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Delineation of the Cardioprotective Agents Found in Red Wine

By

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DECLARATION

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
γ	Gamma
κ	Kappa
ADP	Adenosine diphosphate
AG490	STAT-3 inhibitor
AIDS	Acquired Immunodeficiency Syndrome
Akt	Protein kinase B
AMPK	Adenosine monoshosphate kinase
ASMT	N-Acetylserotonin O-methyltransferase
ATP	Adenosine triphosphate
BCL-2/XL	Anti-apoptotic factor(s)
BSA	Bovine serum albumin
cGMP	Cyclic 3,5-gaunosine monophosphate
CHD	Cardiovascular heart disease
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CVD	Cardiovascular disease
DTT	Dithriothreitol
EDTA	Ethylene glycol tetraacetic acid
EGTA	Ethylene diaminetetraacetic acid
ETC	Electron transport chain
HDL	High density lipoprotein
FAS	Fatty acid synthase
GMP	Guanosine monophosphate
GSK-3β	Glycogen synthase-3β
HEPES	4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid
HHT	Hydro hepatadec atrienoate
HIV	Human Immunodeficiency Virus
HR	Heart rate
HRP	Horseradish peroxidase
ICAM	Intracellular adhesion molecule
IGF-1	Insulin-like growth factor-1
IgG	Immunoglobulin G
IL	Interleukin
IP3	Inositol triphosphate

I/R	Ischemia reperfusion
JAK	Janus kinase
KHB	Krebs Henseleit buffer
LDL	Low density lipoprotein
LIF	Leukaemia inhibitory factor
LVDP	Left ventricular diastolic pressure
LVEDP	Left ventricular end diastolic pressure
LVESP	Left ventricular systolic pressure
MAPK	Mitogen activating protein kinase
MDA	Malonaldehyde
MI	Myocardial infarction
mmHg	Millimetres of mercury
MT1/2/3	Melatonin receptor(s)
NADP	Nicotinamide adenine dinucleotide
NO(S)	Nitric oxide (synthase)
NFĶB	Nuclear factor kappa B
OH	Alcohol
PG1	Prostaglandin I (Prostacyclin)
PGC-1	Proliferator-activated receptor-γ coactivator-1
PI3K	Phosphatidylinositol-3-kinase
PKB/C/G	Protein kinase B/C/G
PMSF	Phenylmethylsulfonyl fluoride
PVC	Premature ventricular contracture
RISK	Reperfusion injury salvage kinase
RPP	Rate pressure product
VEGF	Vascular endothelial growth factor
VF	Ventricular fibrillation
VLDL	Very low density lipoprotein
PKCε	Protein Kinase C
K_{ATP} channel	Potassium adenosine triphosphate channel
TNF α	Tissue necrosis factor alpha
FAAH	Fatty acid amide hydrolase
Apo B	Apoprotein B
STAT-3	Signal Transducer and Activator of Transcription
mPTP	mitochondrial permeability pore

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ABSTRACT

Introduction: Chronic moderate consumption of red wine (2-3 glasses/day) can protect against cardiovascular disease. However, the exact components which are found in the wine, and which are responsible for its cardioprotective effect, still remain to be delineated. The alcohol content and the polyphenol, resveratrol, are thought to contribute to this protection but the biogenic amines, melatonin and ethanolamine, recently identified in the composition of red wine, may also present some prosurvival properties.

Aim: Therefore, the aim of our study was to explore whether resveratrol, alcohol, melatonin or ethanolamine, given chronically at the concentration equivalent as found in 2 glasses of wine/day, may protect the heart against an ischemia-reperfusion insult. Furthermore, we hypothesized that red wine and its cardioprotective components protect via the activation of the transcription factor signal transducer and activator of transcription 3 (STAT-3), a prosurvival factor known to protect against ischemia-reperfusion injuries.

Methods: The drinking water of male Wistar rats was supplemented with a French Cabernet Sauvignon (12% or 6% alcohol by volume), alcohol (6%), resveratrol (7mg/L), ethanolamine (21µg/L) or melatonin (0.75µg/L), to a final concentration corresponding to the concentration found in 2 glasses of wine per day. After 10 days of treatment, hearts were perfused on the Langendorff system and subjected to 30 min global ischemia (I), followed by 60 min of reperfusion (R). Functional parameters were recorded throughout the experiments and infarct size was measured at the end of the protocol. Rate pressure product (RPP: heart rate x left ventricular developed pressure), measured at 60 min of reperfusion, was expressed as a percentage of baseline value. At the end of reperfusion, infarct size was recorded for all hearts.

***Results*:** Control hearts subjected to I/R presented a rate pressure product of 20.5±4.5%. Pre-treatment with wine (6 or 12%) improved the rate pressure product to 40±6% and 43±6%, respectively ($p<0.05$ vs. control).

Neither resveratrol nor alcohol 6% given on their own improved the function of the heart, while ethanolamine and melatonin improved functional recovery to a similar extent to the wine (RPP: 33.9±6.7%, 30.6±2.3%, respectively $p<0.05$ vs. control). Similarly, the pre-treatment with red wine, ethanolamine and melatonin decreased infarct size to (21.5±7.8%,19.0±6.9%, 27.5±7.8%, respectively $p<0.05$ vs. control). Interestingly, Western Blot analysis of the hearts show that pre-treatment with red wine, melatonin or ethanolamine were associated with an increase in STAT-3 phosphorylation, compared with the control groups (+70% for red wine, or +79% for melatonin and +57% ethanolamine). Furthermore, chronic administration of AG490, a STAT-3 inhibitor abolished the protective effect of melatonin, ethanolamine or red wine.

Conclusion: Our novel findings suggest that the biogenic amines, ethanolamine and melatonin, rather than resveratrol or the alcohol content in red wine, contribute to the cardioprotective effect of chronic moderate consumption of red wine and that this protective effect is mediated via the activation of STAT-3. Our findings may lead to the development of novel therapies to protect the heart against ischemia-reperfusion injuries.

CHAPTER 1: INTRODUCTION

1.1. Cardiovascular disease is a burden disease worldwide

Cardiovascular disease (CVD) is responsible for more than 12% of worldwide mortality and it is predicted to become the leading cause of death by 2020, superseding infectious diseases such as Human Immunodeficiency Virus (HIV), tuberculosis (TB) and malaria (WHO 2009). CVD is a major concern in both developed and developing countries. In 2009, an estimated 785,000 Americans will have a new coronary heart attack and about 470,000 will have a recurrent heart attack (Lloyd-Jones et al. 2009). In South Africa, CVD is already the number one killer in the Western Cape Province (= region around Cape Town; see figure 1) (Bradshaw et al. 2003). The main causes for heart disease in South Africa are uncontrolled hypertension and an urban lifestyle including inactivity and eating processed high salt foods. Eighty-eight percent of new heart failure patients in South Africa are black Africans with untreated high blood pressure (Steyn et al. 2005, Stewart et al. 2006).

