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The role of Toll-like receptor 4 (TLR-4) in wine-induced cardioprotection

By

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3. This thesis is my own work.

4. I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own work.

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
κ	Kappa
ω	Omega
μ	Micro/Mu
^oC	Degree Celsius
AIDS	Acquired Immune Deficiency Syndrome
Akt	Protein kinase B
BSA	Bovine serum albumin
b.wt	Body weight
CAD	Coronary artery disease
$CaCl_{2.}H_2O$	Calcium Chloride
CD-14	Cluster of differentiation 14
CF	Coronary flow
CHD	Coronary heart disease
CuZnSOD	Copper –zinc superoxide dismutase
CVD	Cardiovascular disease
DC	Dendritic cells
DTT	Dithiothereitol
ECL	Enhanced chemiluminescence
EDTA	Ethylene glycol tetra-acetic acid
Erk 1/2	P42/P44 extracellular signal-regulated kinases
Foxo 3 a	Forkhead box O3
GPx	Glutathione peroxidase
GSD	Grape seed extract
GSE	Grape skin extract
HDL	High density lipoprotein

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HEPES	4-(2-hydroxythyl)-1-piperazine-ethane sulfonin acid
HIOMT	Hydroxyindol-O-methyl transferase
HIV	Human immunodeficiency virus
HR	Heart rate
HRP	Horse-radish peroxidase
HS	Haemorrhagic shock
Hsp	Heat shock protein
HMGB1	High mobility group box 1
IgG	Immunoglobulin G
IHD	Ischemic heart disease
IL	Interleukin
IRAK	Interleukin-1-receptor-associated kinase
IU	International Unit
JAK	Janus Kinase
KC	Keratinocytes
KCl	Potassium Chloride
KHB	Krebs Henseleit buffer
KH_2PO_4	Potassium dihydrogen phosphate
KO	Knock-out
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
L-NAME	N-ω-nitro-l-arginine-methyl ester
LVEDP	Left ventricular end diastolic pressure
LVESP	Left ventricular end systolic pressure
LVDP	Left ventricular developed pressure
MAPK	Mitogen activating protein kinase
MetS	Metabolic syndrome

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Mg	milligram
$MgSO_4.7H_2O$	Magnesium Sulfate Heptahydrate
min	Minutes
mmHg	Millimetres of mercury
MIP-2	Macrophage inflammatory protein-2
MnSOD	Manganese superoxide
mRNA	Messenger ribonucleic acid
MT 1/2/3	Melatonin receptor (s)
MyD88	Myeloid differentiation primary response gene 88
NaCl	Sodium Chloride
$NaHCO_3$	Sodium Bicarbonate
NAT	N-acetyl transferase
NEDH	insulinoma-bearing New England Deaconess Hospital
NF-κB	Nuclear factor kappa B
ng	nanogram
NHLBI	National Heart Lung and Blood Institute
NO (S)	Nitric oxide (synthase)
6-OHDA	6-hydroxydopamine
oxLDL	Oxidised low density lipoprotein
PAGE	Polyacrylamide gel electrophoresis
PD	Parkinson's Disease
Pg	Pico gram
PI3	Phosphoinositide-3
PMSF	Phenylmethylsulfonyl fluoride
pSTAT-3	Phospho-STAT3
RGJ	Red grape juice
ROI	Reactive oxygen intermediate species

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RPP	Rate pressure product
SAFE	Survivor Activating Factor Enhancement
SCN	Suprachiasmatic nucleus
SDS	Sodium dodecyl sulphate
SHR	Spontaneous hypertensive rats
SOD	Superoxide dismutase
STAT-3	Signal transducer and activator of transcription 3
TBK1	TRAF-family member-associated NF-κB activator-binding kinase 1
TRAF	Tumor necrosis factor receptor-associated factor
TRIF	TIR-domain-containing adaptor protein inducing interferon-β-mediated transcription factor
TLRs	Toll-like receptors
TLR-4	Toll-like receptor 4
TNF-α	Tumor necrosis factor- alpha
WHO	World Health Organisation
Y-pSTAT-3	Tyrosine phospho STAT 3

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Abstract

Introduction: Moderate and chronic consumption of red wine confers cardioprotection. Melatonin, present in wine, may contribute to this cardioprotective effect. Melatonin confers cardioprotection via the activation of tumor necrosis factor-alpha (TNF-α) and the signal transducer and activator of transcription-3(STAT-3), via mechanisms that still remain to be delineated.

Aims: We therefore hypothesise that South African red and white wines confer a cardioprotective effect in relation to their melatonin content. Furthermore, we propose that the cardioprotective effect of melatonin (at a concentration found in red wine) is dependent on the activation of Toll-like receptor 4 (TLR-4) to activate TNF-α/STAT-3.

Methodology: The melatonin content of each wine was measured by high performance liquid chromatography (HPLC) - solid phase extraction (SPE) analysis. The drinking water of male Long Evans rats was supplemented with 7 South African red and white wines from different grape types and different vineyards (one part wine with 7 parts water). After 2 weeks of treatment, hearts were isolated and subjected to ischemia (=heart attack) followed by reperfusion. To explore the role of TLR-4, isolated male Wistar rat hearts were perfused acutely with melatonin (75ng/L) in the presence or absence of a TLR-4 inhibitor (TAK242) (500nM) and subjected to ischemia, followed by reperfusion. Functional parameters were recorded throughout the experiments. Infarct size was analysed on hearts stained with triphenytetrazolium chloride (TTC). Additional pre-ischemic hearts were collected to measure phospho-STAT3 activation by western blot analysis.

Results: Chronic consumption with both South African red and white wines conferred a cardioprotective effect against ischemia/reperfusion. There was no correlation between the cardioprotective effects of each South African wine and their melatonin content. The cardioprotective effect of melatonin was reduced in the presence of the TLR-4 inhibitor. Furthermore, pre-treatment with melatonin with/without TAK242 altered the phosphorylation of STAT-3 in the cytosol.

Conclusion: Our data do not suggest a critical role for melatonin in the cardioprotective effect of South African wines. Furthermore, cardioprotection with melatonin may involve TLR-4 to activate STAT-3.

Word count: 321

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CHAPTER 1: INTRODUCTION

1. Cardiovascular disease

1.1 Definition and incidence of cardiovascular disease worldwide

Cardiovascular disease (CVD) is defined as a group of disorders affecting the heart and blood vessels, specifically coronary heart disease (CHD), cerebrovascular disease (stroke), peripheral artery disease and heart failure. There are several behavioural risk factors linked to the onset of CVD. These risk factors include excessive dietary caloric intake, diet high in salt, diet high in saturated and *trans* fatty acids, excessive alcohol intake, physical inactivity and tobacco smoking (Derman *et al.*, 2008).

The World Health Organisation (WHO) suggests an increase in CVD mortality world-wide. In 2004, CVD accounted for 17.1 million deaths worldwide and in 2030 it is projected that 23.4 million people will die from CVD per year in both developing and developed countries (WHO, 2009).

In Europe, CHD and stroke are responsible for 1.92 and 1.24 million deaths per year, respectively (Rayner *et al.,* 2009*).* More than 80% of CVD deaths occur in the low-middle income populations around the world (WHO, 2009). Therefore, CHD long thought to be a rich man's disease, is obviously a problem for poorer populations as well. The CVD burden in Africa is highlighted by the fact that nearly 20 million people are affected, with prevalence among adults aged 25-64 years living in urban areas (WHO, 2005). Furthermore, it is predicted that ischemic heart disease (IHD) (or myocardial ischemia-defined in section 1.2) will be the leading cause of death in Africa by the year 2030 (Mensah, 2008) **(Figure 1)**.

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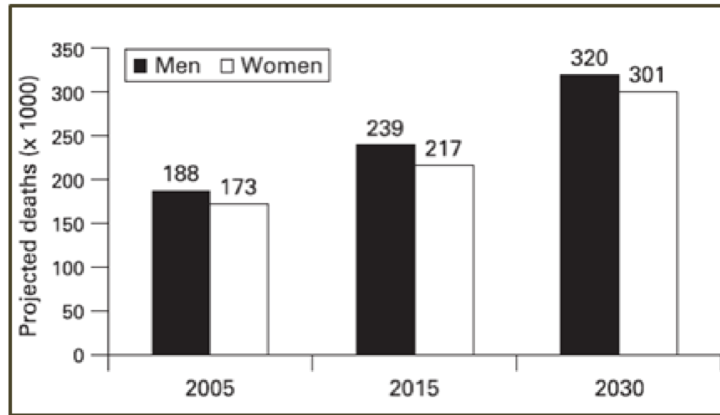

Figure 1: Projections of death by IHD in men and women (according to WHO) in the African region for the years 2005, 2015 and, 2030. (From Mensah, 2008)

In the Western Cape, a region in South Africa, CHD is already the leading cause of death and is superceding the mortality rate of human immune-deficiency virus (HIV)/ Acquired immune deficiency syndrome (AIDS) and other communicable diseases such as tuberculosis (Bradshaw *et al.*, 2008) **(Figure 2)**.

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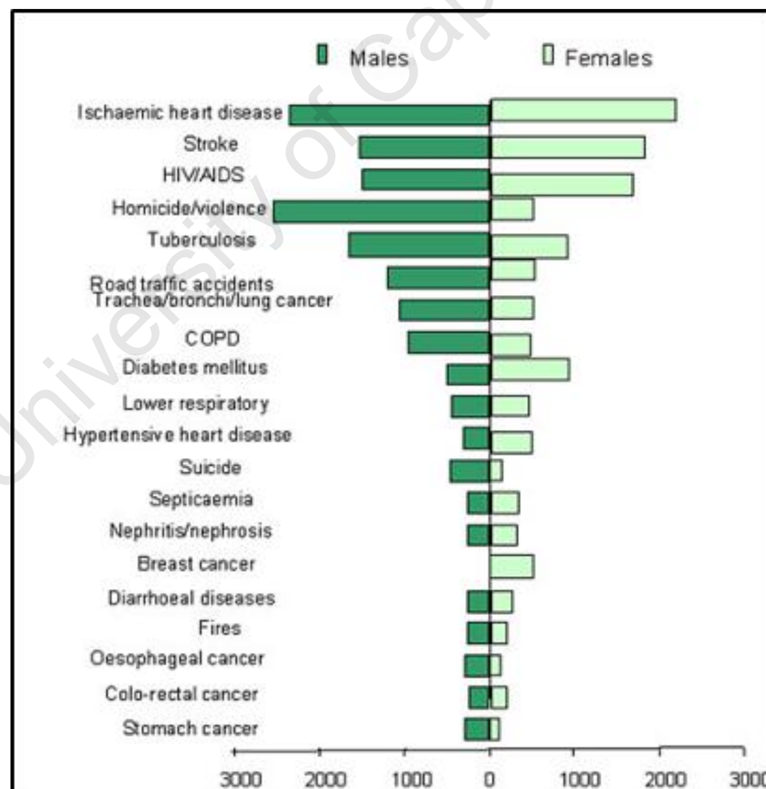

Figure 2: Major causes of death in males and females in the Western Cape region, South Africa, for the year 2005. (From Bradshaw *et al.*, 2008)

South African national prevalence data indicated a shift in dietary intake due to urbanisation among the black population, which represents 3/4 of the total population (Bourne *et al.*, 2002). The notable changes to their diet are increased consumption of fat from 16.4% to 24.2% of total energy and a decreased consumption of carbohydrates from 69.3% to 61.7% of total energy (Bourne *et al.*, 2002). Therefore, the incidence of CVD in developing nations has many other influential factors such as socio-economic status.

1.2 Ischemic heart disease

IHD occurs as a result of a fat build-up (known as atherosclerotic plaque) on the walls of the coronary artery causing an obstruction of flow and narrowing of the coronary artery. The narrowing of the coronary artery results in a reduction of nutrient and oxygen-rich blood flow to cardiac tissue, which is essential for its survival. In the presence of such an environmental change, the cardiac tissue becomes 'ischemic' and eventually dies, forming an infarct **(Figure 3)**.

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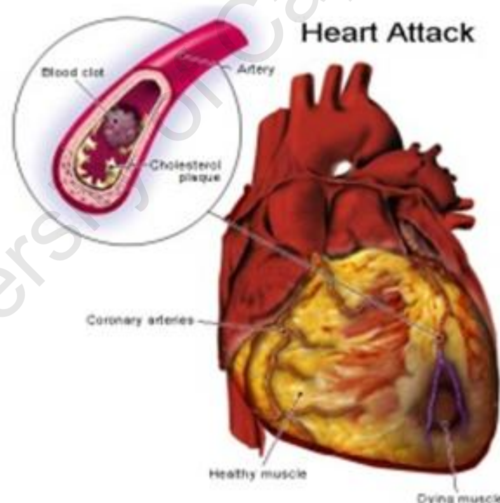

Figure 3: A graphical representation of myocardial infarction resulting from atherosclerotic plaque.

(From http://www.missioncare.com.tw/dis1_e.htm Date accessed: 4.9.11)

In order to rescue the ischemic cardiac tissue from further damage, blood flow has to be restored (=reperfusion) to this area. Restoring the blood flow reduces the amount of dead cardiomyocytes within the cardiac tissue. Reperfusion can be achieved clinically with thrombolytic drugs and/or coronary angioplasty.

Apart from its beneficial outcome, reperfusion has a paradoxical effect by causing further damage to ischemic cardiac tissue. This damage is referred to as "reperfusion injury". During reperfusion, the release of additional cytotoxic compounds, such as free radicals occurs (Kilgore *et al.*, 1993). This is a possible explanation for the occurrence of reperfusion injury resulting in myocardial stunning, no reflow phenomenon, reperfusion arrhythmias, and eventually cardiac cell death (Yellon & Hausenloy, 2007).

Additional preventative and curative therapies limit damage associated with CHD, specifically the damage caused by reperfusion injury. Many epidemiological reports have demonstrated increased serum cholesterol as a substantial predicting risk factor of CHD in humans. The classic heart-diet hypothesis proposed that a diet rich in saturated fat and cholesterol is the primary cause of atherosclerosis and CHD in humans (Gordon, 1988).

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1.3 Diet, a major risk factor for cardiovascular disease

1.3.1 Western diet

The western diet contains high amounts of saturated fats, carbohydrates and low amounts of fresh fruits and vegetables, whole grains, seafood and poultry. Regular consumption of the western diet leads to obesity (Van Dam *et al.*, 2008), an important cardiovascular risk factor for the onset of type-2 diabetes, hypertension, stroke and CHD (see review Yngve, 2009), via multiple mechanisms (see review Hu & Willett, 2002) **(Figure 4)**.

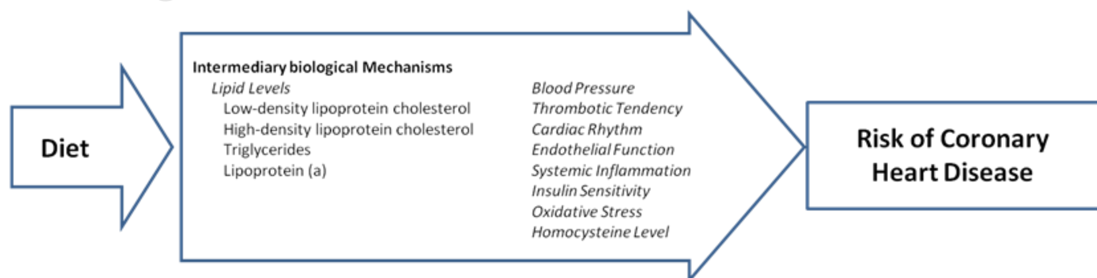

Figure 4: Mechanisms by which diet potentially influences the risk of coronary heart disease.

(Adapted from Hu & Willett, 2002)

A study by Sampey and colleagues has demonstrated the effect of the western diet on weight gain and macrophage infiltration in male Wistar rats, over a period of 10 weeks (Sampey *et al.*, 2011). Animals exposed to the western diet developed hyperinsulinemia (constant elevated levels of insulin in the blood); hyperglycemia (elevated levels of glucose in the blood) and glucose intolerance (inability to properly metabolize glucose) compared to Wistar rats consuming the standard chow (Sampey *et al.*, 2011).

Consumption of the western diet can lead to damage to the vascular system, an important system involved in the transport of nutrients, oxygen and waste removal (Sampey *et al.*, 2011) **(Figure 4)**.

The western diet may inadvertently cause an over-active immune response. Hence, in male C57Bl/6 mice fed a high-fat diet for 3 weeks, an increase in mRNA expression of intercellular adhesion molecular-1 (ICAM-1), a transmembrane adhesion molecule involved in the migration of leukocytes to sites of inflammation in atherosclerotic lesions was observed compared to mice on standard chow diet (Brake *et al.*, 2006). The liver, brown fat and white fat of Wistar rats consuming a western diet had high levels of macrophage present, compared to rats consuming standardised chow (Sampey *et al.*, 2011).

Fortunately, the detrimental effects caused by consumption of the western diet are reversible (Ornish *et al.*, 1990). Conversely, several epidemiological studies have suggested that the Mediterranean diet (high in mono-unsaturated and polyunsaturated fats) is beneficial to cardiovascular health (see review Yngve, 2009; Michel de Lorgeril *et al.*, 1999).

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1.3.2 Mediterranean diet

American physiologist, Dr Ancel Keys in 1958 began a momentous epidemiological study in CHD research, namely the Seven Countries study. This study lasted for 50 years and exhibited a link between CHD mortality and lifestyle factors, focusing on the intake of saturated fatty acids (Keys, 1980). The seven countries involved were Finland, Greece, the United States of America, Italy, Yugoslavia, Netherlands and Japan. The results obtained suggested countries with a high saturated fat intake

had a significantly higher number of CHD deaths in relation to population size (Keys, 1980).

Of these countries, the island of Crete (Greece) had the lowest rate of CHD (Keys, 1980). As such, the Seven Countries Study classified the Cretan diet as the optimal diet and it was named the Mediterranean diet **(Figure 5)** (see review Yngve, 2009).

Figure 5: Typical Mediterranean diet (From http://thelcbridge.com/?p=1277 Date accessed: 30.6.12)

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The Mediterranean diet is primarily vegetarian and includes a diverse range of vegetables, fruits, beans, nuts, seeds and unrefined types of cereals. Foods are predominately high in antioxidants, and olive oil is the primary source of fat (see review Yngve, 2009). Fish is consumed as well as a low intake of meat and dairy foods. Furthermore, the Mediterranean diet has high amounts of mono-unsaturated and n-3 polyunsaturated fatty acids and a low amount of saturated fatty acids (see review Yngve, 2009).

To determine the cardiovascular benefit of the Mediterranean diet, the Lyon Heart Diet study took place (Michel de Lorgeril *et al.*, 1999). Patients surviving their first myocardial infarction placed on a Mediterranean diet showed protection against cardiovascular morbidity and mortality for up to 4 years (Michel de Lorgeril *et al.*, 1999). However, the foremost traditional risk factors such as increased blood cholesterol and increased blood pressure remained unchanged (Michel de Lorgeril *et al.*, 1999). The study concluded that a heart protective diet such as the Mediterranean diet should be a primary, preventative measure to reduce the onset of CVD.

A parallel phenomenon to the Mediterranean diet is the French paradox. In 1991, an American investigative show called *60 minutes* broadcasted a French observation, in which the French have a low incidence of coronary disease, even though they consume more than three times the amount of saturated fat than Americans (see review Constant, 1997). Subsequent epidemiological studies have since then confirmed this observation, named the French paradox (Leger *et al*., 1979).

2. Red wine and its role in cardioprotection

2.1 Clinical evidence for red wine-induced cardioprotection

In 1979, Leger and colleagues were the first to establish an inverse relationship between low-moderate consumption of wine and CAD mortality (Leger *et al*., 1979).

