



UNIVERSITY OF CAPE TOWN
IYUNIVESITHI YASEKAPA • UNIVERSITEIT VAN KAAPSTAD

**The Role of Melatonin
in Red Wine-induced Cardioprotection**

by

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ABBREVIATIONS

2D	2 dimensional mode
3,4,9,5 trihydroxy-trans-stilbene	Resveratrol
5-MCA-NAT	5-methoxy-carbonylation-N-acety tryptamine
α	alpha
$\alpha\beta$	alpha-beta
ADP	adenosine diphosphate
AKt	protein kinase B
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AP-1	activation protein-1
ASMT	s-acetylserotonin-O-methyltransferase
ATP	adenosine triphosphate
β	beta
BAX	Bcl-2-associated X protein
Bcl ₂	B-cell /Lymphoma 2
BKCa	voltage-gated potassium channels
BMI	body mass index
BSA	Bovine Serum Albumin protein
Ca ⁺⁺	calcium
CAD	coronary artery disease
cAMP	cyclic adeno
CAT	catalase
CF	coronary flow
CHD	coronary heart disease
COX-2	cyclooxygenase-2
CVD	Cardiovascular disease
DF	developed force
DRW	dealcoholised red wine
DRY	conserved stretch residue glutamic acid/aspartic
E β ₁	estrogen receptor
E β ₂	estrogen receptor
EF	ejection fraction
ECL	enhanced chemiluminescents
eNOS	endothelial nitric oxide synthetase
ERK	extracellular regulated kinase
ET-1	endothelial-1
FMD	flow mediated dilatation
FOXO	forkhead transcription factors
FS	fractional shortening
GDP	guanine diphosphate
GIT	gastrointestinal system
GPR150	G protein coupled receptor
GPX	glutathione peroxidase
GSK	glycogen synthase kinase
H ₂ O ₂	water
HDL	high density lipoproteins
HF	heart failure
HPT	hypertension

HR	heart rate
HRP	horseradish peroxides
ICAM	intracellular adhesion molecule
IHD	ischemic heart disease
IKK	I κ Bkinase
IL-1	interleukin-1
iNOS	inducible nitric oxide synthase
I/R	ischemia reperfusion
IVS	intraventricular septum
JAK2	janus kinase 2
JNK	c-jun-n-terminal kinase
K _{ATP}	potassium adenosine triphosphate channel
KHB	krebs henseleit buffer
KO	knockout
LDL	low-density lipoproteins
LKB1	liver kinase B1
LVDP	left ventricular developed pressure
LVEDP	left ventricular end diastolic pressure
LVESP	left ventricular end systolic pressure
LVH	pathological left-ventricular hypertrophy
LVIDd	left ventricular internal dimensions diastole
LVPw	posterior wall
MAPK	mitogen-activated kinase
MCP-1	monocyte chemotactic protein
MEK	mitogen extracellular kinase
MEK1	protein kinase 1
MEK2	protein kinase 2
MI	myocardial infarction
MMPs	metalloproteases
MnSOD	manganese superoxide dismutase
mPTP	mitochondrial permeability transition pore
MT1	melatonin receptor 1
MT2	melatonin receptor 2
MT3	melatonin receptor 3
mtor	mammalian target of rapamycin
NFK β	nuclear factor kappa β
NIH	U.S. National Institutes of Health
NO	nitrogen oxide
NRY	encodes for Gprotein coupled receptor conserved amino acid
ONOO	peroxynitrite-
oxLDL	oxidised low density lipoproteins
P38	mitogen activated protein kinase
PAI	plasminogen inhibitor 1
PI3K	phosphoinositide-3-kinase
PKC ϵ	protein kinase C
PMN	polymorphonuclear leukocytes
PTCA	percutaneous transluminal coronary angioplasty
PVC	premature ventricular contraction
PVDF	polyvinylidene fluoride
QR2	quinone reductase 2
RISK	reperfusion injury salvage
ROS	reactive oxygen species
RZR/ROR	termed retinoid receptors
SAFE	survivor activating factor enhancement
SAMP8	senescence-accelerated-prone

SDS	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error mean
SHR	spontaneous hypertensive rats
STAT-3	signal transducer and activator of transcription
TBS	tri-buffered saline
TNF	tumour necrosis factor
TNF α	cytokine tumour necrosis factor alpha
TNFR1	TNF receptor 1
TNFR2	TNF receptor 2
TPA	plasminogen activator
TRX	thioredoxin
TTC	triphenyltetrazolium chloride
UK	United Kingdom
US	United States of America
VCAM	vascular adhesion molecule
VF	ventricular fibrillation
VLDL	very low-density lipoproteins

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Abstract

Introduction: Several epidemiological studies have suggested that the regular moderate consumption of red wine confers cardioprotection. However, the exact components in red wine that confer this effect are unclear. Previous studies performed in our laboratory suggest that neither the well-known polyphenol resveratrol nor alcohol (12%) present in red wine contribute to the cardioprotective effect of red wine when administered chronically, on their own. Therefore, other compounds present in red wine may contribute to its beneficial effects.

Aims: The aim of the study was to explore whether melatonin, recently discovered in red wine, may play a vital role in red wine-induced cardioprotection. Furthermore, we propose that the protective effect may be mediated via the activation of the survivor-acting factor enhancement (SAFE) pathway that involves two integral components, tumour necrosis factor α and signal transducer and activator of transcription 3 (STAT3).

Methods: The drinking water of either male Wistar rats, TNF KO or STAT3 KO mice and their wild-type littermates were supplemented with a French Cabernet Sauvignon (12% alcohol by volume) or melatonin (75ng/l) to a final concentration corresponding to the concentration found in two glasses of wine per day. After 14 days of treatment hearts were subjected to an *ex vivo* or an *in vivo* ischemia reperfusion insult or to a permanent ligation of the left descending coronary artery as a model of ischemic heart failure. Functional parameters and infarct size were assessed.

Main Findings: The chronic moderate consumption of red wine for 14 days reduced infarct size from $60\pm 2.3\%$ to $23.3\pm 1.8\%$ after ischemia reperfusion injury, $p < 0.001$. The pretreatment with melatonin protected to a similar extent as red wine given on its own (infarct size of $20.1\pm 1.7\%$, $p < 0.001$). Interestingly, the cardioprotective effect of red wine was partially abolished with prazosin, a melatonin receptor 3 inhibitor ($40\pm 0.9\%$, $p < 0.001$ vs. wine). Furthermore, the cardioprotective effects of regular moderate consumption of red wine or melatonin were abolished in STAT3 KO and TNF KO mice.

In a model of ischemic heart failure melatonin improved ejection fraction and fractional shortening compared to plain drinking water control in wild-type mice ($p < 0.001$). The

protective effect of melatonin was not abolished in TNK KO and STAT3 KO mice ($p < 0.001$ vs. control), therefore suggesting that the long-term treatment with melatonin in ischemic heart failure protects independently of the SAFE pathway.

Conclusion: Our novel findings suggest that the moderate regular consumption of red wine (equivalent to 2-3 glasses per day) confers cardioprotection, in part due to its melatonin content. The protective effect against ischemia I/R injury of both melatonin and red wine is mediated via the activation of melatonin receptor 3 and the activation of the SAFE pathway while the protective effect of melatonin against ischemic heart failure is independent of the SAFE pathway. Melatonin is a safe, cheap and easily accessible drug that may be considered as an innovative therapeutic agent in the treatment against acute myocardial infarction and ischemic heart failure.

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A. Introduction

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1 Prevalence of cardiovascular disease (CVD) worldwide

CVD is a broad term that describes diseases affecting the heart and its circulatory system. These include pathologies, such as hypertrophy, atrophy, ischemia, hypertension, stroke and inflammatory disease. It is estimated that by 2020, CVD will become the leading cause of death worldwide, and at present it accounts for more than 73% of global mortality (Murray et al., 1996). Over 80% of all CVD deaths occur in low- to middle-income populations (Bradshaw et al., 2005) (see Figure 1).

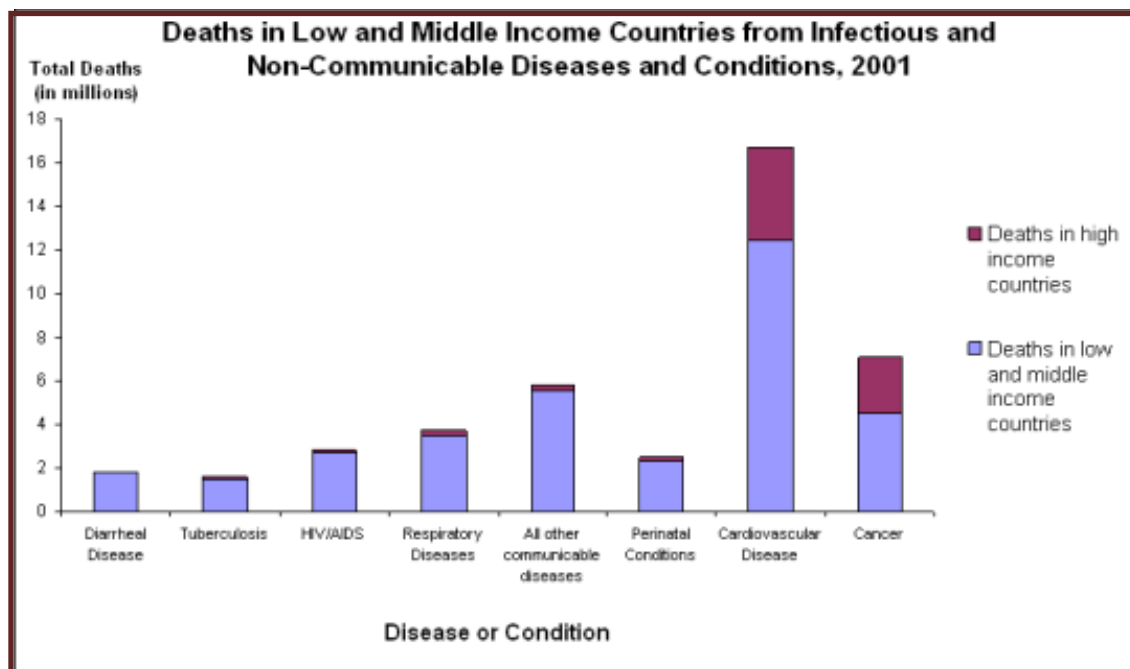


Figure 1: Cardiovascular disease is a growing burden in low- to middle- income countries (WHO 2009).

The cost of managing CVD is staggering, especially in the United States with an estimated cost of \$300 billion annually, which is equal to the entire gross domestic product (GDP) of the African continent (Mensah et al., 2008). CVD affects both developed and developing countries (Steyn et al., 2005a). Developing countries face a double burden, with a high rate of infectious diseases and an increase in chronic diseases of lifestyle, frequently associated with socioeconomic imbalances.

Furthermore, it is predicted that ischemic heart disease (IHD) will be the leading cause of death in Africa by the year 2030 (Mensah et al., 2008). Sub-Saharan Africa is in the first stage of epidemiological transition, with the CVD rate escalating to almost half the rate of CVD in developed countries. Even though human immunodeficiency virus (HIV) is the leading cause of death in this region, CVD is the second overall killer and the first among those of over 30 years of age. There is a profound increase in CVD in urban rather than rural dwellers and the demographic trends also demonstrate a gradual increased risk with age (Sliwa et al., 2008).

In South Africa, IHD is highest among whites, Asians and coloureds, with low mortality rates found in blacks (Steyn et al., 2005b). However, 88% of new heart failure patients in South Africa are blacks with untreated high blood pressure (Steyn et al., 2005; Stewart et al., 2006). In the Western Cape Province (the region around Cape Town), CVD is already the number one killer (Bradshaw et al., 2003). The upward trend of CVD is a result of increased risk factors (see Figure 2).

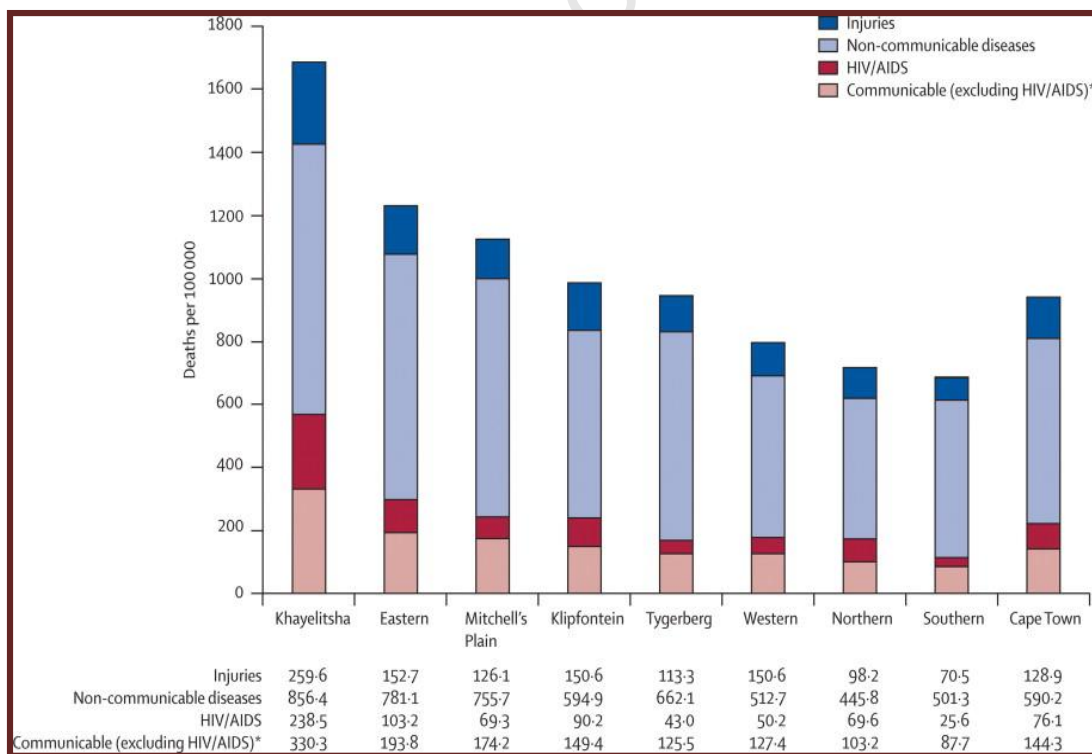


Figure 2: Epidemiological transition in the Western Cape. Non-communicable diseases are on the incline (Mayosi et al., 2009).

1.1 Ischemic heart disease (IHD)

IHD is characterised by the reduced blood flow to the heart. It is an imbalance between supply and demand of oxygen. The coronary artery supplies the heart cells with blood, which contains the necessary nutrients and oxygen for the heart; a partial or complete obstruction in this artery will reduce the blood flow (Opie et al., 2004). An obstruction may develop from atherosclerotic plaque deposition, which partially or wholly blocks a coronary artery.

Atherosclerotic plaque results from an accumulation of oxidised lipoproteins taken up by the macrophage, which develops into yellow fatty streaks on the endothelium. Thereafter, a thin fibrous cap forms and eventually ruptures, subsequently increasing the release of thromboxane- A_3 and serotonin, which in turn, promote platelet aggregation, vasoconstriction and clot formation (thrombus) (Bronte-Stewart et al., 1988). The formation of a thrombus inside a blood vessel obstructs the flow of blood through the circulatory system. This blockage leads to a reduction in the blood flow, nutrients, oxygen supply and vasoconstriction to an area of cardiac tissue (Bronte-Stewart et al., 2008), with the most common causes including anaemia and hypotension (Libby & Aikawa 2002).

Overall, the coronary perfusion is reduced. The area of tissue subjected to reduced flow in such conditions is referred to as 'ischemic' tissue and if left untreated, can result in cell death. Therefore, it is imperative that intervention be timeously introduced to restore muscle function rapidly (Grace 1994, Ovize et al., 1997).

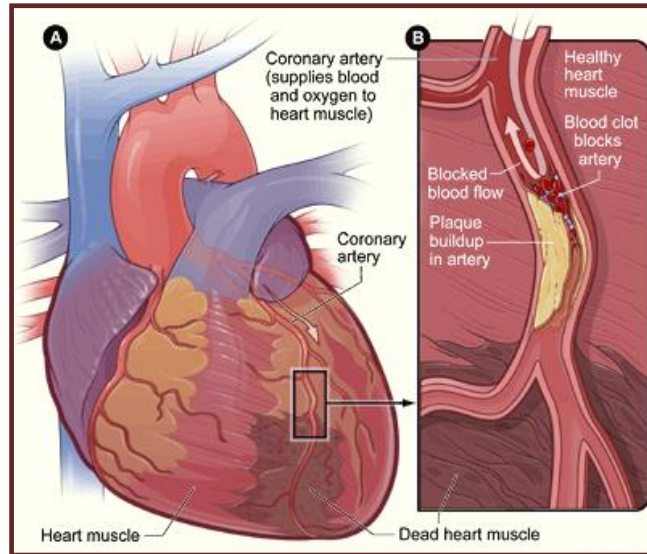


Figure 3: A schematic representation of ischemic heart disease. (www.focalcool.com, accessed 2 Aug 2012)

1.2 Ischemia Reperfusion (I/R) Injury

The only way to salvage the heart from ischemia is to restore the blood flow back into the ischemic area as quickly as possible (i.e. reperfusion), in order to reduce the number of dead cells (forming the infarct) within the myocardium (see Figure 4). However, reperfusion has a paradoxical effect: even though blood flow must be returned for myocardial salvage it also increases oxidative stress (Yellon & Hausenloy 2007). Several debate the existence of reperfusion injury nonetheless; reperfusion has been deemed lethal because of functional changes, contractile impairment, arrhythmias and death to the myocyte (Opie et al., 2004).

The restoration of blood flow to the tissue results in generation of reactive oxygen species (ROS) and calcium overloading (Yellon & Hausenloy 2007; Yellon & Baxter 1999). Increased ROS promotes the formation of oxygen-free radical derivatives which promote endothelial damage. This affects the viable endothelial and myocardial cells, and is termed 'reperfusion injury'. It is estimated that reperfusion injury accounts for approximately 50% of the infarct after the ischemic insult (see Figure 4) (Yellon et al., 2007; Prasad et al., 2009).

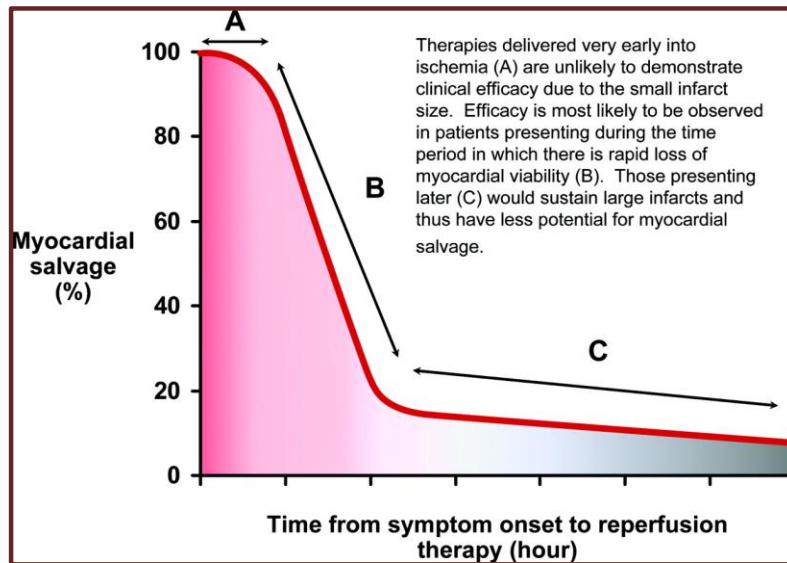


Figure 4: Reperfusion is essential for myocardial salvage. A quicker flow must be returned for myocardial salvage (Prasad et al., 2009).

Other factors that contribute to reperfusion injury aside from ROS; Firstly, when oxygen is supplied to the mitochondria after ischemia there are high levels of calcium in the cardiomyocyte. The mitochondria are responsible for the production of adenosine triphosphate (ATP) in the cell and calcium handling (Farkas et al., 2008). However, there is an imbalance between calcium and sodium exchange at the sarcoplasmic reticulum with the return of blood (Kloner et al., 1993). Sometimes, severe damage to the pump during myocardial infarction encourages an imbalance between intracellular calcium and ATP production. Secondly, during the prolonged ischemia, metabolic acidosis is experienced because of anaerobic metabolism and ATP breakdown. The sodium/hydrogen pump is activated to restore pH, but simultaneously, the sodium/calcium pump is also activated and attempts to remove sodium, which in turn causes calcium overload (Piper et al., 1998). Therefore, the rapid restoration of pH contributes to muscle contracture and worsened reperfusion injury (Ovize et al., 1997). Lastly, the overall normalisation of osmolarity seems to be a key player. It is well known that the sodium/calcium pump regulates the volume of water in the cell and osmotic load. The osmotic load is increased in both the intracellular and interstitial space and the end products of anaerobic metabolism accumulate (Piper et al., 1998; Yellon &

Baxter 1999). During reperfusion, an osmotic gradient develops between the intracellular and extracellular space. Mechanical stress is increased and sarcolemma distribution disrupted in an attempt to restore osmolarity (Piper et al., 1998).

These events are propagated from cell to cell and eventually lead to the progression of necrosis (Garcia Dorado et al., 1997). Therefore, it is critical that reperfusion injury is reduced by the use of pharmacological interventions as well as reducing the risk factors that contribute to the onset of coronary heart disease (CHD) (see Figure 5).

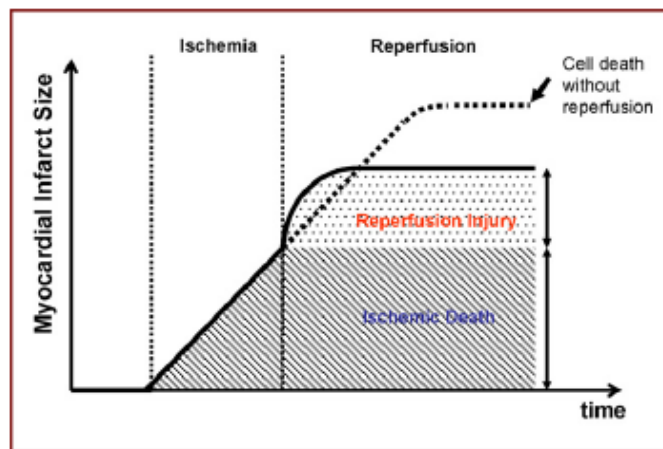


Figure 5: The role of I/R injury in myocardial infarction (Gracia et al., 2006).

If the heart muscle is not reperfused timeously, it can result in permanent damage to the myocardium. With extensive damage, the heart will lose optimal function and change in size and shape (Gracia et al., 2006). The impaired ability of the heart to pump blood to the rest of the body after I/R injury can result in heart failure.

1.3 Ischemic heart failure

Heart failure (HF) is a complex clinical syndrome that results from structural and functional changes that impair the ability of the heart to pump. The heart struggles to support physiological circulation, and there are changes in the peripheral vascular function, as well as the myocardial vasculature (McDonagh et al., 2011). These changes are necessary to maintain cardiac output and peripheral perfusion.

Survivors of a myocardial infarction (MI), even when not complicated, are at a greater risk of developing ischemic heart failure (IHF) (Flaherty et al., 2009). Another study also showed that in 180 patients that died of HF, myocardial infarction was responsible for over 57% of the deaths (Bax et al., 2008). MI-induced HF is a heterogeneous condition with several contributing factors, including changes in hemodynamic function, neurohormonal abnormalities, loss of function, chamber dilation and left-ventricular dysfunction (Chu et al., 2002). The severe loss of cells after MI induces a unique pattern of remodelling and promotes left ventricle (LV) thinning. HF continues to progress with time, ventricular size increases, and the shape, structure and function of the myocardium becomes even more altered (Chu et al., 2002). Myocardial remodelling occurs for months until the heart is completely impaired. An aggressive therapy is required to ameliorate ischemic heart disease and IHF.

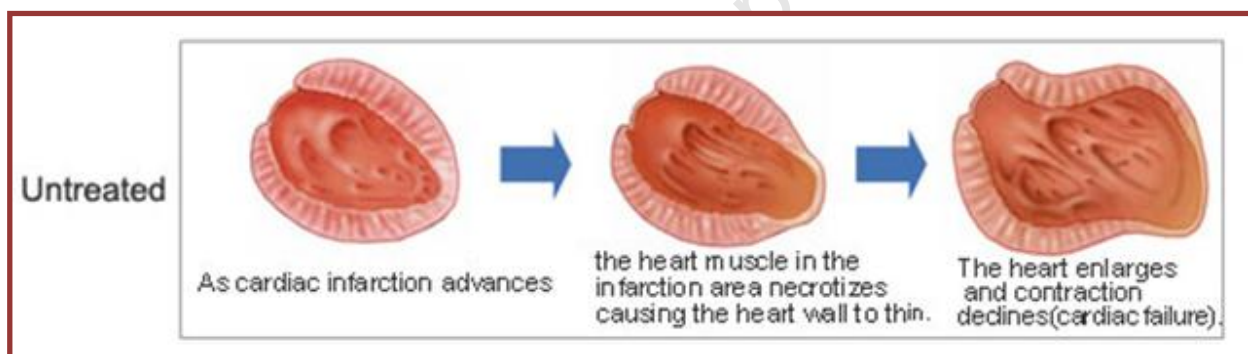


Figure 6: The progression from myocardial infarct to heart failure in untreated heart (www.oxfordjournal.org, accessed 4 Aug 2012).

1.4 Cardiovascular (CVD) risk factors

Classical risk factors for CVD are hypertension, smoking, diabetes mellitus, hypercholesterolemia and genetic predisposition (Opie et al., 2006; Kant et al., 1995).

Hypertension (HPT) is the most common risk factor for both heart failure and IHD in Africa, especially among black Africans, as compared to Western countries, which have effectively controlled hypertension (Bradshaw et al., 2005) (see Figure 7).

A study performed in South Africa on a cohort of 986 subjects over 15 years showed an increased prevalence of high blood pressure by 14.4% in men and 13.7% in women (Steyn et al., 1991). It has been postulated that increased urbanisation, including changes in healthy diets and reduced physical activity, are potential contributors to increased hypertension (Beunza et al., 2010, Mathenge et al., 2010).

Another risk factor is hyperlipidaemia found in patients with metabolic disorders and which are extremely prevalent in developed countries (Mayosi et al., 1998). Hyperlipidaemia is characterised by an excess in cholesterol and free fatty acids in the blood. There is a definite increase in calorie intake greater than 3000 calories per day, predominantly associated with high fats and low fibre intake (Steyn et al., 1991, Hu et al., 1997).

A linear association exists between obesity and a reduction of physical activity (WHO 2000). Sedentary lifestyles are profound among individuals between 30-39 years, with younger people becoming more inactive with time. Other risk factors include diabetes mellitus, with type 2 diabetes on the incline by 2.5% in upper- to middle-class countries (Podar 2001, Hu et al., 2001), and tobacco smoking, which has declined since 1994 (King et al., 1998). A sustained high blood glucose level increases the risk of fat deposition on the endothelial walls and leads to microvasculature complications (Hossain et al., 2007). Alcohol abuse is on the incline, with varying types of alcohol consumed including beer, locally brewed beverages and wine bingeing. Alcohol bingeing, more than 3 glasses/day increases the risk of HPT, CVD and heart failure (Di Castelnuovo et al., 2006). However, several metanalyses display a J-shape curve, where the moderate consumption of alcohol (2-3 glasses/day) is protective, but when consumed excessively the protective effects are lost (Kozarevic et al., 1993, Collins et al., 2009). Potential genetic predisposition may also exist and could increase the probability of CVD independently of the risk factors (Rajith et al., 2004).

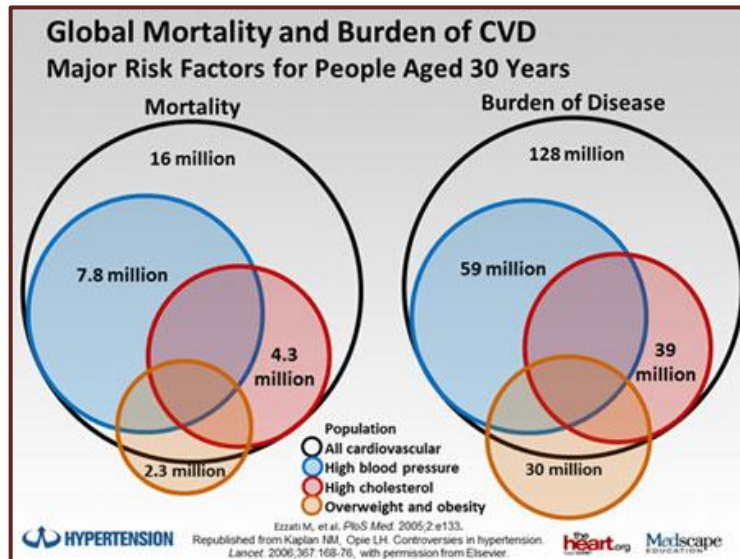


Figure 7: Risk factors that contribute to the development of CVD. (Opie et al., 2006).

1.5 Pathophysiology of ischemic heart disease

The endothelium maintains normal vascular tone and blood fluidity with little or no proinflammatory factors. However, the removal of an obstruction in the heart, such as plaque destabilisation predisposes the heart to increased ROS production, elevated proinflammatory cytokines and increased apoptosis (Libby et al., 2002).

When the endothelium is damaged, vasoreactivity is increased due to the chronic stimulation of the sympathetic system and reduction in nitric oxide release (NO) (Hernandez-Perera et al., 1998). Other studies have shown elevated adhesion proteins as well as increased levels of C-reactive protein in heart failure patients (Walter et al., 2000). Extensive experimental studies in models for diabetes have identified reduced NO as the common cause for endothelial dysfunction (Hattori et al., 1999). When the endothelium is damaged extensively, it eventually leads to cell death and apoptosis.

1.5.1 Apoptosis

After a myocardial infarction, some of the cells survive the prolonged ischemia and lead to cell death, morphological changes are distinct and include apoptosis. In general apoptosis modulates post-ischemic events and predominantly occurs during the reperfusion period, when blood flow is returned to the heart (Baillik et al., 1997,

Yamamoto et al., McCully et al., 2004). It is particularly elevated in the ischemic border and continues to extend after 12 days post infarction (James et al., 1998). Apoptosis is important in the maladaptive process of remodelling and promoted by the activation of pro-apoptotic factors like Bax and inhibition the anti-apoptotic factors such as Bcl₂, but the activation of these factors are dependent on the extent of damage (Lee et al., 2007). Therefore extensive damage promotes caspase release and is an important mode of programmed cell death in heart failure.

1.5.2 Necrosis

Necrosis is generally initialised by non-cellular mechanisms, namely ischemia, trauma and thrombosis, all of which lead to irreversible swelling, energy depletion and cell death (James et al., 1998). Necrosis is characterised by the reduction in oxygenated blood supply to the heart which in turn reduces pH and ATP utilisation (Yamamoto et al., 2011) (see Figure 8). DNA fragmentation and internucleosome generation occur spontaneously as compared to apoptosis (McCully et al., 2004). Furthermore, necrosis is strongly associated with calcium overload that leads to pump dysfunction and cell death (Nakayama et al., 2012). However, it remains unclear whether a distinct variation exists between apoptosis and necrosis and whether these events are characterised discriminately or merely overlap and complement each other were necessary (Yamamoto et al., 2011).

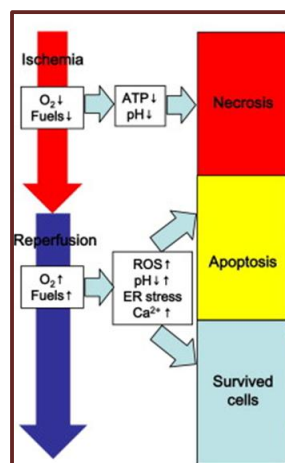


Figure 8: The differentiation between apoptosis and necrosis after ischemia-reperfusion injury (Yamamoto et al., 2011).

1.5.3 Inflammation

Excess levels of tumour necrosis factor alpha (TNF α) are frequently released after ischemia and increase cell death (Kleinbongard et al., 2010). Furthermore, high levels of TNF α are also noted in patients with HF, diabetes, hypertension and atherosclerosis, which all promote cell death. The excessive production of TNF α activates the uncoupling of the β adrenergic receptor, thus decreasing contractility of the heart, and results in cardiac and contractile dysfunction post-ischemia (Gulick et al., 1989). Unfortunately, the down regulation of the contractile tissue increases fibrosis and collagen deposition, especially in a failing heart (Chen et al., 2008). When the myocytes start dying the proinflammatory factors and chemoattractants are released. Muscle contraction is altered and results in left-ventricular dysfunction (Chen et al., 2008).

1.5.4 Cellular response to heart disease

After MI, macrophages, lymphocytes and neutrophils infiltrate the affected myocardium within the first few days (Hoshino et al., 1999). The neutrophils enhance the release of matrix metalloproteases (MMPs), which, in turn, promote the degradation of collagen (Hoshino et al., 1999). Furthermore, the macrophages are mobilised and phagocytose apoptotic cells. Initially, contractile activity is increased to compensate for reduced cardiac output and this will enhance the activation of intracellular signalling molecules including insulin-like growth factor, endothelin, angiotensin-2 and the phosphatidylinositol 3 kinase/ protein kinase B (PI3/Akt) pathway to assist the salvaging of the myocardium from extensive myocardial damage (Hausenloy et al., 2004).

1.6 Current treatment for heart disease

CVD cannot be cured, but it can be managed effectively with a combination of lifestyle changes and correct drug therapy (Pearson et al., 2002). Drug therapy, angioplasty and sometimes surgery are all strategies required to open blocked vessels. Clot formation can be prevented by using antiplatelet and anticoagulant agents however, these agents are not capable of breaking or dissolving existing clots (Huang et al., 2001).

Fibrinolytic agents will dissolve clots rapidly to open the blocked arteries. Plasminogen activator administered intravenously can open up to 80% of blocked arteries, but these agents must be administered early to achieve maximal success (Staat et al., 2005). A more direct approach is percutaneous transluminal coronary angioplasty (PTCA) for the opening of blocked coronary arteries. A thin small plastic catheter with a balloon at one end is guided to the blocked artery, usually via the femoral artery. The balloon is inflated and dislodges the clot. PTCA effectively opens up to 95% of arteries and is used as a long-term plan in conjunction with lifestyle changes and pharmacological interventions to prevent restenosis (Staat et al., 2007). Most interventional strategies pursued for infarcted arteries are PTCA and subsequent stent implantation. Considerable attention has been focused on developing novel strategies to attenuate myocardial I/R and to improve the myocardium post infarction.

