EXPLORING THE CARDIOPROTECTIVE EFFECT OF SYNTHETIC WINE IN LONG EVANS RATS

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ABBREVIATIONS

AAPH	2,2-azobis(2-amidinopropane) dihydrochloride
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BCL-2	B-cell lymphoma-2
BHT	Butylated hydroxytoluene
Ca	Calcium
cAMP	cyclic adenosine monophosphate
CF	Coronary flow
cGMP	cyclic guanosine monophosphate
CHD	Coronary heart disease
CVD	Cardiovascular disease
FC	Folin Ciocalteu
GAE	Gallic acid equivalent
GI	Global ischemia
GPCR	G protein coupled receptor
GSH	Glutathione
HDL	High density lipoprotein
HR	Heart rate
HSP	Heat shock protein
IHD	Ischemic heart disease
IR	ischemia-reperfusion
K	Potassium
LDH	lactate dehydrogenase
LDL	low density lipoprotein
LMIC	Low-and middle income countries
LVDP	Left ventricular developed pressure
LVEDP	Left ventricular end diastolic pressure
LVESP	Left ventricular end systolic pressure
MDA	Malondialdehyde
Mel	Melatonin
MI	Myocardial Infarction
ml	Milliliters
mM	Millimolar
MT1/2/3	Melatonin receptor(s)
Na	Sodium

NAD	Nicotinamide adenine dinucleotide
NCD	Non-communicable disease
NIH	National Institutes of Health
NO	Nitric Oxide
NOS	Nitric oxide synthase
ORAC	Oxygen radical absorbance capacity
pH	Hydrogen potential
PKC	Protein kinase C
R	Reperfusion
Resv	Resveratrol
RGJ	Red grape juice
RISK	Reperfusion Injury Salvage Kinase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPP	Rate pressure product
S	Stabilization
SAFE	Survivor Activating Factor Enhancement
SEM	Standard error of the mean
SOD	Superoxide dismutase
SPT	8-(p-sulfophenyl)theophylline
SSA	Sub-Saharan Africa
STAT-3	Signal transducer and activator of transcription-3
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TE	Trolox equivalent
TNFα	Tumour necrosis factor alpha
TTC	Triphenyltetrazolium chloride
USA	United States of America
WHO	World Health Organisation
YPD	Yeast peptone dextrose

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ABSTRACT

Background: Moderate and chronic consumption of red wine protects against cardiovascular disease. Wine is a complex matrix containing multiple molecules whose concentrations can vary from one bottle to another. Therefore, the delineation of the putative cardioprotective components in wine such as alcohol, resveratrol and melatonin is very challenging when using commercially available red wine.

Aim: We aimed to use synthetic wine, whose composition is well characterized, to explore whether the presence of alcohol, resveratrol and melatonin (as found in commercial wines) contributes to the cardioprotective effect of chronic and moderate consumption of red wine (equivalent to 2 glasses of wine/day) in an animal model. Additionally, we hypothesized that synthetic wine enriched with resveratrol and melatonin confers cardioprotection via improvement of overall antioxidant profile.

Methods: The drinking water of male Long Evans rats was supplemented with synthetic wine (12% alcohol v/v) with/without resveratrol (100µg/L) and/or melatonin (0.075µg/L) to a final concentration corresponding to the concentration found in 2 glasses of wine per day. After 14 days of treatment, hearts were perfused on the Langendorff system and subjected to 30 minutes global ischemia followed by 60 minutes of reperfusion. Functional parameters were recorded throughout the experiments and infarct size was measured at the end of the protocol. Functional recovery (heart rate x left ventricular developed pressure), measured at 60 minutes of reperfusion, was expressed as a percentage of baseline value. Blood plasma was collected when harvesting the heart to measure total antioxidant capacity, lipid peroxidation (thiobarbituric acid reactive substances (TBARS) assay), superoxide dismutase (SOD) and catalase activity.

Results: Control hearts subjected to ischemia/reperfusion presented a functional recovery of 11±2%. Pre-treatment with synthetic wine with/without melatonin or resveratrol did not improve functional recovery (15±6%,12±1%, 19±4% respectively, n.s. vs control). However, addition of both melatonin and resveratrol in synthetic wine improved functional recovery to 32±5% ($p<0.01$ vs control). No significant changes were identified amongst the treatments when quantifying antioxidant enzyme activity in the form of SOD or catalase. However, synthetic wine enriched with both melatonin and resveratrol significantly increase lipid peroxidation (TBARS: 16.3±0.9 µmol/mL vs. 8.5±0.4 µmol/mL for control; $p<0.01$).

Conclusion: In conclusion, our data strongly suggest that the presence of melatonin and resveratrol in wine is required for cardioprotection with chronic moderate consumption of wine. Our data do not provide a role for the antioxidant activities of the wine as a possible mechanism for cardioprotection. Our findings support that the combination of melatonin with resveratrol, at the concentration found in wine, may be considered as a therapeutic agent for cardioprotection.

A. INTRODUCTION

1.1 Prevalence of cardiovascular disease

Non-communicable diseases (NCDs) can be defined as all the diseases, which are non-infectious and non-transferable among people. NCDs include heart disease, most cancers, diabetes, chronic kidney disease and Alzheimer's disease amongst others. The World Health Organisation (WHO) reports that NCDs are the leading cause of death in the world, representing over 60% of all deaths (WHO, 2011). Of the 57 million global deaths in 2008, 36 million were due to NCDs. NCDs have surpassed communicable diseases as the world's major disease burden, with cardiovascular disease (CVD) remaining the principal global cause of death, accounting for 17.3 million deaths per year (Smith, Collins et al. 2012) (Figure.1).

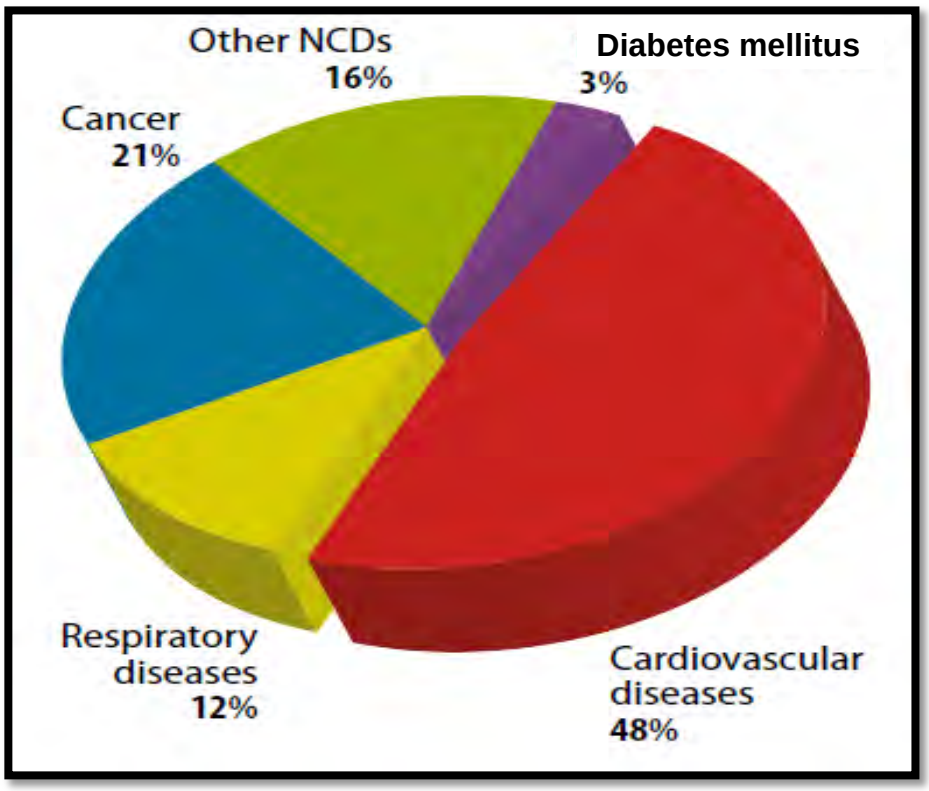

Figure 1: Distribution of global non-communicable disease by cause of death in both sexes

From *WHO/WHF/WSO: Global Atlas on Cardiovascular Disease Prevention and Control. Geneva. World Health Organisation. 2011*

CVD is expected to grow to more than 23.6 million by 2030 if no adequate prevention/therapies are put in place (Mendis, Puska et al. 2012). CVD associated morbidity

predominantly affects both men and women in low-and middle income countries (LMIC) (Figure.2) where 80% of these deaths occur predominantly at younger ages compared with higher income countries (Abegunde, Mathers et al. 2007). CVDs have reached epidemic proportions in Sub-Saharan Africa (SSA) (Alberts, Urdal et al. 2005, Gersh, Sliwa et al. 2010, Ikem, Sumpio 2011). Previously, conditions such as ischemic heart disease (IHD) and angina were considered a rarity in SSA (Walker, Sareli 1997, Seedat, Mayet et al. 1992). However, there has been a recent increase in both prevalence and incidence in the number of patients presenting with IHD (Mensah 2008), in part as a result of decreased access to effective and equitable healthcare services in those countries (Abegunde, Mathers et al. 2007, Smith, Collins et al. 2012). Other key factors underlying the increasing prevalence of CVD in developing countries such as South Africa include: the ongoing change in nutrition patterns, the increase in weight and obesity, the decrease in physical activity and high levels of stress as well as the increase of urbanization (Alberts, Urdal et al. 2005). These lifestyle factors, associated with obesity and poor nutrition, lead to the emergence of well determined risk factors for IHD (Yusuf, Hawken et al. 2004).

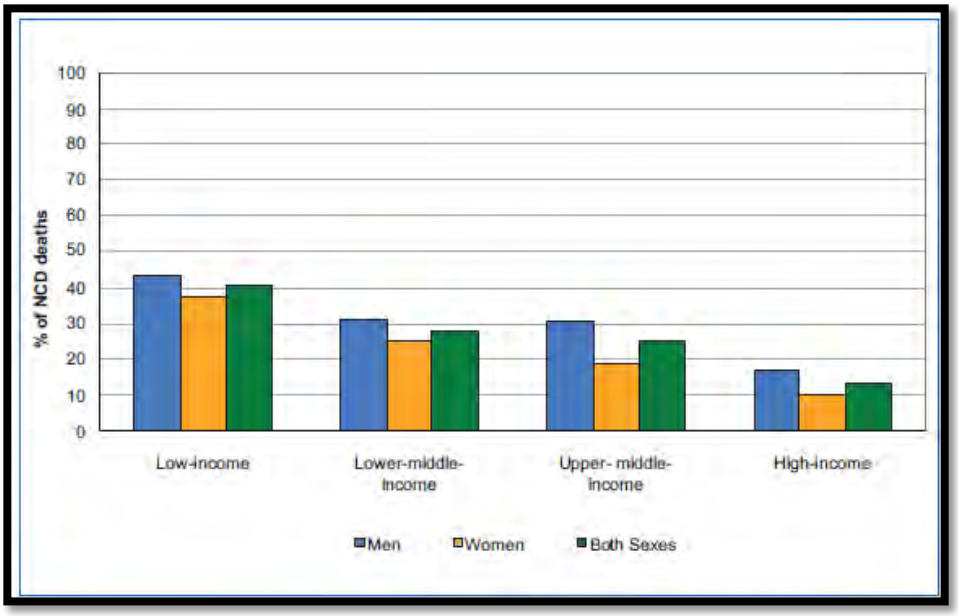

Figure 2: Proportion of deaths due to CVD by country income level

From (Laslett, Alagona et al. 2012)

In South Africa, although the continued epidemic of human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) is responsible for 29%and 36% of deaths in men and women, respectively (Shisana, Rehle et al.2013), CVD represents a major burden. IHD, hypertension and stroke account for more than a third of deaths in the population older than 65 years (Gaziano, Thomas A 2010). The burden of CVD is predicted to increase

substantially in South Africa over the next decade if measures are not taken to slow down this burden (Mayosi, Flisher et al. 2009).

1.2 Ischemia/ reperfusion

1.2.1 Definition

IHD is a condition that affects the supply of blood to the myocardium. Myocardial ischemia occurs when blood flow to the myocardium is decreased by a partial blockage of the coronary arteries and thus, reduces the myocardium oxygen supply. Complete blockage of the coronary arteries results in deficient oxygenation and nutrient supply to the cardiomyocytes, leading to damage and necrosis of the tissue, which is known as a myocardial Infarction (MI) (Opie & Seedat, 2005)(Figure.3).

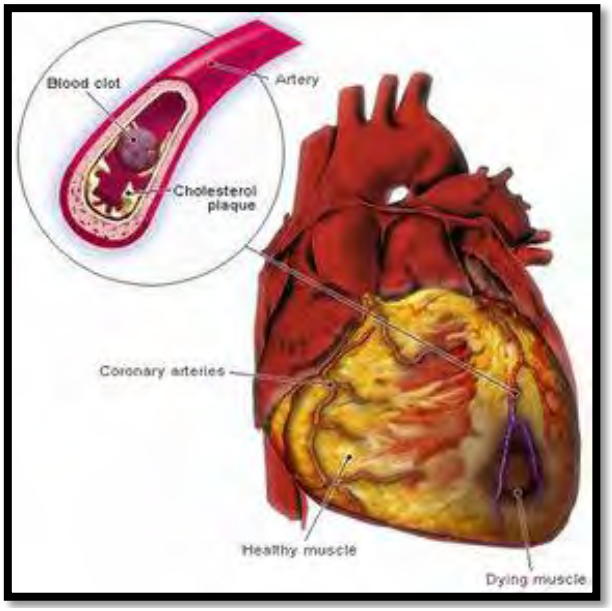

Figure 3: Graphical representation of acute myocardial infarction

From: www.medicinenet.com/heart_attack/page2.htm

In a large majority of cases, myocardial ischemia is confined to specific regions of the myocardium and is termed regional ischemia. However, there are some conditions which can lead to the entire myocardium becoming ischemic (global ischemia), such as open-heart surgery in patients undergoing coronary artery bypass grafting, valve replacement therapy or heart transplant. While restoring blood flow to the ischemic area is essential to save threatened cardiomyocytes either by the use of pharmacological therapy with thrombolytics or through physical means with angioplasty, reperfusion is paradoxically associated with a

cascade of deleterious effects in cardiomyocytes leading to ischemia/reperfusion (IR) injury (Yellon, Hausenloy 2007).

1.2.2 Pathophysiology

Ischemic injury

The absence of oxygen halts cellular oxidative phosphorylation, leading to mitochondrial membrane depolarization, adenosine triphosphate (ATP) depletion, and inhibition of mitochondrial contractile function (Lesnefsky, Tandler et al. 1997, Reimer, Hill et al. 1981). This process is aggravated by the breakdown of available ATP as a result of ATP hydrolysis and increase in mitochondrial inorganic phosphate (Halestrap, Clarke et al. 2004). In the absence of oxygen, cardiomyocyte metabolism changes to anaerobic glycolysis, resulting in accumulation of lactate which reduces intracellular pH (Avkiran, Marber 2002, Kloner, Bolli et al. 1998). These changes lead to modifications in cardiomyocyte physiology and structure, including mitochondrial and sarcolemmal injury and alterations in intracellular calcium handling (Meissner, Morgan 1995). If the ischemic insult is limited in time, the damage is reversible, and restoration of blood flow during this period will lead to recovery of normal function. However, if ischemia persists for an extended period of time, this damage becomes irreversible and cell death occurs. Therefore, early restoration of oxygenated blood to the ischemic myocardium is required to limit infarct size (Simoons, Brand et al. 1985). Ironically, the return of blood can cause further cardiac damage and is referred to as reperfusion injury.

Reperfusion injury

The reintroduction of oxygen and energy into an ischemic cellular environment triggers additional events that produce further cardiomyocyte damage collectively called reperfusion injury. Several factors contribute to reperfusion injury. They include aggregation of inflammatory mediators (Frangogiannis, Smith et al. 2002), calcium overload (Zimmerman, Hülsmann 1966, Garcia-Dorado, Ruiz-Meana et al. 2012) and oxidative stress (Garlick, Davies et al. 1987, Zweier, Flaherty et al. 1987) (Figure. 4). Oxidative stress is a disturbance in the balance between the production of free radicals and antioxidant defences (Sies 2000). Reperfusion of ischemic cells results in the formation of potentially toxic free radicals, including hydroxyl radicals and superoxide anions. Free radicals harm cardiomyocytes directly by altering membrane proteins and phospholipids leading to metabolic and structural changes (Tsai, Sun et al. 2008, Kaplan, Babusikova et al. 2003). For instance, oxygen radicals injure the sarcolemma and may impair contractile function of the cardiomyoctye

(Verma, Fedak et al. 2002).The role of free radicals as a source of significant myocardial damage is further illustrated by studies demonstrating that free radical scavengers, such as superoxide dismutase, administered during reperfusion help preserve myocardial function (Salvemini, Cuzzocrea 2002).

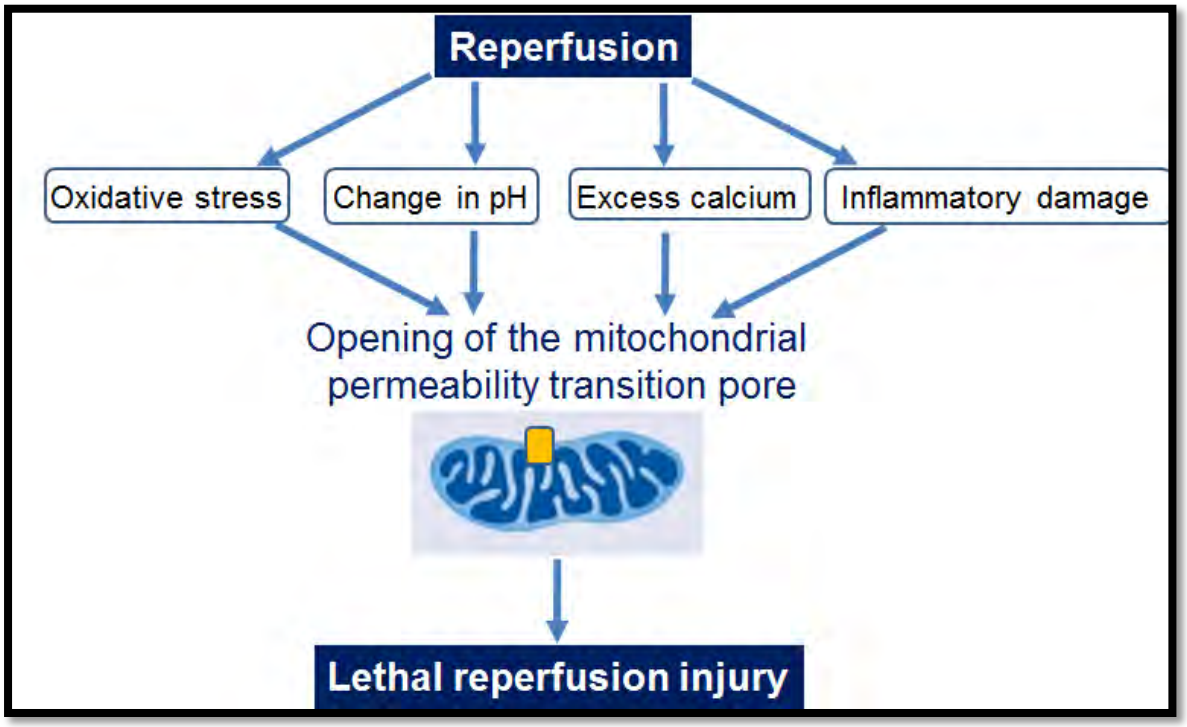

Figure 4: Graphical representation of the pathogenesis of reperfusion injury

From (Lecour, Opie et al. 2012)

Furthermore, during an ischemic period, intracellular calcium increases due to impaired calcium handling and sarcolemmal damage (Meissner, Morgan 1995). This process can be worsened with reperfusion. The restoration of a normal extracellular pH after reperfusion produces a hydrogen gradient across the cell membrane. The sodium/hydrogen exchanger is activated and causes an influx of sodium into the cytosol. Under physiological conditions, the resulting increase in intracellular sodium would be corrected by the sodium/potassium ATPase. However, this channel may not function normally after a period of ischemia due to a lack of energy and structural damage. In this setting, the sodium excess causes the sodium/calcium channel to run in reverse, producing an influx of calcium into the calcium-overloaded cell. Although the pathophysiology of reperfusion injury offers itself to potential therapeutic strategies, few therapies have made their way into clinical practice. A possible

reason for this effect is that various mechanisms contribute to the consequences of IR injury. Thus, the impact of a therapy targeted to a single component of the pathophysiology may be weakened in clinical practice.

Oxidative stress

Oxidative stress is essentially an imbalance between the production of free radicals and the ability of the body to counteract or detoxify their harmful effects through neutralization by antioxidants (Sies 2000). Free radicals are oxygen containing molecules that have one or more unpaired electrons, making them highly reactive with other molecules. Free radicals can chemically interact with cellular components such as DNA, proteins or lipids and steal their electrons in order to become stabilized. This, consequently, destabilizes the cellular components or molecules which then seek an electron from another molecule, triggering a large chain of free radical reactions. Oxygen by-products are relatively unreactive but some of these can undergo metabolism within the biological system to give rise to highly reactive oxidants. For instance, the diatomic oxygen molecule has two unpaired electrons, if this molecule accepts an electron the product is a superoxide radical. Many free radicals have important intermediates such as hydrogen peroxide and peroxynitrite which are not free radicals but which are highly reactive and may be responsible for some of the biological effects attributed to free radicals.

Free radicals and their non-radical reactants are recognized as critical mediators of cardiac injury during ischemia and reperfusion. They have been implicated in cardiac cell death, post ischemic contractile dysfunction and in chronic cardiovascular diseases. The main source of free radicals in IR injury is the mitochondrial electron transport chain. During the reduction of molecular oxygen into biological energy through the four mitochondrial complexes the reduction process is not completely efficient and 1%-4% of available oxygen is normally incompletely reduced and leaks from the electron transport chains in the form of a superoxide radical. This process however becomes significantly accelerated at suboptimal oxygen tensions or after mitochondrial injury and is believed to be the primary source of ROS during IR injury. Cellular hypoxia decreases the activity of cytochrome oxidase. When oxygen is reintroduced, leakage of free radicals from proximal complexes is greatly accelerated. Although it was previously believed that ROS formation occurred primarily or solely at reoxygenation after ischemia, it is now known that significant formation of ROS occurs during ischemia from residual superoxide. This has been demonstrated in cardiomyocytes (Vanden Hoek,Li et al.1997) and in the whole heart (Kevin, Camara et al.2002,Kevin, Novalija et al.2003). Most peroxide is dismutated by manganese superoxide dismutase in the mitochondrial matrix to hydrogen peroxide, which easily diffuses through

mitochondrial membranes. The remainder exits the mitochondria through anion channels in the mitochondrial membrane and is then rapidly converted to hydrogen peroxide in the cytoplasm, either spontaneously, or when catalyzed by copper superoxide dismutase. Hydrogen peroxide is reduced to water and oxygen by catalase and glutathione peroxidase. Alternatively, hydrogen peroxide reacts with transition metals, to generate hydroxyl radical.

Possible future cardioprotective therapies

Currently, there are few promising therapies that can effectively protect the heart against IR injury. Novel approaches as potential adjunctive therapies to current reperfusion strategies such as coronary angioplasty and thrombolytics are required to provide further cardioprotection in the setting of MI to reduce morbidity and mortality. The discovery of ischemic pre-conditioning and post-conditioning have emerged as promising experimental therapies against IR injury. In each case, protection is conferred by repeated short bouts of ischemia interspersed with bouts of reperfusion either before or after a potential lethal ischemia (Murry, Jennings et al. 1986, Zhao, Corvera et al. 2003). Experimental and clinical evidence suggests that both of these strategies are promising therapies to protect the heart against IR injury (Murry, Jennings et al. 1986, Zhao, Corvera et al. 2003, Staat, Rioufol et al. 2005, Thibault, Piot et al. 2008). The signalling pathways that mediate ischemic pre- and post-conditioning can be initiated by a large variety of agents including adenosine (Liu, Thornton et al. 1991), bradykinin (Goto, Liu et al. 1995), sphingolipids (Lecour, Smith et al. 2002) and insulin (Jonassen, Sack et al. 2001). These protective effects are mostly mediated via the activation of Reperfusion Injury Salvage Kinase (RISK) (Yellon, Hausenloy 2007, Yellon, Baxter 1999) and Survivor Activating Factor Enhancement (SAFE) (Lecour 2009) pathways (Figure.5). The RISK pathway includes activation of the prosurvival kinases Akt and extracellular regulated kinase 1/2 (Erk1/2) at the time of reperfusion while the SAFE pathway includes the activation of the cytokine tumour necrosis factor alpha (TNFα) and the transcription factor signal transducer and activator of transcription-3 (STAT-3) (Hausenloy, Lecour et al. 2011).