The study conducted in 18 countries portrayed a strong negative correlation between alcohol intake and IHD mortality and further concluded that the effect observed was largely due to the consumption of wine (Leger *et al*., 1979). Patients who drank red wine had a lower incidence of coronary disease, compared to patients who drank liquor (spirits) or beer (Klatsky & Armstrong, 1993). In another study conducted in 40 countries (excluding France & Finland), the cardioprotective effect of red wine against CHD positively correlated with the consumption of large amounts of saturated fat (Artaud-Wild *et al*., 1993). Hence, additional components to that of alcohol contributed to the cardioprotective effect of red wine (Siemann & Creasy, 1992).

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2.2 Evidence for red wine-induced cardioprotection

In 2000, Sato and colleagues suggested that red wine has a cardioprotective action against ischemia/reperfusion injury (Sato *et al*., 2000). Male Sprague-Dawley rats treated acutely with red wine extract (1µg/ml) for 15 min and subjected to an ischemia/reperfusion insult displayed a significant reduction in infarct size, compared to rats which only received water (Sato *et al*., 2000). Similarly, spontaneous hypertensive rats treated acutely with non-alcoholic red wine extract (50µg/ml) for 10 min before and after ischemia demonstrated a cessation in contractile dysfunction of the heart, compared to controls (Fantinelli & Mosca, 2007). This suggests that red wine could potentially contain non-alcoholic components, which can be cardioprotective.

Most studies that have explored the cardioprotective effect of red wine have only focussed on red wine extracts and not the wine itself. Recently, Lamont *et al.*, demonstrated that a chronic pre-treatment of red wine (for 10 days) at a concentration equal to 2-3 glasses/day deemed beneficial in male Long Evans rats subjected to ischemia/reperfusion injury (Lamont *et al.*, 2012). Several studies both clinical and experimental have shown a difference between red wine and white wine in cardioprotection.

2.3 Red wine vs. White wine

In 2003, Wollny and colleagues observed that male Wistar rats treated with red wine for 10 days were prevented from suffering experimental thrombosis (Wollny *et al.*, 2003). However, this observation was absent in male Wistar rats pretreated with white wine (Wollny *et al.*, 2003). Interestingly, both red wine and white wine improved fibrinolytic factors in healthy volunteers (Mansvelt *et al.*, 2002). These conflicting reports indicate that further investigation is required to clearly establish whether white wine plays a role in platelet activity.

CAD patients consuming either red wine (4 ml/kg) or white wine (4 ml/kg) with a light meal had an improved flow mediated dilation of the brachial artery after 360 minutes (Whelan *et al.*, 2004). Male hypercholesterolemic rabbits pretreated with red wine (4ml/kg/day) had an increase in the expression of endothelin-1 (Zhou *et al.*, 2003). Therefore, wine has an influence on endothelium activity even under pathological conditions such as the presence of excessive plasma cholesterol. However, the consumption of red, but not white wine for 28 days caused an increase in total plasma antioxidant levels in healthy volunteers (Velden *et al.*, 2002).

Sprague-Dawley male rats treated chronically with white wine for 30 days followed by an ischemia/reperfusion insult presented a significant reduction in infarct size after 24 hours compared to controls (Thirunavukkarasu *et al.*, 2008). Furthermore, fractional shortening and ejection fraction of white wine-treated hearts significantly improved, compared to the controls (Thirunavukkarasu *et al.*, 2008). This cardioprotective effect was mediated via the activation of the Akt ((Akt)/Forkhead box O3 (Foxo3a)/ nuclear factor kappa B (NFkB) survival pathway (Thirunavukkarasu *et al.*, 2008).

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Differences in the protection observed between red wine and white wine could be partly attributed to the process of wine making. The production of red wine originates from the pulp of red/black grapes (which includes the skin and seeds) (Dharmadhikari, 2000). The must (i.e. the juice and pulp of freshly pressed grapes) is fermented via the same process involved in the making of beer and liquor (Dharmadhikari, 2000). Fermentation is complete via conversion of sugars to ethyl alcohol and carbon dioxide (Dharmadhikari, 2000). Conversely, the production of white wine is mostly from red grapes but, can be produced from any grape type (Dharmadhikari, 1999). The juice obtained from crushed grapes is fermented and practically no contact with skin or presence of seeds occurs (Dharmadhikari, 1999). In addition, grape type, soil, climate and viticultural practices all influence the composition and quality of the wine (Dharmadhikari, 1999).

Since the French Paradox is solely based on the consumption of red wine, most research studies explaining the cardioprotective effect of wine are done with red wine.

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2.4 Cardioprotective components found in red wine

2.4.1 Alcohol/Ethanol

i. Definition

Ethanol, produced by the fermentation of sugars and starches or chemical synthesis, is the intoxicating ingredient found in alcoholic beverages. It is a 2-carbon alcohol with the molecular formula of CH_3CH_2OH **(Figure 6)**.

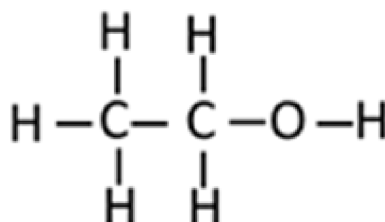

Figure 6: Diagram depicting the structure for Ethanol (EtOH). (From Zulfah Albertyn, 2012)

The effect of alcohol consumption in society has been a challenge for centuries. Many studies have proved that excessive alcohol consumption has substantial health implications as it causes the development of a variety of diseases and

disorders i.e. alcoholism (Holbert & Tueth, 2004), CVD (Jackson *et al.*, 1991), alcoholic liver disease (O'Shea *et al.*, 2010), and chronic pancreatitis (Vonlaufen *et al.*, 2007). Interestingly, of these diseases and disorders, research in the literature (epidemiological and animal models) has proved that a low-moderate consumption of alcohol can reduce the incidence of CVD (see review Rimm *et al.*, 1996), thus suggesting that alcohol can have a paradoxical effect.

ii. Clinical evidence for alcohol-induced cardioprotection

a. Moderate alcohol consumption and CVD incidence

Several epidemiological studies have proposed a correlation between moderate alcohol consumption (spirits, beer, and wine) and the incidence of CVD, with an inverse relationship between alcohol consumption and CVD incidence in both male and females (see review Rimm *et al.*, 1996) **Refer to Table 1**.

Table 1: Several studies displaying a correlation between moderate alcohol consumption and CVD mortality. (From Rimm *et al.*, 1996)

Table 1—*Ecological studies of consumption of specific types of alcoholic drink and correlation with mortality from heart disease*

Study	Correlation			Comment
	Wine	Beer	Spirits	
St Leger *et al* 1979[5] Per capita consumption (by county) *v* ischaemic heart disease mortality, 1970. Men and women aged 55-64 in 18 developed countries	Men −0·70 Women −0·61	Men 0·23 Women 0·31	Men −0·26 Women −0·32	Not adjusted
LaPorte *et al* 1980[6] Per capita consumption (by county) *v* coronary heart disease mortality, 1972. Men aged 55-64 in 20 countries	−0·62	0·15	−0·29	Adjusted for meat consumption
Werth 1980[7] Per capita consumption (by state) *v* coronary heart disease mortality, 1969-78. Men and women in USA	−0·49 to −0·58 (P<0·001) for each year between 1969 and 1978	Not given	Not given	Not adjusted
LaPorte *et al* 1981[8] Per capita consumption (by state) *v* coronary heart disease mortality, 1970. Men and women in USA	White men 0·05 White women 0·17	White men 0·11 White women 0·09	White men 0·19 White women 0·24	Adjusted for age
Schmidt *et al* 1981[9] Per capita consumption (by state) *v* coronary heart disease mortality, 1970. Men and women in USA	−0·28 (P<0·05)*	−0·06 (NS)*	−0·26 (P<0·05)*	Adjusted for cigarette smoking
Nanji 1985[10] Per capita consumption (by country) *v* ischaemic heart disease mortality, 1970. Men in 27 developed countries	−0·75 (P<0·001)	0·60 (P<0·001)	No association	Drink type presented as % of total alcohol
Nanji *et al* 1986[11] Change in per capita alcohol intake (by country) *v* change in ischaemic heart disease mortality, 1970-80. Men in 22 countries	−0·50 (P<0·01)	0·32 (P<0·05)	Not given	Not adjusted
Hegsted 1988[12] Per capita consumption (by country) *v* coronary heart disease mortality, 1971, 1973. Men in 18 countries	P<0·01 (inverse)	NS	NS	Adjusted for saturated and polyunsaturated fat. Predictive equation almost identical when total alcohol used instead of each drink type
Renaud *et al* 1992[13] Per capita consumption (by country) *v* coronary heart disease mortality, 1987. Men and women aged 35-64 in 17 countries	−0·87 (P<0·0001)	NS	NS	Adjusted for dairy fat intake
Artaud-Wild *et al* 1993[14] Per capita consumption (by country) *v* coronary heart disease mortality, 1977. Men aged 55-64 in 40 countries	−0·16 (NS)	Not given	Not given	Total alcohol significantly correlated with mortality for all countries (*r*=0·35) but significantly inversely correlated with mortality in countries with high cholesterol and saturated fat intake
Criqui *et al* 1994[15] Per capita consumption (by country) *v* coronary heart disease mortality, 1965, 1970, 1980, 1988. Men and women aged 35-74 in 21 countries	1965 P=0·07 (inverse) 1970 P<0·01 (inverse) 1980 P<0·01 (inverse) 1988 P=0·12 (inverse)	1965 NS 1970 P=0·09 (inverse) 1980 NS 1988 NS	1965 P=0·12 (inverse) 1970 NS 1980 NS 1988 NS	Each year had a separate model which controlled for % calories for animal fat, vegetables, and fruit
La Porte *et al* 1980[6] **(time trend analysis)** Per capita consumption (by state across years) *v* coronary heart disease mortality, 1950-75. Men and women in USA	−0·41	−0·61	−0·07	After incorporating 5 year lag, change in consumption of beer and cigarettes were strongest predictors of changes in mortality

*Interstate correlations.

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The J- or U-shaped mortality curve portrays the relationship of alcohol consumption and CHD mortality. This mortality curve displays a modest intake of alcohol as being beneficial whereas either no consumption of alcohol (abstainers) or excessive amounts of alcohol consumption is harmful (Jackson *et al.*, 1991). This effect differs in men and women. There was a beneficial effect observed in men who consumed up to three drinks/day (or ~35g/day) and in women who consumed up to two drinks/day (or ~25g/day) **(Figure 7)** (see review Di Castelnuovo *et al.*, 2006).

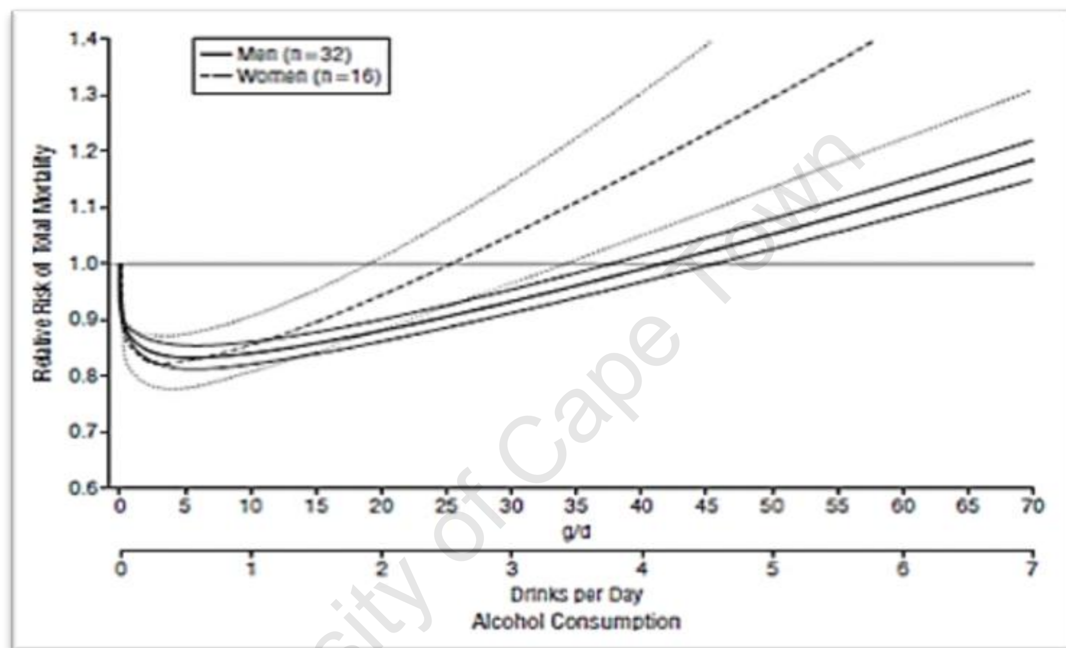

Figure 7: Graphic representation of J-mortality curve for alcohol consumption between men and women (From Di Castelnuovo *et al.*, 2006)

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Interestingly, the J- or U-shaped mortality curve has confounding factors. The benefit of moderate alcohol consumption in females and males was only 18% and 17%, respectively (see review Di Castelnuovo *et al.*, 2006). Although the inverse relationship between alcohol consumption and mortality is valid, the benefit observed from this effect is related to the survival of patients with CVD (see review Di Castelnuovo *et al.*, 2006). Therefore, this effect could possibly benefit patients who had or potentially will have a myocardial infarction or an underlying angina.

Men enrolled in the Physicians Health study that had a previous myocardial infarction and who consumed low-moderate amounts of alcohol had a lower incidence of total mortality than abstainers or those who rarely consumed alcohol did (Muntwyler *et al.*, 1998). Furthermore, patients who consumed alcohol in moderation before the onset of a myocardial infarction had a reduced mortality

compared to heavy drinkers or abstainers (Mukamal *et al.*, 2001). Therefore, the evidence favours a positive role for moderate alcohol consumption in the reduction of CVD incidence.

iii. Evidence for alcohol-induced cardioprotection

Hyperlipidemic mice consuming alcohol at various concentrations displayed an attenuation in atherosclerotic fatty streak formation compared to untreated controls (Emeson *et al.*, 1995). Similarly, hypercholesterolemic swine consuming alcohol only, demonstrated a significant reduction in intramyocardial fibrosis after chronic ischemia compared to control hypercholesterolemic swine (Lassaletta *et al.*, 2012).

Male Hartley guinea pigs underwent a chronic and moderate treatment with 10% ethanol for 3-12 weeks followed by exposure of the hearts to 45 min no flow ischemia (Miyamae *et al.*, 1997). These hearts exhibited improved left ventricular developed pressure (LVDP) compared to control animals receiving only water (Miyamae *et al.*, 1997). Male Sprague-Dawley rats treated with ethanol over a period of 16 weeks and subjected to 45 min no flow ischemia significantly improved their LVDP compared to control rats (Miyamae *et al.*, 1998). Thus, chronic consumption of alcohol was beneficial for the contractility of the heart after ischemia/reperfusion injury. Furthermore, male Sprague-Dawley rats on an isocaloric liquid diet (similar calorie quantities in protein and carbohydrates with ethanol being 36% of the diet) for 8-10 weeks were preconditioned and subsequently subjected to 35 min global ischemia. The functional recovery of these animals improved compared to the control rats, which were only preconditioned (McDonough, 1997). Therefore, chronic alcohol treatment improves the contractility and functionality of the heart after an ischemic insult.

iv. Does the presence of alcohol solely contribute to the cardioprotective effect of red wine?

Rats pre-treated with red wine containing either 12% or 6% alcohol exhibited a similar protective effect against ischemia/reperfusion injury (Lamont *et al.*, 2012). These animals had an improved functional recovery in relation to the untreated controls (Lamont *et al.*, 2012). Conversely, the rats pre-treated with alcohol extracted from the wine (12% or 6%) did not achieve protection against ischemia/reperfusion injury (Lamont *et al.*, 2012). This data, therefore, suggests that

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alcohol does not solely contribute to the cardioprotective effect of red wine against ischemia/reperfusion injury.

Interestingly, red grape juice (RGJ) has similar cardioprotective properties to that of red wine. Patients undergoing hemodialysis who consumed RGJ for 14 days had a significant reduction in plasma monocyte chemoattractant protein 1 concentration and low-density lipoprotein (LDL) concentration (Castilla *et al.*, 2006). In addition, patients displayed higher levels of high-density lipoprotein (HDL) compared to patients not consuming RGJ (Castilla *et al.*, 2006). Therefore, alcohol is not the sole contributor in red wine-induced cardioprotection.

2.4.2 Resveratrol

Polyphenols are naturally abundant in most common foods such as fruits, vegetables, coffee, tea, chocolate and soy (Scalbert & Williamson, 2000). The literature contains abundant evidence displaying the antioxidative effects of polyphenols. Most research extrapolating the physiological effects of polyphenols arose from red wine extract. In red wine, there are several grape polyphenols, including resveratrol (stilbene), flavonoids and its derivatives, flavons, flavonols and anthocyanins (see review Lecour & Lamont, 2011). Of these grape polyphenols, resveratrol is by far, the most studied.

i. Definition

Resveratrol (3, 5, 4'-trihydroxy-*trans*-stilbene) is a stilbenoid (type of natural phenol) and a phytoalexin (a substance produced by plant tissue in response to contact with a parasite). Resveratrol has two forms, *cis*-resveratrol and *trans*-resveratrol **(Figure 8)**.

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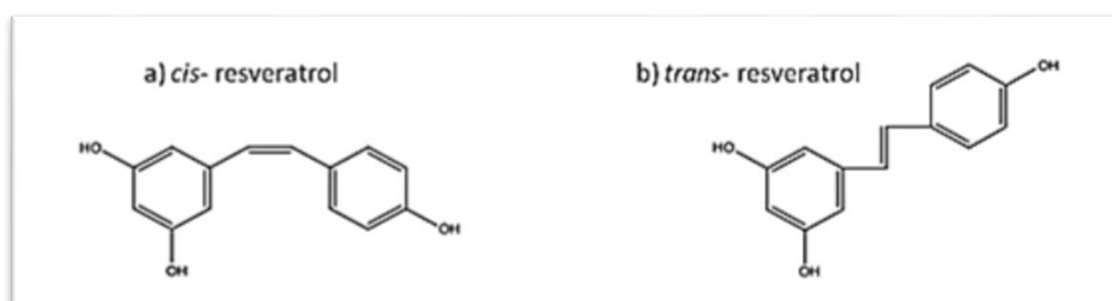

Figure 8: Diagram depicting the derivatives of resveratrol, *cis*-resveratrol and *trans*-resveratrol (From White *et al.*, 2009).

Many plant species produce resveratrol as a defence mechanism against pathogens such as bacteria and fungi. Takaoka first discovered resveratrol in the roots of *Veratrum grandiflorum* (white hellebore) (Takaoka, 1939). In 1976, Langcake and Price discovered *trans*-resveratrol in the leaves of *Vitis vinifera,* as a result of Vitaceae infection or ultra violet light exposure (Langcake *et al*., 1976).

As resveratrol can be found primarily in the skins of grapes, a higher content of resveratrol is found in red than in white wine.

ii. Evidence for resveratrol-induced cardioprotection

In 1993, Frankel and colleagues demonstrated that resveratrol, present in wine, attenuated the peroxidation of plasma LDL (Frankel *et al*., 1993). Therefore, this study was the pioneer in determining how red wine had a superior protective effect, compared to other alcoholic beverages.