In 1986, the phenomenon of ischemic preconditioning was discovered by Murray and colleagues, whereby 4-5 minutes of ischemia followed by intermittent reperfusion was induced prior to the prolonged ischemic insult and it was shown to reduce infarct size by 30% (Murray et al., 1986). Intermittent reperfusion appeared to decrease the adverse effects of lethal reperfusion injury (Murray et al., 1986). Given these findings a similar protocol was carried out after the prolonged ischemic insult and was subsequently termed ischemic postconditioning (Zhao et al., 2003; Mykytenko et al., 2008). The protective effects of ischemic postconditioning were comparable to ischemic preconditioning (Skyschally et al., 2009; Tsanget al., 1994). Postconditioning reduces infarct size in both large and small animals by 30% to 80% (Yellon & Hausenloy 2007). Preconditioning and postconditioning are strategies that can be used in a clinical setting to treat against MI and HF. Interestingly, there are multiple intrinsic survival pathways that can be deployed to salvage the heart after I/R injury.

2 Intrinsic cardiac prosurvival pathways

2.1. Reperfusion injury salvage pathway

The reperfusion injury salvage (RISK) pathway involves the activation of phosphoinositidol kinase (PI3K), protein kinase B (Akt), extracellular regulated kinase (ERK), mitogen extracellular kinase (MEK) and glycogen synthase kinase (GSK), which are capable of protecting against I/R injury (Yellon et al., 1999; Hausenloy et al., 2004). The activation of this pathway will result in the closing of the mitochondrial permeability transition pore (mPTP) (Juhaszvoa et al., 2004). It has been suggested that the PI3K/Akt and ERK can be activated prior to the ischemic insult as well as at the onset of reperfusion to confer cardioprotection (Omura et al., 1999). Therefore, the early activation can act as a primer for kinase phosphorylation at the onset of reperfusion (Hausenloy et al., 2005). Interestingly, the inhibition of PI3K/Akt with wortmanin or LY294002 in the isolated rat heart increases infarct size (Tsang et al., 2004). Similarly, inhibition of MEK/ERK 1/2 pathways with PD98059 increased infarct size after I/R injury (Yang et al., 2004). By 2007, some of the intermediate targets for the RISK pathway that were identified are protein kinase G (PKG), protein kinase C (PKC) and glycogen synthase kinase-beta (GSK3 β) (Hausenloy et al., 2007).

The endpoint for the RISK pathway is thought to be the inhibition of the opening of mPTP. The structure of this mPTP still needs to be delineated but its opening results in the release of cytochrome C from the mitochondria, leading to apoptosis (Hausenloy et al., 2004). Recently, it has been suggested that there are other alternative pathways that also protect the heart, such as the survivor activating factor enhancement (SAFE) pathway.

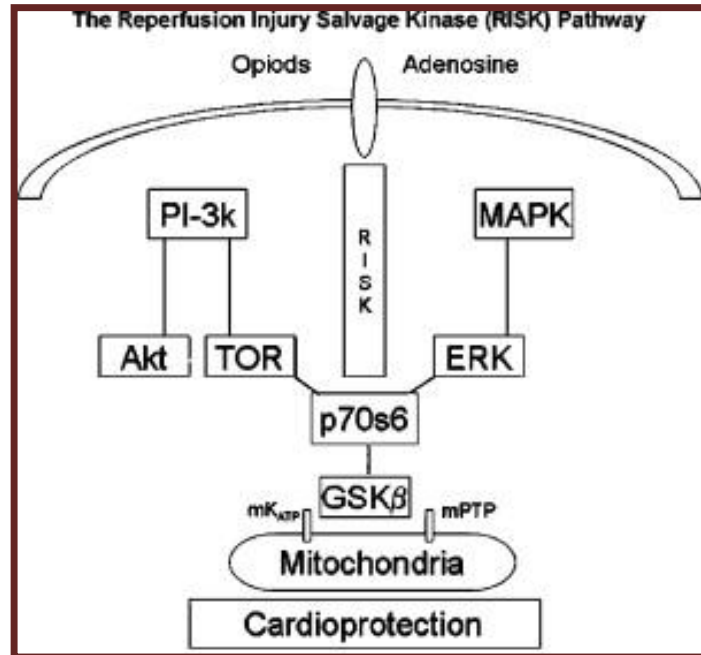


Figure 9: Activation of the RISK (reperfusion injury salvage kinase) pathway for myocardial salvage. Opioids and adenosine activate the RISK pathway with either PI3K/Akt (phosphoinositol kinase)/protein kinase B or MAPK/ERK (mitogen activating kinase/extracellular kinase) activation which in turn target the mitochondria as the endpoint to confer cardioprotection (Gross & Auchampach. 2007)

2.2. Survivor Activating Factor Enhancement (SAFE) pathway

Another key prosurvival pathway is the SAFE pathway (Lecour et al., 2009). The constituents are TNF α , which initiates the activation of the innate immune system and the well-known protective signaling molecule, signal transducer and activator of transcription 3 (STAT3).

2.2.1. TNF alpha and its role in the SAFE pathway

The first line of defence after an ischemic insult is the activation of the innate immune system. It is an adaptive process that mobilises the infiltration of cytokines such as TNF α (Kleinbongard et al., 2010). Several studies have demonstrated the pleiotropic effects of TNF α , exhibiting protective qualities at low concentrations and detrimental effects at higher concentrations (Lecour et al., 2002).

However, the good versus the bad effects of TNF α are not only concentration-dependent but also receptor-dependent (Lacerda et al., 2009; Lecour et al., 2002).

There are two types of receptors: TNF α receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) (Baxter et al., 1999). Receptor stimulation is dependent on other inflammatory cytokines, including interleukin-1 (IL-1), IL-10, IL-4 and growth factors (Manna et al., 1998). TNF α binds to either receptor in a dose- and time-dependent manner (Deucher et al., 2007) (see Figure 10). When bound to TNFR1 it stimulates activation protein-1 (AP-1), IK beta complex (IKK) which, in turn, activates nuclear factor kappa B (NFKB) (Haudek et al., 2001). NFKB regulates the inflammatory response and further increases the release of TNF α (Haudek et al., 2001). The continuous stimulation of TNFR1 propagates the activation of pro-apoptotic factors like JNK and exacerbates the effects of MI, HF and atherosclerosis (Dorg et al., 2002, Kin et al., 2008). However, the outcome after activation of TNFR2 is vastly different.

TNFR2 is activated by inflammatory cytokines and growth factors, but its response is mediated via the activation of Janus Kinase 2 (JAK2), which in turn, phosphorylates and activates the signal transducer and activator of transcription (STAT3) (Frias et al., 2009; Lacerda et al., 2009). In addition to this, TNFR2 also stimulates PI3/Akt and ERK prosurvival signalling molecules (Kleinbongard et al., 2010). Interestingly, the inhibition of TNFR1, with specific antibodies, in both rabbits and dogs, increases infarct size after I/R injury (Dorge et al., 2002). However, the complete inhibition of TNF α has led to controversial data, in one study there was a reduction in infarct size after ischemia-reperfusion injury, in TNF-deficient mice. On the contrary, another study showed that mice without TNF α were worse off compared to their wild-type littermates (Dawn et al., 2004; Labruto et al., 2005). Hence the presence of TNF, even in small quantities, is necessary for heart function (see Figure 10). In 2002, the exogenous administration of TNF α at low concentration prior to the prolonged ischemia conferred cardioprotection in an isolated rat heart model (Lecour et al., 2002). Other experiments demonstrated that the low concentration of TNF α improved mitochondrial respiration and reduced

mitochondrial swelling; and suggested that the protective effect of TNF α may be mediated via the transient closure of the mPTP (Lacerda et al., 2010).

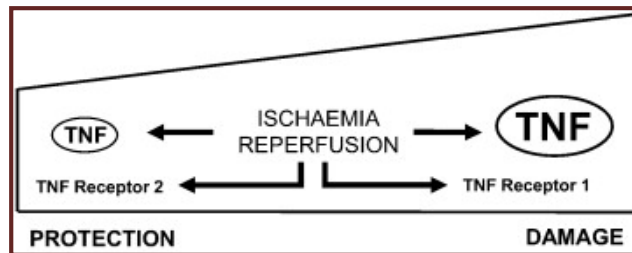


Figure 10: The pleiotropic effects of TNF α (tumour necrosis factor) in protecting against I/R. (Lecour 2009).

2.2.2. STAT3 and its role in the SAFE pathway

STAT3 is one of seven STAT isoforms found in the heart (Soon et al., 2006). STAT-1 and STAT-3 (Xuan et al., 2001; Yang et al., 2008), have opposing effects; STAT-3 activation is antiapoptotic and STAT-1 activation proapoptotic (Hilfiker-Kleiner et al., 2004). Mice with STAT3 depleted in the cardiomyocytes eventually develop heart failure (Hilfiker-Kleiner et al., 2004; Jacoby et al., 2003).

In the SAFE pathway, TNF α binds to its receptor 2 which leads to the activation of STAT3 after the ligand binding of Janus Kinase 2 (JAK), a tyrosine kinase, see figure 11. It is postulated that the activation of JAK may promote the phosphorylation of STAT3 on the tyrosine residue (Xuan et al., 2001). STAT3 then homodimerises and translocates from the cytosol to the nucleus, where it is further phosphorylated on a serine residue 705 (pSTAT) and becomes activated as a transcription factor for prosurvival genes such as manganese superoxide dismutase (MnSOD) and inducible Nitric Oxide Synthase (iNOS) (as reviewed by Boengler et al., 2008a; Boengler et al., 2008b).

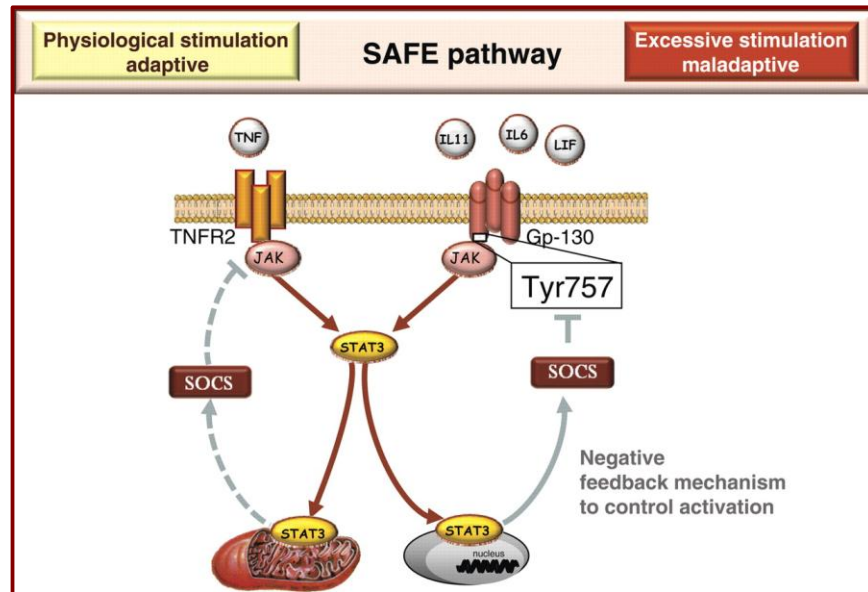


Figure 11: The role of the SAFE pathway in myocardial salvage. Cytokine, TNF α binds to its receptor and in turn, activates JAK which leads to the activation and translocation of STAT3 from the cytosol to nucleus or the mitochondria pathway).IL (interleukin) Gp 130 (G coupled protein receptor), soc (suppressor cytokine signaling). (Lecour & James. 2011).

The presence of STAT-3 in the mitochondria has recently been discovered, leading to the updated pathway which proposes that the location of STAT-3 phosphorylation may determine its fate (Mykytenko et al., 2008, Bolli et al., 2001). Furthermore, recent evidence has suggested that STAT-3 may not even need to be phosphorylated or dimerised in order to enter the nucleus, although tyrosine phosphorylation is still required for transcriptional activity (Liu et al., 2005). Thus, in the new model pSTAT bound on the tyrosine residue translocates to the nucleus and acts as a transcription factor, while pSTAT bound on the serine translocates to the mitochondria (Liu et al., 2005), where it promotes cell survival, most likely via mPTP activation (Boengler et al., 2008b) has an anti-apoptotic effect; however, more studies are required (Liu et al., 2005). Nonetheless, it is clear that STAT3 is a powerful signalling molecule that is activated to protect the heart and is closely integrated in the SAFE pathway. There are a variety of agents that activate STAT3 such as insulin (Fuglestege et al., 2008), ethanolamine, a component of red wine (Kelly et al., 2010), sphingosine-1-phosphate, a component of HDL (Somers et al., 2011) and interleukin 6 (Kurdi et al., 2007), an

inflammatory cytokine. Another potential activation of the SAFE pathway is red wine and its cardioprotective compounds, see figure 12.

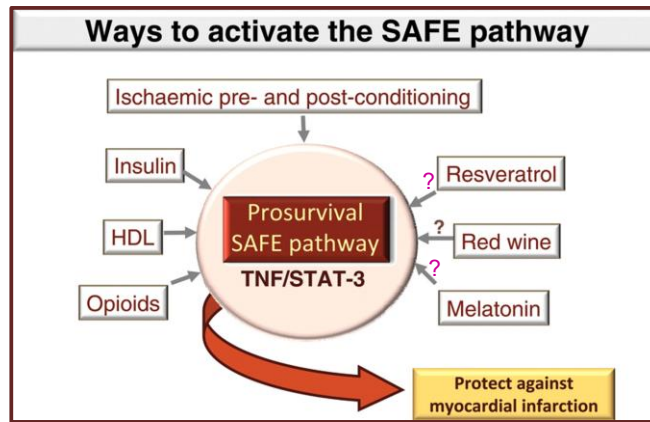


Figure 12: Several components stimulating the SAFE pathway. (Lecour & James 2011).

3. Red wine and cardioprotection

Daily moderate consumption of red wine is associated with reduced cardiovascular events compared to those who abstain or binge-drink (WHO 2002). Drinking wine with food may also be of benefit as it slows the absorption of ethanol (Kozarevic et al., 1983).

3.1 The history of red wine

Wine describes a class compound of yeast fermentation pressed from grapes, from the genus *Vitis* (Murche et al., 2010). Interestingly, wine can be traced back to the ancient civilization with the earliest known production in 4500BC in ancient Greece, Thrace and Rome (Berkowitz et al., 1996; Soleas et al., 1997). Red wine has been a socioreligious symbol for decades and is believed to have magical medicinal value. The benefit of red wine was first proposed by the father of medicine, Hippocrates of Kos in Greece, and was not researched for many years (Soleas et al., 1997). The interest in the protective qualities of red wine re-emerged in 1992 as an extremely well-researched topic, after epidemiological studies tantalised the world with the premise that moderate red wine consumption could offer cardioprotection (St Leger et al., 1992).

3.2 The French paradox

In France, wine traces back to the 6th century BC, and over 50-60 million hectolitres of wine are produced per year (Berkowitz et al., 1996). During the Middle Ages some of the best vineyards were conserved and maintained by monks for celebrations and generating a steady income during turbulent times. As time progressed, it became a social grace to drink wine and many vineyards were later owned by nobility (Berkowitz et al., 1996). In France, wine consumption is clearly a part of their national heritage and an important part of their culture that is valued, nurtured and rarely observed consumed in excess or as binge-drinking. Therefore, it is easy to understand that the French habitually drink wine with their meals.

Epidemiological studies demonstrate a strong inverse association between moderate red wine consumption and CVD in France when compared to other European countries, North America and Australasia (St Leger et al., 1979). Even though the French consume foods which are saturated in fat, the risk of CVD is reduced compared to other Westernised countries (see Figure 13). A possible reason is the regular consumption of red wine with meals. This observation was consequently termed as the French paradox (Renaud et al., 1992).

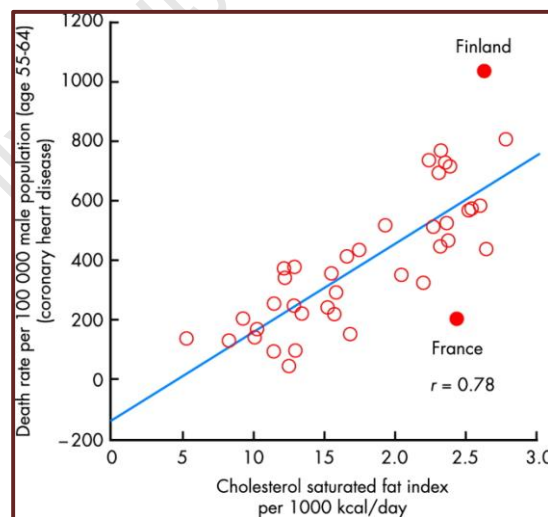


Figure 13: The French paradox. The French population has a lower risk of coronary heart disease even though the cholesterol intake is high (Ferrieres et al., 2004).

A study conducted in more than 36 000 men in the eastern part of France between 1978 and 1983, demonstrated that men drinking between 2 to 3 glasses/day of red wine had 30% fewer deaths from all-cause mortality compared to non-drinkers or excessive drinkers (that drank more than the prescribed, 2-3 glasses/day) (Renaud et al., 1999). Klatsky reported that moderate alcohol consumption from whatever source reduced the risk of CVD compared to teetotallers (Klatsky et al., 1993).

These studies were further corroborated by Kannel and Eddison, who also found that moderate alcohol consumption improves cardiovascular outcome (Kannel & Eddison 2007; Doll et al., 2007). Interestingly, those that consumed red wine had a superior cardioprotective effect compared to other alcoholic beverages in reducing CVD (Doll et al., 2007).

However, these epidemiological studies need to be interpreted with caution as differences in alcohol consumption may also reflect differences in nutritional intake, lifestyle and behaviour (Empana et al., 2011). Indeed, regular wine drinkers consume more fruits, salad, vegetables, fish and olive oil (Tjonneland et al., 1999). Also, wine drinkers exercise more, smoke less and they have a higher level of education than non wine drinkers (Mukamal et al., 2005).

3.3 Red wine confers cardioprotection

In survivors of a first MI, the association between red wine intake and the risk of recurrence of MI during a four-year follow-up demonstrated a reduction of the adjusted risk of cardiovascular complications by 59% in patients, drinking an average of four glasses per day (De Lorgeril et al., 2002). A metaanalysis of 51 studies concluded that moderate red wine consumption reduced the risk of CVD by 20% (Smitko et al., 2005). Furthermore, regular red wine consumption improves the outcome of both MI patients and diabetic patients.

In animal models very few studies have demonstrated wine that has been administered on its own to determine its protective effect. The only experimental studies to date that compared the differences in red wine vs. white wine in an isolated heart model demonstrated that the cardioprotective effects of white wine were mediated by the

presence of the polyphenols tyrosol and hydroxytyrosol (Dudley et al., 2008). However, this study has been withdrawn from Pubmed due to fraudulent activity.

Most studies have administered either alcohol or polyphenol extracts independently (Das et al., 1999). Therefore, the key attributes of red wine are unclear; it may be the alcohol and the polyphenol content or a synergy of the two that promote the protective effect of red wine, which remains elusive.

3.4 Mechanisms for red wine-induced protection

3.4.1 Anti-oxidant effect

Red wine has a variety of attributes: it is an anti-oxidant, reduces low-density lipoproteins (LDL) oxidation, and improves cytokine release and eicosanoid response thereby reducing inflammation (Handelman et al., 1993). It is believed that the polyphenol content in wine permits its anti-oxidant ability (Frankel et al., 1993). Red wine scavenges free radicals both directly and indirectly. The polyphenols in red wine facilitate reduced hydrogen peroxide (H_2O_2) formation by quenching the electronically excited compounds (Rodrigo et al., 2011). After the consumption of 300ml of wine, the anti-oxidant capacity in human plasma increased to 18%, an effect was comparable to the effect of vitamin C (Rodrigo et al., 2011). In human studies, both the anti-oxidant qualities of regular moderate consumption of white, rose and red wine was assessed (Coimbra et al., 2005). The findings demonstrate strong anti-oxidant ability in red wine when compared to the other beverages. In addition to this the production of H_2O_2 and phospholipase C diminished after red wine consumption (Tozzi Ciancarelli et al., 2011). Red wine prevents the accumulation of the by-products (such as malonaldehyde and hydroxyl ions) from lipid peroxidation and improves the endothelial function (Tozzi Ciancarelli et al., 2011).

3.4.2 Vascular effect

Vascular endothelial function plays a crucial role in the pathogenesis of atherosclerosis. The structural integrity and function must be maintained by promoting factors such as endothelin-1 (ET-1, vasoconstrictor) and plasminogen inhibitor 1 (PAI, vasodilator) (Kiviniemi et al., 2010). Furthermore, the consumption of red wine suppresses ET-1 and

induces vasorelaxation in the blood vessels (Kiviniemi et al., 2010, Crozier et al., 2011). Red wine improved coronary flow velocity (Shimada et al., 2010). Furthermore, the polyphenols present in red wine prevented the migration of oxidised LDL to the macrophages thereby, limiting both the progression and formation of plaque (Maxwell et al., 1994). Red wine also inhibits the NFkB in the mononuclear cells and in human cells (Pellegatta et al., 2003) as well as the migration of monocyte chemotactic protein (MCP-1) and vascular smooth muscle cells to the intima (Pellegatta et al., 2003). Thus red wine displays strong antithrombotic action. In the Health Physicians study, light to moderate drinkers had a reduced fibrinogen (which is synthesised in the liver to reduce coagulation) reduced Von Willebrand factor (key glycoprotein involved in haemostasis) and diminished fibrinogen activator (Mukamal et al., 2001).

3.4.3 Anti-platelet effect

Platelet aggregation is the clustering together of thrombocytes to prevent bleeding. The formation or presence of a blood clot in a blood vessel is termed a thrombus. In the *ex vivo* assessment of platelet response in healthy patients, red wine inhibited platelet aggregation mediated by the presences of the polyphenols. Thromboxane, produced from arachadonic acid in the eicosanoid pathway and 5-lipoxygenase, was also reduced (Tozzi Ciancarelli et al., 2011). E-selectin and IL-6 were increased after red wine consumption and may have an effect on lipid responsiveness by improving the hemostatic profile (Tozzi Ciancarelli et al., 2011). In addition, aggregation in response to adenosine diphosphate (ADP) and thrombin in human platelets is strongly inhibited by red wine (Pace-Asciak et al., 1993). Other studies debate whether it is the polyphenol content in the wine or the ethanol content that explains the decrease in platelet aggregation (Soleas et al., 1997).

3.4.4 Anti-cholesterol effect

Regular consumption of red wine reduces LDL, also commonly termed, the “bad cholesterol” decreases lipid peroxidation and increases plasma concentration of high density lipoproteins, HDL also commonly termed the “good cholesterol” (Frankel et al., 1993; Kaplan et al., 2004). In addition, the red wine modified and enriched the

composition of HDL in polyunsaturated phospholipids such as omega-3 fatty acids, which are said to be beneficial against coronary artery disease (Perret et al., 2002, Fabrizio et al., 2007).

In hypercholesterolemic rabbits, the chronic moderate consumption of red wine improved flow-mediated dilatation (FMD) and increased the release of nitric oxide (NO) (Wallerath et al., 2003). Thus red wine consumption mitigates the effects of a high-cholesterol diet. Clinical studies conducted in patients with metabolic syndrome assessed red wine intake with a questionnaire and then assessed lipid profile. In these patients a glass of red wine a day reduced the triglyceride and total cholesterol level (Davis et al., 2002).

3.5 The role of alcohol and red wine-induced cardioprotection

There are more than 1000 compounds present in red wine, but alcohol and polyphenols are thought to be the major contributors to the protective effects of red wine. The production of red wine is from an assortment of grape cultivars, under varying climatic conditions in different geographical locations. The grapes have different maturity levels for vinification and ageing. Therefore, red wine is extremely heterogeneous in chemical composition, flavour, taste and appearance. The extraction process for red wine is prolonged with extensive fermentation. The fermentation process increases the phenolic content by 40-60% (Crozier et al., 2011). Ageing and maturity of the wine is further enhanced when stored in an oak barrel, which can also contribute to improving the phenolic content (Jimenez-Moreno et al., 2003, Iriti et al., 2010). In white wine, the phenolic yield is reduced compared to red wine, due to the removal of grape skins during the winemaking process. The alcohol content in red wine is crucial for the stability, ageing, and gustatory properties of the wine. It plays a role in the extraction of pigments and tannins during the fermentation of the skin and seeds of grapes (Das et al., 1999). Red wine consists of 81% water, 12% alcohol, 1% sugar, 5% sulphites, 1% grape thaumatin, 1.5% acid (i.e. gallic acid), 1% vitamins, 1% minerals and 1% phenolic compounds.

It is surprising that the phenolic content is so low, but this will differ from wine to wine and also emphasises the importance of the fermentation process as well as the synergy of all the compounds in red wine that contribute to its cardioprotective effect.

3.5.1 Alcohol-induced health benefit

Several epidemiological studies including, the Framingham study and Copenhagen City Heart trial, have shown that there is a consistent inverse relationship between alcohol consumption and mortality, whereby a low to moderate consumption of alcohol during meals promotes beneficial effects (Hulley et al., 1981). However, the excessive consumption of alcohol or binge drinking has a linear association between the drinks consumed and increases the risk of mortality and disease (Gordon & Kannel 1984; Doll et al., 1994; Thun et al., 1997) and the alcohol effect can be described as a J-shaped curve (see Figure 14). It is recommended for men and women to have 1 to 3 glasses of alcohol per day to confer cardioprotection and teetotallers have neither an increased nor a decreased risk of CVD (Thornton et al., 1983).

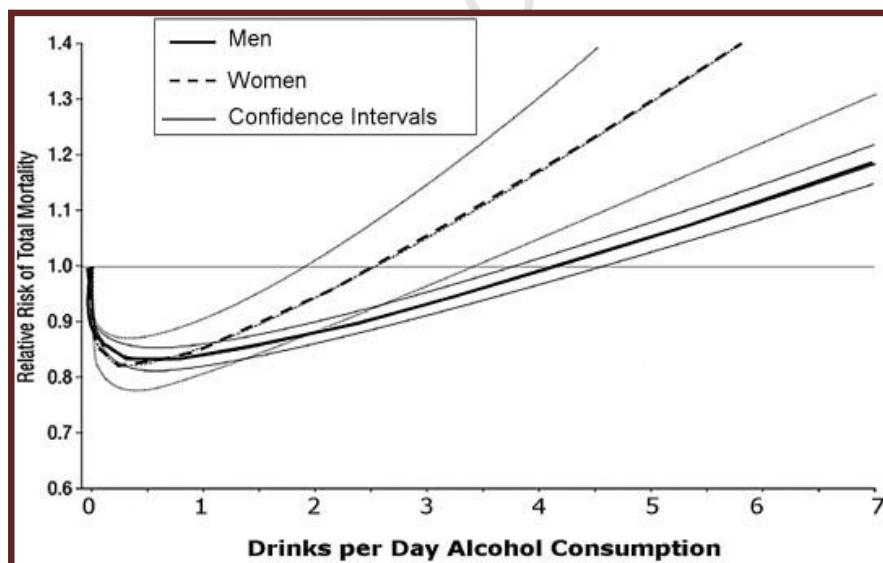


Figure 14: The J Shape mortality curve. 1-3 glasses per day reduces relative risk of mortality (Keefe et al., 2007).

In a multinational study, lack of alcohol intake increased risk factors for Type 2 diabetes, emphasising that cardioprotection conferred from alcohol consumption affects triglycerides and insulin levels (Hu et al., 2001).

However, the cessation of alcohol will result in the loss of all beneficial effects within 24 hours (Jackson et al., 1992). Klurfield et al. compared different alcoholic beverages, including beer, white wine, red wine, whiskey and ethanol on its own, and found that red wine conferred cardioprotection to a greater extent and its beneficial properties were deemed superior compared to the other beverages (Klurfield et al., 1981).

3.5.2 The alcohol protects against I/R

Regular moderate consumption of alcohol from whatever source can confer cardioprotection. The health professionals' study suggested that 2-3 drinks per day reduce the risk of MI (Mukamal et al., 2003). Similar data were obtained in the physicians' health study, which showed reduced risk of CVD with alcohol consumption (Cramargo et al., 1997). In the Framingham cohort the risk for congestive heart failure was reduced relative to alcohol intake (Cramargo et al., 1997). However, whether one alcoholic beverage had superior protection remained elusive. Experimentally, mice drinking water supplemented with ethanol ranging between (2.5-36%) for six weeks, showed profoundly reduced infarct size after I/R *ex vivo*. The heart's recovery was improved and creatin levels reduced (Jackson et al., 1992).

Acute ethanol (10mM) administration 20 minutes prior to I/R reduced infarct size and conferred protection *ex vivo* (Fuchs et al., 2007). On the contrary, 0.8 g/kg administered 20 minutes prior to I/R did not reduce infarct size and there was no hemodynamic variation *in vivo* (Rakotovac et al., 2004). These results are well supported in the literature and further demonstrated that the protective effect of ethanol was lost at high concentrations, thus the cardioprotective effect of alcohol is dose-dependent (Rakotovac et al., 2004). In these studies, moderate alcohol consumption-sustained cardioprotection required the activation of the mitochondrial potassium adenosine triphosphate (K_{ATP}) channels, as reviewed by (Collins et al., 2009). Mice treated with 10% alcohol for 12 weeks protected the hearts against I/R injury via the activation of NO (Zhou et al., 2002b). Similarly, rats that were treated with 18% alcohol for eight weeks in drinking water improved post-ischemic systolic and diastolic pressure and it also

increased cardiovascular resistance via the activation of protein kinase C (PKC ϵ), see review (Collins et al., 2009).

3.6 Does alcohol contribute to red wine induced cardioprotection

Healthy patients younger than 40 years were divided into two groups: red wine with or without alcohol (Agewall et al., 2000). Acute red wine ingestion (250m ℓ) increased blood flow and heart rate. After the acute ingestion of dealcoholised red wine there was an increase in the flow-mediated dilation in the brachial artery, but not the other hemodynamic parameters (Agewall et al., 2000). Many speculate that purple grape juice may have similar effects to dealcoholised red wine. In hypercholesterolemic subjects that received 250m ℓ of red wine vs. 500m ℓ of grape juice for 14 days, the flow-mediated dilation was improved in both red wine and grape juice drinkers compared to placebo controls (Fremont et al., 1999). However, red wine significantly increased endothelium-independent vasodilation. Grape juice reduced intracellular adhesion molecule (ICAM) but this effect was not observed with red wine consumption on its own. Therefore, hypercholesterolemic patients exhibit improved vasodilation after red wine or grape juice consumption (Coimbra et al., 2005). In humans, supplementation with grape juice promotes endothelial relaxation and inhibits LDL oxidation (Vinson et al., 2001).

Experimentally, hyperlipidemic mice fed on grape juice, red wine or dealcoholised wine had significantly higher HDL levels compared to the ethanol groups or water controls (Dai et al., 1997). Furthermore, experimental studies performed during research for my Masters degree demonstrated that red wine with a reduced alcohol content (from 12% to 6%) still conferred cardioprotection to a similar extent as the unaltered red wine (Lamont et al., 2012). Therefore, red wine-induced protection goes beyond the alcohol content and may be attributed to other components, such as polyphenols.

3.7 Resveratrol and red wine

Resveratrol (3,4,9,5 trihydroxy-trans-stilbene) is a common phytoalexin produced in response to stress in specific plants (see Figure 15 below). It is present in several foods such as grapeskins, peanuts, strawberries and red wine (Mukherjee et al., 2009).

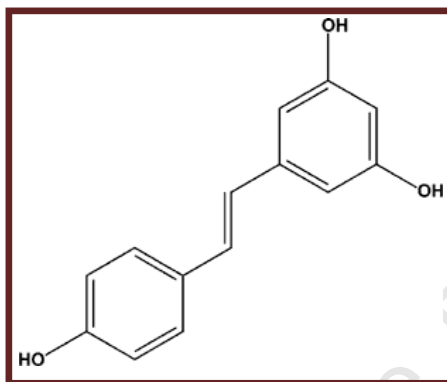


Figure 15: Schematic presentation of the chemical structure of resveratrol (own drawing).

Resveratrol exhibits a wide range of biological and pharmaceutical properties. It promotes vasodilation via nitric oxide activation (Wong et al., 2011), inhibits platelet aggregation, increases HDL cholesterol (Wang et al., 2005) and therefore serves as a strong cardioprotective agent. These properties have propelled a great interest in the health benefit of resveratrol found in wine. It has been speculated that high resveratrol levels in red wine (in comparison to other beverages) may be responsible for its cardioprotective effect. The concentration of resveratrol in the grape is dependent on the type of grape and can range from 0.1-14 mg/ml of fresh weight (Bertelli et al., 1998).

The consumption of half a bottle of Brazilian red wine containing the stilbene resveratrol at a concentration of 11mg/ml would result in a blood concentration of between 4µM and 10µM (Bertelli et al., 2002). The concentration of 10µM is the minimum resveratrol concentration required to exert a biological effect *in vitro* (Pellegatta et al., 2003).

3.7.1 Resveratrol confers cardioprotection

Preconditioning the heart with resveratrol ($10\mu\text{M} = 2.3 \text{ mg}/\ell$) improves post-ischemic ventricular recovery, reduces infarct size and decreases cardiomyocyte apoptosis (Hattorri et al., 2002). The administration of resveratrol ($10\mu\text{M}$), 30 minutes prior to I/R, protects the heart via increased expression of adenosine A_1 and adenosine A_2 receptors (Das et al., 2005).

Resveratrol modulates prosurvival-signalling pathways and activates antiapoptotic molecules like Bcl₂, Akt and the forkhead transcription factors (FOXO) after I/R injury (Das et al., 2005).

Furthermore, resveratrol promotes the translocation of Bax, the proapoptotic factor and reduces the release of cytochrome c and caspase 8 from the mitochondria (Xi et al., 2009). The activation of these pathways is dose-dependent.

Interestingly, the protective effect of resveratrol can only be observed at low doses between (2.5 - 5mg/kg/ day) with an improvement in post-ischemic ventricular function (Das et al., 2006). A high dose of resveratrol (50mg/kg) deteriorates heart function and increases infarct size (Das et al., 2005). On the other hand, in cancer cells, resveratrol at a high concentration also promotes a death signal, which is of benefit in this pathology (Das et al., 2005). Therefore, resveratrol (at varying concentrations) activates diverse pathways which affect different pathologies. In cardioprotection, a low concentration of resveratrol is preferred and can be well correlated with the notion that red wine is only cardioprotective with regular moderate consumption.

Resveratrol has pleiotropic effects that can be extended to a variety of pathologies. In the brain, chronic resveratrol (30mg/kg) treatment improves brain metabolism, increases glucose, ATP and increases energy exchange after I/R injury. Furthermore, resveratrol increases neuroprotective agents such as adenosine, inosine, hypoxanthine and xanthine (Dong et al., 2008). Therefore, even in a neurological model, resveratrol protects effectively against IR injury (Dong et al., 2008).

3.7.2 Resveratrol may improve the effects of heart failure (HF)

The major risk factors contributing to HF are hypertension, diabetes, obesity and coronary heart disease (Dong et al., 2008; Das et al., 2010). Early resveratrol administration reduces these effects in several studies (Dong et al., 2008; Das et al., 2010). Interestingly, resveratrol treatment improves vascularisation in the infarcted myocardium via the activation of thioredoxin (TRX) (Hoshino et al., 2007). Resveratrol (10 μ M) regulates TRX and activates anti-apoptotic, anti-inflammatory pathways via hemoxygenase-1 activation, thus the activation of TRX may reduce the effects of HF and cardiomyopathy (Hoshino et al., 2007).