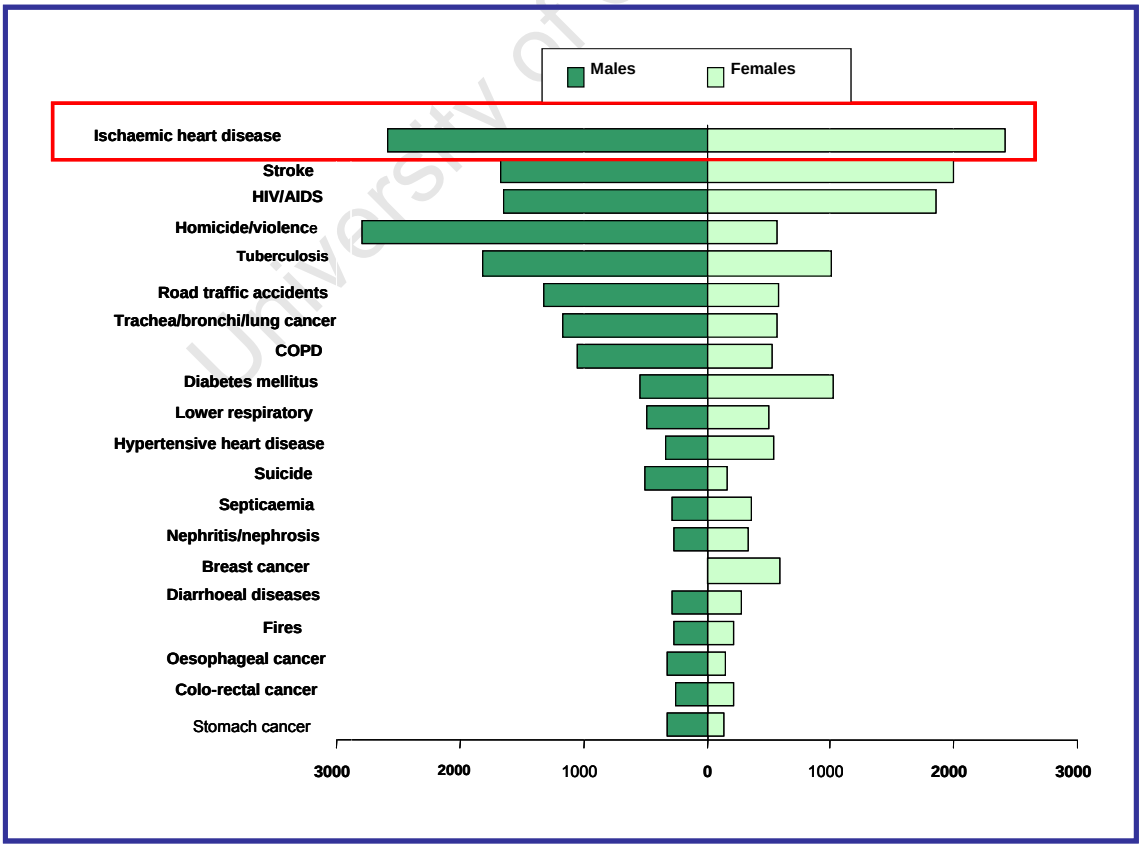


Figure 1: *A graphic representation of the burden of cardiovascular disease in the Western Cape region of South Africa.* (Bradshaw et al. 2003)

Coronary heart disease occurs when an atherosclerotic plaque accumulates and partially or wholly blocks a coronary artery. The formation of a blood clot (thrombus) inside a blood vessel obstructs the flow of blood through the circulatory system. This blockage leads to a reduction in the blood flow, nutrients, oxygen supply and vasoconstriction to an area of cardiac tissue. The area of tissue subjected to reduced flow in such conditions is referred to as 'ischemic' tissue and if left untreated, can result in cell death.

The only way to salvage the heart is to restore the blood flow back (= reperfusion) into the ischemic area as quickly as possible in order to reduce the number of dead cells (forming the infarct) within the myocardium (see figure 2). This can be done with thrombolytic drugs or coronary angioplasty.

The benefit of reperfusion, however, comes at a price. Paradoxically, the restoration of blood flow to the ischemic tissue often results in additional damage to the tissue, known as "reperfusion injury" (Grace 1994). This damage manifests itself as stunned myocardium, arrhythmias, endothelial damage and a significant increase of up to 50% of the final size of the infarct (Yellon, Hausenloy 2007).

Additional therapies are required to limit the damage associated with coronary heart disease and recent evidence suggests that chronic and moderate consumption of red wine may protect the heart against cardiovascular disease.

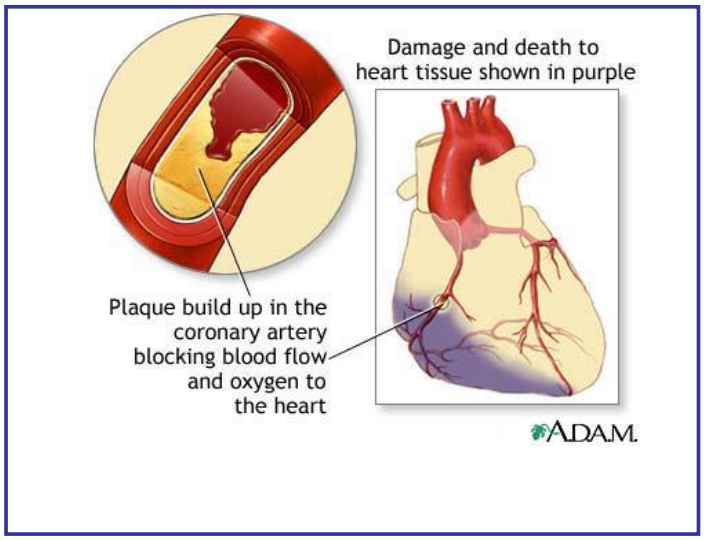


Figure 2: *A graphic representation of myocardial infarction. (From www.adam.about.com/encyclopedia/Acute. MI. html)*

1.2. Wine is a cardioprotective agent

Although consumption of high amounts of wine (as well as other alcoholic beverages) remains a definite risk to health, consumption of lower amounts may not be harmful and even be considered as beneficial**.**

1.2.1. The French paradox

In 1979, St Leger and Colleagues were first to describe an inverse relationship between consumption of wine and deaths from cardiovascular disease in Europe, North America and Australasia (St Leger, Cochrane & Moore 1979a, St Leger, Cochrane & Moore 1979b). In 1992, Renaud and De Lorgeril emphasized the fact that a higher consumption of wine in France compared with other Western countries may contribute to explain the French Paradox, whereby French have a low coronary heart disease death rate despite high intake of dietary cholesterol and saturated fat (Renaud, de Lorgeril 1992).

A study conducted in the Eastern part of France between 1978 and 1983 in more than 36 000 men, demonstrated that men drinking between 2 to 4 glasses/day suffered 30% fewer deaths from all causes compared with non-drinkers or drinkers with more that 4 glasses/day (Renaud et al. 1999).

However, attributing the cardioprotective effect to the red wine leads to controversies as all the facets of wine drinkers' behaviour cannot be summed up. Hence, differences in alcohol consumption may also reflect differences in nutritional intake. Indeed, wine drinkers consumed more fruits, salad, vegetables, fish and olive oil (Tjonneland et al. 1999). Also, wine drinkers exercise more (such as gardening), smoke less and they have a higher level of education (Mukamal et al. 2005a, Mukamal et al. 2005b).

Also, the drinking pattern maybe of importance. Hence, a daily consumption of wine is associated with a better outcome than only occasional or weekend drinking (WHO, 2002). Drinking wine with food may also be of benefit as it slows the absorption of ethanol.