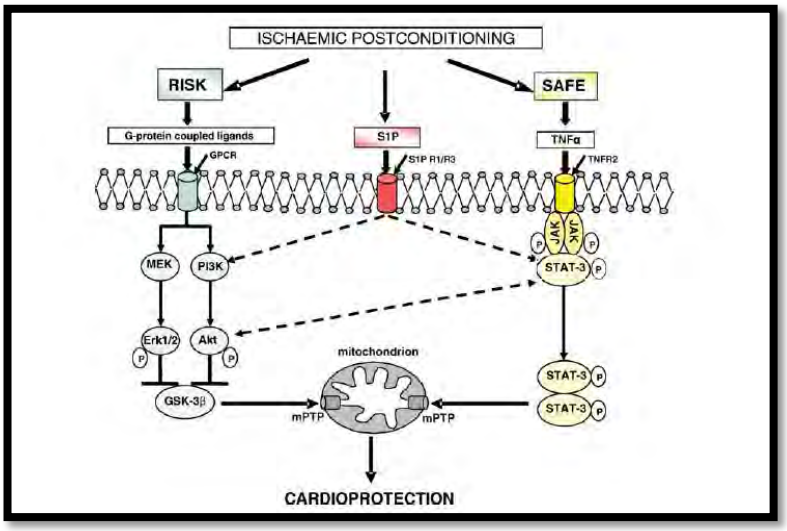

Figure 5: Schematic diagram showing the RISK and SAFE pathway. Both pathways may confer cardioprotection

From Lacerda, Somers et al. 2009

Additionally, ischemic conditioning can also be applied remotely when it is performed by small episodes of ischemia-reperfusion in an organ separate to the heart (Przyklenk, Bauer et al. 1993). However, despite an improved understanding of the pathophysiology of IR injury and encouraging preclinical trials of multiple agents, most of the clinical trials to prevent reperfusion injury have been disappointing. This could be attributed to several reasons; one being that the presence of comorbidities may impact the efficacy of the treatment see review (Heusch 2013). For instance, a study by Engbersen and colleagues demonstrated that although type 1 diabetes patients were more tolerant to forearm IR injury compared to healthy controls, the efficacy of ischemic preconditioning was reduced in patients with type 1 diabetes mellitus (Engbersen, Riksen et al. 2012). Despite these problems, adjunctive therapies to limit IR injury remain an active area of investigation as there is a need for alternative therapies which could limit the damage of IR injury. Thus, targeting lifestyles would present a major benefit as it is inexpensive relative to medication and therefore would be a better approach for low and middle income countries (LMIC).

1.3 Lifestyle factors for cardiovascular disease

The majority of CVD is caused by risk factors that can be controlled, treated or modified such as high blood pressure, cholesterol, overweight/obesity, tobacco use, lack of physical activity and diabetes (Yusuf, Hawken et al. 2004, Steyn, Sliwa et al. 2005). However, there are also some major CVD risk factors that cannot be controlled which include age, gender and family history (Jousilahti, Vartiainen et al. 1999).

With all the factors considered, an unhealthy lifestyle can contribute up to 80% of cardiovascular deaths, whilst modest reductions in risk-associated behaviours can have exponential benefits (Cheng, Zhao et al. 2009). For example, a 0.5% reduction in risk factors can result in as much as a 23% decrease in mortality.

The role of diet is crucial in the development and prevention of CVD. Diet is one of the major factors that can change an individual's risk of acquiring CVD. IHD has a low incidence in some developed countries such as Italy and France, leading to a higher life expectancy in Mediterranean areas compared to Northern European countries and the United States of America (USA) (Martínez-González, García-López et al. 2011, Pierucci, Misciagna et al. 2012). Diet and lifestyle related factors are suggested to be responsible for this advantage (Estruch, Ros et al. 2013). The role of diet in IHD has been well documented for the past century and substantial evidence about the protection by some food items and nutrients is currently available (Valls-Pedret, Lamuela-Raventós et al. 2012, Urpi-Sarda, Casas et al. 2012). The Mediterranean diet, first studied by Keys and Grande in 1959 as the traditional dietary pattern found in areas of Southern Italy and Crete, has attracted significant interest (Keys, Anderson et al. 1965). The traditional Mediterranean diet is characterized by high intake of olive oil, nuts, vegetables, and cereals, a moderate intake of fish and poultry, a low intake of dairy products and wine in moderation (Huxley, Clifton 2013, Willett, Sacks et al. 1995). Several observational studies and secondary prevention trials such as the Lyon diet heart study have consistently shown that adherence to the Mediterranean diet has considerable benefit with respect to cardiovascular risk (Sofi, Abbate et al. 2010, Michel de Lorgeril, Salen et al. 1999). Likewise, the INTERHEART study found five protective factors which may guard against CVD and include: maintaining an ideal weight, regular exercise, not smoking, eating a diet rich in fruit and vegetables as well as a moderate intake of alcohol (2-3 glasses/day) (Yusuf, Hawken et al. 2004) (Figure.6). Until recently, alcohol consumption was frequently overlooked as an important part of the diet. Alcohol, more specifically wine, is an essential component of the Mediterranean diet.

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Lifestyle 'big five'	% of protection from deaths	Mechanism
1. Not smoking	28%	Protects arteries
2. Exercise (30 min or more per day)	17%	Slows the heart, lowers BP
3. Ideal weight	14%	Avoids toxic chemicals released from fat cells
4. Ideal diet	13%	High unsaturated fatty acids, high fruit and vegetables, low red meat
5. Modest alcohol	7%	Anti-stress; alcohol improves blood cholesterol patterns
All five	**79%**	

(Deaths from all causes, including heart attack, stroke, and cancer)

Figure 6: Five lifestyle changes that can protect against cardiovascular death

From Opie.L ,2011 , *Living longer, living better: exploring the heart-mind connection* , Oxford University Press, Oxford.

1.4 Red wine as a cardioprotective agent

1.4.1 Definition of red wine

Wine is an alcoholic beverage made from fermented grapes. Although the primary ingredients of wine include grape berry components including water, the end product yields a complex composition of compounds mostly as a result of the fermentation process. The final product can contain multiple chemical compounds varying in amounts from one part per hundred to parts per billion. To date, more than 1000 compounds have been identified in wine (Soleas, Diamandis et al. 1997). The water content represents from 80 to 85% of the wine total mass and is principally derived from grape berries. The alcohol content differs amongst wines from 9% to 16% and is achieved with fermentation by yeast which converts sugars into alcohol and carbon dioxide. The most abundant alcohol in wine is ethanol. Under standard fermentation conditions, it can sometimes accumulate to nearly 16%, but generally, ethanol concentration ranges between 10 and 13%, depending mainly on the sugar content of the grape, the temperature and the yeast strain. Phenols are derived from the seeds, skin and vine stems and can be produced by yeast metabolism. Phenols affect the taste, appearance, fragrance and antimicrobial properties of the wine. Other compounds found in wine include: organic acids, glycerol, biogenic amines minerals and amino acids, most of these compounds are found in low concentrations of not more than 100mg/L (Waterhouse, 2002) (Figure.7).

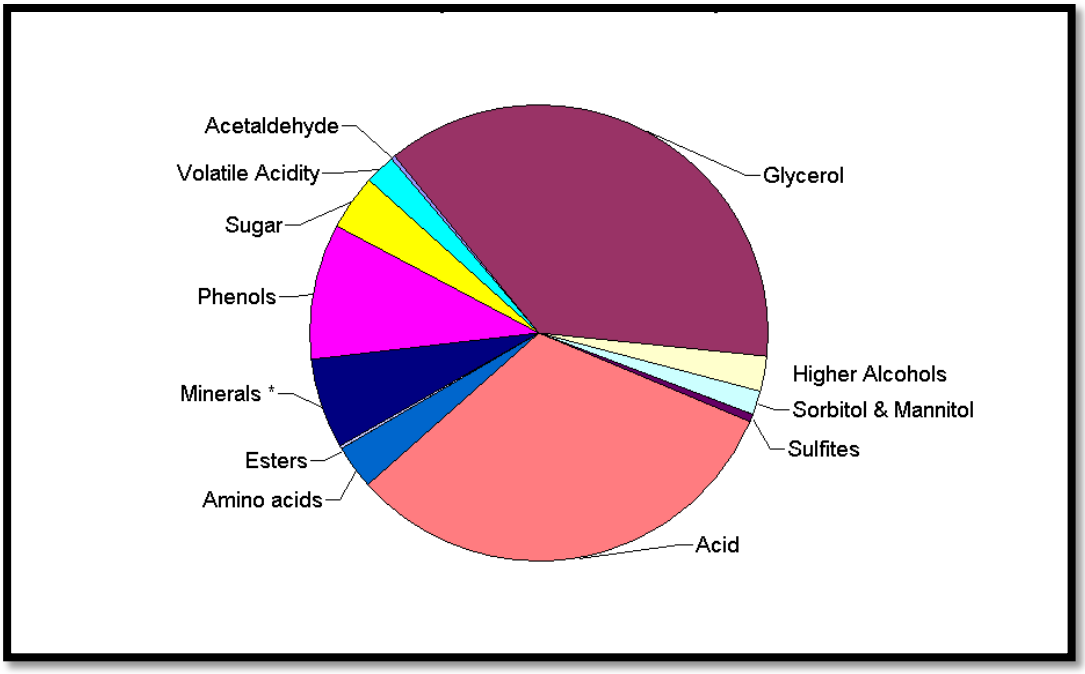


Figure 7: Pie chart showing red wine component composition

From waterhouse.ucdavis.edu/whats-in-wine/red-wine-composition

1.4.2 Cardiovascular benefit of red wine

In recent years, the benefits of daily moderate consumption of alcoholic beverages, particularly of red wine, in the prevention of heart disease has received increasing attention and debate (Klatsky, Armstrong et al. 1990). Alcohol intake from any type of alcoholic beverage appears to be beneficial, but some studies suggest that red wine confers additional health benefits (Thornton, Symes et al. 1983). The cardioprotective effects of red wine have been accredited mostly to several polyphenolic antioxidants. The proposed mechanisms for the observed cardioprotective effects have included, amongst others, inhibition of low density lipoprotein (LDL) cholesterol (Frankel, German et al. 1993), increased high density lipoprotein (HDL) cholesterol (Gaziano, Buring et al. 1993), reduction or inhibition of platelet aggregation (Renaud, Beswick et al. 1992), increased free radical scavengers (Sánchez-Moreno, Larrauri et al. 1999, Saint-Cricq de Gaulejac, Glories et al. 1999) and the increased activation in expression of several cardioprotective oxidative stress inducible proteins including heat shock proteins (HSPs) (Sato, Maulik et al. 2002).

1.4.3 Epidemiological evidence of red wine-induced cardioprotection

1.4.3.1 The French paradox

In 1819, cardiologist Dr Samuel Black noticed a high incidence of coronary obstructions at autopsies in Ireland, however, there was an unexplainable rarity of reports of such obstructions coming from France. He attributed this difference to ‘the French’ habits and

modes of living' see review (Evans 1995). In 1979, St Leger and colleagues drew attention to the cardioprotective properties of wine when they described an inverse relationship between wine consumption and the risk of mortality from CVD in several countries from North America and Europe (St Leger, Cochrane et al. 1979). Almost two decades later in 1991, a popular investigative documentary television program in the United States, called *60 minutes*, introduced to the public that chronic moderate consumption of red wine in France could be responsible for the low incidence of coronary heart disease in this country, despite an increased intake of saturated fat comparable to other developed countries (Renaud, de Lorgeril 1992) (Figure.8). This observation became known as the "French paradox" and was first published in the Lancet in 1992 (Renaud, de Lorgeril 1992).

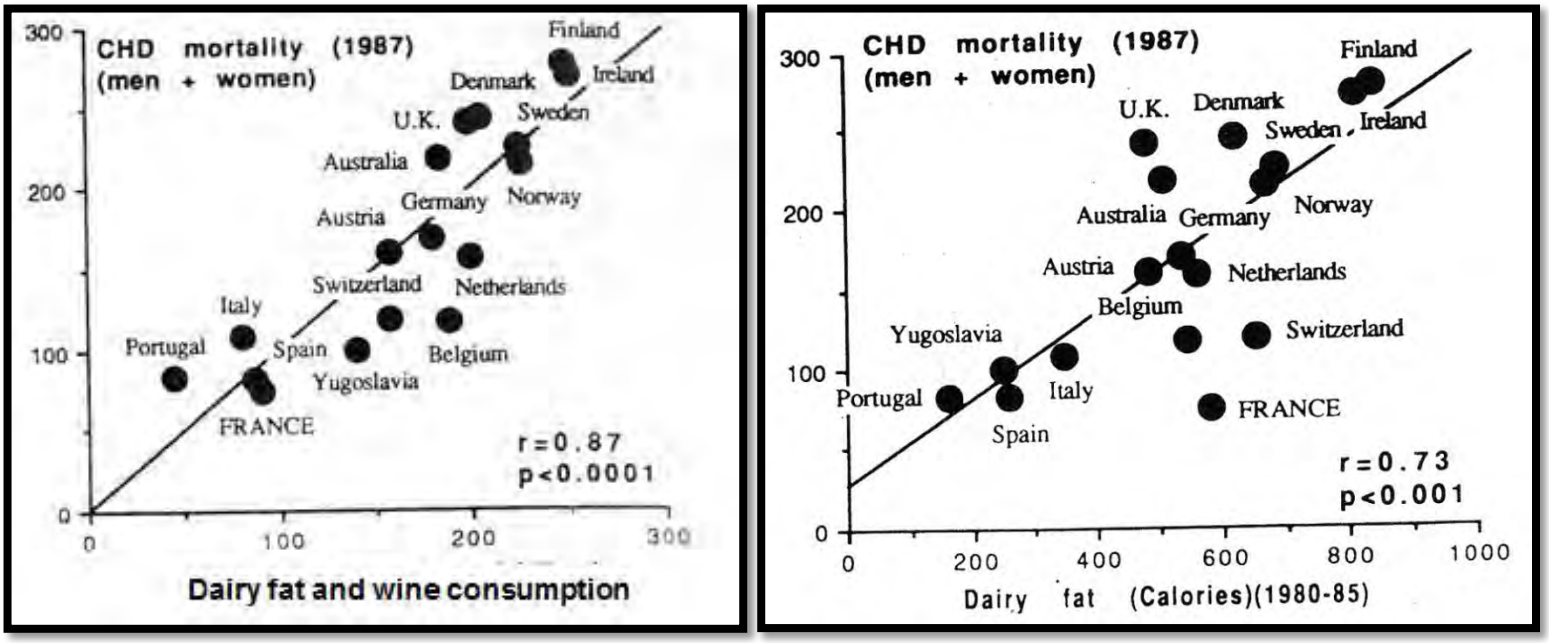


Figure 8: Graph showing the low mortality rate of CHD in France in comparison to other European countries despite similar intake of high saturated fats

From Renaud and de Lorgeril, Lancet, 1992

It is possible that the supposed protection conferred by red wine may result from a complex and partially understood association of wine intake with medical, psychosocial, religious and/or demographic confounding factors. Since the possibility of a randomized controlled study is low, the relationship between wine intake and the supposed lower risk of CVD requires careful analysis. Growing evidence supports that red wine might afford a degree of

coronary protection in part due to multiple confounding factors. For example, moderate wine drinkers may represent as a proxy of higher socioeconomic status, superior heath status and lower CV risk (Hansel, Thomas et al. 2010). In addition, it has been demonstrated that moderate wine drinkers consume a healthier diet when compared with heavy drinkers or abstainers (Ruidavets, Bataille et al. 2004, Johansen, Friis et al. 2006).

1.4.4 Experimental evidence of red wine induced cardioprotection

A few studies have demonstrated the cardioprotective effect of red wine against IR in an isolated rat heart model. An acute treatment of red wine extract (1µg/ml) protects rat hearts against IR injury by reducing infarct size as well as an improving developed pressure compared to the control rats (Sato, Ray et al. 2000). The cardioprotective effect of red wine was further illustrated in a study which examines whether the flesh and seeds of red grapes possess any cardioprotective abilities. Hence, rats chronically fed with flesh of grapes (2.5mg/kg) or seeds of grapes (2.5mg/kg) for 30 days are protected against IR injury, as demonstrated by improved post-ischemic ventricular recovery and reduced myocardial infarct size compared to the control groups treated with water only (Falchi, Bertelli et al. 2006). These studies demonstrate that red grapes contain components that are cardioprotective independent of fermentation derived molecules which could be responsible for this protective effect.

Unfortunately, most of these studies have investigated the cardioprotective effect of red wine with particular interest in specific components of red wine and not the wine in its entirety. One of the few studies that investigated the cardioprotective effect of whole red wine was conducted by Lamont and colleagues who demonstrated that chronic pre-treatment with red wine (for 10 days) at a concentration equivalent to 2-3 glasses/day was beneficial in male Long Evans rats exposed to IR injury (Lamont, Blackhurst et al. 2012).

1.5 Possible cardioprotective components in red wine

Red wine contains a complex mixture of bioactive compounds, including flavonols, monomeric and polymeric flavanoids, highly coloured anthocyanins, biogenic amines and phenolic acids. Studies have shown that some of these compounds have health advantages see review (Tsuda 2012, Xiao, Peng et al. 2011). To date, there have been three main

components in red wine that have been suggested to elicit cardioprotection: alcohol, resveratrol and melatonin. Each will be reviewed in detail, specifically with regards to biological mechanisms supporting the cardiovascular benefits of these components in moderate consumption of wine.

1.5.1 Alcohol

1.5.1.1 Epidemiological evidence

Substantial evidence suggests the consistent negative correlation between alcohol consumption and the incidence of CVD. Numerous studies from the late 1970s onwards have reached a consensus that people who consume one to two drinks per day have a lower CVD risk compared with abstainers and binge drinkers (Figure.9), a relationship described as a J-shaped or U-shaped curve (St Leger, Cochrane et al. 1979, Connor 2006). Moderate alcohol consumption mostly equivalent to 1 drink per day for women and 2 drinks per day for men has been found to decrease the incidence and adverse consequences of heart disease in several epidemiological studies (Mukamal, Chung et al. 2006). The definition of one alcohol drink varies by country and publication. Terms such as light, moderate and heavy drinking are unclear. For instance, one drink is 8 g of ethanol in England, 12 g in USA, and 20-24g in Japan. According to Dietary Guidelines for Americans, moderate drinking is no more than 1 drink (12 g of ethanol) per day for women and no more than 2 drinks (24 g of ethanol) per day for men (McGuire 2011).

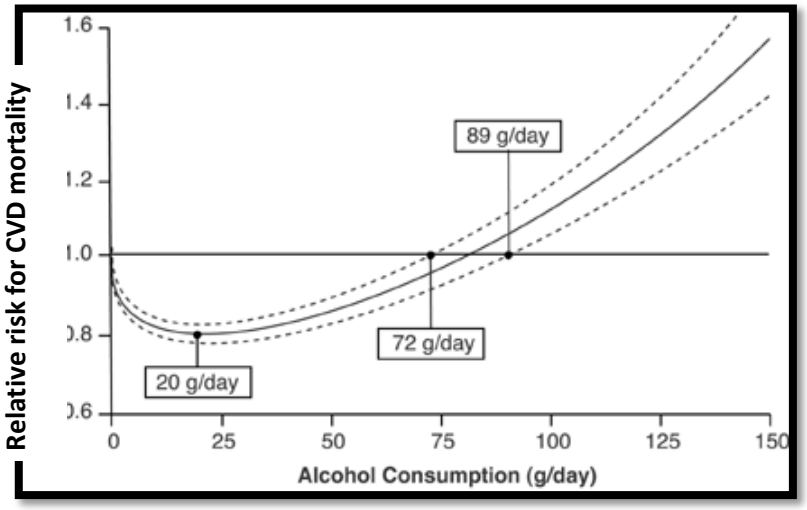


Figure 9: A graphical representation of J-shaped mortality curve for alcohol consumption.

From Corrao, Rubbiati et al. 2000

This phenomenon has been further illustrated by a study from Gronbaek et al, who investigated the relationship between various types of alcoholic beverages and mortality in a population comprised of men and women between ages 30 to 79 (Gronbaek, Deis et al. 1995). The findings included the reduction of the relative risk of death from 1.00 in abstainers to 0.4 for those who drank 3 to 5 glasses of wine per day. With regards to the intake of beer, 3 to 5 bottles per day conferred a reduction in risk of 0.72 compared to abstainers. In contrast, consumption of 3-5 drinks of spirits per day was linked with increased mortality. Furthermore, the study concluded that light and moderate wine drinking is associated with dose-dependent decrease in all-cause mortality that is attributed to a decrease in cardiovascular-related disease. The health benefits and mechanisms observed might be heavily influenced by social, genetic and environmental factors. Hence, a recent study by Leong et al demonstrated that alcohol consumers living in South Asia and the Middle East, in contrast to the rest of the world, did not display protection against MI (Leong, Smyth et al. 2014). In some instances, populations from particular South Asian countries showed significantly elevated risk after adjusting for quality of diet, body composition and classic vascular risk factors. This study suggests that the negative effects of alcohol are not exclusive to frequent binge drinkers but, in addition, can extend to light-to-moderate drinkers. Thus, the beneficial effects of alcohol intake in human health should be better defined, and additional research is required before any suggestions can be made to initiate light-to-moderate consumption of alcohol.

1.5.1.2 Experimental evidence of alcohol induced cardioprotection

Animal experiments have been performed to mimic human drinking patterns in order to investigate whether moderate alcohol consumption could protect the heart against IR injury. There is evidence that long-term alcohol consumption may improve survival after myocardial infarction. Miyamae et al, found that prolonged consumption of 10% ethanol protects against IR injury in guinea pig hearts (Miyamae, Diamond et al. 1997). Particularly, hearts isolated from animals fed with ethanol for 3-12 weeks demonstrated better recovery and less myocyte damage after IR injury compared to controls receiving water only. The authors attributed the cardioprotective effect to an ethanol-induced adenosine receptor activation, an important mediator of ischemic pre-conditioning. Furthermore, Kobuyashi et al, demonstrated that ethanol added to the buffer of perfused rat hearts prior to anoxia, followed by reoxygenation decreased myocardial injury (Kobayashi, Ashraf et al. 1987). However, this study did not determine whether chronic ethanol consumption produced protection against reperfusion injury in the absence of ethanol. Upon review of varied literature, the trend appears to be that the concentration of ethanol required to produce an adaptive biological

response is inversely correlated to the duration of exposure (Diamond, Gordon 1994). Studies by Miyamae et al have shown that doses as low as 2.5% and 5% ethanol produced partial cardioprotection after 3 weeks of exposure, however full protection is maximal after 6 weeks of treatment independent of the dose of alcohol given (Miyamae, Diamond et al. 1997). Logically, higher concentrations of ethanol produced maximum protection at 3 weeks and this was sustained as long as ethanol was consumed for a period of 12 weeks.

1.5.1.3 Does alcohol contribute to red wine-induced cardioprotection?

Although multiple experimental and clinical studies support a cardiovascular benefit of chronic consumption of alcohol, other studies strongly suggest that the cardioprotective effect of red wine goes beyond alcohol content. A study conducted by Keevil et al, showed substantial inhibition of platelet activity in healthy humans after drinking two cups of purple grape juice for one week (Keevil, Osman et al. 2000). A study on coronary heart disease patients showed that 250mL of de-alcoholized Greek red wine was able to decrease arterial stiffness (Zilkens, Burke et al. 2005). Interestingly, red grape juice had similar cardioprotective properties to that of red wine. Patients undergoing hemodialysis and who consumed red grape juice for 14 days, had a significant reduction in plasma monocyte chemoattractant protein 1 concentration and LDL concentration (Castilla, Echarri et al. 2006). In addition, patients displayed higher levels of HDL compared to patients not consuming red grape juice. Experiments conducted at the Hatter Institute did not demonstrate any cardioprotective effect of alcohol in isolated hearts subjected to IR injury after 2 weeks of feeding with alcohol equivalent to 2-3 glasses of wine/day (Lamont, Blackhurst et al. 2012). Rats pre-treated with alcohol only (6% and 12%) extracted from red wine did not attain protection against IR injury compared to the untreated controls. However, rats pre-treated with red wine containing either 6% or 12% alcohol demonstrated similar cardioprotection against IR injury. Therefore suggesting that alcohol is not the sole contributor in red wine induced cardioprotection.