Pathological left-ventricular hypertrophy (LVH) is a devastating condition that increases susceptibility to IHD and HF. LVH is characterised by increased myocardial mass modulated by the prohypertrophic pathways such as liver kinase B1/ adenosine monophosphate (LKB1/AMP)-activated protein kinase (AMPK). Chronic resveratrol administration (2.5mg/kg/daily) inhibits LKB1/AMPK signaling and activates mammalian target of rapamycin (mTOR) and p-70ribosomal s6kinase (p70S6K) thereby, reducing the effects of LVH (Dong et al., 2008). Therefore, resveratrol treatment presents as an adjunct therapy for patients and may lessen the risk of IHD and HF.

Experimentally, the direct impact of resveratrol on HF *in vivo* demonstrates that resveratrol (10 μ M) suppress the progression of HF via the activation of the longevity protein sirtuin1 an effect that and may be attributed to the upregulation of MnSOD via FOXO3a activation (Chui et al., 2010 ISHR abstract).

3.8 Mechanisms for resveratrol

3.8.1 Resveratrol and its anti-oxidant properties

Resveratrol is a potent scavenger for intracellular reactive oxygen species (ROS) (Bhat et al., 2002), see Figure 16. Although it possesses anti-oxidant properties *in vivo*, it is a weak scavenger *in vitro* (Bhat et al., 2002). This anti-oxidant effect could explain the inhibition of LDL oxidation (Frankel et al., 1993).

Resveratrol has a very high binding affinity for VLDL and LDL. At the onset of administration, it scavenges the free radicals in an aqueous solution and then traps the polyunsaturated fatty acids within the membrane. It prevents lipid peroxidation by chelating copper and not iron, thereby accelerating the removal of copper from LDL particles without affecting iron absorption in the microsome (Fremont et al., 1999). Moreover, resveratrol reduces the formation of thiobarbituric acid-reactive substance and has a strong inhibitory effect on the superoxide radicals and H₂O₂ produced by the macrophages (Frankel et al., 1993).

After the administration of resveratrol to aortic endothelial cells, the production of reactive oxygen species (ROS) from polymorphonuclear leukocytes (PMN) is fully segmented (Leonard et al., 2003). Granulocyte with lobes in the nuclei are reduced, as well as the release of elastase and b-glucuronidase. For adhesion-mediated function PMN must bind to b₂ integrin MAC-1, but this binding is prevented with resveratrol administration (Pellegatta et al., 2003). Furthermore, resveratrol inhibits both the intracellular adhesion molecule (ICAM) and the vascular adhesion molecule (VCAM) via the activation of NFkB, an indirect marker for oxidative stress, in the presence TNF α (Ferrero et al., 2003).

3.8.2 Resveratrol and endothelial function

Resveratrol protects the myocardium against I/R, in both an *in vitro* and *in vivo* setting via NO activation, see figure 16 (Hattorri et al., 2002, Wallerath et al., 2003). However, resveratrol fails to protect in NO-deficient mice against I/R injury (Hattorri et al., 2002, Wallerath et al., 2003). Clinically, impaired FMD in the brachial artery is characterised by the loss in the endothelial dependent vascular smooth muscle, increased oxidative stress and eNOS uncoupling (Wong et al., 2008) (see Figure 16) In experimental studies resveratrol promotes vasorelaxation via the activation of NO and improves FMD (Bhatt et al., 2011). In a study, a sample size of 40 obese patients with a body mass index (BMI) between 25 and 35 were separated and treated daily with varying doses of resveratrol tablets (0, 30, 90, 270 mg).

The findings demonstrate that in obese patients daily consumption of resveratrol improves flow-mediated dilatation in a dose-dependent manner (Wong et al., 2008). Interestingly, a tablet that optimises the absorption and bioavailability of resveratrol has been developed to improve its natural effect at a lower concentration (Fujitaka et al., 2011).

Hence, Longevenix is a modified resveratrol microcapsule in plant starches and dextrans, used to prevent phytoisomerisation and therefore improves resveratrol half-life. The tablet consists of 100 mg resveratrol, vitamin D3 and quercetin (Fujitaka et al., 2011). Longevenix was administered to 34 patients that were diagnosed with the metabolic syndrome and were already on standard treatment (ACE inhibitors, angiotensin receptor || blockers and statins) (Fujitaka et al., 2011). In conjunction with this, Longevenix was administered for three months and stopped for three months to determine whether the effect persists. There were no changes with blood pressure, HDL, LDL or BMI. However, a prominent increase in FMD was displayed after the three months but not sustained. Thus the study supports the notion that resveratrol improves endothelial function (Fujitaka et al., 2011), activates NO, but its effect on hypertension, a major contributor to vascular disease and oxidative stress, is unknown.

Recently, an experimental study demonstrated that resveratrol (50mg/l), given in the drinking water of rats) improves endothelial function and attenuates the rise in blood pressure in hypertensive rats (Pellegeta et al., 2003). Furthermore, the early chronic treatment of resveratrol prevents e-NOS uncoupling, reduces H₂O₂, and improves the percentage change of relaxation in the mesenteric artery rings (Bhatt et al., 2011). It has been reported that the vascular oxidative stress precedes development of hypertension in spontaneous hypertensive rats (SHR) (Nabha et al., 2005). The study highlights the importance of early resveratrol treatment in reducing the effects of endothelial dysfunction. Overall, resveratrol prevents eNOS uncoupling and endothelial dysfunction and attenuates the development of hypertension, a major contributor to IHD and HF.

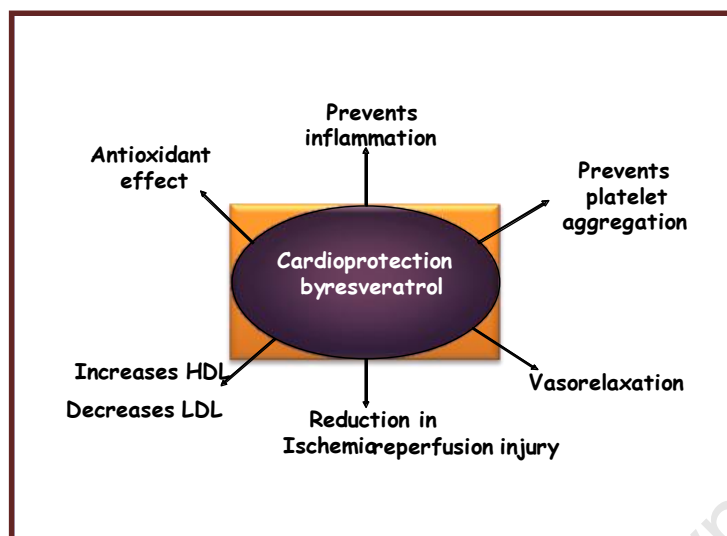


Figure 16: Cardioprotective actions of resveratrol (adapted from Vidavavular et al., 2009).

4 Melatonin Structure and Function

Melatonin (N acetyl-5-methoxytryptamine) is synthesised by the pineal gland and regulates circadian rhythm (Cajochen et al., 2003). It is involved in multiple processes (Tan et al., 1993) and has been suggested to have strong anti-oxidant, anti-inflammatory, vascular and anti-ischemic effects. However, melatonin capsules are freely available and are commonly used for jet lag and insomnia (Cajochen et al., 2003, Singh & Kumar 2009).

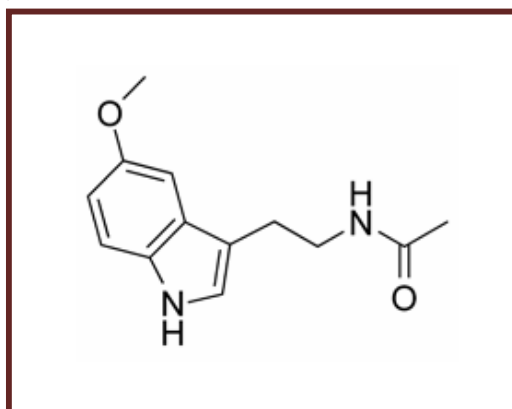


Figure 17: Schematic presentation of the chemical structure of melatonin (own drawing).

4.1 Melatonin and biosynthesis

Melatonin is produced in several tissues including, the pineal gland, retina, lens and gastrointestinal tract (Dowling et al., 2005). Melatonin is both lipid and water soluble which easily facilitates its movement across the cell membrane. After its release, it gains access to various tissues and cellular components (Galli-Carminati et al., 2009).

It is synthesised from the amino acid tryptophan (via synthesis of serotonin). Serotonin is converted to melatonin in the presence of N-acetyltransferase and s-acetylserotonin-O-methyltransferase (ASMT) (Lane & Moss 1985). Its production by the pineal gland is regulated by the hypothalamus which receives information from the retina about the daily pattern of light and darkness (Dowlin et al., 2005). Light suppresses the production of melatonin while darkness stimulates its production. Exposure to excessive light in the evening or too little light during the day can disrupt the body's normal melatonin cycle. Melatonin levels oscillate from 40 pg/ml during the day to 80 pg/ml at night (Hardeland et al., 2003). The exogenous administration of melatonin (supraphysiological levels), for jet lag or insomnia, does not interfere with the normal production of melatonin.

Of note, there are no studies that demonstrate melatonin toxicity even at higher concentrations and this encourages its clinical use without side effects (Jahke et al., 1999).

4.2 Melatonin and its receptors

Melatonin is mediated via its G-coupled protein receptors. Melatonin receptor 1 (MT1) and 2 (MT2) are G-coupled protein receptors. These receptors have a distinct encodes the Gprotein receptors, aspartic (NRY motif) rather than a conserved amino acid glutamic/aspartic (DRY motif). The receptors are glycosylated in their N-terminus. PKC, casein 1, 2 and PKA participate at the regulation of the receptor (Rodriguez et al., 2004). The activation of the MT1 or MT2 facilitates the dissociation of alpha (α) and beta-gamma ($\beta\gamma$) dimers from the G-coupled protein receptor (Ekmekcioglu et al., 2001)..

MT1 regulates the rhythmicity of the circadian clock expression via the activation of adenylyl cyclase which in turn, inhibits c β fibre activation in the hypothalamus (Hattori et al., 1998). The binding of melatonin to its receptor 1 leads to the activation of mitogen-activated kinase (MAPK). MT1 tentatively activates the nuclear receptor RZR/ROR (termed retinoid receptors) and it is suggested that the anti-oxidant effect of melatonin is mediated via the interaction of MT1 and RZR/ROR (Rodriguez et al., 2004). This co-localisation with the RZR/ROR receptor modulates calcium release via Ca⁺⁺/calmodulin signalling (Rodriguez et al., 2004) MT2 regulates the circadian rhythm via the activation of guanylyl cyclase (Masana et al., 2002).

Melatonin receptor 3 (MT3) is a putative receptor with a distinct pharmacological profile from MT1 and MT2. It was identified in the hamster brain that MT3 and quinone reductase 2 (QR2), both bind to the iodolmelatonin with the same pharmacological profile (Masana et al., 2002). Evidence suggests that MT3 is actually QR2 present as a cytosolic enzyme and is not membrane-bound. The complete identity of MT3 membrane binding sites with the quinone reductase 2 remains enigmatic (Dubovovich & Markowska, 2005; Ekmekcioglu.2006)

Melatonin receptors are located in the vascular beds. MT1 and MT2 are detected in the cerebella arteries in humans. Melatonin modulates both vasoconstriction and vasodilation depending on which receptor mediates the response (Marillet et al., 2009, Masna et al., 2002). In the rat caudal artery, melatonin potentiates the adrenal nerve stimulation. Vasoconstriction was mediated via the reduction in cAMP phosphorylation of the voltage-gated potassium channels (BKCa) through MT1 predominantly present in the smooth muscles and regulates estrogen release (Marillet et al., 2009, Girgert et al., 2009). MT1 also leads to the activation of protein kinase 1 and 2 (MEK1, MEK2). Furthermore, MT1 is present in the hippocampus and it is proposed to regulate blood flow as well as diurnal blood pressure fluctuation (Pong et al., 2002). Conversely, MT2 is involved in the reduction of vascular tone (Pong et al., 2002). Melatonin bound to MT2 increases blood flow to the skin and may regulate heat loss. This receptor is important for the adaptations required for seasonal change.

MT3 seems to play a role in the regulation of the immune system as well as regulating ocular pressure, but its exact function in the heart is unclear. MT3 prevents the recruitment of leukocyte adhesion molecules (Mailliet et al., 2005, Menendez-Palaez et al., 1993).

4.2.1 Melatonin confers cardioprotection

Many studies enumerate the cardioprotective effect of melatonin against ischemic damage and altered physiology. Tan et al, showed that Langendorff perfused hearts that underwent 10-minute ischemia followed by 10-minute reperfusion, had increased ventricular fibrillation (VF) and premature ventricular contraction (PVC) by 80% (Tan et al., 1993). However, melatonin treatment (1 μ M, 10 μ M, 50 μ M) decreased both VF and PVC. This was compared to the effect of vitamin C (500 μ M). Vitamin C was significantly less effective than melatonin in reducing VF and PVC. Similarly, Kaneko et al. found that melatonin (100 μ M) prior to I/R also reduced VF and improved the overall function of the left ventricle. Other studies with melatonin and its analogue, 5-methoxy-carbonylation-N-acetyltryptamine (5-MCA-NAT) protected against I/R injury (Lopez et al., 2003, Szarszoi et al., 2001). The protective effect of melatonin *ex vivo* was corroborated with *in vivo* studies. All melatonin-treated rats had improved VF and reduced mortality rates (Lagneux et al., 2000).

In rats, the long-term effects of melatonin were evaluated 24 hours after melatonin administration (2.5 or 5.0 mg/kg, ip) or after the oral supplementation of melatonin in the drinking water for seven days (20 or 40 μ g/ml) (Lochner et al., 2006). These results suggest that melatonin induces long-term protection as evidenced by the reduction of infarct size. The cardioprotective effect of melatonin persisted for two to four days after discontinuation of treatment. Chronic administration of melatonin (10mg/kg) for one month after mice were born modulated the expression of senescence-accelerated-prone mice (SAMP8) and reduced the A β fibres (Gutierrez et al., 2008). The oxidative stress was also markedly reduced and enhanced the activation of Bcl-2 (Forrest et al., 2007). The protective effects of melatonin can reduce I/R injury in other organs including the brain, liver, kidney, placenta and GIT (Forrest et al., 2007). Patients with rheumatoid

arthritis treated with melatonin (10mg/day) given orally had reduced inflammatory cytokines (Maney et al., 1996).

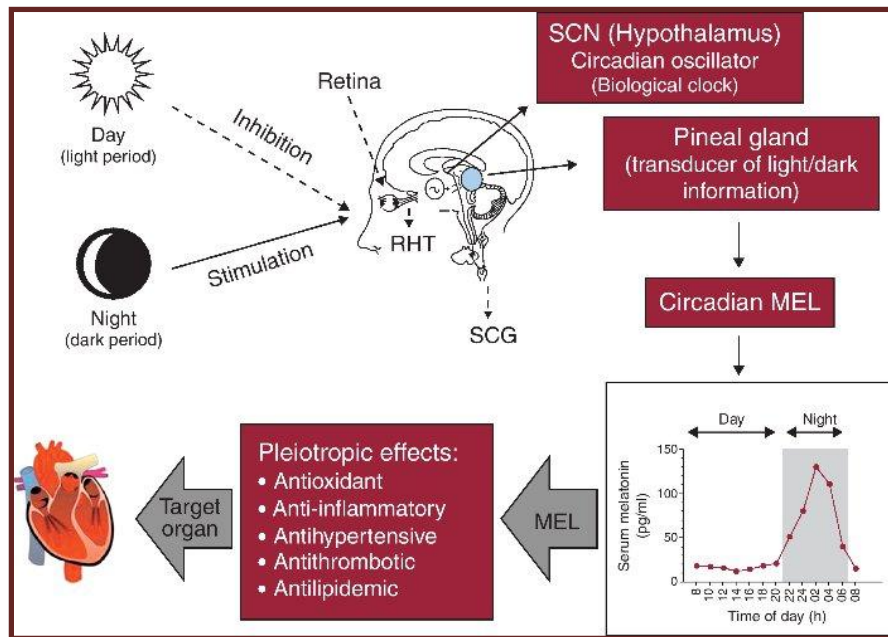


Figure 18: The cardioprotective properties of melatonin. SCN (suprachiasmatic nucleus), Mel(melatonin),SCG (superior cervical ganglion), RHT (retinohypothalamic tract) (Dominguez-Rodriguez et al., 2012).

4.3 Mechanism of melatonin-induced cardioprotection

Melatonin can scavenge free radicals directly by inhibiting their formation or alternatively via the upregulation of anti-oxidant enzymes, thus improving its anti-oxidant capabilities (Zang et al., 1998). The direct scavenging effects of melatonin are receptor-independent and the effects of melatonin as an antioxidative are receptor-dependent (Tan et al., 1993).

4.3.1 Melatonin and its other effects

Melatonin does not possess a hydroxyl group, like other anti-oxidants such as vitamin E, and hinders the formation of lipid peroxidation by curtailing the initial factors that promote oxidation, and not via the direct breaking of the chain (Karbownik et al., 2000). A study comparing melatonin and vitamin E found that melatonin was twice more effective in scavenging free radicals (Pieri et al., 1996). Even melatonin's metabolite 6-

hydroxymelatonin acts as a powerful free radical scavenger and promotes detoxification (Scinivasan et al., 2002). Melatonin detoxifies H_2O_2 directly and increases glutathione peroxidase (GPX), catalase (CAT), superoxide dismutase (SOD) and MnSoD (Reiter et al., 2003). Melatonin modulates Ca^{++} signals because of its fixation to calmodulin which influences adenylyl-cyclase and phosphodiesterase (Petrosillo et al., 2006, Petrosillo et al., 2009). Furthermore melatonin can regulate the release of Ca^{++} directly by acting on the voltage gated Ca^{++} channel. Furthermore, melatonin increases the efficiency of the electron transport chain by lowering e-leakage from complex 4 and in turn, reduces free radical release and therefore, improves mitochondrial function (Chen et al., 2005, Okatani et al., 2003). Also, melatonin reduces the effects of reperfusion by regulating the production of cardiolipin and in turn regulates the opening of the mitochondrial permeability pore (mPTP) (Petrosillo et al., 2009). The inhibition of the mPTP promotes cardioprotection (Petrosillo et al., 2009). Therefore, any drug such as melatonin that influences the mitochondrial permeability pore may mitigate the adverse effects of acute myocardial infarction.

Atherosclerosis is the abnormal development and progression of cholesterol deposits in the tunica intima of arteries.

The development of the plaque involves the activation of inflammatory cytokines and oxidised LDL (Pieri et al., 1996). According to the oxidative modification hypothesis LDL becomes mildly oxidised in the arteries and leads to the recruitment of a variety of vascular cells. These cells activate the monocytes chemotactic protein 1 (Pieri et al., 1996). Consequently, the LDL is recognised by the scavenger receptors on the macrophage. The macrophage internalises the LDL and forms a foam cell. It is not subject to negative feedback and thus increases the uptake of cholesterol (Okatani et al., 2000). LDL has direct chemotactic effects on the monocyte and promotes the binding of the monocyte to the endothelium walls (Kelly et al., 1997). The LDL becomes trapped in the sub-endothelial space because LDL inhibits regress of the arterial wall. This is supported by the extensive presence of LDL in the atherosclerotic lesion. Also LDL is oxidatively altered *in vitro*. Melatonin inhibits oxidised LDL *in vitro* in a dose-

dependent manner (Duell et al., 1998). Other studies support these findings and suggest that melatonin precursors (such as L-tryptophan and serotonin) as well as the breakdown products (such as niacin or quinolinate) also inhibit oxidation of LDL (Duell et al., 1998). Melatonin reduces the total cholesterol plasma levels and decreases very low-density lipoproteins, (VLDL) in hypercholesterolemic rats (Kelly et al., 1997).

4.3.2 Melatonin and other mechanisms

Melatonin treatment lessens inflammation by reducing the oxidative stress. It targets the binding of leukotriene B4 (LKB4) to the endothelial cells and reduces the mobilisation of NFkB and in turn prevents the recruitment of ICAM, VCAM and P-selectin (Kelly et al., 1997). Melatonin targets chronic inflammation by blocking the translocation of NFkB and reduces ROS formation in the cells (Li et al., 2005). Melatonin also inhibits cyclooxygenase (COX-2) and inducible nitric oxide synthetase (iNOS) (Deng 2006). In patients with rheumatoid arthritis, patients treated with melatonin (10mg/day) had reduced inflammatory cytokines. In elderly patients melatonin (5mg/day) reduces the effects of hypertension compared to placebo (Lusardi et al., 2002).

Clinically, melatonin reduces the effects of oxidative stress in humans subjected to cardiopulmonary bypass surgery (Ochoa et al., 2003). Whether it is the reduction in melatonin that causes disease or the disease that reduces melatonin levels remain unclear. Moreover, melatonin given at the onset of major surgery also reduced the by-products of lipid peroxidation in the erythrocyte membrane and in the blood (Ochoa et al., 2003). Hypertension is one the leading risk factor for IHD and HF (Steyn et al., 2005). Pinealectomised rats have diminished melatonin levels and are strongly associated with vasoconstriction and temporary hypertension (Stewart et al., 2006). However, these effects were reversed with melatonin administration (Zanoboni et al., 1967). The role of endogenous melatonin in cardioprotection is further illustrated in pinealectomised mice that present with a mortality rate of 63% compared to zero mortality in the intact mice. Interestingly, continuous exposure to light both day and night prevents the nocturnal increase of melatonin, suppresses circadian heart rate and augments blood pressure, resulting in melatonin-deficient hypertension (Rezzani et al.,

2010, Paulis et al., 2010). SHR with left ventricular hypertrophy were treated with melatonin and improved cardiac function but did not alter the left ventricular relative weight (Zanoboni et al., 1967). Clinical studies in hypertensive patients demonstrated that chronic melatonin treatment (2.5 mg/day) significantly reduced blood pressure after three months (Scheer et al., 2004). These results strongly suggest that melatonin plays a major role in the regulation of hypertension. It has been shown in an experimental model that pharmacological concentrations of melatonin are receptor-dependent and are mediated via the activation of the Akt/Erk pathway (Genade et al., 2008).

4.3.3 Melatonin is present in red wine

Melatonin is synthesised by various plants such as rice, berries and grapes (Iriti 2009, Hattori et al., 1995). In 2006, Italian researchers found the presence of melatonin in extracts from different wine grapes including Nebbiolo, Croatina, Sangiovese, Merlot, Mrzemino, Cabernet Franc, Cabernet Sauvignon and Barbera (Iriti et al., 2010). Melatonin was found in a high concentration in red wine ranging from 50pg/m μ l to 200pg/m μ l. Interestingly, the amount of melatonin is lower in white wine (range: 10-50pg/m μ l) and even less in liquors (range: 5-20pg/m μ l) (Guerrero et al., 2008) (see Figure 19).

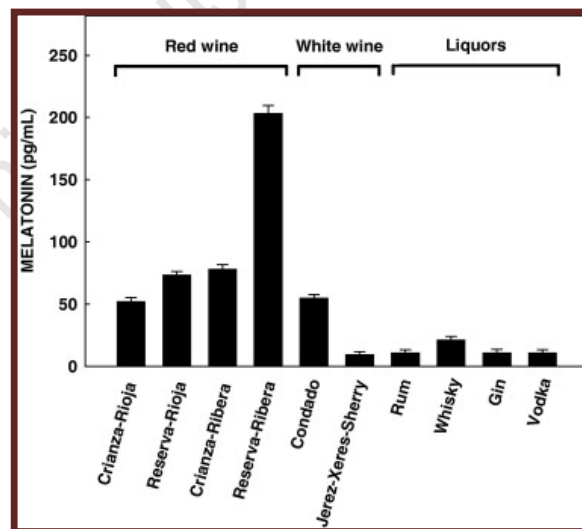


Figure 19: Melatonin concentration in varying alcoholic beverages (Guerrero et al., 2008).

It is likely that cultivar, agro-meteorological conditions (Iriti et al., 2010), vintage and wine-making procedures contribute to the difference in concentration of melatonin between the wines (Iriti et al., 2010). Ironically, the anti-mould fungicide benzothiadiazole, which wineries spray on their plants to protect the grapes, seems to increase the concentration of melatonin (Iriti et al., 2010). Interestingly, Guerrero et al., (2008) demonstrated that serum melatonin was significantly increased in humans, one hour after an intake of 100 ml of red wine. However, for unknown reasons, this article has been withdrawn at the request of the editor of the relevant journal.

University of Cape Town

B. Aims and hypothesis

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1 Aims

Ischemic heart disease (IHD) is a global burden and additional therapies are urgently required to assist in the prevention and curing of the disease. Both epidemiological studies and experimental studies have shown that the regular moderate consumption of red wine confers cardioprotection against IHD. Delineating the cardioprotective components present in wine that contribute to this cardioprotective effect may lead to the development of novel therapies against IHD. The presence of melatonin, a biogenic amine present in foods, but also produced naturally in the body to regulate sleep, has recently been identified in red wine (Guerrero et al., 2008). Melatonin protects against I/R injury but whether it contributes to the cardioprotective effect of red wine remains unclear (see Figure 20).

We therefore hypothesised that the presence of melatonin in red wine contributes to the cardioprotective effect of the regular moderate consumption of red wine against IHD. We further suggest that this cardioprotective effect may be mediated via the activation of the SAFE pathway that involves the modulation of TNF α and STAT3.

To investigate our hypothesis the following objectives were pursued

1.1 Objectives

- 1) Using an *in vitro* model of I/R our objective is to demonstrate that the acute treatment with melatonin, at concentration found in red wine, can confer cardioprotection. We further propose that this cardioprotective effect is mediated via the activation of the SAFE pathway (Chapter 1).
- 2) Using both an *ex vivo* and an *in vivo* model of I/R we aim to demonstrate that the chronic and moderate consumption of red wine or melatonin protect against I/R injury via the activation of the SAFE pathway. Furthermore, melatonin receptor inhibitors were used to explore the exact role of melatonin in red wine-induced cardioprotection (Chapter 2).

- 3) Using an *in vivo* mouse model of ischemic heart failure we aimed to demonstrate that the cardioprotective effect of a low and chronic treatment with melatonin can be extended to ischemic heart failure. (Chapter 3).

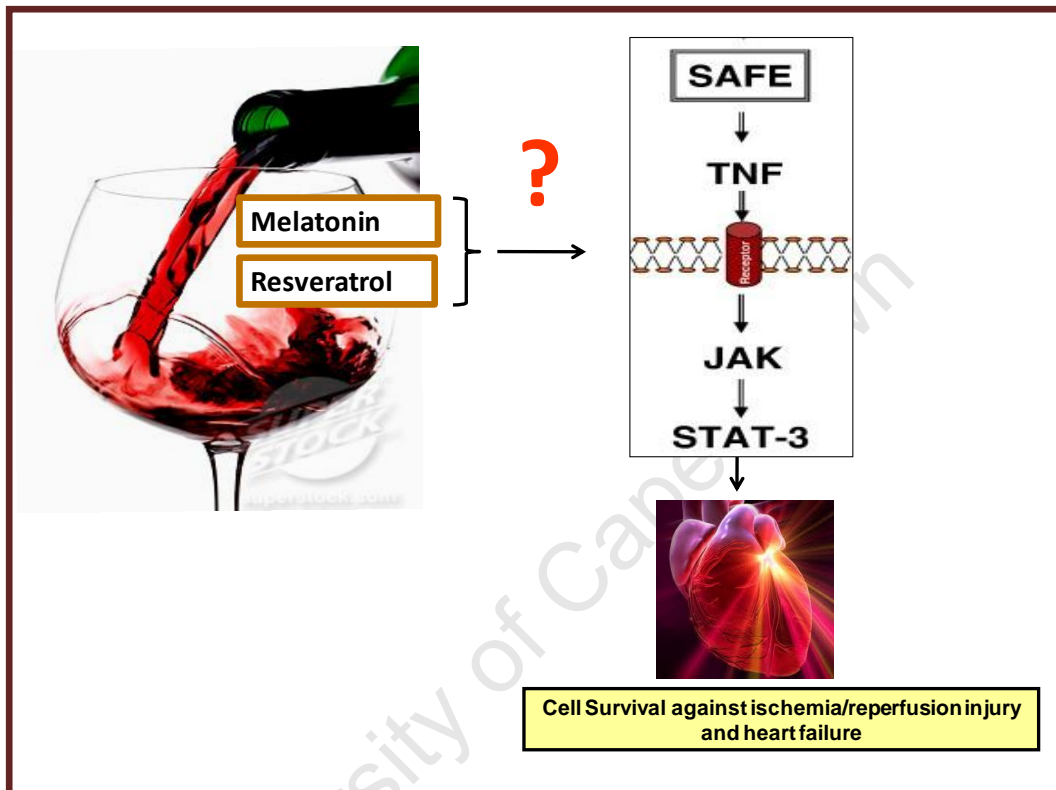


Figure 20: Hypothetical mechanism for red wine-induced cardioprotection mediated via the activation of the SAFE pathway.

C. MATERIALS AND METHODS

University of Cape Town

1 Animals used

Experiments were conducted on either male Wistar rats weighing 230-300g or mice ranging from 12-14 weeks, and were performed in accordance with the *Guide for Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH publication No. 85(23), revised 1996). All procedures were approved by the Animal Research Review Committee of the University of Cape Town (applications; 011/026 “Cardioprotection with red wine and its active components in an *in vivo* mouse model of heart attack/HF” 008/026 entitled “Delineation of mechanisms for wine-induced cardioprotection”, 007/012 “Delineation of resveratrol-induced cardioprotection” 005/016 “The cardioprotective effect of alcohol-free wine”

2. *In vitro* mouse model of I/R injury

2.1 Types of mice

Homozygous TNF deficient mice (TNF receptor 2 $-/-$), and their respective wild-type littermate control Black 6 x 129 (TNF-WT) were donated by Dr Muazzam and Professor Bernhard Ryffel from the Department of Immunology and Infectious Diseases, University of Cape Town.

Takeda et al. showed that whole-body STAT3 knockout (KO) mice resulted in the death of the embryo between 6.5- 7.5 days of gestation (Takeda et al., 1997). Thus STAT3 is crucial for the early development of the embryo. Consequently, the STAT3 knockout (KO) mice were created as cardiomyocyte-specific, deficient mice achieved by using the Cre-Lox P system of conditional gene ablation.

Cardiomyocyte-specific STAT3 deficient (STAT 3 $-/-$) mice were from a C57 Black 6 background created at the Hatter Institute by crossing homozygous floxed STAT3 mice with a heterozygous myosin light chain 2 ventricle (MLC2V)-driven Cre-recombinase mice, as described by Smith et al., 2002.

2.2 The Langendorff perfusion isolated mouse heart model

The Langendorff perfusion system is a retrograde perfusion system, with the heart cannulated at the aorta and maintained at constant pressure. It was first discovered in 1895, by the German physiologist Sir Oskar Langendorff who isolated and perfused mammalian heart preparations (Langendorff et al., 1895). It is widely adopted in cardiac physiology for the examination of contractile strength, heart rate and other vascular effects. The simplicity of the model has led to its frequent use in both basic and pre-clinical drug research and experiments can be conducted on both rats and mice.

2.3 Preparation of the Langendorff system

The system was flushed several times with boiling water to remove any bacteria and endotoxins. Krebs Henseleit Buffer (KHB) was prepared daily because if kept for a longer period of time the calcium would precipitate and cause a blockage in the perfusion system. The KHB was made as described in the table 1, in mM to make 5L of water. The composition of KHB was set to mimic body plasma as much as possible to keep the heart viable outside the body. The different compounds were added to 4L of distilled water, with calcium and glucose added after the other compounds had been oxygenated with (95%O₂ and 5% CO₂). Thereafter the pH of the KHB was maintained at 7.4 and the solution made up to a final volume of 5L with distilled water. The KHB was further oxygenated for 20 minutes before use. The Powerlab Data Acquisitions software™ (ADInstruments™) was used to measure functional parameters which were calibrated daily with a sphygmomanometer for accurate readings.

Table 1: The composition of KHB for mouse perfusion.

Krebs-Henseleit perfusion buffer	
mM	Grams (g)/ℓ
118.0 NaCl	34.5
24.0 NaHCO ₃	10.1
4.0 KCl	1.5
1.0 NaH ₂ PO ₄	0.7
1.2 MgCl ₂	1.2
2.5 CaCl	1.85
0.5 EDTA	0.95
10.0 Glucose	9.0

2.4 Mounting of the heart on the system

Adult male TNF α $-/-$, TNF R2 $-/-$ and cardiomyocyte specific STAT3 $-/-$ mice and their wild-type littermates were anaesthetised with 60 mg/kg intraperitoneal sodium pentobarbitone mixed with 20 international units (I.U.) of heparin. To determine adequate anaesthesia prior to the opening of the chest, the pedal test was implemented whereby the complete lack of a reflex was an indicator for adequate anaesthesia. The sternum and the costal cartilage were excised for the complete view of the mediastinum. The mouse hearts were rapidly removed from the chest cavity and placed in cold KHB buffer, to reduce enzymatic activity. The heart was cannulated via the aorta, and perfused retrogradely using the Langendorff perfusion system at a constant pressure (100 cm H₂O), at 37°C by inserting a fine thermo-coupled wire monitored on a digitron 2600T (Torquay, UK).

The developed tension was assessed, with a weight that created a force of 2g to work against, by utilising a rigid lightweight lexan coupling rod connected to a force placement transducer (Grass FT03C, MA, USA) (see Figure 21). A 4.0 suture attached to a 20mm atraumatic needle was threaded 1 mm from the tip of the apex of the heart

and fastened firmly onto the coupling rod. All the hearts were equilibrated to pull at 2g weight and were paced at 560bpm. Tension output was recorded on the Powerlab chart recorder (ADI Instruments, Australia) as described by Smith et al., 2002. The isolated mouse hearts were left to calibrate for 20 minutes prior to 35 minutes of global ischemia (total cessation of perfusate by closing the three-way stopcock to prevent flow from the aortic cannula; in addition, KHB was added into the jacket to maintain temperature of 37°C at all times) followed by 45 minutes reperfusion (Smith et al., 2002). Cardiac parameters were monitored throughout the experiments and included heart rate (HR), coronary flow (CF) and developed force (DF).

2.5 Exclusion criteria

Mice that did not comply with the following criteria were removed from the study; (1) Coronary flow must be a minimum of 1.5ml/minute and a maximum of 5.0ml/minute,(2) Heart rate should be a minimum of 460–600 beats/min, (3) Developed force must be greater than or equal to 4mg. An exclusion criterion provides a baseline for consistency and standardisation of each heart mounted onto the system. At the end of the experiments, the hearts were removed and 1ml of 1,2,3 triphenyleterazolium chloride (TTC, 1% phosphate buffer, ph 7.4) was infused into the hearts through the stopcock. The hearts were frozen at -4°C and kept for a maximum of one week before infarct size analysis.

