the involvement of catecholamine release as a stimulus. Our overall aim with the experiments contained in this thesis was to, even to some small degree, increase the understanding of the mechanisms by which TS is induced, and how the myocardial damage is engendered. Broadly, our results reaffirm the central role of catecholamines and inflammation in the pathogenesis of TS.

Reviewing the literature and incorporating our own findings, we have created a proposed pathogenic mechanisms schematic (Figure 7.1). As with many of the other animal models of TS, and clinical reports from the literature, the “cascade” begins with catecholamine administration or release

(experimentally through bolus dose administration[222, 234]) and clinically seen as an acute stress response[72, 76]. From there, activation of α- and βAR occurs in response to catecholamines, with apparent paradoxical resultant impairment of inotropic status. Catecholamines, through αAR or βAR-mediated actions on mitochondria, stimulate increased O_2^- production and oxidative stress[244, 246]. Another outcome of βAR stimulation is NOS activation, with both β_2- and β_3AR linked to NOS[211, 257, 261, 326], while other inflammatory stimuli have been shown to increase iNOS expression[254]. In the presence of increased O_2^- and NO production, there is spontaneous $ONOO^-$ formation[255], which we believe to be a crucial point in the biochemical cascade caused by a catecholamine "surge".

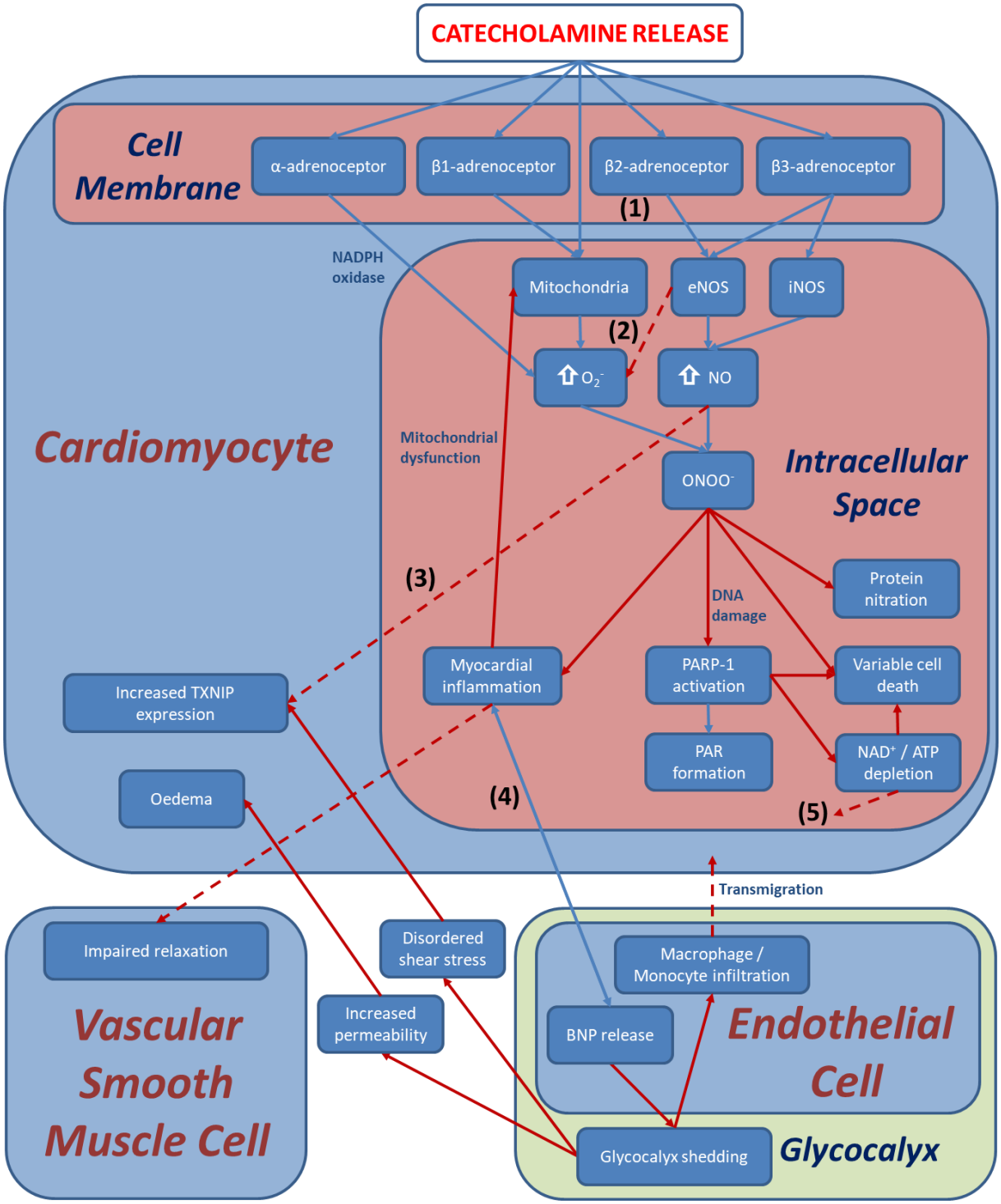


Figure 7.1. Proposed pathway schematic for the pathogenesis of TS: integrated schema incorporating all results from this thesis.
(1) = Biased Gi-mediated post-receptor signalling
(2) = Potential eNOS "uncoupling"
(3) = Diminution in NO availability favours TXNIP expression
(4) = Relationship between inflammation and BNP release is bidirectional: BNP suppresses inflammation, while inflammation induces BNP formation/release
(5) = Negative inotropic effects

From this point on, our findings lead us to believe that oxidative and nitrosative stress result in the myocardial dysfunction associated with TS, reflecting (*inter alia*): glycocalyx shedding[96], impaired cardiac energetic function[102, 272], oedema and myocardial inflammation[92]. The finding that PARP-1 inhibition in our model resulted in limitation of functional impairment suggests that PARP-1 activation and subsequent NAD^+/ATP depletion in the production of PAR[264] suggests energetic impairment may play a crucial role in inotropic status in the acute phase of TS. On the other hand, non-selective inhibition of NOS resulted in amelioration of ISO-induced mortality in our model, which provides evidence that NOS activation plays a critical role in survival. To that end, our results add significantly to the literature regarding TS pathogenesis, and as with other recent results[234] suggest a crucial role for oxidative and nitrosative stress.

7.1.2 Therapeutic implications

These will be reviewed in greater detail below. In Figure 7.2, I provide a repeat concept diagram of the pathogenesis of TS, coupled to potential therapeutic modalities at myocardial and vascular levels, together with agents to avoid.