1.5.2 Resveratrol

1.5.2.1 Definition and structure

Resveratrol (3,5,4'-trihydroxystilbene) was first isolated from the roots of white hellebore in 1940, and later, in 1963, from the roots of *Polygonum Cuspidatum,* a plant used in traditional Chinese and Japanese medicine (Nonomura, Kagawnaa et al.1963). Resveratrol is a stilbenoid, a type of natural phenol and a naturally occurring phytoalexin produced by a wide variety of plants in response to stress, injury, ultraviolet (UV) irradiation and fungal infection as part of their defence mechanism (Langcake, Pryce 1976). Resveratrol can be obtained exogenously from various dietary sources which include red grapes, peanut butter, dark chocolate and legumes (Cassidy, Hanley et al. 2000).

Resveratrol initially generated modest interest until 1992, when it was suggested to explain the cardioprotective effects of red wine (Siemann, Creasy 1992). Subsequently, multiple studies have shown that resveratrol can prevent or slow down the progression of a wide variety of diseases including cancer (Jang, Cai et al. 1997), atherosclerosis (Wang, Zou et al. 2005), heart failure (Rimbaud, Ruiz et al. 2011) and IHD (Ray, Maulik et al. 1999).

Resveratrol exists as two geometric isomers, a *cis* and a *trans* form (Figure.10). Trans-resveratrol is suggested to be the isomer that contributes to the cardioprotective properties of red wine (Orallo, Alvarez et al. 2002) through endothelium-dependent vasorelaxant activity, possibly mediated by nitric oxide (NO) release from endothelial cells (Fitzpatrick, Hirschfield et al. 1993).

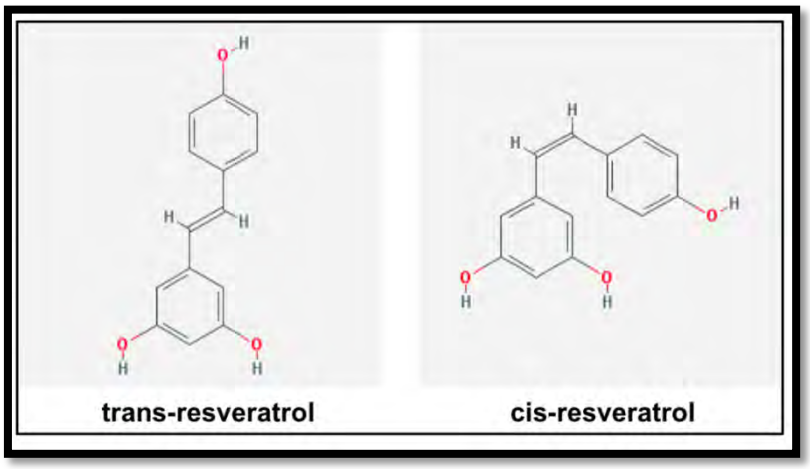


Figure 10: A graphical representation of the bioactive conformations of resveratrol.

From Smith, Andreansky 2013

Cis-resveratrol is not a natural constituent of grape berries. However, *cis*-resveratrol has been detected in all wine analyses (Siemann, Creasy 1992). It is likely that cis-resveratrol derives from its *trans* isomer during vinification. Fresh grape skins contain 50 to 100 mg resveratrol per gram (Jeandet, Sbaghi et al. 1995), however, resveratrol concentrations show large variation in numerous types of wine (Figure.11). Concentrations of resveratrol in wine depend on multiple factors which include geographical origin (Goldberg, Ng et al. 1996), wine type (Threlfall, Morris et al. 1999) and oenological practices (Jeandet, Bessis et al. 1995, Soleas, Goldberg et al. 1995). Another major factor is the fermentation process, contact with grape skins is important because resveratrol is largely produced by the skin and not the pulp of grapes which would explain its negligible concentration in white wine (Jeandet, Bessis et al. 1995).

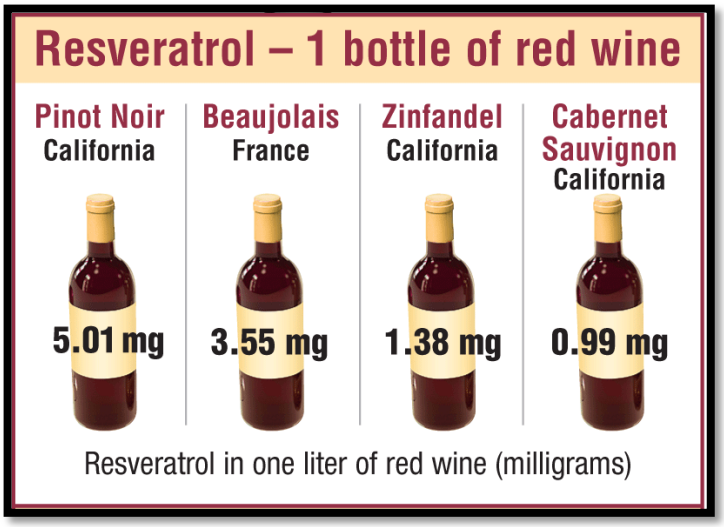


Figure 11: Different types of red wine and their resveratrol concentrations

From www.Nutritionexpress.com

1.5.2.2 Cardiovascular benefit of resveratrol

Many studies have demonstrated that resveratrol has a wide range of pharmacological properties. In the cardiovascular system, resveratrol is suggested to mediate its cardioprotective effects through several mechanisms such as its antioxidant activity, inhibition of platelet aggregation and anti-inflammatory activity (Figure.12).

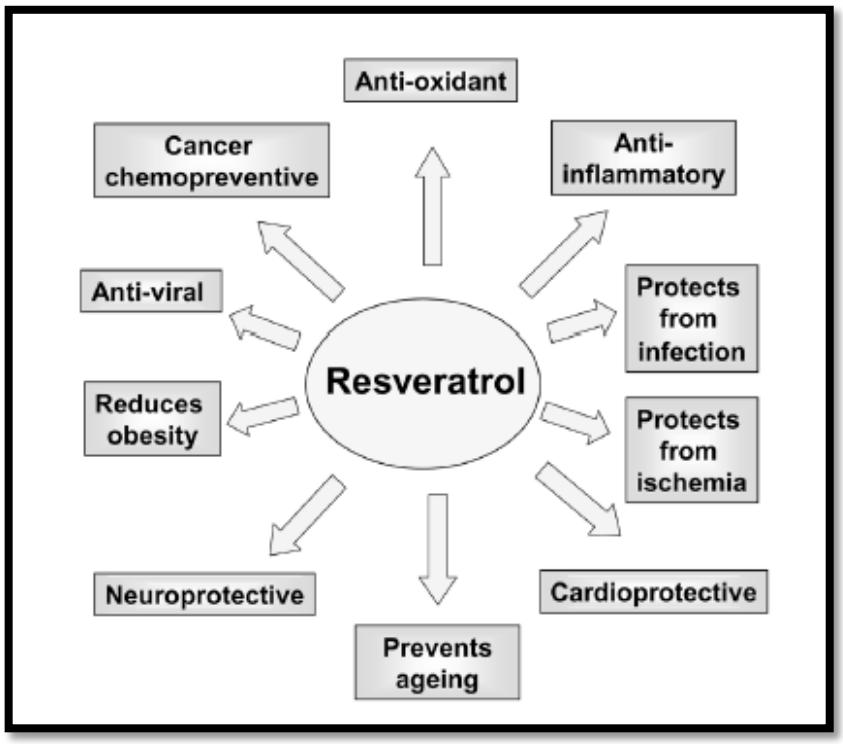


Figure 12: The multiple effects of resveratrol on cardiovascular health and disease

Resveratrol has been shown to be effective in protecting against IR injury. In a study conducted by Mokni and colleagues, rats which were pre-treated with resveratrol (25mg/kg/day) for seven days and subjected to IR injury demonstrated cardioprotection as shown by improved post-ischemic ventricular recovery, improved antioxidant enzyme activity and reduced myocardial lipid peroxidation (Mokni, Hamlaoui et al. 2013).This effect was thought to be mediated by a reduction in reactive oxygen species (ROS) production. In another study, when resveratrol (100μmol/L) was administered prior to cardiomyocytes being subjected to two hours of simulated ischemia, there was increased cell viability by preventing apoptosis via increasing the expression of B-cell lymphoma 2, an anti-apoptotic factor (Shen, Wu et al. 2012). Additionally, there was a decrease in lactate dehydrogenase (LDH) release and increase in adenosine triphosphatase activity. These effects were mediated by activation of the cyclic guanosine monophosphate pathway and protein kinase c (PKC), a well-known mediator in ischemic preconditioning.

In 2007, a study conducted by Penumathsa and colleagues highlighted the effect of resveratrol against IR injury. 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 being exposed to 30 minutes of global ischemia (Penumathsa, Thirunavukkarasu et al. 2007). Resveratrol-treated rat hearts displayed a significant reduction in infarct size, as well as improved functional recovery, compared to untreated hypercholesterolemic rat hearts after an IR insult. 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, Mustafi et al. 2011). These findings suggest that resveratrol protects the heart against the detrimental effects of IR injury. Although many studies have confirmed the cardioprotective effects of resveratrol, most of them have used a concentration far larger than the resveratrol concentration found in red wine (0.5 to 13.5mg/L).

1.5.2.3 Does resveratrol contribute to red wine cardioprotection?

Lamont and colleagues observed that an acute treatment of resveratrol (2.3mg/L) corresponding to the concentration found in red wine significantly reduced infarct size in mouse hearts, but not in tumor necrosis factor (TNF) receptor 2 knockout or STAT3-deficient mice (Lamont, Somers et al. 2011). This data suggests that resveratrol protects via the SAFE prosurvival signalling pathway. In addition, when rats were pretreated with resveratrol (7mg/L) chronically for 10 days, resveratrol failed to improve post-ischemic functional recovery or reduce infarct size (Lamont 2009). Despite abundant experimental studies that have been carried out in animal models, investigations regarding the safety and beneficial effects of resveratrol in humans through randomized clinical trials are rare. Recently, a study conducted by Semba and colleagues involving 800 people from the Chianti region of Italy investigated whether dietary resveratrol had any links with cancer and CVD death rates (Semba, Ferrucci et al. 2014) . The study found that the risk of death during the nine-year follow-up period was no different for people with the highest levels of metabolites (breakdown products) of resveratrol in their urine, compared to people with the lowest levels. There were no differences in the risk of CVD. However, one of the limitations of the study was that resveratrol levels were measured using 24 hour urine samples that looked for breakdown products of resveratrol and this may not be representative of the participants' usual pattern of consumption of red wine, berries and chocolate. Moreover, a recent study suggests that resveratrol could counteract the benefits of cardiovascular exercise in older men. The objective of the study was to investigate the effects of resveratrol supplements during high-intensity exercise. For the study, the men were required to increase their exercise levels and carry out high-intensity interval training three times a week for 4 weeks (Gliemann, Schmidt et al. 2013). In addition, the men were randomized to receive either a placebo or a 150-mg dose of resveratrol each day. The results showed that after 4 weeks, the physical fitness of the men who received resveratrol supplementation did not improve. However, those who received the placebo saw some benefits associated with physical activity, such as an increase in superoxide dismutase 2 (SOD2) gene expression associated with heart protection during exercise. However, the limitations of the study included a small

sample size and did not control for confounding drug use. Furthermore, when resveratrol is given at a high dose it can become a pro-oxidant and could possibly cause damage in the heart. A study demonstrating this was conducted by Gurusamy and colleagues who found that resveratrol-induced autophagy occurred when resveratrol was given at higher doses (100mg/kg/day) (Gurusamy, Lekli et al. 2010).This effect was mediated by inhibiting the expression of rictor, a component that activates prosurvival kinase AKT. Of note, the field of resveratrol research has been tainted by scientific fraud by Dr Dipak Das who was charged with 145 cases of fabricated or false data (Naik 2011, Sen 2012). This has led to many of his scientific articles being retracted.

1.5.3 Melatonin

1.5.3.1 Definition and structure

Melatonin (*N*-acetyl-5-methoxytryptamine) (Figure.13) was first isolated and identified in bovine pineal tissue in the late 1950's by Lerner and colleagues (Lerner, Case et al. 1958). One of the earliest findings regarding the production of melatonin in the pineal gland was that it is primarily synthesized and secreted at night and that the circadian rhythm of melatonin is determined by the light-dark cycle (Reiter 1995). Apart from the pineal gland, multiple organs have the capability of producing melatonin including the gastrointestinal tract (Bubenik 2002) and the melanocytes in the skin (Slominski, Tobin et al. 2008). In more recent years, melatonin has demonstrated multiple functions, which include; strengthening the immune system (Maestroni 2001), slowing down cellular aging (Bonilla, Medina-Leendertz et al. 2002) as well as regulating hormones involved in sexual maturation and reproduction in females (Cavallo, Ritschel 1996) and regulation of leptin in the gastrointestinal tract (Rasmussen, Boldt et al. 1999). Melatonin can be sourced exogenously from certain foods such as cherries (Burkhardt, Tan et al. 2001), rice (Hattori, Migitaka et al. 1995) and meat (Tan, Zanghi et al. 2014). It can also be bought over-the-counter as a supplement to alleviate jet lag (Herxheimer, Petrie 2002) and most importantly can be sourced from red wine (Rodriguez-Naranjo, Gil-Izquierdo et al. 2011a). Melatonin is found in *Vitis vinifera* seeds during the onset of ripening of the grapeberries (Vitalini, Gardana et al. 2011). Melatonin content in wine varies, depending on the grape type (Iriti, Rossoni et al. 2006), its environment, genetics, harvesting process and storage (Lachman, Šulc et al. 2009). 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, Rossoni et al. 2006). Treatment of grape vines with benzothiadiazole, a plant defence

activator, results in an increase in the amount of melatonin in the skins of these grape berries. The presence of melatonin has been detected in both white and red South African wines, with large differences in quantites of melatonin from various wine estates (Albertyn 2012). 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). 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 dry land areas (eg. Robertson Valley).

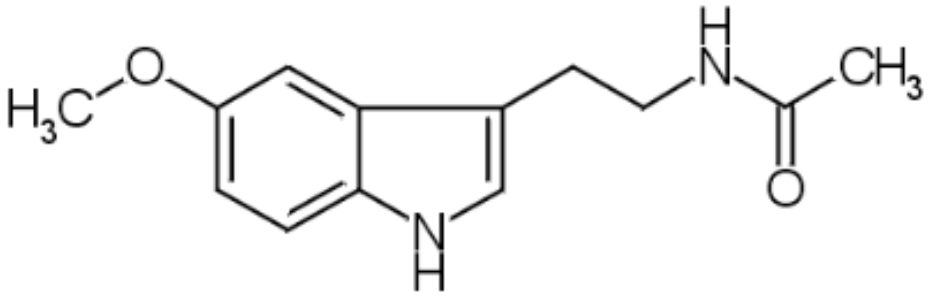


Figure 13: The molecular structure of melatonin

Biosynthesis and physiological mechanism of action

In the biosynthesis of melatonin, tryptophan is first converted by tryptophan hydroxylase to 5-hydroxytryptophan, which is decarboxylated to serotonin. The synthesis of melatonin from serotonin is catalyzed by two enzymes, arylalkylamine *N*-acetyltransferase which catalyzes the N-acetylation of serotonin to N-acetylserotonin. This enzyme controls the circadian rhythm of melatonin production by the pineal gland in all vertebrates. Its enzyme activity is highest at night time and its activity decreases upon exposure to light. Hydroxyindole-o-methyltransferase is the last enzyme of the melatonin biosynthesis pathway which catalyzes the transfer of a methyl group from S-adenosyl-L-methionine onto N-acetyl-serotonin to produce melatonin (Figure.14) (Sugden 1989).

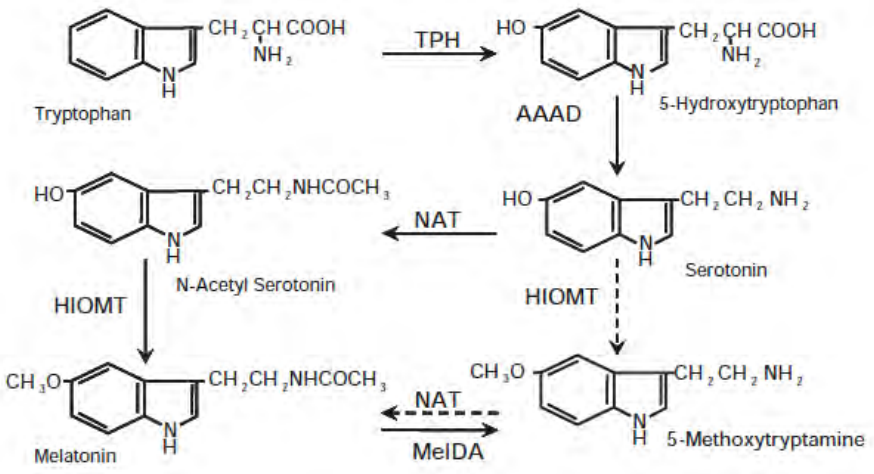


Figure 14: The classic biosynthetic pathways of melatonin in vertebrates

From Tan, Manchester et al.2007

Melatonin exerts its influence through membrane receptors. The three major membrane receptors with varying affinities for melatonin are currently identified as melatonin 1 (MT1) and melatonin 2 (MT2) (Morgan, Barrett et al. 1994), as well as relatively unknown melatonin 3 (MT3), which shows homology to human quinine reductase 2, a detoxification enzyme (Mailliet, Ferry et al. 2004). Receptors MT1 and MT2 are members of the G protein coupled receptor (GPCR) family with seven transmembrane domains. Depending on the specific cell, melatonin activates a variety of different second messenger cascades after it binds to the membrane receptor. Melatonin receptors are present in various localizations of the cardiovascular system and both MT1 and MT2 have been found to be highly expressed in sections of isolated coronary arteries, aorta and left ventricular specimens from healthy hearts as well as patients with dilated and ischemic cardiomyopathy (Ekmekcioglu, Haslmayer et al. 2001). When melatonin binds onto MT1 receptors, it has a vasoconstrictive effect (Ting, Dunn et al. 1997) in contrast to MT2 receptors which has a vasodilatory effect. This demonstrates that melatonin has the potential to regulate blood pressure (Masana, Doolen et al. 2002). The activation of melatonin receptors results in a decrease in cyclic adenosine monophosphate (cAMP) and in phospatidylino-inositol-4,5-bisphosphate hydrolysis, which leads to vasoconstriction (Paulis, Simko 2007) .

1.6.3.2 Cardiovascular benefit of melatonin

In 1985, Muller and colleagues found that the likelihood for a MI to occur peaks between 9 am and 11 am (Muller, Stone et al. 1985). During the rest of the day, MI occurs at a similar rate. This suggests that the occurrence of a MI is dependent on the circadian rhythm.

The production of melatonin is influenced by the detection of light and dark by the retina of the eye. For instance, the production of melatonin is inhibited when the retina detects light

and is stimulated in the absence of light. Thus, melatonin production is lower during the daytime than at night-time see review (Reiter 1991).There is a correlation between MI incidence and presence of melatonin. A study by Dominguez-Rodriguez and collegues demonstrated that acute MI is associated with a nocturnal serum melatonin insufficiency as well as increased oxidative stress (Domínguez-Rodríguez, Abreu-González et al. 2002). Patients diagnosed with acute MI had lower glutathione peroxidise levels and did not show diurnal variation. In addition, lipid peroxidation levels in acute MI patients were increased and diurnal variation was also lost.

Melatonin, given at varying concentrations (1, 10 and 50 μM) attenuated cardiac arrhythmias in an isolated male Sprague-Dawley rat heart model (Tan, Manchester 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 IR injury (Szárszoi, Asemu et al. 2001, Dobsak, Siegelová et al. 2003). In an *ex vivo* setting, Lee and colleagues established the cardioprotective effect of melatonin against IR injury (Lee, Chen et al. 2002). Male Sprague Dawley rat hearts treated with melatonin (1.0 and 5.0 mg/kg) 10 min before occluding the left anterior descending artery and 45 min reperfusion, had a significant reduction in infarct size, reduced tachycardia and fibrillation, compared to the control group. Melatonin (10mg/kg) treatment of male Sprague Dawley rats for 4 weeks subjected to IR injury reduced the infarct size and LDH release, compared to vehicle treated hypoxic rats (Yeung, Hung et al. 2008). 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 LDH release, an indicator of necrosis, compared to untreated controls (Petrosillo, Colantuono et al. 2009).

1.6.3.3 Does melatonin contribute to red wine-induced cardioprotection?

Recent research conducted within the Hatter Institute for Cardiovascular Research in Africa has suggested the role of melatonin in red wine-induced cardioprotection. Chronic pre-treatment with melatonin at the concentration found in red wine, protected the heart against IR injury (Lamont 2009). Likewise, isolated rat hearts treated acutely with melatonin at a concentration similar to that found in red wine (75 ng/L), demonstrated a significant reduction in infarct size in compared to untreated control hearts after an IR insult (Lamont, Somers et al. 2011). In STAT-3 knockout or TNF-α knock-out mice, pretreatment with melatonin (75ng/L) failed to reduce the infarct size after an IR insult. 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. These data suggest that melatonin confers cardioprotection against IR injury via the SAFE pathway. In another study conducted within

the Hatter Institute, the aim was to explore whether South African red and white wines confer a cardioprotective effect in relation to their melatonin content (Albertyn 2012). The results demonstrated that chronic moderate pre-treatment with both South African red and white wines improved the cardiac function of rats subjected to IR injury. However, after measuring the melatonin content in each South African wine, the data did not show a relationship between the melatonin content present in the wine and its' cardioprotective effect. As wine may contain varying concentrations of melatonin and also other potential cardioprotective molecules from one bottle to another, it is difficult to establish if melatonin plays a vital role in red wine induced cardioprotection. Thus, in order to validate the role of melatonin in wine-induced cardioprotection, we propose the use of a synthetic wine, whose composition is fully known and controlled.

B. AIMS AND OBJECTIVES

Aim and objectives

A large number of epidemiological studies have demonstrated that chronic and moderate consumption of wine is associated with reduced cardiovascular disease. Elucidating the components found in wine which contribute to this cardioprotective effect may lead to the development of novel therapies against ischemic heart disease. Wine contains alcohol and natural antioxidant compounds, including resveratrol and melatonin, that have all been suggested to possess cardioprotective properties.

Red wine is a highly complex matrix which contains over a thousand different molecules whose concentrations vary from one bottle of wine to another. Therefore, the delineation of the exact components in wine which confers cardioprotection is very challenging when using commercially available red wine. In the wine biotechnology field, the preparation of synthetic wine, with a chemical composition which is perfectly known and controlled, is often used to better understand the wine making process. This medium is designed to reflect the main nutrients used by yeast that are present in freshly prepared, highly clarified, grape juice.

In the present study, we therefore hypothesised that synthetic wine, enriched with cardioprotective components such as resveratrol and/or melatonin, contributes to the cardioprotective effect of chronic and moderate consumption of wine.

To explore this hypothesis, the following objectives will be pursued:

1. In collaboration with the Wine Biotechnology Institute at the University of Stellenbosch, we will prepare a synthetic wine whose chemical and functional properties will be characterised.
2. To investigate the effect of chronic and moderate consumption of synthetic wine enriched with melatonin and/or resveratrol in an *ex vivo* rat model of IR injury. Rats will be pretreated with water or synthetic wine containing or not melatonin and/or resveratrol. After 14 days of treatment, the hearts will be isolated and subjected to a global ischemia-reperfusion insult. Functional parameters and infarct size will be assessed as an endpoint
3. To investigate the effect of a chronic treatment of synthetic wine with/without resveratrol and/or melatonin on antioxidant status in plasma of rats pretreated for 14 days. Antioxidant capacity (ORAC, catalase and SOD assay) and markers of oxidative stress (TBARS assay) will be assessed in the plasma with the aim to elucidate if cardioprotective effect is mediated by an improvement of the antioxidant profile.