1.2.2. Red wine vs. white wine vs. other alcoholic drinks

In a Danish study conducted on more than 24 000 men and women living in Copenhagen, 1 to 3 glasses of wine intake reduced the risk of dying from cardiovascular disease by half compared with non-drinkers, whereas beer and spirit drinkers did not experience this advantage (Gronbaek 2000, Gronbaek et al. 2000). Similar data were found in a French study (Renaud et al. 1999) and in Northern California (Klatsky 2003, Klatsky et al. 2003). In contrast, type of alcohol made no difference to its protective effect on myocardial infarction in more than 38 000 participants in the US male health professions study over 12 years of follow-up (Mukamal et al. 2003). In reality, data suggesting a better benefit of wine vs. beer may reflect the fact that wine drinkers have a better lifestyle than beer drinkers (Wannamethee, Shaper 1999).

The unique protective effects of wine have been attributed to several polyphenols, especially resveratrol which is present in high concentration in red wine but not in white wine. This is explained by the fact that the grape skin, which contains a high concentration of polyphenols, is retained during the fermentation process of the red wine, while it is discarded for the white wine. Thus, the protective effect of red wine is suggested to be greater than white wine (van Velden, Mansvelt & Troup 2002).

However, several recent experimental studies indicate that white wine could have cardioprotective abilities similar to those of red wine and it is suggested that this cardioprotective effect is mediated by the presence of the polyphenols tyrosol and hydroxytyrosol (Dudley et al. 2008).

1.2.3. Main physiological properties of red wine

A regular consumption of red wine in humans (375 mL/day of red wine for 2 weeks) is associated with a strong antioxidant effect (Fuhrman, Aviram 2001). This antioxidant effect may contribute to the cardioprotective effect of red wine by reducing the oxidative stress that occurs in various pathophysiological conditions such as ischemic heart disease, arteriosclerosis, heart failure, hypertrophy and arrhythmias (see review (Opie, Lecour 2007). Regular consumption of red wine reduces low density lipoproteins (LDL also called the "bad cholesterol") and lipid peroxidation and increases plasma concentration of high density lipoproteins (HDL also called the "good cholesterol") (Fuhrman, Lavy & Aviram 1995). In addition, the red wine modified and enriched the composition of HDL in polyunsaturated phospholipids such as omega-3 fatty acids, which are said to be beneficial against coronary artery disease (Perret et al. 2002). Red wine also inhibits smooth muscle cell proliferation in a dose-dependent manner, via the activation of phosphoinositol 3 phosphate kinase and p38 mitogen activated protein kinases (Araim et al. 2002). In addition, aggregation in response to adenosine diphosphate (ADP) and thrombin in human platelets is strongly inhibited by red wine (Pace-Asciak et al. 1995).

1.2.4. Red wine and anti-ischemic properties

In survivors of a recent myocardial infarction event, the association between red wine intake and the risk of recurrence during a 4-year follow-up demonstrated a reduction of the adjusted risk of cardiovascular complications by 59% in patients, with an average drinking of four glasses per day (de Lorgeril et al. 2002).

Chronic and moderate consumption of red wine is thought to contribute to cardioprotection and many experimental studies using the isolated rat heart model have explored the role of red wine against ischemia-reperfusion. However, all these studies were performed by giving acutely either red wine polyphenols extracts (Das et al. 1999) or some components of the wine such as resveratrol or ethanol (Ray et al. 1999b); see review (Das, Maulik 2006b). In the literature, none of the studies gave the original red wine orally, in conditions corresponding to a moderate and chronic consumption of red wine. Therefore, the relevance to a clinical setting of these studies is very limited.

1.3. Cardioprotective components found in red wine

Wine is a complex nutriment based on grape juice, in which alcohol has formed following natural fermentation. Wine contains more than 500 compounds, some originating from the grapes and some metabolic by-products of yeast activity during fermentation. Most of these compounds are present in very low concentrations, but a few occur at concentrations above 100 mg/mL. These include water, alcohol, organic acids, sugars and glycerol (see figure 3).

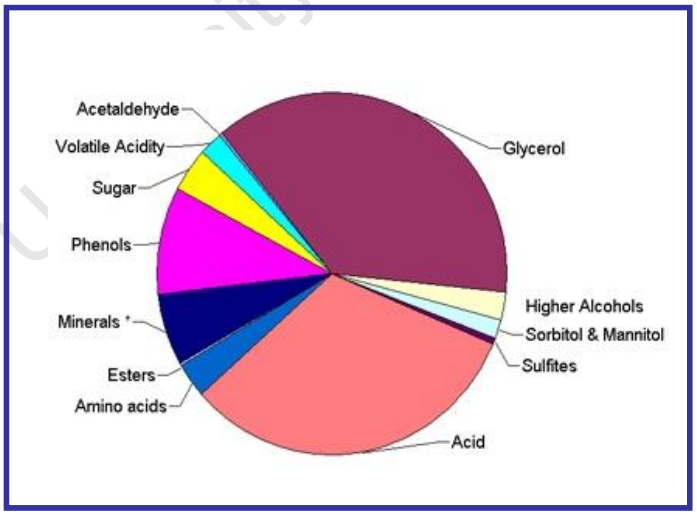


Figure 3: *The main components of red wine. Theworldwidewine.com*

Here, we will review four compounds found in red wine and that we suggest may play a role in red wine-induced cardioprotection: alcohol, resveratrol, melatonin and ethanolamine.

1.3.1. Alcohol-induced health benefit

The most important alcohol in wine is ethanol, with concentrations ranging from 10 to 14%. Ethanol is crucial for the stability, aging, and gustatory properties of the wine. It plays a role in the extraction of pigments and tannins during the fermentation of the skin and seeds of grapes (Das, Maulik 2006a) (Das et al. 1999).

1.3.1.1. The J-shaped mortality curve

Several epidemiological studies, the Framingham and Copenhagen City Heart trial, have shown that there is a consistent inverse relationship between alcohol consumption and mortality, whereby a low to moderate consumption of alcohol during meals promotes beneficial effects (Hulley, Gordon 1981, Thornton, Symes & Heaton 1983). However, the excessive consumption of alcohol or binge drinking increases the risk of mortality and disease (Gordon, Kannel 1984, Doll et al. 1994, Thun et al. 1997) and the alcohol effect can be described as a J-shaped curve (see figure 4). It is recommended for men and women to have 2 to 3 glasses of alcohol per day to confer cardioprotection and teetollars have neither an increased nor a decreased risk of CVD (Thornton, Symes & Heaton 1983).

In a multinational study, lack of alcohol intake increased risk factors for type 2 diabetes, emphasizing that cardioprotection conferred from alcohol consumption affects triglycerides and insulin levels (Hu et al. 2001). However, the cessation of alcohol will result in the loss of all beneficial effects within 24 hours (Jackson, Scragg & Beaglehole 1992). The ill effects of binge drinking counter the inverse proportion of cardioprotection for moderate drinkers who consume alcohol with meals. However, there is no relationship with alcohol consumption during meals in American men who predominantly consume beer and liquor (Kozarevic et al. 1983).