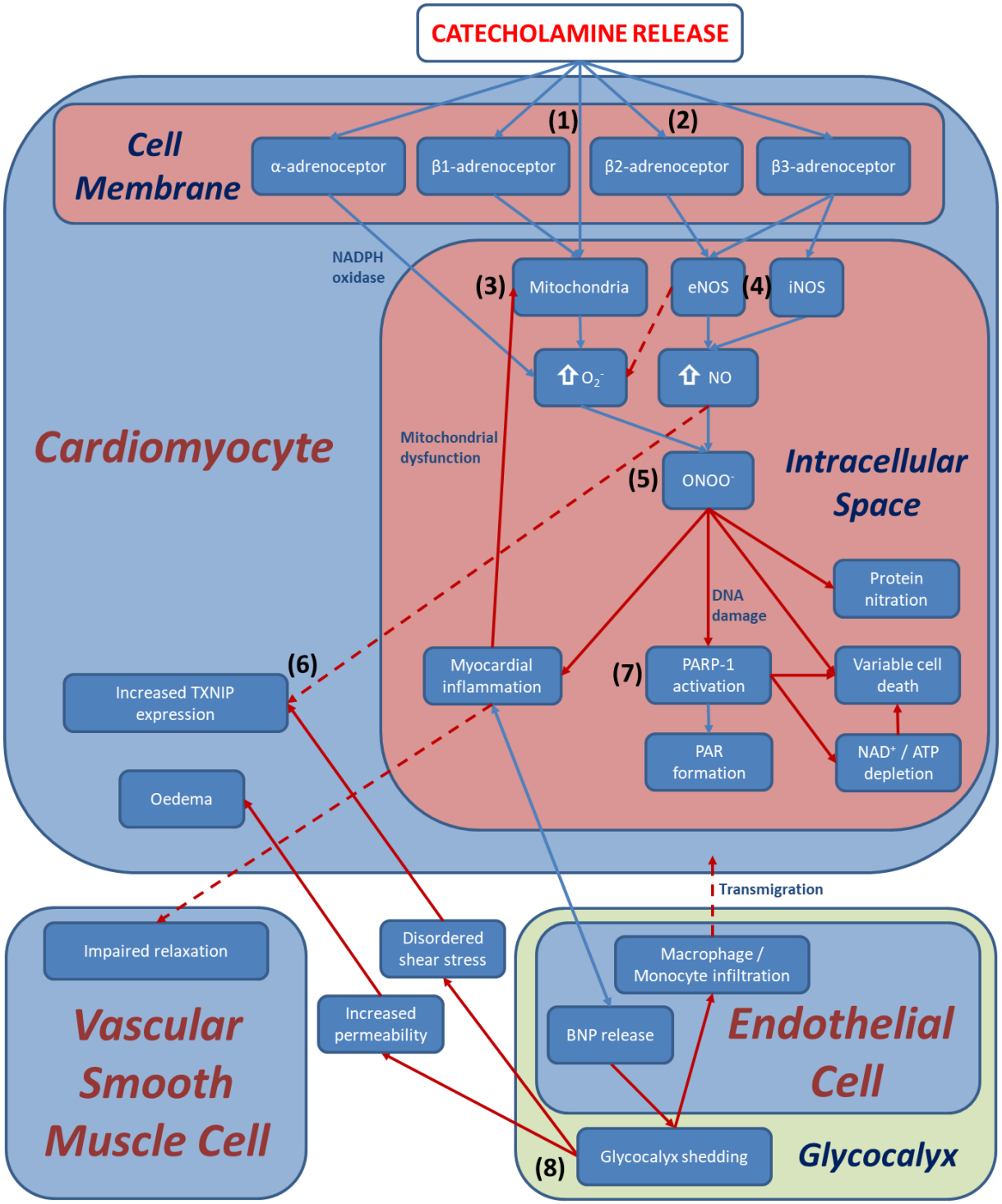


Figure 7.2. Proposed pathogenic mechanisms schematic, coupled to potential therapeutic modalities at myocardial and vascular levels, together with agents to avoid. + = potentially useful, - = potentially harmful
(1) = β-adrenoceptor agonists (-)
(2) = Selective β_2-adrenoceptor blockade (+ and -)
(3) = Mitochondrial antioxidants (+)
(4) = NOS inhibition (+ and -)
(5) = $ONOO^-$ decomposition catalysts (+)
(6) = Verapamil / diltiazem (not acutely, +)
(7) = PARP-1 inhibition (+)
(8) = Metalloproteinase inhibitors (+)

7.2 Alternative investigations with this model

These results outlined above certainly show not only the robustness of this ISO-induced rat model of TS, but also its utility in the testing of potential therapeutics. There are of course many further experiments that we would have liked to have embarked on, given the time and/or resources. This section will briefly discuss what we might have done with this model of TS.

7.2.1 Further pathway delineation

Building on results from Chapter 5, the measurement of myocardial energetics in these animals would have added substantial clarity to understanding the role of PARP-1 activation, and its role in the impairment of systolic function. To achieve this, measurement of ATP and PCr/ATP ratios would have given us greater insight into high energy phosphate use after catecholamine surge, and to what extent this plays a role in the pathogenesis of TS. The measurement of PCr/ATP ratio has been previously performed in rats[327], and more significantly, in TS patients, who displayed impaired cardiac energetic status[102, 272]. In one study phosphocreatine/gamma-adenosine triphosphate ratio (PCr/γATP) ratio was obtained non-invasively by 31P-magnetic resonance spectroscopy and showed not only impairment at acute presentation, but also an abnormal result at 4-month follow up[102]. In another study with a mean follow up time of 20 months, patients still displayed disordered cardiac energetics, compared to age-, sex- and comorbidity-matched patients, suggesting the development of persistent heart failure phenotype[272].