In 2007, a study conducted by Penumathsa and colleagues highlighted the effect of resveratrol against ischemia/reperfusion injury (Penumathsa *et al*., 2007). Male hypercholesterolemic Sprague-Dawley rats were fed a 2% cholesterol diet for 8 weeks, followed by a chronic treatment of resveratrol (20mg/kg/day) for 2 weeks before the onset of 30 min global ischemia. Resveratrol treated rat hearts displayed a significant reduction in infarct size, as well as myocardium functionality, compared to untreated hypercholesterolemic rat hearts after ischemia/reperfusion injury (Penumathsa *et al*., 2007). *In vitro* human cardiac specimens treated with resveratrol (10 μM) and placed in a microperfusion chamber, displayed a significant reduction in apoptosis, compared to control cardiac specimens (Usta *et al*., 2011). Therefore, it is suggested that resveratrol potentially protects the heart against the detrimental effects of a heart attack.

iii. Does the presence of resveratrol contribute to the cardioprotective effect of red wine?

Many experimental and clinical data suggest that resveratrol concentration is a key factor in red wine-induced cardioprotection. However, many studies observing the cardioprotective effect of resveratrol tend to use a concentration far greater than the resveratrol concentration found in red wine (2.3mg/L). For instance, patients undergoing primary CVD prevention participating in a randomized, triple-blinded, placebo-controlled trial for 6 months were placed in three groups: the placebo

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group, the grape extract (no resveratrol) group and the grape extract (8 mg/day with resveratrol) group (Tomé-Carneiro *et al.*, 2012). After 12 months, the grape extract group displayed a reduction in high-sensitivity C-reactive protein; compared to the placebo group and the grape extract group (Tomé-Carneiro *et al.*, 2012).

In 2011, Lamont and colleagues observed an acute resveratrol treatment (at a concentration found in red wine) protected isolated Wistar rat hearts against ischemia/reperfusion injury by reducing the infarct size compared to the control rat hearts (Lamont *et al.*, 2011). However, chronic resveratrol treatment (at a concentration found in red wine) abolished the cardioprotective effect of resveratrol against ischemia/reperfusion injury (Lamont *et al.*, 2012). These data, therefore, suggest that additional components contribute to red wine-induced cardioprotection. Resveratrol content differs from one red wine to another (Goldberg *et al.*, 1995) and, therefore, each wine could potentially demonstrate a different degree of cardioprotection.

3. Melatonin and cardioprotection

3.1 Definition

Melatonin (N-acetyl-s-methoxytryptamine) **(Figure 9)**, discovered in 1958 (Lerner *et al.*, 1958), is a natural hormone found in both animals and plants as a biogenic amine.

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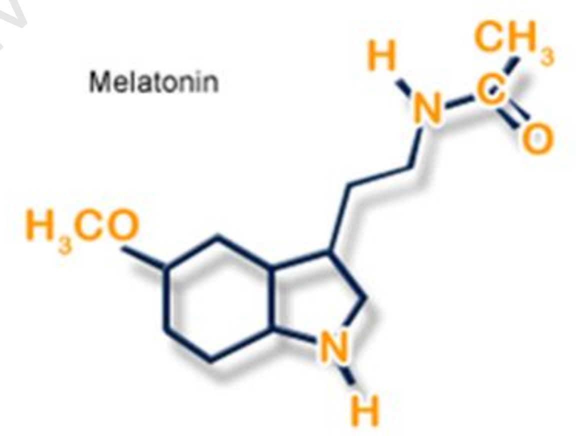

Figure 9: Molecular structure of melatonin. (From: hbcprotocols.com; Date accessed: 9.4.12)

The main physiological function of melatonin is to control the circadian rhythm of the body (Cajochen *et al.*, 2003). The circadian rhythm regulates the sleeping pattern by affecting the temperature of the body (see review Dawson & Encel, 1993). Other physiological functions of melatonin include the secretion of luteinizing hormone during ovulation (Voordouw *et al.*, 1992) and the control in energy balance (Bartness *et al.*, 2002).

3.1.1 Biosynthesis of melatonin

In mammals, melatonin is primarily produced in pinealocytes found in the pineal gland **(Figure 10)**.

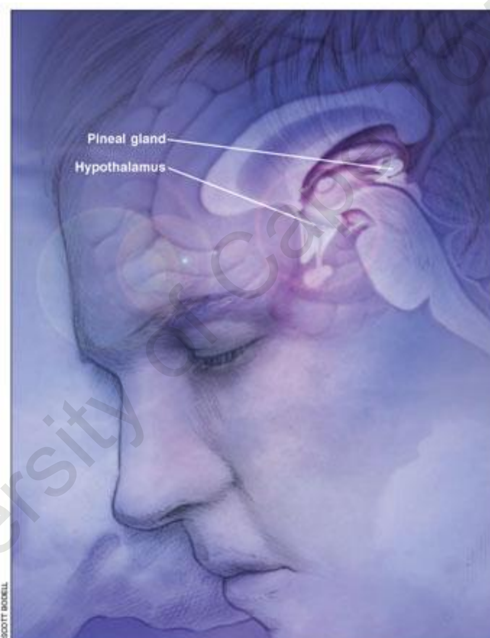

Figure 10: Location of pinealocytes in the brain.
(From http://faroucheombre.wordpress.com/category/5ht1/ Date accessed: 3.7.12)

It can also be produced in other organs such as the retina (Tosini & Menakar, 1998), the gastrointestinal tract (Bubenik *et al.*, 1977), the bone marrow cells (Conti *et al.*, 2000), and blood platelets (Champier *et al.*, 1997). Melatonin synthesis starts off by an uptake of L-tryptophan from circulation. Next, is the conversion of L-tryptophan to serotonin via hydroxylation and carboxylation. N-acetyl transferase (NAT), a rate-limiting enzyme, converts serotonin to N-acetyl-serotonin. Lastly, hydroxyindol-O-methyl transferase (HIOMT) methylates N-acetyl-serotonin to produce melatonin (Axelrod, 1974).

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Melatonin is released into the blood stream (Tan & Khoo, 1981; Bruce *et al.*, 1991) and the cerebrospinal fluid (Tricoire *et al.*, 2002). This process, initiated by the daily light-dark pattern, is perceived by the retina. The presence/absence of light regulates the production of melatonin (see review Reiter, 1991). The optic nerve sends the signal to the suprachiasmatic nucleus (SCN) which regulates the production of melatonin and is located in the anterior hypothalamus, superior to the optic chiasm (Reppert *et al.*, 1988; Weaver *et al.*, 1996). The mean production rate of melatonin can be calculated as approximately 30µg/day in mammals (Lane & Moss, 1985). The melatonin production rate in adult humans is between 30-120 pg/ml/night time and 10-20 pg/ml/daytime (Geoffriau *et al.*, 1999) **(Figure 11)**.

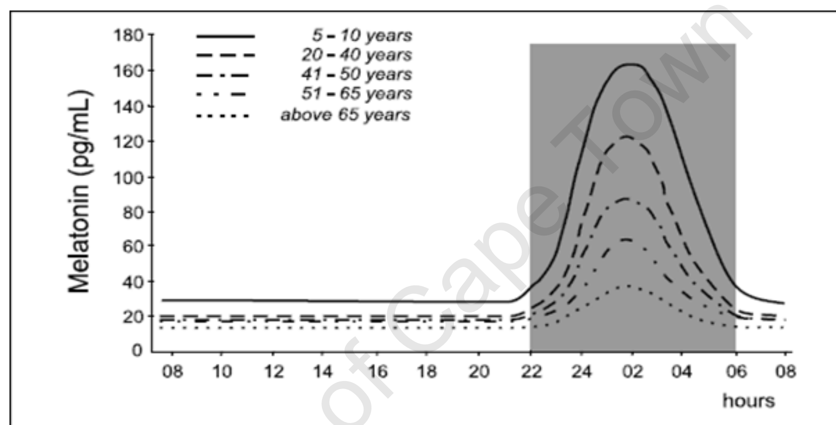

Figure 11: Circadian profiles of serum melatonin concentrations in humans at various ages: grey area = period of darkness (From Karasek & Winczyk, 2006).

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Melatonin has a biexponential decay, starting with a half-life of 2 min, followed by a second metabolic half-life of 20 min (Geoffriau *et al.*, 1999). The biosynthesis of melatonin in plants is different to that of mammals as a different rate-limiting step (tryptophan decarboxylase) exists to synthesise serotonin (Schröder *et al.*, 1999).

3.1.2 Melatonin receptors

Since its discovery in 1958, melatonin has contributed to many biological functions in the body and these effects are mostly observed by the activation of melatonin receptors on varying cell types. Melatonin acts through its two G-protein coupled membrane receptors; melatonin-type 1, melatonin-type 2 (MT-2) (see review Ekmekcioglu, 2006) and melatonin-type 3 “receptor”, a protein which is part of the quinine reductase family (Mailliet *et al.*, 2004). Melatonin receptors are commonly

expressed alone or together in different tissues. Functional melatonin receptors were discovered in the cardiovascular system, more specifically in the cardiac ventricular wall (Ekmekcioglu *et al*., 2003; Ekmekcioglu *et al*., 2001) and the arteries (Masana *et al*., 2002). However, the location of MT-2 receptor expression is restricted to certain organs and primarily expressed in the brain (Hunt *et al*., 2001; Fujieda *et al*., 2000).

A well-known function of melatonin is to regulate sleep in mammals, as well as acting as a strong antioxidant, a molecule which can inhibit the oxidation of other molecules.

3.1.3 Main physiological actions of melatonin

i. Regulation of sleeping pattern

Sleep is defined as a natural, periodic state of rest for the mind and body. In 1985, Borbely and colleagues proposed a circadian influence on sleep and wakefulness, defined as a periodic state during which one is conscious and aware of the world (Borbely *et al*., 1985).

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As mentioned previously, the release of melatonin into the blood stream is initiated by the daily light-dark pattern perceived by the retina (Reppert *et al*., 1988; Weaver *et al*., 1996). Under normal conditions, the SCN resets on a daily basis due to light inputs from the retina during the day, as well as melatonin secretion from the pineal gland during the dark cycle (Cassone *et al*., 1986).

ii. Antioxidative effect of melatonin

In 1993, melatonin was found to be a potent endogenous free radical scavenger, different to that of other antioxidants i.e. vitamin E (Tan *et al*., 2000).

Melatonin is a highly lipophilic molecule (Costa *et al*., 1995) and hydrophilic molecule (Shida *et al*., 1994). Thus, it easily and efficiently crosses morphophysiological barriers, i.e. nucleus, cytosol and cellular membranes (Reiter, 1998). Melatonin can easily cross the blood-brain barrier, a useful characteristic to remove free radicals present in the brain (Menendez-Pelaez *et al*., 1993). As a broad-spectrum antioxidant, melatonin can directly scavenge reactive oxygen and nitrogen species (see review Reiter *et al*., 2003). Of these free radicals, the hydroxyl radical is highly toxic and interaction with melatonin results in the formation of cyclic

3-hydroxymelatonin (Tan *et al.*, 1998), a free radical scavenger (López-Burillo *et al.*, 2003). Melatonin can neutralize hydrogen peroxide (Tan *et al.*, 2000), superoxide anion radical (Marshall *et al.*, 1996), nitrogen monoxide (Mahal *et al.*, 1999) and peroxynitrite (Zhang *et al.*, 1999). However, the mechanisms involved remain unclear.

Another antioxidative property of melatonin is its ability to stimulate the production/expression of antioxidative enzymes. These antioxidative enzymes play a key defensive role in free radical damage, which is dependent on the oxidative profile of the cell (Sewerynek *et al.*, 1995). Five antioxidative enzymes have been investigated in relation to melatonin: manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (CuZnSOD), catalase, glutathione peroxidase (GPx) (Antolin *et al.*, 1996) and glucose-6-phosphate dehydrogenase (Bilici *et al.*, 2001). Treatment with melatonin prevented the decrease in expression levels of neuronal GPx, CuZnSOD and MnSOD in rats administered the neurotoxin 6-hydroxydopamine (6-OHDA) (Mayo *et al.*, 2002).

The direct scavenging effects of melatonin are receptor-independent. However, the effect of melatonin on antioxidative activity is dependent on the interaction of melatonin, with its receptors on both the nucleus and cell membrane (see review Reiter *et al.*, 2003).

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3.2 Cardioprotective effect of melatonin

3.2.1 Hypertension

Hypertension is one of the main risk factors of CVD (Steyn *et al.*, 2005). The effect of melatonin in a hypertensive setting is paramount. Rats subjected to N-ω-nitro-l-arginine-methyl ester (L-NAME)-induced hypertension exhibited a restoration of the nitric oxide (NO) pathway in the presence of melatonin (Paulis *et al.*, 2010). Spontaneous hypertensive rats with endothelial dysfunction demonstrated the normalization of inducible NO, compared to untreated normotensive rats (Rezzani *et al.*, 2010). These findings propose a positive role for melatonin in the control of hypertension.

3.2.2 Atherosclerosis

Atherosclerosis is a disorder specifically affecting medium and large arteries. Fat, cholesterol and other substances build-up along the wall of the arteries to form hard structures called plaque. This plaque eventually blocks the artery. Plaque development requires the activation of inflammatory cytokines and oxidised LDL (Pieri *et al.*, 1996). The presence of oxidised LDL in human umbilical arteries causes a vasospastic effect (Okatani *et al.*, 2000). Treatment with 10 µM of melatonin significantly reduced this vasospastic effect of oxidised LDL (Okatani *et al.*, 2000).

3.2.3 Metabolic syndrome

In accordance to the National Heart Lung and Blood Institute, metabolic syndrome (MetS) is characterised as a group of risk factors that increase the risk of CVD (NHLBI, 2011). There are 5 MetS risk factors; large waistline ("abdominal obesity"), high triglyceride level, low HDL levels, high blood pressure and high fasting blood sugar (NHLBI, 2011). Patients suffering from MetS are at a higher risk of dying from CVD and CHD (Malik *et al.*, 2004). Consequently, earlier diagnosis of MetS can reduce the incidence of CVD in this group.

A treatment with melatonin (5mg/day) for 2 months in MetS patients improved catalase activity, decreased screen lipid peroxidation, decreased LDL levels and decreased blood pressure (Koziróg *et al.*, 2011). Furthermore, melatonin can potentially improve insulin activity and the lipid profile in MetS patients. Nocturnal melatonin-insulin ratio, which is classified as melatonin levels and insulin levels measured at 3 am, established a negative relationship with LDL and a positive relationship with HDL levels in MetS patients (Robeva *et al.*, 2008). Therefore, melatonin has the ability to reduce the onset of MetS risk factors.

3.2.4 Ischemia/Reperfusion

In 1985, Muller and colleagues found that the likelihood for myocardial infarction to occur peaks between 9 am and 11 pm. During the rest of the day, myocardial infarction occurs at a similar rate **(Figure 12)** (Muller *et al.*, 1985). Melatonin production is lower during the daytime than at night-time (see review Reiter, 1991). Thus, a possible correlation between myocardial infarction incidence and presence of melatonin could be observed.

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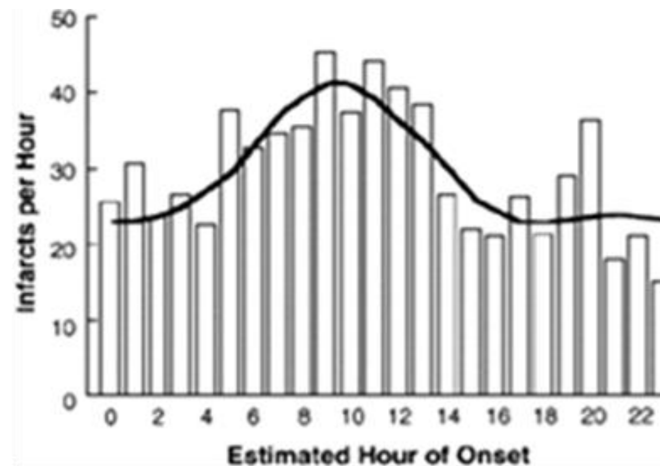

Figure 12: Circadian variation in the frequency of onset of myocardial infarction. (From Muller *et al*, 1985)

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Melatonin, given at varying concentrations (1, 10 and 50 μM) attenuated cardiac arrhythmias in an isolated male Sprague-Dawley rat heart model (Tan *et al.*, 1998). Furthermore, 10 μM of melatonin and 50 μM of melatonin reduced reperfusion ventricular induced fibrillation and arrhythmias in male Wistar rats subjected to ischemia/reperfusion injury (Szárszoi *et al.*, 2001; Dobsak *et al.*, 2003). This evidence suggests that melatonin, at these concentrations in an *in vitro* setting, has a significant cardioprotective effect. In an *in vivo* setting, Lee and colleagues established the cardioprotective effect of melatonin against ischemia/reperfusion injury (Lee *et al.*, 2002). Male Sprague Dawley rat hearts treated with melatonin (1.0 and 5.0 mg/kg) 10 min before left artery coronary occlusion and 45 min reperfusion, had a significant reduction in infarct size, reduced tachycardia and fibrillation, compared to the ischemia/reperfusion control group (Lee *et al.*, 2002).

Male Wistar rats subjected to 30 min global ischemia and treated acutely with melatonin (50μM) had a significant reduction in infarct size with a reduction in lactate dehydrogenase (LDH) release, an indicator of necrosis, compared to untreated controls (Petrosillo *et al.*, 2009). Melatonin (10mg/kg) treatment of male Sprague Dawley rats for 4 weeks subjected to ischemia/reperfusion injury reduced the infarct size and LDH release, compared to vehicle treated hypoxic rats (Yeung *et al.*, 2008).

In 2000, Lagneux and colleagues observed the antioxidative effect of both melatonin and a melatonin structurally-related indole compound (5-methoxy-carbonylamino-N-acetyl-tryptamine), against ischemia/reperfusion injury (Lagneux *et al.*, 2000). Male Wistar rats pre-treated with melatonin (10 mg/kg intraperitoneally) and 5-methoxy-carbonylamino-N-acetyl-tryptamine (10 mg/kg intraperitoneally), followed by 30 min regional ischemia, had a significant reduction in infarct size compared to vehicle treated rats (Lagneux *et al.*, 2000).

3.3 Mechanisms of melatonin-induced cardioprotection

Adult insulinoma-bearing New England Deaconess Hospital (NEDH) rat ventricular cardiomyocytes under hypoxic conditions showed accumulation of calcium with a generation of hydrogen peroxide. However, melatonin treatment (50 μM and 100 μM) attenuated these effects (Salie e*t al.*, 2001). Furthermore, melatonin has the potential to control the expression of NF-κB genes by interacting with calmodulin (Benítez-King *et al.*, 1996; see review Tomás-Zapico & Coto-Montes., 2005). Thus, melatonin regulates the expression of immune cells and antioxidative enzymes.

Interestingly, melatonin has the ability to activate the well-known reperfusion injury salvage kinase (RISK) pathway to protect the heart against ischemia/reperfusion injury. This pathway involves the rapid activation of pro-survival signalling cascades phosphatidylinositol-3-OH kinase (PI3K)-Akt and p42/p44 extra-cellular signal-regulated kinases (Erk 1/2) at the onset of reperfusion (see review Hausenloy & Yellon, 2004). Chronic treatment of melatonin (4mg/kg) for 16 weeks in diet-induced obese male Wistar rats increased Akt activation during reperfusion (Nduhirabandi *et al.*, 2011). In addition, melatonin (50 μM) administered acutely in an isolated male Wistar rat heart model increased Akt expression with a reduction in the pro-apoptotic kinase, p38 Mitogen-activated protein kinase (MAPK) (Genade *et al.*, 2008).

Recently, Lamont and colleagues observed the potential of melatonin to activate a novel protective pathway called the survivor activating factor enhancement (SAFE) pathway via tumor necrosis factor-alpha (TNF-α) and the signal transducer and activator of transcription 3 (STAT-3) (Lamont *et al.*, 2011).

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3.4 Melatonin confers cardioprotection via the SAFE pathway

3.4.1 Definition of the SAFE pathway

The SAFE pathway **(Figure 13)**, recently described as a novel cardioprotective pathway, involves the activation of STAT-3. STAT-3 activation occurs following the binding of TNF-α and TNF-receptor 2 (Lecour *et al.*, 2009).