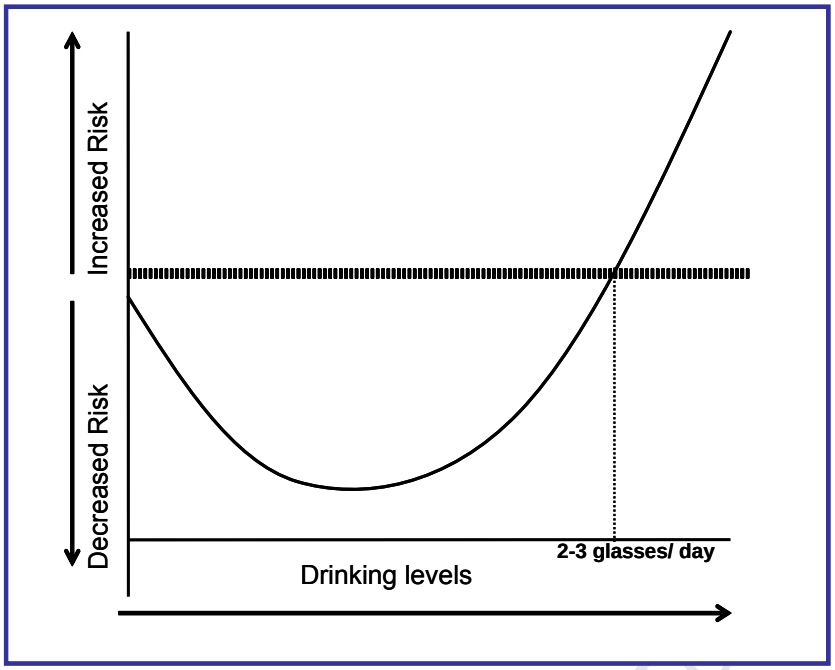


Figure 4: *A graphic representation of the J-shaped mortality curve illustrating that the moderate consumption of alcohol has been shown decrease the risk of mortality, but high levels of alcohol consumption has the adverse effects (Adapted from Gordon and Kannel, 1984).*

1.3.1.2. Mechanisms involved in alcohol-induced cardioprotection

Moderate alcohol consumption, 1 drink or less per day for women and 2 drinks or less per day for men, has been found to reduce the incidence and adverse consequences of heart disease in several epidemiological studies, see review (Mukamal et al. 2006). There is a great focus on the identification of intracellular signalling pathways activated by moderate alcohol consumption that preserve viability and contractility of the cardiac tissue. Adult rodent protocols have been performed to simulate human drinking patterns to test whether moderate alcohol intake could protect the heart against I/R injury. In these studies, moderate alcohol consumption-sustained cardioprotection required the activation of the mitochondrial K_{ATP} channels, see review (Collins et al. 2009). Mice treated with 10% alcohol for 12 weeks protected the hearts against ischemia reperfusion injury via the activation of NO (Zhou, Karliner & Gray 2002b). Similarly, rats that were treated with 18% alcohol for 8 weeks in drinking water improved post-ischemic systolic and diastolic pressure and it also reduced cardiovascular resistance via the activation of protein kinase C (PKCε), see review (Collins et al. 2009).

1.3.1.3. Alcohol free wine can confer cardioprotection

The contribution of alcohol in red wine-induced cardioprotection is questioned by studies performed on de-alcoholized red wine. In patients with coronary artery disease, 250 mL of de-alcoholized Greek red wine decreased arterial stiffness (Zilkens et al. 2005). Similarly, brachial artery flow-mediated vasodilation was improved after drinking de-alcoholized red wine (Rosenkranz et al. 2002) or grape juice (Cruz et al. 2006). Also, grape juice and red wine reduced LDL susceptibility to oxidation while ethanol failed to do so (Cruz et al. 2006). Therefore, these data strongly suggest a cardioprotective effect of red wine beyond alcohol.

1.3.2. Resveratrol induced health benefit

The richest source of resveratrol and its analogs occur naturally in many plant species, including berries, grapevines and the roots of *Polygonum cuspidatum* cultivated in China and Japan. Resveratrol (3,4,5-trihydroxy-trans-stilbene) is a phytoalexin and has been reported to exhibit a wide range of biological and pharmacological properties. It has been speculated that dietary resveratrol could be an explanation for the so-called 'French paradox' as it may act as a cardioprotective agent via its antioxidant properties promoting nitric oxide production, inhibiting platelet aggregation and increasing HDL cholesterol.

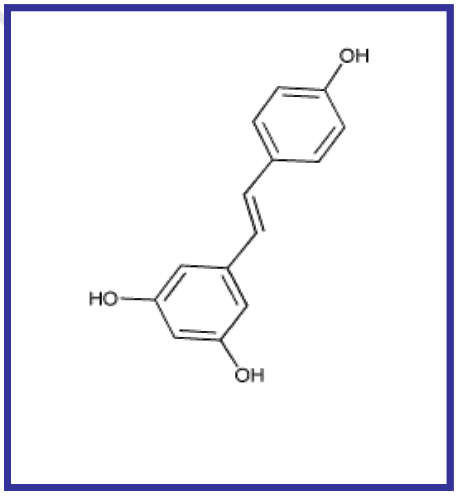


Figure 5: *The molecular structure of resveratrol*

1.3.2.1 Resveratrol and wine

Both isoforms cis and trans-forms of resveratrol exist in wine. It is believed that they are in equilibrium and exhibit similar biological properties. However, there are varying concentrations of resveratrol in different wines. A possible reason for this is that resveratrol is synthesized in response to environmental stressors, which include water deprivation, UV radiation and fungal infection, see review (Das, Maulik 2006a). Resveratrol is one of the several stilbenes found in wine and probably the most researched. The consumption of half a bottle of Brazilian red wine containing a stilbene of 11mg/mL would result in a blood concentration of between 4µmol/L and 10µmol/L, see review (Opie, Lecour 2007). The concentration of 10µmol/L is the minimum resveratrol concentration required to exert a biological effect *in vitro* (Pellegatta et al. 2003). This study raises the possibility that therapeutic resveratrol levels could be reached by consuming red wine.

***Table 1**: Different types of red wine, their countries of origin and their content in resveratrol adapted from (see review: Opie and Lecour, 2007).*

Wine varietal	***Country of origin***	***Resveratrol concentration***
Pinot noir	Australian	13.4 mg/L=59 µmol/L
Pinot noir	Californian	5.5 mg/L
Pinot noir	Burgundy	4.4 mg/L
Pinot noir	Spanish	5.1 mg/L
Cabernet Sauvignon	Australian	1.7 mg/L
Cabernet Sauvignon	California	0.9 mg/L
Rhone valley reds	France	3.6 mg/L
Bordeaux reds	France	3.9 mg/L
Red, varietal not stated	Switzerland	5.0–12.3 mg/L
Red, varietal not stated	Brazil	18.0 mg/L
Red grape juice	–	0.5 mg/L

1.3.2.2 Main physiological properties of resveratrol

a) Antioxidant properties

Resveratrol is responsible for scavenging intracellular reactive oxygen species (ROS). Although it possesses antioxidant properties *in vivo,* it is a weak scavenger *in vitro* (Bhat, Kosmeder & Pezzuto 2001). This antioxidant effect could explain the inhibition of LDL oxidation (Frankel et al. 1993). Furthermore, resveratrol has the ability to inhibit intracellular adhesion molecule (ICAM) and nuclear factor beta (NFkB) which is an indirect marker for oxidative stress (Leonard et al. 2003). The activation of NFkB by tissue necrosis factor alpha (TNFα) is attenuated by resveratrol administration (Leonard et al. 2003). Furthermore, resveratrol inhibits angiotensin–induced hypertrophy. This effect is also thought to be related to its antioxidant properties because the inhibition is associated with a reduced production of ROS, see review (Das, Maulik 2006a).