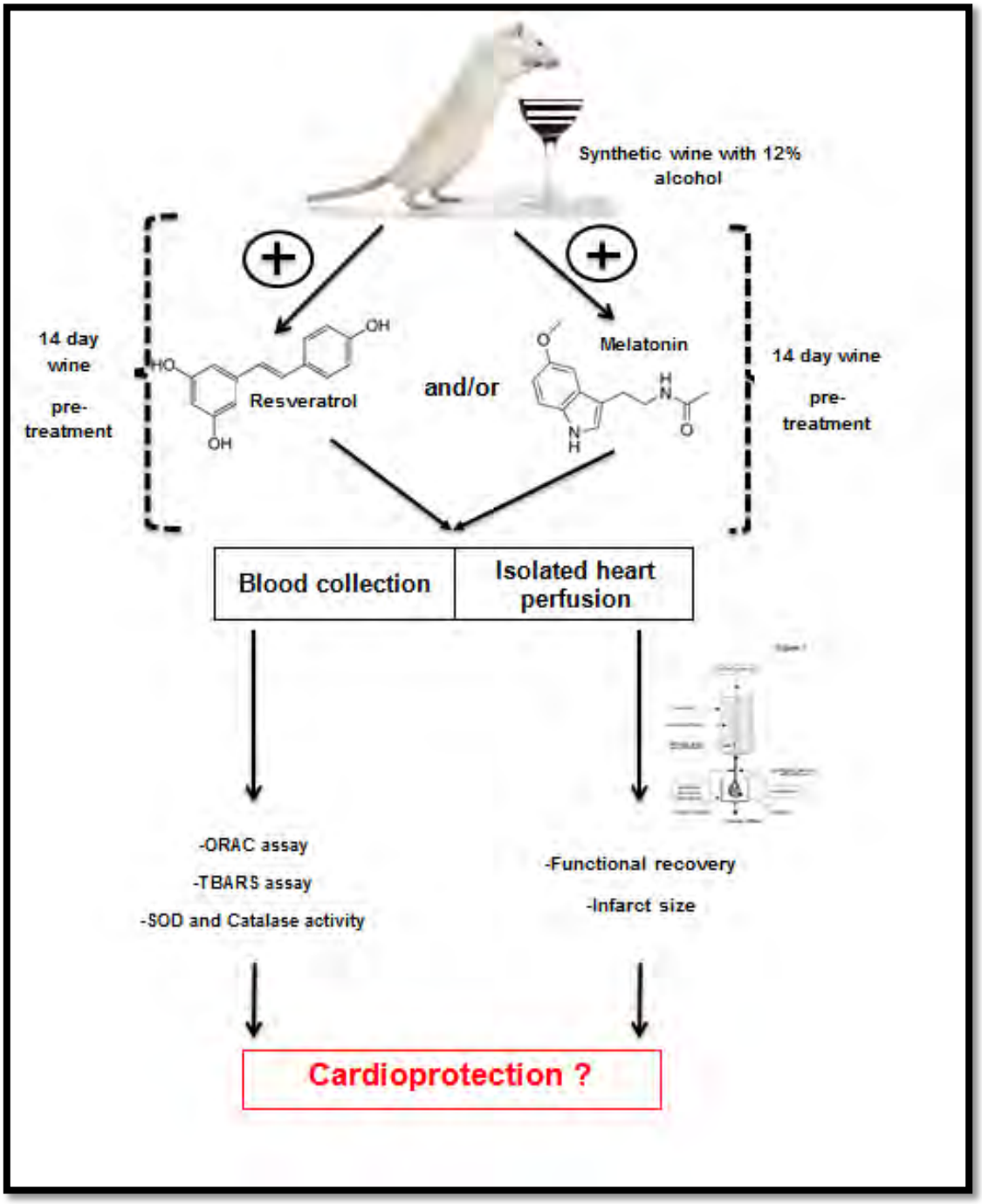


Figure 15: A simplified diagram which illustrates a hypothetical setting whereby enriching synthetic wine with resveratrol and/or melatonin may contribute to the cardioprotective effect of chronic moderate consumption of wine

ORAC- Oxygen radical absorbance capacity

TBARS- Thiobarbituric acid reactive substances

SOD-Superoxide dismutase

C. MATERIALS AND METHODS

1 Production and validation of synthetic wine

1.1 Production of synthetic wine

Wine is an extremely complex matrix combining over a thousand different chemicals in it. In order to assess the effect of alcohol, resveratrol and/or melatonin in wine-induced cardioprotection, a simplified synthetic wine, whose composition is perfectly controlled was designed. Sugars (glucose and fructose) are used as the main energy source and organic acids as well as amino acids are used as nitrogen sources.

The preparation of the synthetic wine is based on a method previously described by Henschke and colleagues (Henschke, Jiranek 1993). The composition of the synthetic wine is described in Appendix A. Briefly, sugars, organic acids, organic salts, amino acids, trace elements, vitamins and lipids were added into 600mL of deionised water. The pH of the medium was adjusted to 3.3 by adding potassium hydroxide and the volume was increased to 1L with deionised water before inoculation.

Yeast strain and fermentation conditions

The yeast strain used in this study was VIN13 (Anchor yeast, South Africa), a diploid *saccharomyces cerevisiae* strain used in industrial wine fermentations. Yeast cells were cultivated at 30°C in Yeast Peptone Dextrose (YPD) synthetic media 1% yeast extract (Biolab, Midrand, South Africa), 2% peptone, and 2% glucose (Sigma-Aldrich, Germany). Solid medium was supplemented with 2% agar (Biolab, South Africa). Fermentation bottles were inoculated with YPD cultures in the exponential growth phase to an OD_{600} of 0.1. All fermentations were carried out under anaerobic conditions in 1L round-bottom flasks and sealed with rubber stoppers to provide better exclusion of oxygen with a carbon dioxide outlet. The fermentation temperature was maintained at approximately 25°C, and no continuous stirring was performed during the course of the fermentation. The fermentations followed a time course of 23 days, and the bottles were weighed daily to assess the progress of fermentation. As the sugars were converted into alcohol and carbon dioxide by the yeast, the fermentation flask would weigh less on each day. The fermentation was considered complete when residual sugars in wine were lower than 2g/L and the fermentation flask weight stabilized. Alcohol content was measured and equalled to 12% (v/v). Once primary fermentation was completed there is a need for confirmation of the success of the fermentation process. The central Analytical Facility in the Wine biotechnology institute at the University of Stellenbosch completed these analyses including a test for residual sugars to confirm the dryness of wine and cessation of fermentation. The results of an analysis of the synthetic wine used in this study are shown in Table 1. Once full

analysis of the synthetic wine was complete, the wine was degassed with nitrogen before it was aliquoted into standard Bordeaux 750mL wine bottles and sealed with screw-top caps. The wine bottles were subsequently stored at 4°C until they were used for further experiments.

Table 1: Post-fermentation analysis of the synthetic wine obtained from the Central Analytical Facility

Acetic acid(g/L)	D-Fructose(g/L)	D-Glucose(g/L)	Glycerol(g/L)
0.8	0.0187	-0.0042	6.006

A wine is considered dry when residual sugars (D-fructose and D-glucose) are less than 1g/L, in order to avoid re-fermentation of yeast. Analysis of volatile acids establishes a baseline that enables monitoring of potential spoilage problems throughout the storage life of a specific wine. At the post-fermentation stage, acetic acid, the main component of volatile acidity, may be formed by oxidation of ethanol in an aerobic environment. The acceptable upper limit for volatile acidity is 1.2g/L (Zoecklein, Fugelsang et al. 1990).

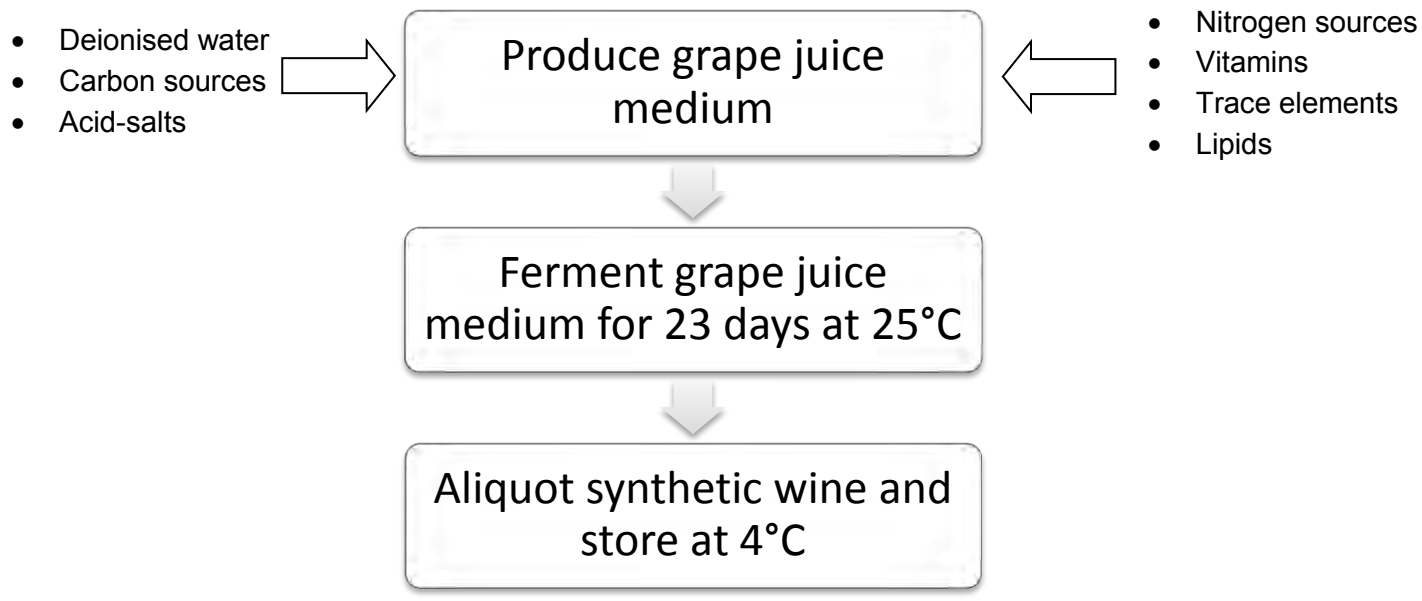


Figure 16: Simplified schematic diagram summarising the different steps in the production of the synthetic wine

Functional assays of synthetic wine

A large number of epidemiological studies have demonstrated that moderate consumption of red wine is associated with reduced mortality and reduced IHD, an association popularized as the 'French paradox". Although red wines do not contain significant amounts of antioxidant vitamins such as vitamin C and E, their protective effects have been largely ascribed to phenolic components. It has been proposed that phenols act as antioxidants. Phenols have shown to be effective radical scavengers, with respect to lipid, protein and deoxyribonucleic acid (DNA) oxidation, which is determined mostly by their reactivity as hydrogen or electron donating agents.

Red wine contains wood and yeast derived phenols, in addition to the large numbers of phenol compounds which originate from grapes, particularly the skins which are removed during the vinification of white wine. Thus, because of the different types of grapes used and the different production processes implemented, there are some fundamental differences in white and red wine composition in terms of the nature of polyphenols.

In order to investigate the effect of synthetic wine-induced protection, it is essential to characterize the chemical and functional properties of the synthetic wine.

1.2 Determination of concentration of total phenolic compounds in synthetic wine

The Folin-Ciocalteu assay is the most commonly used procedure to determine the total phenolic content of food extracts. The concentration of synthetic wine phenolics was measured by using the Folin-Ciocalteu (F-C) method adapted from Waterhouse (Waterhouse 2002).The F-C assay is an *in vitro* colormetric assay based on the reduction of a phosphotungstate –phosphomolybdate complex by phenolic compounds.
Wine samples (20µL) and gallic acid standards (20µL) were mixed with 1.58mL water in 5mL plastic tubes. Undiluted commercially available F-C reagent (100µL) was added to the tube and the mixture was vortexed and left at room temperature for 5 minutes. An aqueous sodium carbonate (20% m/v) solution (300µL) (refer to Appendix B) was added to the tube, vortexed and incubated at 40ºC for 30 minutes. The absorbance of the mixture was determined by spectrophotometry (Spectramax Plus 384, Molecular Devices, Labotec, South Africa) using Softmax Pro (Version 4.4) software at 765nm. A calibration curve was prepared using gallic acid ranging from 0 to 500 mg/L (Figure.17). The results were expressed in mg/L of gallic acid equivalents (GAE). Each sample and standard was measured in triplicate.

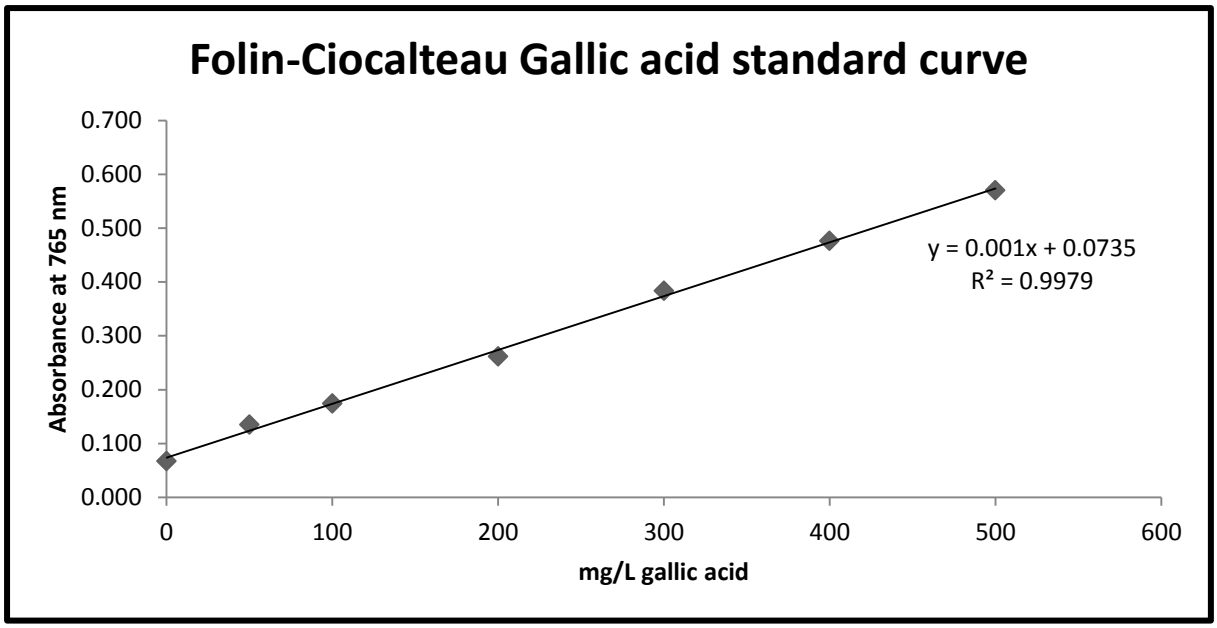


Figure 17: Standard calibration curve of gallic acid to determine total phenolic content in synthetic wine

1.3 Measurement of total antioxidant capacity in the synthetic wine by the oxygen radical absorbance capacity (ORAC) assay

Total antioxidant capacity was performed in synthetic wine using methods previously described by Cao and collegues (Cao, Alessio et al. 1993) using the oxygen radical absorbance capacity (ORAC) assay. This assay evaluates the intrinsic antioxidant protective effect of samples against oxidative degradation of a fluorescent molecule, fluorescein (3,6, dihydroxyspiro(isobenzofuran-1(3H), 9(9H)(xanthen)(disodium) (Sigma Aldrich, Germany), following mixing with AAPH (2,2′-azobis(2-amidinopropane)dihydrochloride), a peroxyl radical generator. The fluorescence intensity decreases as the oxidative damage of the free radicals released by AAPH progresses (Figure.18).

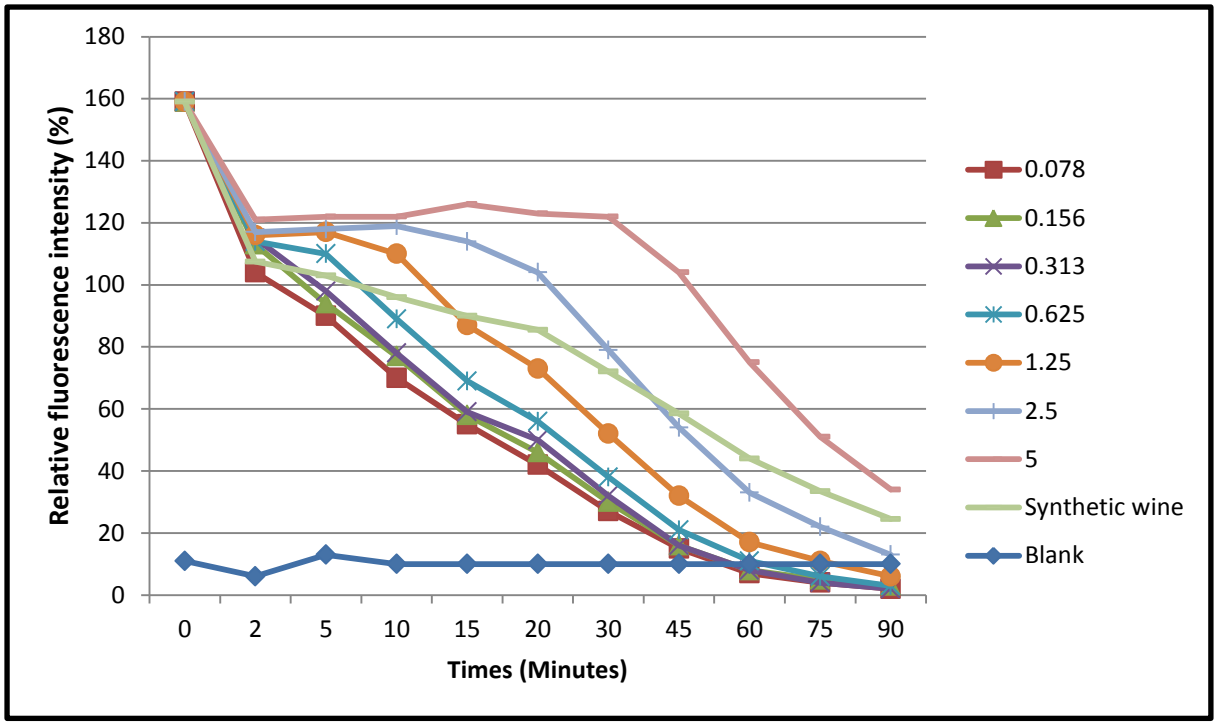


Figure 18: Decrease in fluorescence over time with different concentrations of the vitamin E analogue trolox.

The protection against the free radicals by the synthetic wine is compared to known concentrations of the vitamin E water-soluble analogue Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylicacid (Sigma-Aldrich, Germany). The reactions were carried out in phosphate buffer (75mM, pH 7.4) and the final reaction volume was 300µL. To each well of a 96-wellplate was added with diluted wine samples or trolox standards (50µL), phosphate buffer (50µL) and fluorescein (100µL) (refer to Appendix C). The mixture was pre-incubated for 15 minutes at 37°C. AAPH solution (100µL; 32.1µM final concentration) was added rapidly using a multichannel pipette. The plate was automatically agitated prior to each reading. The plate was immediately placed in the Varian Cary eclipse fluorescence spectrophotometer reader (Set point tech., South Africa) and the fluorescence was recorded every 5 minutes for the first 30 minutes and every 15 minutes thereafter for the duration of the experiment. Fluorescence readings were carried out using excitation and emission filters at 485nm and 520nm, respectively. A blank using phosphate buffer in place of the wine sample, and eight calibration solutions using Trolox (between 1 and 8 nmol/mL final concentration) were analysed in the same run. AAPH and trolox solutions in phosphate buffer (75 mM,pH 7.4) were prepared daily and fluorescein was diluted from a stock solution (1.196mM) in the same phosphate buffer that was stored at 4°C. All sample reactions were measured in duplicate.

A standard curve was generated using the known concentrations of trolox, compared to the area under the curve (Figure.19).

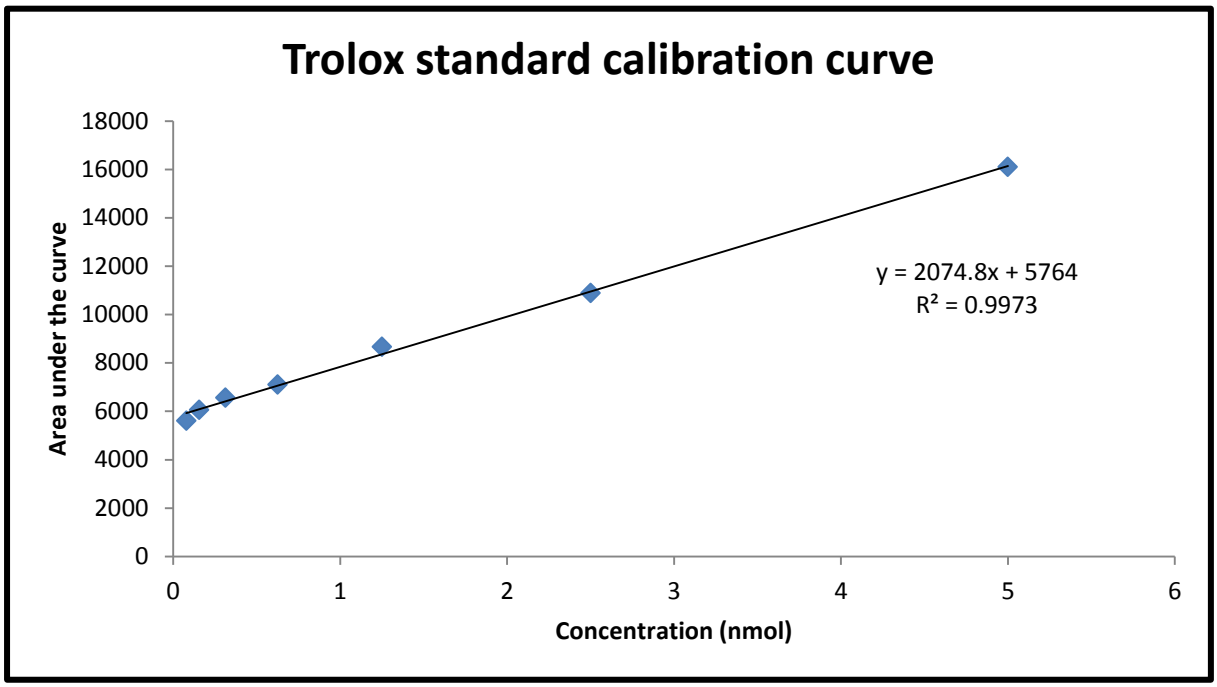


Figure 19: The area under each curve was for each concentration trolox in nmol was used to generate a standard curve

The area under the curve is proportional to the antioxidant capacity as a small area indicates faster decay and less protection against the free radicals generated by AAPH. The area under the decay curve over time of the wine samples was calculated using Microsoft Excel and the concentration of trolox equivalents of antioxidant capacity in nmol/mL was extrapolated from the trolox standard curve.

2 Testing the cardioprotective effect of synthetic wine

2.1 Animals

All experimental procedures were carried out with the approval of the faculty of Health Sciences Animal Ethics committee, University of Cape Town (Application 013/015 “Exploring the cardioprotective effect of synthetic wine using Long Evans rats”). All protocols were carried out in compliance with the guide for care and use of Laboratory Animals published by the U.S National Institute for Health (NIH publication No. 85(23), revised 1996). Male Long Evans rats (150-170g) were bred and obtained from the University of Cape Town animal unit. Animals were allowed access to food *ad libitum* but water or treatment was given according to body weight. The temperature was maintained at 22-24°C and lighting at 100-300 lux. Rats were housed in a 12-hour light and dark cycle, with lights on at 06h00.

2.2 Experimental design

In order to assess the cardioprotective effect of synthetic wine with varying wine components, Long Evans rats were pre-treated with synthetic or water for a period of 14 days. For this study, a total of 57 rats were divided into eight groups (Figure.20): Control (n=12), synthetic wine only (n=6), synthetic wine enriched with resveratrol only (n=7), synthetic wine enriched with melatonin only (n=7), synthetic wine enriched with both melatonin and resveratrol (n=6), water enriched with resveratrol only (n=5), water enriched with melatonin (n=8) and water enriched with both melatonin and resveratrol (n=6) .The treatment solutions were prepared by adding one part of synthetic wine or distilled water enriched with either resveratrol (100µg/L) or melatonin (75ng/L) (refer to Appendix F) to seven parts of drinking water as previously described (Lamont, Blackhurst et al. 2012). When the treatment was given to the rats, both body weight and the amount of drinking water consumed by each rat was taken into account to ensure that the amount given per day corresponded to an equivalent of 2 glasses of wine/day which would mimic the human setting. The rats were treated for a period of 14 days. On the 15th day, rats were anaesthetised, and *ex vivo* hemodynamic measurements were taken using the isolated Langendorff heart system.