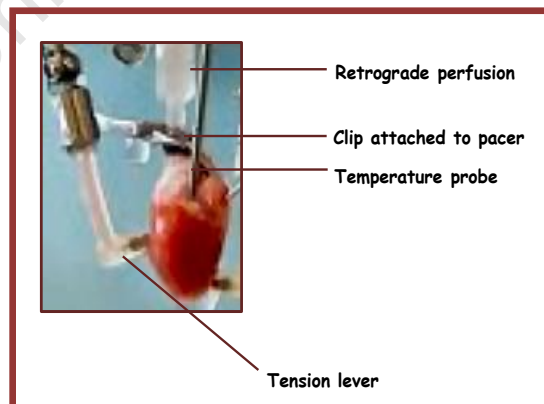


Figure 21: Mouse heart mounted on the Langendorff apparatus. The heart is retrogradely perfused via the aorta.

3. Isolated rat heart model

Laboratory rats are derived from the wild Norway rat *Rattus Norvegicus*. Rats were first domesticated for laboratory use in 1828 and utilised for research (Farkas et al., 2008). Wistar rats are mutant albino rats belonging to the same strain. Wistars are currently the most popular rat strain used for laboratory research and are characterised by their wide heads, long ears and long tail. They are easily bred and commercially available in large quantities, small in size with minimal maintenance cost and are well characterised anatomically, physiologically and genetically and therefore perfect for research (Farkas et al., 2008).

3.1 Preparation of Langendorff perfusion system

The hearts were mounted onto the perfusion system and perfused retrogradely through the ascending aorta, which forces the aortic valve closed and shunts the blood toward the coronary ostia into the coronary arteries. The perfusion system must remain at a constant pressure (100 cm H₂O), at 37°C, with KHB equilibrated with O₂/CO₂ 95:5%. To prepare the KHB different compounds were added to 4L of distilled water, with calcium and glucose added after the other compounds had been bubbled with (95%O₂ and 5% CO₂), see table 2. Thereafter the pH of the KHB was maintained at pH of 7.4 and the solution made up to 5L with distilled water. The composition of the rat KHB was slightly different to the mouse system. These conditions are set to mimic bodily function as much as possible to keep the heart viable outside the body and are similar to the mouse model as described in this section.

Table 2: The composition of the KHB for rat perfusion.

KHB perfusion buffer	
mM	Grams (g)/5L
118.0 NaCl	34.63
25.2 NaHCO ₃	10.5
4.7 KCl	1.77
1.2 MgSO ₄ .7H ₂ O	1.47
1.2 KH ₂ PO ₄	0.8
1.2 CaCl ₂ .2H ₂ O	1.0
11.0 Glucose	10.99

Before mounting the heart the system was flushed with boiled distilled water daily to remove any bacteria and endotoxins from the system as mentioned in the mouse model. A fresh batch of KHB was made daily, as keeping it for more than a day leads to the precipitation of calcium or glucose, which is detrimental to the heart. After the KHB was made to a final volume of 5L it was further oxygenated for 20 minutes before use, as mentioned in the mouse model. The Powerlab Data Acquisitions Software™ (ADInstruments™) was used to measure functional parameters which were calibrated daily with a sphygmomanometer for accurate readings.

3.2 Mounting of the rat heart onto the Langendorff perfusion system

Rats were anaesthetised with a 60 mg/kg intraperitoneal injection of sodium pentobarbitone and mixed with 200 IU heparin. The animals were placed in a quiet room and left for 15 minutes to acclimatise and before anaesthesia for stress reduction. Stress causes the release of catecholamines and other circulatory factors which can affect the functioning of the heart, which can affect experimental outcome. The depth of anaesthesia was assessed with the pedal reflex, whereby the paw of the animal was pinched and its perception of pain was observed.

Thoracotomy was performed when the rat was deemed unconscious. A forward incision of the skin at the xyphoid-sternum (sternotomy) was delicately performed. The incision continued along the proximal ends of the left and right costal margins. The thoracic cavity was opened by and the pericardium surrounding the heart removed thereby, exposing the beating heart in the chest cavity. The heart was firmly cradled and hosted between the thumb and the index finger and removed with one quick precise incision without inflicting damage to the aorta. The isolated heart was immediately placed into ice-cold KHB to arrest the heart.

The heart was mounted onto the cannula with two pairs of forceps stretching the aorta on either side to ensure a snug fit onto the cannula while perfusate dripped from the cannula to enable a fluid-fluid connection. The heart was temporarily secured with a bulldog clamp in order to keep it in place. Thereafter, a thin suture tied around the aorta connected to the cannula was used to securely fasten the heart, see figure 22. The pulmonary artery was punctured to release the pressure build-up in the coronary ostia. Subsequently, the left ventricle was exposed with a small excision of fat deposits surrounding the left atria, to enable the balloon insertion into the left ventricle for hemodynamic measurements. The intraventricular balloon attached to a catheter filled with distilled water was connected to a pressure transducer. Inside the left ventricle, the balloon was inflated and filled with distilled water, thus creating a closed system to measure the pressure experienced by the left ventricle, see figure 23.

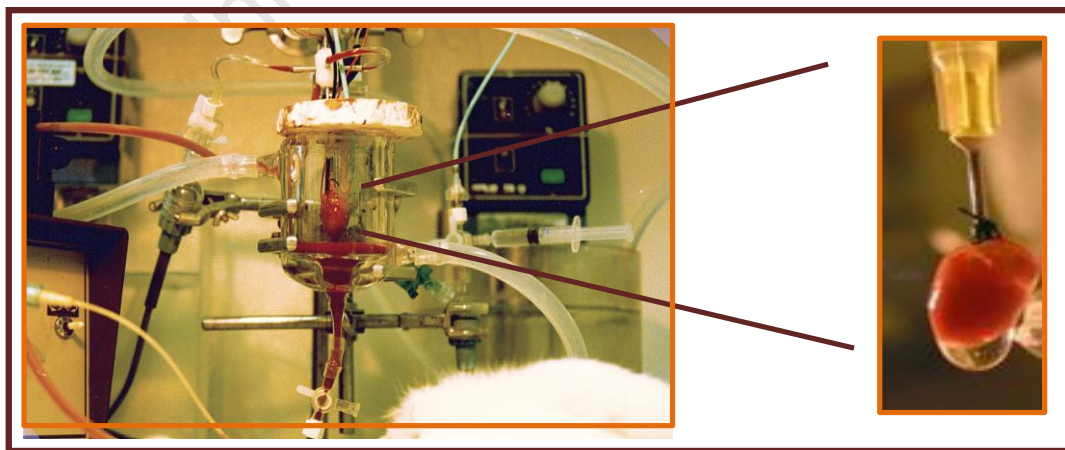


Figure 22: Rat heart mounted on the Langendorff perfusion apparatus.

The left ventricular end diastolic pressure was adjusted between 4 and 12mmHg and the hemodynamic parameters were assessed as follows.

Cardiac parameters were monitored throughout the experiments and included heart rate (HR), left ventricular developed pressure (LVDP: difference between left ventricular end systolic pressure (LVESP), end diastolic pressure (LVEDP) and the coronary flow (CF). Functional recovery was expressed as LVDP X HR at a specific time point compared to the stabilisation

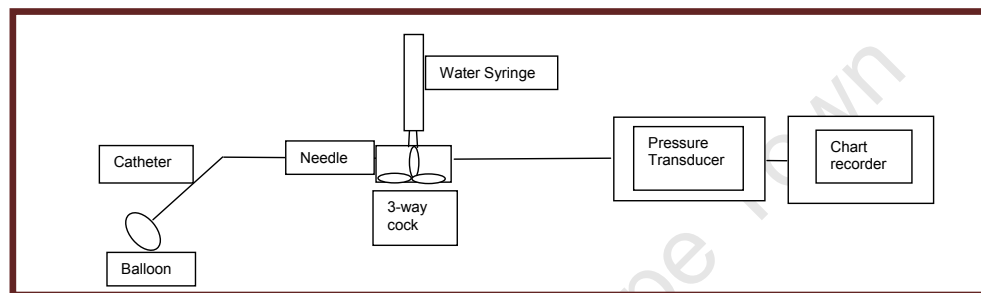


Figure 23: Schematic representation of the balloon catheter. (AD Instruments, London).

3.3 Exclusion criteria

Rats that did not comply with the following criteria were removed from the study: (1) Left ventricular pressure must be higher than 80mmHg; (2) Coronary flow must be a minimum of 8ml/minute and a maximum of 16ml/minute; (3) Heart rate should be a minimum of 240 beats/minute.

3.4 Ischemia reperfusion models

Ischemia reperfusion models were ideal to study cardiac physiology for the examination of contractile strength, heart rate, coronary flow and other vascular effects. The model was used to test drugs of interest in protecting against I/R.

3.4.1 Regional Ischemia

In regional ischemia, a 3/0 silk suture was placed around the left ascending coronary artery to form a snare to simulate ischemia. Drugs of interest were administered prior to the ischemic insult and were directly administered to the heart through a side pump attached to the system.

Thereafter, the snare was tightened and blocked off, the flow of buffer to that specific region of the heart, thus ischemia was induced for 30 minutes. KHB was returned and the heart was reperfused for 120 minutes. Clinically, this setting mimics the blockade of an artery in the heart which eventually leads to myocardial infarction. At the end of the protocol the snare was tightened and 1% Evans blue was perfused through the three-way stopcock into the non-ischemic areas of the heart. The Evans blue, is a monochromatic stain, blue in colour and used to delineate the dead cells; in the ischemic area, the non-ischemic area or the area at risk. Thereafter, the heart was removed and stored at -4°C for infarct size analysis.

3.4.2 Global ischemia

Hearts were equilibrated and subjected to 30 minutes global ischemia followed by 60 minutes of reperfusion. Global ischemia was used for all the chronic studies and was achieved by the complete cessation of perfusate together with additional KHB within the jacket to maintain temperature at 37°C . In this setting the drugs administered to salvage the heart were given chronically for 14 days, prior to the global ischemia. At the end of reperfusion, hearts were removed and stored at -4°C for infarct size analysis. Global ischemia mimics the complete cessation of flow of blood as attained during coronary bypass or heart transplants in the clinical setting.

All pharmacological agents were purchased from Sigma Chemicals Company (St Louis, MO, USA), if not stated otherwise. Luzindole and prazosin, a melatonin inhibitor was obtained from Tocris bioscience (London, England).

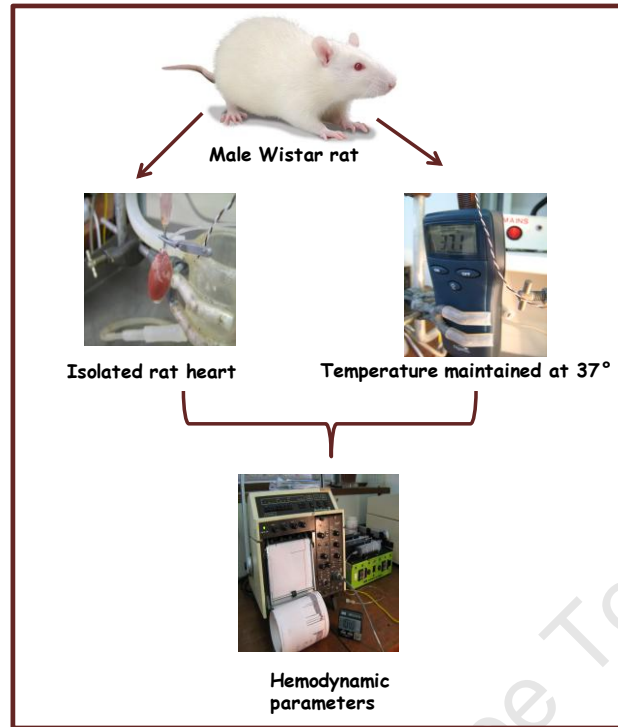


Figure 24: Isolated rat hearts were mounted onto the Langendorff system and hemodynamic parameters were assessed.

4. In vivo mouse model of I/R injury

Male mice (12-14 weeks) were anaesthetised with a combination of ketamine (0.06mg/ml) and xylazine (2mg/ml); the final concentration of both was 0.01mg/ml and the body temperature was maintained at 37 degrees (Lim et al., 2007). Non-recovery procedures used ketamine/xylazine as the anaesthetic of choice based on the following criteria: (1) rapid induction for a relatively long duration (\pm 90 minutes); (2) excellent muscle relaxation; (3) a dissociative anaesthetic; (4) disruption of pain transmission and suppression of spinal cord activity with some action on opioid receptors (Green et al., 1981). The ketamine/xylazine was used for acute experiments because it has a less pronounced cardioprotective effect (Roth et al., 2002).

However, for recovery animals (permanent ligations for ischemic HF), 4% isoflurane, prior to intubation this was reduced after intubation to 2% isoflurane mixed with oxygen were administered based on the following criteria: (1) the cardiopulmonary depression is not as extensive as gases that require inhalation; (2) reduced sensitisation of heart, by a

reduction in the catecholamines; (3) reduced respiratory depression; (4) rapid induction and recovery (Green et al., 1981). It has been suggested by that isoflurane used in long-term studies has a less pronounced cardioprotective effect because there is sufficient time for drug metabolism and excretion (Flecknell, 1996).

After the administration of anaesthesia (either xylazine/ketamine or isoflurane) the pedal reflex test was performed to ensure deep sleep. Thereafter, a tracheotomy was performed. The mice were intubated for artificial respiration at 120 strokes/minute and 220 μ l stroke volume using a minivent (Kent Scientific; Torrington CT). A left anterior thoracotomy was performed in the third intercostal space (retractors were used for clear visualisation of the heart) and the pericardium was removed. The left ascending coronary artery was ligated with ligature, 8.0 ethilon, nylon suture black filament, 2mm below the tip of the left auricle. A snare was created with a small piece of rubber tubing to create the ischemic insult for 30 minutes and then released and reperfused for 120 minutes. The experiments were terminal and the mice were sacrificed. At the end of the protocol, 200-500 μ l of Evans blue of which was injected directly into the left ventricle of the heart until the extremities of the mouse changed colour. The heart was squeezed and rinsed vigorously in saline to remove blood clots. Thereafter, the hearts were stored at -4°C and sectioned a week later.

For the long-term studies, IHF was assessed; the left ascending coronary artery was permanently ligated (Salto-Tellez et al., 2004). The ribs were sutured together with 5.0 prolene blue monofilament polypropylene suture and the chest closed. Thereafter, the isoflurane flow was ceased and the mice were ventilated on oxygen only, until fully conscious. The mice were kept and assessed daily for 28 days.

Buprenorphine (0.05mg/kg, ip) was administered as an analgesic with a single dose given before surgery and administered every 12 hours post operation for 5 days (Adamson et al., 2010). Buprenorphine has an antinociceptive effect and is considered a multimodal analgesic due to its action on the opioid receptors.

The maximum efficacy is between 3-6 hours and has a bioavailability of 24-72 hours after initial administration (Adamson et al., 2010). Prior to the ligation and before the termination hemodynamic function was assessed with an echocardiogram.

5. Infarct size assessment

At the end of the experiments, the hearts that underwent global ischemia were removed from the perfusion system and stained with either triphenyltetrazolium chloride (TTC, 1% in phosphate buffer, pH7.4). TTC reacts with ubiquinone reductase (NADPH) in live tissue and stains the cells with a brick red colour, on the contrary the infarcted tissue lacks NADPH and remains white. The hearts were frozen overnight and sectioned perpendicular to the apex, into 1mm slices and placed into 10% formalin for 12 hours.

Thereafter, the sectioned hearts were kept for one week. The hearts were stained with warm TTC (1%) solution poured into petri dishes. The frozen hearts were slightly defrosted and sectioned, once all the residual water had been removed the hearts were placed into the petri dishes with TTC solution and were maintained for 15-20 minutes at 37°C. Thereafter, the TTC solution was decanted and 10% formalin was added for 12 hours at room temperature.

The hearts, whether stained with TTC alone or Evans blue, were mounted in between two glass plates at a distance of 0.5mm for compaction for clear digital scanning. Infarct size was quantified with computerised planimetry (Planimetry+, Boreal software, Norway).

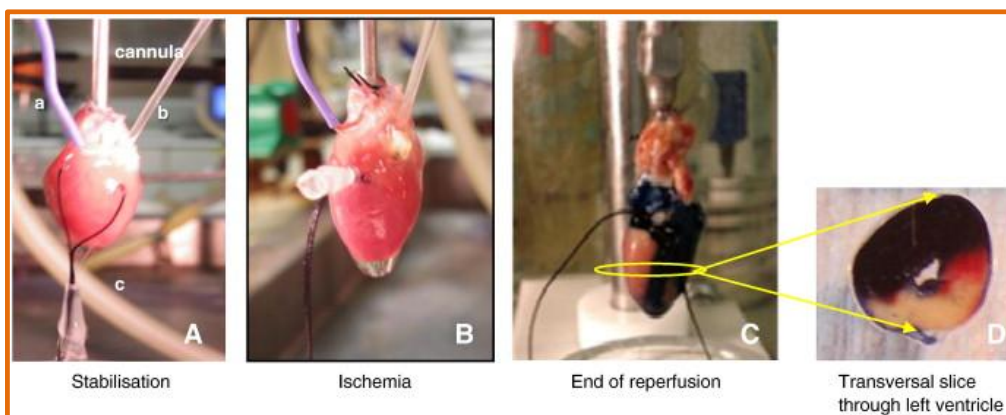


Figure 25: A graphical representation of Evans blue and TTC staining for infarct size.

6. Western Blotting

Rat hearts were harvested and perfused as previously described. The hearts were subjected to different acute treatments (control, melatonin and resveratrol) and freeze-clamped prior to the ischemic insult (after removal of atria). An additional group that was chronically pre-treated with red wine was collected at 15 minute into reperfusion, for later Western Blot analysis to explore the levels of total and phosphorylated STAT-3 on the tyrosine residue in the heart.

6.1 Nuclear and cytosolic protein extraction

Frozen tissue was wrapped in foil and pulverized with a hammer and liquid nitrogen. The powdered tissue (200mg) were homogenized by Polytron, at setting 4, with 900 μ l lysis buffer (20mM HEPES, 2.5mM MgCL₂, 100 μ M EDTA, 20mM β - glycerophosphate, 0.05% Triton x-100 (cytosolic extract), 1% Triton x-100(nuclear extract), dithiothereitol (DTT), phenylmethylsulfonyl fluoride (PMSF) and NaCl and centrifuged at 10000g for 5 minutes at 4°C with the lysis buffer. The supernatant (crude cytosolic extract) was removed and corresponded to the crude cytosolic extract. Thereafter, 500 μ l, resuspension buffer, was added to the remaining pellet and homogenized and centrifuged at 15000g for 30 minutes, see table 3. The supernatant was removed and corresponded to the crude nuclear extract.

Table 3: Compounds required to make up lysis buffer and resuspension for the extraction process.

Lysis buffer			Resuspension buffer		
Solution (30mℓ)	μl	Final Concentration	Solution (10mℓ)	μl	Final Concentration
1M Hepes, ph 7.9	600	20mM	1M Hepes, ph 7.9	200	20mM
2M MgCl ₂	37.5	2.5mM	2M MgCl ₂	12.5	2.5mM
500mM EDTA	6	100μM	500mM EDTA	2	100μM
100mM glycerolphosphate	6000	20mM	100mM glycerolphosphate	2000	20mM
TritonX-100(100%)	15	0.5%	TritonX-100(100%)	100	1.0%
100 mM DTT	1500	500μM	100 mM DTT	1500	500μM
100mM NaVO ₄	30	100μM	100mM NaVO ₄	10	100μM
EDTA protease inhibitor (tablet)	1200	-	EDTA protease inhibitor (tablet)	400	-
100 mM PMSF	300	1mM	100 mM PMSF	100	1mM
5M NaCl	450	75mM	5M NaCl	150	75mM
dH ₂ O	19862	-	dH ₂ O	6526	-

6.2 Protein quantification

The Lowry Assay was used to quantify the concentration of proteins after the extraction process (Lowry et al.,1951). Bovine Serum Albumin (BSA) standard curve ranged from a concentration of 5-200mg/ℓ and the absorbance was measured at 250 nm; see details below. The aliquots of 5μl of nuclear and cytosolic of protein was added to 995 μl of distilled water. A standard curve was prepared using bovine serum albumin (Sigma, Germany) diluted to provide a concentration ranging from 10-200μg/ml. CTC reagent (colour reactive agent): solution a (Na₂CO₃ in 20g/100mℓ in double distilled water, ddH₂O) was added to solution b (0.2g CuSO₄.5 H₂O + 0.4 g K₂ tartate in 100mℓ ddH₂O)

and constantly stirred to prevent precipitation. The 10% SDS was made by diluting 20% SDS solution (w/v). The 0.4g of sodium hydroxide (NaOH) was added to 200ml ddH₂O.

Equal volumes of all three reagents were mixed immediately and prepared before use. All the samples were quantified in duplication. 5µl of protein of aliquot either nuclear or cytosolic origin was added to 995µl of sterile distilled water (1:200 dilution). 1ml of CTC reagent was added to each tube and mixed immediately and incubated at room temperature for 10 minutes. 500µl of folins reagent (1:5 dilution, Merck Germany) was added immediately. Folins agent has a short half-life of 8 seconds and must be used quickly, so the samples were incubated for 30 minutes. Thereafter, the optical density was measured at 750nm on Varian 130 dual beam spectrophotometer (Peterson 1977). Bovine serum albumin protein (BSA, Sigma Germany) was used for the standard curve and diluted to a concentration ranging from 10-200µg/ml and was assayed in the same manner as samples.

6.3 Sulphate dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) of extracted proteins

Lysates, 100µg, were diluted in Laemli sample buffer and boiled for 5 minutes. 100µg of proteins were separated on a 10% sodium dodecyl sulphate (SDS PAGE) gel, using standard Bio-Rad Mini-PROTEAN II System for two hours at 120 volts before being transferred to nitrocellulose membrane (Amersham Bioscience Hybond PRPN 303F) overnight.

6.4 Immuno-blotting and detection

The membranes were stained with a Ponceau Red stain (Ponceau's solution sigma, USA P7170) to check for equal loading. Membranes were blocked with 5% milk in Tris buffered saline (TBS-Tween) (0.1% Tween) for 3 hours. The membranes were probed with primary antibodies phospho-STAT3 (overnight) and total-STAT3 (2 hour) or β-actin, overnight at 4°C.

The primary antibody (total STAT (H-190) sc-7179 Santa Cruz Biotechnology rabbit polyclonal, phospho-STAT3 (Tyr 705) sc-7993 Santa Cruz Biotechnology goat

polyclonal, was washed off with TBS -T (0.1% Tween), three times for 5 minutes. The membranes were probed with the secondary antibody (Biotechnology, Donkey anti-rabbit IgG-HRP sc-2313 Santa Cruz Biotechnology, USA) for 1 hour. Detection of immune reagent bands were accomplished with enhanced chemiluminescence (ECL). The protein bands were normalised to Ponceau and β -actin

The total-STAT3 antibody was prepared by adding, STAT3 antibody rabbit polyclonal IgG 1:4000 in 5% (w/v) fat free milk. The secondary antibody was left on the membrane for 2 hours at room temperature (anti-rabbit) 1:3000 in 5% (w/v) fat free milk. The primary antibody for phosphorylated STAT3 is a phosphopplus STAT3 (Tyr 705) rabbit polyclonal IgG 1:1000 in TBST. The secondary antibody is left after the primary antibody and left for 2 hours at room temperature. All of the antibodies were purchased from Cell signalling, USA.

After the tSTAT3 and pSTAT3 were complete the emission of light was based on the interaction between the Luminol and the horseradish peroxides (HRP) conjugated to the secondary antibody for qualitative or semi-quantitative analysis. Relative densitometry was determined with use of a computerised software package, UVIBAND.

7. Mouse echocardiography

A transthoracic echocardiograph, using the GE Vivid E machine, is well established as a non invasive diagnostic procedure. It is used to assess cardiac morphology and performance. An ultrasound is a beam that is radiated, reflected and refracted by a small object. It travels poorly through air and dense substances, including lung and bone, thus a medium for transduction of the signal is required for its propagation. The intensity of the signal is directly associated with the acoustic impedances (density) of the medium. Reflection is also related to the thickness of the target, the angle of incidence of the beam penetrating the heart and the probe. The beam must be focused on a specific area. The beam width and frequency influence the resolution of the image obtained. Resolution is the ability to separate two targets as one distinct echo, to the extent that the systems gain button on the echo machine influences beam width and is

directly associated with the resolution of the image. Several factors must be considered for accurate ultrasound imaging: a high frequency transducer (GE i12L-RS linear array probe and it has a frequency range of 5.0 to 13.0 MHz), accurate positioning of the probe at midpapillary level, and the appropriate use of the gain controls are critical in obtaining a clear image (Hoit B. 2006) .

There are two different echo modalities for capturing an image, M mode and 2 dimensional modes (2D). The M mode, details the motion and structure along the ultrasound beam. Stationary structures generate horizontal lines. The distance from the top to bottom of the image indicates the depth and movement (Gardin et al.,1995). The 2D mode is a cross section of the image at different positions and usually used for the delineation of anatomical structures for quantifying the volumes in the chambers (Kumar and Clarke, 2005).Therefore, mouse echocardiography has become an essential non-invasive tool for experimental cardiovascular biology by providing a conceptual framework that transparently translates from the mouse to the human.

7.1 Procedure for mouse echocardiography

Transthoracic echocardiographies are performed in unconscious mice. The mice inhale 2% isoflurane mixed with oxygen delivered via a nose cone. The heart rate is usually maintained between 250-400 beats/minute. The chest hairs are removed by shaving, the mice are placed onto a heating pad and maintained at 37°C. Thereafter, the limbs of the mice are securely tape down. All mice are imaged in the left lateral decubitus position. Initially, 2D images are taken for the anatomical location of the heart to ensure that all the readings are taken at a mid papillary level. This is done in the parasternal short axis view and projected in the M mode for assessment of wall motion and wall thickness. In the M mode the cursor must be placed perpendicular to the left ventricular septum and left posterior wall. Upon completion the isoflurane was ceased and only oxygen administered for recovery.

7.2 Echocardiographic assessment of cardiac parameters in the mouse

Left ventricular systolic dysfunction is perceived to be a hallmark for cardiac disease. Ejection fraction (EF) and fractional shortening (FS) are indicators of wall function in the

absence of abnormalities. These readings are normally taken in M mode by comparing the end diastolic dimensions and the end systolic dimensions. Therefore, FS is defined as the percentage reduction in cavity size. This is an easy method of assessment for FS but presents with inaccuracy for measuring EF because it does not take into account reduced regional function of either the mid or apical myocardium which may be due to infarction. Thus the EF is a mere estimation of the left ventricular volumes from systole to diastole and is derived from planimetered measurements. In mice the cavity obliteration is 90%, which is far greater than in humans and is rarely used for assessment (Sahn De Maria., 1978). Other measurements for assessing wall thickness are intraventricular septum (IVSs) and the posterior wall (LVPw). The end diastolic measurements are IVSd, LVPwd and the left ventricular internal dimensions (LVIDd) are all assessed. FS is calculated as $[(LVIDd - LVIDs) / LVIDd] \times 100\%$. All the left ventricular measurements are averaged from leading edge to leading edge. Also the heart rate was tracked with a minimum of 3-5 consecutive beats required for measurements.

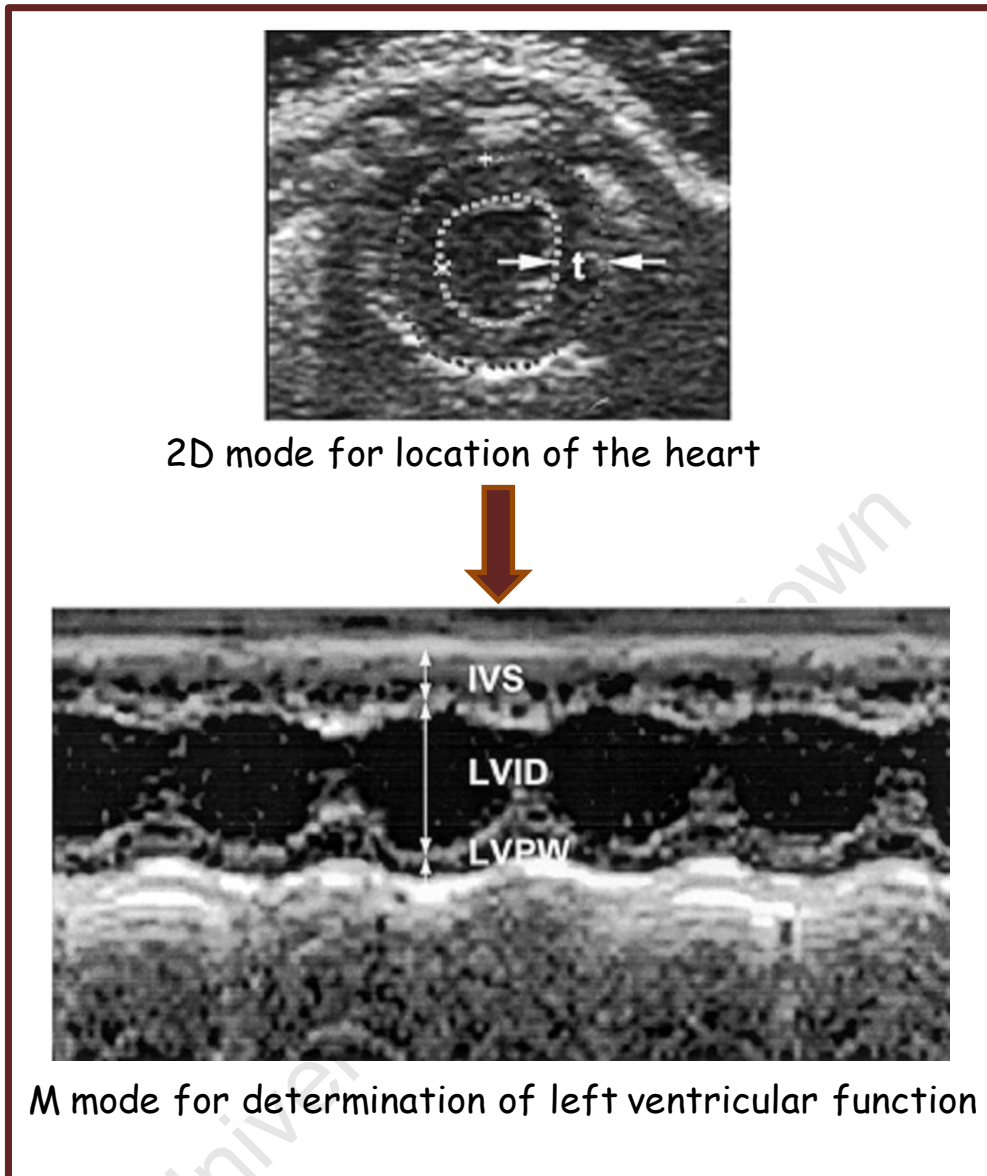


Figure 26: A graphical representation of the echocardiographic measurements in the left ventricle in a mouse heart. IVS (intraventricular septum), LVID (left ventricular internal diameter), LVPW (left ventricular posterior wall thickness) 2D (2 dimensional), M mode (measurement mode, 1 dimensional) (Rottman et al., 2007).

D. Chapter 1: Acute administration of melatonin or resveratrol protect via the activation of the SAFE pathway

Part of this work was previously published in Lamont et al., Is red wine a SAFE sip away from cardioprotection? Mechanisms involved in resveratrol and melatonin-induced cardioprotection. 2011.J of Pineal Research.50:374-380.

1. Introduction

Regular and moderate consumption of wine reduces the risk of cardiovascular disease (St Leger 1979, Alexy et al., 2009). The red wine hypothesis proposes that red wine is superior to white wine in conferring cardiovascular benefits (Opie & Lecour, 2007). Resveratrol, a polyphenol predominantly present in red wine, raises a strong argument for the benefits of red wine thus supporting the red wine hypothesis. Resveratrol in red wine varies from 0.5 to 13.5mg/ℓ and confers anti-ischemic effects via nuclear mechanisms involving anti-oxidant properties as well as via the activation of prosurvival pathways, such as the PI3-kinase/Akt pathway (Dudley et al., 2009).

Interestingly, another component present in red wine that has recently been discovered is melatonin. It is more prominent in red wine as compared to white wine and other liquors and therefore, favourably supports the red wine hypothesis. Melatonin is a natural compound well known to regulate the circadian rhythm in mammals; it is also synthesized in various plants and is often used in the treatment for jet lag or insomnia (Cajochen et al., 2003). Melatonin concentration in red wine varies from 50 to 200ng/ℓ (Guerrero et al., 2008). After the consumption of 100mℓ of red wine the melatonin levels in patients are significantly increased in the blood plasma (Guerrero et al., 2008). Melatonin is a powerful anti-oxidant and is 60 times more potent than fat soluble vitamin E (Tan DX, 1993). Furthermore, experimental data suggest that higher concentrations of melatonin (40μg/ℓ) administered acutely to a rat heart on a working heart model confers anti-ischemic effects (Lochner et al., 2006). However, its mechanisms of cardioprotection still remain poorly understood.

Recently, a novel intrinsic prosurvival pathway was discovered to protect against IR injury and was termed the survivor activating factor enhancement (SAFE) pathway. The pathway involves the activation of the innate immune system, TNFα and the downstream activation of STAT3. However, it is unknown whether resveratrol and melatonin (components predominately found in red wine) protect via the activation of this powerful pathway (see Figure 27).

We hypothesised that melatonin (75ng/l) or resveratrol (10uM=2.3mg/l) administered acutely, at similar concentrations to that found in red wine, can protect against ischemia-reperfusion injury in an isolated rat heart model. Genetically modified animals were used (TNF receptor 2 deficient mice or STAT3 deficient mice) to demonstrate that the acute administration of both melatonin and resveratrol, as present in red wine, can protect via the activation of the SAFE pathway (see figure 27).

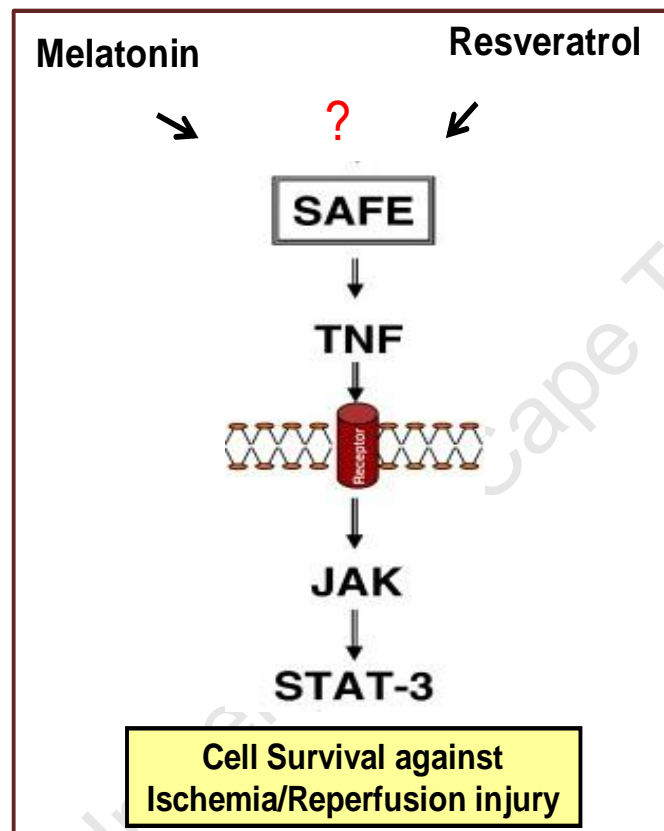


Figure 27: Schematic hypothesis. That melatonin and resveratrol given at low concentration protect against I/R injury and to determine whether this effect is mediated via the activation of the SAFE pathway. SAFE (Survivor Activation Factor Enhancement), JAK (Janus Kinase), STAT3 (Signal Transducer and Activator of Transcription).