b) Metabolic properties

Resveratrol has the ability to shift the physiology of middle-aged mice fed with a high caloric diet towards that of the mice on a standard diet and significantly increase their survival (Baur et al. 2006). Interestingly, the metabolic effects of resveratrol include an increased insulin sensitivity, reduced insulin-like growth factor-1 (IGF-1) levels, increased adenosine monophosphate activated kinase (AMPK) and perioxisome proliferator activator receptor γ coactivator 1α (PGC-1 α) activity with increased mitochondrial number and improved motor function, see review (Opie, Lecour 2007).

c) Anti-aging properties

Resveratrol is the only molecule that consistently prolongs lifespan across all species (Wood et al. 2004, Viswanathan et al. 2005). The polyphenol has increased lifespan of yeast *C elegans* and *Drosophilia,* see review (Valenzano, Cellerino 2006a). The administration of resveratrol directly mixed with the daily food of fish starting after the 4th week of life extended from 27 weeks to 59 weeks until death (Valenzano, Cellerino 2006a). Resveratrol induced dose-dependent life extension. Furthermore, resveratrol retarded the

onset of age-dependent cognitive function and locomotive deficit and prevented the neuro-degeneration (Valenzano et al. 2006b).

1.3.2.3 Resveratrol and the cardiovascular system

a) Vascular properties

Nitric oxide (NO) is a key player in vasorelaxation. When less oxygen is available in the cell, trans-resveratrol will have vasorelaxing properties on the endothelium of the heart. In the pulmonary artery, a high secretion of NO is detected but, when the endothelium was removed, vasorelaxation was lost with resveratrol, implying that resveratrol confers cardioprotection via NO (Cishek et al. 1997). Resveratrol increases 3,5-guanosine-monophoposphate (cGMP) with an increase in nitric oxide in cultured pulmonary endothelial cells (Klinge et al. 2003). In the presence of a NO inhibitor, monomethyl-L-arginine and N-G-methyl-L-arginine, in the working rat heart model, and resveratrol no longer conferred cardioprotection against ischemia reperfusion (Hattori et al. 2002).

b) Anti-ischemic properties

Several studies conducted on the isolated rat heart model and *in vivo* have demonstrated that resveratrol has the ability to protect the ischemic myocardium through NO activation (Hattori et al. 2002). Hence, preconditioning the heart with 2.3mg/L of resveratrol given prior to the ischemic insult, provided cardioprotection as evidenced by improved postischemic ventricular recovery, reduced infarct size, and decreased cardiomyocyte apoptosis (Hattori et al. 2002). This effect is mediated via a reduction in reactive oxygen species. Interestingly, inducible nitric oxide synthase (iNOS) knockout mouse hearts could not be preconditioned with resveratrol (Imamura et al. 2002). Also, resveratrol (2.3mg/L), given before a 30 min ischemia and 2 hours reperfusion insult, protects the heart through increased expression of adenosine A_1 and adenosine A_2 receptor (Das et al. 2005). Resveratrol induced the expression of anti-apoptotic Bcl-2 along with the phosphorylation of cyclic AMP response element binding protein, Akt and Bad, see review (Das, Maulik 2006b). These results indicate that resveratrol pharmacologically preconditions the heart via the activation of prosurvival

pathways. Recent studies have also demonstrated that resveratrol protects the heart against reperfusion injury (Xi et al. 2009). In isolated rat hearts exposed to 30 min ischemia and 2 hours reperfusion, the resveratrol given at the onset reperfusion, enhanced glycogen synthase-3β (GSK-3β) phosphorylation and its translocation from the cytosol to mitochondria by targeting the inhibition of the mitochondrial permeability transition pore (mPTP) (Xi et al. 2009). However, the concentration of resveratrol used in these experiments is far higher than the quantity of resveratrol given by consuming 2 glasses of red wine per day. In addition, no experiments have shown a cardioprotective effect of resveratrol by chronically administering resveratrol at an equivalent concentration to the concentration found in red wine. There is no evidence that strongly suggests that resveratrol can account for the cardioprotective effect of red wine.

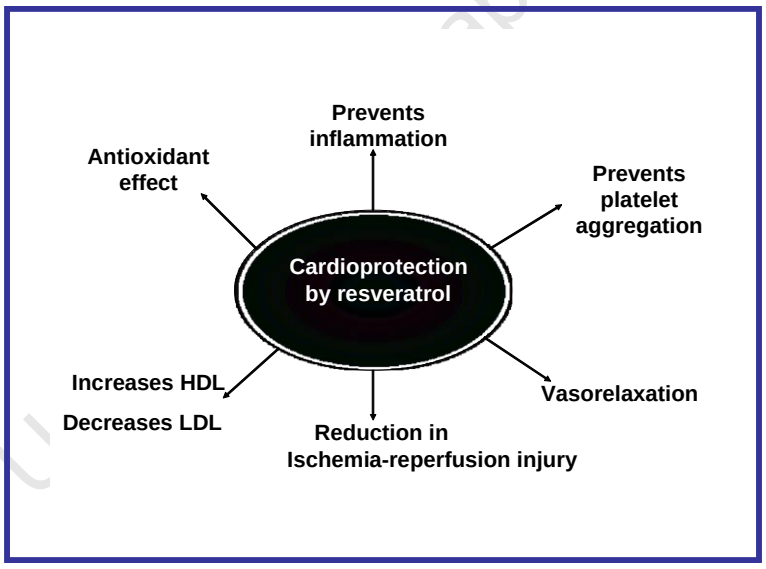


Figure 6: *Schematic representation of the proposed cardiovascular mechanisms of resveratrol. HDL=high density lipoproteins, LDL=low density lipoproteins. Adapted from Das et al., 2006*

1.3.3. Melatonin induced health benefit

In 2006, melatonin was identified as another phytochemical present in grapes (Iriti 2009). Melatonin, also known chemically as N-acetyl-s-methoxytryptamine, is a naturally occurring hormone found in humans.

Its main function in the body is to control the body's circadian rhythm (Kumar, Singh 2009) and to regulate other hormones (i.e. female reproductive hormones) (Girgert et al. 2009). It has strong antioxidant properties that may contribute to its cardioprotective effect (Tan et al. 1993).