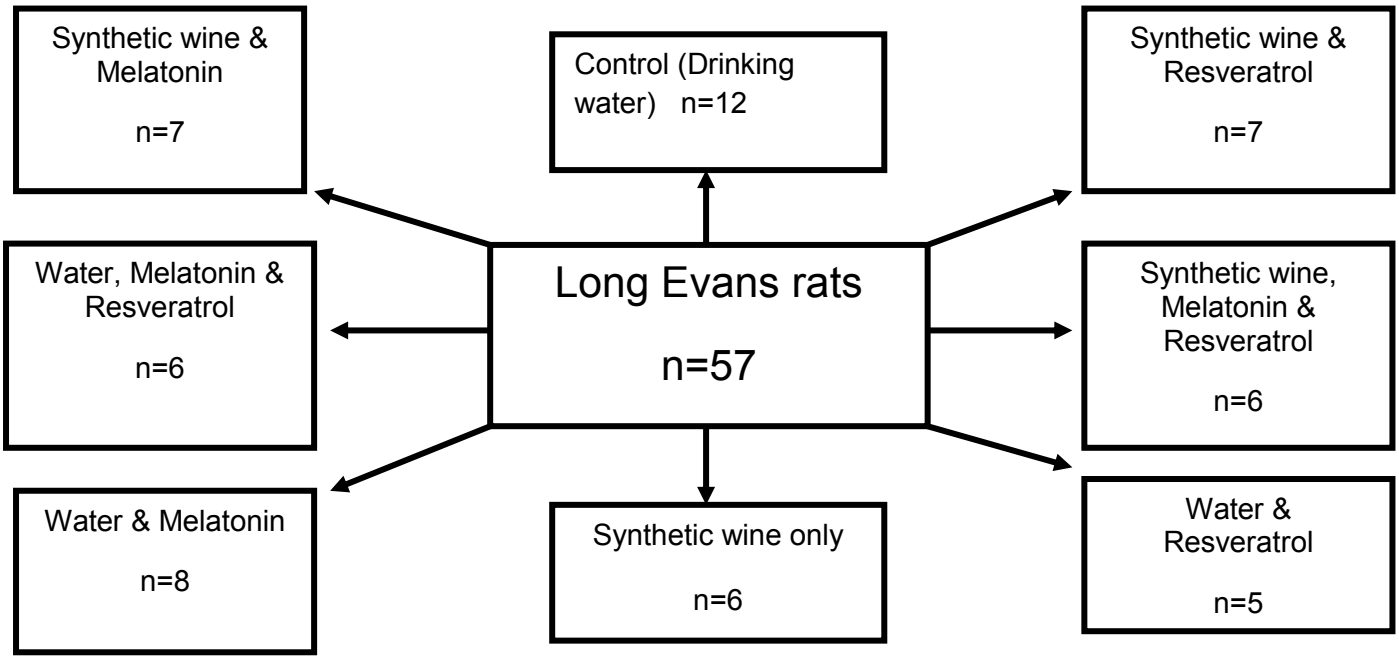


Figure 20: A schematic diagram showing the different treatment groups used to assess the effect of chronic moderate consumption of synthetic wine on ischemia/ reperfusion injury

2.3The Langendorff heart preparation as a model for the study of cardiovascular disease

The isolated retrograde perfused mammalian heart system was first pioneered by Oscar Langendorff in 1895. The Langendorff system is a retrograde perfusion system which is frequently used in cardiac physiology to study cardiac metabolism, contractile function and coronary flow regulation.

Before calibrating the Langendorff system, the apparatus was washed out with boiling distilled water several times to remove bacteria and debris. Once the apparatus was fully cleaned the Krebs Henseleit buffer (refer to Appendix D) was prepared. The molecular composition of the buffer resembles that of blood plasma in order to successfully perfuse the heart *ex vivo.*The Langendorff heart preparation entails the cannulation of the aorta which is then attached to a tank containing Krebs fluid oxygenated with 95% oxygen and 5% carbon dioxide. The oxygenated Krebs fluid is delivered in a retrograde direction down the aorta at a constant pressure maintained at 100cm with water. The aortic valves are forced shut and the perfusion fluid is directed into the coronary ostia, in that way perfusing the entire ventricular mass of the heart, draining into the right atrium via the coronary sinus.

The general procedure for Langendorff perfusion entails excision of the heart from the donor anaesthesized animal. Immediately after excision, the heart is immersed in ice cold Krebs fluid before cannulation and vascular perfusion. A water-jacketed reservoir positioned above the aortic cannula, contains the Krebs fluid which is oxygenated through a sintered glass gas distributor. The aorta is then eased over the end of the cannula, taking precaution not to insert the cannula too far into the aorta. Once the heart is securely attached to the cannula, any excess tissue is trimmed away. Once cannulation is completed and coronary perfusion is initiated, contractile function and regular heart rhythm will return within a few seconds. Throughout this time, various instrumentation is taken. Contractile activity of the heart, is assessed through insertion of a compliant intraventricular balloon. The balloon is inflated with water from a syringe until a left ventricle end diastolic pressure of between 4-12mm Hg is obtained. Once the balloon is in position, left ventricular systolic, diastolic and developed pressure can be recorded. Once instrumentation of the heart has been completed, a temperature regulated heart chamber should be placed around the heart to maintain the temperature at 37°C.

2.4 Experimental Langendorff perfused isolated rat heart preparation.

The Long Evans rats were anaesthetised with sodium pentobarbitone (60mg/kg) and heparin (200I.U.) intraperitoneally. Prior to anaesthesia, animals were placed in a quiet room for 15 minutes to acclimatise them to the new environment. The depth of anaesthesia was judged by the absence of the pedal withdrawal reflex, whereby the paw of the rat was pinched and the perception of pain was observed. A thoracotomy was performed when the rat was deemed to have reached surgical anaesthesia. An incision of the skin below the sternum was delicately performed. The incision was continued along the proximal ends of the left and right costal margins. The thoracic cavity was opened and the pericardium surrounding the heart was removed, exposing the beating heart in the chest cavity. The heart was gently cradled and hoisted between the thumb and the index finger and removed with one quick precise cut without inflicting damage to the aorta. The isolated heart was immediately placed into ice-cold Krebs buffer [NaCl 118.5mM, $NaHCO_3$ 25mM, KCl 4.7mM, $MgSO_4.7H_2O$1.2mM, KH_2PO_4 1.2mM, Glucose 11.1mM, and $CaCl_2.2H_2O$1.3mM] as previously described (Krebs, Henseleit 1932) (for preparation and abbreviations refer to Appendix D). Blood was removed from the rat chest cavity and stored in a tube containing heparin (200 I.U). These tubes were centrifuged immediately at a speed of 2000g for 10 minutes to separate the plasma from the serum. The plasma was immediately stored at -80ºC and later used for oxidative stress analyses.

Subsequently, the heart was mounted retrogradely onto the cannula of the Langendorff apparatus within 3 minutes with two pairs of forceps stretching the aorta on either side to ensure a fit onto the cannula (Figure.21).

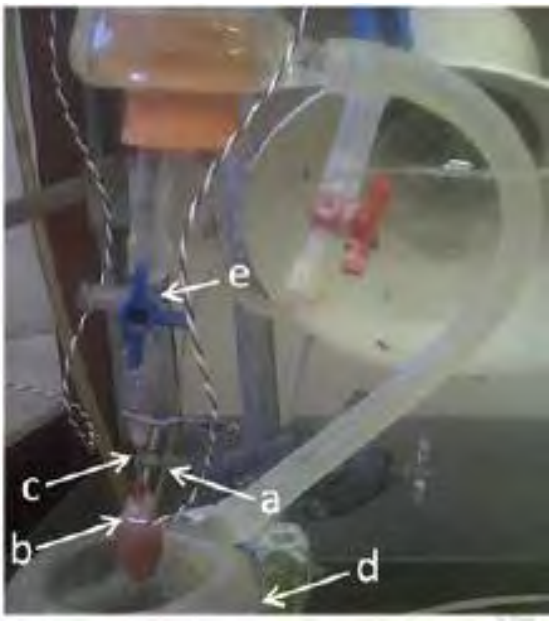


Figure 21: A Langendorff-perfused rat heart. Figure key: (a) intraventricular balloon inserted into the left ventricle and mounted on a 21-gauge bevelled needle; (b) thermal couple for continual temperature monitoring inserted in pulmonary artery; (c) cannula whereby heart is mounted onto system; (e) a 2-way tap placed between the bubble trap and cannula and (d) water-jacketed reservoir

This procedure was carried out under low flow to enable a fluid-fluid connection. The heart was temporarily secured with a bulldog clamp in order to keep it in place. Thereafter, a thin suture was tied around the aorta to secure the heart onto the cannula. The left ventricle was exposed with a small excision of the atrial appendage surrounding the left atria, to enable the insertion of a balloon. The intraventricular balloon was attached to a catheter filled with water which was connected to a pressure transducer (MLT0670, Lasec, South Africa) on a PowerLab system (ADInstruments, Australia) for hemodynamic measurements. Inside the left ventricle, the balloon was inflated by filling it with water, thus creating a closed system to measure the pressure experienced by the left ventricle.

2.5 Experimental protocol

All hearts were stabilized (S) for a period of 30 minutes and consequently subjected to 30 minutes of global ischemia (GI) followed by 60 minutes of reperfusion (R) (Figure.22).

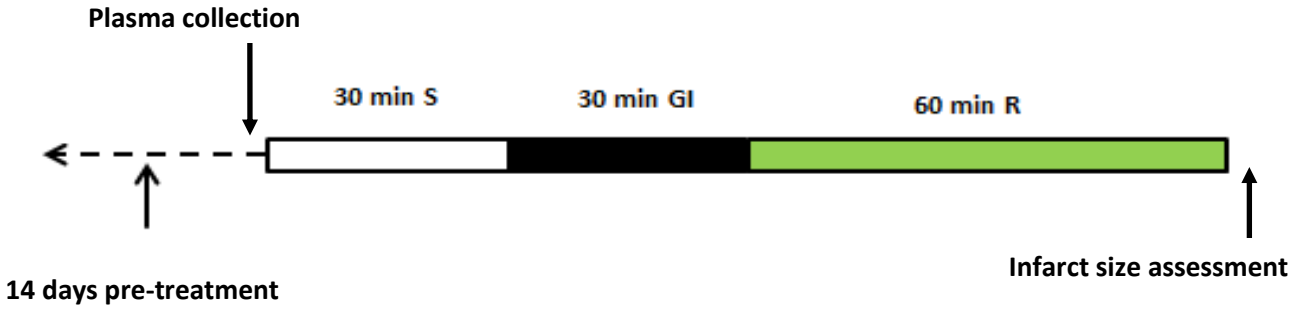


Figure 22: A schematic representation of perfusion protocol

Global ischemia was achieved by complete cessation of perfusate while reperfusion was restored by re-introducing perfusate. Temperature was measured with a thermocoupled wire (Physitemp, NJ, United States of America) inserted into the pulmonary artery, and connected to a Digitron 2600T temperature sensor (Torquay, United Kingdom)(Figure.23). Temperature was controlled at 37°C with a water jacket and a desk lamp. At the end of reperfusion, hearts were removed and stored at -80ºC before staining and infarct size analysis.

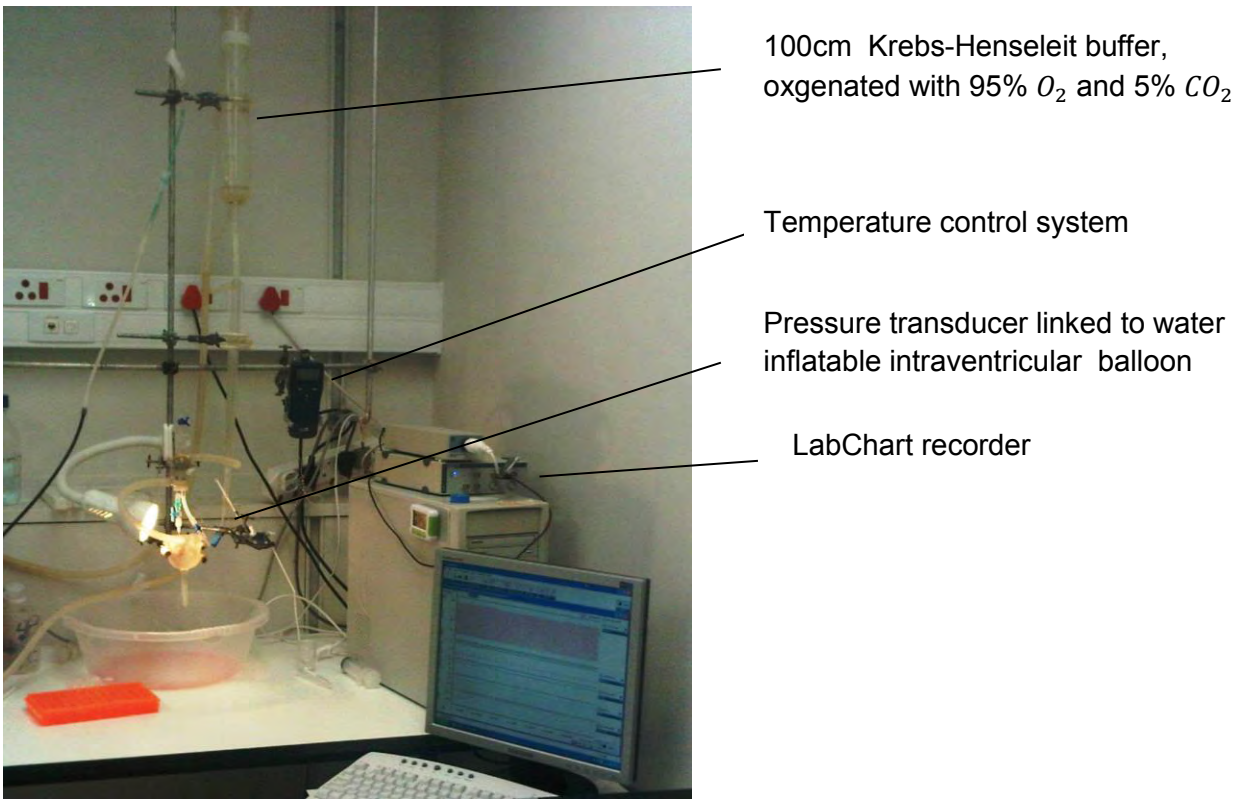


Figure 23: Langendorff perfusion retrograde apparatus

2.6 Hemodynamic parameters

Hemodynamic parameters were monitored throughout the experiments with LabChart Pro 7 software (ADInstruments, Australia) and included heart rate (HR) expressed in beats/min , left ventricular developed pressure at mmHg (LVDP: difference between left ventricular end

systolic pressure (LVESP) and end diastolic pressure (LVEDP)) and the coronary flow (CF) expressed in ml/min. Functional recovery was assessed through the rate pressure product (RPP) which was calculated as LVDP X HR at the stabilisation period compared to the end of reperfusion. CF was measured with Falcon™ 15mL Conical Centrifuge Tubes.

Functional parameters were measured at 5, 10, 20 and 30 minute intervals during the stabilisation period to ensure the inclusion criteria were met.

Functional parameters were also measured after 5,15,30,45 and 60 minutes of reperfusion.

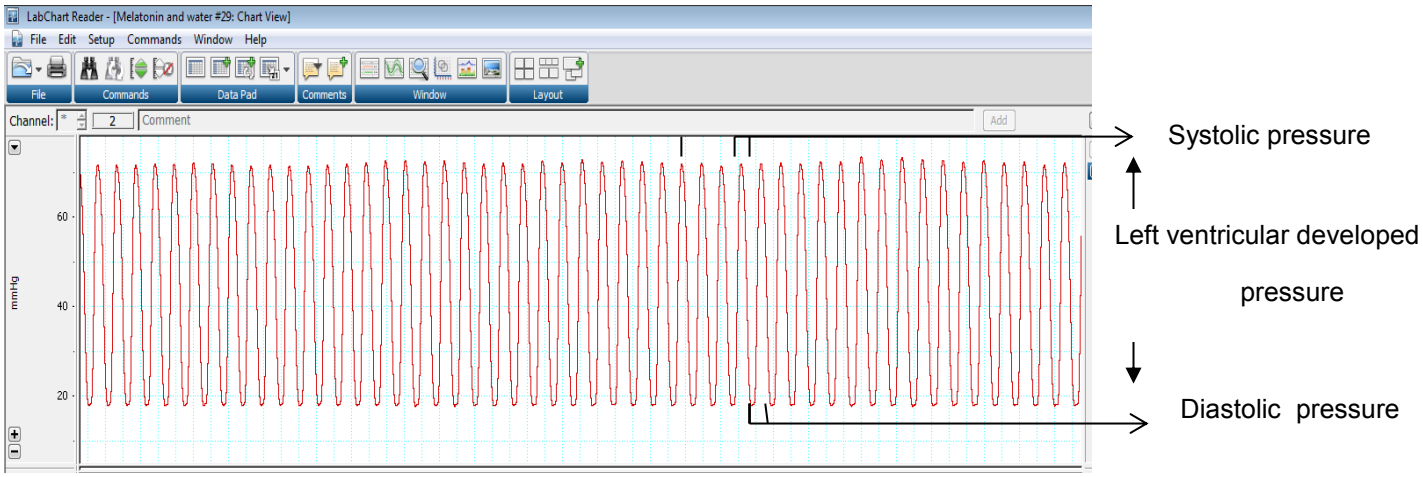


Figure 24: Labchart trace showing various hemodynamic parameters recorded during experimental protocol

2.7 Exclusion criteria

Rats that did not meet the terms of the following criteria were not included in the study:

A. The heart rate should fall between 240-360 beats/min
B. The rate × pressure product must fall within the range 21 000-36 000
C. Left ventricular end diastolic pressure of 2-12mm Hg
D. The developed pressure should fall between 70-120mm Hg
E. The coronary flow rate should be between 8-18 ml/min

2.8 Infarct size

Hearts were sectioned transversely at 2mm thickness and stained by immersion into 2,3,5-Triphenyltetrazolium chloride solution (TTC) (1% in phosphate buffer) at pH 7.4 for 10 minutes at 37ºC. TTC is a redox indicator used to differentiate between metabolically active

and inactive tissue. TTC is a colourless solution which is enzymatically reduced to a brick red precipitation of formazan dye in living tissues with intact mitochondrial respiration due to the activity of various dehydrogenases in the presence of the electron donor NADH. After TTC staining, necrotic zones remain as pale areas of the heart as a result of enzymatic wash-out in damaged cells. The slices were then fixed in 10% formalin for 24 hours and scanned using a flatbed scanner. Infarct size was quantified by with both computerised planimetry (Summa sketch II; Summa Graphics) and Image J (National Institute of Health, United States of America).

3 Analysis of total antioxidant capacity of blood plasma

There is strong evidence to suggest that increased intracellular ROS are a central cause of myocardial injury. The changes associated with myocardial injury result, in part in an imbalance between the formation of oxidants and the availability of endogenous antioxidants. Experiments were performed using the ORAC, thiobarbitituric acid reactive substances (TBARS), catalase and superoxide dismutase assay to measure the total antioxidant capabilities and levels of oxidative stress in plasma from animals that received different treatments for 14 days.

3.1 Protein quantification

The Lowry Assay was used to quantify the concentration of plasma proteins (Lowry, Rosebrough et al. 1951). A series of dilutions of 0.3mg/ml bovine serum albumin was prepared to create a standard curve with concentrations which ranged from 0.03 to 1.15mg/ml. A volume of 200µL of sample or standard was added to 1ml of freshly mixed complex-forming reagent, subsequently the solution was left to stand at room temperature for 10 minutes. Afterwards, 100µL of diluted folin reagent was added. The mixture was left to stand at room temperature for a further 30 minutes. The absorbance was read at 750nm for protein concentration below 500ug/mL or at 550nm if the protein concentration was between 100µg/ml and 2000 µg/ml.

The standard curve of absorbance as a function of initial protein concentration was used to determine the unknown protein concentrations.

3.2 Sample preparation: protein extraction

Plasma samples were thawed slowly and mixed well on a vortex. To extract the protein from the plasma, 200µl ice cold ethanol (100%) was added to 100µl of plasma. The mixture was then centrifuged at 12000 rpm at 4°C for 5 minutes. The protein free supernatant was diluted 363x in the phosphate buffer (Refer to Appendix E). Plasma antioxidant capacity was determined by ORAC assay using deproteinized plasma using the same method as previously described in section 1.3 of this chapter.

3.3 Thiobarbituric acid reactive substances (TBARS) assay

The thiobarbituric acid reactive substances (TBARS) are formed as part of lipid peroxidation and may be detected using thiobarbituric acid (TBA) as a reagent. The assay mostly measures malondialdehyde (MDA) in the plasma, a low molecular weight end product of lipid peroxidation of polyunsaturated fatty acids. MDA and other TBARS in the plasma condense with two equivalents of thiobarbituric acid to give a red derivative that can be assayed spectrophotometrically. In order to measure the amount of MDA in the rat plasma, the TBARS assay was performed according to the method of (Jentzsch, Bachmann et al. 1996)Jentzsch (Jentzsch, Bachmann et al. 1996).

Plasma was mixed with butylated hydroxytoluene (BHT) and 50µL orthophosphoric acid in 2mL Eppendorf micro test tubes and vortexed for 10 seconds. 25µL TBA reagent was added and the mixture was vortexed again. The reaction mixture was then heated at 90ºC for 45 minutes. The tubes were put on ice to stop the reaction. After cooling to room temperature for 5 minutes, TBARS were extracted once with 500µL n-butanol. To facilitate phase separation, 50µl saturated sodium chloride solution was added, and the test tubes were centrifuged at 2000 rpm for 2 minutes in a centrifuge. 300µL of the upper butanol phase was placed in a flat-bottom 96-well plate. Absorption was read at 535nm on a spectrophotometer (Spectramax Plus 384, Molecular Devices, Labotec, South Africa) using Softmax Pro (Version 4.4) software

3.4 Catalase activity assay

The catalase activity assay is based on the principle that catalase, an antioxidant enzyme, catalyses the reduction of hydrogen peroxide to water (Aebi 1984) and (Claiborne 1985). For this assay, 30% hydrogen peroxide was diluted to a concentration of 12mM by adding

150µL of hydrogen peroxide to 100mL of phosphate buffer. The phosphate buffer was prepared by adding KH_2PO_4(50mM) to Na_2HPO_4(50mM) to a final volume of one litre and concentration of 50mM (ph 7.0). All the samples were diluted to a ratio of one in ten. 241µL of phosphate buffer was added into the first two wells and 241µL of the hydrogen peroxide was added to the next two wells. In the remainder of the wells, 10µL of the diluted samples was added as well as 10µL of phosphate buffer.

The 96-well plate (Co-star,Lasec,SA) was inserted into the spectrophotometer (Spectramax Plus 384, Molecular Devices, Labotec, South Africa) and a reading was recorded at 240nm to confirm that the optical density of the diluted hydrogen peroxide was approximately 0.52±0.025. Thereafter, 221µL of the diluted hydrogen peroxide was added to each well and the timer was started. A reading was recorded (at time point zero) and exactly one minute later another reading was taken. The zero minute reading was subtracted from the one minute reading. The difference between these two values was used to calculate the final catalase activity which was expressed as international units per milligram protein.

3.5 Superoxide dismutase (SOD) activity assay

Plasma superoxide dismutase (SOD) activity was measured by using a method adapted from ((Dalloz, Maingon et al. 1999). The first step was to prepare all components of the assay including the phosphate buffer (50mM), xanthine (2mM), ammonium sulphate (3mM), superoxide dismutase (stock solutions for the purpose of the standard curve)(5000IU/mL), xanthine oxidase solution and the reaction solution. The second step was to determine the reaction slope of the xanthine oxidase solution, during which the volume of xanthine oxidase was either decreased or increased to bring the reaction slope to approximately 0.025±0.027. To do this, 290µL of the reaction solution was added to wells A1 and A2 of a Greiner 96-well plate, together with 6µL of phosphate buffer. Lastly, 3.5µL of xanthine oxidase was added and the timer was started. Absorbance was read using a spectrometer (preheated to 37ºC) at 550nm, with a micro plate data acquisition program Softmax Pro (Version 4.4) software.
This was considered the reading at time point zero and after exactly three minutes, another reading was recorded. The reaction slope of the xanthine oxidase was then calculated by subtracting the first reading from the last. Once this slope was adjusted to approximately 0.025±0.027, the rest of the assay was performed.
The third step was to prepare the range of SOD dilutions in order to generate the SOD standard curve. The SOD stock solution was used to prepare five standards: zero IU/mL

(0μL SOD in 5mL phosphate buffer), 10IU/mL (10μL SOD in 4990μL phosphate buffer), 20 IU/mL (20μL SOD in 4980μL), 40IU/mL (40μL SOD in 4960μL phosphate buffer), 80IU/mL (80 μL SOD in 4920μL phosphate buffer). In a clean 96 well plate, 3.5 μL of each standard was pipette into wells A1 to A10 in a duplicate manner. In these wells 290 μL of reaction solution was also added as well as 6 μL of phosphate buffer. During the fourth step of the assay, 3.8 μL of each blood plasma sample was added to the separate well. After all the additions were made, 3.5μL of xanthine oxidase was added to all the wells containing the SOD and a reading was taken using a spectrophotometer. A reading was recorded at time point zero with a micro plate data acquisition program and exactly three minutes later another reading was recorded. The final reaction slope was determined and the final plasma SOD activity was expressed in international units per milligram protein

4. Statistical analysis

All values were presented as mean ± standard error mean (SEM). Column statistics were done to confirm if the data passed normal distribution with the Shapiro-Wilk normality tests. For comparison of more than two groups, data were analysed by one-way analysis of variance (ANOVA), followed by a Tukey-Kramer post hoc comparison using GraphPad Prism 3.0. Differences within the data were considered statistically significant when $P < 0.05$.