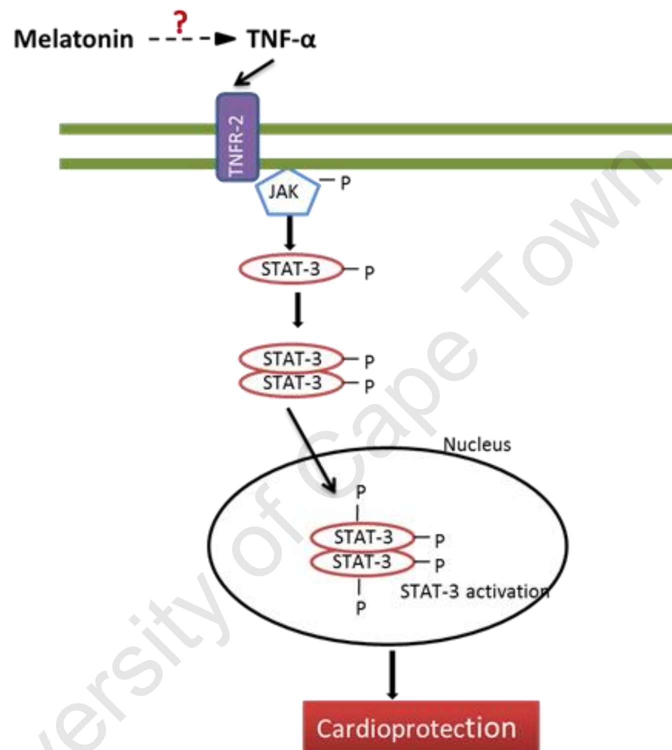

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Figure 13: Illustration of the SAFE pathway. (From Zulfah Albertyn, 2010)

An acute treatment of exogenous TNF-α (0.5 ng/ml) prior to a myocardial ischemia/reperfusion insult in an isolated male Long Evans rat heart model displayed a significant reduction in infarct size compared to controls (Lecour *et al.*, 2002). Interestingly, male Long Evans hearts preconditioned with TNF-α (0.5 ng/ml) demonstrated a reduction in infarct size as well as phosphorylation of STAT-3 (Lecour *et al.*, 2005). To protect patients against ischemia/reperfusion injury, activation of the SAFE pathway can become a therapeutic tool. However, high levels of TNF-α in the circulatory system could be potentially harmful to the patient, by causing, for example, liver injury (McClain and Cohen, 1989). Nevertheless, there are several pharmacological agents such as opioids (Gross *et al.*, 2006);

insulin (Fuglesteg *et al.*, 2008), sphingosine-1-phosphate (Kelly *et al.*, 2008) and melatonin (Lamont *et al.*, 2011) which have the ability to activate the SAFE pathway and mediate cardioprotection against ischemia/reperfusion injury.

i. Melatonin reduces TNF-α level

The immune system has many factors which can play major roles in the functioning of the heart. Melatonin, more specifically, affects the release of pro-inflammatory cytokines i.e. interleukin-6 (IL-6) and TNF-α.

Acute exercise has a profound effect on the production of TNF-α, IL-6 and activation of NF-κB. However, in the presence of either melatonin these effects are entirely or partially inhibited (Veneroso *et al.*, 2009). An additional study using a haemorrhagic shock (HS) model provides further insight into the relationship between melatonin and TNF-α. HS is the sudden and rapid loss of a significant amount of blood, which affects the perfusion commands required for normal cellular activity in the body. HS-induced rats demonstrated an increase in TNF-α and IL-6 at 1 hour and up to 48 hours after HS, with an increased heart rate (Yang *et al.*, 2009). However, treatment with 10 mg/kg of melatonin suppressed the release of serum TNF-α and IL-6 in these rats (Yang *et al.*, 2009).

Interestingly, melatonin displays a protective role in septic shock. In endotoxemic rats melatonin prevented circulatory failure, as well as improved the survival rate in mice treated with high doses of lipopolysaccharide (LPS) (Wu *et al.*, 2001). There was a significant drop in serum TNF-α levels in endotoxemic rats treated with melatonin compared to the untreated controls (Wu *et al.*, 2001).

Therefore, melatonin can reduce the production of large amounts of TNF-α produced in pathophysiological conditions.

ii. Melatonin requires TNF-α for cardioprotection

Paradoxically, Lamont and colleagues have shown that, in the absence of TNF-α (as in TNF-α knock-out mice), melatonin failed to protect against an ischemia/reperfusion insult (Lamont *et al.*, 2011). An acute dose of melatonin (75ng/L) administered to TNF-α knock-out mice showed no difference in infarct size when compared to wild type mice treated with melatonin (Lamont *et al.*, 2011).

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This finding suggests that circulating TNF-α (at small concentrations) could possibly play a cardioprotective role in melatonin-induced cardioprotection against ischemia/reperfusion injury.

3.4.2 JAK/STAT in ischemia/reperfusion injury

i. Definition of the JAK/STAT pathway

The signal transducing and activator of transcription (STAT) proteins are a family of cytoplasmic transcription factors mediating intracellular processes activated by cytokines at the surface receptor of cell membranes. There are several known relatives of the STAT family, STAT-1, STAT-2, STAT-3, STAT-4, STAT-5a, STAT-5b and STAT-6 (Xuan *et al.*, 2001). Induction of apoptosis simulated by ischemia upregulates the expression of STAT-1 in cardiomyocytes (Stephanou *et al.*, 2000). Conversely, STAT-3 demonstrates a cardioprotective effect by decreasing apoptotic cell death in cardiomyocytes (Lu *et al.*, 2008). STAT-3 activation occurs when a cytokine such as TNF-α binds to the receptor, which activates Janus Kinase-2 (JAK-2) or mitogen activating protein kinase (MAPK). Phosphorylation of STAT-3 causes the formation of homo- or heterodimers of STAT-3 (see review Rawlings *et al.*, 2004) which translocates to the nucleus for the activation of stress responsive gene transcription (Carballo *et al.*, 1999).

During ischemia/reperfusion injury STAT1, STAT3, STAT5a, and STAT6 expression occurs in the rat myocardium (Mascareno & Siddiqui, 2000).

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ii. JAK/STAT3 and ischemia/reperfusion injury

The first detection of STAT-3 activation following a myocardial infarction occurred in 2001. An increase in the phosphorylation of STAT-3 up to 24 hours occurred after ligation of the left coronary artery in rats (Negoro *et al.*, 2001). Endothelial-derived STAT-3 is essential in post-ischemic myocardial function (Wang *et al.*, 2007). Indeed, endothelial STAT-3 knock-out mice displayed a drop in myocardial functional recovery in response to ischemia/reperfusion injury and linked to increased levels of LDH release (Wang *et al.*, 2007). Consequently, STAT-3 knock-out mice displayed elevated levels of p38 MAPK (Wang *et al.*, 2007). A key role for the JAK/STAT signalling pathway is in ischemia, pharmacological preconditioning

(Lecour *et al*., 2005; Xuan *et al*., 2001) and postconditioning (Lacerda *et al*., 2009; Boengler *et al*., 2008).

3.4.3 Role of the SAFE pathway in melatonin-induced cardioprotection

Melatonin interacts with TNF-α and activates the SAFE pathway. In STAT-3 knock-out or TNF-α knock-out mice, pre-treatment with melatonin (75ng/L) failed to reduce the infarct size after an ischemia/reperfusion insult (Lamont *et al*., 2011). In addition, male Wistar rat hearts subjected to an acute administration of melatonin (75ng/L) had a significant expression of STAT-3 in the nucleus of cardiomyocytes (Lamont *et al*., 2011). Thus, melatonin can activate the SAFE pathway and confer cardioprotection against ischemia/reperfusion injury.

3.5 Does melatonin contribute to the cardioprotective effect of wine?

3.5.1 Presence of melatonin in red wine

Many food products contain melatonin. The first edible plant in which melatonin was discovered was tomatoes (*Solanum ly-copersicum* L., 'syn. *Lycopersicon esculentum* Mill.') (Dubbels *et al*., 1995). Melatonin concentration varies dependent on developmental stage but, it is generally found at higher concentrations in the seeds (Okazaki & Ezura., 2009). Melatonin was also found to be present in *Vitis vinifera* seeds during véraison, "the onset of ripening"/"change of colour of the grape berries" (Vitalini *et al*., 2011; Murch *et al*., 2010).

In addition, melatonin content varies, depending on the grape type, its environment, genetics, harvesting process, storage and agrometeorological conditions. In 2006, Iriti and colleagues measured the melatonin content in eight different wines. The berry skin of the Nebbiolo contained the highest melatonin concentration (428.3±32.1pg/ml), whereas the Cabernet Franc contained the lowest concentration (2.4±0.6pg/ml) (Iriti *et al*., 2006). During field treatments of grape vines with benzothiadiazole (BTH), a plant defence activator, there was a significant increase in the amount of melatonin in the skins of these grape berries (Iriti *et al*., 2006) **(Table 2)**. A merlot treated with BTH demonstrated a 2.7-fold increase in melatonin content in the berry skin, compared to no treatment with BTH (Iriti *et al*., 2006).

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Table 2: Melatonin concentration in eight grape cultivars examined and in two plasma samples used as a control (From Iriti *et al*., 2006)

Sample	Melatonin[a] ($pg\,ml^{-1}$)
Plasma control 1	10.6 ± 3.2
Plasma control 2	134.7 ± 10.3
Barbera	25.5 ± 2.5
Croatina	304.6 ± 23.4
Cabernet Sauvignon	183.6 ± 18.8
Cabernet Franc	2.4 ± 0.6
Marzemino	14.9 ± 4.1
Nebbiolo	428.3 ± 32.1
Sangiovese	255.5 ± 16.3
Merlot	114.2 ± 7.2
Merlot, BTH-treated	301.5 ± 22.5

Current literature evidence on the amount of melatonin present in South African red wine and white wine is poor. Interestingly, half an hour after consumption of red wine, an increase in serum melatonin levels can be detected in humans (Guerrero *et al*., 2008).

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3.5.2 Melatonin, at the concentration found in red wine, confers cardioprotection

Isolated rat hearts treated acutely with melatonin at a concentration similar to that found in red wine (75 ng/L), portrayed a significant reduction in infarct size in relation to untreated control hearts after an ischemia/reperfusion insult (Lamont *et al*., 2011). Hence, resveratrol, at a concentration found in red wine is not the only beneficial molecule to protect against a heart attack, as melatonin also showed beneficial effects.

Recent research conducted within the Hatter Institute for Cardiovascular Research in Africa has confirmed the role of melatonin in red wine-induced cardioprotection. Chronic pre-treatment with melatonin for 2 weeks, at the concentration found in red wine, protected the heart against ischemia/reperfusion injury (Lamont *et al*., 2011). Interestingly, this protective effect was abolished in the presence of the melatonin receptor 3 antagonist (Prazosin) (unpublished data), indicating that melatonin plays a vital role in red wine-induced cardioprotection via the activation of this receptor.

4. Toll-like receptor 4 and cardioprotection

4.1 Classification of Toll-like receptor 4

Toll-like receptor 4 (TLR-4) is part of a family of 10 Toll genes found in the human genome (Du *et al.*, 2000). It is a transmembrane receptor, activated mainly by lipopolysaccharide (LPS) (gram-negative bacteria) (Poltorak *et al.*, 1998). It can also be activated by paclitaxel, and several endogenous heat shock proteins (HSPs), fibronectin, heparin sulfate, high mobility group box 1 protein (HMGB1) and hyaluronan, a naturally occurring glycosaminoglycan, which behaves as a lubricating, binding and protective agent in cells.) **(Table 3).**

Table 3: Representation of ligands activating TLR-4. (From Chao *et al.*, 2009; modified by: Zulfah Albertyn, 2010)
See abbreviation list

TLRs	Endogenous ligands	Cells	End points
TLR-4	Hsp-22	Macrophages	IL-6
	Hsp-60	Fibroblasts	P38, JNK1/2, ERK 1/2, IKK
		Macrophages	TNF, NO
	Hsp-70	HEK293	NF-κB
		Fibroblasts	NF-κB
		Macrophages	TNF
	Fibronectin (extra domain A)	HEK293	NF-κB
	Fribinogen	Macrophages	MCP-1
	Hyaluronic acid (oligosaccharide)	Dendritic cells	TNF, pMAPK, NF-κB
	Heparin sulphate (polysaccharide)	Dendritic cells	DC maturation
	Hyaluronan	Macrophages	MIP-2, TNF, KC
	Lung surfactant protein A	Macrophages	NF-κB activity
		Ovary cells	TNF, IL-10

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Activation of TLR-4 results in the release of antimicrobial peptides, inflammatory cytokines (i.e. TNF-α), chemokines and co-stimulatory molecules, which initiate an innate immune response.

4.1.1 TLR-4 signalling pathways

TLR-4 activates 2 pathways: the myeloid differentiation primary response gene 88 (MyD88)-dependent and/or–independent pathway. Upon TLR-4 activation,

recruitment and activation of several downstream kinases occur via a set of adaptor proteins. The downstream kinases are interleukin (IL)-1 receptor-associated kinase (IRAK)-1, IRAK-4, TNF receptor-associated factor (TRAF)-family member-associated NF-κB activator-binding kinase 1 (TBK1) and, TIR-domain-containing adaptor protein inducing interferon-β-mediated transcription factor (TRIF) (see review Chao *et al*., 2009).

All TLRs activate the MyD88-dependent pathway; however, TLR-4 and TLR-3 also activate the MyD88-independent pathway. Indeed, MyD88-deficient mice exposed to all 10 TLRs demonstrated no increase in TNF-α, IL-6 or IL-12 (Akira *et al*., 2001). On the other hand in the presence of LPS, these MyD88-deficient mice expressed NF-κB (Kawai *et al*., 2001) **(Figure 14),** proposing that TLR-4 activates both MyD88-dependent and independent pathways.

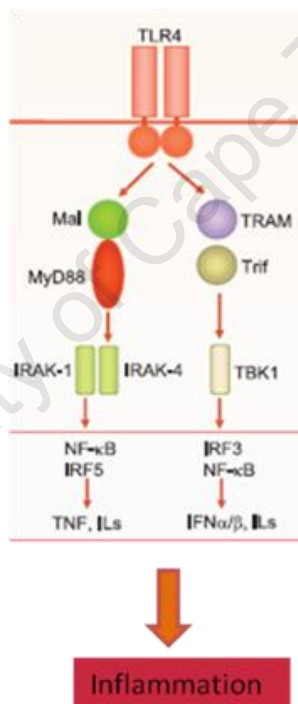

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Figure 14: Illustration depicting TLR-4 activation by 2 pathways (From Chao *et al*., 2009; modified by: Zulfah Albertyn, 2010)

4.2 TLR-4 in ischemia/reperfusion injury in the heart

According to the literature, the role of TLR-4 against ischemia/reperfusion injury in the heart is conflicting in nature. Some research studies suggest that TLR-4 is deleterious, whereas other studies suggest that TLR-4 is cardioprotective.

4.2.1 Harmful effect of TLR-4 in ischemia/reperfusion injury

Fallach and colleagues revealed the presence of TLR-4 in myocardial ischemia (Fallach *et al.*, 2010). In this study, wild-type (C57BL) mice challenged with LPS or myocardial infarction displayed increased TLR-4 expression, reduced cardiac function and raised levels of myocardial TNF-α and IL-1β prior to myocardial leukocyte infiltration (Fallach *et al.*, 2010). Furthermore, TLR-4 knock-out mice displayed superior myocardial function, as well as significantly smaller infarcts, compared to wild-type (C57BL) mice after myocardial infarction (Fallach *et al.*, 2010).

Many studies have linked TLR-4 signalling to cardiac dysfunction (Cha *et al.*, 2008). However, there is some debate relating to the protective effect of TLR-4 in cardiovascular function. TLR-4 defective (C3H/HeJ) hearts administered with TNF-α and IL-1β, demonstrated a reduction in cardiac functionality similarly to that of control hearts (Cha *et al.*, 2008).This suggests a protective function of TLR-4 in ischemia/reperfusion injury in an isolated heart model. However, using the same model and strain of mice, Hua and colleagues showed a decrease in myocardial injury in TLR-4 knock-out mice subjected to ischemia/reperfusion injury (Hua *et al.*, 2007). The same group also detected the activation of the PI3K/Akt signalling pathway in TLR-4 knock-out mice (Hua *et al.*, 2007). Mice defective in TLR-4 (C3H/HeJ) represented with a 40% reduction in infarct size compared to control mice (Chong *et al.*, 2004). Nevertheless, a study by Kim and colleagues observed that, TLR-4 knock-out mice do not exhibit improved cardiac function, even though infarct size was reduced (Kim *et al.*, 2007).

4.2.2 TLR-4 -induced cardioprotection

LPS, a TLR-4 agonist, can potentially protect against ischemia/reperfusion injury if given prior to an ischemic insult. This mimics a preconditioning effect resulting in a

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reduction in infarct size in mice with a normal TLR-4 expression compared to TLR-4 deficient mice (Pradillo *et al.*, 2009). Furthermore, activation of TLR-4 via preconditioning by HMGB1 could also be beneficial (see review Yang *et al.*, 2010). In contrast, TLR-4 knock-out mice subjected to an ischemia/reperfusion insult failed to be preconditioned with HMGB1 compared to wild-type mice (Izuishi *et al.*, 2006). A protective role for TLR-4 in ischemia/reperfusion injury could, therefore, depend on the preconditioning capabilities of its ligands.

4.3 TLR-4 and melatonin

A recent study forged a link between TLR-4 expression and the pineal gland. Da Silveira Cruz-Machado and colleagues validated the presence of TLR-4 in the pineal gland (Da Silveira Cruz-Machado *et al.*, 2010). Here, LPS induced activation of TLR-4 in the pineal gland and triggered the NF-κB pathway, which leads to increased TNF-α expression (Da Silveira Cruz-Machado *et al.*, 2010). Melatonin could potentially protect against septic shock. Endotoxemia induced in Wistar-Kyoto rats by LPS injection followed by melatonin treatment (1-3mg/kg/day) displayed an attenuation of circulatory failure as well as improved survival rates (Wu *et al.*, 2001). Serum TNF-α levels decreased in endotoxemic rats treated with melatonin compared to controls (Wu *et al.*, 2001).

The data above suggest a possible link between melatonin, TLR-4 activation and TNF-α. However, the role of TLR-4 in melatonin-induced cardioprotection against ischemia/reperfusion injury is unknown.

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OBJECTIVES

Several epidemiological and experimental studies have proposed a cardioprotective effect for the chronic and moderate consumption of red wine. However, the cardioprotective effect of South African wines is yet to be tested and whether red wine has a better cardioprotective effect than white wine is unclear.

Melatonin, a powerful cardioprotective agent, was recently discovered in wine, but whether melatonin contributes to the cardioprotective effect of a chronic and moderate consumption of wine is unknown.

Melatonin protects against ischemia/reperfusion injury by activating the SAFE pathway, via mechanisms that still require to be delineated.

In the present study, we propose that South African (red and white) wines confer a cardioprotective effect, function to their melatonin content. Furthermore, we suggest that the cardioprotective effect of melatonin, at the concentration found in red wine, is dependent on the activation of TLR-4 and its ability to activate the SAFE pathway.

To explore this hypothesis, we used the Langendorff perfusion isolated rat heart model. The objectives of this study were as follows:

1. To determine whether South African wines exert a cardioprotective effect, male Long Evans rats were chronically pre-treated with red and white South African wines for 14 days and subjected to ischemia/reperfusion injury on the 15th day **(Figure 15)**. The melatonin content in the wine was measured to explore a possible correlation between the melatonin content and the cardioprotective effect of the wines.