2. Methods

2.1 Animals

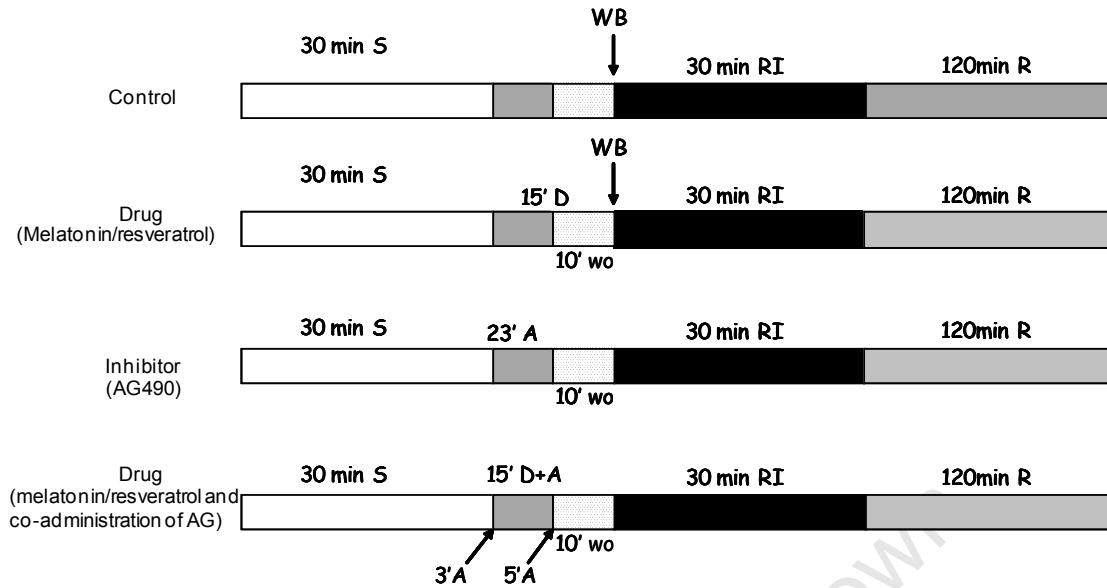
All the experiments conducted on male rats (230-300g) or mice (12-14 weeks) were performed in accordance with the *Guide for Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH publication No. 85(23), revised 1996). All procedures were approved by the Animal Research Ethic Committee (UCT), 009/026 "Delineating the cardioprotective effects of red wine."

2.2 Experimental model

Wistar rats (230-300g) were anaesthetised with 60mg/kg intraperitoneal sodium pentobarbitone and were given an intravenous injection of 200IU heparin. The hearts were removed from chest cavity rapidly and perfused retrogradely, using the Langendorff perfusion technique as previously described in the methods section.

2.3 Isolated rat heart perfusion protocol

The perfusion protocol is shown in Figure 28. All rat hearts were stabilised for 30 minutes and subjected to a standard 30 minute regional ischemia (RI) with a 3/0 silk suture that was placed around the left coronary artery to form a snare. After the occlusion, the heart was reperfused for 120 minutes. Either, melatonin (75ng/l) or resveratrol (2.3mg/l), were perfused for 15 minutes followed by 10 minutes wash-out period prior to the regional ischemia. Additional groups were perfused with AG490 (100 nM), an inhibitor of the JAK/STAT-3 pathway that was administered on its own or dually with either melatonin or resveratrol. AG490 was perfused for three minutes on its own followed by a 15 minute co-administration with melatonin or resveratrol, followed by a further five minutes of AG490 alone (see Figure 29).



S(stabilisation), D (drug), WO (wash out), A (AG490, STAT3 inhibitor), RI (regional ischemia), R (reperfusion)

Figure 28: Schematic representation of experimental protocol. Rat hearts were subjected to 30 minutes stabilisation followed by 30 minutes of regional ischemia and 120 minutes reperfusion. Prior to the ischemic insult melatonin (75ng/l) or resveratrol (2.3mg/l) was administered for 15 minutes either with or without AG490.

2.3.1 Isolated TNF R2-/- and STAT-3 -/- knockout heart protocol

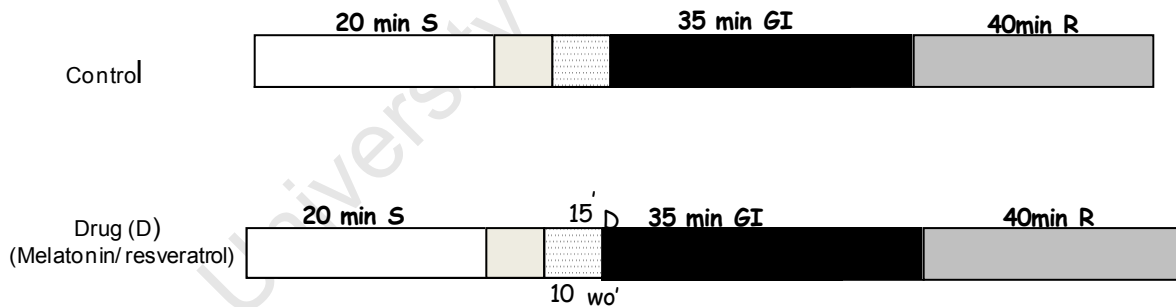


Figure 29: Schematic representation of experimental protocol in isolated heart model. Mouse hearts were subjected to 20 minutes stabilisation followed by 35 minutes global ischemia and 45 minutes reperfusion. Prior to the ischemic insult, melatonin or resveratrol were administered for 15 minutes followed by a 10min wash-out. S (stabilisation), min (minutes), GI (global ischemia, R(reperfusion). D(drug), 10'wo (10 minutes wash-out).

TNF receptor-2-deficient mice (TNFR2^{-/-}) and their littermate controls (TNF-WT), cardiomyocyte-specific STAT-3-deficient mice (STAT-3^{-/-}) and their littermate controls were used, as previously described. All mice were (12–16 weeks of age) anaesthetised (sodium pentobarbitone, 60mg/kg i.p.) and mounted on a Langendorff perfusion system. After a 30 minutes equilibration, the hearts underwent 35 minutes of global ischemia followed by 45 minutes of reperfusion (see Figure 29). Hearts were pretreated with either 75ng/l melatonin or 2.3mg/l resveratrol for 15 minutes followed by a 10 minutes washout period prior to global ischemia. At the end of each experimental protocol, the infarct size was assessed by TTC staining and infarct size was determined with using computerised planimetry, as detailed in the Methods section.

2.4 Western Blots analysis

Before the regional ischemic insult, the ventricular tissue from control, melatonin or resveratrol pretreated rat hearts, were excised, freeze clamped in liquid nitrogen and stored at -80°C. Phosphorylated states of STAT-3 (phospho-STAT-3 Tyr 705) and total levels of STAT-3 were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with antibodies from cell signalling technology, as previously described in the methods section. Equal loading was verified with Ponceau staining and levels of phosphorylated proteins were normalised to their total protein levels performed in the same samples and under the same conditions but on a separate membrane.

3. Statistical analysis

Data are presented as mean \pm SEM. N=6 per group. Comparisons between multiple groups were performed by one-way ANOVA followed by the Dunnet's post hoc test (Graph Pad Instat). A value of $p < 0.05$ was considered as statistically significant.

4. Results

4.1 Melatonin fails to protect in TNFR2^{-/-} and STAT3^{-/-} mice

In Figure 30, isolated mouse hearts were subjected to 35 minutes of global ischemia followed by 45 minutes of reperfusion. The wild-type mice presented with an infarct of $69.0 \pm 3.3\%$ of the total heart. Melatonin pre-treatment reduced infarct size to $24.5 \pm 2.1\%$ ($p < 0.001$ compared with the ischemic control). TNFR2-deficient mice had an infarct size of $61.8 \pm 3.2\%$ (ns. vs. wild-type control). Melatonin did not protect in TNFR2-deficient mice (ns vs TNFR2 control). Melatonin pre-treatment protected in STAT3 wild-type mice and presented with an infarct of $19.2 \pm 2.5\%$. In STAT3-deficient mice melatonin failed to protect (ns. vs wild-type control) (see Figure 30).

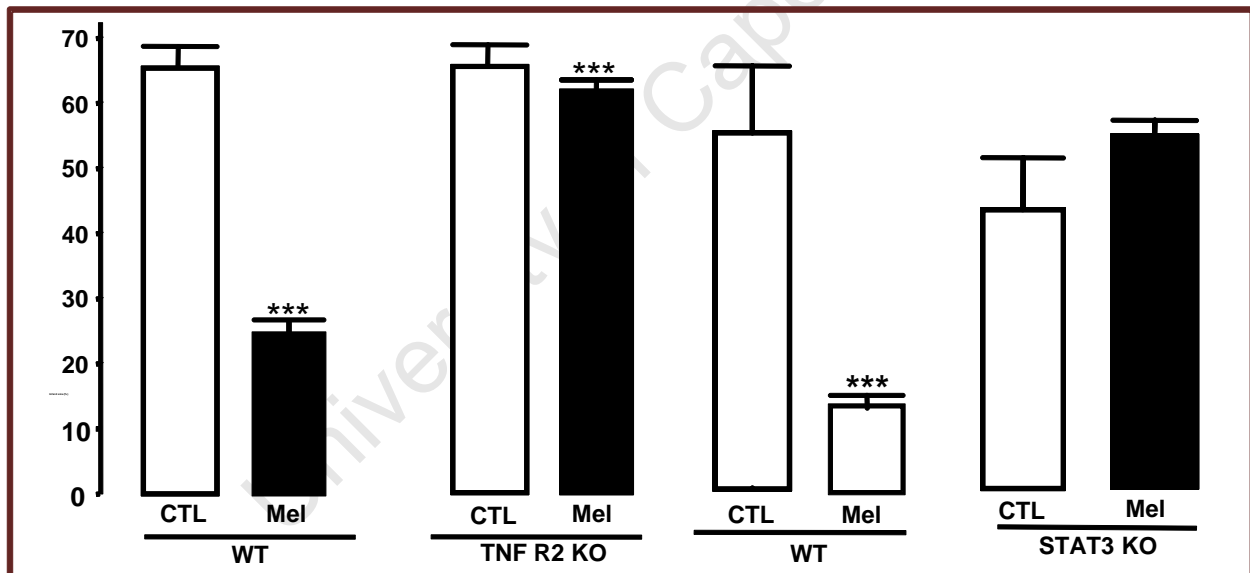


Figure 30: Melatonin (75ng/l) protects against I/R. The protective effect of melatonin is abolished in TNFR2 and STAT3KO. *** $p < 0.001$ vs control

4.2 Melatonin fails to confer cardioprotection in the presence of the STAT 3 inhibitor (AG490)

Isolated rat hearts subjected to 30 minutes of regional ischemia and 120 minutes of reperfusion had an infarct of $44.4 \pm 2.8\%$ (Figure 31). Pre-treatment with melatonin reduced the infarct size to $25.0 \pm 2.9\%$ ($p < 0.001$ versus control group).

However, addition of AG490, the STAT-3 inhibitor, with melatonin abolished the protective effect of melatonin (Mel + AG = $36.83 \pm 8.7\%$, ns versus control group). AG490 given on its own did not alter the infarct size compared to the control group.

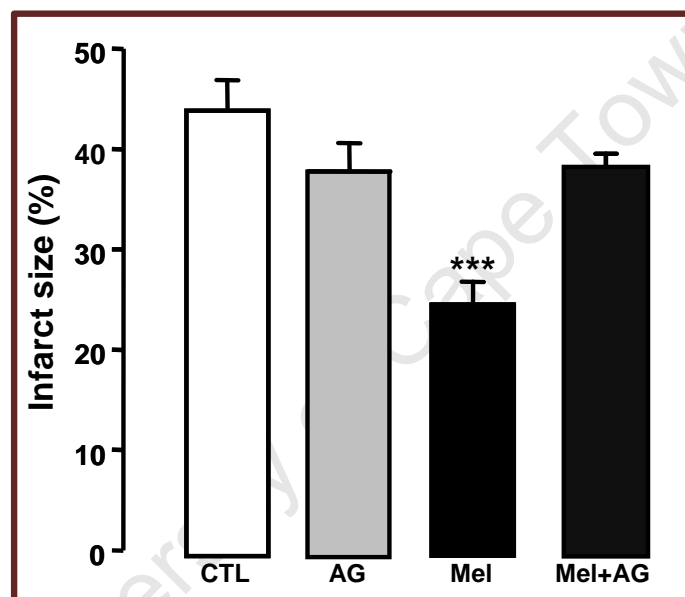


Figure 31: The protective effect of melatonin is attenuated in the presence of AG490, STAT3inhibitor, in isolated rat hearts subjected to I/R. *** $p < 0.001$ vs.control, CTL (control), Mel+AG (coadministration of melatonin and AG490) *Important note: these experiments were presented in my Master's thesis but are presented here for better comprehension of the study.*

4.3 Melatonin increases STAT-3 phosphorylation in isolated rat hearts

In the isolated rat heart, phosphorylated levels of STAT3 were assessed following 15 minutes administration of melatonin prior to the ischemic insult. In the cytosol, there was no significant difference in phosphorylation of STAT3 between the control groups and the hearts treated with melatonin. However, in the nucleus, melatonin increased the phosphorylation of STAT3, on the tyrosine residue, by 75% compared to control (** $p < 0.01$) (See Figure 32).

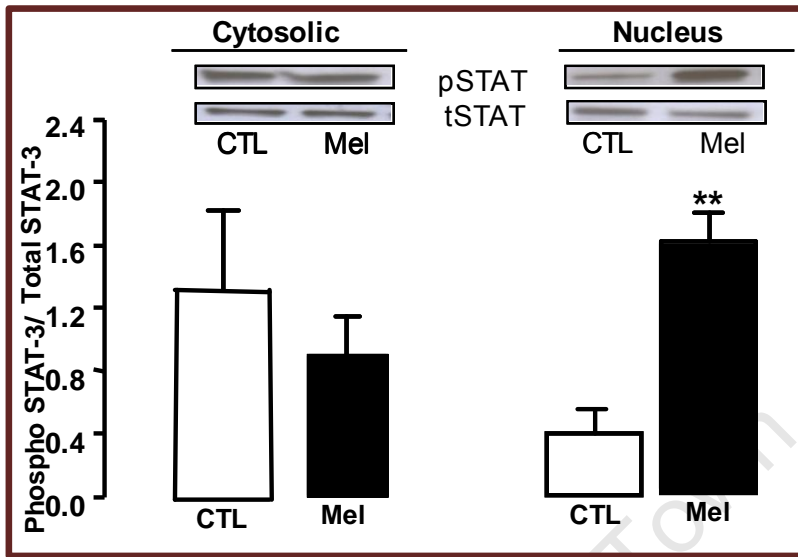


Figure 32: Role of melatonin (75ng/l) on STAT3 phosphorylation **p<0.01 vs control (n=4). CTL (Control), Mel (Melatonin), pSTAT3 (phosphorylated STAT3 Tyr 705), tSTAT3 (total STAT3). Important note: these experiments were presented in my Master's thesis but are presented here for better comprehension of the study.

4.4 Resveratrol fails to confer cardioprotection in TNF2^{-/-} and STAT3^{-/-} mice

In Figure 33, isolated mouse hearts were subjected to 35 minutes of global ischemia followed by 45 minutes of reperfusion. The wild-type mice presented with an infarct of 69.5±2.1 of the total heart. Resveratrol pre-treatment reduced infarct size to 25.2±2.5% (p<0.001 compared with the ischemic control). TNFR2-deficient mice had an infarct size of 65.2±2.3% (ns. vs. wild-type control). Resveratrol did not protect in TNFR2-deficient mice (ns vs TNFR2 control). Resveratrol pre-treatment protected in STAT3 wild-type mice and presented with an infarct of 25.2±2.5% (p<0.001 vs. wild-type). In STAT3-deficient mice melatonin failed to protect (ns. vs. wild-type control) (see Figure 33).

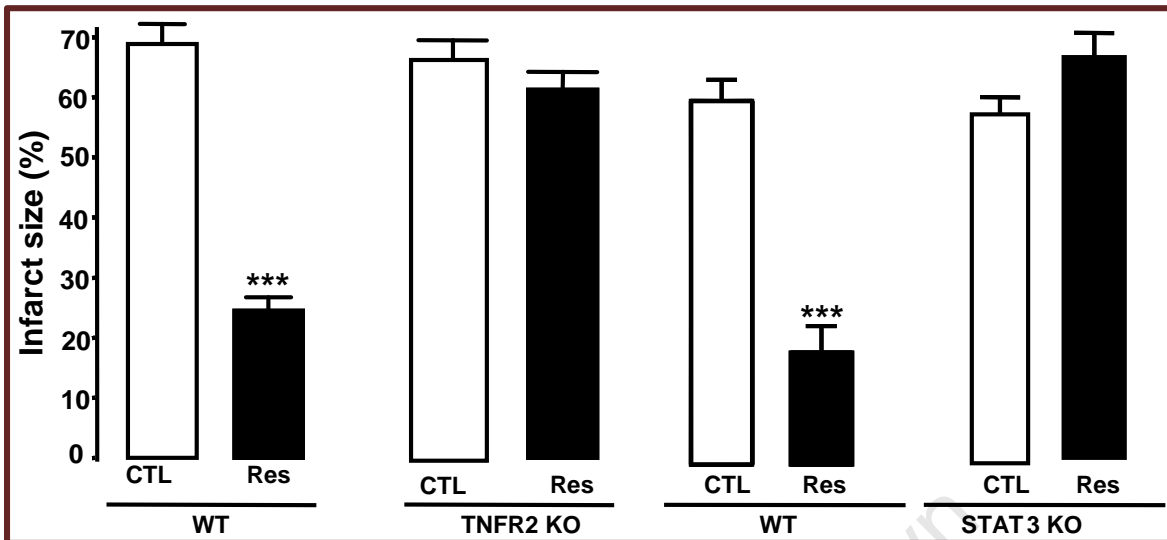


Figure 33: Resveratrol (2.3mg/l) protects against I/R. The protective effect of resveratrol is abolished in TNFR2 KO and STAT3KO. ***p <0.001 vs relative control.

4.5 Resveratrol Fails to confer Cardioprotection in the presence of the STAT3 inhibitor (AG490)

Isolated rat hearts subjected to 30 minutes of regional ischemia and 120 minutes of reperfusion had an infarct of $48.8 \pm 4.0\%$ (Figure 33). Pre-treatment with resveratrol reduced the infarct size to $15.0 \pm 1.0\%$ ($p < 0.001$ vs. control). However, coadministration of AG490, the STAT-3 inhibitor, with resveratrol abolished the cardioprotective effect of resveratrol (Res + AG = $49.5 \pm 6.9\%$, ns vs control group) (see Figure 34). AG490 given on its own did not alter the infarct size compared to the control group (ns vs. control).

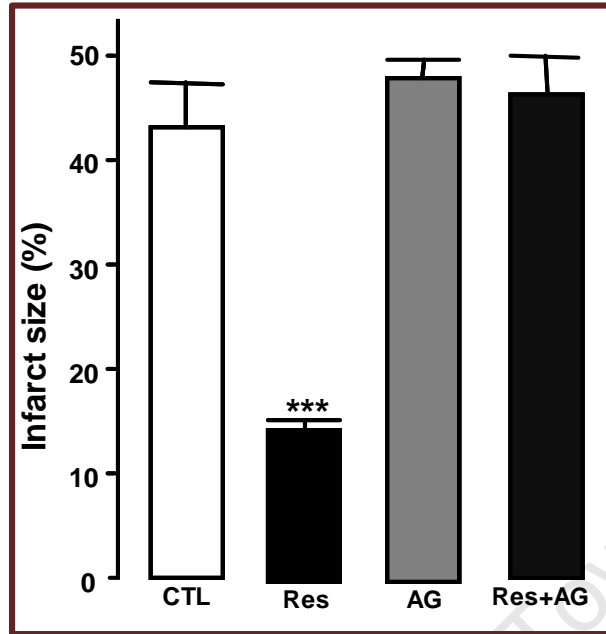


Figure 34: The protective effect of resveratrol is attenuated in the presence of AG490 in isolated rat hearts subjected to I/R. CTL (Control), AG (AG490 STAT3 inhibitor), Res (Resveratrol), Res+AG (Co-administration of resveratrol and AG490). *** $p < 0.001$ vs. control.

4.6 Resveratrol increases STAT-3 phosphorylation in isolated rat hearts

In the isolated rat heart phosphorylated levels of STAT3 were assessed following 15 minutes administration of resveratrol. In the cytosol, there was an increase in phosphorylated STAT3, on the tyrosine residue, by 50% between the control groups and those treated with resveratrol ($*p < 0.05$). In the nucleus there was a 71% increase in pSTAT3 with resveratrol treatment compared to control ($**p < 0.01$) (see Figure 35).

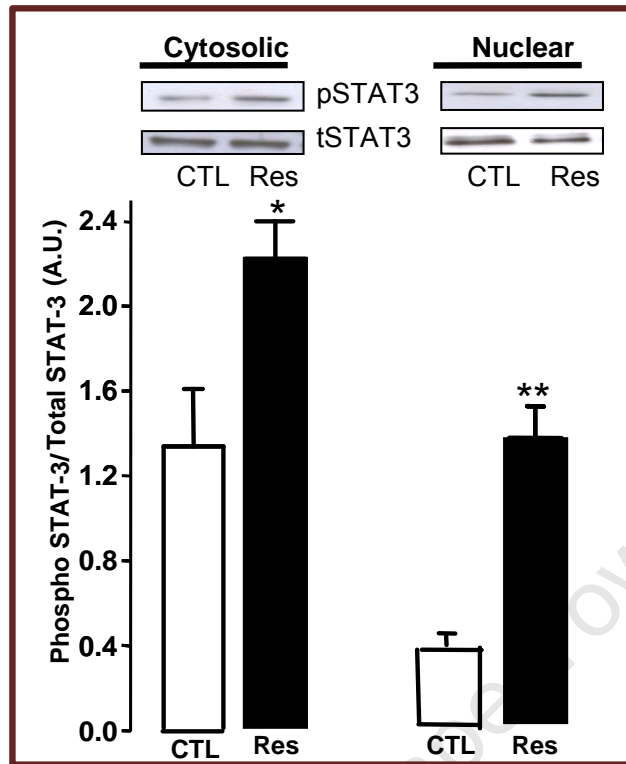


Figure 35: Role of resveratrol (2.3mg/l) on cytosolic and nuclear phosphorylation of STAT3. Phosphorylated STAT3 levels are increased in the cytosol (*p<0.05 vs. control) and in the nucleus 15 minutes after treatment **p<0.01 vs respective control (n=4). CTL (Control), Res (Resveratrol), pSTAT3 (phosphorylated STAT3 Tyro 705), tSTAT3 (total STAT3).

5. Discussion

The present study demonstrates that a very low concentration of melatonin, corresponding to the concentration found in red wine, protects the heart against an IR injury. Furthermore, we demonstrate that both melatonin and resveratrol confer protection via the activation of the newly discovered SAFE pathway which involves the activation of TNFR2 and STAT3. Hence, both resveratrol and melatonin failed to protect against ischemia-reperfusion in hearts of TNFR2 deficient mice or STAT3-deficient mice. Similarly, the protection with resveratrol or melatonin in rat hearts was abrogated in the presence of AG490, an inhibitor of the STAT3 pathway. Furthermore, both resveratrol and melatonin pre-treatment were associated with an increased activation of STAT3 in the nucleus therefore suggesting an activation of STAT3 in the myocardium of treated rats.

5.1 Melatonin, given at a similar concentration as present in red wine, can confer cardioprotection

The cardioprotective effect of melatonin against IR was previously reported both in vitro and in vivo at concentration ranging from 1-50 μM (0.22mg/l -11mg/l) (Tan et al., 1998). These concentrations are up to a 1000 times higher than the physiological mammalian blood levels of melatonin which will generally oscillate between 10ng/l during the day and 200ng/l at night (Kaneko et al., 2000). The novelty of our work was to demonstrate that a physiological concentration of melatonin (75ng/l), corresponding to the concentration found in natural food products such as red wine, can also protect the heart against an I/R insult. Our data support previous work demonstrating that endogenous melatonin contributes to cardioprotection with infarct size measured in pinealectomised rats subjected to ischemia-reperfusion being increased compared to normal rats (Hardeland et al., 2005). Melatonin is commercially available and prescribed to regulate the sleeping pattern at a dose ranging between 1 and 3mg per day. This dose of melatonin would require drinking more than 1000l of wine per day! We acknowledge the fact that the amount of melatonin given directly to the isolated heart is higher than the amount that the heart would receive after ingestion of red wine, but half

an hour after drinking 100ml of red wine, the amount of melatonin in the blood is increased by approximately 20% (Guerrero et al., 2008).

5.2 Melatonin protects via the activation of the SAFE pathway

As ROS contribute to I/R injuries, the cardioprotective effect of melatonin has been essentially attributed to its anti-oxidant capacities by scavenging ROS (Tan et al., 1998), limiting mitochondrial electron leakage and inhibiting the opening of the mitochondrial permeability transition pore opening (Tengattini et al., 2008). Melatonin can exert some cardioprotective properties independently of its anti-oxidant effect, by activation of the protein kinase B or inhibition of the proapoptotic p38 mitogen-activated protein kinase (Genade et al., 2008).

Activation of the prosurvival intrinsic SAFE pathway is required for the protection of both ischemic pre- and postconditioning, two powerful phenomena to protect against IR injury (Suleman et al., 2008), and also multiple cardioprotective drugs such as sphingosine-1 phosphate, ethanolamine, insulin, bradykinin or opioids also protect via this pathway (Somers et al., 2010). Using genetically modified animals, our data demonstrate for the first time that melatonin-induced cardioprotection requires the activation of two major components of the SAFE pathway, namely the cytokine TNFR2 and the transcription factor STAT3.

5.3 Resveratrol protects against I/R injury via the SAFE pathway

In patients with coronary artery disease, dealcoholised red wine (DRW) decreased arterial stiffness (Wang et al., 2005) and in apolipoprotein E deficient mice, DRW limits atherosclerosis, thus providing conclusive evidence that red wine properties “go beyond alcohol” (Wang et al., 2005).

Our data confirm numerous previous reports demonstrating that a concentration of resveratrol at 2.3mg/l protects against ischemia-reperfusion injury. Again, a limitation of our study and others is the fact that the amount of resveratrol given directly to the isolated heart is higher than the amount that the heart would receive after ingestion of red wine. Following an acute oral administration of red wine in rats (4 ml of an Italian

red wine containing 6.5mg/l), the resveratrol concentration peaked at 20 μ g/l in the plasma (Bertelli et al., 1998). However, chronic red wine consumption may be associated with higher concentrations than those measured with an acute treatment and several studies have demonstrated that resveratrol as low as 0.22mg/l can exert a number of biological effects (Bhat et al., 2001). Additional *in vivo* and chronic animal studies are required to confirm that this effect can account for the cardioprotective effect of red wine.

The mechanisms of protection for resveratrol have been extensively studied but still remain uncertain. Well known for its anti-oxidant properties (Hattorri et al., 2002), the anti-ischemic effect of resveratrol is thought to be mediated via activation of nitric oxide, adenosine, Akt, Bcl-2 and inactivation of pro-apoptotic factors such as Bad and glycogen synthase kinase 3 β (Xi et al., 2009). Our data delineate a novel prosurvival pathway that resveratrol can activate to limit IR injury, and we demonstrate that resveratrol can target the immune system to limit IR damage by activating TNF α .

As both resveratrol and melatonin, at concentrations as found in red wine confer cardioprotection by activation of the SAFE pathway, we suggest that moderate consumption of red wine protects against IHD by activation of the SAFE pathway. However, additional experiments conducted *in vivo* with a chronic consumption of melatonin, resveratrol or red wine will be required to confirm our statement.

Melatonin appears to be superior to resveratrol because a far larger quantity of resveratrol is required to obtain a protective effect. This may be due to the varying bioavailability of each compound present in red wine. Also, there may be a variety of interactions between the compounds present in red wine which are required to confer cardioprotection. However, the synergistic effects of these compounds remain elusive and require further investigation.

5.4 Summary

In summary, the cardioprotective effect of an acute treatment with melatonin or resveratrol (at concentrations similar to red wine) is mediated via the activation of the SAFE pathway. Therefore, it is suggested that the moderate consumption of red wine may protect against IHD through the activation of the SAFE pathway via melatonin and resveratrol (see figure 36). However, additional experiments conducted *in vivo* with the regular chronic moderate consumption of melatonin, resveratrol or red wine will be required to confirm our statement.

5.5 Limitations

A major limitation of this study is that an isolated heart model was utilised. Therefore, the exact interaction of each compound and its bioavailability or half life in the body is unknown. Furthermore, the compounds were given acutely therefore further investigation is required pertaining to the long term beneficial effects of melatonin and resveratrol. In spite of these limiting factors, the study is still novel in demonstrating the mechanism involved in the cardioprotective effects of the individual compounds, resveratrol and melatonin. It may explain the mechanism involved in red wine induced cardioprotection.

6. Conclusion

In conclusion, our data strongly suggest that low concentrations of melatonin and resveratrol given acutely can protect the heart against I/R injury and may contribute to the cardioprotective effects of red wine. Furthermore, we have delineated a novel mechanism by which melatonin and resveratrol protect the heart via the activation of the powerful prosurvival SAFE pathway, which involves the activation of both TNF α and STAT3. Our data provide exciting novel insight for the use of natural compounds in the treatment of cardiac disease.

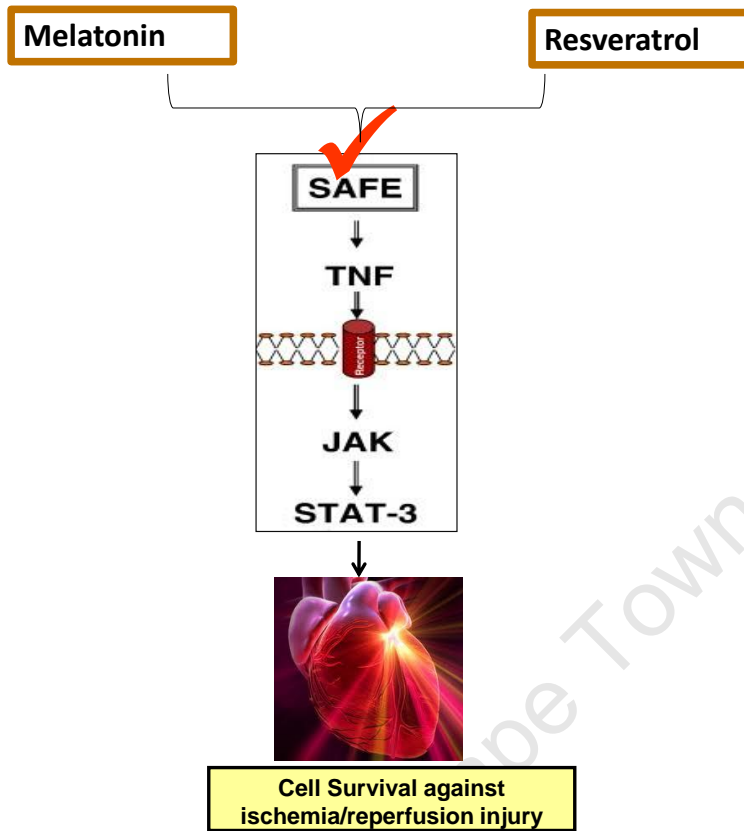


Figure 36: Melatonin and resveratrol induced cardioprotection is mediated via the activation of the SAFE pathway.

**E. Chapter 2: The role of melatonin in
red wine induced cardioprotection
against I/R injury.**

1. Introduction

Several epidemiological studies have shown that the moderate and chronic consumption of red wine confer cardioprotection (Maier-Krenz et al., 2012). However, the exact components present in red wine that contribute to this effect remain unclear. Resveratrol, alcohol (12%) and melatonin are the three potential candidates that may contribute to the beneficial effects of red wine induced cardioprotection. In Chapter 1, our data demonstrate that the acute administration of melatonin or resveratrol, at a concentration found in red wine, could confer cardioprotection in an *ex vivo* model of I/R injury. However, a major limitation of the study was that the experiments were performed *ex vivo* and melatonin and resveratrol were given directly to the isolated rat or mouse hearts. Therefore, the bioavailability of these compounds in the blood stream and the break down in the body were not taken into consideration. The interaction with hormones and the nervous system should also be mentioned as melatonin released from the pineal gland, in the brain. Therefore, whether the chronic administration of melatonin and resveratrol could protect in an *in vivo* setting remains unclear and raises the question as to whether melatonin present in red wine may contribute to the protective effect of red wine. However, it is difficult to credit melatonin exclusively, for the cardioprotective effects of red wine and it is likely that a synergy of components enables the beneficial effects of red wine.

Previous findings obtained during my Master's degree demonstrated that neither resveratrol, given as a chronic treatment for 10 days at similar concentration to that found in red wine, nor alcohol (6%), protected against I/R injury in an isolated rat heart model, see figure 37 (Lamont et al., 2012). However, the chronic moderate consumption of alcohol (12%) equivalent to that found in red wine was not tested.

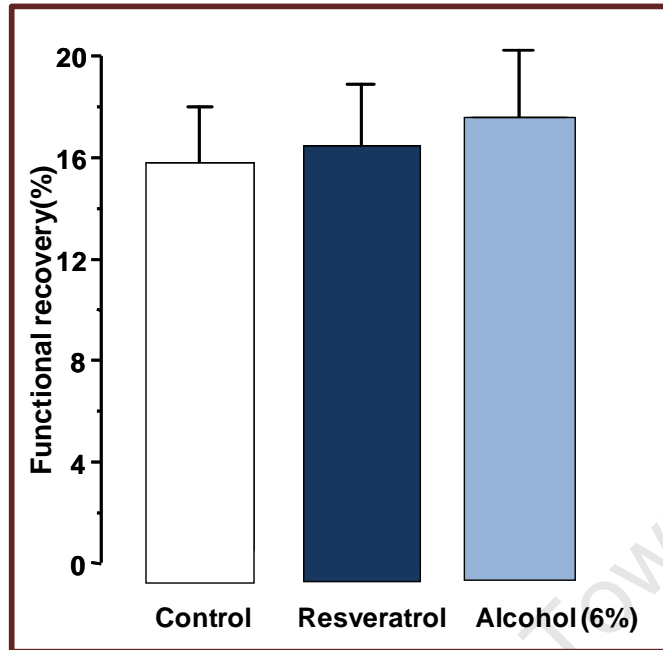


Figure 37: Effect of chronic pretreatment with resveratrol (2.3mg/l) and alcohol (6%). Resveratrol or alcohol did not improve functional recovery in an isolated rat heart model subjected to I/R. n=6 per group (Lamont et al.,2012).