5. Chemical agents

Unless stated in the text, all reagents were supplied by Sigma-Aldrich, Munich Germany.

D. RESULTS

1. Determination of stability of synthetic wine over 20 weeks

The concentration of phenolic compounds and total antioxidant capacity was determined over the course of 20 weeks in order to assess the stability of the synthetic wine. Phenols are effective radical scavengers, during lipid, protein and DNA oxidation. In addition, phenolic compounds may act as secondary antioxidants. Rather than scavenging free radicals themselves, they are able to regenerate antioxidants vitamins E and/or C when they accept or donate electrons from a free radical. It was therefore important to analyse the stability of the antioxidant functionality of the synthetic wine during the course of the project.

1.1 Determination of the total phenol content in synthetic wine

The concentration of total phenolic compounds of the synthetic wine was determined using the Folin-Ciocalteu method at 4 different time points of storage over 20 weeks (Figure. 25). Results showed no statistically significant difference between the 4 time points. The values were determined from a regression equation calibration curve for each week and were expressed in gallic acid equivalents (GAE) per volume of wine. The total phenol concentration ranged from 40.0±3.1 to 53.0±7.0 mg/mL GAE.
The intra- assay coefficient of variation was 8.0 %

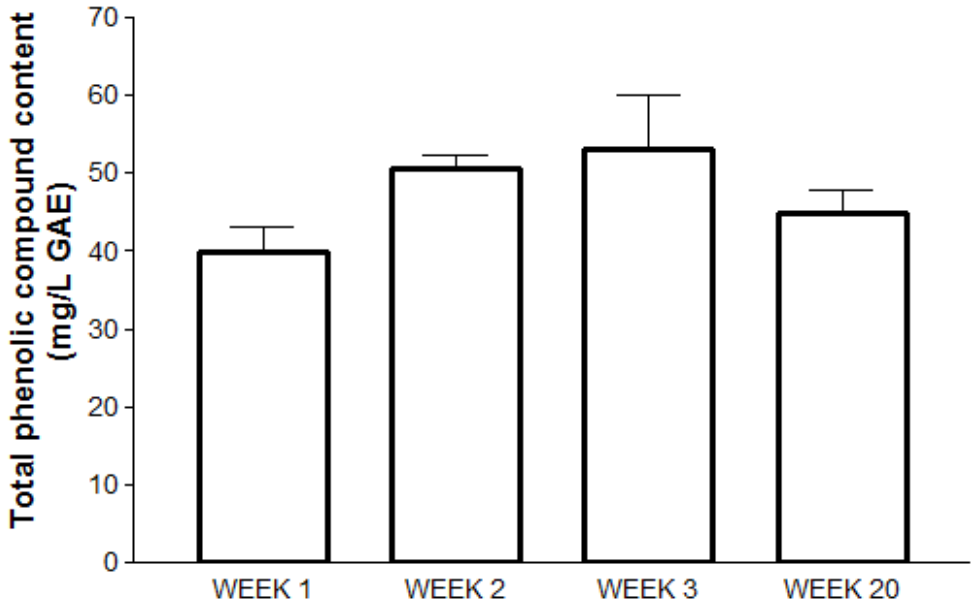


Figure 25*:* The total phenol content in synthetic wine over a period of 20 weeks. n=3
GAE-Gallic acid equivalents

1.2 Determination of the total antioxidant capacity of synthetic wine

The total antioxidant capacity of the synthetic wine was assessed using the ORAC assay. The ORAC values of the synthetic wine at 4 different time points over 20 weeks ranged from 8.9±0.1 to 10.7±0.4 µmol/mL Trolox equivalents (TE) (Figure.26). There were no statistically significant differences in the total antioxidant capacity between the 4 time points of storage. The intra- assay coefficient of variation was 5.9 %.

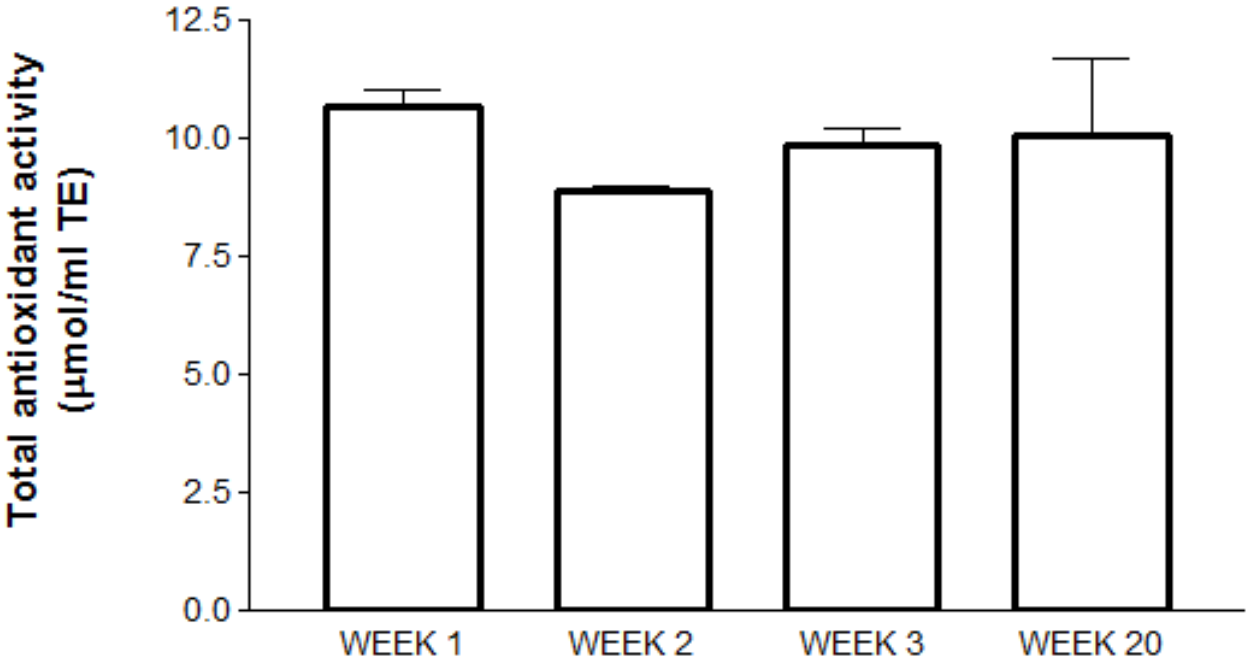


Figure 26*:* The total antioxidant capacity of synthetic wine over 20 weeks. n=4
TE-Trolox equivalents

1.3 Determination of the total antioxidant capacity of synthetic wine enriched with resveratrol and/or melatonin

The total antioxidant capacity of both water and synthetic wine enriched with melatonin and/or resveratrol was determined in order to assess functionality before feeding to animals. The ORAC values ranged from 9.2±0.7 to 15.5±1.9 µmol/mL Trolox antioxidant equivalents (TE) (Figure.27). There was significant difference in the total antioxidant capacity between water and all the other different treatments.

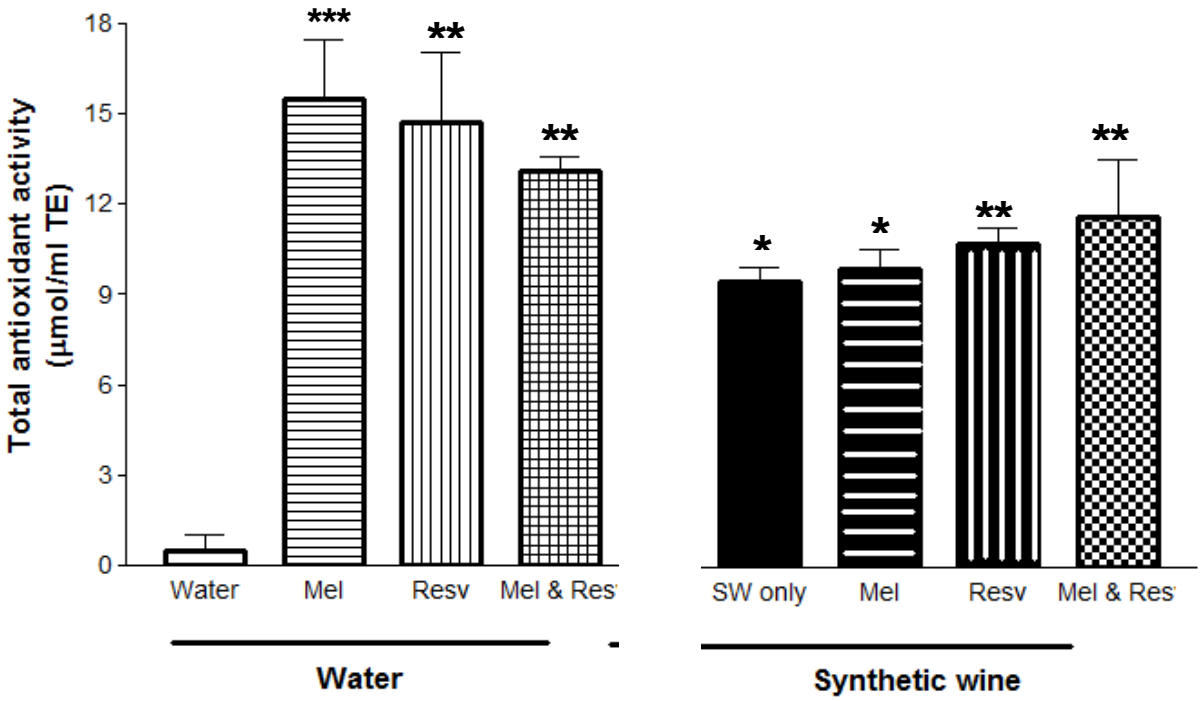


Figure 27*:* The total antioxidant capacity of synthetic wine and water enriched with melatonin (Mel) and Resveratrol (Resv) n=2 Mel- Melatonin Resv-Resveratrol SW-Synthetic wine ***p<0.05 vs. Water, ****p<0.01 vs. Water *****p<0.001 vs. Water n=4

TE-Trolox equivalents

2. Effect of synthetic wine enriched with melatonin and/or resveratrol in isolated hearts subjected to an ischemia/reperfusion insult

2.1 Effect of chronic consumption of synthetic wine with melatonin and/or resveratrol on heart rate

Prior to an ischemic insult, control hearts had a heart rate of 278±8 beats/min (Table 2). This is consistent with findings previously described in the literature (Lecour, Smith et al. 2002). Pre-treatment with melatonin or resveratrol with and without synthetic wine had no significant effect on the pre-ischemic values of heart rate compared to the control group. After 30 minutes of ischemia and 60 minutes of reperfusion, the control hearts had a heart rate of 256±15 beats/min ($p>0.05$ vs. pre-ischemia). None of the treatments affected the heart rate at the end of reperfusion compared to the control group.

Table 2: Effect of chronic consumption of synthetic wine with melatonin and/or resveratrol on heart rate (beats/min) in isolated rat hearts subjected to ischemia/reperfusion injury

Heart rate (beats/min)	Pre-ischemia	Reperfusion 5 min	Reperfusion 15 min	Reperfusion 30 min	Reperfusion 45 min	Reperfusion 60 min
Water (Control)	278± 8	242 ± 44	250± 36	303± 42	308± 57	256± 15
Water & melatonin	276± 12	131 ± 26	146± 35	288± 20	300± 15	301± 12
Water & resveratrol	292± 17	214 ± 95	309± 81	326± 34	337± 41	280± 12
Water, resveratrol & melatonin	276± 17	151 ± 49	241± 39	242± 41	262± 45	269± 20
Synthetic wine	270± 7	162 ± 52	179± 40	174± 20	221± 22	228± 19
Synthetic wine & melatonin	266± 7	220 ± 57	127± 29	234± 34	225± 17	208± 43
Synthetic wine & resveratrol	278± 11	123 ± 35	232± 67	243± 31	250± 18	244± 13
Synthetic wine, resveratrol & melatonin	281± 14	144 ± 38	234± 25	255± 35	263± 27	276± 17

Control n=12, Synthetic wine n=6, SW & M-synthetic wine & Melatonin n=7 wine, SW & R-synthetic wine & Resveratrol n=7, W & M-Water & Melatonin n=8, W & R-Water & Resveratrol n=5, W,M & R-Water,Melatonin & Resveratrol n=6, SW, M & R-Synthetic wine, melatonin and Resveratrol n=6.

2.2 Effect of chronic consumption of synthetic wine enriched with melatonin and/or resveratrol on left ventricular developed pressure (LVDP) in hearts subjected to ischemia/reperfusion injury

At baseline, control hearts had a LVDP of 88±2 mmHg (Table 3). Pre-treatment with melatonin or resveratrol with and without synthetic wine had no significant effect on the pre-ischemic LVDP values compared to the control group. After 30 minutes of ischemia and 60 minutes of reperfusion, the control hearts had a LVDP of 12±2 mmHg (#p<0.05 vs. pre-ischemia). After 60 minutes of reperfusion, pre-treatment with synthetic wine did not improve the LVDP compared to controls. Addition of melatonin or resveratrol in the synthetic wine did not alter the LVDP. Pre-treatment with melatonin or resveratrol without synthetic wine did not display a significant improvement in the LVDP compared to the untreated controls. After 30, 45 and 60 minutes of reperfusion, pre-treatment with synthetic wine enriched with both resveratrol and melatonin improved LVDP to 24±5mmHg, 30±5mmHg and 29±5mmHg respectively, (*p<0.05 vs. control.). After 45minutes of reperfusion, water enriched with both resveratrol and melatonin significantly improved LVDP to 31± 8mmHg (*p<0.05 vs. control) however, this effect was lost at 60 minutes.

Table 3: Effect of chronic consumption of synthetic wine or water enriched with melatonin and/or resveratrol on LVDP (mmHg) in isolated rat hearts subjected to ischemia/reperfusion injury

LVDP (mmHg)	Pre-ischemia	Reperfusion 5 min	Reperfusion 15 min	Reperfusion 30 min	Reperfusion 45 min	Reperfusion 60 min
Water(Control)	88± 2	2± 1	6± 2	6± 2	10± 3	12± 2#
Synthetic wine	94± 6	9± 4	9± 2	13± 5	14± 5	15± 5#
Synthetic wine & melatonin	82± 2	2± 1	2± 1	6± 1	9± 2	10± 2#
Synthetic wine & resveratrol	83± 5	3± 2	4± 2	10± 2	16± 3	18± 4#
Synthetic wine, resveratrol & melatonin	88± 4	4± 2	8± 4	24± 5*	30± 5*	29± 5*#
Water & melatonin	79± 5	4± 1	4± 2	11± 4	18± 4	20± 4#
Water & resveratrol	85± 4	3± 2	5± 2	17± 3	22± 6	22± 4#
Water, resveratrol & melatonin	88± 4	3± 2	6± 3	20± 6	31± 8*	28± 7#

Control n=12, Synthetic wine n=6, SW & M-synthetic wine & Melatonin n=7 wine, SW & R-synthetic wine & Resveratrol n=7, W & M-Water & Melatonin n=8, W & R-Water & Resveratrol n=5, W,M & R-Water,Melatonin & Resveratrol n=6, SW, M & R-Synthetic wine, melatonin and Resveratrol n=6.

*p<0.05 vs. the control group at same time of reperfusion

#p<0.05 60 minutes of reperfusion vs. pre ischemia

2.3 Effect of chronic consumption of synthetic wine enriched with melatonin and/or resveratrol on functional recovery in hearts subjected to ischemia/reperfusion injury

After 60min of reperfusion the control hearts had a functional recovery of 11±2% (which is expressed as a percentage of baseline).The pre-treatment with resveratrol significantly enhanced the functional recovery compared with control groups (*p <0.05) and protected to a similar degree to animals pre-treated with melatonin only (25±3% vs. 26±5%). Synthetic wine alone did not significantly improve the functional recovery after 60 minutes of reperfusion compared with controls (15±6% vs. control). Addition of either melatonin or resveratrol in the synthetic wine did not significantly improve the functional recovery compared to the untreated control hearts. Pre-treatment with synthetic wine enriched with both resveratrol and melatonin enhanced the functional recovery compared with control groups (**p<0.01) and protected to a similar degree to water enriched with both resveratrol and water (functional recovery: 32±5% vs. 32±8%).

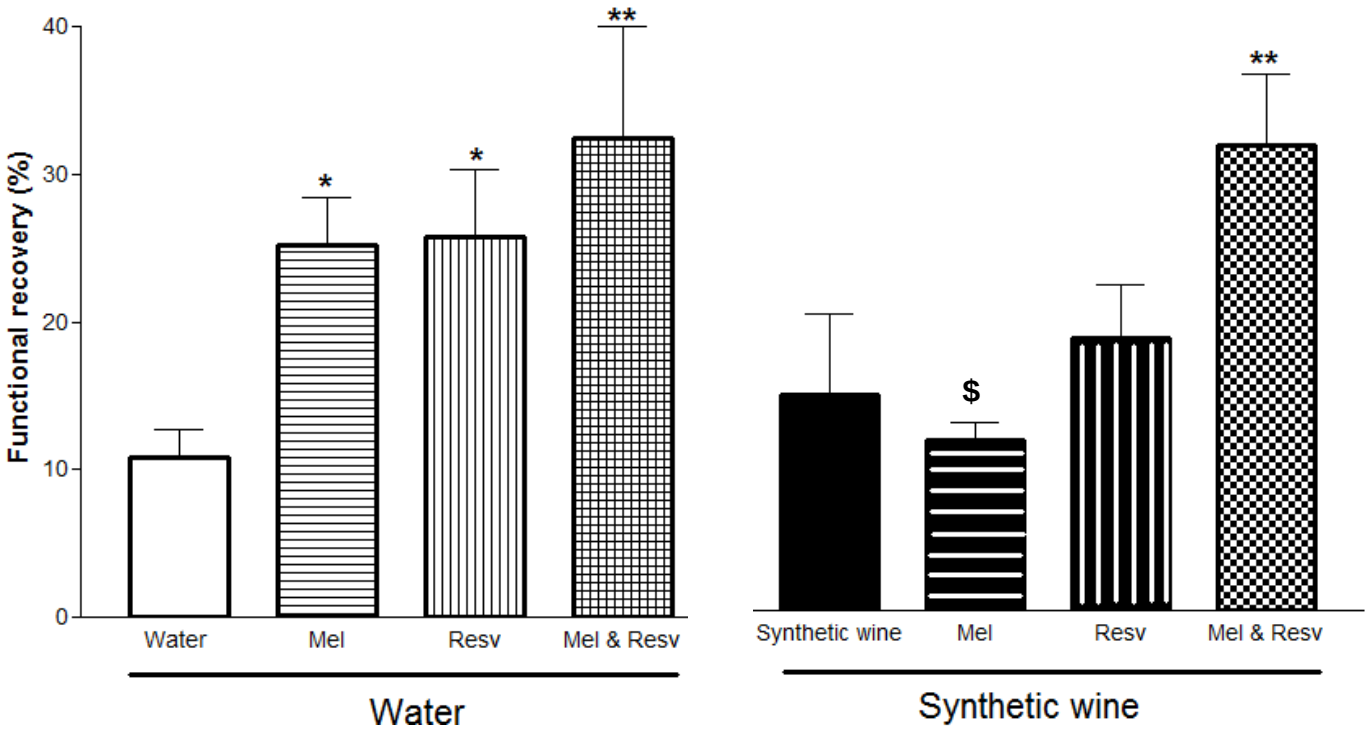


Figure 28: Effect of chronic consumption of synthetic wine enriched with melatonin(Mel) and/or Resveratrol(Resv) on functional recovery after 60 minutes of reperfusion. Control n=12, Synthetic wine n=6, SW & M-synthetic wine & Melatonin n=7 wine, SW & R-synthetic wine & Resveratrol n=7, W & M-Water & Melatonin n=8, W & R-Water & Resveratrol n=5, W,M & R-Water,Melatonin & Resveratrol n=6, SW, M & R-Synthetic wine, melatonin and Resveratrol n=6. *p<0.05 vs. control **p<0.01 vs. Control $p<0.05 vs. Water or synthetic wine enriched with melatonin and resveratrol

2.4 Effect of chronic consumption of synthetic wine or water enriched with melatonin and/or resveratrol on coronary flow in hearts subjected to ischemia/reperfusion injury

At baseline, control hearts had a coronary flow of 12.0 ±1.2 mL/min (Table 4). This is consistent with findings previously described in the literature (Lecour, Smith et al. 2002). Pre-treatment with synthetic wine or water with/ without melatonin and/or resveratrol had no significant effect on the pre-ischemic values of coronary flow compared to the control group. After 30 minutes of ischemia and 60 minutes of reperfusion the control hearts had a coronary flow of 6.4±0.5 mL/min ($p<0.05$ vs. pre-ischemia) none of the treatments affected coronary flow at the end of reperfusion compared to the control group.

Table 4: Effect of chronic consumption of synthetic wine enriched with melatonin and/or resveratrol on coronary flow (mL/min) in isolated rat hearts subjected to ischemia/reperfusion injury

Coronary flow (mL/min)	Pre-ischemia	Reperfusion 5 min	Reperfusion 15 min	Reperfusion 30 min	Reperfusion 45 min	Reperfusion 60 min
Water(Control)	12.3± 1.2	5.0± 0.7	6.3± 0.7	5.1± 0.6	6.1± 0.5	6.1± 0.5#
Synthetic wine	12.1± 0.1	5.2± 0.5	5.2± 0.7	5.4± 0.7	6.3± 0.8	6.1± 0.6#
Synthetic wine & melatonin	13.6± 0.6	6.4± 0.5	6.0± 0.5	7.0± 0.4	7.1± 0.5	7.3± 0.3#
Synthetic wine & resveratrol	12.5± 1.0	5.1± 0.7	6.4± 0.8	6.1± 0.8	6.1± 0.9	6.0± 0.6#
Synthetic wine, resveratrol & melatonin	14.4± 1.1	3.3± 0.8	5.5± 0.9	7.4±0.2	7.2±0.3	7.4± 0.2#
Water & melatonin	13.3± 1.2	6.3± 1.0	7.1± 1.0	7.3± 0.8	7.0± 0.9	7.1± 0.8#
Water & resveratrol	14.7± 1.7	4.4± 0.5	6.3± 0.7	7.1± 0.5	7.4± 0.5	7.2± 0.5#
Water, resveratrol & melatonin	13.1± 0.9	4.2± 0.1	6.4± 0.2	7.1±0.5	7.3±0.7	7.1±0.5#

#p<0.05 60 minutes of reperfusion vs. pre ischemia

Control n=12, Synthetic wine n=6, SW & M-synthetic wine & Melatonin n=7 wine, SW & R-synthetic wine & Resveratrol n=7, W & M-Water & Melatonin n=8, W & R-Water & Resveratrol n=5, W,M & R-Water,Melatonin & Resveratrol n=6, SW, M & R-Synthetic wine, melatonin and Resveratrol n=6.

2.5 Effect of chronic consumption of synthetic wine enriched with melatonin and/or resveratrol on infarct size

In order to quantify the total infarct area, two different forms of computerised planimetry were used namely Image J (NIH) and a digitizing tablet (SummaSketch graphics). The analysis was performed by a researcher blinded to the treatments.

2.5.1 Infarct size measured with IMAGE J analysis

The control hearts subjected to 30 min global ischemia followed by 60 min of reperfusion had an infarct size of 49.2±4.7%. Pre-treatment with synthetic wine did not reduce infarct size compared to controls (44.0±8.0% vs. control). None of the treatment combinations significantly reduced the infarct size (Figure.29).