The next experiment that would have added substantially to our understanding of TS from this rat model is the addition of specific β_2-/β_3AR blockade. Our data, and those of other groups, have shown that catecholamines act on βAR and initiate the TS. Experimentally, β-blockade has had mixed results: - One study showed improvement in haemodynamics with pre-treatment using a specific β_2-blocker[231] and another showed pre-treatment with non-selective and β_1-blockers improved survival[229], while selective β_2AR blockade with ICI-118,551 in two other studies was shown to reduce the functional impairment caused by catecholamines, but also to substantially increase mortality[100, 222]. Clinically, it

appears that β-blockers are not useful in limiting in-hospital acute mortality[328] or recurrence[126]. It remains possible that the use of selective β-blockers may be beneficial and merit further investigation.

Although an expected result to some extent, the clear association of nitrosative stress with TS in both patient samples (Chapter 3) and in our rat model (Chapters 4-6) has wide-reaching implications in elucidating the pathogenesis of TS. Furthermore, in proinflammatory conditions, such as those described in TS, production of O_2^- and NO can increase significantly, which results in enormous elevation of $ONOO^-$ formation[329]. The use of a peroxynitrite decomposition catalyst (PNDC) would give great insight into the specific role of $ONOO^-$-mediated nitrosative stress, and if PNDCs could be useful in the treatment of TS. Positive results from experimental work with PNDCs, such as the finding that neutralisation of $ONOO^-$ resulted in prevention of myocardial dysfunction and inflammation in endotoxemic rats[330], suggest that they may indeed be worthy of further assessment. Although there are currently no clinically-available PNDCs, there is ongoing substantial research and development occurring in this area. For a more detailed review of available PNDCs and their therapeutic potential see Slosky *et al*, 2015[329].