Therefore, whether melatonin (75ng/l) or alcohol (12%) administered chronically in an *in vivo* model could protect the heart against I/R requires investigation. Our experiments conducted in Chapter 1 demonstrated that the acute treatment with melatonin protected against I/R injury via the activation of the SAFE pathway, which involves the activation of TNF α and STAT3. However, whether the regular consumption of red wine or melatonin could protect via the activation of the SAFE pathway is unknown.

2. Aim

The aim of the present study was to explore whether the chronic and moderate consumption of melatonin, given at a dose equivalent to the concentration obtained after 2-3 glasses of red wine per day, may protect against I/R injury via the activation of the SAFE pathway. Furthermore, we used inhibitors of melatonin receptors to explore whether melatonin may contribute to the cardioprotective effect of chronic and moderate consumption of red wine (see Figure 38).

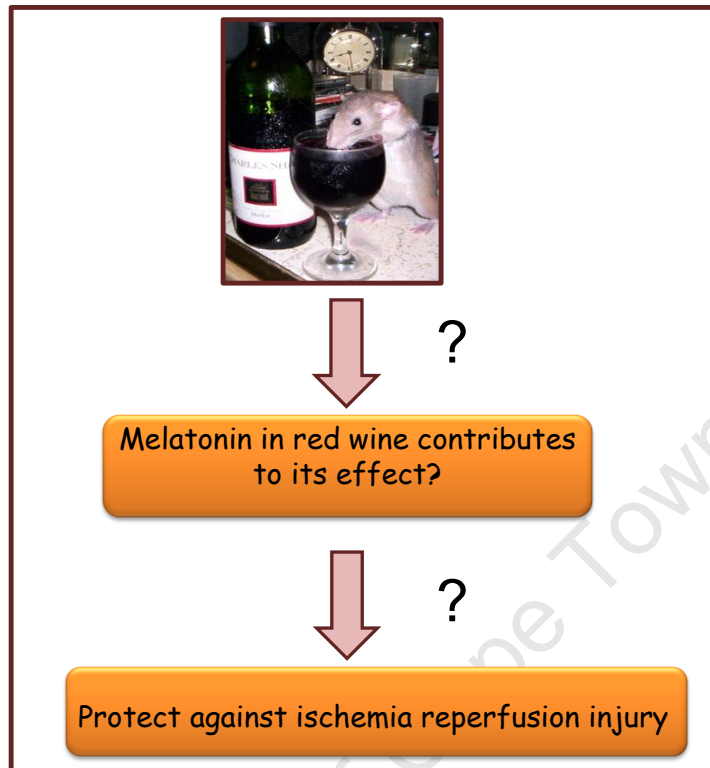


Figure 38: Schematic of hypothesis: we propose that the chronic and moderate consumption of red wine confers cardioprotection via melatonin and the activation of the SAFE pathway.

3. Materials and methods

3.1. Animals

All the experiments were conducted on male rats or mice and were performed in accordance with the *Guide for Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH publication No. 85(23), revised 1996). All procedures were approved by the Animal Research Ethics Committee, University of Cape Town, under the ethics number 011/026, "Delineating the cardioprotective effect of red wine and its components in an *in vivo* mouse model of I/R injury and IHF."

3.2. Chronic treatment with wine, alcohol (12%) and melatonin

The drinking water for controls was supplemented with either red wine (French Cabernet Sauvignon), alcohol 12% directly extracted from red wine, or melatonin

(75ng/l) for 14 days. The different drinking solutions were prepared by adding one part of red wine, alcohol (12%) or melatonin to seven parts of drinking water (equivalent of 2-3 glasses of red wine/day relative to body weight), as previously described (Lamont et al., 2012). The addition of the drugs had no effect on the liquid consumption of the rats or mice. All the solutions were made fresh daily and preliminary data were performed to ensure the well-being of the animals and that the drinking behaviour remained unaltered. Thereafter, the hearts were isolated and mounted onto the Langendorff perfusion system or an *in vivo* I/R injury was performed, as described in methods section. To explore the role of melatonin in red wine-induced cardioprotection, luzindole (melatonin 1 and 2 inhibitor, administered 1mg/kg, ip) or prazosin (2.5mg/kg, ip) were given for 14 days in the presence or absence of red wine or melatonin (see Figure 39).

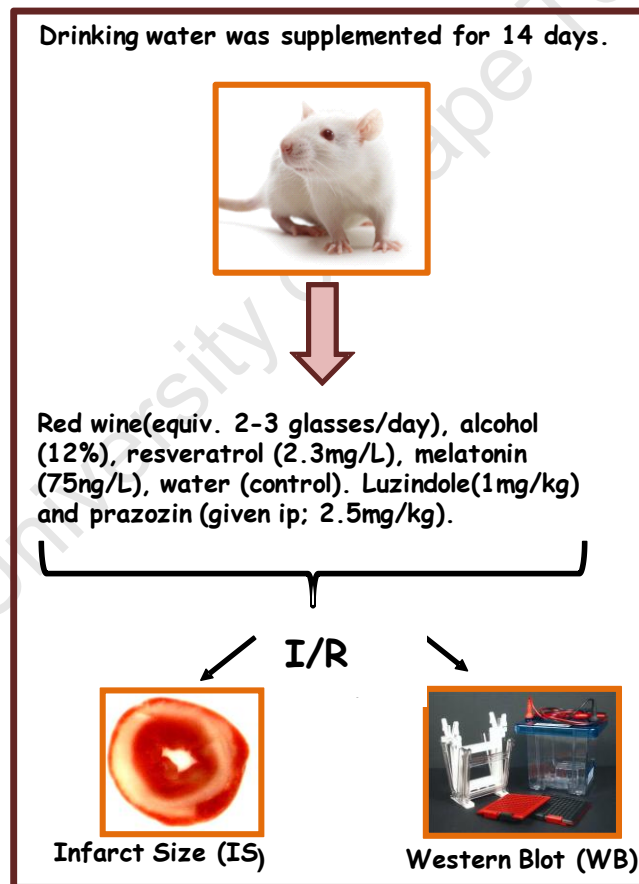


Figure 39: Design of study male rats or mice drinking water was supplemented with either red wine, melatonin or alcohol 12 (%) for 14 days. Infarct size was assessed at the end of each perfusion protocol and on a separate set of heart western blots were performed.

3.3. Experimental protocol

Wistar rats (230-300g) were treated for 14 days; thereafter the rats were anaesthetised with 60mg/kg intraperitoneal sodium pentobarbitone and 200 IU heparin. Hearts were excised rapidly and perfused retrogradely using the Langendorff perfusion technique at a constant pressure (100 cm H₂O) as described previously in the methods section.

3.3.1. Experimental protocol for in vitro model of I/R in isolated rat heart model

After the chronic pretreatment, all rats underwent 30 minutes stabilisation followed by 30 minutes global ischemia (at 37°C) and 60 minutes reperfusion. The different treatments were as follows, see fig 39; rats were treated with either: red wine equivalent to 2-3 glasses/day; alcohol (12%) melatonin (75ng/l); prazosin (2.3 mg/kg/day, ip), a melatonin receptor 3 inhibitor in the presence or absence of red wine or melatonin; luzindole, a melatonin receptor 1&2 inhibitor (1mg/kg/day, i.p.), also given in the presence or absence of red wine or melatonin. An additional group was treated with AG 490, an inhibitor of JAK/STAT-3 (10mg/kg, given i.p.) on its own, or co-administered with red wine.



Figure 40: Schematic representation of the experimental protocol. Rat hearts were subjected to 30 minutes of S(stabilisation) and underwent 30 minutes of global ischemia followed by 60 minutes of reperfusion. At the end of the experiments the hearts were removed for infarct size.

The functional parameters were measured throughout the protocol. At the end of the protocol infarct size was assessed. Additional groups were performed for Western Blot analysis; hearts were collected prior to the ischemic insult, as mentioned in the methods section.

3.3.2 Experimental protocol for in vitro I/R injury in an isolated mouse model

TNF-deficient mice and their littermate controls (TNF-Wild types), as well as STAT-3 cardiomyocyte-specific mice and their littermate controls were used. All mice were used between 12-16 weeks. The mice were pretreated for 14 days with either red wine, melatonin or plain drinking water, as described in section 3.1.1 of this chapter. The mice were anaesthetised and mounted onto the perfusion system, as described in the methods section. Thereafter, the hearts underwent 30 minutes stabilisation followed by 35 minutes global ischemia at 37°C and 40 minutes reperfusion.



Figure 41: Schematic representation of the experimental protocol. Mouse hearts were subjected to 20 minutes S (stabilisation) and underwent 35 minutes of global ischemia followed by 40 minutes of reperfusion. Infarct size was assessed at the end of all experiments.

3.1.3 Experimental protocol for in vivo mouse I/R model

C5BL6 mice were pre-treated for 14 days with either: red wine, melatonin (75ng/l) or water (as described in section 3.1.1). Mice were anaesthetised with a combination of ketamine (0.06mg/ml) and xylazine (2mg/ml); the final concentration of both was 0.01ml/g (Lim et al., 2007). The mice were intubated for artificial respiration, at 120 strokes/minutes and 220ul stroke volume, using a minivent and supplemental oxygen was supplied. Thereafter, a left anterior thoracotomy was performed in the third intercostal space. The left ascending coronary artery was ligated with (8.0 ethilon, nylon suture black filament), 2 mm below the tip of the left auricle. A snare was created for 30 minutes of ischemia and then released for 120 minutes of reperfusion. Evans blue was injected into the left ventricle to delineate dead tissue. The hearts were stored at -4° and later sectioned and stained with TTC, as previously described in the methods section.

4 Western Blots analysis

Before the ischemic insult, the ventricular tissue from control, melatonin- or resveratrol-pretreated rat hearts, were excised, freeze-clamped in liquid nitrogen and stored at -80°C . Phosphorylated states of STAT-3 (phospho-STAT-3 Tyr 705) and total levels of STAT-3 were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis with antibodies from cell signalling technology, as previously described in the methods section. Equal loading was verified with Ponceau staining and levels of phosphorylated proteins were normalised to their total protein levels performed in the same samples and under the same conditions but on a separate membrane.

Statistical analysis

Data are presented as mean \pm SEM. N=6 per group. Comparisons between multiple groups were performed by one-way ANOVA followed by the Dunnet's *post hoc* test (Graph Pad Instat). A value of $p < 0.05$ was considered as statistically significant.

6 Results

6.1. Alcohol (12%) fails to protect the rat heart against I/R injury

Prior to the ischemic insult the control hearts presented a left ventricular developed pressure (LVDP) of 87 ± 1 mmHg, heart rate (HR) of 340 ± 17 beats/minute and a coronary flow (CF) of 13 ± 1 ml. The treatment with either red wine or alcohol (12%) had no effect on the hearts prior to I/R. However, after 30 minutes of ischemia and 30 minutes of reperfusion the values were reduced for the control hearts to; LVDP (23 ± 3 mmHg), HR (227 ± 20 beats/minute) and CF (5.5 ± 0.3 ml). Red wine pre-treatment improved LVDP to 38 ± 3 mmHg ($*p < 0.05$ vs. control). Pre-treatment with alcohol (12%) had no effect on the functional parameters after ischemia-reperfusion injury (ns. vs. control). Of note, the treatment with red wine or alcohol did not affect the body weights of the animals.

Table 4: Haemodynamic parameters of isolated perfused rat hearts subjected IR and pretreated with wine or alcohol

	Pre-ischemic	Reperfusion 5 minutes	Reperfusion 30 minutes
LVDP (mmHg)			
Control	87 ± 1	16 ± 2	23 ± 3
Wine	88 ± 3	26 ± 3*	38 ± 3*
Alcohol 12%	87 ± 6	10 ± 4	17 ± 2
Heart rate (bts/minute)			
Control	340 ± 17	192 ± 32	227 ± 20
Wine 12%	300 ± 9	250 ± 37	253 ± 29
Alcohol 12%	328 ± 15	232 ± 66	286 ± 36
Coronary Flow (mℓ/minute)			
Control	13.0 ± 1.1	7.3 ± 0.4	5.5 ± 0.3
Wine 12%	11.8 ± 1.4	10.0 ± 0.9	6.7 ± 0.4
Alcohol 12%	10.4 ± 0.6	9.2 ± 0.5	7.0 ± 0.6

Parameters measured prior to ischaemia (pre-ischaemic), after 5 and 30 minutes of reperfusion. Values are mean ± standard error of mean (SEM) ($n=6$). LVDP (left ventricular developed pressure), HR (heart rate), CF (coronary flow), bts/minute (beats/ minute). * $p<0.05$ vs. control

6.2. Chronic and moderate administration of red wine and melatonin confer cardioprotection in both an *in vivo* and an *ex vivo* model

In the *in vivo* model, mouse hearts were pretreated with either red wine or melatonin for 14 days. Thereafter, the mice were anaesthetised, ventilated and the chest was opened. The hearts underwent 35 minutes ischemia and 120 minutes reperfusion. The mice that were given plain drinking water presented an infarct of $60.0\pm 2.3\%$. The red wine dramatically reduced infarct size from $60.0\pm 2.3\%$ to $23.3\pm 1.8\%$ compared to

controls ($p < 0.001$). Similarly, melatonin given on its own reduced infarct size to a similar extent to red wine $20.1 \pm 1.7\%$, ($p < 0.001$ compared to control), see figure 42. These data were confirmed in an ex vivo model, where the mouse hearts were isolated and mounted onto the perfusion system and subjected to 35 minutes of ischemia and 40 minutes of reperfusion. Hearts that were pretreated with red wine had a smaller infarct than the control group of ($19.8 \pm 2.5\%$ and $47.3 \pm 3.5\%$ respectively, $p < 0.001$). Similarly, melatonin reduced infarct size to 17.9 ± 2.0 versus the control ($p < 0.001$). Of note, the treatment with red wine or melatonin did not affect the weights of the animals.

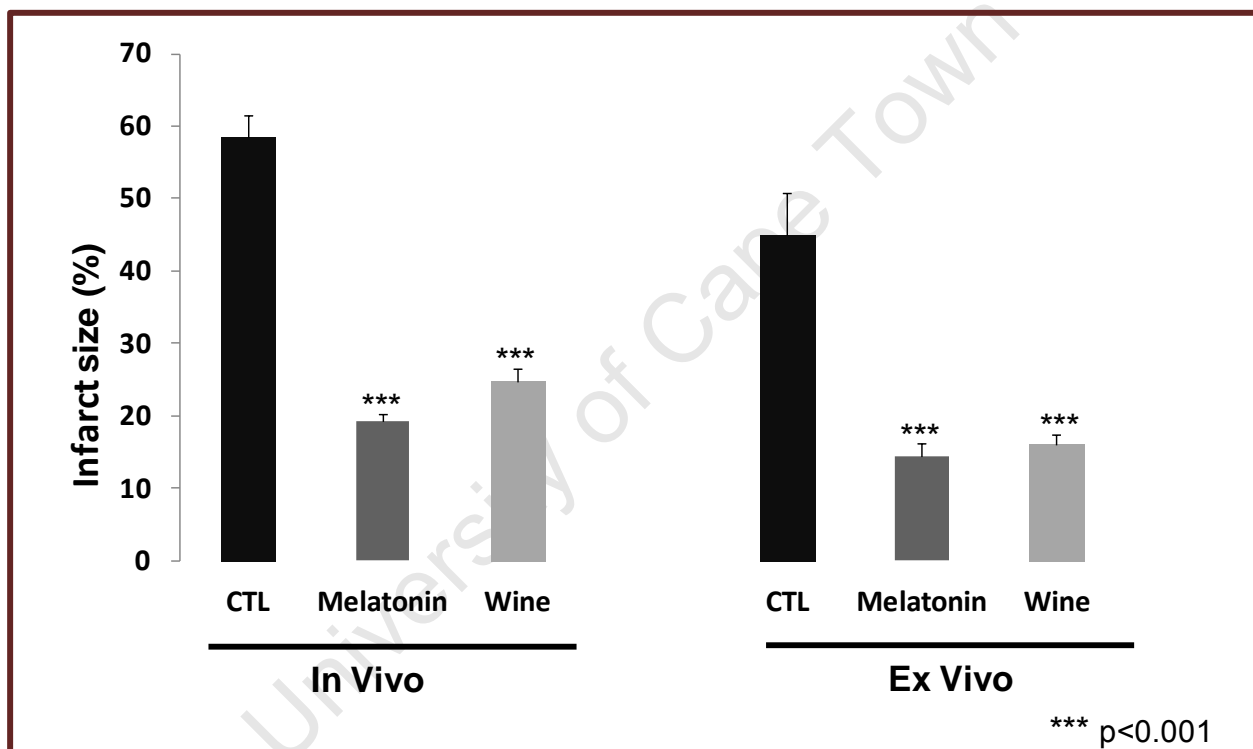


Figure 42: Red wine and melatonin protect against I/R in an in vivo and an ex vivo mouse model. CTL (Control). *** $p < 0.001$ vs. respective control.

6.3. The effect of melatonin receptors inhibitors on red wine-induced cardioprotection.

To explore whether melatonin may contribute to red wine induced cardioprotection, rats were treated for 14 days with either wine or melatonin in the presence or absence of the melatonin inhibitors (luzindole, inhibits receptor 1 and 2 or prazosin, inhibits receptor 3

all administered ip) and then subjected to an I/R injury on the isolated rat heart perfusion model.

6.3.1. The effect of luzindole of red wine induced protection

The chronic moderate consumption of red wine reduced infarct size from $58.8 \pm 3.8\%$ to $19.2 \pm 2.6\%$ ($p < 0.01$ vs. control) (see Figure 43). Similarly, melatonin reduced infarct size to $19.9 \pm 4.0\%$ ($p < 0.01$ vs. control). Luzindole on its own was similar to the control (ns vs. control). Melatonin lost its infarct sparing ability in the presence of luzindole and presented with an infarct of $58.9 \pm 5.0\%$ (ns vs. control). On the contrary, the protective effect of red wine persisted in the presence of luzindole (ns vs. control). The chronic moderate consumption of red wine reduced infarct size from $58.8 \pm 3.8\%$ to $19.2 \pm 2.6\%$ ($p < 0.01$ vs. control) (see Figure 43). Similarly, melatonin reduced infarct size to $19.9 \pm 4.0\%$ ($p < 0.01$ vs. control).

6.3.2. The effect of prazosin on red wine induced protection

In separate experiments control hearts had an infarct of $80 \pm 4.1\%$ (see Figure 44). The prazosin treatment on its own presented with an infarct of $79.8 \pm 3.0\%$ (ns vs. control), these data were performed separately and are displayed on a separate graph. When prazosin was coadministered with red wine, the protective effect of red wine was attenuated ($p < 0.001$ vs. red wine). Similarly, the protective effect of melatonin was attenuated with prazosin treatment compared to melatonin given on its own ($p < 0.01$ vs. melatonin). The treatment with prazosin alone was missing in figure 43 and additional experiments were performed several months later using a different perfusion system. Although infarct size was increased when compared to controls on figure 43, prazosin alone had no effect compared to controls (figure 44). Of note, none of the treatments affected the body weights of the animals prior to I/R.

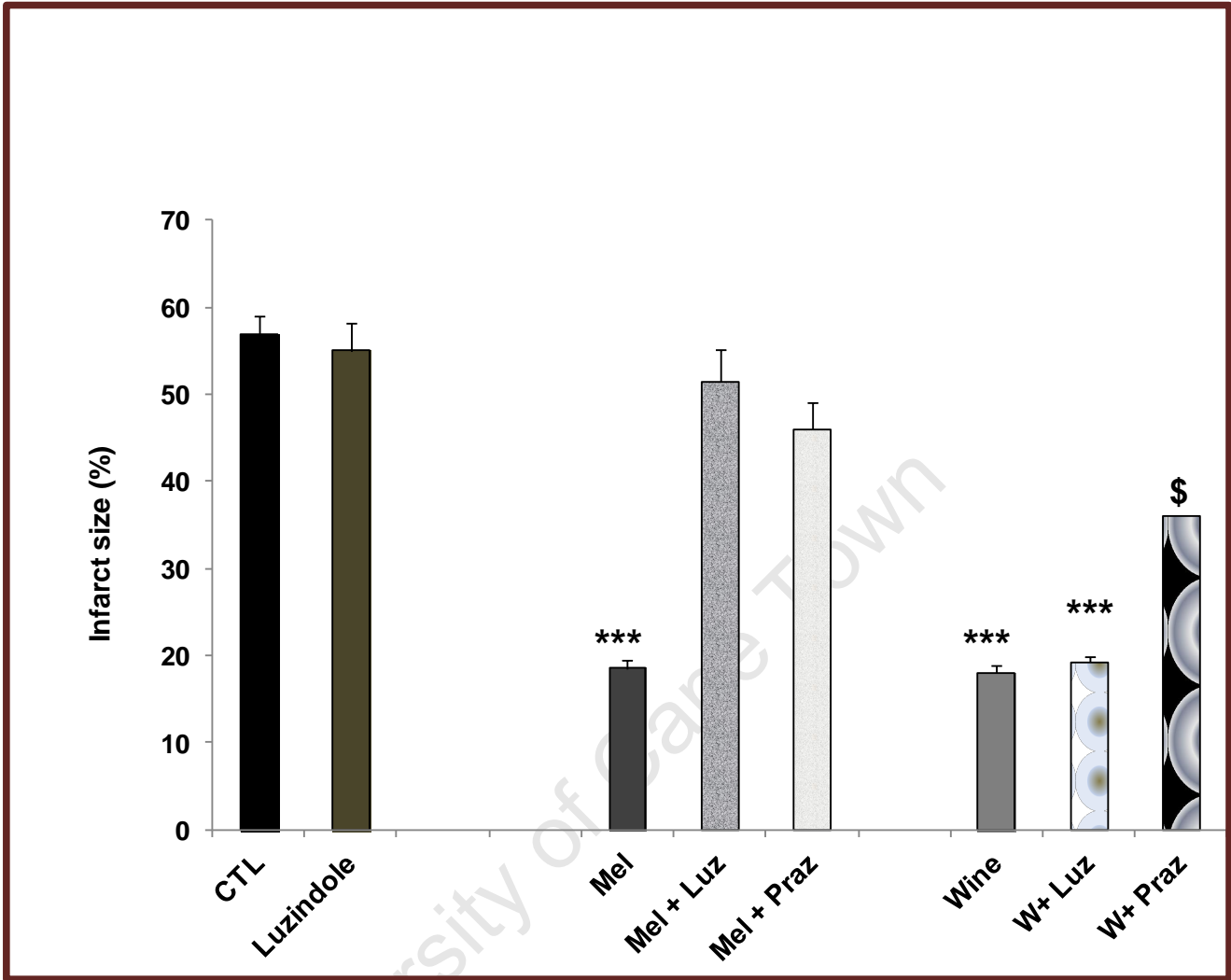


Figure 43: The effect of luzindole on red wine induced cardioprotection in the isolated rat heart model. CTL (Control), Mel (Melatonin), Luz (Luzindole), W (Wine).***p<0.01 vs. control, ++ p<0.01 vs. melatonin, \$ p<0.01 vs wine.

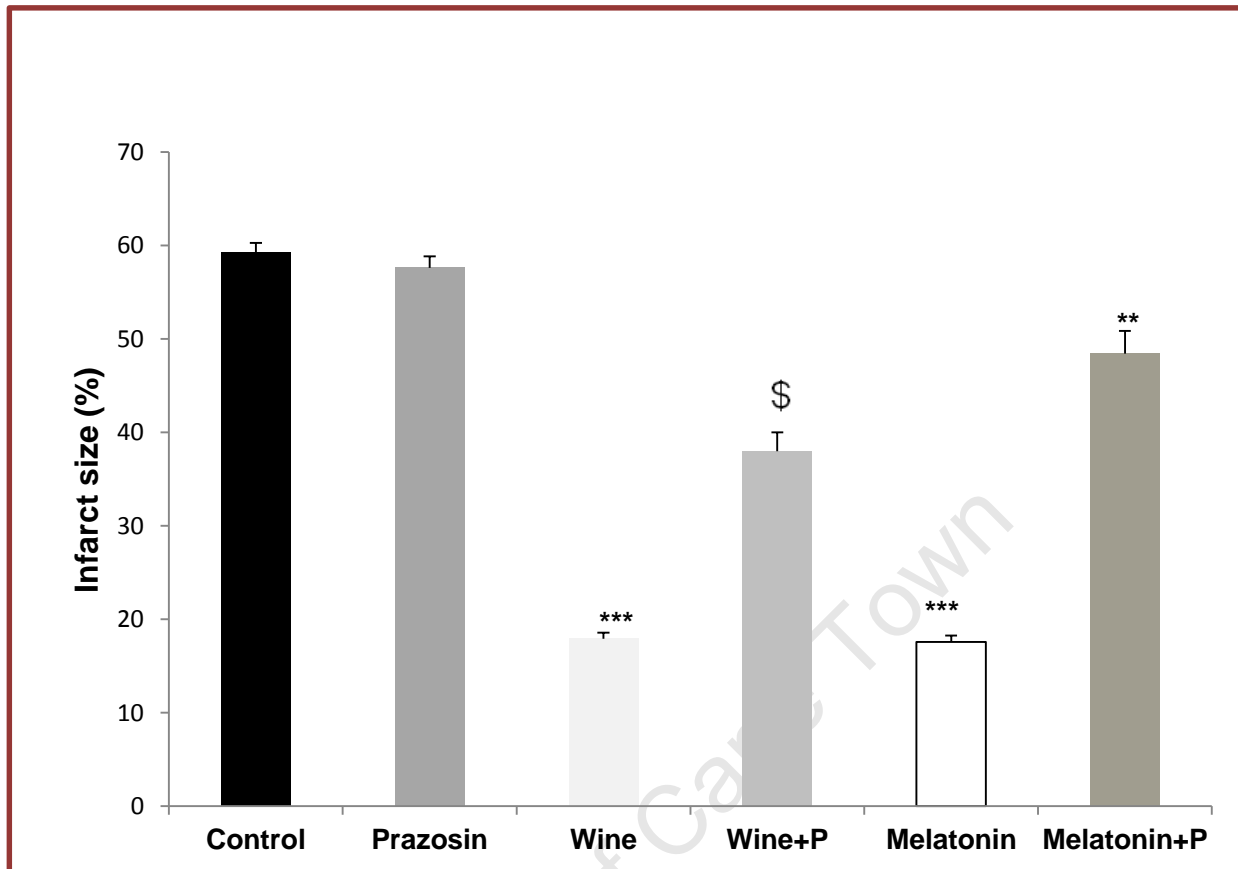


Figure 44: The effect of prazosin on red wine induced cardioprotection in the isolated rat heart model. ***p<0.001 vs. control, \$p<0.001 vs. wine, ** p<0.001 vs melatonin. These experiments were performed several months later on a different perfusion protocol as compared to figure 43.P (Prazosin).

6.4. Chronic treatment with red wine or melatonin fails to confer cardioprotection in TNF receptor 2 deficient mice

To explore the role of the SAFE pathway in red wine or melatonin induced cardioprotection, we used in TNFR2 knockout mice subjected to an *ex vivo* model of I/R injury.

In Figure 45, isolated wild type control mice received plain drinking water and were subjected to 35 minutes of global ischemia followed by 45 minutes of reperfusion presented an infarct of 49.9±5.0%. Red wine pre-treatment for 14 days reduced infarct size in wild-types to (19.9±4.0%; p<0.001 vs. control). Similarly, melatonin pre-treatment reduced infarct size to 15.3±3.2% compared to the ischemic control.

The protective effect of both red wine and melatonin was lost in TNF receptor 2-deficient mice (ns. vs. control).

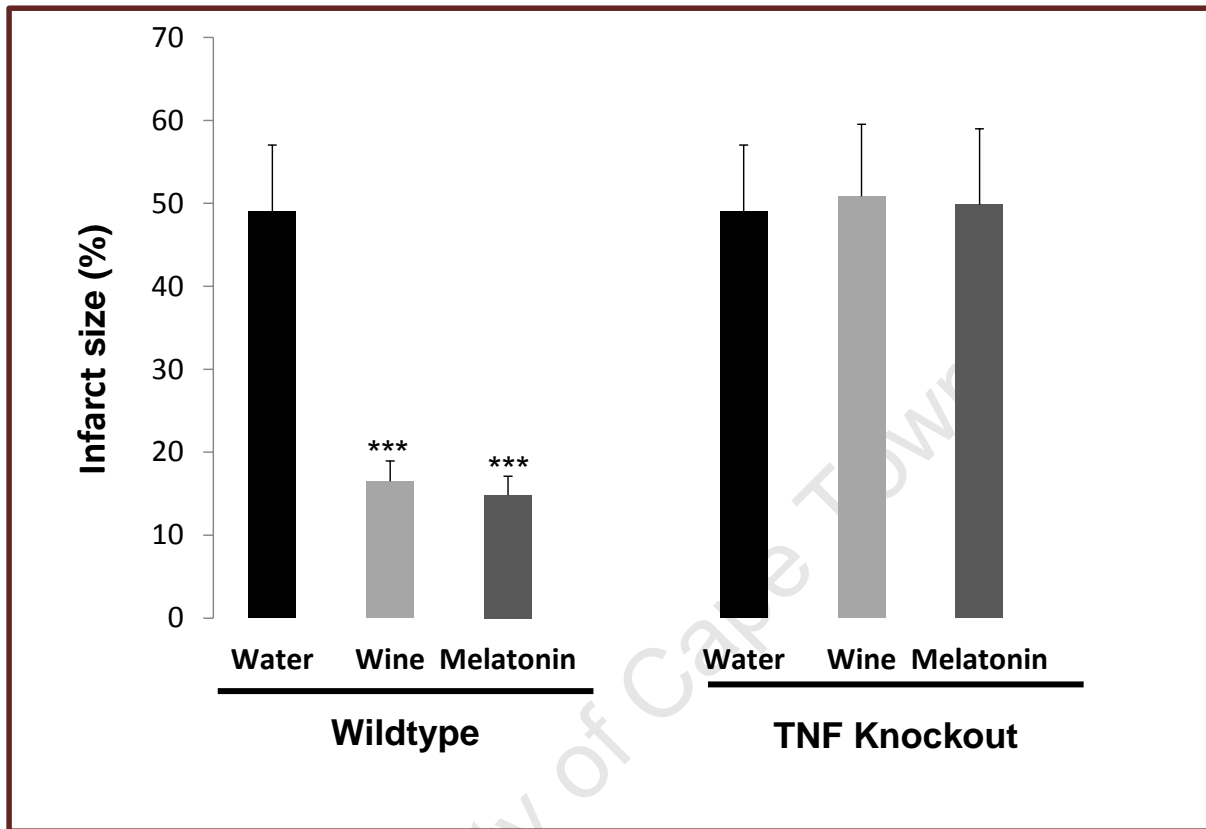


Figure 45: The cardioprotective effect of red wine and melatonin is lost in TNF receptor 2 deficient mice in an *ex vivo* model of I/R. (n=6 for all groups). ***p<0.001 vs water CTL (control).

6.5. Chronic treatment with red wine and melatonin fails to confer cardioprotection in STAT3 knockout mice

In figure 46, isolated wild type control mice that received plain drinking water and that were subjected to 35 minutes of global ischemia followed by 45 minutes of reperfusion presented an infarct of 49.7±4.5%. Red wine pre-treatment for 14 days reduced infarct size in wild-type hearts to (18.7± 3.2%; p<0.001 vs. control). Similarly, melatonin pre-treatment reduced infarct size to 15.8±3.8% compared to the ischemic control (p<0.001). The protective effect of both red wine and melatonin was lost in STAT3-deficient mice (ns. vs. control).

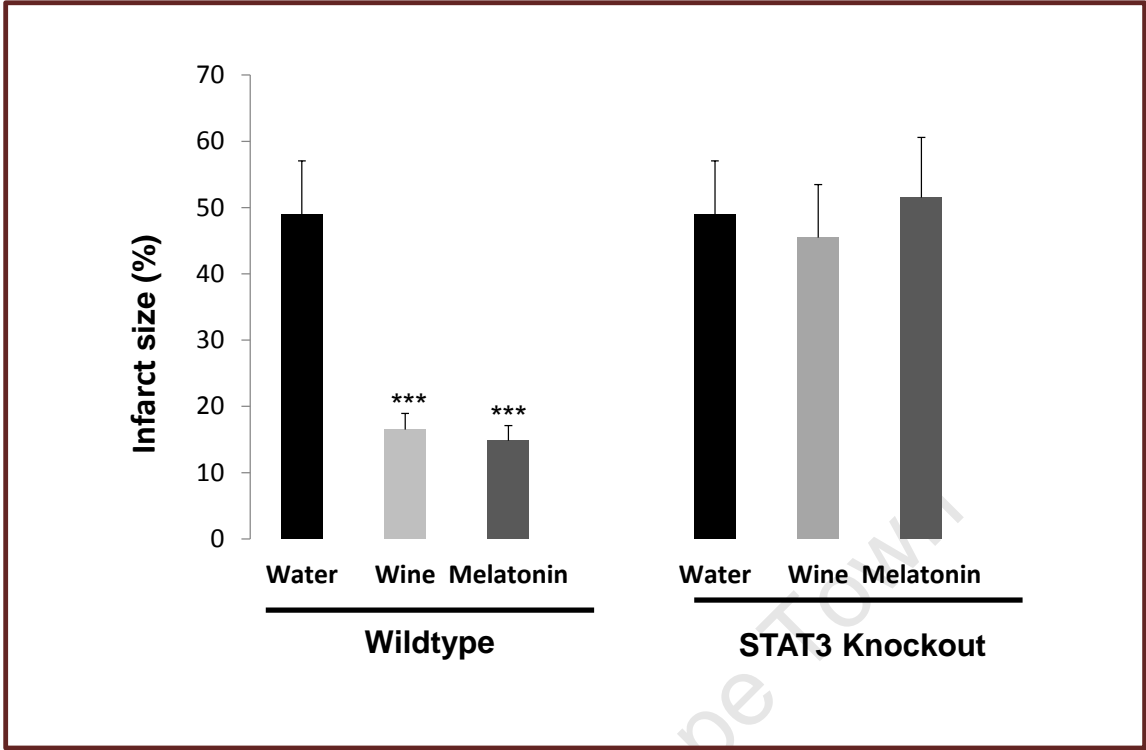


Figure 46: The cardioprotective effect of red wine and melatonin is lost in STAT 3 cardiomyocyte deficient mice subjected to an ex vivo model of I/R injury. (n=6 for all groups). ***p<0.001 vs. water control

7. Discussion

7.1. Summary

The results show that the regular moderate consumption of melatonin, similar to that present in red wine, and red wine on its own (equivalent to 2-3 glasses/day) protect the heart against I/R injury in both an *in vivo* and an *ex vivo* setting. The protective effect of both red wine and melatonin were partially abolished with prazosin. Therefore suggesting that melatonin contributes to the cardioprotective effect of regular moderate red wine consumption, against I/R injury furthermore our data conducted in the TNFR2- and STAT3-deficient mice suggest that the protective effect may be mediated via the activation of the newly discovered SAFE pathway.