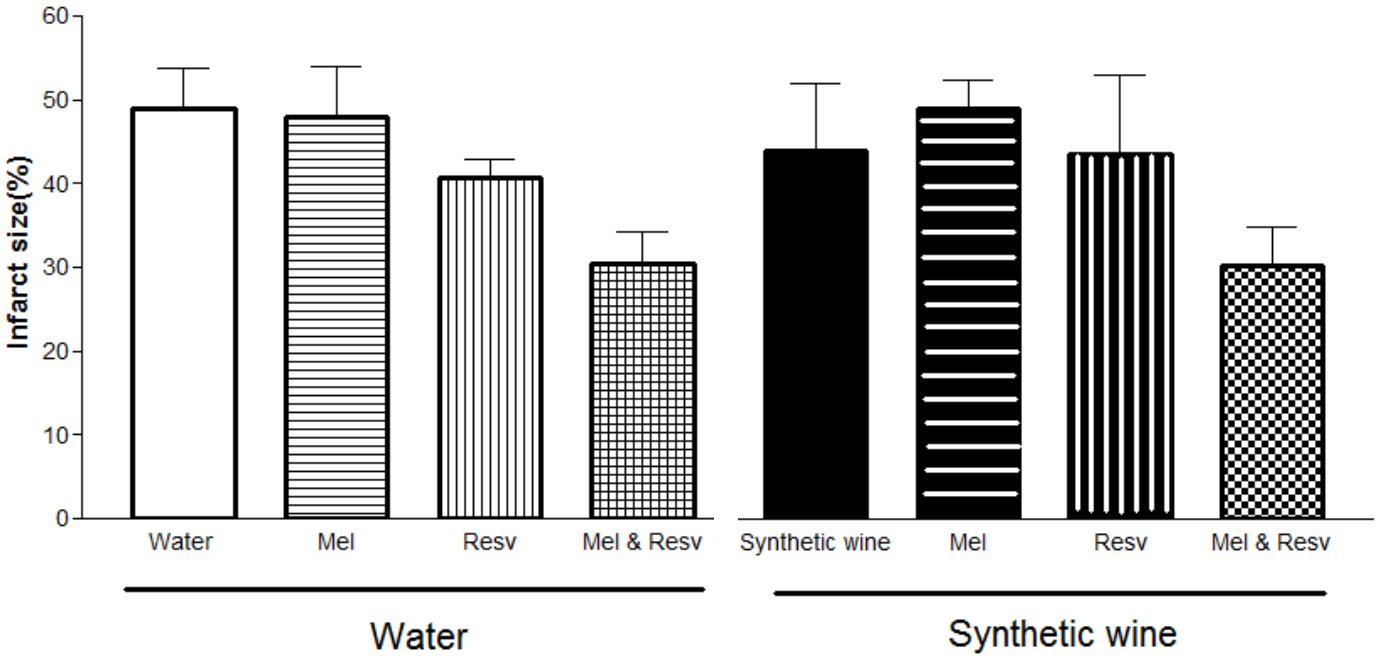


Figure 29*:* Effect of chronic consumption of synthetic wine enriched with melatonin(Mel) and/or Resveratrol(Resv) on infarct size after 60 minutes of reperfusion using Image J. Control n=12, Synthetic wine n=6, SW & M-synthetic wine & Melatonin n=7 wine, SW & R-synthetic wine & Resveratrol n=7, W & M-Water & Melatonin n=8, W & R-Water & Resveratrol n=5, W,M & R-Water,Melatonin & Resveratrol n=6, SW, M & R-Synthetic wine, melatonin and Resveratrol n=6.

2.5.2 Infarct size measured with digitized tablet (SummaSketch graphics)

The control hearts subjected to 30 min global ischemia followed by 60 min of reperfusion had an infarct size of 61.2±4.0% using a digitized tablet. Pre-treatment with synthetic wine did not reduce infarct size compared to controls (53.4±8.0% vs control). None of the treatment combinations significantly reduced the infarct size (Figure.30).

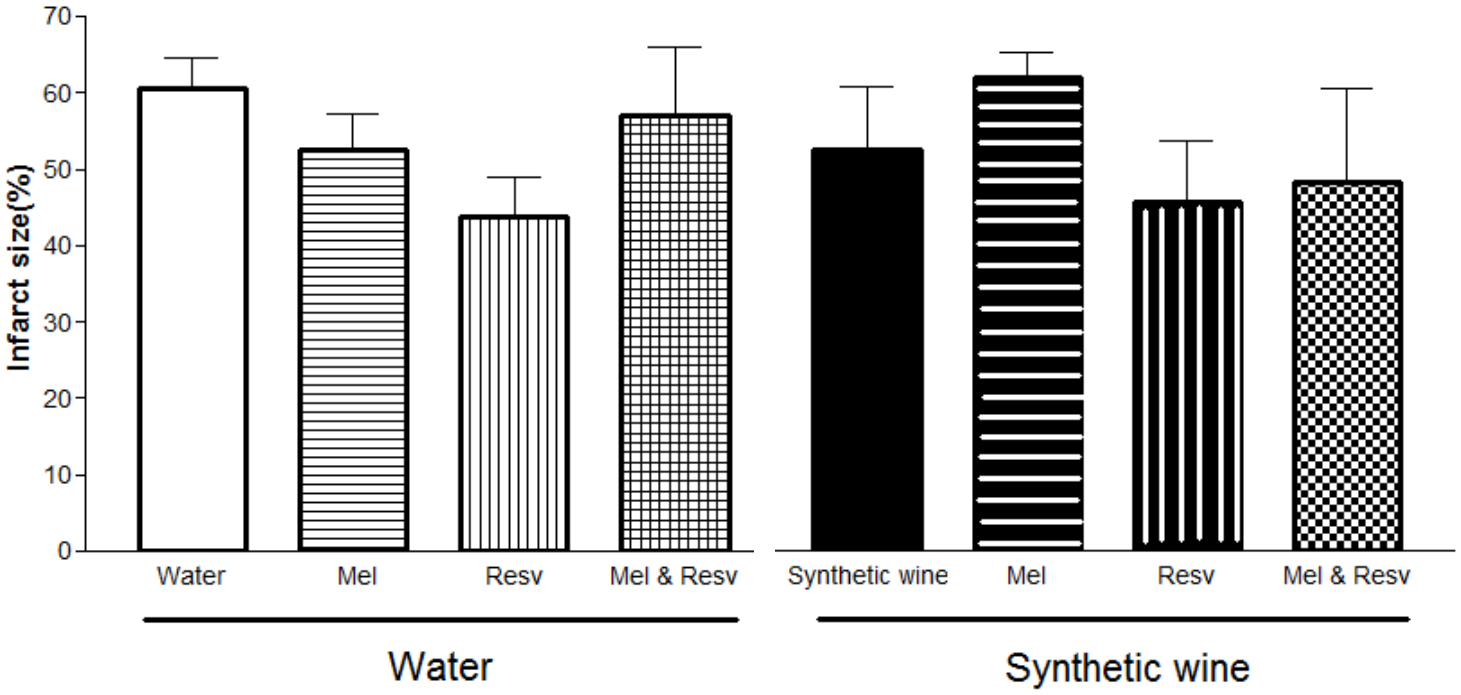


Figure 30*:* Effect of chronic consumption of synthetic wine enriched with melatonin(Mel) and/or Resveratrol(Resv) on infarct size after 60 minutes of reperfusion using a digitized tablet. Control n=12, Synthetic wine n=6, SW & M-synthetic wine & Melatonin n=7 wine, SW & R-synthetic wine & Resveratrol n=7, W & M-Water & Melatonin n=8, W & R-Water & Resveratrol n=5, W,M & R-Water,Melatonin & Resveratrol n=6, SW, M & R-Synthetic wine, melatonin and Resveratrol n=6.

3. Analysis of the antioxidant effect of synthetic wine with and without melatonin and Resveratrol on rat plasma antioxidant activity

3.1 Effect of chronic consumption of synthetic wine enriched with melatonin and/or resveratrol on plasma total antioxidant activity

The total antioxidant capacity of the rat plasma after various treatments was assessed by using the ORAC assay (Figure.31). Control animals had a total antioxidant capacity of 9.4±2.2 µmol/mL trolox equivalents (TE). There was no difference between animals pre-treated with synthetic wine compared to the control group (15.2±3.6 µmol/mL vs control). Synthetic wine enriched with either melatonin or resveratrol did not alter the total antioxidant activity relative to the control group. The ORAC values did not differ between animals pre-treated with water enriched with melatonin (7.7±2.5 µmol/mL) and water enriched with resveratrol (6.5±2.4 µmol/mL). However, animals pre-treated with synthetic wine and water enriched with both resveratrol and melatonin demonstrated a significant reduction in total antioxidant capacity relative to animals treated with synthetic wine only (**p<0.01;1.1±2.9 mol/mL, 1.0±0.4 µmol/mL vs synthetic wine). Similarly, animals pre-treated with synthetic wine and water enriched with both resveratrol and melatonin showed a significant reduction in total antioxidant capacity relative to animals treated with synthetic wine enriched with melatonin only (**p<0.001; 1.1±2.9 µmol/mL, 1.0±0.4 µmol/mL vs 15.4±3.3).

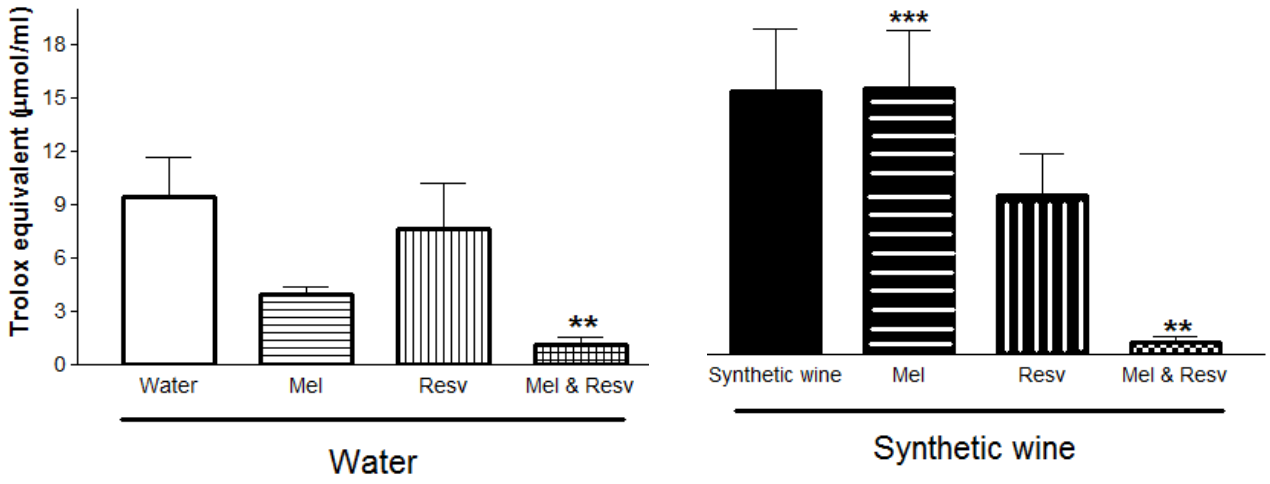


Figure 31*:* Oxygen radical absorbance capacity (ORAC) assay to determine the plasma antioxidant capacity in Trolox equivalents (µmol/mL). Mel-Melatonin and Resv-Resveratrol.**p<0.01 vs. Synthetic wine, ***p<0.001 vs. Synthetic wine enriched with both resveratrol and melatonin/Water enriched with both resveratrol and melatonin. Control n=12, Synthetic wine n=6, SW & M-synthetic wine & Melatonin n=7 wine, SW & R-synthetic wine & Resveratrol n=7, W & M-Water & Melatonin n=8, W & R-Water & Resveratrol n=5, W,M & R-Water,Melatonin & Resveratrol n=6, SW, M & R-Synthetic wine, melatonin and Resveratrol n=6.

3.2 Effect of chronic consumption of synthetic wine enriched with melatonin and/or resveratrol on plasma levels of oxidative stress markers

To measure markers of oxidative stress in the plasma after various treatments, a marker of lipid peroxidation, the TBARS assay measuring plasma MDA concentrations was carried out. Water treated animals had a MDA concentration of 11.5±1.1 µmol/L (Figure.32). There was no significant difference between animals pre-treated with synthetic wine compared to the control group (12.6±2.2 µmol/L vs control). Addition of either melatonin or resveratrol in the synthetic wine did not significantly alter MDA levels relative to the control group (8.5±0.4 µmol/L & 11.7±1.6µmol/L, respectively). Furthermore, the MDA concentrations did not significantly differ between animals treated with water enriched with melatonin (9.8±0.9 µmol/L) and water enriched with resveratrol alone (9.76±0.69 µmol/L). Animals pre-treated with synthetic wine combined with both resveratrol and melatonin displayed significantly higher MDA concentrations relative to animals treated with synthetic wine and melatonin only (16.3±0.9 µmol/L vs synthetic wine and melatonin). In addition, animals treated with water combined with resveratrol and melatonin showed significantly increased MDA concentrations compared to animals treated with synthetic wine enriched with melatonin (16.6±0.1 µmol/L vs 8.5±0.4)

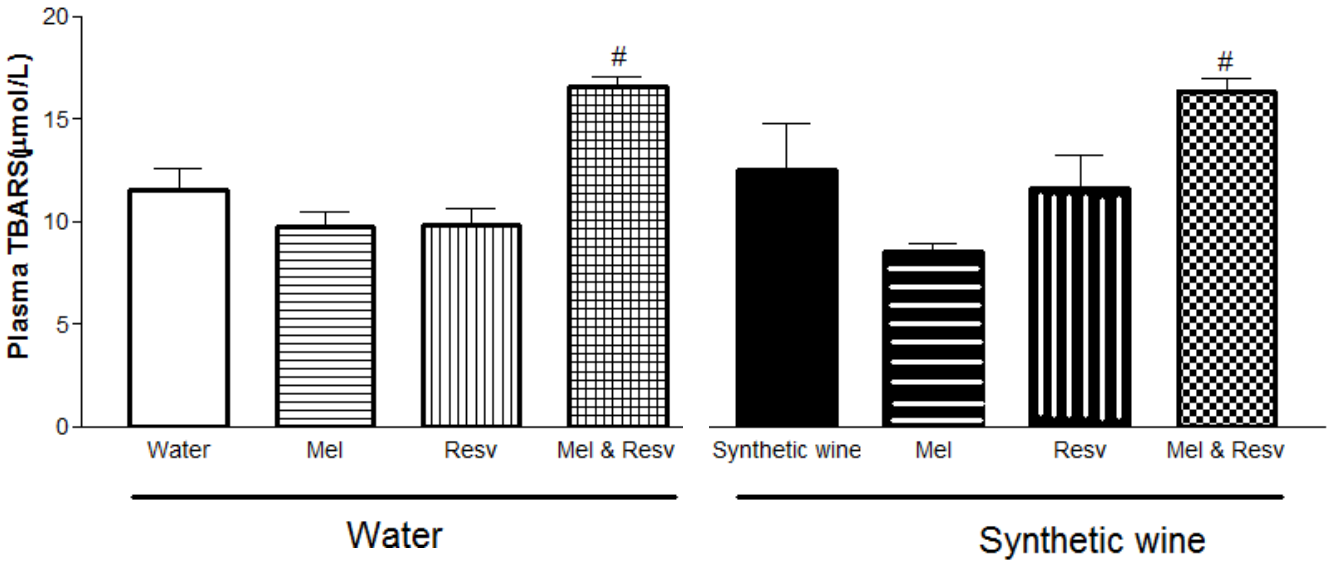


Figure 32: Results of Thiobarbituric acid reactive substances assay (TBARS) assay for the measurement of malondialdehyde (MDA) in rat plasma. Mel-Melatonin and Resv-Resveratrol. #p<0.01 vs. Synthetic wine and melatonin. Control n=12, Synthetic wine n=6, SW & M-synthetic wine & Melatonin n=7 wine, SW & R-synthetic wine & Resveratrol n=7, W & M-Water & Melatonin n=8, W & R-Water & Resveratrol n=5, W,M & R-Water,Melatonin & Resveratrol n=6, SW, M & R-Synthetic wine, melatonin and Resveratrol n=6.

3.3 Effect of chronic consumption of synthetic wine or water enriched with/without melatonin and/or resveratrol on plasma antioxidant enzyme catalase activity

Catalase activity of the plasma obtained from various treatment groups was assessed. Results shown in Figure 32 illustrate control animals had a total catalase activity of 39.89±9.47 IU/mg protein. There was no statistically significant difference between animals pre-treated with synthetic wine compared to the group treated with water only (25.5±11.5 IU/mg protein vs control). Addition of melatonin or resveratrol in the synthetic wine did not significantly alter catalase activity relative to the control group (28.7±5.3 IU/mg protein & 36.9±8.3IU/mg protein, respectively). Furthermore, the catalase activity did not differ between animals treated with melatonin alone (29.2±7.2 IU/mg protein) and resveratrol alone (24.7±6.6 IU/mg protein). Synthetic wine or water enriched with melatonin and resveratrol did not significantly change the catalase activity of the plasma obtained from the respective groups.

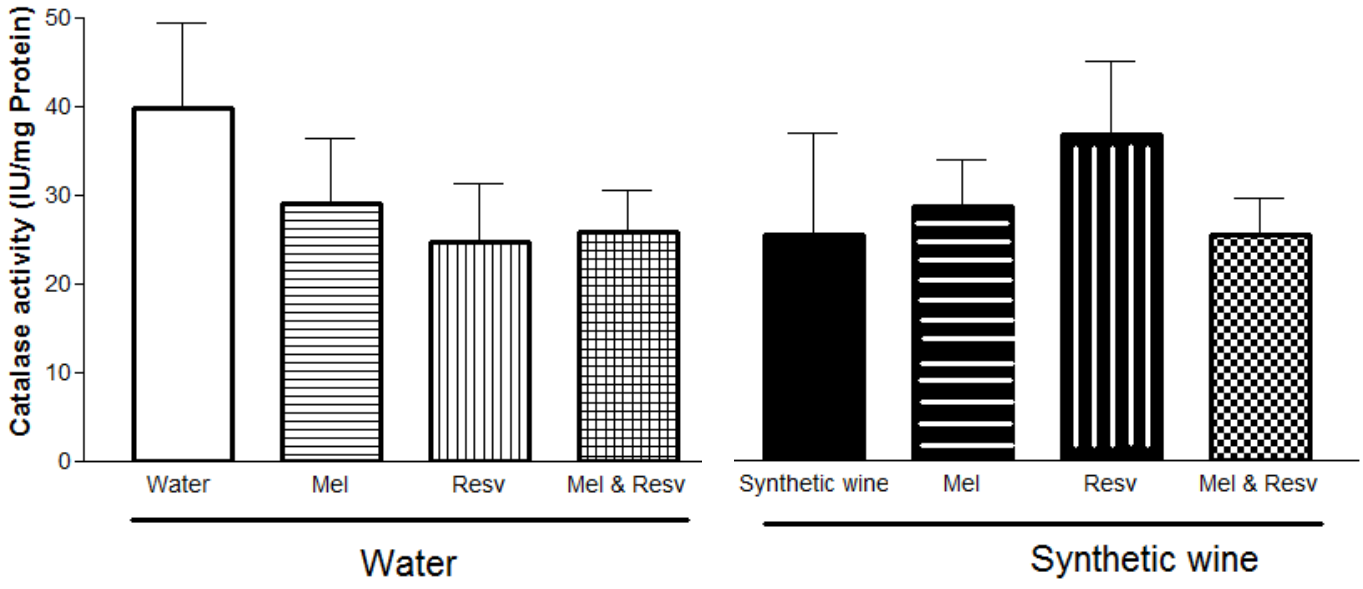


Figure 32: Effect of chronic consumption of synthetic wine or water enriched with/without melatonin(Mel) and/or Resveratrol(Resv) on Catalase activity in rat plasma. Control n=12, Synthetic wine n=6, SW & M-synthetic wine & Melatonin n=7 wine, SW & R-synthetic wine & Resveratrol n=7, W & M-Water & Melatonin n=8, W & R-Water & Resveratrol n=5, W,M & R-Water,Melatonin & Resveratrol n=6, SW, M & R-Synthetic wine, melatonin and Resveratrol n=6.

3.4 Effect of chronic consumption of synthetic wine or water enriched with/without melatonin and/or resveratrol on plasma antioxidant enzyme superoxide dismutase (SOD) activity

SOD activity of the plasma obtained from various treatment groups was assessed. Results shown in Figure.33 illustrate control animals had a total SOD activity of 6.6±0.8 IU/mg protein. There was no statistically significant difference between animals pre-treated with synthetic wine compared to the control group (7.4±0.5 IU/mg protein vs control). Addition of melatonin or resveratrol in the synthetic wine did not significantly alter SOD activity relative to the control group (6.0±0.3 IU/mg protein & 6.2±0.7 IU/mg protein, respectively). Furthermore, SOD activity did not differ between animals treated with melatonin alone (7.7±0.4 IU/mg protein) and resveratrol alone (7.0±0.3 IU/mg protein). Synthetic wine or water enriched with both melatonin and resveratrol did not significantly change the SOD activity of the plasma obtained from the respective groups.

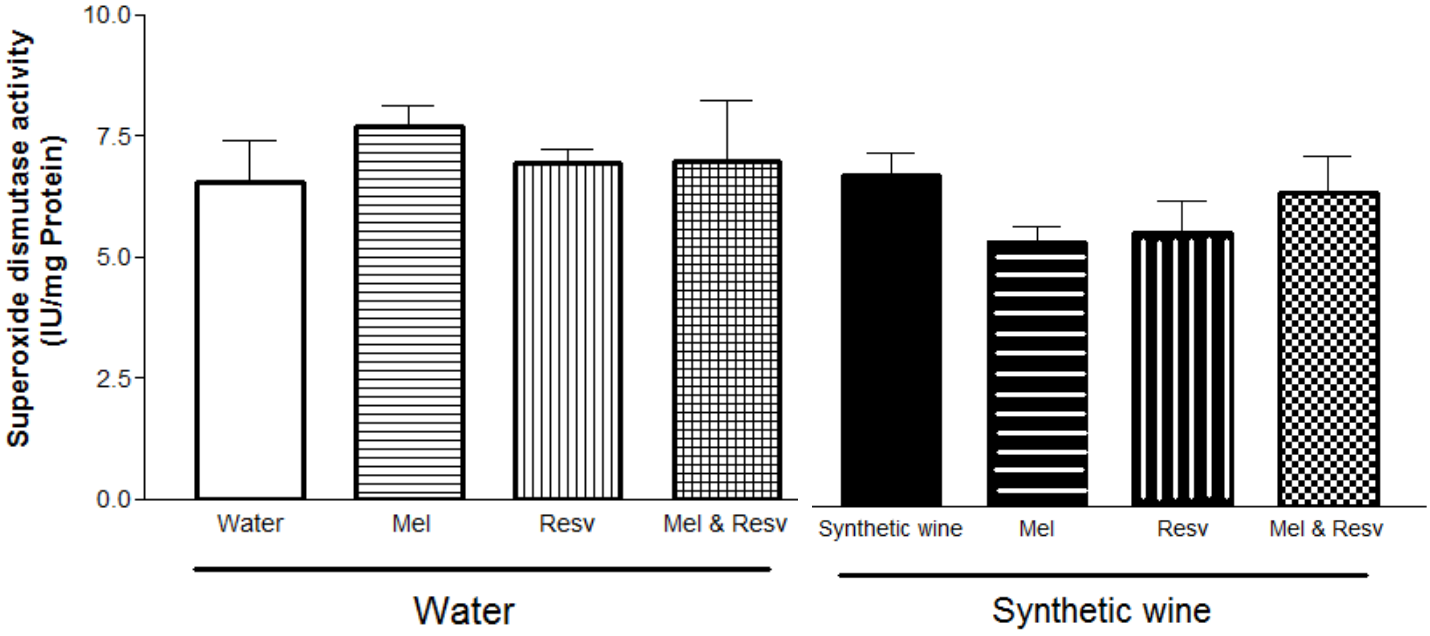


Figure 33: Effect of chronic consumption of synthetic wine or water enriched with/without melatonin (Mel) and/or Resveratrol (Resv) on superoxide dismutase activity in rat plasma. Control n=12, Synthetic wine n=6, SW & M-synthetic wine & Melatonin n=7 wine, SW & R-synthetic wine & Resveratrol n=7, W & M-Water & Melatonin n=8, W & R-Water & Resveratrol n=5, W,M & R-Water,Melatonin & Resveratrol n=6, SW, M & R-Synthetic wine, melatonin and Resveratrol n=6.