Finally, for a more complete understanding of the results we found during these experiments, selective blockade of TXNIP would have proven to be useful. We found increased myocardial accumulation of TXNIP in the hearts of our TS rats after catecholamine exposure (see Chapter 4). TXNIP is an endogenous inhibitor of the antioxidant thioredoxin, an inflammatory mediator, and critical in the regulation of glucose. TXNIP may also suppress NO generation and is known to activate the NLPR3 inflammasome[320]. It thus represents a possible contributor to prolongation of inflammatory responses post initial catecholamine release (currently unexplained). As calcium antagonists such as verapamil and diltiazem suppress TXNIP expression, these should be considered for long-term treatment following TS. Experimentally, investigation of the role of TXNIP in the model could be achieved utilising a TXNIP DNAzyme, as previously mentioned, such as in Tan *et al* (2015)[323]. For a more comprehensive review of TXNIP and its role in health and disease, please see Chong *et al* (2014)[268].

7.2.2 Time course delineation

One of the major questions that these experiments left unanswered was in relation to the time course of TS, and specifically what is occurring the acute and sub-acute phases. Although minor dose-ranging and time-course investigations were made before beginning these experiments, they were only based on echocardiography and would not have represented a true indication of the peak of myocardial damage/dysfunction. Clinically, little is known about the pathogenesis of the ultra-acute phase, with the inherent difficulties of collecting data during this phase, though there may be opportunities with "secondary TS" associated with medical or surgical emergencies for example where patients would already be in hospital. This means that for the most part animal models are the most attractive option for investigating the earliest phase of TS, and it would be pertinent to compare data from our 24-hour timepoint to that of earlier time points, for example, within the first hour and at approximately two and six hours post catecholamine exposure. An excellent review of the available literature in 2014 attempted to delineate the time-course of TS, however the data available at that stage were from studies that generally only investigated short time points <3 hours[331]. Most significantly, in relation to our findings, the impact of nitrosative stress and/or inflammation was not investigated here.

Our results with two potential therapeutic options (Chapters 5 and 6) also raise questions regarding timing and length of therapy. As our putative therapeutic agents were given 30 minutes prior to catecholamine exposure, we cannot definitively say that PARP-1 or NOS inhibition reverses the damage/dysfunction caused. Clinically, this is of course of critical importance, as there is no way to predict attacks of TS and limit the damage caused by them. Another issue, briefly touched on in Chapter 6, is the length of time during which these agents exert their beneficial effects. Specifically, would continuous infusion result in better outcomes at the 24-hour time point that we investigated?

With these questions in mind, it then follows that an attractive option for investigation during this early time point and with different therapeutic modalities is a working heart model. To date (and to our knowledge) there have not been any studies involving a working heart model of TS. The closest appears to be that of Willis *et al* (2015)[230]. The researchers in this study used the Langendorff apparatus

to mount rat hearts two weeks after ISO overdose and study myocardial oxygen consumption and myocardial performance index in response to different doses of ISO. The results showed that ISO-overdose hearts had a limited response to ISO, displaying an apparent inefficient coupling between contraction and energy production, which suggests that these "*hearts required more respiratory activity to generate contractile force at any given ISO concentration*"[230]. This certainly appears to be an area of substantial opportunity.

7.3 How could this be coupled with future clinical investigations?

To date there are no reported data from controlled clinical trials involving patients with TS. A combined view of the currently presented animal data and the available clinical data suggest the following categories for future controlled clinical investigation: -

1) <u>Prevention/treatment of early hypotension/shock:</u>

   Shock represents the main cause of in-hospital death for TS patients and its cause is uncertain. It is increasingly likely that it represents fluid extravasation due to glycocalyx shedding. Particularly since catecholamine administration is inappropriate in this circumstance, three options may be beneficial: -

   i) limitation of glycocalyx shedding (e.g. doxycycline)

   ii) stabilisation of vascular reactivity to NO (e.g. N-acetylcysteine[332])

   iii) NOS inhibition

2) <u>Limitation of myocardial damage:</u>

   Following the results of the experiments in Chapter 4, clinical trials of PARP-1 inhibitors seems appropriate

3) <u>Acceleration of resolution of vascular and myocardial inflammation:</u>

   It is now established, especially from the work of Dawson *et al* (2015), that episodes of TS are followed by energetic impairment lasting at least four months[102], with ongoing contractile dysfunction[94], and possibly some myocardial fibrosis[272]. It is possible that the initial

catecholamine “surge” results in longstanding impairment of catecholamine uptake into the synaptic cleft. Irrespective of the cause, there seem to be two options therapeutically: -

i) Prolonged treatment with NO-potentiating anti-inflammatory agents, such as ACE inhibitors

ii) Treatment with agents which improve myocardial energetics without intersecting directly with PARP-1 (e.g. mitochondrial anti-oxidants, CPT-1 inhibitors)