7.2. Melatonin and red wine confers cardioprotection *in vivo* and *ex vivo*

Over the past few years, there has been a growing interest in red wine-induced cardioprotection. Evidence from epidemiological studies demonstrates reduced cardiovascular risk in France even though they consume foods that are high in saturated fat (St Leger et al., 1979). Experimentally, most studies exploring the role of red wine are completed on red wine extracts, such as the popular polyphenol resveratrol, but not the red wine itself (Bertelli et al., 1998, Covas et al., 2010). Very high concentrations of resveratrol (10 μ M), which are not within the expected therapeutic range after 2-3 glasses of red wine/day are administered, therefore, suggesting, the presence of other components in red wine that confer its protective effect. Melatonin administration at a high concentration of 40 μ g/ ℓ on the working heart model, protects against IR injury and reduces infarct size (Lochner et al., 2006). In the previous chapter, we have suggested that low concentrations of melatonin, similar to that found in red wine (75ng/ ℓ) can protect against IR injury (Lamont et al., 2011). Melatonin is present in both red and white wine, but is more predominant in red wine (Guerrero et al., 2009); therefore it has been suggested that the protective effect of wine may be dependent on the melatonin concentration. Our present data show that, the regular and chronic administration of melatonin for 14 days mimics the human setting as far as possible. It has been shown for the first time to our knowledge that the regular moderate daily

consumption of melatonin at low concentrations can protect the heart against ischemia-reperfusion injury to a similar extent as red wine given on its own, in both an *ex vivo* and an *in vivo* model. The dose used in our model is almost a thousand times lower the pharmacological doses used in melatonin tablets ranging from 1-3mg. Furthermore, it is important to note that our concentrations of melatonin correspond to physiological ranges of plasma melatonin levels (Kaneko et al., 2000).

7.3. The role for melatonin in red wine induced cardioprotection is mediated via melatonin receptor 3

MT1 and MT2 are G protein couple receptors that inhibit adenylyl cyclase activity in different parts of the body, including the brain, heart, liver, kidney and retina (Zhao et al., 2002). Melatonin receptor 3 (MT3) is a putative receptor widely spread in the hamster brain and peripheral tissues, but very little is known about the beneficial properties of this receptor.

7.3.1. The effect of luzindole on red wine induced cardioprotection

It is well documented that melatonin-induced protection is mediated via either melatonin 1 or 2 receptors (MT1 and MT2) (Genade et al., 2008). It has even been postulated that it is not a membrane-bound receptor but rather a QR enzyme that influences vasoconstriction and immunological function in humans (Mailliet et al., 2005) Firstly, we explored the role of MT1 and MT2, with an antagonist luzindole, in red wine-induced protection. It has been suggested that luzindole may have anti-oxidant effect on its own (Mathes et al.,2008), therefore different concentrations were used to ensure no cardioprotective properties of the inhibitor on its own (high concentrations 5mg/kg done during my Masters research ,and lower concentrations 1mg/kg in the present study). Luzindole inhibited the protective effect of melatonin suggesting that melatonin on its own may protect via MT1 or MT2, but failed to block the protective effect of red wine, therefore suggesting that the protective effect of low concentration of melatonin in red wine is not mediated via MT1 or MT2 and stimulation of these receptors may be dose specific. The pharmacodynamics of luzindole in conjunction with the various components in red wine is unknown and may reduce the efficacy of the inhibitor.

Furthermore, if any of the compounds in the red wine share a similar anatomical structure to luzindole there may be competition for the same site and if so wine induced cardioprotection elicited in the presence or absence of luzindole.

7.3.2. The effect of prazosin on red wine-induced cardioprotection

It has been demonstrated that at high concentrations (40 μ g/l) the cardioprotective effect of melatonin is receptor dependent (Genade et al., 2008). Consequently we explored the role of MT3. To determine the role of the putative MT3, prazosin was administered in the presence or absence of red wine or melatonin. The infarct-sparing ability of melatonin was abolished in the presence of prazosin. Furthermore, the protective effect of red wine was diminished, with melatonin inhibition. The protective effect of melatonin may be dependent of its receptors, if MT3 is actually an unbound enzyme. The fact that prazosin partially attenuated the infarct sparing capacity of red wine suggest that a synergy of other compounds present in red wine may contribute to its cardioprotective effect. Interestingly, prazosin also inhibits the adrenoreceptors, located in the vascular smooth cells (Dubocovich & Markowska 2005). These receptors are responsible for the vasoconstrictive effect of norepinephrine, and when inhibited with prazosin blood pressure, anxiety and sleep disorders are diminished (Galli-Carminati et al., 2009). Interestingly, the production of melatonin at night is triggered by the release of norepinephrine which triggers the sympathetic nerves in the pineal perivasculature. This promotes the activation of the α and β adrenoreceptors and the secondary messenger cyclic adenosine diphosphate, which drives the production of melatonin (Sun et al., 2002; Gonzalez et al., 2012) The adrenoreceptors play a key role in the regulation of the 5 hydroxyserotonin (5-HT), the precursor for melatonin formation. The β adrenoreceptor is where 5-HT is synthesised and α adrenoreceptors is where 5-HT is released. When 5-HT is released, it must undergo decarboxylation to form N-acetylserotonin, the rate limiting step during melatonin biosynthesis (Sun et al., 2002; Gonzalez et al., 2012). Thus, the inhibition of the adrenoreceptors with prazosin will reduce the synthesis and release of 5-HT, and in turn, will reduce melatonin synthesis. Thus the effect of prazosin on melatonin may be independent of its melatonin receptors as it may directly affect the production of endogenous melatonin. This remains unclear

and requires further investigation with ex vivo experiments. Nonetheless, pharmacological inhibitors are non selective so to determine the exact role of the melatonin receptors; melatonin receptor knockout mice should be used. Unfortunately there are no MT3 knockout mice available.

7.4. Melatonin- and red wine-induced cardioprotection is mediated via the SAFE pathway

Melatonin is a powerful anti-oxidant 100x more effective than vitamin E (Tan et al., 1999). Melatonin protects against I/R injury. This protective effect may be mediated by melatonin anti-oxidant qualities. In 2010, it was demonstrated that melatonin can exert its protective effect through the inhibition of the mitochondrial permeability pore opening (Tengetti et al., 2008). Other studies have shown that melatonin can protect via the activation of the PI3k/Akt pathway and this protective effect is mediated via its receptors, MT1 and MT2 (Genade et al., 2008). In Chapter 1, we have shown that melatonin activates the SAFE pathway via the innate immune system, TNF α and its downstream target STAT3 (Lamont et al., 2011). Our present data demonstrate for the first time that the moderate daily consumption of red wine or low doses of melatonin requires the activation of the prosurvival SAFE pathway for cardioprotection. This effect is most likely mediated via the putative MT3 receptor. Furthermore, a well known downstream target of the SAFE pathway is the activation of the mPTP pore (Boengler et al., 2008), it can be postulated that a low dose of melatonin as attained by the regular and moderate consumption of red wine may protect via the closing of the mPTP.

8. Limitations of the study

Red wine and melatonin were diluted in the drinking water of either mice or rats. The body weight and normal consumption of each animal was taken into careful consideration prior to administration. However, there were 3-4 animals in each cage so the exact consumption by each animal remains unknown. For future studies oral gavage would be a better alternative to ensure standardized treatment and to better mimic a clinical setting. Furthermore, the melatonin concentration should be measured in the blood plasma after red wine consumption to better determine melatonin's bioavailability.

These studies also used relatively young healthy rats/mice in the model of I/R injury but in reality, people who suffer from an acute myocardial infarction are typically middle to old aged with, hypertension or obesity. Therefore, the contraindications of moderate red wine or melatonin consumption due to possible interaction with medications, including β -blockers, aspirin or statin therapy are unknown and require further investigation.

9. Summary and Conclusion

Our data provide strong evidence that the daily moderate consumption of red wine can protect the heart against I/R injury. Also, melatonin administration at concentrations corresponding to the one found in red wine can confer cardioprotection. Most importantly the inhibition of melatonin in red wine, results in the attenuation of red wine induced protection, thus strongly suggesting that melatonin is a key player but not the only component that contributes to red wine-induced cardioprotection. Furthermore, the cardioprotective effect of both melatonin and red wine are mediated via the activation of the prosurvival SAFE pathway. Our data provide a safe therapy in treating cardiovascular disease in low- and high-income settings as melatonin is a cheap compound that is widely available over the counter in many countries, see figure 47.

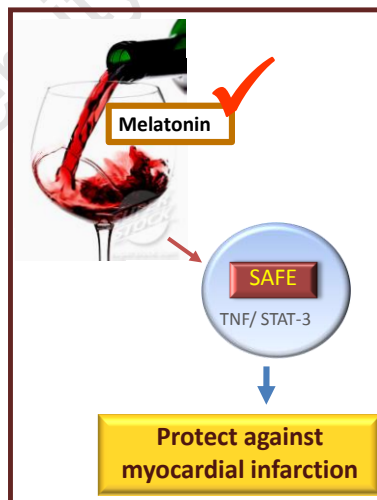


Figure 47: Melatonin contributes to red wine induced protection via the activation of the SAFE pathway.

F. Chapter 3: Melatonin protects in a model of ischemic heart failure

University of Cape Town

1. Introduction

HF is a complex syndrome involving a variety of structural and functional changes. These changes impair the ability of the heart to pump and eventually render the heart incapable of maintaining normal physiological circulation (McDonagh et al., 2011). Patients with an acute myocardial infarction will often develop ischemic heart failure. Unfortunately, there are no real therapies for the prevention of IHF.

Previous studies performed in chapter 1, showed that an acute perfusion of melatonin, can protect the heart against ischemia-reperfusion injury. In Chapter 2, we have demonstrated that a regular consumption of melatonin, given at physiological concentrations for 14 days, reduced I/R injury in an isolated heart model. These findings were further corroborated in an *in vivo* model of I/R. Interestingly, this protective effect was mediated via the activation of the prosurvival signalling pathway, the survivor activating factor enhancement (SAFE) pathway. STAT3, a major component of the SAFE pathway may play a pivotal role in HF as STAT3 deficient mice develop HF. Therefore, whether, the SAFE pathway could protect in a model of ischemic HF requires investigation.

In the literature patients with CAD and MI exhibit a reduction in melatonin levels at night (as reviewed by Paulis & Simko, 2007). Long-term studies performed in patients with high blood pressure displayed an improved outcome with melatonin supplementation (2.5mg/day for 14 days) (as reviewed by Paulis & Simko 2007; Paulis et al., 2010). However, whether chronic melatonin administration could be used as a therapy in ischemic HF remains unclear and whether the protective effect could be mediated via the chronic action of the SAFE pathway is unknown.

2. Aim

Therefore, the aim of the current study was to extend the findings from a model of I/R to a model of ischemic HF and to determine whether chronic treatment with melatonin, at the concentration found in 2-3 glasses of red wine per day, can confer cardioprotection

against ischemic HF. Furthermore, we propose that melatonin-induced cardioprotection in ischemic HF may be mediated via the modulation of the SAFE pathway.

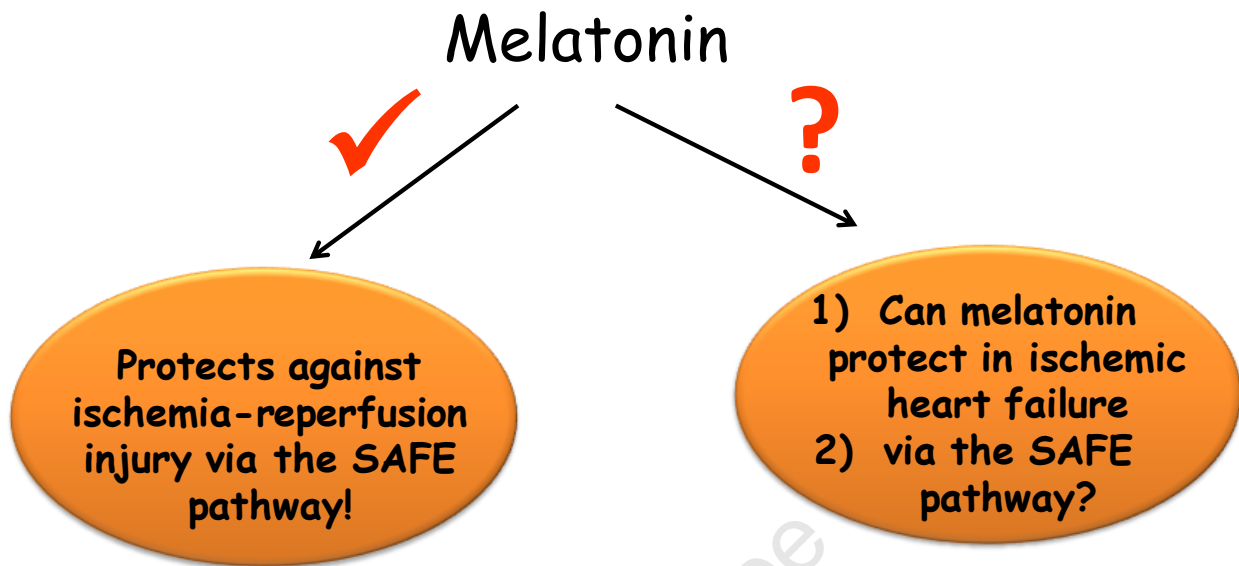


Figure 48: Proposed hypothesis: Can melatonin protect in a model of ischemic HF via the SAFE pathway.

3. Methods

3.1. Animals

All the experiments conducted on mice (12-14 weeks) were performed in accordance with the *Guide for Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH publication No. 85(23), revised 1996). All procedures were approved by the Animal Research Ethics Committee (UCT) under the ethics number 011/026, "Delineating the cardioprotective effects of red wine and its active components in an in vivo mouse model of ischemic HF". Animals had access to food and water ad libidum.

3.2. Heart failure model

After the administration of anaesthesia (isoflurane mixed with oxygen), a tracheotomy was performed and the mice were intubated. With a lack of a pedal reflex, a left anterior

thoracotomy was performed in the third intercostal space and the pericardium removed. The left ascending coronary artery was ligated as described in Chapter 2.

3.2.1. Experimental protocol

TNF^{-/-} mice, STAT3^{-/-} mice their littermate controls and C5/BL6 mice were pre-treated for 5 days with either: water, melatonin (75ng/ℓ), water and AG490, in the presence or absence of melatonin (10mg/kg, ip). To assess the hemodynamic function the mice underwent echocardiography prior to the permanent ligation, for baseline values with or without treatment. Thereafter, the left ascending coronary artery was ligated and the mice were further treated with plain drinking water, melatonin with or without AG490. After 28 days, the mice were anaesthetised with isoflurane and oxygen. Another echocardiogram was performed prior to termination. The heart was excised and stored in 10% formalin for future histological assessment. Body weight, heart weight and tibia length were all assessed as measures for HF.

Statistical analysis

Data are presented as mean ± SEM. N=6 per group. Comparisons between multiple groups were performed by one-way ANOVA followed by the Dunnet's post hoc test (Graph Pad Instat). A value of $p < 0.05$ was considered as statistically significant.

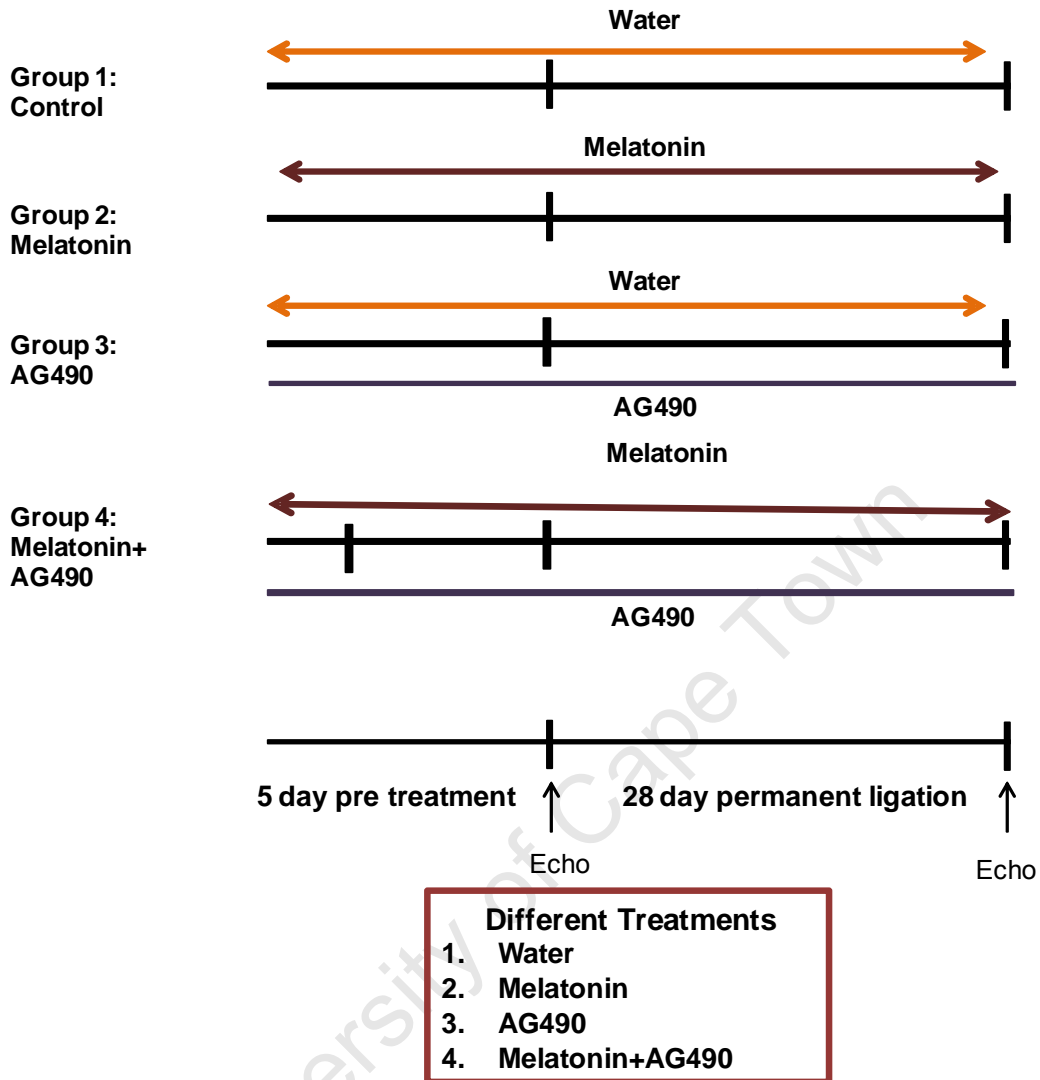


Figure 49: Experimental protocol melatonin was administered in the drinking water of wild-types, STAT3 KO or TNF KO mice as well as wild-type littermate controls. In the C5BL6 mice, melatonin was administered in the presence or absence of AG490 (10mg/kg), a STAT3 inhibitor five days prior to the permanent ligation and throughout the 28-day period, post ligation.

4. Results

4.1. The cardioprotective effects of melatonin against ischemic HF in TNF KO mice

4.1.1. Heart weight/Body weight

As depicted in the Table 5, the wild-type controls that were given plain drinking water that were subjected to a 28-day permanent ligation, had a heart-weight/body-weight (HW/BW) ratio of 11.9 ± 2.3 . The melatonin treatment in wild-type mice reduced HW/BW to 6.7 ± 3.1 (* $p < 0.05$ vs. control). In the TNF KO mice that were given plain drinking water presented with a HW/BW was 11.5 ± 1.4 . TNF KO that had been treated with melatonin had a reduced HW/BW of 6.5 ± 2.6 ($p < 0.05$ vs. control).

4.1.2. Tibia length/Body weight

As depicted in the Table 5, the wild-type controls that were given plain drinking water that were subjected to 28-day permanent ligation, had a tibia-length/body-weight (TL/BW) ratio of 82.9 ± 2.2 . The melatonin treatment in wild-type mice reduced TL/BW to 62.3 ± 1.8 (* $p < 0.05$ vs. control). In TNF KO mice that were given plain drinking water had TL/BW of 77.2 ± 3.0 . The TNF KO that had been treated with melatonin had a reduced HW/BW of 58.4 ± 2.9 (* $p < 0.05$ vs. control).

Table 5: The heart weight/body weight (HW/BW) vs. tibia length/body weight (TL/BW) in TNF KO and wild-type littermate controls

Wildtypes		TNF KO		Wildtypes		TNF KO	
Control	Melatonin	Control	Melatonin	Control	Melatonin	Control	Melatonin
Heart Weight/Body Weight (ratio)				Tibia length/Body Weight (ratio)			
11.9 ± 2.3	* 6.72 ± 3.1	11.5 ± 1.4	* 6.55 ± 2.6	82.9 ± 2.2	* 62.3 ± 1.8	77.2 ± 3.0	* 58.4 ± 2.9

* $p < 0.05$ vs control

4.1.3. Echocardiography

Hemodynamic parameters of all mice were assessed by echocardiography. Mouse hearts were subjected to a permanent ligation. In Figure 50, the wild-type mice given plain drinking water presented with an EF of $89.1 \pm 1.5\%$ and a FS of $54.0 \pm 2.0\%$. Melatonin treatment had no effect on the wild-type mice prior to ligation (ns. vs. control). In Figure 51, TNF KO mice that received plain drinking water presented with an EF of $91.1 \pm 1.0\%$ and FS of $54 \pm 2.9\%$. Melatonin treatment had no effect on the TNF KO mice prior to ligation (ns. vs control).

In Figure 51, the wild-type controls had an EF of $49.3 \pm 0.9\%$ and FS of $23 \pm 2.8\%$, post ligation. The wild-types that received melatonin treatment had an improved EF of $74.4 \pm 3.1\%$ and FS of $38.9 \pm 2.8\%$ ($p < 0.001$ vs control). The TNF KO mice that received plain drinking water had an EF of $60 \pm 4.0\%$ and FS of $28.3 \pm 1.9\%$. The TNF KO that received melatonin treatment had an improved EF of $82 \pm 1.4\%$ and FS of $45 \pm 1.9\%$ ($p < 0.001$ vs. controls) (see figure 52).

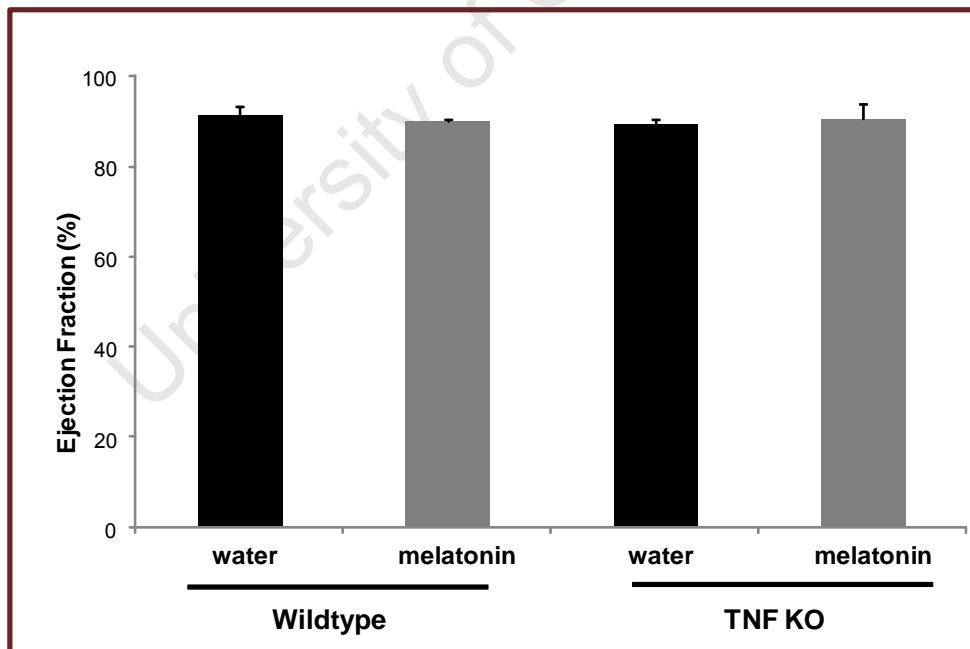


Figure 50: EF (ejection fraction) prior to ligation. WT (wild-types) and TNF KO mice were treated with either water or melatonin for 5 days.

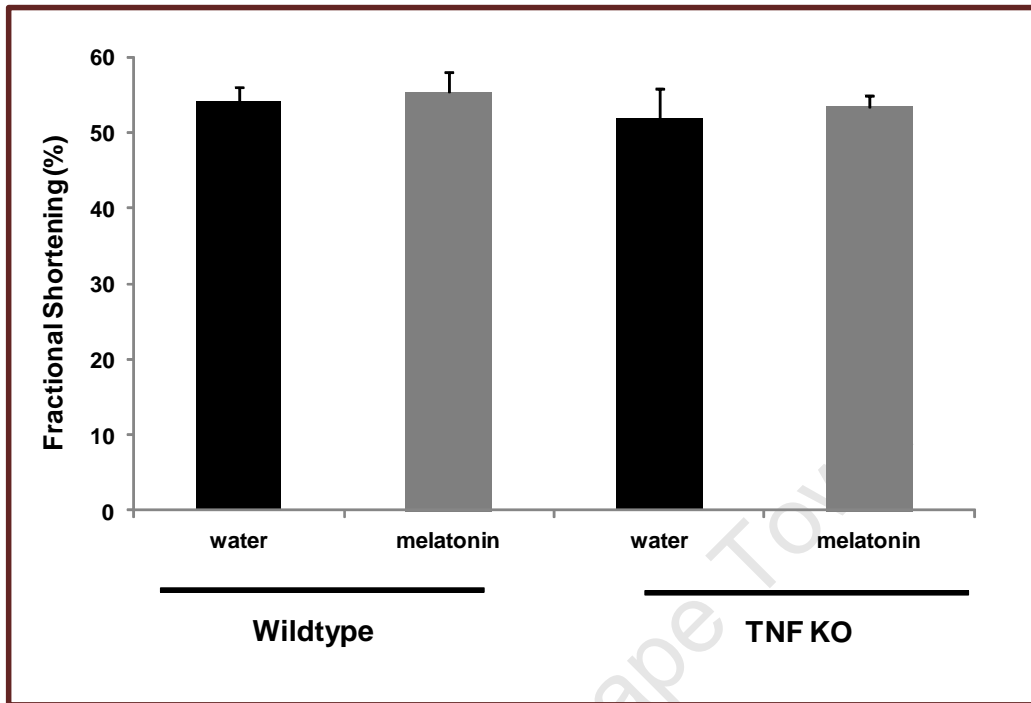


Figure 51: FS (fraction shortening) prior to ligation. WT (wild-types) and TNF KO mice were treated with either water or melatonin for five days.

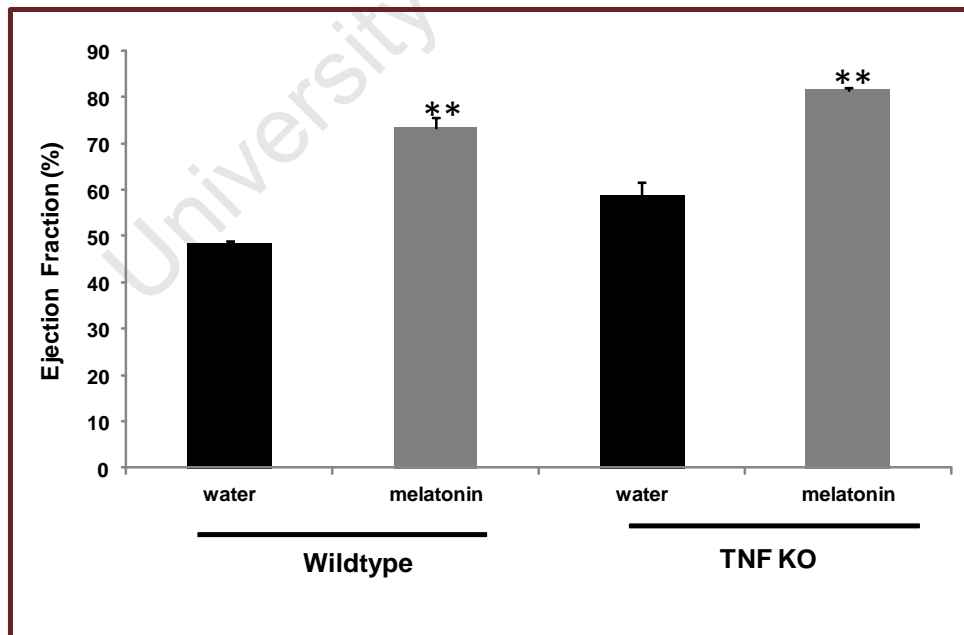


Figure 52: Ejection fraction after 28 days of permanent ligation. WT (wild-types) and TNF KO mice were treated with either water or melatonin for 28 days. **p<0.001 vs. respective water controls

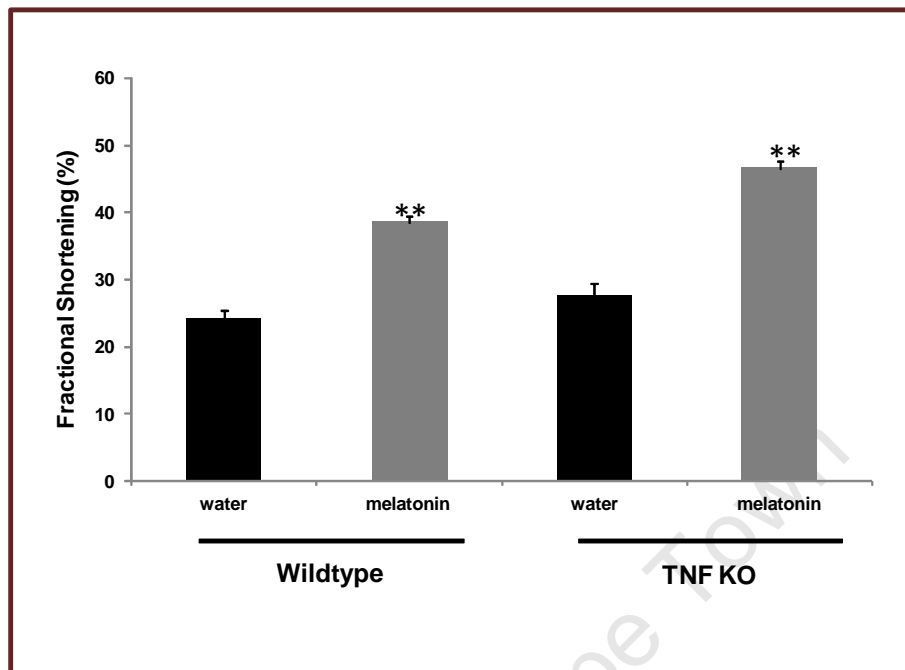


Figure 53: Fractional shortening (FS) after 28 days of permanent ligation. WT (wild-types) and TNF KO mice were treated with either water or melatonin for 28 days. ** $p < 0.001$ vs. respective water controls.

4.4. The cardioprotective effects of melatonin protects against ischemic HF in STAT3KO mice

4.4.1. Heart weight/body weight

As depicted in the Table 6, the wild-type controls that were given plain drinking water that were subjected to a 28-day permanent ligation, had a heart-weight/body-weight (HW/BW) ratio of 12.1 ± 0.3 . The melatonin treatment in wild-type mice reduced HW/BW to 6.4 ± 1.9 (* $p < 0.05$ vs. control). In the STAT3 KO mice that were given plain drinking water HW/BW, was 9.7 ± 3.2 . STAT3 KO that had been treated with melatonin had a reduced HW/BW of 6.6 ± 2.8 (* $p < 0.05$ vs. control).

4.4.2. Tibia length/Body weight

As depicted in the table 6, the wild-type controls that were, given plain drinking water that were subjected to a 28 day-permanent ligation, had a tibia-length/body-weight (TL/BW) ratio of 61.0 ± 2.6 . The melatonin treatment in wild-type mice reduced TL/BW to 68.1 ± 4.0 . In STAT3 KO mice that were given plain drinking water had a TL/BW of

89.3±3.5. STAT3 KO treated with melatonin was similar to the plain drinking water control.

Table 6: The heart weight/body weight (HW/BW) vs. tibia length / body weight (TL/BW) in STAT3 KO and wild-type littermate controls.

Wildtypes		STAT3 KO		Wildtypes		STAT3 KO	
Control	Melatonin	Control	Melatonin	Control	Melatonin	Control	Melatonin
Heart Weight/Body Weight (ratio)				Tibia length/Body Weight (ratio)			
12.1±0.3	6.4 [*] ±1.9	9.7±3.2	6.6±2.8	61.0±2.6	68.1±4.0	89.3±3.5	70.2±2.8

*p<0.05 vs control

4.4.3. Echocardiography

Hemodynamic parameters of all mice were assessed by echocardiography. Mouse hearts were subjected to a permanent ligation. In Figure 54, the wild-type mice given plain drinking water presented an EF of 89.1± 1.5% and a FS of 54.0±2.0%. Melatonin treatment had no effect on the wild-type mice prior to ligation (ns. vs. control). In Figure 54, STAT3 KO mice that received plain drinking water presented with an EF of 91.1± 1.0% and FS of 54±2.9%. Melatonin treatment had no effect on the TNF KO mice prior to ligation (ns. vs control).

In Figure 56, the wild-type controls had an EF of 49.3±0.9% and FS of 23±2.8%, post ligation. The wild-types that received melatonin treatment had an improved EF of 74.4±3.1% and FS of 38.9±2.8% (p<0.001 vs control). The STAT3 KO mice that received plain drinking water had an EF of 60±4.0% and FS of 28.3±1.9%. The STAT3 KO that received melatonin treatment had an improved EF of 82±1.4% and FS of 45±1.9%, post ligation (p<0.001 vs. controls) (see Figures 56 and 57).