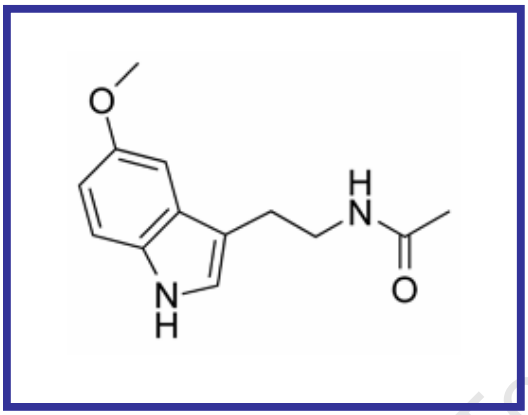


Figure 7: *The molecular structure of melatonin.*

1.3.3.1 Melatonin and wine

Melatonin is synthesised by various plants such as rice. In 2006, Italian researchers found the presence of melatonin in extracts from different wine grapes including Nebbiolo, Croatina, Sangiovese, Merlot, Mrzemino, Cabernet Franc, Cabernet Sauvignon and Barbera. Melatonin was found in a high concentration in red wine ranging from 50pg/mL to 200pg/mL. Interestingly, the amount of melatonin is lower in white wine (range: 10-50pg/mL) and even less in liquors (range: 5-20pg/mL) (Guerrero et al. 2008). It is likely that cultivar, agro-meteorological conditions (Burkhardt et al. 2001), vintage and wine-making procedures contribute to the difference in concentration of melatonin between the wines (Burkhardt et al. 2001). Ironically, the anti-mould fungicide benzothiadiazole, which wineries spray on their plants to protect the grapes, seems to increase the concentration of melatonin (Burkhardt et al. 2001).

Interestingly, Guerrero et al. 2008 demonstrated that serum melatonin was significantly increased in humans, one hour after an intake of 100 mL of red

wine. However, for unknown reasons, this article has been withdrawn at the request of the editor.

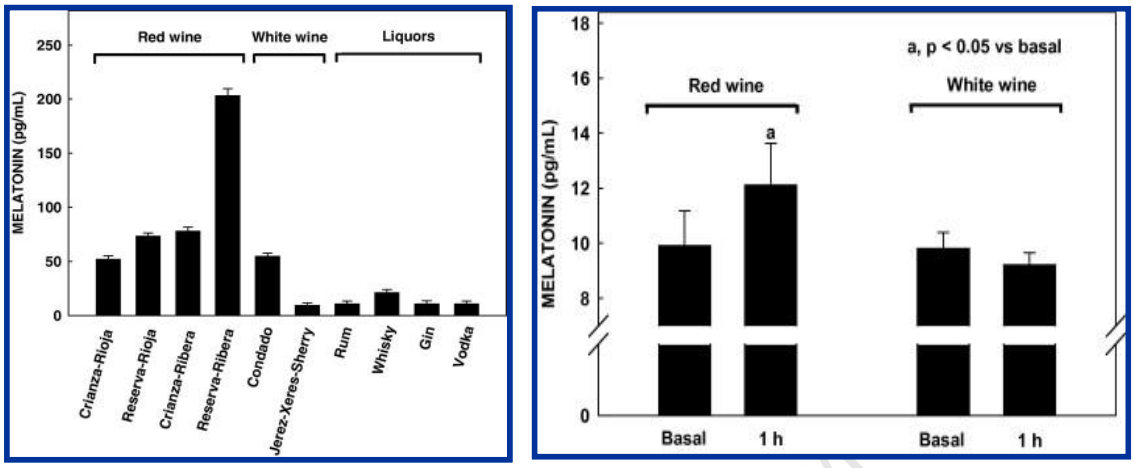


Figure 8: *Melatonin concentrations found in differing alcoholic beverages: red wine, white wine and liquors (*Guerrero et al. *2008).*

1.3.3.2 Biosynthesis of melatonin

Melatonin is produced by pinealocytes in the pineal gland and also by the retina, lens, gastrointestinal tract and other tissues such as the skin (Karasek, Winczyk 2006). It is synthesised from the amino acid tryptophan (via synthesis of serotonin). Serotonin is converted to melatonin in the presence of N-acetyltransferase and s-acetylserotonin-O-methyltransferase (ASMT). Its production by the pineal gland is regulated by the hypothalamus which receives information from the retina about the daily pattern of light and darkness. Light suppresses the production of melatonin while darkness stimulates its production. Exposure to excessive light in the evening or too little light during the day can disrupt the body's normal melatonin cycle.

The mean production rate of endogenous melatonin has been calculated as 30µg per day with a half-life of 30-60 min, see review (Karasek, Winczyk 2006).

1.3.3.3 Physiological properties of melatonin

Since its discovery in 1958, many biological functions have been attributed to melatonin and most of its effects are produced through the activation of the

melatonin receptors. Melatonin acts through a series of targets, melatonin receptor 1, 2 or 3 (MT-1, 2, 3) (Mailliet et al. 2005) and its cardioprotective effect is suggested to be dependent of receptors 1 and 2 (Genade et al. 2008). In the heart and in the arteries, melatonin receptors were discovered in 2002 (Pang et al. 2002, Masana et al. 2002). Melatonin forms part of the system that regulates the circadian cycle by chemically causing drowsiness and lowering the body temperature.

In 1993, powerful antioxidant properties of melatonin were discovered (Tan et al. 1993). Melatonin, a lipid- and water-soluble compound, easily and rapidly enters all cells and subcelullar compartments, including the nuclei and mitochondria (Karbownik, Reiter 2000). Melatonin can act as a free radical scavenger by directly scavenging reactive oxygen species and reactive nitrogen species, see review (Tengattini et al. 2008). Hence, melatonin can neutralize the highly toxic hydroxyl radical (·OH) to generate the product cyclic-3-hydroxymelatonin (Karbownik, Reiter 2000) Melatonin can also neutralize the superoxide anion radical (Reiter et al. 2001), the hydrogen peroxide (Tan et al. 2000), the nitrogen monoxide (Mahal, Sharma & Mukherjee 1999) and peroxynitrite (Srinivasan 2002), but the mechanisms remain unclear. Beside its free radical scavenging properties, melatonin has the ability to regulate the antioxidant enzyme activities such as superoxide dismutase, catalase, and glutathione peroxidase, see review (Tengattini et al. 2008). Both melatonin receptors (MT1 and MT2) seem to be involved in this indirect antioxidative effect (Tomas-Zapico, Coto-Montes 2005). Interestingly, melatonin may also regulate the activity of pro-oxidative enzymes such as nitric oxide synthase (Karbownik, Reiter 2000).

Table 2: *The different types of ROS/RNS/enzyme and the respective effect of melatonin. Adapted from (*Tengattini *et al.,* 2009).

ROS/RNS/enzyme	Effect of melatonin
ROS/RNS	
Hydrogen peroxide	↓
Hydroxyl radical	↓
Singlet oxygen	↓
Nitric oxide	↓
Peroxynitrite anion	↓
Antioxidative enzyme	
Superoxide dismutase	↑
Catalase	↑
Glutathione peroxidase	↑
Glutathione reductase	↑
Glucose-6-phosphate dehydrogenase	↑
Gamma-glutamylcysteine synthase	↑
Pro-oxidative enzymes	
Nitric oxide synthase	↓
Lipo-oxygenase	↓

The antioxidant properties of melatonin may reduce damage caused by some type of Parkinson's disease and it may increase longevity (Jung-Hynes, Ahmad 2009). Melatonin acts as an endogenous sleep-inducing agent and reduced concentration may result in lowered sleep propensity which is associated with advanced aging. Decreased melatonin results in the inhibition of longevity protein sirtuin-1 (SIRT1) which has an antiproliferative response in aging via the resynchronization of the circadian clock (Jung-Hynes, Ahmad 2009).