7.4 Major concerns/limitations

7.4.1 The rat model

To begin this section of the thesis, there must of course be discussion of the rat model, and some of the inherent issues therein. The specific problems with the model are: -

a) The relatively low ISO dose compared to other models. This was out of necessity in fact, as during initial dose-ranging experiments, higher doses of ISO were associated with unacceptably high mortality in our aging female rats. The question arises though if myocardial damage is related to catecholamine dose size.
b) Single echocardiography time-point. It would have been beneficial, certainly when assessing the impact of our two potential therapeutic modalities, to investigate the time-course of LV dysfunction.
c) Intraperitoneal injection. Is this route of administration the most effective in initiating TS-like changes in rats?
d) Administration of therapeutics before ISO. This scenario is not clinically relatable and was used due to technical issues with administering ISO first, followed by the chosen therapeutic.
e) Radial strain is not the most accurate analysis available. The use of radial strain was favoured over global longitudinal strain or strain rate, for example, due to the quality of images

acquired. Future investigations utilising contemporary echocardiography systems, or even small animal MRI would add significantly to the reliability of the results obtained.

f) Tissue harvesting methods. For immunohistochemistry, hearts were cut circumferentially to separate apex from base. In this technique, there is potential for "mid-LV" tissue to either be assigned to apex or base. Perhaps a better technique would involve cutting hearts longitudinally, which would allow for visualisation of potential accumulation gradients within the myocardium.

g) Impact of specific anaesthetic agents. In these experiments, we have utilised isoflurane as the anaesthetic given to rats before/during echocardiography. Redfors *et al* (2016) studied the effect of different anaesthetic agents in ISO treated rats, with the main finding that isoflurane was cardioprotective vs pentobarbital or ketamine[226]. Intriguingly, Oras *et al* (2017) also found that isoflurane was cardioprotective, with the added finding that it reduced NO and ROS levels in macrophages when compared to propofol-anaesthetised rats[233]. For a more complete review of catecholamine-induced rat models please see Appendix 1.

Despite these limitations, our rat model has been shown to be effective in mimicking the clinical characteristics of TS, allowing us to confidently relate results from the experiments in this thesis to TS in humans.

7.4.2 Partition of the vascular from the myocardial components of TS

It is clear from the current experiments that TS is associated with: -

1) myocardial inflammation
2) vascular endothelial dysfunction

The latter is paradoxical in the sense that during recovery patients exhibit supranormal NO signalling[283]. However, in the current study, only thoracic aortic vascular smooth muscle was evaluated. In vivo, there will be complex interactions between coronary reactivity and myocardial

contractile status, governed by coronary autoregulation and by the Gregg phenomenon[333]. These interactions are difficult to evaluate, and remain to be formally considered in the rat model.

One type of experiment to consider in this regard would be examination of contractile function in paced papillary muscles: this would remove both heart rate and coronary flow effects from consideration of contractility. Another potential option would be ultrathin myocardial slices, as used by Watson *et al*, 2019, which combine "the benefits of a complex multicellular three-dimensional preparation with the simplicity of acquiring structural and functional data from a two-dimensional monolayer"[334].

7.4.3 Does the therapeutic approach have to be mechanism specific?

Throughout this thesis, mechanistic studies have been performed, and the results are easily translated into potential therapeutic options (see Figure 7.2). However, it is conceivable that valid therapeutic options for TS might be only incidentally related to pathogenesis.

For example, the documented energetic impairment in the TS myocardium[94, 272] probably reflects PARP-1 effect. However, energetics may be improved by other approaches, such as CPT-1 inhibition with perhexiline, which activates a "Randle shift" in myocardial metabolism[335] resulting in increased ATP generation from carbohydrates.

7.4.4 What about the association with cancer?

The association of TS with cancer has been recognised for a long time (relative to first reports of TS) and is well documented in the literature[336-338]. What is not yet well understood is the connection between the two, and to what extent they interact with each other. There has been suggestion that the presence of cancer may actually lower the threshold for stress stimuli and/or may heighten cardiac adrenoceptor sensitivity. Intriguingly, a retrospective study of 154 TS patients, of whom 44 had cancer, showed that malignancies appeared to significantly enhance neurohormonal activation and the

inflammatory response during the acute phase of TS[336]. Analysis of these data also revealed that the presence of malignancies were an independent predictor of early cardiac death and overall mortality during follow-up. Myocardial inflammation has consistently been found to be associated with TS, and could represent a potential link between these two conditions, as cancers are known to stimulate inflammatory responses[336]. There remain, however, many unanswered questions relating to this association which warrant further research.