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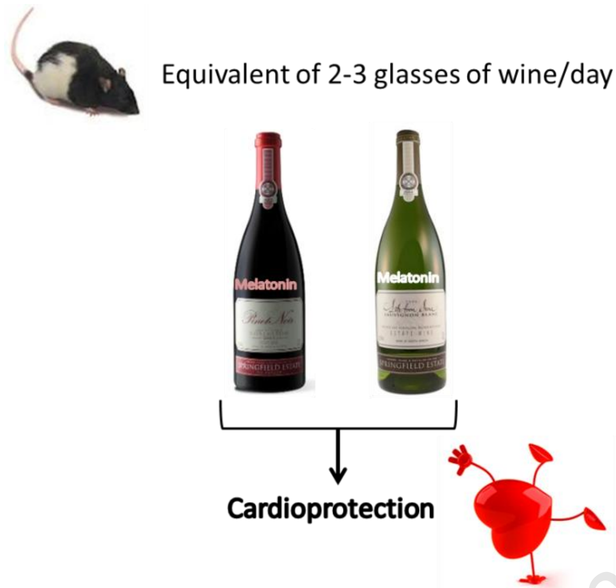

Figure 15: Hypothetical view of melatonin (present in wine)-induced cardioprotection

(Diagram by: Zulfah Albertyn, 2012; Rat image from: http://www.lookfordiagnosis.com/mesh_info.php?term=Ratas +Long-Evans&lang=2, Access date: 12.8.12; red wine image from: http://www.topwinesa.com/in_the_ spotlight.html Access date: 12.8.12; white wine image from: http//:www.wine-searcher.com/find/springfield +life+ stone+sauvignon+blanc, Access date: 12.8.12; heart image from: http://www.tellmeaboutdiabtes.com/ 4239/dash-diet-best-for-type-2-diabetes-management/, date accessed: 12.8.12)

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2. To understand the role of TLR-4 in melatonin-induced cardioprotection, the cardioprotective effect of melatonin against ischemia/reperfusion injury was explored in an isolated perfused heart model in the presence of a TLR-4 inhibitor (TAK242). To explore a link between the SAFE pathway and TLR-4, Western blot analysis was conducted **(Figure 16)**.

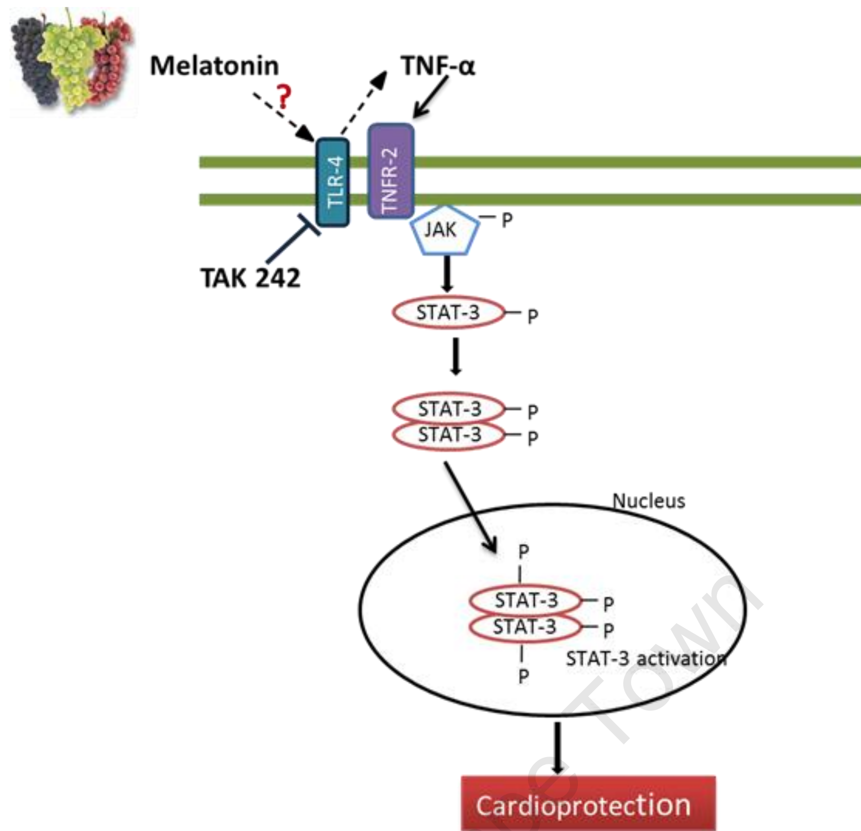

Figure 16: Hypothetical protective pathway: Wine-induced cardioprotection by the interaction of melatonin with TLR-4 is mediated by the SAFE pathway. (From Zulfah Albertyn, 2012; Grape image from http://grapesweb.com/., date accessed :3.7.12)

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CHAPTER 2: MATERIALS AND METHODS

2.1 Wine used in the study

Table 4: Classification of each wine

Grape varietal & vintage	Vineyard	Area,City	Country
Cabernet Sauvignon 2007	Paetzold	Bordeaux	France
Pinot Noir 2009	Paul Cluver	Elgin, Cape Town	South Africa
Pinot Noir 2010	Springfield	Robertson, Cape Town	South Africa
Nebbiolo 2010	Idiom	Helderberg, Cape Town	South Africa
Sauvignon Blanc 2010	Springfield	Robertson, Cape Town	South Africa
Syrah 2009	Mulderbosch	Stellenbosch, Cape Town	South Africa
Chardonnay 2008	Meerhof	Malmesbury, Cape Town	South Africa
Cabernet Sauvignon 2010	First Cape	Cape Town, Cape Town	South Africa

2.2 Determination of melatonin content in wine

2.2.1 Melatonin content in wine measured by High performance liquid chromatography (HPLC) with solid phase extraction (SPE)

The melatonin content in each South African wine (and one French wine) was estimated by the method described by Mercolini *et al.*, 2008 and was performed in the laboratory of Dr M Iriti at the University of Milan, Italy.

HPLC is a chromatographic technique, which separates a mixture of compounds to identify, quantify and purify the individual components of the mixture. Wine samples were pre-treated for the SPE procedure by centrifugation and the supernatant was filtered through a Surtorius (Göttingen, Saxony, Germany) syringe membrane filter with a pore size of 0.2μm. The SPE was carried out on a Varian (Walnut Creek, California, USA) BondElut C18 cartridge (100mg, 1l) by means of a VacElut (Varian) apparatus.

Calibration curves were established to validate the method. A mixture of standard analyte solutions (50 μl) at seven different concentrations containing the internal standard (Mirtazapine) was added to 500 μl of each simulated wine. This mixture underwent SPE pre-treatment and was injected into the HPLC system. The entire procedure was carried out in triplicate for each concentration. The analyte/internal standard peak area ratios (pure numbers) were plotted against the corresponding concentrations of analytes (expressed as ng/ml) and the calibration curves constructed by means of the least-square method.

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The standard solutions of some natural compounds normally found in wine were injected into the HPLC. The resulting chromatograms were checked for possible interface from the detected compounds. Polyphenolic content in wine samples was measured according to the method of Singleton & Rossi, 1965.

2.3 Animals

All the experiments were conducted and performed on male Wistar rats or male Long Evans rats (240-300g), in accordance with the Guide for Care and Use of Laboratory animals published by the U.S. National Institute of Health (NIH publication No. 85(23), revised 1996). This study required two different strains of rat, Wistars as well as Long Evans. Firstly, due to the availability of animals at the time of our study, we used Wistar rats for our acute study with melatonin and Long Evans rats for our chronic study with wine. Secondly, previous chronic experiments performed in our laboratory with red wine were done on Long Evans rats while acute studies with melatonin were done using Wistar rats. We, therefore, wanted to continue this study with both strains in order to compare the present data with that of our previous studies. All procedures were approved by the Animal Research Review Committee of the University of Cape Town (application; 011/031 entitled "The role of Toll-like receptor 4 in wine-induced cardioprotection".)

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2.4 The Langendorff perfused isolated rat heart model

2.4.1 History

The Langendorff perfused isolated rat heart model was established more than 100 years ago. Carl Ludwig and Elias Cyon created the first isolated perfused frog heart preparation in 1866. H. Newell Martin (in 1883) and Oscar Langendorff (in 1895), developed perfusion systems for isolated mammalian hearts (see review Zimmer 1998). This methodological procedure/technique has allowed cardiovascular researchers to be able to study myocardial function, regulation of coronary blood flow and cardiac metabolism.

2.4.2 Apparatus and Technique

The heart is cannulated via the aorta. Attached to the cannula is a reservoir filled with oxygenated perfusate. The perfusate is pumped through the aorta retrogradely at 37°C, which refers to the reverse flow of blood to the heart **(Figure 17)**. As the

perfusate does not flow in the normal circulatory pattern, this marks an inability for the left ventricle to generate a pressure volume. The downward flow of the perfusate causes closure of the aortic valves as well as perfusion of the coronary ostia, thus leading to perfusion of the entire ventricular mass of the heart.

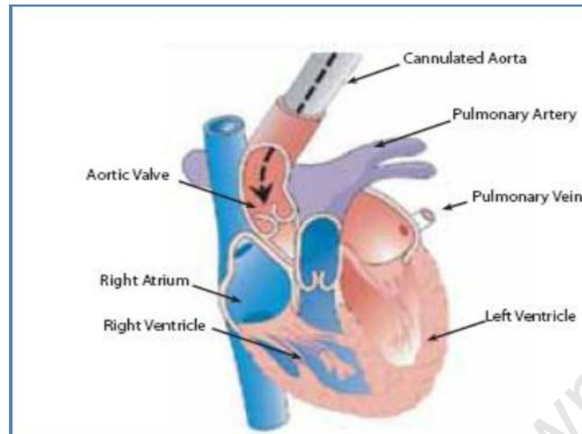

Figure 17: A graphic representation of retrograde perfusion in Langendorff mode. (ADInstruments™, London)

The perfusate is delivered to the heart at a constant hydrostatic pressure (100 cm in H_2O).

Maintenance of a constant pressure is achieved due to a constant adjustment of the reservoir level by means of a pump. Krebs-Henseleit buffer (KHB), an appropriate perfusate containing the necessary nutrients, is required for the heart to beat for a maximum of 4 hours before the onset of oedema. KHB is equilibrated with O_2/CO_2 95:5%, respectively and has the following composition (mM): 118.0 NaCl, 25.2 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4.7H_2O$, 1.2 KH_2PO_4, 1.2 $CaCl_2.H_2O$, and 11.0 Glucose, pH of 7.4 (see appendix p84 and abbreviation list p ix, x, xi)

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2.4.3 Isolation of the heart

i) Before mounting the heart on the system

The Langendorff apparatus was flushed with boiled distilled water daily to remove any bacteria and endotoxins from the system. KHB was made daily, as keeping it for more than a day will lead to the precipitation of calcium or glucose which is detrimental to the heart. KHB must be oxygenated for 20 minutes before use. The powerlab data acquisitions software™ (ADInstruments™, Cape Town, South Africa) was used to measure functional parameters and was calibrated daily with a sphygmomanometer for accurate readings.

Rats were anaesthetised with 60 mg/kg intraperitoneal injection of sodium pentobarbitone mixed with 200 IU heparin, an anticoagulant to reduce the formation of an emboli in the cardiac vasculature. The animals were placed in a quiet room to be anaesthetised as to reduce the animals stress levels. Stress causes the release of catecholamines and other circulatory factors which can affect the functioning of the heart, thus the presence of stress can in turn affect an experiment. The effect of the anaesthesia was checked with the pedal reflex, whereby the paw of the animal was pinched and its perception of pain was observed.

ii) Harvesting of the heart

Removal of the heart for isolation requires the animal to be completely unconscious (disappearance of pedal pain withdrawal reflex). Thoracotomy was done to remove the heart from its cavity. A forward incision of the skin at the xyphoid-sternum (sternotomy) continued along the lateral ends of the left and right costal margins. The thoracic cavity was opened by removal of the outer pleural membrane, which connects the ribcage and diaphragm, and thus resulting in exposure of the beating heart. The researchers' thumb (of the left hand) is placed firmly under the heart, and with both the index and middle finger the heart is cradled. The heart was gently lifted followed by one precise incision to remove the heart without damaging the aorta or cutting the aorta too short. Upon its removal, the heart was immediately placed into ice cold KHB to arrest the heart (inhibiting any enzymatic reactions taking place and degradation of any protein within the cardiomyocytes).

iii) Cannulation of the heart onto the system

A pair of tweezers were used to mount the heart onto the cannula (which was connected to the perfusion system and the perfusate was dripping from the cannula to enable a fluid-fluid connection). This action must be done carefully to prevent damage to the aorta. The heart was clamped to the cannula by means of a bulldog clip in order to keep the heart in place. Immediately, a thin suture tied around the aorta connected to the cannula was used to firmly anchor the heart. In addition, the pulmonary artery was punctured to release the pressure build-up in the heart. Subsequently, the left ventricle was exposed by snipping away some of the left atria, to allow the insertion of the deflated intraventricular balloon into the left ventricle **(Figure 18)**. 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.

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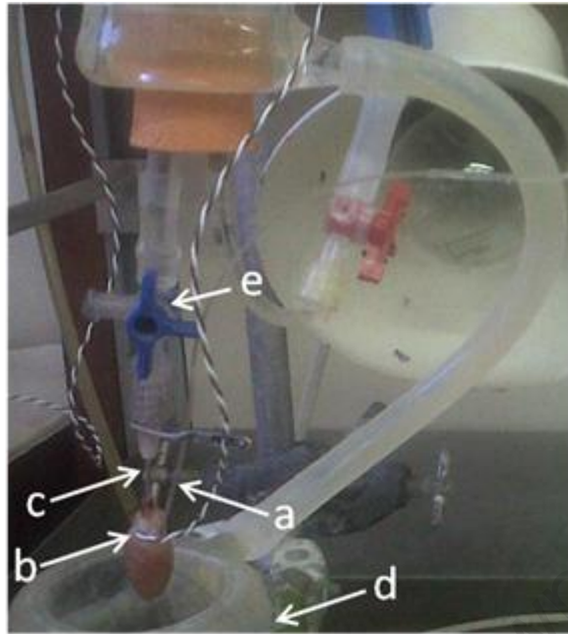

Figure 18: A Langendorff-perfused rat heart. During perfusion the heart is placed in a water-jacketed reservoir (d) to keep the temperature of the heart constant. Figure key: (a) intraventricular balloon inserted into the left ventricle and mounted on a 21-gauge bevelled needle; (b) thermal couple for continual temperature monitoring; (c) cannula whereby heart is mounted onto system; (e) a 2-way tap placed between the bubble trap and cannula, which serves to switch off the buffer during global ischemia. (From Zulfah Albertyn, 2012)

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2.4.4 Functional parameters

Functional parameters measured throughout all experiments with the intraventricular balloon are: left ventricular end diastolic pressure (LVEDP), left ventricular end systolic pressure (LVESP), left ventricular developed pressure (LVDP= difference between LVESP and LVEDP), heart rate (HR) and the coronary flow (CF). Rate pressure product (RPP) was expressed as LVDP×HR for each time point.

i) Functional recovery was calculated as follows:

LVDP×HR (at specific time points)/ LVDP×HR (at stabilisation period) =X × 100= % functional recovery

ii) Exclusion criteria

Rats not complying with the following criteria were removed from the study:

1) LVDP must meet the minimum requirement of 80 mmHg.

2) CF must be a minimum of 8ml/minute and a maximum of 16 ml/minute.

3) Heart rate should be a minimum of 220 beats/minute and a maximum of 400beats/minute.

2.5 Langendorff perfusion protocol

- **Global ischemia**

Long Evans male rats (160g-180g) were pre-treated (orally) for 14 days either with water or with 7 selected South African wines or one French wine. On the 15th day, hearts were harvested and perfused via the Langendorff perfusion system. The selected SA wines were from various vineyards within the Western Cape region, South Africa.

Every day for 14 days the drinking water used for controls were supplemented by one of the 7 South African wines or French wine mentioned above. Hence, the wine-drinking water solution was made up fresh everyday and if not, rats will not drink it due to the taste. Furthermore, the different wine solutions were prepared by adding 1 part of wine to 7 parts drinking water (Wollny *et al.*, 2003), equivalent to 2 glasses wine/day for human beings. Preliminary studies have shown that addition of wine to the water does not affect the drinking behaviour of the rats (Lamont *et al.*, 2012). Therefore, 5ml of wine was diluted with 35ml drinking water, and each rat drinks a maximum of 40ml fluid /day (previous study in our laboratory). In addition, the wine was given to the experimental rats early afternoon, therefore in the evening, when they were wide-awake they would have fluid available.

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Once on the Langendorff perfusion system, all rat hearts were equilibrated for 30 min and were consequently subjected to 30 min global ischemia followed by a 60 min reperfusion period. Global ischemia occurred by complete cessation of the perfusate to the heart (closing the 3-way stop cock) and additional KHB was placed within the jacket which stabilised the temperature of the heart at 37°C for a period of 30 min. Upon reperfusion, the flow is returned to the heart by releasing KHB present inside the jacket followed by opening the 3-way stopcock.

The functional parameters (LVESP, LVEDP, LVDP, HR, and CF) of the heart were monitored **(Figure 19)**.

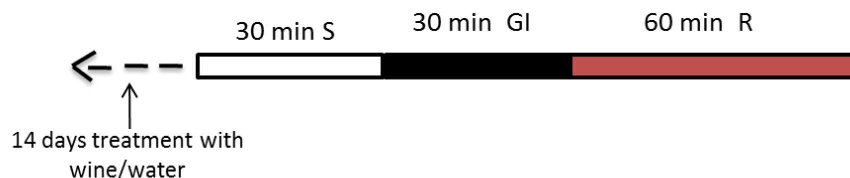

Figure 19: Schematic representation of the perfusion protocol for wine-induced cardioprotection in global ischemia/reperfusion injury. Abbreviations used: S=Stabilization, GI=Global Ischemia, R=Reperfusion, I.S=Infarct Size.

Melatonin (75ng/L) was perfused for 15 min followed by a 10 min wash out period before global ischemia as previously described (Lamont *et al.*, 2011). Additional groups were perfused with TAK242 (500nM), a TLR4 inhibitor. TAK242 was perfused for 23 min on its own followed by a 5 min wash-out period before global ischemia **(Figure 20).**

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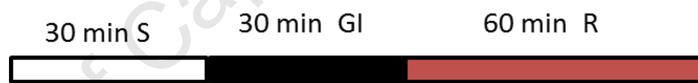
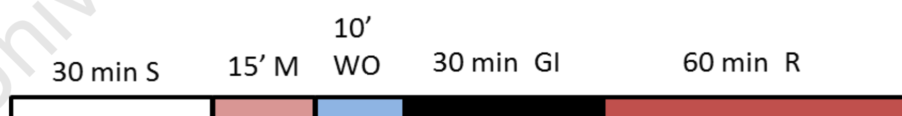
Group 1: Control; n=5



Group 2: Acute treatment with melatonin (75 ng/L); n=3



Group 3: TAK242 (500 nM); n=3

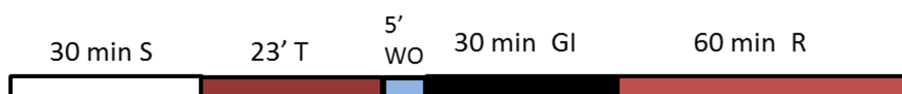


Figure 20: Schematic representation of the perfusion protocol for melatonin-induced cardioprotection in global ischemia/reperfusion. Abbreviations used for all the protocols: S=Stabilisation, M=Melatonin, WO=Wash out, GI=Global Ischemia, R= Reperfusion, T=TAK 242

- **Regional ischemia**

Regional ischemia occurred by placing a 6/0 silk suture around the left coronary artery to form a snare. Before the onset of ischemia, both ends of the suture were placed together and pulled through two shortened 1 ml pipette tips. This occurred until the tips could withstand the beating of the heart and remain as such throughout the entire ischemic period (30 min). After the occlusion, hearts were reperfused for 120 min by simply removing the tips and gently moving the suture in a left to right movement.