The activation of the melatonin's receptors may play a role in the process of learning and memory and the treatment of melatonin may decrease the evolution of cognitive impairment in Alzheimer patients (Olcese et al. 2009).

Recent data also suggest that deficiency in melatonin may be involved in the pathology of autism spectrum disorders **(**ASD).

Parents of autistic patients have a lower level of melatonin associated with a lower activity of the ASMT gene, which encodes for the last enzyme of melatonin synthesis (Galli-Carminati, Deriaz & Bertschy 2009, Golnik, Ireland 2009).

Melatonin may play an important role in metabolic regulations of the body. After activation of its membrane receptor 1 (MT1), melatonin activates insulin receptors and upregulates insulin-stimulated leptin expression (Alonso-Vale et al. 2005).

1.3.3.4 Melatonin and the cardiovascular system

a) Melatonin and blood pressure

Hypertension is one of the most prevalent risk factors for cardiovascular disease (Steyn et al. 2005, Stewart et al. 2006). The effect of melatonin on blood pressure is paramount. Pinealectomy rats have decreased levels of melatonin associated with vasoconstriction and temporary hypertension. However, the administration of melatonin in these animals reversed the effects (Zanoboni, Zanoboni-Muciaccia 1967). Interestingly, continuous exposure to light both day and night prevents the nocturnal increase of melatonin, suppresses circadian heart rate and augments blood pressure, resulting in melatonin deficient hypertension. Spontaneous hypertensive rats with left ventricular hypertrophy were treated with melatonin, which improved cardiac function but did not alter the left ventricular relative weight (Zanoboni, Zanoboni-Muciaccia 1967). These results strongly suggest that melatonin plays a major role in the regulation of hypertension.

b) Melatonin and atherosclerosis

Atherosclerosis is the abnormal development and progression of cholesterol deposits in the tunica intima of arteries. The development of the plaque involves the activation of inflammatory cytokines and oxidized LDL, (Pieri et al. 1996). Melatonin inhibits oxidized LDL *in vitro* in a dose-dependent manner (Duell et al. 1998).

Other studies support these findings and suggest that melatonin precursors (such as L-tryptophan and serotonin) as well as the breakdown products (such as niacin or quinolinate) also inhibit oxidation of LDL (Pieri et al. 1996). Melatonin reduces the total cholesterol plasma levels and decreases very low-density lipoproteins, (VLDL) in hypercholesterolemic rats (Kelly, Loo 1997).

c) Melatonin and ischemia/reperfusion

(i) Acute administration

In 1998, Tan and Colleagues used an isolated heart model to demonstrate that concentrations of melatonin ranging from 1-50μM, protected the heart against ischemia reperfusion-induced arrhythmias, including decreasing the severity and reducing their incidence and duration (Tan et al. 1998). Interestingly, this protective effect was more potent than the well-known antioxidant vitamin C.

Administration of melatonin also protected the isolated rat heart by reducing ventricular fibrillation and partially restoring ventricular function (Kaneko et al. 2000). This protection was associated with the reduction in free radicals released and a decrease in lipid peroxidation (Kaneko et al. 2000). Melatonin (1 or 10mg/kg; ip), given to rats 30 min prior to an ischemia-reperfusion *in vitro,* reduced infarct size (Lagneux et al. 2000). The protective effect of melatonin proposed to be mediated via its antioxidant effect on intracellular calcium accumulation (Salie et al. 2001). Melatonin-induced protection is also associated with the activation of Akt and inhibition of the pro-apoptotic kinase, p38MAPK during early reperfusion (Genade et al. 2008). Furthermore, melatonin possesses an anti-adrenergic action via nitric oxide synthase and guanylyl cyclase activations (Genade et al. 2008).

(ii) Chronic administration

A chronic treatment with melatonin has the ability to reduce infarct size (Lochner et al. 2006). The long term effects of melatonin were evaluated 24 hours after melatonin administration (2.5 or 5.0 mg/kg, ip) or after the oral supplementation of melatonin in the drinking water for 7 days (20 or 40μg/mL)

(Lochner et al. 2006). The results obtained suggested that melatonin induces long-term protection evidenced by the reduction of infarct size.
The cardioprotective effect of melatonin persisted for 2-4 days after discontinuation of treatment.

However, these concentrations used in these studies are superior to the concentration of melatonin that would be found in regular consumption of 2 glasses of wine per day (concentration of melatonin in red wine = 0.75µg/L) and no studies have explored whether melatonin from wine may contribute to the cardioprotective effect of regular consumption of red wine.

1.3.4. Ethanolamine induced health benefit

Ethanolamine, also called 2-aminoethanol or monoethanolamine, is an organic compound that is both a primary amine (due to the amino group in its molecule) and a primary alcohol (due to a hydroxyl group).

1.3.4.1. Ethanolamine and wine

Exogenous ethanolamine can be obtained from many foods and beverages including wine (Hernandez et al. 2007) and soya products (Imaizumi et al. 1989). It is found in both red and white wine, at a concentration ranging from 4.5 to 7 µg/L in white wine and from 13.5 to 25 µg/L (mean concentration of 21µg/L) in red wines (Hernandez et al. 2007), but these concentrations may vary during wine aging, the climate, the type of soil, the type of grapes and the fermentation process used (Jimenez Moreno, Torrea Goni & Ancin Azpilicueta 2003). Hence, certain types of yeasts used for the fermentation process will influence the formation of ethanolamine more than other type of yeasts (Jimenez Moreno, Torrea Goni & Ancin Azpilicueta 2003).

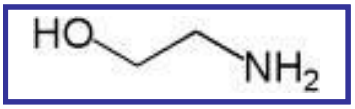


Figure 9: *The molecular structure of ethanolamine.*

1.3.4.2. Physiological properties of ethanolamine

In the body, little is known about the physiological properties of ethanolamine. This biogenic amine is synthesized as a downstream product of anandamide (an endogenous cannabinoid), in the presence of the enzyme fatty acid amine hydrolase (FAAH).

Ethanolamine stimulates the phospholipids synthesis such as phosphatidylethanolamine, phosphatidylcholine and sphingomyelin, major constituents of the phospholipid membrane (Sundler, Akesson 1975).

The presence of ethanolamine in the circulation is thought to act as a humoral hepatotrophic factor in the early stages of liver regeneration following hepatectomy (Kume, Sasaki 2006, Kume, Sasaki & Kano-Sueoka 2006).

Recent evidence suggests that ethanolamine may have anti-cholesterol properties. Interestingly, hypercholesterolemic rats fed with a high fat/high cholesterol diet and water containing up to 1mg/mL of ethanolamine, decreased VLDL cholesterol and LDL cholesterol in a dose dependent manner (Kume, Sasaki 2006). Levels of serum HDL cholesterol levels remained unchanged. The anti-hypercholesterolemic effect of ethanolamine is suggested to be mediated via the downregulation of ApoB in the liver (Kume, Sasaki 2006). In brain cells, ethanolamine can protect from low serum-induced apoptosis via inactivation of the caspases 3/7 activities (Matas et al. 2007).

1.3.4.3. Ethanolamine and the heart

No previous studies have explored the role of ethanolamine in the heart. However, anandamide, the precursor of ethanolamine's formation in the body, can protect the isolated heart against coronary occlusion. Hence, anadamide (1μM), given 5 min before ischemia until the end of reperfusion, reduced the infarct size (Underdown, Hiley & Ford 2005). This effect is suggested to be partly mediated via a cannabinoid receptors-independent pathway (Underdown, Hiley & Ford 2005) and therefore may involve ethanolamine.