7.4.5 How/why do psychiatric illnesses predispose?

Similar to the aforementioned association of TS and cancer, there has been an increasing number of reports linking psychiatric illness and TS[339]. In a literature review on the topic Nayeri *et al* (2018) found that when controlling for age and sex, there is a higher prevalence of pre-existing psychiatric illness in TS patients compared to controls with acute coronary syndrome[340]. Of these illnesses, both anxiety and mood disorders were linked to TS, but association with other psychotic and schizophrenic-spectrum disorders remains unknown. There have been reports that administration of both serotonin-norepinephrine reuptake inhibitors and selective norepinephrine reuptake inhibitors have been the trigger for TS, suggesting the endogenous increase in serum epinephrine as the pathogenic mechanism[340]. Although this fits with the catecholamine-triggered TS hypothesis, it does not completely explain the association, and would certainly benefit from further investigation.

Of note, a recent short communication from Hiestand *et al* (2018) investigated the presence of structural abnormalities in the brain of patients with TS, utilising MRI. They found anatomical differences between TS patients and healthy control subjects in the limbic network, influential in the control of emotional processing, cognition and the autonomic nervous system. As this was a cross-sectional study, cause and effect cannot be elucidated, however, this provides an interesting pathogenic mechanism.

7.4.6 Type 2 error

One of the major weakness of most forms of animal experimentation is the evaluation of inadequate numbers to see the "full picture". In the current study, all sample size calculations related to changes in apical radial strain, which itself was only chosen as primary end-point because of its considerable reproducibility.

Conceptually, it would have been far better if 3-NT expression were the main end-point in Chapter 4, PCr/ATP ratio in Chapter 5 and again 3-NT in Chapter 6. This was not done.

However, the issue of Type 2 error most obviously requires consideration regarding: -

1) Mortality effects of 3AB
2) Inotropic effects of L-NAME

7.4.7 How important is monocyte/macrophage infiltration?

It is known that monocyte/macrophage infiltration is a predictable consequence of glycocalyx shedding[293]. In turn, this cellular inflammatory infiltrate may theoretically modulate inflammatory activation in surrounding myocardium, either positively or negatively[341]. This was not investigated in the current studies: the issue is important because it relates ultimately to the potential utility of doxycycline and other inhibitors of glycocalyx shedding.

7.5 Final conclusion

The experiments that underpin this thesis help to inform understanding of the pathogenesis of TS, and also offer substantial potential for therapeutic advancement. There are understandably limitations to the work, frankly summarised above, but publications arising from this work represent a major advance in the literature, as the first reports of nitrosative stress in association with TS. In a condition which currently has no definitive therapeutic strategy, the findings from this set of experiments and the conclusions drawn from them certainly present a number of viable and exciting research opportunities for the future.

Appendix

Table 1. Chronological summary of studies of Takotsubo Syndrome utilising (i) animal models and (ii) pharmacological stimuli for development of TS

Study, **Species (sex),** **Agent used (Dose)**	**Intervention**	**Results**
Izumi *et al*, 2009[221] Monkey (M) Epinephrine (2 x 10µg/kg/min)	β-blocker (Metoprolol)	- β-blocker metroprolol administration after epinephrine improved LVEF and diminished epinephrine-induced cardiomyocytolysis in monkeys killed 24hrs post epinephrine. - 9/25 monkeys died during/shortly after first or second epinephrine infusion. VF was the most common cause of death with haemorrhage though tracheal cannula, pulmonary oedema and pulmonary haemorrhage found during autopsy. - A subset of epinephrine + metoprolol or epinephrine + saline monkeys were killed 30 days post epinephrine, showing that LV dysfunction was reversible in both groups.
Paur *et al*, 2012[222] Rat (M) Epinephrine (78.4µg/kg) Norepinephrine (1.43 $\times 10^{-7}$ mol/100 g)	- Gi protein inhibitor (Pertussis toxin) - p38 MAPK antagonist (SB203580) - β_2AR antagonist (ICI-118,551) - β-blocker (propranolol) - β-blocker (carvedilol) - Calcium sensitiser (levosimendan)	- Main finding was biased agonism of epinephrine only, not norepinephrine, for β_2AR-Gs at low concentrations and for Gi at high concentrations. - Apical cardiomyocytes had increased β_2- to β_1AR ratio and were more responsive to adrenergic stimulation. - Pre-treatment with pertussis toxin (Gi protein inhibitor) abolished negative epinephrine effect. - Echo parameters recorded every five minutes post-catecholamine for 60min giving time course of LV dysfunction – initiated ~15min, reaching a nadir between 20-25min. - Epinephrine alone mortality was ~45%, ~90% for SB203580, 100% for ICI-118,551 and 0% for levosimendan. Death occurred early within