Melatonin (75ng/L) was perfused for 15 min followed by a 10 min wash out period before regional ischemia. Additional groups were perfused with TAK242 (500 nM). TAK242 was perfused for 23 min on its own followed by a 5 min wash-out period before regional ischemia. When TAK242 and melatonin were perfused together, TAK242 was perfused for 3 min alone followed by 15 min perfusion with melatonin, and 5 min of TAK242 alone. For western blot analysis additional hearts were perfused following the protocol above. However, all the hearts were collected before the onset of ischemia **(Figure 21)**.

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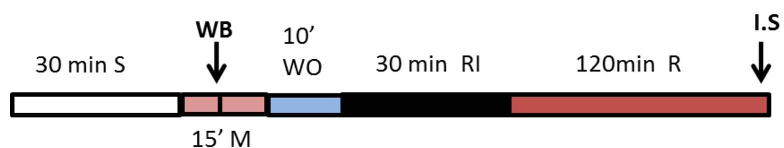
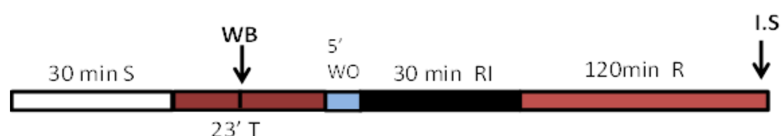
Group 1: Control; n=18

Group 2: Acute treatment with melatonin (75ng/L); n=7



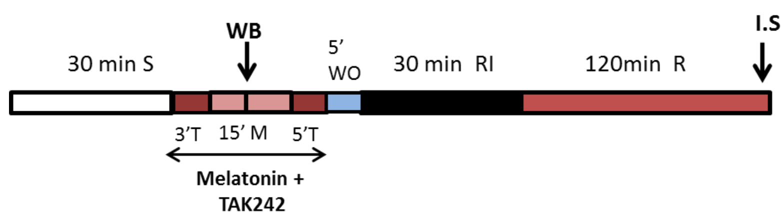
(For Western blot analysis, tissue was collected after 7 min of melatonin perfusion.)

Group 3: Acute administration of TAK242 (500 nM); n=5



(For Western blot analysis, tissue was collected after 10 min of TAK242 perfusion.)

Group 4: Acute co-administration of melatonin (75ng/L) and TAK 242(500 nM); n=7



(For Western blot analysis, tissue was collected after 7 min of melatonin + TAK 242 perfusion)

Figure 21: Schematic representation of the perfusion protocol for TLR-4-induced cardioprotection via melatonin in regional ischemia/reperfusion. Abbreviations used for all the protocols: S=Stabilisation, M=Melatonin, T=TAK 242, WO=Wash out, RI=Regional Ischemia, R=Reperfusion, I.S=Infarct size analysis, WB=Collection of tissue for western blot

2.6 Infarct size

At the end of the reperfusion period, the coronary artery was reoccluded and 0.5 ml of 2% Evans blue was perfused slowly through the heart to delineate the area at risk. All hearts were frozen for 24hours at -20°C and then cut into 2mm thick slices. The slices were stained by incubation in sodium phosphate buffer containing 1% w/v of triphenytetrazolium chloride pH 7.4 at 37°C for 15 min. Slices were fixed in 10% v/v formaldehyde solution for 24hours for visualisation. Infarct size and the area at risk were determined with planimetry (Planimetry+, Boreal Software, Norway) as described previously (Lacerda *et al*., 2009). Infarct size was expressed as a percentage of the area at risk (Lecour *et al*., 2005).

2.7 Western blots

Additional hearts were perfused for western blot analysis. Rat hearts were harvested and perfused with different acute treatments (melatonin, TAK242 and melatonin + TAK242). The control hearts were collected before ischemia, the melatonin hearts were collected after 7 min of melatonin perfusion, the TAK242 hearts were collected after 10 min of TAK242 perfusion and hearts perfused with a combination of melatonin with TAK242 were collected after 7 min of melatonin perfusion **(Figure 22)**. Hearts were then freeze clamped prior to the onset of

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ischemia (after removal of atria). Hearts were stored at -80 ^{o}C for later use in the western blot analysis to explore the levels of total and tyrosine phosphorylated STAT-3 (Y-pSTAT-3) in the heart.

2.7.1 Protein extraction

Frozen tissue was pulverised with a hammer. The powdered tissue (~ 100 mg) was homogenised with a Polytron in a solution which consists of 20mM HEPES, 2.5mM MgCl2, 100µM EDTA, 20mM β- glycerophosphate, 0.05% Triton X-100 (cytosolic extract), 1% Triton X-100 (nuclear extract), 500µM dithiothereitol (DTT), 1mM phenylmethylsulfonyl fluoride (PMSF) and 75mM NaCl and centrifuged at 10000g for 5 min. The supernatant was removed and characterised as the crude cytosolic extract. Thereafter, 300µl of lysis buffer was added to the pellet and centrifuged at 15000g for 30 min. The supernatant was removed and was characterised as the crude nuclear extract.

2.7.2 Protein quantification

The Lowry Assay was used to quantify the concentration of proteins (Lowry *et al.*, 1951). Bovine Serum Albumin (BSA) standard curve ranged of concentrations 0, 10, 60, 100 g/L and the absorbance was measured at 250 nm.

2.7.3 SDS-PAGE of extracted proteins

Lysates were diluted in Laemmli sample buffer and boiled for 5 min. 100µg of proteins were separated on 10% sodium dodecyl sulphate (SDS) PAGE using standard Bio-Rad Mini-PROTEAN II System for two hours at 120 volts and transferred to PVDF membrane (Amersham Bioscience Hybond PRPN 303F) overnight **(Figure 22)**.

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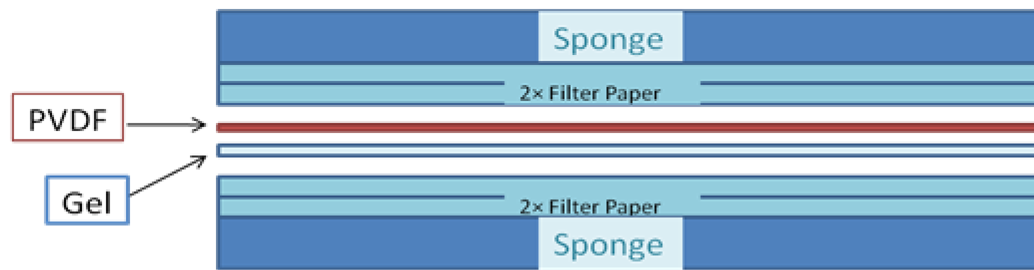

Figure 22: Schematic representation of electro-blotting transfer procedure. PVDF: Polyvinylidene fluoride (From Zulfah Albertyn, 2010)

2.7.4 Immuno-blotting and detection

The membranes were stained with Ponceau Red stain (Ponceau solution, Sigma-Aldrich, Schnelldorf, Germany) to check for equal loading. Membranes were blocked with 5% milk in tri-buffered saline and 0.1% Tween (TBS-Tween) for two hours. The membranes were probed with primary antibodies for p-STAT-3 (200µg/ml) and total-STAT-3 (200µg/ml) or β-actin (200µg/ml) overnight at 4°C. The primary antibody p-STAT-3Tyr 705(sc-7993, rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, California, USA) or total-STAT-3 (H-190, sc-7179, rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, California, USA) was washed off with TBS-T three times for 5 min. The membranes were then probed with the secondary antibody (donkey anti-rabbit IgG-HRP, sc-2313, 200µg/0.5ml, Santa Cruz Biotechnology, Santa Cruz, California, USA) for 1 hour. The secondary antibody was washed off with TBS-T three times for 5 min before protein detection. The membranes were then stripped before being probed with β-actin. The membranes were washed twice with distilled water for 5 min. The membranes were then stripped with 0.2 M NaOH at room temperature for 5 min, followed by two washes with distilled water for 5 min. The membranes were probed with β-actin overnight at 4°C. The β-actin primary antibody (c-11, sc-1615, goat polyclonal IgG (200µg/ml), Santa Cruz Biotechnology, Santa Cruz, California, USA) was washed off with TBS-T three times for 5 min before incubation with the secondary antibody for one hour. The secondary antibody was washed off with TBS-T (0.1% Tween), three times for 5 min before protein detection. Detection was accomplished with enhanced chemiluminescence (ECL). The emission of light was based on the interaction between luminal and the horse-radish peroxidase (HRP) conjugated to the secondary antibody for qualitative or semi-quantitative analysis. Relative densitometry was determined with the use of a computerised software package, JPEG.

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2.8 Statistical analysis

All values are expressed as the mean±SEM. Multiple comparisons were made with one-way analysis of variance (ANOVA) followed by a post (Dunnett) statistical test. The statistical analysis was implemented in Graph pad Instat. Statistical significance was set at $*p<0.05$ and $**p<0.01$.

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CHAPTER 3: RESULTS

3.1 South African wines and Cardioprotection

3.1.1 Melatonin Content

The presence of melatonin in South African wines was determined via HPLC-SPE analysis **(Table 5)**.

Table 5: Melatonin content (pg/mL) of South African wine and French wine.

Wine	Melatonin (pg/mL wine)
Bordeaux Cabernet Sauvignon 2007: French	50± 20
Paul Cluver Pinot Noir 2009	100 ± 20
Springfield Pinot Noir 2010	0 ±0
Idiom Nebbiolo 2010	490 ± 90
Springfield Sauvignon Blanc 2010	340 ± 80
Mulderbosch Syrah 2009	290 ± 50
Meerhof Chardonnay 2008	70 ± 10
First Cape Cabernet Sauvignon 2010	90 ± 20

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The content of melatonin in South African wines varied function to the grape variety. It was detected in both red and white wines.These amounts are similar to those described by Iriti et al that were detected in Italian wines (Iriti *et al.*, 2006). Interestingly, the two Pinot Noirs used were from different estates and the melatonin content could only be detected in the Paul Cluver Pinot Noir 2009.

3.1.2 Cardioprotective effect of South African wines in isolated hearts subjected to ischemia/reperfusion injury.

i. Cardioprotective effect of South African wines on the haemodynamic parameters of the heart

To explore the cardioprotective effect of South African wines, rats were pre-treated with wine (equivalent to 2-3 glasses/day) for 14 days and on the 15th day hearts were subjected to 30 min of global ischemia followed by 60 min of reperfusion on the isolated Langendorff perfusion system.

a.The effect of a chronic pre-treatment with South African wine on left ventricular developed pressure (LVDP) in hearts subjected to ischemia/reperfusion injury.

Table 6: The effect of chronic pre-treatment of wine on left ventricular developed pressure (mmHg) in isolated hearts subjected to ischemia/reperfusion injury

Global ischemia: Chronic treatment with wine Haemodynamic Parameters				
LVDP (mmHg)	Pre-Ischemic	5' Reperfusion	30' Reperfusion	60' Reperfusion
Control	88.8±2.4	12.7±3.5	16.0±3.4	23.2±4.0
Bordeaux Cabernet Sauvignon 2007- French	97.4±4.8	8.8±3.4	16.8±2.2	28.9±4.4
Paul Cluver Pinot Noir 2009	93.1±3.3	19.5±5.7	30.7±7.7	46.1±6.6 **
Springfield Pinot Noir 2010	94.5±3.1	9.5±2.9	19.6±4.0	37.5±2.0
Idiom Nebbiolo 2010	83.4±2.4	14.6±6.1	24.5±2.9	34.3±3.6
Springfield Sauvignon Blanc 2010	88.9±0.68	11.6±1.9	19±3.5	33.6±3.7
Mulderbosch Syrah 2009	86.1±4.0	10±3.1	23.1±7.0	44.1±2.0 *
Meerhof Chardonnay 2008	92.2±6.4	13.0±3.3	23.9±2.5	34±5.4
First Cape Cabernet Sauvignon 2010	91.3±3.1	13.7±3.7	30.7±5.0	36.3±4.4

Parameters measured prior to ischemia (pre-ischemic), parameters measured after ischemia at 5, 30 and 60 min reperfusion of LVDP= Left Ventricular Developed Pressure. *$p<0.05$ vs. control group at the same time point, **$p<0.01$ vs. control group at the same time point

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Pre-treatment with all the South African wines did not alter the LVDP compared to the untreated control hearts prior to ischemia. After 30 min of ischemia, the untreated control hearts had a drop in LVDP. After 5 min of reperfusion and at 30 min of reperfusion, pre-treatment with South African wines did not improve LVDP compared to the untreated controls.

Pre-treatment with Paul Cluver Pinot Noir 2009 and Mulderbosch Syrah 2009 significantly improved the LVDP at 60 min of reperfusion compared to untreated control hearts (p=0.0037 and p=0.0083, respectively). At the same time point, pre-treatment with; Bordeaux Cabernet Sauvignon 2007, Springfield Pinot Noir 2010, Idiom Nebbiolo 2010, Springfield Sauvignon Blanc 2010, Meerhof Chardonnay 2008 and First Cape Cabernet Sauvignon 2010 did not significantly improve the LVDP compared to the untreated control hearts **(Table 6)**.

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b.The effect of a chronic pre-treatment with South African wines on the heart rate in hearts subjected to ischemia/reperfusion injury.

Table 7: The effect of chronic pre-treatment of wine on heart rate (beats/min) in isolated hearts subjected to ischemia/reperfusion injury

Global ischemia: Chronic treatment with wine Haemodynamic Parameters				
Heart Rate (beats/minute)	Pre-Ischemic	5' Reperfusion	30' Reperfusion	60' Reperfusion
Control	277.0±8.9	126.0±27.1	180.3±22.6	162.1±24.9
Bordeaux Cabernet Sauvignon 2007- French	301.9±12.0	177.7±71.3	262.5±14.0 *	265.5±11.3*
Paul Cluver Pinot Noir 2009	259.5±8.7	207.3±30.0	224.1±15.9	238.3±10.2*
Springfield Pinot Noir 2010	261.2±13.5	217.3±38.6	237.5±13.1	249.8±12.2*
Idiom Nebbiolo 2010	292.9±6.7	129.8±58.0	260.3±18.8	249.8±25.6
Springfield Sauvignon Blanc 2010	291.7±27.4	245.9±37.3	277.6±25.2 *	272.1±20.5**
Mulderbosch Syrah 2009	310.5±15.1	174.4±53.6	241.7±16.3	273.4±12.5**
Meerhof Chardonnay 2008	254.7±11.6	200.0±49.7	193.9±18.2	194.7±19.4
First Cape Cabernet Sauvignon 2010	256.3±12.5	143.5±15.8	233.9±14.6	251.3±18.0*

Parameters measured prior to ischemia (pre-ischemic), parameters measured after ischemia at 5, 30 and 60 min reperfusion of heart rate (beats/min). *p<0.05 vs. control group at the same time point, **p<0.01 vs. control group at the same time point

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Pre-treatment with all the South African wines did not alter the heart rate (beats/min) compared to the untreated control hearts, prior to ischemia. After 5 min of reperfusion, untreated control hearts had a drop in heart rate. At 30 min of reperfusion, the untreated control hearts displayed a slight increase in heart rate. Pre-treatment with Bordeaux Cabernet Sauvignon 2007 and Springfield Sauvignon Blanc 2010 significantly improved the heart rate compared to the untreated control hearts (p<0.05 Vs. Control). At the same time point, pre-treatment with Paul Cluver Pinot Noir 2009, Springfield Pinot Noir 2010, Idiom Nebbiolo 2010, Mulderbosch Syrah 2009, Meerhof Chardonnay 2008 and First Cape Cabernet Sauvignon 2010 did not significantly improve the heart rate compared to the untreated control hearts.

At 60 min of reperfusion, the untreated control hearts displayed a slight increase in heart rate. At 60 min of reperfusion, pre-treatment with the Bordeaux Cabernet

Sauvignon 2007, Paul Cluver Pinot Noir 2009, Springfield Pinot Noir 2010, Springfield Sauvignon Blanc 2010, Mulderbosch Syrah 2009 and First Cape Cabernet Sauvignon 2010 significantly improved the heart rate (p=0.0010) compared to the untreated control hearts. At the same time point, pre-treatment with Idiom Nebbiolo 2010 and Meerhof Chardonnay 2009 did not significantly improve the heart rate compared to the untreated control hearts **(Table 7)**.

c.The effect of a chronic pre-treatment of South African wines on rate pressure product (RPP) in hearts subjected to ischemia/ reperfusion injury.

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After 5 min of reperfusion, the untreated control hearts had a rate pressure product % (expressed as a percentage of baseline value before ischemia) or functional recovery % of 7.4±1.6%. After 5 min of reperfusion, pre-treatment with Paul Cluver Pinot Noir 2009 significantly improved the rate pressure product to 17.4±3.5% compared to the untreated control hearts (*p<0.05). At the same time point, pre-treatment with Bordeaux Cabernet Sauvignon 2007, Springfield Pinot Noir 2010, Idiom Nebbiolo 2010, Springfield Sauvignon Blanc 2010, Mulderbosch Syrah 2009, Meerhof Chardonnay 2008 and First Cape Cabernet Sauvignon 2010 did not significantly improve the rate pressure product compared to the untreated control hearts.

At 30 min of reperfusion, the untreated control hearts had a slight increase in the rate pressure product to 12.2±2.4%. Pre-treatment with Paul Cluver Pinot Noir 2009 significantly improved the rate pressure product to 29.1±7.0% compared to the untreated control hearts (*p<0.05). At the same time point, pre-treatment with Bordeaux Cabernet Sauvignon 2007, Springfield Pinot Noir 2010, Idiom Nebbiolo 2010, Springfield Sauvignon Blanc 2010, Mulderbosch Syrah 2009, Meerhof Chardonnay 2008 and First Cape Cabernet Sauvignon 2010 did not significantly improve the rate pressure product compared to the untreated control hearts.

At 60 min of reperfusion, the untreated control hearts had a rate pressure product of 17.5±3.0%. Pre-treatment with Paul Cluver Pinot Noir 2009, Springfield Pinot Noir 2010, Mulderbosch Syrah 2009 and First Cape Cabernet Sauvignon 2010 significantly improved the rate pressure product to 46.0±6.5%, 38.4±2.9%, 45.5±2.9% and 39.3±5.0% respectively, compared to the untreated control hearts

(**p<0.01). At the same time point, pre-treatment with Bordeaux Cabernet Sauvignon 2007, Idiom Nebbiolo 2010, Springfield Sauvignon Blanc 2010 and Meerhof Chardonnay 2008 did not significantly improve the rate pressure product compared to the untreated control hearts **(Figure 23)**.