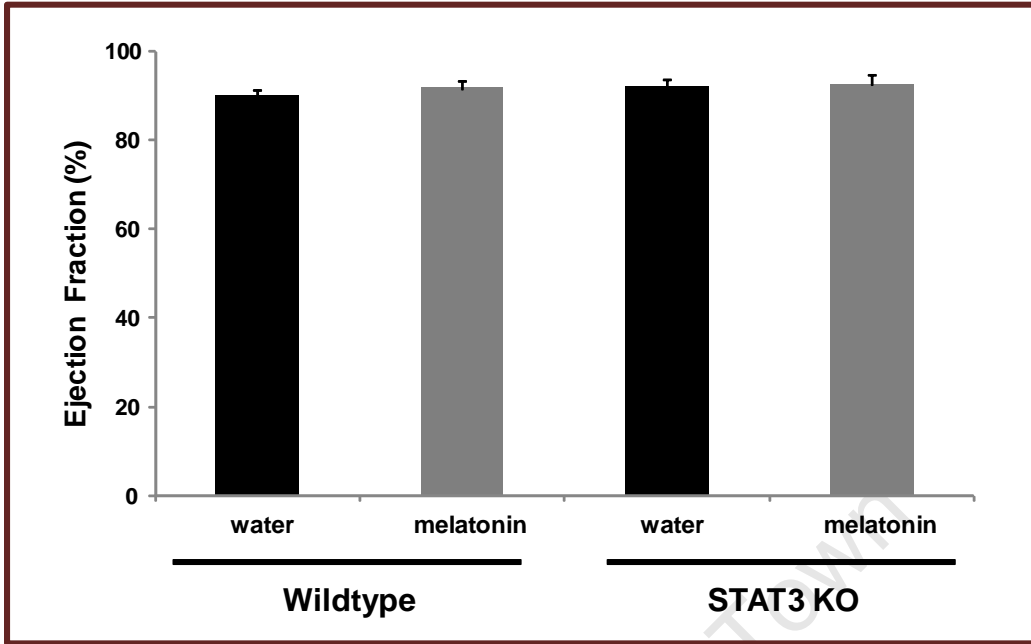


Figure 54: EF (ejection fraction) prior to ligation. WT (wild-types) and STAT3 KO mice were treated with either water or melatonin for five days.

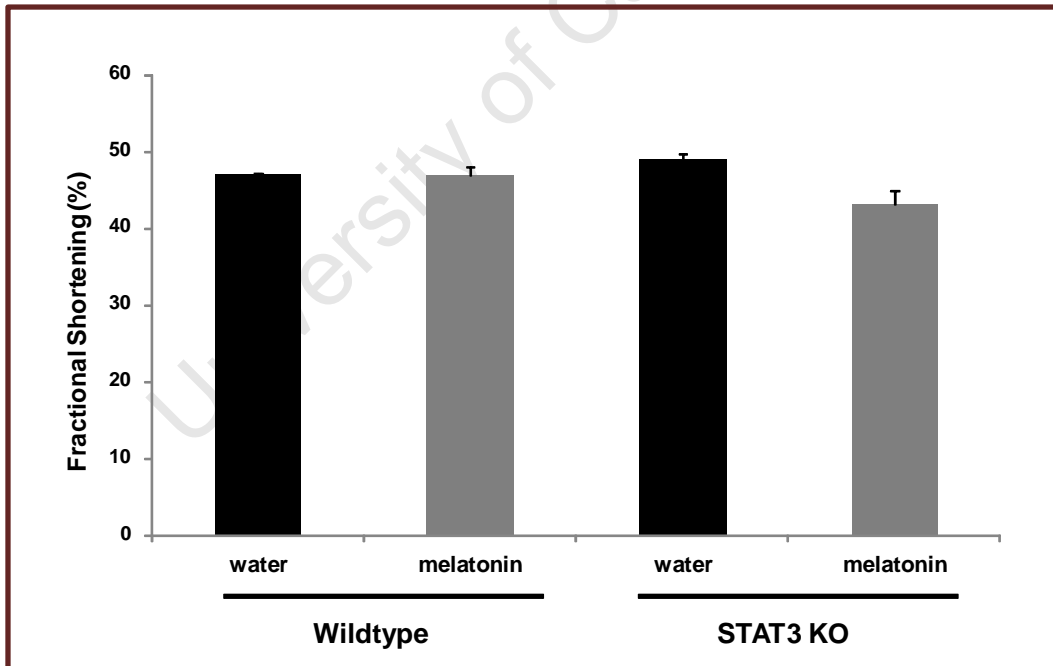


Figure 55: FS(fractional shortening) prior to ligation. WT (wild-types) and STAT3 KO mice were treated with either water or melatonin for five days.

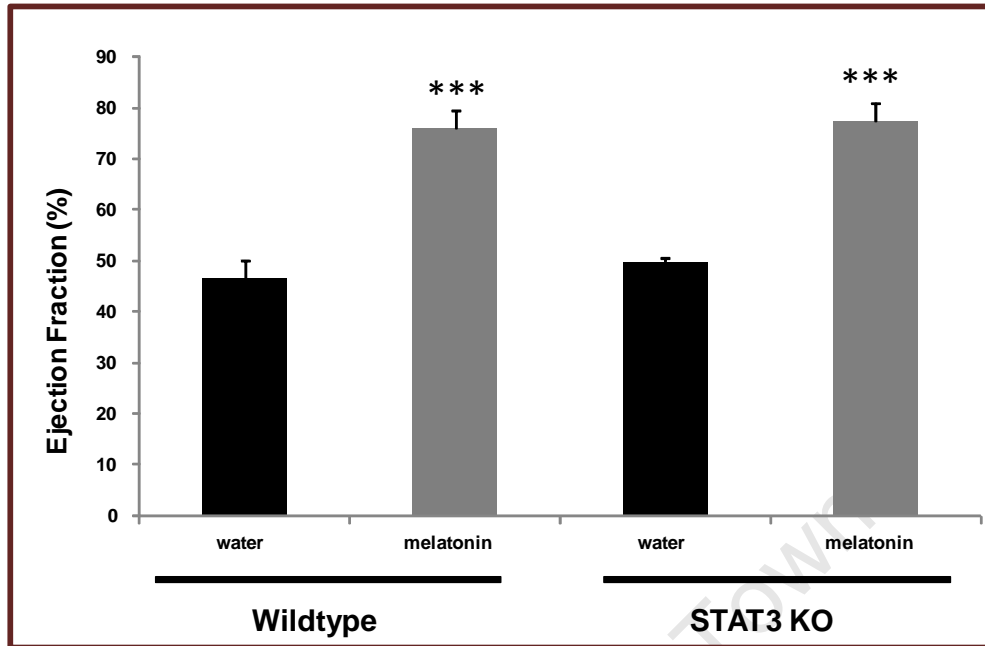


Figure 56: . Ejection fraction after 28days of permanent ligation. WT (wild-types) and STAT3 KO mice were treated with either water or melatonin for 28 days. ***p<0.001 vs.repective water controls

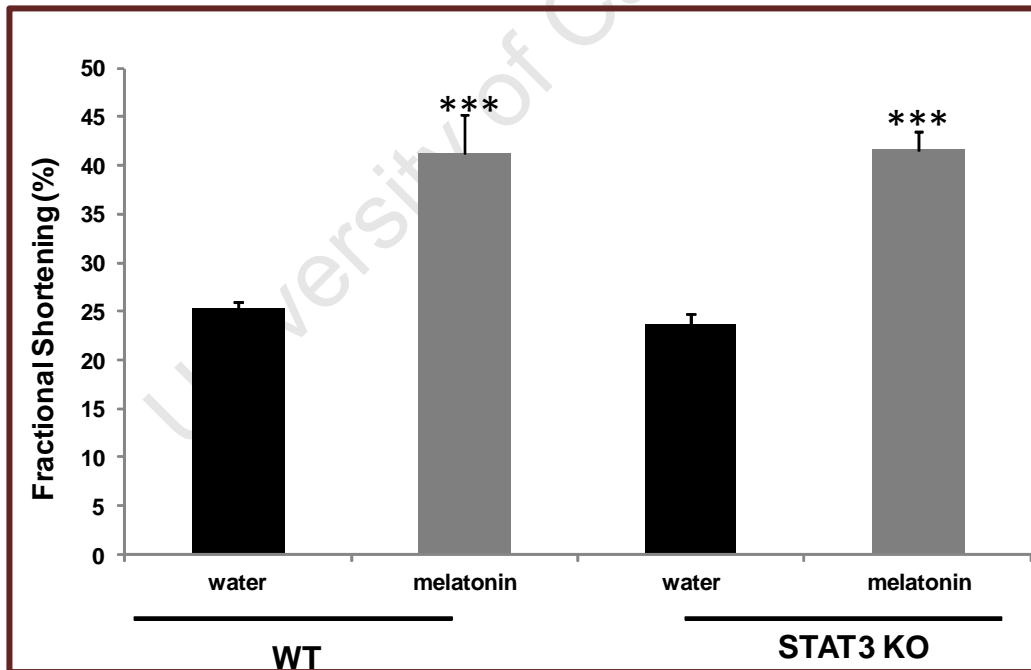


Figure 57 : FS (fractional shortening) after 28days of permanent ligation. WT (wild-types) and STAT3 KO mice were treated with either water or melatonin for 28 days. ***p<0.001 vs.repective water controls

4.5. Inhibition of STAT3 with AG490 does not abolish Melatonin induced cardioprotection

4.5.1. Heart weight/Body weight

As depicted in the table 7, controls that were given plain drinking water were subjected to 28 day permanent ligation, had a heart-weight/body-weight (HW/BW) ratio of 12.0 ± 1.9 . The melatonin treatment in wild-type mice reduced HW/BW to 6.1 ± 3.8 (* $p < 0.05$ vs. control). AG490, a STAT3 inhibitor had a HW/BW of 10.2 ± 3.6 . Coadministration of melatonin and AG490 had a HW/BW of 6.6 ± 4.0 (ns vs. control).

4.5.2. Tibia length/Body weight

As depicted in the Table 7, the controls given plain drinking water were subjected to 28 day permanent ligation, had a tibia length /body weight (TL/BW) ratio of 55.0 ± 2.3 . The melatonin treatment had no significant effect (ns. vs. control). The coadministration of AG490 had no effect on tibia length (ns vs control). However, the coadministration of melatonin and AG490 reduced TL/BW to 70.0 ± 0.8 (* $p < 0.05$ vs. control).

Table 7: The heart weight/body weight (HW/BW) vs. tibia length/body weight (TL/BW) in wild-type littermate mice.

Water		AG 490		Water		AG 490	
Control	Melatonin	Control	Melatonin	Control	Melatonin	Control	Melatonin
Heart Weight/Body Weight (ratio)				Tibia length/Body Weight (ratio)			
12.0 ± 1.9	$6.1 \pm 3.8^*$	10.2 ± 3.6	6.6 ± 4.0	55.0 ± 2.3	$69.5.1 \pm 3.0$	89.0 ± 2.5	$70.0 \pm 0.8^*$

* $p < 0.05$ vs control

Control mouse hearts that were, given plain drinking water were subjected to a permanent ligation had an EF of $48 \pm 3\%$ and FS of $20 \pm 3\%$ (see Figures 59 and 60). Pre-treatment with AG490 had no effect (ns vs. control). Melatonin treatment on its own improved EF from $48 \pm 3\%$ to $80 \pm 1\%$ ($p < 0.001$). Treatment with melatonin also improved FS from $20 \pm 3\%$ to $40 \pm 3\%$ compared to controls. However, addition of AG490, with

melatonin did not abolish the protective effect of melatonin and presented with an EF of $75 \pm 2\%$ (ns versus melatonin group) and FS of $38.2 \pm 2.8\%$.

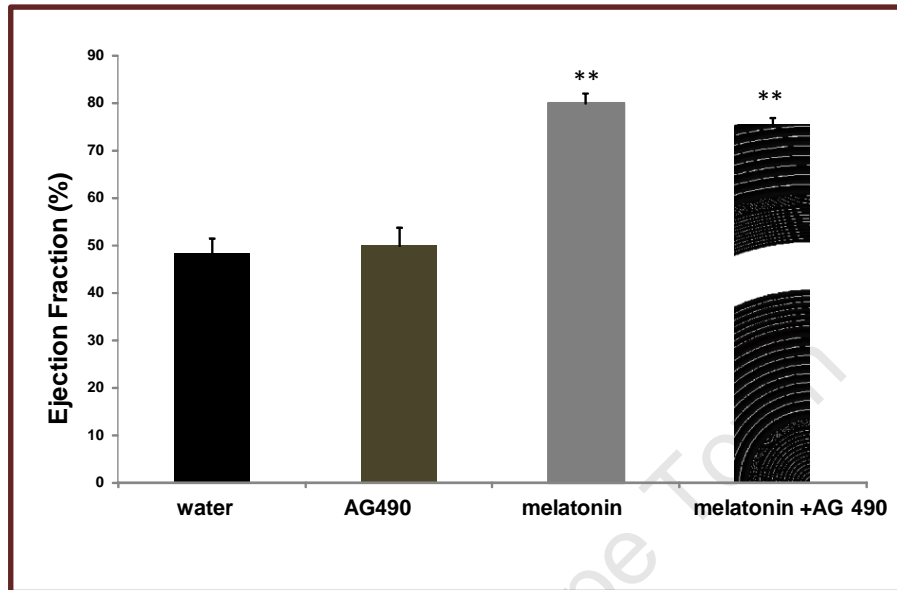


Figure 58: EF (ejection fraction) was assessed by echocardiography after 28 days ligation in the presence or absence of AG490 in wild-types mice receiving either water or melatonin treatment..

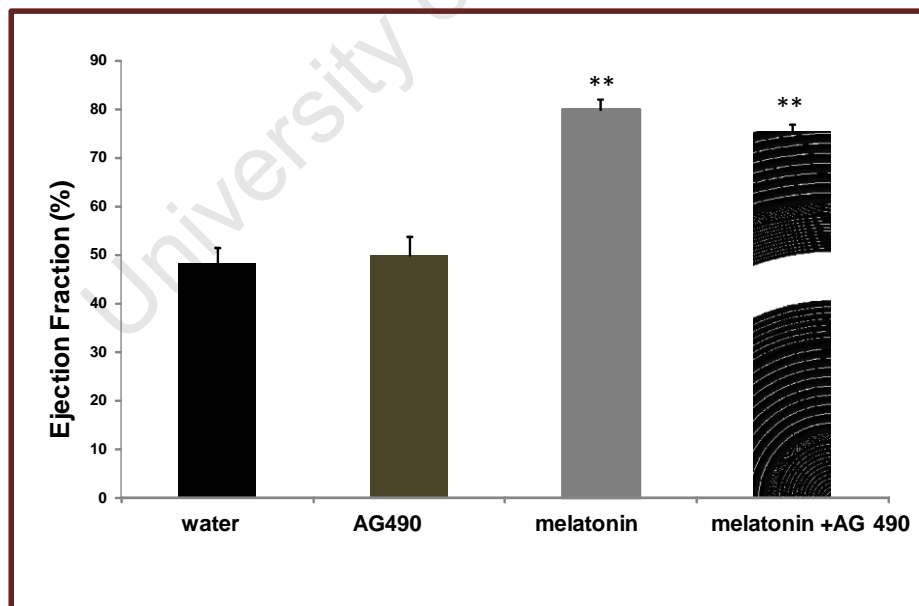


Figure 59: FS (fractional shortening) was assessed by echocardiography after 28 days ligation in the presence or absence of AG490 performed in wild-types mice receiving either water or melatonin treatment.

5. Discussion

The previous chapters have shown that melatonin administered acutely and chronically (over a 14-day period) protects the heart in a model of I/R injury. The present chapter extends our findings to a model of IHF. We have shown that the chronic moderate consumption of melatonin given daily at physiological concentrations can ameliorate the detrimental effect of HF using STAT3KO and TNFKO mice, our data suggest that this cardioprotective effect of melatonin is independent of the SAFE pathway

5.1. Validation of IHF model

The mice underwent a permanent ligation of the left descending coronary artery, 2 mm below the auricle, and were maintained for 28 days. Although this model is commonly used and described in the literature, it is questionable whether 28 days is sufficient for the progression of HF (Gao et al., 2000). HF is a complex syndrome that develops over time: the heart function becomes impaired and fails to pump. In humans, a heart beating with an EF of 50% is fully functional, thus the EF must drop below 50% for impairment (Chu et al., 2009). In IHF the infarct progresses and leads to myocyte damage, reduced contractility and eventually the dysfunction of the left ventricle (Chu et al., 2009). The size and location of the infarct will influence the progression of HF. Therefore, in the current study it would be imperative that infarct size be measured, to give a better indication of the development of IHF. Furthermore, in the current experiments conducted in the STAT3 KO, TNF KO and their wild-type littermates and wild-type mice with the C5BL6 background, all presented with a mean ejection fraction of $\pm 50\%$ post infarction but with a significant drop in FS to $\pm 20\%$. Therefore the mouse hearts are functioning at a capacity of 50% and would not be considered failing in a human setting (Fox et al., 2001). In a mouse model of IHF, the EF decreases to $\pm 30\%$ before it is considered failing. However, in the mouse, the extent of typical cavity obliteration is far greater than in man, so that the normal EF is near 90%. It is therefore much more common to use FS% as the basic measure of ventricular function and is the most common method for the analysis of left ventricular function in mice (as reviewed by Rottman et al., 2007; Chu et al., 2002). Furthermore, even after a permanent ligation a

mouse heart continues to beat at 400-600 beats/minute as compared to a human heart 60-90 beats/minute. The elevated heart rate in mice highlights differences in calcium handling and muscle contraction (Barry et al., 1999). Thus distinct variations persist in a mouse model when compared to clinical setting (Chu et al., 2002). Again, this raises the question whether the model used in our study is sufficient to mimic HF or merely an illustration of the progression toward HF. In the literature, most mouse studies utilise aortic banding to induce an abnormal pressure overload, these mice develop HF within weeks (Barry et al., 1999). Therefore, for future studies a different model of HF could be utilised to determine whether the outcome remains the same. Another alternative would be to keep mice for an extended time frame to fully declare the progression of HF.

5.2. Melatonin protects against IHF

In our previous study, in Chapter 2, the daily and moderate consumption of melatonin at physiological concentrations protected the heart against I/R injury in an isolated mouse model as well as *in vivo*. There have been many studies that demonstrate the cardioprotective effects of melatonin, at high concentrations (Lochner et al., 2006, Tan et al., 1993, Tan et al., 1998) but studies explore the cardioprotective role of melatonin in IHF. Interestingly, there are clinical trials that have suggested a relationship between melatonin and coronary heart disease (Brugger et al., 1995). Brugger et al., found reduced melatonin levels in the plasma, at night (02:00h) in patients with coronary artery disease (Brugger et al., 1995). It would be of future importance to measure the level of melatonin in IHF patients.

In this chapter the long-term consumption of melatonin reduced the HW/BW ratio in wild-type mice when compared to the plain drinking water controls. The heart weight to body ratio were first measured in 1884 to draw correlations between increased heart mass and hypertrophy (Bergman et al., 1884, Chu et al., 2002). Furthermore, melatonin administration reduced the HW/BW ratio in TNFKO, STA3KO and their respective wild-type littermates therefore these hearts had not developed compensatory hypertrophy and are fairly functional.

The hemodynamic parameters were also measured in all mice. Prior to the ligation there was no difference between the plain drinking water controls and the melatonin treated groups. However, after 28 days the controls given the drinking water had a reduced ejection fraction and fractional shortening of 50%. The wild-type mice given melatonin treatment also had a pronounced improvement in ejection fraction and fractional shortening. Interestingly, this protective effect of melatonin persisted in STAT3KO and TNFKO mice. Melatonin protected even in the presence of AG490 the STAT3 inhibitor.

5.3. Melatonin confers cardioprotection via STAT3

Low concentrations of melatonin administered daily protects in wild-type mice in the presence or absence of AG490, the JAK/STAT3 antagonist. Both the EF and FS were improved in melatonin-treated groups compared to the water controls that received no treatment. This may imply that the protective effect of melatonin is independent of STAT3. However, AG490 is a non-selective antagonist for JAK2 and not STAT3, thus further investigations are required to verify these results (Schaefer et al., 1995). Another alternative would be to administer a more specific inhibitor such as Stattic, a direct STAT3 antagonist.

In the wild-type littermates melatonin preserves EF and FS compared to the controls that received plain drinking water. Surprisingly, this protective effect persisted in cardiomyocyte specific STAT3 KO mice, contrary to the studies performed *in vitro* (Lamont et al., 2010). A possible reason for this is that the *in vitro* studies were performed acutely; a once-off bolus dose of melatonin was administered. In the current model the same dose of melatonin was administered chronically over a 28-day period. The literature has demonstrated that excess levels of STAT3 may induce apoptosis, hypertrophy and HF (Oshimo et al., 2005, Lecour & James. 2011). The JAK/STAT3 pathway is an intricate pathway that requires cytokine activation (Rodig et al., 1998). In this model the inflammatory mediators are elevated, which in turn leads to the chronic or excessive stimulation of STAT3 which can promote the onset of HF (Sood et al., 2006). Alternatively, the chronic activation of the JAK/STAT3 pathway can lead to the up-

regulation of NF κ B which in turn promotes the activation of metalloproteinase release and apoptosis (Gross et al., 2006 and Kunisada et al., 2003). Thus in this model of IHF, it is fitting that melatonin protects in the STAT3 KO mice, because there is no excessive accumulation STAT3, therefore confirming the initial findings that demonstrated the protective effect of melatonin in the presence or absence of AG490.

5.4. Melatonin protects in TNF KO mice

TNF α is one of the cytokines activated during ischemia-reperfusion injury (Lecour et al., 2005, Lacerda et al., 2009, Deucher et al., 2007) and contributes to cardiac dysfunction and apoptosis (Dorge et al., 2002). However, the release of TNF α is both adaptive and maladaptive: it evokes a healing response as well as attacking the invaders (Lecour & James 2011). Nonetheless, the different roles of TNF α are both concentration- and receptor- dependent. It has been shown that high concentrations of TNF α are detrimental, whereas lower concentrations of TNF α are beneficial (Lecour et al., 2005). Furthermore, TNF receptor 1 is believed to activate a death signal because it stimulates the release of caspase 3 and 8 which in turn promote the release of cytochrome C, which are proapoptotic factors. On the contrary, TNF receptor 2 activates antiapoptotic factors including the JAK/STAT pathway and confers cardioprotection.

A remarkable feature about IHF is that the syndrome is accompanied with both a systemic and local inflammatory response and contributes to the progressive deterioration of the heart (Chen et al., 2008). In the Vesnarinone (VEST) trial a strong correlation between high levels of TNF and functional stages of HF were identified, with poorer prognosis for those with high TNF levels (Deswal, 2001). This may be due to a negative inotropic action and disturbance of the β adrenoreceptor sensitivity caused by excessive TNF release (Deswal et al., 2001). In patients with myocardial infarction, TNF levels are also increased (Cesari et al., 2003). Interestingly, in the current study, melatonin protected in the wild-type mice when compared to those that drank plain drinking water. Surprisingly, melatonin induced cardioprotection persisted in TNF KO mice with improved EF and FS. A possible reason for this is that melatonin also exerts an effect on the adrenoreceptors especially via its receptor 3 as demonstrated in the

chronic studies performed in the previous chapter. Thus in the absence of TNF α melatonin restores the optimal function of the adrenoreceptors and confers cardioprotection (Deswal et al., 2001). However, in an isolated heart model an acute dose of melatonin was administered prior to I/R and the protective effect of melatonin was lost in TNF-deficient mice but this was not a long term model. Thus, in IHF there is a chronic release of cytokines with increased hemodynamic overload (Kleinbongard et al., 2010, Dorge et al., 2002). The invasion of cytokines into the myocardium are prevented in TNF KO mice therefore, the cytokine levels are not excessive as normally displayed. In light of this it is understandable that melatonin successfully protects in TNF KO mice. Therefore, the effects of IHF, hypertrophy, inflammation, apoptosis and overall cell death are reduced without the binding of TNF α to its receptor 1 (Chrysohou et al., 2009).

5.5. Melatonin protects independently of the SAFE pathway

The two integral parts of the survivor activating factor enhancement (SAFE) pathway are TNF α and STAT3. This pathway has been shown to be cardioprotective in I/R injury (Lacerda et al., 2009, Lecour S. 2009). However, in a model of IHF both STAT3 and TNF are upregulated; the chronic stimulation of these compounds can lead to hypertrophy (Kunidisada et al., 2001). In experimental IHF, TNFR1 and TNFR2 have opposing effects on heart remodelling and hypertrophy (Schulz et al., 2009). Therefore, drugs used for the treatment of HF should be specific to enhance the activation TNFR2. Furthermore there is evidence that the chronic stimulation of gp130-mediated activation of JAK/STAT3 promotes cardiac inflammation, adverse remodelling and HF in mice with MI (Kleiner et al., 2010). Our data show that, melatonin protects independently of the SAFE pathway in a model of IHF as chronic activation of the SAFE pathway is likely to worsen HF. Somehow melatonin has been able to adapt to this physiological condition and “switch off” the SAFE pathway to confer cardioprotection. The mechanism involved in this regulation still remain to be delineated but may involve the Toll like receptor activation known to regulate TNF α . The mechanism by which melatonin protects against IHF still remain to be delineated. Melatonin is known to activate Akt (Genade et al.,

2008) but, again, chronic stimulation of Akt can lead hypertension and therefore worsen IHF (Buss et al., 2012). Further studies will be required to determine the exact mechanism in melatonin induced cardioprotection against IHF and it is likely that melatonin protects via its direct anti-oxidant effect (Mahal et al., 1999).

In conclusion, our data demonstrate that melatonin is a powerful, cheap and safe drug to consider in patients with IHF.

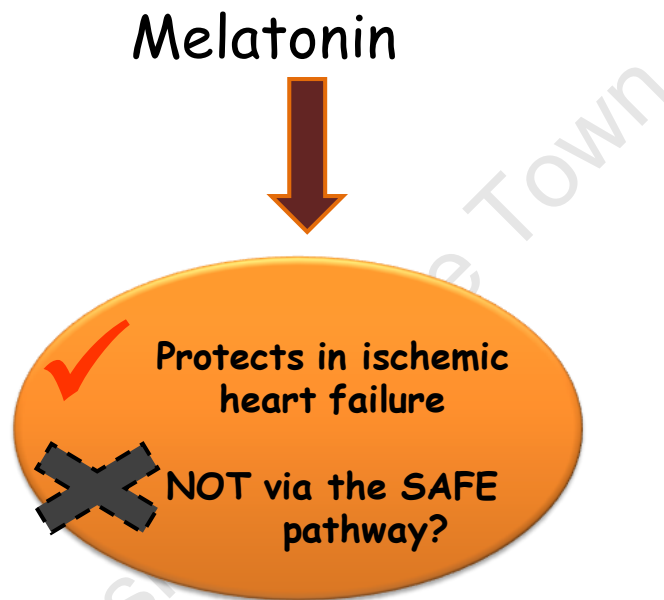


Figure 60: Melatonin confers cardioprotection in a model of ischemic HF but not via the SAFE pathway.

E. Final Discussion Closing Remarks

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Several epidemiological studies have shown that the regular moderate consumption of red wine reduces the effects of cardiovascular disease, as demonstrated in the French population (St Leger et al., 1979, de Lorgeril et al., 2002). Our study has demonstrated that the chronic and moderate consumption of red wine, equivalent to 2-3 glasses of red wine/day, can protect against ischemia-reperfusion injury both in an isolated heart model and *in vivo*. Many postulated that the presence of resveratrol predominantly present in red wine, may explain the cardioprotective effect of red wine. However, high concentrations of resveratrol have been given experimentally and not the actual concentration as that found in red wine (Bauer et al., 2002 and Pellegatta et al., 2003).

In Chapter 1, the acute administration of resveratrol (10 μ M) protected against IR injury in the isolated heart model. In contrast, the chronic and regular administration of resveratrol, given at the concentration to that found in red wine did not protect against IR injury. Hence, our data therefore suggest that other components may contribute to the beneficial effect of red wine. In 2008, Guerrero and collaborators found that melatonin was present in red wine (Guerrero et al., 2008). One hour after the consumption of 100m ℓ of red wine the melatonin levels in the blood plasma were increased (Guerrero et al., 2008). Our findings demonstrate that melatonin, given at a similar concentration to 2-3 glasses of red wine/day, protected against I/R injury to a similar extent as red wine given on its own. To investigate the role of melatonin in red wine induced cardioprotection, luzindole (melatonin receptor 1 & 2 inhibitor) and prazosin (melatonin receptor 3 inhibitor) were used. The literature has shown that melatonin given at higher concentrations (40 μ g/ ℓ) exert its protective effects via the activation of melatonin receptor 1 and 2 (Genade et al., 2008). However, the inhibition with luzindole in our study reduced the infarct sparing ability of melatonin but failed to abolish the protective effect of red wine. Prazosin abolished the protective effect of melatonin and partially attenuated the cardioprotective effect of red wine. Thus melatonin present in red wine is one of the key cardioprotective components in the red wine that confer its effect via the activation of MT3, this was a major milestone in our study. However, melatonin is not the only compound present

in red wine that confers a cardioprotective effect. Red wine contains 1000 different compounds, including alcohol, resveratrol and melatonin which may have different effects when supplemented together. It is therefore possible that a synergistic action, between several compounds contribute to the beneficial effects of red wine. Previous studies show that melatonin at high concentrations protects via the activation of the Akt/Erk pathway (Genade et al., 2008). Furthermore, melatonin inhibits the opening of the mitochondrial permeability pore (Petrosillo et al., 2009) a downstream target of the SAFE pathway. Our study demonstrated for the first time that red wine induced cardioprotection was modulated by melatonin and that this protective effect was mediated via the activation of the powerful prosurvival SAFE pathway. The SAFE pathway involves the activation of the innate immune system (TNF α) and STAT3. We have shown that melatonin, at the concentration found in red wine which is similar to physiological concentrations, protects against I/R injury. However, whether this protective effect could be extended to a model of IHF, with long term melatonin administration, required investigation.

In chapter 3, we explored the cardioprotective effect of melatonin in IHF. Interestingly, in our study the long term use of melatonin in a model of IHF improved ejection fraction and fractional shortening when compared to the mice that received plain drinking water. However, the protective effect of melatonin persisted in both TNF α and STAT3 KO mice, contrary to previous findings in chapter 1. Thus the long term administration of melatonin in IHF protects independently of the SAFE pathway. A possible reason for this is, excess release of TNF α is mediated via TNF receptor 1 in HF (Deswal et al., 2001). Also it has been suggested that excessive STAT3 release promotes a death signal in the heart (Kunisade et al., 2000). In summary, we demonstrate the significance of melatonin as a vital component present in red wine that contributes to its beneficial effect. Furthermore, the protective effect of both red wine and melatonin are mediated via the activation of the SAFE pathway, in a model of myocardial infarction. In a long term model, the bioavailability of the compounds in the wine and rate of absorption, in the gut may have an effect on the mechanism of

protection. Thus, this may give a possible explanation why the long-term administration of melatonin protects independently of the SAFE pathway in IHF.

The main novel findings:

- 1) Regular and moderate consumption of red wine, equivalent to 2-3 glasses of red wine/day confers cardioprotection against ischemia-reperfusion injury.
- 2) Melatonin, at the low concentration as found in red wine, contributes to red wine-induced cardioprotection against I/R injury.
- 3) Red wine and melatonin-induced cardioprotection against I/R injury are mediated via the activation SAFE pathway (TNF α and STAT3 activation).
- 4) Melatonin, at the concentration found in red wine, protects in a model of IHF.
- 5) Melatonin induced cardioprotection against IHF is independent of the SAFE pathway activation

Future directions

Melatonin is a cheap compound that is easily accessible, over the counter and may be used as an adjunctive therapy for patients with ischemic HF. It would be of interest to measure melatonin in the blood samples of HF patients to confirm reduced melatonin secretion. To prove the cardioprotective effect of melatonin in red wine induced cardioprotection we have used non specific melatonin inhibitors. To further explore our hypothesis melatonin knockout mice would be ideal. However, MT3 knockout mice are not available because this receptor has not been fully characterized. Therefore, in collaboration with University of Stellenbosch we aim to use synthetic wine and compare the cardioprotective effects with or without melatonin.

Since both red wine and melatonin protect via the activation of the SAFE pathway it is imperative to explore what lies upstream and downstream of the SAFE pathway.

Preliminary studies performed have shown that the infarct sparing ability of melatonin is abolished in the presence of a toll like receptor 4 inhibitor which may act as a potential upstream activator of the SAFE pathway is toll like receptor 4. Furthermore, we have also started to explore the idea of the mitochondria as the end target for melatonin induced cardioprotection, these data are incomplete and are not shown.

In conclusion, melatonin is a pleiotropic compound that can exert a variety of protective effects via different mechanisms. Thus, the use of such a compound in developing countries where both IHD and HF are on the incline may be of benefit to alleviate the burden of CVD. Furthermore, melatonin is cheap, safe and easily accessible to various socioeconomic groups.

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F. Publications and conference outputs

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Publication list

- Lamont KT, Blackhurst D, Marais D, and Lecour S. Lowering the degree of alcohol in red wine does not alter its cardioprotective effect. SAMJ. 2012, 102: 6.
- Lamont KT, Sarin S, Lacerda L, Opie LH, Lecour S. Is red wine a SAFE sip away from cardioprotection? Mechanisms involved in resveratrol and melatonin-induced cardioprotection. J of Pineal Res. 2011, 50:374-380.
- Lecour S and Lamont KT. Natural polyphenols and cardioprotection. Mini reviews in Medicinal Biochemistry. 2011, 14: 1191-9.
- Opie LH, Lamont KT and Lecour S. Wine and heart health: learning from the French Paradox. South African Heart Journal 2011, 8: 172-77.
- Kelly RF, Lamont KT, Somers S, Hacking D, Thomas P, Opie LH, Lecour S. Ethanolamine is a novel STAT-3 dependent cardioprotective agent. Basic Res Cardiol. 2010 Nov; 105(6): 763-70.

Conference outputs

- Lamont KT, Opie LH, Lecour S: Regular moderate consumption of red wine confers cardioprotection via melatonin and STAT3 activation, European heart Society, Paris 2011 (Poster).
- Lamont KT, Opie LH, Lecour S: Sleep your way to cardioprotection: Melatonin is responsible for red wine induced protection, International Society of Heart Research, Japan. 2010 (Poster). JMCC 48:S1-S185.
- Lamont KT, Opie LH, Lecour S. Melatonin activates the transcription factor STAT3: its role in red wine induced cardioprotection. Physiological Society of Southern Africa, Stellenbosch , 2009 (oral presentation) .
- Lamont KT, Opie LH, Lecour S. Melatonin found in red wine: just a sleep away from protection. South African Heart ,2009 (oral presentation)6;250-300.
- Lamont KT, Kelly RF, Opie LH, Lecour S. Revisiting the cardioprotective components in red wine, MRC Research day, 2008 (Oral Presentation).

- Lamont KT, Opie LH, Lecour S. Rooting out the active cardioprotective component in red wine. South African Heart Journal, 2008 (oral presentation).

Accolades awarded

- Award for best presentation of the Honours project presentation, 2007.
- Award for 2nd best oral presentation at Medical Research Day, 2008.
- Award for best oral presentation at the Physiological Society of Southern Africa, 2009.
- Award for best basic science presentation at South African Heart Association, 2009.
- Award for best basic science at South African Heart Association , 2010.
- Award for best Moderated Poster ESC, Paris 2011.
- Award for best Science presentation, Bernard Pimstone prize , UCT 2011.

G. References

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