		10min, caused by cardiogenic shock rather than primary VF. - ICI-118,551 treatment resulted in less negative inotropy with increased mortality, levosimendan (cAMP-independent inotrope) however reversed dysfunction without increased mortality.
Shao *et al*, 2013[224] Mouse (?) Isoprenaline (400mg/kg)		- ISO induced TS-like dysfunction on echo two hours post injection. Function was normalised at one-week post injection in surviving animals. - Patchy fibrosis noted at 10 days post ISO. - Severe lipid accumulation at two hours post ISO, normalised at 10 days. - Lipotoxicity was closely associated with catecholamine-induced myocardial dysfunction. - Mortality ~39% in wild type mice at one-week post ISO. There was significantly lower mortality in mice overexpressing ApoB lipoprotein, ~27%.
Shao *et al*, 2013[223] Mouse (?) Isoprenaline (400mg/kg)	Adenosine	- Global and posterior regional wall function were significantly better in adenosine-treated mice two hrs post-ISO. - Echo at baseline, 2hrs, 24hrs and every day until day 10, with the beneficial effect of adenosine vs isoprenaline alone present until day four post-ISO. - Fibrosis and intramyocardial lipid content reduced in heart tissue of adenosine pre-treated mice compared to ISO.
Shao *et al*, 2013[100] Rat (?) Isoprenaline (25, 50, 100, 150, 300, 450 or 600 mg/kg)	- Gi-protein inhibitor (pertussis toxin) - β_2AR antagonist (ICI-118,551) - β_2AR agonist (clenbuterol)	- Continuous echo for first 120min post-ISO showed peak LV dysfunction at ~80min, plateauing after. - β_2AR blockade or Gi-pathway inhibition associated with less akinesia but higher acute mortality. - 7-day mortality was 50% in rats receiving 50mg/kg ISO alone, 0% within 2 hours. - Cardiac function and fibrosis normalised in surviving rats at 10 days post-ISO.

		- Severe lipid accumulation was present in all akinetic rat LV segments, with little intracellular lipid after recovery. These findings were consistent with patient biopsy samples. - Intracellular glycogen stores were depleted in akinetic rat LV segments.
Redfors *et al*, 2014[228] Rat (M) Isoprenaline (50mg/kg)		- Contrast echocardiography was performed at baseline and repeated at 5, 10, 20, 30, 40, 50, 60, 70, 80, and 90 min post-ISO. - Apical perfusion was not impaired in the early phase of TS in this rat model. - Histological investigation showed no structural damage of myocardial vessels.
Redfors *et al*, 2014[225] Rat (M) Dopamine (25mg/kg), Isoprenaline (50mg/kg), Phenylephrine (1mg/kg), Epinephrine (1mg/kg), Norepinephrine (1mg/kg)	- Phenylephrine - Hydralazine - Nitroprusside	- All catecholamines induced TS-like regional dysfunction on echocardiography 90min post-injection. - ISO resulted in low BP + apical dysfunction, all other catecholamines resulted in high BP + basal dysfunction. - Hydralazine/nitroprusside infusion caused decreased BP and switch to apical dysfunction in epinephrine/norepinephrine treated rats. - Phenylephrine administration with ISO increased BP and prevented apical dysfunction. - Mortality within 90min stated for each dose of catecholamine in titration studies. 0% for chosen dose of ISO (50mg/kg).
Sachdeva *et al*, 2014[229] Rat (M) Isoprenaline (25, 50, 85, 100, 170, 200, 400, and 600 mg/kg)	- β-blocker (propranolol) - β_1-blocker (metoprolol)	- Pre-treatment with β-blockers improved survival but did not affect structural and functional alterations. - Mortality in dose ranging study was 42% within 24hrs post-ISO. At selected dose of 100mg/kg in second protocol, ISO mortality was 40% within 24hrs. - Histology and electron microscopy showed necrosis, sarcomeric disruption and disarray,