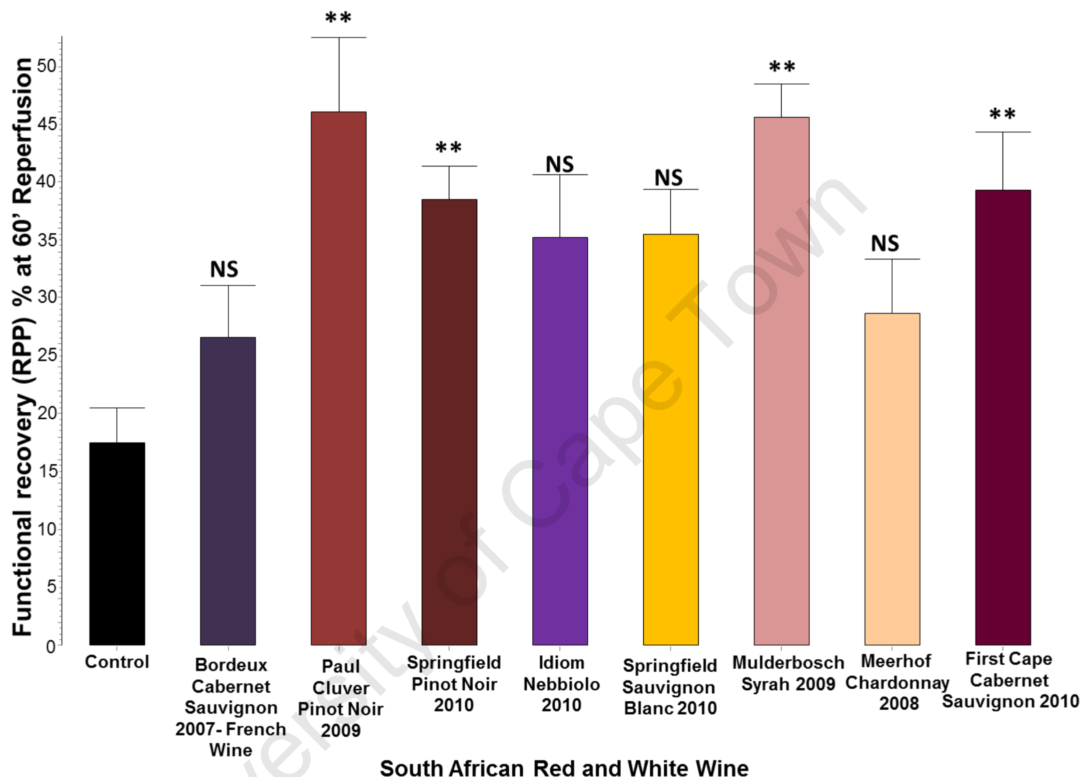

Figure 23: The effect of a chronic consumption of wine on functional recovery (RPP %) during 60 min of reperfusion. Control: n=14, Bordeaux Cabernet Sauvignon 2007 (French wine): n=5, Paul Cluver Pinot Noir 2009: n=10, Springfield Pinot Noir 2010: n=7, Idiom Nebbiolo 2010: n=4, Springfield Sauvignon blanc 2010: n=4, Mulderbosch Syrah 2009: n=5, Meerhof Chardonnay 2008: n=7, First Cape Cabernet Sauvignon 2010: n=6; **p<0.01 vs. Control group at the same time point, NS: Not Significant

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d. Correlation between melatonin content and cardioprotective effect in South African wines

Figure 24 demonstrates the relationship between the melatonin content in South African wines and the functional recovery subsequent to animals pre-treated with wine subjected to an ischemia/reperfusion insult. It was observed that there is no obvious correlation between the melatonin content in South African wines and the functional recovery of the treated hearts.

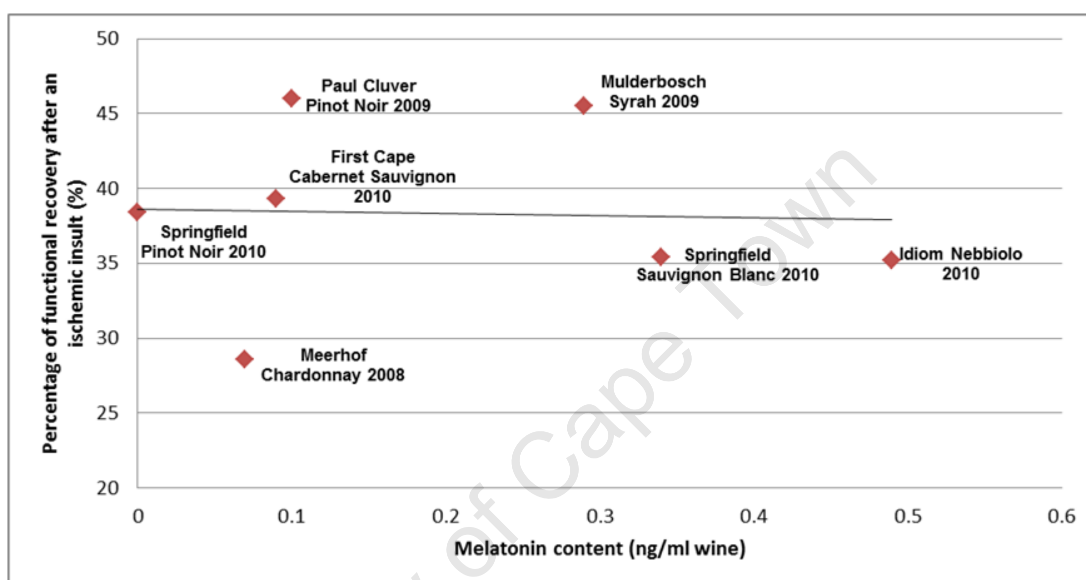

Figure 24: The relationship between functional recovery (RPP) expressed in % after an ischemic insult and melatonin (ng/mL wine) concentration in South African wines.

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3.2 Role of Toll-like receptor 4 in cardioprotection

To explore the role of Toll-like receptor 4 (TLR-4) in melatonin-induced cardioprotection, rat hearts perfused with an acute treatment of either melatonin alone, TLR-4 inhibitor (TAK242) alone or with both melatonin and TAK242 underwent ischemia/reperfusion injury. Subsequently, all hearts were subjected to either 30 min of global ischemia followed by 60 min of reperfusion or 30 min of regional ischemia followed by 120 min of reperfusion.

3.2.1 Effect of TLR-4 on functional recovery

- **Global ischemia**

Table 8: The effect of TLR-4 on isolated Wistar rat hearts subjected to global ischemia/reperfusion injury.

	Heamodynamic parameters: Global Ischemia			
	Pre-Ischemic	**5' Reperfusion**	**30' Reperfsusion**	**60' Reperfusion**
LVDP (mmHg)				
Control	90.4±4.3	8.8±2.3	8.0±3.6	20.0±1.3
Melatonin	90.7±3.5	16.0±10.6	25.3±7.1 *	41.0±3.6 **
TAK242	84.0±2.3	14.7±1.3	38.7±6.1 *	48.0±4.0 **
Heart Rate (beats/min)				
Control	328.0±15.0	248.0±32.0	192.0±54.3	272.0±20.0
Melatonin	293.3±13.3	173.3±104.1	280.0±23.1	266.7±13.3
TAK242	280.0±0.0	213.3±26.7	240.0±23.1	293.3±13.3
Coronary Flow (ml/min)				
Control	9.2±0.8	6.5±0.4	8.0±6.3	7.6±0.8
Melatonin	10.5±1.8	7.9±1.3	8.7±0.7	7.2±0.6
TAK242	8.0±0.0	8.4±0.6	8.5±0.5	7.6±1.2
Rate Pressure Product (beats/min × mmHg)				
Control	29792.0±2447.7	1952.0±352.0	2176.0±1160.0	5376.0±337.9
Melatonin	26507.0±373.3	3360.0±1730.7	7413.3±2503.8	10960.0±1257.3 **
TAK242	23520.0±646.6	3093.3±384.6	9440.0±1710.8 *	14133.0±1584.8 **

Parameters measured prior to ischemia (pre-ischemic), parameters measured after ischemia at 5, 30, and 60 min of reperfusion (post-ischemic). LVDP= left ventricular developed pressure, *$p < 0.05$ vs. Control group at the same time point,**$p < 0.01$ vs. Control group at the same time point

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Hearts perfused acutely with either melatonin (75ng/L) or TAK242 (500nM) did not alter the LVDP (mmHg) compared to the control hearts prior to ischemia. After 30 min of global ischemia and 5 min of reperfusion, hearts perfused acutely with either melatonin or TAK242 did not alter the LVDP compared to the control hearts. At 30 min of reperfusion, hearts perfused acutely with either melatonin or TAK242 displayed a significant improvement in LVDP compared to the untreated control hearts (*$p<0.05$). At 60 min of reperfusion hearts perfused acutely with either melatonin or TAK242 displayed a significant improvement in LVDP compared to the untreated control hearts (**$p<0.01$) **(Table 8)**.

Prior to global ischemia, 5 min reperfusion, 30 min reperfusion and 60 min reperfusion; hearts perfused acutely with either melatonin or TAK242 did not alter the heart rate (beats/min) compared to the untreated control hearts.

An acute perfusion with either melatonin or TAK242 did not alter the coronary flow at baseline, 5 min reperfusion, 30 min reperfusion and 60 min reperfusion compared to the untreated control hearts **(Table 8)**.

Before the onset of global ischemia, the control hearts had a rate pressure product of 29792.0±2447.7 beats/min×mmHg. Hearts perfused acutely with either melatonin or TAK242 did not alter the rate pressure product compared to the untreated control hearts prior to ischemia (p=N.S). After 30 min of global ischemia and 5 min of reperfusion, the control hearts had a rate pressure product of 6.51±1.01 %. Hearts perfused acutely with either melatonin or TAK242 did not alter the rate pressure product compared to the control hearts (p=N.S). After 30 min of reperfusion, the control hearts had a rate pressure product of 7.04±3.27 %. An acute treatment with either melatonin or TAK242 significantly improved the rate pressure product % ($p<0.05$ Vs. control)

At 60 min of reperfusion, the control hearts had a rate pressure product of 18.27±1.11 %. Hearts perfused acutely with either melatonin or TAK242 improved the rate pressure product compared to the control hearts (41.49±5.39 % and 60.33±5.39 %, respectively) (**$p<0.01$) **(Figure 25)**.

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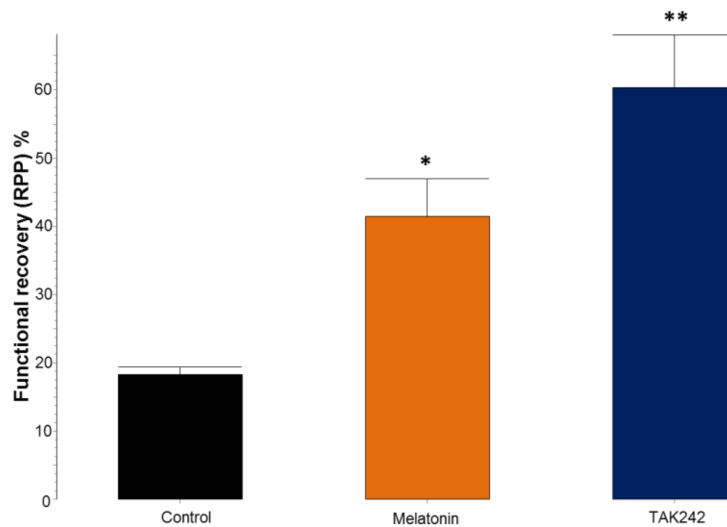

Figure 25: The effect of an acute treatment of melatonin (75ng/L) and TAK242 (500nM) on rate pressure product (expressed as a percentage of baseline) after 60 min of reperfusion. Control: n=5, Melatonin: n=3, TAK242: n=3; *$p<0.05$ Vs. Control at the same time point; **$p<0.01$ Vs. Control at the same time point

Since the TAK inhibitor exerts a cardioprotective effect on its own, the melatonin + TAK242 group could not be studied further and we, therefore, changed our model to a regional ischemia model to test the role of TLR-4 in melatonin-induced cardioprotection.

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- **Regional ischemia**

Table 9: The effect of TLR-4 on isolated Wistar rat hearts subjected to regionalischemia/reperfusion injury.

	Haemodynamic parameters					
	Pre-Ischemia	30' Ischemia	5' Reperfusion	30' Reperfusion	60' Reperfusion	120' Reperfusion
LVDP (mmHg)						
Control	89.73±2.14	59.07±5.58	77.15±7.68	73.64±7.02	71.82±5.91	64.19±5.92
Melatonin	88.84±1.71	48.8±6.60	65.34±13.33	65.56±11.18	59.93±10.98	54.59±10.68
TAK 242	92.84±6.94	55.84±14.66	63.14±16.52	73.46±3.78	53.78±14.21	60.24±56.70
Melatonin + TAK 242	91.57±4.95	55.39±6.23	67.27±13.87	74.93±7.72	69.49±6.87	65.03±6.66
Heart Rate (beats/min)						
Control	305.27±6.43	272.62±18.36	281.01±18.93	287.48±18.98	300.46±8.50	287.93±11.36
Melatonin	305.54±15.46	303.31±22.22	250.56±47.70	296.64±14.44	299.07±13.48	311.73±17.96
TAK 242	307.84±16.36	251.72±65.34	262.82±68.17	318.76±19.76	250.66±65.75	285.38±50.83
Melatonin + TAK 242	300.57±16.69	275.94±25.44	209.66±48.14	287.9±20.08	269.43±26.48	282.24±20.75
Coronary Flow (ml/min)						
Control	9.07±0.32	5.36±0.61	8.4±0.77	7.29±0.68	7.6±0.67	6.82±0.59
Melatonin	10.29±0.62	5.49±0.58	6.97±1.31	8.74±0.80	7.4±0.40	5.71±1.04
TAK 242	9.6±0.96	6.56±1.50	7.2±2.11	8.32±1.27	5.76±1.46	6±1.12
Melatonin + TAK 242	10.11±1.13	4.81±1.17	5.83±1.72	6.29±1.31	6.34±0.74	5.71±0.61

Parameters measured prior to ischemia (pre-ischemic), parameters measured after ischemia at 5, 30, 60 min and, 120 min of reperfusion (post-ischemic). LVDP= left ventricular developed pressure

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Hearts perfused acutely with melatonin (75ng/L), TAK242 (500nM) or a co-administration of melatonin with TAK242 did not alter the LVDP compared to the control hearts prior to ischemia, at 30 min of reperfusion, at 60 min of reperfusion and at 120 min of reperfusion **(Table 9)**.

Preceding the onset of regional ischemia, hearts perfused acutely with melatonin (75ng/L), TAK242 (500nM) or a co-administration of melatonin with TAK242 did not alter the heart rate compared to the control hearts prior to ischemia, at 30 min of regional ischemia, at 30 min of reperfusion, at 60 min of reperfusion, and at 120 min of reperfusion **(Table 9)**.

At baseline, 30 min of regional ischemia, 30 min of reperfusion, 60 min of reperfusion, and 120 min of reperfusion, hearts perfused acutely with melatonin (75ng/L), TAK242 (500nM) or a co-administration of melatonin with TAK242 did not alter the coronary flow compared to the untreated control hearts **(Table 9)**.

3.2.2 TLR-4 and its effect on infarct size

After control hearts were subjected to 30 min of regional ischemia followed by 120 min of reperfusion, they presented an infarct size (infarct area/ area at risk of heart × 100) of 62.6±2.7%. The hearts perfused acutely with melatonin significantly reduced the infarct size to 34.7±2.8% compared to the control hearts (**p<0.01). The hearts perfused with TAK242 did not reduce the infarct size (68.6±5.3%) compared to the control hearts (62.6±2.7 %) (p=0.98) and the infarct sparing effect of melatonin was reduced to 49.2±6.5% (*p<0.05 vs. Control) in the presence of TAK242 **(Figure 26)**.

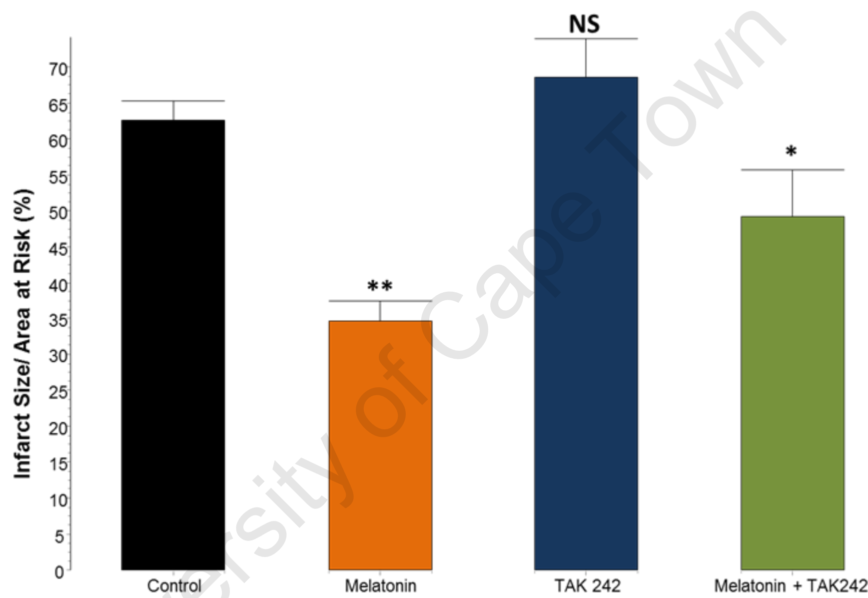

Figure 26: The effect of an acute administration of TAK242 with or without melatonin on infarct size. Regional Ischemia: Control, n=18; Melatonin n=7, TAK242 n=5, Melatonin+TAK242 n=7 (**p<0.01 vs. Control; *p<0.05 vs. Control)

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3.2.3 Western blot analysis

To explore the role of TLR-4 in melatonin-induced cardioprotection via activation of the SAFE pathway, isolated rat hearts were perfused acutely with melatonin in the presence or absence of TAK242. Before the onset of ischemia hearts were collected to determine the phosphorylation of STAT-3 at its tyrosine residue in both the cytoplasm and nucleus.

Research in the literature, has shown that after ischemia/reperfusion injury, there is a translocation of STAT-3 from the cytosol to the nucleus (Fuglestag *et al*., 2008; Lacerda *et al*., 2009). As such, Western blot analysis was performed in both the nucleus and cytosolic fractions.

- **STAT-3 phosphorylation in the cytoplasm**

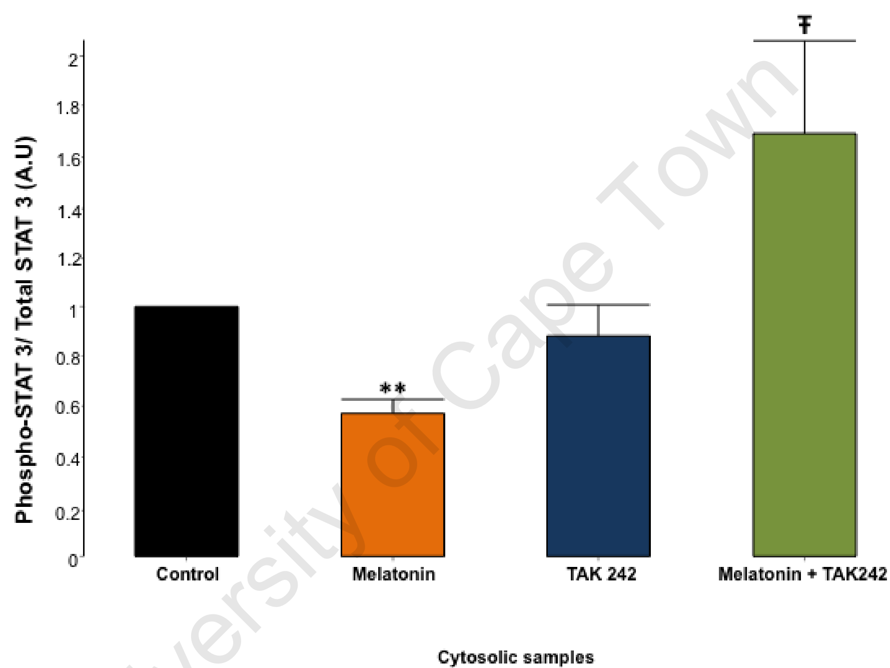

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Figure 27: The effect of melatonin with or without TAK242 on tyrosine phospho-STAT-3 (Y-p-STAT3) activation in the cytoplasm. n= 4 for all groups. A.U= Arbitrary Units, **p<0.01 vs. Melatonin + TAK at the same time point, Ŧp<0.05 vs. Control at the same time point

The phosphorylation of STAT-3 at its tyrosine residue was detected in the cytosol in hearts subjected to ischemia/reperfusion injury.There was a reduction in STAT-3 levels in the cytosol in hearts perfused with melatonin compared to untreated control hearts. Hearts perfused acutely with TAK242 did not demonstrate an increase in phosphorylation of STAT-3 at its tyrosine residue compared to the control hearts. Interestingly, hearts perfused acutely with melatonin in the presence or absence of TAK242 had an overall significant effect on the phosphorylation of STAT-3 at its tyrosine residue in the cytoplasm (p=0.0113). Furthermore, hearts co-administered

with melatonin and TAK242 caused a significant phosphorylation of STAT-3 at its tyrosine residue in the cytoplasm compared to the control hearts **(Figure 27)**.