E. DISCUSSION

4.1 Summary of results

The aim of our study was to use synthetic wine whose composition is well characterized to explore whether the presence of alcohol, resveratrol and melatonin (as found in commercial wine) contributes to the cardioprotective effect of chronic and moderate (2 glasses wine/day) consumption of red wine in an animal model. Additionally, we hypothesized that synthetic wine enriched with these components confers cardioprotection via improvement of overall antioxidant profile.

In this study, we have confirmed that chronic and moderate consumption of resveratrol or melatonin, given in water for 14 days, has the ability to protect the heart against IR injury in an animal model. Synthetic wine, given on its own for 14 days, did not confer any cardioprotective benefit. However, a chronic treatment of synthetic wine enriched with both resveratrol and melatonin conferred cardioprotection against IR injury. Surprisingly, this cardioprotective effect was lost when the synthetic wine was given with resveratrol or melatonin only.

A chronic treatment with synthetic wine enriched with both melatonin and resveratrol did not affect plasma antioxidant enzymes activities and plasma lipid peroxidation in rats. These data therefore do not support that red wine protects via a modulation of the antioxidant profile.

Our data therefore suggest that the presence of both melatonin and resveratrol is critical for chronic and moderate consumption of red wine-induced cardioprotection.

4.2 Stability of synthetic wine

A synthetic wine is traditionally used in wine biotechnology to optimize yeast fermentations and to assess the efficiency of various yeast strains. To the best of our knowledge, this is the first study to use synthetic wine in order to investigate its effect against IR injury. Since there was virtually no information available on the chemical and functional properties of the synthetic wine, the first objective of our study was to investigate synthetic wines stability and functionality before feeding it to our animals. The total antioxidant capacity and total phenol of the synthetic wine remained relatively the same throughout the 20 week storage period. These measures therefore suggest that the synthetic wines chemical and functional properties remain constant throughout the duration of the project and this information was useful to avoid any confounders due to artefacts of ageing in the wine.

4.3 Cardioprotection with synthetic wine

4.3.1 Alcohol fails to induce cardioprotection

Alcohol is thought to contribute to the cardioprotective effect of red wine against IR injury. In this study, we were able to demonstrate that synthetic wine, an alcohol based matrix, did not confer protection against IR injury. This result suggests that alcohol solely does not play a vital role in wine induced cardioprotection.

Previous studies have demonstrated that protection conferred by alcohol against IR injury proceeds through an ischemic preconditioning-like mechanism which involves activation of protein kinase C (PKC) and that this is dependent on the time and duration of ethanol exposure. Hence, a study has shown that rats treated with 36% alcohol for 16 weeks and ginea pigs treated with 20 % alcohol for 16 weeks protected the hearts against IR injury (Miyamae, Camacho et al. 1998). Likewise, guinea pigs fed with 2.5% alcohol for four months showed improved recovery of post-ischemic left ventricular developed pressure and decreased necrosis compared to control ginea pigs fed with water (Miyamae, Domae et al. 2003). In contrast, in our study the chronic consumption of synthetic wine (12%) for 14 days did not improve functional recovery nor reduce infarct size. Possibly, if the rats were pre-treated for a longer duration or given the treatments through oral gavage, cardioproection would have been observed. Of note, in our study, infarct size was quantified using two well

established planimetry techniques, Image J and Summa sketch. Both techniques showed similar patterns in measuring the level of necrosis in the heart tissue. However, Image J computerised planimetry in our opinion, is more objective relative to Summa sketch since it detects infarct size based on threshold settings predetermined by the program. In contrast, Summa sketch detection of infarct size is dependent on the skill of the person analysing the hearts.

4.3.2 Resveratrol protects isolated hearts against ischemia/reperfusion injury

Substantial experimental evidence has demonstrated that acute administration of resveratrol can provide cardioprotection against IR injury. Resveratrol given at a concentration of 228µg/L elicits its cardioprotective effect by improving post-ischemic ventricular function and aortic flow, reduces infarct size and translocates glycogen synthase kinase 3 β (GSK-3 β) from the cytosol to the mitochondria presumably by targeting the mitochondria permeability transition pore (mPTP)(Xi, Wang et al. 2009) (Ray, Maulik et al. 1999a). However, most studies administered resveratrol at a much higher concentration than that found in wine. Lamont and colleagues were able to observe a cardioprotective effect when resveratrol (2.3mg/L) was given acutely to hearts exposed to IR injury. Furthermore, resveratrol was shown to protect through the activation of the SAFE pathway (Lamont, Somers et al. 2011). However, when rats were pre-treated with resveratrol (7mg/L) chronically for 10 days, Resveratrol failed to improve post-ischemic functional recovery or reduced infarct size (Lamont 2009b).

To date, there have not been any studies that explored the cardioprotective effect of chronic administration of resveratrol against IR injury. To the best of our knowledge, our study is the first to demonstrate that the chronic consumption of resveratrol at a concentration of 100µg/L can protect against IR injury. We observed that hearts pre-treated with resveratrol independent of synthetic wine showed improved functional recovery after IR insults. However, our antioxidant and oxidative stress data do not show significant differences between resveratrol and other treatment groups, suggesting that resveratrol might elicit its cardioprotective effects independent of its' direct radical scavenging effect. Treatment of normal rats with resveratrol does not affect lipid peroxidation, as reflected by the presence of TBARS (Turrens, Lariccia et al. 1997). However, in spontaneously hypertensive rats, pre-treatment with resveratrol (2.3mg/body weight) for 8 weeks significantly reduced oxidative stress and inflammation (Thandapilly, Louis et al. 2013). In addition, obese rats

supplemented with resveratrol for 2 months had a higher survival rate than their control counterparts after isoproterenol injection (60 mg/kg) and this effect was largely associated with increased antioxidant enzyme activities and reduced oxidative damage (Avila, Marques et al. 2013). These studies show that resveratrol can suppress pathological increases in the peroxidation of lipids and other macromolecules *in vivo.* However, it is possible that resveratrol exerts its cardioprotective effect through NO and adenosine. Indeed, in a similar model to ours, rats provided with resveratrol (1mg/body weight) for 15 days showed improved recovery in function and coronary flow of isolated hearts even 24 hours after resveratrol treatment was stopped (Bradamante, Barenghi et al. 2003). This protective effect was hindered by L-NAME, a nitric oxide synthase (NOS) inhibitor or 8-(p-sulfophenyl)theophylline (SPT), an adenosine receptor antagonist. This could possibly suggest that the activation of adenosine receptors play a role in the delayed effects of resveratrol. Surprisingly, the addition of resveratrol in synthetic wine did not improve post-ischemic left ventricular function. This could be due, in part, to a quenching effect of the synthetic wine, however, more studies would have to be done in examining interactions between properties of synthetic wine.

4.3.3 Melatonin protects isolated heart against ischemia/reperfusion injury

Previous studies have shown that melatonin administered both chronically and acutely reduced myocardial IR injury in an isolated heart model. Indeed, isolated hearts exposed to an acute concentration (100μM) of melatonin demonstrated a significant reduction in MDA, improve left ventricular function and decreased duration of ventricular tachycardia and ventricular fibrillation (Kaneko, Okumura et al. 2000). In an *in vivo* model, rats exposed to IR injury and treated with melatonin (10mg/kg) before ischemia showed a significant reduction in MDA levels, infarct size and increased glutathione (GSH) levels (Sahna, Parlakpinar et al. 2005). However, the concentration of melatonin used in these studies is much higher than the concentration found in red wine, which usually ranges between 74ng/mL to 423 ng/mL subject to the grape variety (Rodriguez-Naranjo, Gil-Izquierdo et al. 2011b).

In our experiments, chronic consumption of melatonin given alone at a concentration of 75ng/L was sufficient to improve functional recovery as previously described size (Lamont 2009b). However, the treatment failed to reduce infarct size. Previous studies in our lab have demonstrated a reduction in infarct size after pre-treated with melatonin (75ng/L) for 10 days (Lamont 2009). However, in our experiments we failed to observe a reduction in infarct size

after 14 days of pre-treatment. This could be attributed to the levels of stress the rats were exposed to at the time of these experiments as the building in which the rats were housed was undergoing extensive renovations and the noise may have significantly altered the behaviours of the experimental subjects. In addition, our oxidative stress and antioxidant data does not show significant differences between melatonin and the control group, thus suggesting that the free radical scavenging properties of melatonin may not play a major role in its cardioprotective effect in our model. It is possible melatonin conferred its effect through melatonin receptors. Previously, it has been shown that melatonin mediates its cardioprotective effect by activating pro-survival RISK and SAFE pathways during reperfusion and inhibiting the opening of the MPTP (Smith, Dixon et al. 2010). Lamont and colleagues demonstrated convincingly the significance of melatonin receptors in melatonin induced cardioprotective by demonstrating that luzindole, selective melatonin receptor antagonist, abolished it cardioprotective effect against IR injury (Lamont 2009).

4.3.4 Combination of resveratrol and melatonin induces additional cardioprotection

Red wine has been shown to be cardioprotective against IR injury. Multiple studies have demonstrated the protective effect of red wine using red wine extracts, singular components of red wine such as resveratrol or ethanol (Sato, Ray et al. 2000) or whole red wine (Lamont, Blackhurst et al. 2012, Lamont 2009). A previous study in our lab surprisingly demonstrated an inverse relationship between resveratrol and melatonin content i.e South African wines with high melatonin content had a low resveratrol content and conversely (Albertyn 2012). Nonetheless, wines which displayed this relationship showed improved functional parameters after IR injury. This demonstrates melatonin and resveratrol in wine may act synergistically to provide a cardioprotective effect against IR injury. One of the chief limitations of this study was that it was difficult to attribute the cardioprotection to melatonin specifically as wine is a complex matrix with multiple components which may be responsible for wine-induced cardioprotection.

In our experiments, a chronic pre-treatment of synthetic wine enriched with both melatonin and resveratrol at concentrations 75ng/L and 100µg/L respectively, was enough to further improve post-ischemic functional recovery compared to groups which either received melatonin or resveratrol only. This effect could be attributed to melatonin binding onto

quinone reductase 2, a third melatonin binding site (MT3). Interestingly, a recent study has shown that quinone reductase 2 has a similar affinity for resveratrol and melatonin (Ferry, Hecht et al. 2010). We speculate that the synergy of both compounds leads to the activation of quinone reductase 2 as well. This could partially explain why wine-induced cardioprotection was not eradicated in the presence of luzindole, a non-selective inhibitor which binds onto MT1 and MT2, but protection was lost in melatonin-induced cardioprotection when luzindole was co-administered (Lamont 2009). It would be interesting to investigate whether the administration of both luzindole and S29434, a novel competitive inhibitor of quinine reductase 2 (Reybier, Perio et al. 2011) may alter synthetic wine enriched with both melatonin and resveratrol induced cardioprotection. Thus, co-administration of melatonin and resveratrol, given at concentrations similar to those found in red wine, had an synergistic effect and protected the myocardium against IR injury by improving functional recovery to a better degree than their own individual parts. Interestingly, rats pre-treated with synthetic wine (12% in alcohol) enriched with either resveratrol or melatonin did not demonstrate improved post-ischemic functional recovery or reduced infarct size. Perhaps components within the synthetic wine have a quenching effect on melatonin and resveratrol and thus these two components are unable to fully execute their cardioprotective capabilities.

4.4 Role of antioxidants in synthetic wine induced cardioprotection

Reperfusion injury worsens myocardial injury and considerable evidence attributes free radicals either produced by the cardiomyocyte or by inflammatory cells (Bolli, Jeroudi et al. 1989) as an early event in IR injury. Once produced, free radicals can lead to cellular damage through multiple pathways including direct damage to membranes (Toyokuni 1999) or indirect damage through activation of pro-apoptotic pathways (Bialik, Cryns et al. 1999). While using antioxidants to scavenge free radicals may be a potential approach to reduce myocardial IR injury, multiple clinical trials using antioxidant therapies have been largely disappointing see review (Steinhubl 2008).

Well known components of red wine such as resveratrol and melatonin are suggested to protect against IR injury through their antioxidant properties. In this study, chronic consumption of the various treatments did not increase antioxidant enzymes catalase and SOD activity or reduce the amount of lipid peroxidation. In addition, most of the treatments did not significantly increase the total antioxidant capacity of the animals receiving the treatment. Surprisingly, animals pre-treated with synthetic wine or water enriched with both melatonin and resveratrol demonstrated significant increases in lipid peroxidation and a

significant decrease in their total antioxidant capacity. Paradoxically, these are the two groups which demonstrated improved post-ishemic recovery relative to the animals which received water only. Our experiments were conducted in baseline conditions and it would be of interest to explore the antioxidant status of the rats in both plasma and heart tissue following an IR insult. Of note, free radicals have been implicated as mediators of diverse physiologic and pathophysiologic events however, there is growing evidence which suggests that free radicals may have a dual effect, becoming protective in some pathological conditions. ROS generation has been suggested to mediate in ischemic preconditioning see review (Yellon, Downey 2003) and the role of free radicals in TNFα induced cardioprotection (Lecour, Rochette et al. 2005).

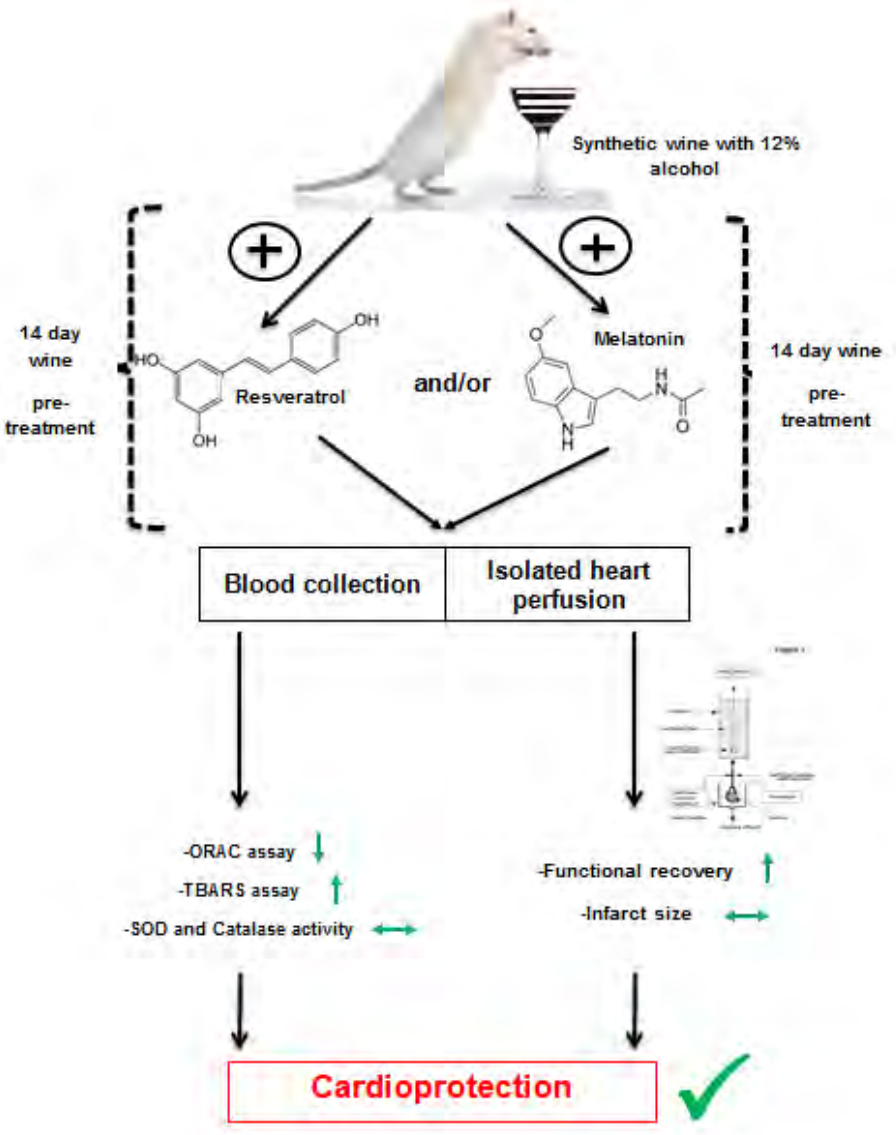
4.5 Limitations and future prospects

a) It would be of interest to simulate our experiments using an in vivo model of IR injury, as the isolated heart does not take into consideration the confounding effects of other organs systemic and circulating neuro-hormonal factors.
b) The oxidant and antioxidant results predominantly did not show any level of significance. It would have been interesting if animal tissue such as the heart and liver were examined to see if there were any changes in the pro- and anti-oxidant status. In addition, if hearts subjected to IR injury were examined for changes in the oxidative profile, there could have been more robust differences in our measures.
c) During the duration of this project, the building in which our animals were being housed was being refurbished. The noise from the on-going construction could have stressed the animals and thus, might have been responsible for the lack of differences between the groups when measuring infarct size.
d) Most of the results demonstrated large variation within groups. If the sample size within each group were to be increased statistical significance could possibly be reached
e) There was no way to ascertain if all the rats in one cage received the same amount of treatment in a day. Thus, giving the rats their treatment through oral gavage would be a more accurate method.
f) In this study, we only used resveratrol and melatonin to enrich the synthetic wine. There are other components which could be added into the matrix such as ethanolamine which could be a step further in mimicking the composition of wine.

g) This study demonstrated synthetic wine enriched with both resveratrol and melatonin improved post-ischemic functional recovery. However, we did not elucidate through which mechanism it achieves this effect. It would be of interest if we could use both melatonin inhibitors luzindole and S29434 and examine if the treatment elicits it effect through pro-survival SAFE and RISK pathways.

4.6 Conclusion

In conclusion, our data strongly suggest that chronic and moderate consumption of synthetic wine enriched with both melatonin and resveratrol protects against IR injury. Our data did not support a role for alcohol in red wine-induced cardioprotection. Our findings support the use of dietary concentrations of combined resveratrol and melatonin as a safe, inexpensive and simple therapy against ischemic heart disease.



F. APPENDICES

APPENDIX A

Production of synthetic wine

Component	Amount per litre
Carbon sources	
Glucose	100 g
Fructose	100 g
Acids	
PotassiumTartrate	2.5 g
L-Malic acid	3.0 g
Citric acid	0.2 g
Salts	
Dipotassium phosphate	1.14 g
magnesium sulfate heptahydrate	1.23 g
Calcium Chloride Dihydrate	0.44 g
Nitrogen sources	
Ammonium phosphate dibasic	500 mg
Alanine	100 mg
Arginine	750 mg
Asparagine	150 mg
Aspartic acid	350 mg
Glutamine	200 mg
Glutamic acid	500 mg
Glycine	50 mg
Histidine	150 mg
Isoleucine	200 mg
Leucine	300 mg
Lysine	250 mg
Methionine	150 mg
Phenylalanine	150 mg
Proline	500 mg
Serine	400 mg
Threonine	350 mg
Tryptophan	100 mg
Tyrosine	20 mg
Valine	200 mg

Trace elements	
Manganese(II)Chloride	200 µg
Tetrahydrate	135 µg
Zinc chloride	30 µg
Iron(II) chloride	15 µg
Copper(II) chloride	5 µg
Cobalt(II) nitrate hexahydrate	30 µg
Sodium molybdate dihydrate	25 µg
Potassium iodate	10 µg
Vitamins	
Myo-Inositol	100 mg
Pyridoxine.HCL	2 mg
Nicotinic acid	2 mg
Ca Pantothenate	1 mg
Thiamin.HCL	0.5 mg
Para-aminobenzoic acid.K	0.2 mg
Riboflavin	0.2 mg
Biotin	0.125 mg
Folic acid	0.2 mg
Lipids/Oxygen	
Ergosterol	10 mg
Tween 80	0.5 mL
Air saturated or	0-9 ppm
O_2 free	
pH	3.2 - 3.5

APPENDIX B

Folin-ciocalteau assay

a) 20% (m/v) Gallic acid in ethanol

0.5 g of Gallic acid in 10mL of ethanol

b) Gallic acid standard curve:

100µL of Gallic acid + 9900µL distilled water=500mg/L

400µL 0f Gallic acid + 100µL distilled water=400mg/L

300µL of Gallic acid + 200µL distilled water=300mg/L

200µL of Gallic acid + 300µL distilled water=200mg/L

100µL of Gallic acid + 400µL distilled water=100mg/L

50µL of Gallic acid + 450µL distilled water=50mg/L

0µL of Gallic acid + 500µL distilled water=0mg/L

c) 20% (m/v) Sodium carbonate (Na_2CO_3)

Measure 20g anhydrous Na_2CO_3 in 80mL distilled water and bring to boil.

After cooling, add few crystals of Na_2CO_3 and leave for 24 hours. Filter and add distilled water to 100mL.

APPENDIX C

Oxygen radical absorbance capacity (ORAC) assay

a) **Phosphate buffer(0.075M pH=7.4):**

0.75M K_2HPO_4 .$3H_2O$ (MW= 228.23: 85.6g →500mL Distilled water)

0.75M $Na2H_2P0_4.H_2O$ (MW= 137.99: 51.8g →500mL Distilled water)

90mL K_2HPO_4 +24mL $Na2H_2P0_4$

Make up to 900mL then pH , make up to 1L

b) **Trolox(6-OH-2,5,7,8-tetromethylchroman-2-carboxylic acid) standard curve**

0.005g Trolox in 200µl ethanol = 100mM

1. 100µl + 9.9 µl buffer =1000µM
2. 1mL + 9mL buffer =100µM
3. 300µl +300 µl buffer =50 µM
4. 300µl +300 µl buffer =25 µM
5. 300µl +300 µl buffer =12.5 µM
6. 300µl +300 µl buffer =6.25 µM
7. 300µl +300 µl buffer =3.13 µM
8. 300µl +300 µl buffer =1.37 µM

c) **Fluorescein (3,6-dihydroxyspiro(isoberyofuran-1(3H),9(9H)-xanthen) (disodium)**

Stock 1: 0.0225g in 50mL buffer (0.0011959 mol/L)

Stock 2: 50µl stock 1 in 10mL buffer 1 in 10m/L buffer (5.98µmol/L)

Working solution: 320µl stock 2 in 20mL buffer (95.7nmol/L)

d) **AAPH: (2,2'-Azobis(2-amidinopropane) dihydrochloride)**

NB: Prepare immediately before use in phosphate buffer pre washed to 37°C

32.1 µmol per well

0.696 + 7840 warm buffer

APPENDIX D

Krebs Henseleit buffer for Langendorff perfusion (5L)

	Weight(g)
Sodium chloride-NaCl	34.63
Sodium bicarbonate-$NaHCO_3$	10.5
Sodium chloride-KCl	1.77
Magnesium Sulfate Heptahydrate-$MgSO_4.7H_2O$	1.47
Monopotassium phosphate-KH_2PO_4	0.80
Glucose	10.99
Calcium chloride-$CaCl_2.2H_2O$	1.00

APPENDIX E

Triphenyltetrazolium chloride staining for defrosted heart sections

Solution 1: 100mM Monobasic sodium phosphate

15.6g NaH_2PO_4 in 1000mL distilled water

Solution 2: 100mM Dibasic sodium phosphate

14.2g Na_2HPO_4 in 1000mL distilled water

Method used for 1% Triphenyltetrazolium chloride solution

Mix 4 parts solution 2 : 1 part solution 1 and titrate to pH 7.4

Add 250 mg TTC in 25ml triphenyltetrazolium buffer solution

APPENDIX F

Solubility of compounds at 20°C

- Resveratrol : 0.03g/L in water at 20°C
  50.0g/L in ethanol at 20°C
- Melatonin: 2g/L in water at 20°C
  182g/L in ethanol at 20°C

Preparation of Melatonin (75 ng /L)

- Measure 15mg into 200ml of solvent (ethanol) to create stock solution **(A)**
- Pipette 10µL of solution **(A)** into 10000µL of ethanol to create solution **(B)**
- Pipette 10 µL of solution **(B)** into 10000µL of synthetic wine to create solution **(C)**
- Dilute 1 part of solution **(C)** into 7 parts of distilled water

Preparation of Resveratrol (100µg/L)

- Measure 10mg in 100ml of ethanol to create stock solution **(A)**
- Pipette 10 µL of solution **(A)** into 10000µL of wine to create solution **(B)**
- Dilute 1 part solution **(B)** into 7 parts of distilled water

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