		inflammatory cell infiltration, collagen deposition and profuse oedema in myocardium.
Redfors *et al*, 2014[226] Rat (?) Isoprenaline (50mg/kg)	K_{ATPm} channel blocker (glyburide)	- Main finding was that isoflurane was cardioprotective vs pentobarbital or ketamine. - This cardioprotection was not mediated via K_{ATPm} channels.
Redfors *et al*, 2015[227] Rat (M) Isoprenaline (50mg/kg)	-Norepinephrine -Phenylephrine	- Using the same TS model as in previous studies by this group vs an AMI rat model. - Main finding was that vasopressor treatment was associated with increased mortality in TS.
Willis *et al*, 2015[230] Rat (M) Isoprenaline (67mg/kg)		- Mitochondrial dysfunction and oxidative stress were associated with ISO injection. - Mortality ~20% in first 48h, none from 48h to 4wk end point. - Echo at 2-weeks showed systolic and diastolic dysfunction which had resolved by follow-up 4-week timepoint. - Ex vivo studies showed impaired contractile-metabolic coupling during physiological β-adrenergic stimulation. - In vitro studies showed no change in cAMP or PKA activity in ISO treated cells compared to controls, as well as dysfunctional systolic and diastolic Ca^{2+} handling.
Cao *et al*, 2015[231] Rat (F) Epinephrine (8.56×10^{-8} mol/100 g (156.8ug/100g))	-β_2AR antagonist (ICI118,551) -β_2AR agonist (clenbuterol) -estradiol	- Model using epinephrine alone or with sham surgery or ovariectomised rats. A catheter was placed into LV to assess haemodynamics. -H&E staining showed no structural change in epinephrine treated rats compared to sham or ovariectomised alone groups. - Lack of estrogen resulted in more serious cardiac dysfunction and injury. - Estrogen supplementation improved haemodynamic inhibition.

		- Pre-treatment with ICI118,551 returned all the hemodynamic indicators to the baseline level. - Estrogen increased the plasma concentration of cAMP and the phosphorylation of PKA, with plasma cAMP, estrogen, epinephrine and norepinephrine measured by ELISA, while Gαs and Gαi, PKA and phosphor-PKA measured by western blot.
Oras *et al*, 2017[233] Rat (M) Isoprenaline (50mg/kg, 100mg/kg)		- Cardioprotective effects of isoflurane vs propofol or ketamine + midazolam. - Proteomic analysis showed up-regulation of pathways involved in inflammation, coagulation, endocytosis and lipid metabolism. - LV ejection fraction was higher, and extent of LV akinesia was lower with isoflurane, when compared with the propofol and/or the ketamine + midazolam groups. - Echo at 90min then hearts removed. - Experiment 1: 10% mortality pre-echo (1 animal in isoflurane group, two animals each in other anaesthetic groups). Experiment 2: 9% mortality (one animal in each group). - NO and ROS levels in macrophages were decreased in isoflurane treated rats compared propofol group.
Oras *et al*, 2017[232] Rat (M) Isoprenaline (50mg/kg)	Isoflurane	- Early treatment with isoflurane attenuates the LV akinesia and improves survival. - Echo performed at 90 minutes post-ISO and at 48 hours. - Stroke volume, cardiac output and LV ejection fraction were higher in the early treatment groups vs. controls. - Overall mortality 56% at 48hrs, 20% in pre and 30min isoflurane groups and 33% in 10min isoflurane group. ISO alone (control) and longer isoflurane associated with higher mortality (86%).

Zhang *et al*, 2017[234] Rat (M) Isoprenaline (50mg/kg)	Sodium hydrosulfide (NaHS)	- Intervention with NaHS reversed dysfunction and ISO-induced oxidative stress. - Catheter inserted into LV was used to record haemodynamics consecutively for 150min post-ISO with echo prior and for first 150min post ISO. - NaHS pre-treatment reduced malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) concentrations in plasma and myocardial tissue. - H&E staining showed no structural differences in ISO vs control tissue. - ISO alone mortality ~35%, ameliorated with 100umol/kg NaHS. - NaHS inhibited cell apoptosis in the cardiac tissue of TS rats. - Long term NaHS showed decreased H_2O_2 and MDA levels, and increased GSH and SOD levels compared to ISO alone.
Kolodzinska *et al*, 2018[235] Rat (F) Isoprenaline (150mg/kg)		- Investigation of time course of Toll-like receptors (TLRs) and inflammatory cell infiltration, along with apoptosis in endothelial cells. - Hearts collected 24, 48, 72 hours and seven days. - At 24hrs necrosis, cardiomyocyte vacuolisation, lipid accumulation, mononuclear cell influx, mitochondrial damage and oedema were seen. - At 8 days there was myonecrosis, interstitial spaces filled with collagen, inflammatory cell infiltration, intramyofiber and interstitial oedema, and marked vacuolization was seen. - TLR2, TLR4, TLR6 protein expression was up-regulated in cardiomyocytes, fibroblast-like cells, CD68-positive cells and mononuclear cells. - No significant differences in plasma concentration of epinephrine and norepinephrine in control and TS rats. - No associations between catecholamine concentration and TLR or apoptosis expression.

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