- **STAT-3 phosphorylation in the nucleus**

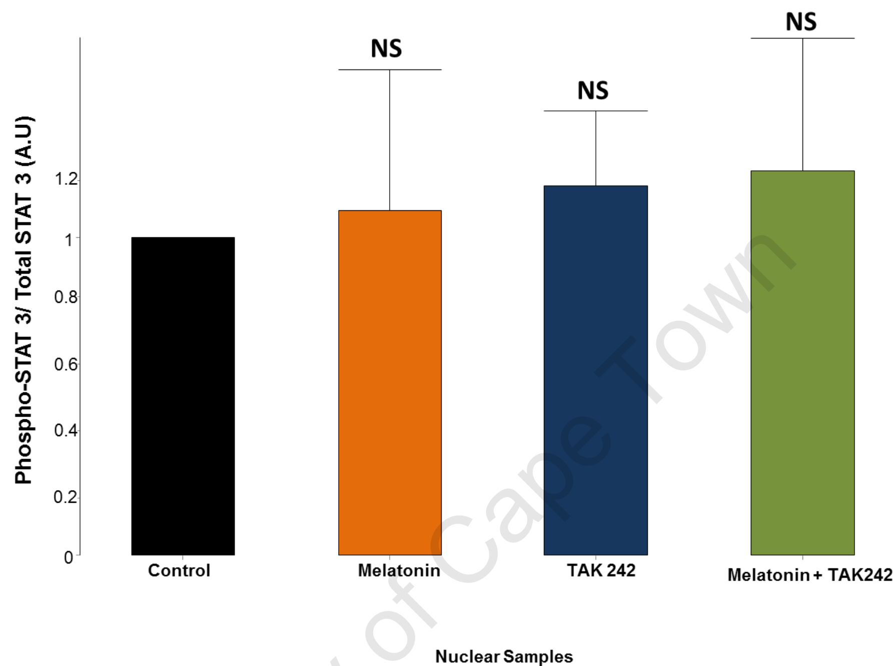

Figure 28: The effect of melatonin with or without TAK242 on tyrosine phospho-STAT-3 (Y-p-STAT3) activation in the nucleus. n= 4 for all groups. NS= Not Significant, A.U= Arbitrary Units

The hearts pre-treated with melatonin in the presence or absenceTAK242 had no significant effect on the phosphorylation of STAT-3 at its tyrosine residue in the nucleus (**Figure 28)**.

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CHAPTER 4: DISCUSSION

4.1 Summary of results

The aim of this work was to explore whether South African red and white wines confer a cardioprotective effect in relation to their melatonin content. Furthermore, we aim to explore the role of TLR-4 in melatonin-induced cardioprotection. Our results demonstrate that a chronic and moderate pre-treatment with both South African red and white wines improves the cardiac function of rats subjected to a myocardial ischemia/reperfusion injury. After measuring the melatonin content in each South African wine, our data did not support a relationship between the melatonin content present in the wine and their cardioprotective effect. Our experiments conducted with an acute treatment of melatonin (75 ng/L-concentration found in red wine) suggest that the cardioprotective effect of melatonin involves the activation of TLR-4. Hence, a co-treatment of a TLR-4 inhibitor (TAK242) with melatonin abolished the cardioprotective effect of melatonin. In addition, the inhibitor reduced the phosphorylation of STAT-3, therefore, suggesting that melatonin may activate STAT-3 via TLR-4 activation. This study provides a novel mechanistic approach into wine-induced cardioprotection and offers novel therapeutic tartgets against myocardial ischemia/reperfusion injury.

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4.2 South African wine confers a cardioprotective effect against ischemia/reperfusion injury

4.2.1 South African wine improves cardiac function after ischemia/reperfusion injury

To the best of our knowledge, our study was the first to demonstrate the cardioprotective effect of South African red wines and white wines against ischemia/reperfusion injury. In our study, the wine consumed by the rats was diluted with their drinking water. Previous studies, delineating the role for wine induced-cardioprotection, used grape seed extract or resveratrol and not wine. A study conducted in our laboratory has exhibited, for the first time, the role of wine-induced cardioprotection after a chronic and moderate pre-treatment of a French wine

(Lamont *et al.*, 2012). Adding red wine by gavage would have better mimicked the human drinking pattern of red wine, but for practical reasons, adding the wine to the drinking water daily was the best choice.

We investigated the cardioprotective effect of eight wines, of which seven were South African (from a coastal to an inland location) and, one from France (Bordeaux) (used as a positive control). All the wines were from different vintages ranging from 2008 to 2010.

In our study, we observed that hearts pre-treated with either South African red or white wines improved the haemodynamic parameters (i.e. heart rate, LVDP and functional recovery: expressed as a percentage of baseline value before ischemia) of the heart after an ischemia/reperfusion insult.

Contrary to the general concept that only red wine is cardioprotective, this study observed that South African white wines protected the heart against ischemia/reperfusion injury to a similar extent than red wines.However, not all wines confer protection. This finding could be due, in part, to the characteristics of each wine, which can be largely influenced by the agrometeorological conditions (i.e. influential factors in agricultural crop development are the weather, climate, horticulture, animal husbandary and forestry). According to Conradie and colleagues, the physical property of the soil plays a pivotol role in the quality of wine (Conradie *et al.*, 2002). Furthermore, the environment in which the grapes grow in play a vital role in developing the characteristics of each wine (Dirninger *et al.*, 1998). Upon production of the must, yeasts added to the wine for the fermentation process can influence the quality of wine, as well (Romano *et al.*, 2003). There are several vineyards in the Western Cape region of South Africa. The location of these vineyards varies, geographically, from areas close to the coast (eg. Hermanus) to in land areas (eg. Robertson Valley). This current study found that coastal wines and inland wines could be cardioprotective to a similar extent.

However, our study has shown that pre-treatment with the Paul Cluver Pinot Noir 2009 or the Mulderbosch Syrah 2009 improved the heart rate, LVDP and functional recovery compared to the untreated controls. Pretreatment with most of the other studied wines, either only improved the heart rate or the LVDP compared to the

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untreated controls. Chardonnay and Nebbiolo failed to protect the heart. This evidence suggests that South African wines confer different degrees of cardioprotection against ischemia/reperfusion injury.

4.2.2 Melatonin content in South African wines and its role in cardioprotection

To delineate the exact components that may explain the cardioprotective effect of wine, the melatonin content in each studied wine was measured. Research in the literature proposes that the amount of melatonin content present in each wine could be influenced by, grape growth stage at time of harvest (Vitalini *et al*., 2011), environmental stresses (Dirninger *et al*., 1998), agrometeorological conditions (Burkhardt *et al*., 2001) fermentation process (Rodriguez-Naranjo *et al*., 2011; Gomez et al., 2012), wine storage, vintage and pesticide treatment (Iriti *et al*., 2006).

The literature reports comparable amounts between white and red wine (see review Fernández-Mar *et al*., 2012; Stege *et al*., 2010; Rodriguez-Naranjo *et al*., 2011). In our present study, we confirmed these studies.

In our study, the highest melatonin content was present in the Idiom Nebbiolo 2010. Iriti and colleagues explored the melatonin content in different Italian wines and found that the melatonin content was higher in an Italian Nebbiolo than the other Italian wines (Iriti *et al*., 2006). Amongst the two Pinot Noirs used in our study we observed the absence of melatonin in the Springfield Pinot Noir 2010. Another finding in this study is that of the varying concetrations of melatonin found in the two white wines. As discussed previously, according to Vitalini and colleagues, the presence of melatonin in grapes are greatest in the seeds (Vitalini *et al*., 2011). In contradiction, another study proposes that, the presence of melatonin only starts to occur during the wine making process, specifically during the fermentation stage (Rodriguez-Naranjo *et al*., 2011). This evidence indicates that the grape varietal is not a definitive means of suggesting that melatonin would be present in certain wines.

Furthermore, in this study, we found that the melatonin content in the wine did not correlate with the functional recovery results obtained in pre-treated rat hearts at 60 min of reperfusion. Consequently, the amount of melatonin present in the wine could have been inadequate to attain a positive correlation with the functional recovery of the heart after an ischemic insult. Interestingly, recent research

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proposes that there is structurally similar melatonin isomers expressed during the fermentation stage in wine. Unfortunately, its physiological role is yet to be explored (see review Tan *et al.*, 2012), but it would be of interest to explore whether a correlation between the amount of melatonin isomers in wine and a cardioprotective effect of the wine exists.

Research studies displaying the cardioprotective effect of wine denote alcohol and resveratrol as the cardioprotective components found in wine. The amount of alcohol present in each wine in this study was similar. Therefore, we commend that alcohol by its self could not have caused one wine to be more cardioprotective than the other. A measurement for resveratrol content was performed in the wines of the current study, as many studies have proposed resveratrol to be cardioprotective against ischemia/reperfusion injury.

Interestingly our study revealed an inverse correlation between melatonin content and resveratrol was determined. South African wines (red and white) with high melatonin content had a low resveratrol content and vice versa (data not shown). The Springfield Sauvignon Blanc had high melatonin content but low resveratrol content; nevertheless, this wine markedly improved the functional parameters of the heart after ischemia/reperfusion injury. Also, the Paul Cluver 2009 had low melatonin content with high resveratrol content and improved the functionality of the heart after an ischemic insult. From the evidence above, melatonin and resveratrol present in wine may interact synergistically to provide a cardioprotective effect against ischemia/reperfusion injury.

4.3 Role of TLR-4 in melatonin-induced cardioprotection

To determine the role of TLR-4 in melatonin-induced cardioprotection, a TLR-4 inhibitor, namely TAK242, was used in this study. TAK242 inhibits TLR-4 selectively and in a concentration-dependent manner (Kawamoto *et al.*, 2008).

An acute treatment with melatonin or TAK242 significantly improved the LVDP and functional recovery at 60 min of reperfusion in a global ischemia model. In support

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of our findings, Cha *et al.*, showed that TLR-4 deficient hearts subjected to global ischemia/reperfusion injury displayed a significant improvement in both LVDP and functional recovery postischemia compared to wild-type hearts (Cha *et al.*, 2008). However, there was no significant improvement in the haemodynamic parameters when a regional ischemia model was used.

The model of global ischemia mimics clinical conditions of cardiac arrest observed during cardiac surgery. Cardiac arrest accounts for sudden cessation of pumping action of the heart as well as a disappearance in arterial blood pressure which causes either ventricular fibrillation or ventricular standstill. On the other hand, the model of regional ischemia mimics clinical conditions of acute myocardial infarction. Thus, the physical strain inflicted on the heart differs between global ischemia and regional ischemia. This physical strain causes an infarction of cardiac tissue either globally or regionally. Therefore, our data suggest that the role of TLR-4 may differ in regional versus global ischemia.

In a regional ischemia model, TAK242 inhibited melatonin-induced cardioprotection. In the presence of melatonin with TAK242, the infarct size of hearts subjected to ischemia/reperfusion injury was reduced compared to those treated with melatonin alone. To the best of our knowledge, the data in this study shows for the first time that TLR-4 is a possible down-stream target for melatonin-induced cardioprotection.

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4.4 TLR-4 plays a role in activation of the SAFE pathway

There are many research studies in the literature focussing on possible downstream targets activated by TLR-4 in response to ischemia/reperfusion injury. In the absence of TLR-4, hearts subjected to ischemia/reperfusion injury resulted in a reduction of pro-inflammatory response activators such as JNK, (Shimamoto *et al.*, 2006; Chong *et al.*, 2004) and NFκB (Shimamoto *et al.*, 2006). These data, therefore, proposes that TLR-4 could possibly promote ischemic injury via its ability to activate inflammatory signalling molecules.

In the presence of LPS following ischemia/reperfusion injury, TLR-4 promotes cardiomyocyte protection via a TRIF-independent mechanism (Wang *et al.*, 2011) as well as Akt phosphorylation (Ha *et al.*, 2008; Li *et al.*, 2004).

Moreover, TLR-4 activation increases the expression of cytokines such as TNF-α, which subsequently leads to inflammation. A recent study has implicated TNF-α expression in the activation of the JAK/STAT-3 pathway, which are key cardioprotective components in the activation of the SAFE pathway (Lamont *et al.*, 2011). Here, the hearts of TNF-α knock-out mice and STAT-3 knock-out mice failed to be protected against ischemia/reperfusion injury in the presence of melatonin (Lamont *et al.*, 2011), therefore, suggesting a link between TNF-α expression and melatonin.

Our study, suggests that melatonin-induced cardioprotection via TLR-4 activation and subsequent activation of tyrosine-p-STAT-3 (Y-pSTAT-3). There was no evident translocation of Y-pSTAT-3 from the cytosol to the nucleus in any of the treated groups. However, we observed that an acute treatment with melatonin resulted in a reduction of Y-pSTAT-3 in the cytosol compared to the control. Furthermore, an acute treatment with TAK242 caused a slight increase in Y-pSTAT-3 in the cytosol compared to an acute treatment with melatonin. Interestingly, an acute co-administration of melatonin with TAK242 displayed an increase in the phosphorylation of Y-pSTAT-3 in the cytosol, compared to the control group. Therefore, TLR-4 activation could in part, be dependent on the presence of melatonin.

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4.5 Limitations to the study

In this study, an *in vitro* system was used to determine the effect of a chronic consumption of wine on cardioprotection; these data would need to be confirmed using an *in vivo* model of ischemia/reperfusion injury to take into account the effect from blood components and other organs.

In addition, measurement of melatonin levels in the wine could not provide us with clear evidence for its role in wine-induced cardioprotection.

As the content of other components is different from one wine to the other, these components may also play a role in wine-induced cardioprotection. Furthermore, we used an acute treatment of melatonin to explore the mechanisms involved in melatonin-induced cardioprotection. These experiments would need to be confirmed in a chronic setting.

To determine the role of TLR-4 in melatonin-induced cardioprotection, we used a pharmacological inhibitor, which may have underlying functions that are still unknown. The use of TLR-4 knock-out mice should be considered.

Similarly, the time point chosen to collect tissue for Western blot analysis may not have been optimal to detect phospho-STAT-3. Different time points should be further explored. Moreover, we observed phospho-STAT-3 translocation in the cytoplasm, which suggests that the phospho-STAT-3 might have translocated to the mitochondria. To confirm this translocation, phospho-STAT-3 within the mitochondria sub-fraction should be measured.

Furthermore, in our study, the experimental sample size was small and to gain statistical significance we would need to increase the sample size.

4.6 Future prospects

To confirm the role of melatonin in wine-induced cardioprotection, we propose that the use of a synthetic wine, with or without melatonin. The cardioprotective effect of the wine containing melatonin could be compared to a wine having the same composition, but lacking melatonin. A melatonin inhibitor can be given in conjunction with the synthetic wine.

These experiments should be counducted in TLR-4 knock-out models to confirm the role of TLR-4 in wine-induced cardioprotection.

To assess the role of SAFE pathway, tissue collection can be performed for western blot analysis with the suggestions mentioned earlier. Thus, the protein expression levels of TNF-α, NF-κB can be assessed as well as both serine-p-STAT3 and Y-p-STAT3 should be detected in the different cellular subfractions.

4.7 Conclusion

In conclusion, this study is the first to show the protective role of both red and white South African wines against myocardial ischemia/reperfusion injury. Our data do not suggest a critical role for melatonin in the cardioprotective effect of South African wines. However, melatonin-induced cardioprotection may involve TLR-4 by activating STAT-3 and hence the SAFE pathway. The understanding of these cardioprotective pathways may lead to the development of novel therapeutic targets against IHD.

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APPENDIX: SOLUTIONS AND BUFFERS

1. HEMODYNAMIC PARAMETERS OF ISOLATED PERFUSED RAT HEARTS

1.1 KREBS HENSELEIT BUFFER FOR LANGENDORFF PERFUSION

NaCl	118.0 mM
$NaHCO_3$	25.2mM
Glucose	11.0 mM
KCl	4.7 mM
$MgSO_4.7H_2O$	1.2 mM
KH_2PO_4	1.2 mM
$CaCl_2.2H_2O$	1.2 mM
pH 7.4	

2. SOLUTIONS AND BUFFERS FOR PROTEIN EXTRACTION AND QUANTIFICATION

2.1 PROTEIN EXTRACTION

2.1.1 LYSIS FOR PKB/IRSI (30ml)

Tris-HCl, pH 7.4; EGTA	3ml
HEPES (20mM)	1.8ml
EDTA (100µM)	600µl
β-glycerophosphate (20mM)	6ml
NaCl (75mM)	0.1125ml
PMSF (1mM)	60µl
DTT (0.5mM)	1.5ml
Triton X-100 (0.05%-cytosolic)	45µl
Triton X-100 (1%-nuclear)	10ml

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2.2 PROTEIN QUANTIFICATION

2.2.1 CTC REAGENT FOR PROTEIN ASSAY

(a) Na_2CO_3	20g
(b) $CuSO_4.5H_2O$	0.2g

K_2Tatrat

Add dH_2O up to 100ml for both (a) and (b) separately.

Add (a) to (b) slowly, while mixing to prevent precipitation.

2.2.2. 10% SDS

SDS	20g

Add dH_2O up to 200ml.

2.2.3. 0.2M NaOH

NaOH	0.4g

Add dH_2O up to 200ml.

3. SOLUTIONS AND BUFFERS FOR SODIUM DODECYL SULPHATE POLYACRLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

3.1. 10% AMMONIUM PERSULPHATE (APS)

APS	20g

Add dH_2O up to 200ml.

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3.2. 3X Laemelli (loading buffer)

Tris	3.03g
SDS	8.8g
Glycerol	20g
Bromophenol Blue	0.025g
Na in dH_2O	75ml

Adjust pH to 6.6 with HCl.

Add 150µl of β-mercaptoethanol in 850µl of the above solution.

3.3. 10X Running (Tank) buffer

Tris	60.6g
Glycine	288g
SDS	20g

Add dH_2O up to 2L.

3.4. 10% Resolving gel

dH_2O	9.8ml
1.5M Tris-HCl, pH 8.8	5ml
20% SDS	100µl
Acrylamide	5ml
10% APS	100µl
TEMED	40µl

3.5. 10% Stacking gel

dH_2O	7.5ml
0.5M Tris-HCl, pH 8.8	3ml
20% SDS	60µl

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Acrylamide	1.5ml
10% APS	30µl
TEMED	40µl

4. SOLUTIONS AND BUFFERS FOR WESTERN BLOTTING AND IMMUNODETECTION

4.1 Transfer buffer

Tris	6.06g
Glycine	28.8g
20% methanol	400ml

Add dH_2O up to 1L.

4.2. 10X Tris-buffered saline (TBS)

Tris 48.4g

Dissolve in 500ml dH_2O and adjust pH to 7.6 by adding HCl.

NaCl 160g

Add dH_2O up to 1L.

4.3. TBS-Tween 20 (0.01%)

Tween 20	1ml
TBS	1000ml

4.4. Blocking solution (5% milk powdered solution)

Powdered milk	5g
TBS-T solution	100ml

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