Further studies are necessary to delineate the exact mechanisms involved in the protective effect.

1.4. The JAK/STAT-3 pathway protects against ischemia-reperfusion

The Janus kinase / Signal transducer and activator of transcription-3(STAT-3) pathway is a novel signal transduction pathway which, upon its action, activates numerous genes, see review (Lecour 2009a, Lecour 2009b). In the heart, the activation of this pathway has been implicated in cardiac hypertrophy (Kurdi, Booz 2007) and ischemia-reperfusion (Lecour et al. 2005b); see review (Lecour 2009a, Negoro et al. 2000, Omura, Yoshiyama & Yoshikawa 2000).

1.4.1. Definition of the JAK/STAT-3 pathway

STAT are a family of cytoplasmic transcription factors mediating intracellular processes that are activated by cytokines at the surface receptor. There are several known relatives to the STAT family, STAT1, STAT2, STAT-3, STAT4, STAT5a, STAT5b and STAT6. In the heart, STAT activity is limited to STAT1 and STAT-3 (McCormick et al. 2006). STAT1 has been demonstrated to have apoptotic effects, whereas STAT-3 is involved in cardioprotection (Hattori et al. 2001). STAT-3 activation can occur when a cytokine binds to a specific receptor activating Janus Kinases-2 (JAK2) or mitogen activating protein kinases (MAPK). Upon its phosphorylation, STAT-3 will form dimers and translocate to the nucleus. Studies have shown that STAT-3 can also translocate to the mitochondria after phosphorylation on the tyrosine site. Hence, in heart mitochondria isolated from STAT-3 deficient mice the activity of complex I and III of the electron transport chain (ETC) was decreased. The mitochondrial respiration was altered with a decrease in complex I and III activity of the ETC (Gough et al. 2009, Wegrzyn et al. 2009).

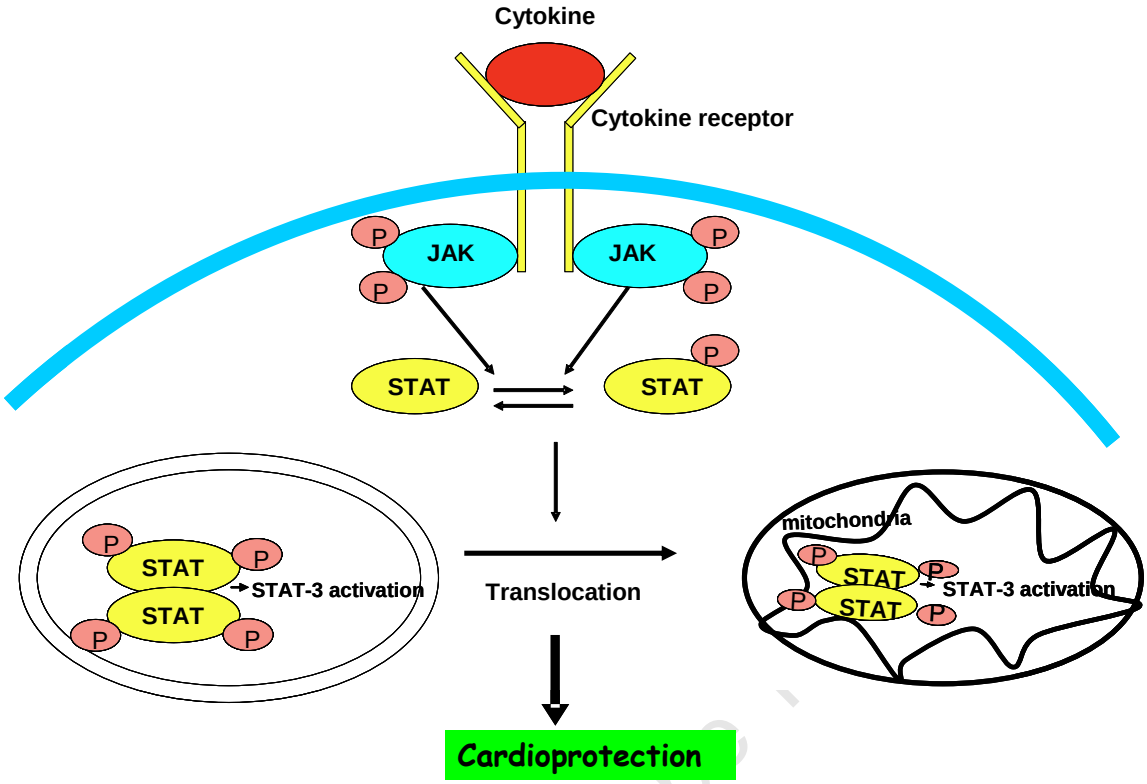


Figure 10: *Schematic representation of the JAK-STAT pathway (adapted from Cell Signalling catalogue 2007).*

1.4.2. JAK/STAT-3 pathway protects against ischemia-reperfusion

The activation of STAT-3 following myocardial infarction was first demonstrated in 2001, with an increase in phosphorylation of STAT-3 which was observed up to 24 hours after ligation of the left coronary artery of rats (Xuan et al. 2001, Yang et al. 2008).Transgenic mice with cardiac specific 10 fold over-expression of STAT-3 had significantly reduced infarct size after 1 hour ischemia and 2 hours of reperfusion as well as another set of transgenic mice exposed to 30 min ischemia followed by 24 hours reperfusion compared to non-transgenic mice(Xuan et al. 2001, Yang et al. 2008). A key role for the JAK/ STAT signalling pathway has been demonstrated in ischemia and pharmacological preconditioning (Xuan et al. 2001, Lecour et al. 2005a) and postconditioning (Lacerda et al. 2009, Boengler et al. 2008a) see review (Boengler et al. 2008b).

1.4.3. Possible downstream targets of JAK/STAT-3

Downstream effectors of the JAK/STAT-3 pathway target the mitochondrial potassium channel ($K_{/ATP/}$) (Mykytenko et al. 2008). The opening of the mitochondrial permeability transition pore in the inner membrane of the mitochondria is responsible for the uncoupling of proteins, the efflux released from cytochrome c and several other apoptotic factors that eventually lead to cell death (Hausenloy, Yellon 2003, Hausenloy, Duchen & Yellon 2003). There are several other downstream targets that have been identified including proteins that are involved in cell survival and proliferation such as Bcl-2, Bcl-xL, Mcl-1, Fas, p21 and growth factors like VEGF. STAT-3 plays a crucial role in a preconditioning setting by reducing the pro-apopototic factors such as Bax and Bad and enhances the activation of Bcl-2 see review (Lecour 2009a, Lecour 2009b).

1.5. Objectives of the study

AIM

The aim of our study was to explore whether resveratrol, alcohol, melatonin or ethanolamine, given chronically at the concentration equivalent as found in 2 glasses of wine/day, may protect the heart against an ischemia-reperfusion insult.

Furthermore, we hypothesized that red wine and its cardioprotective components protect via the activation of the transcription factor signal transducer and activator of transcription 3 (STAT-3), a prosurvival factor known to protect against ischemia-reperfusion injuries.

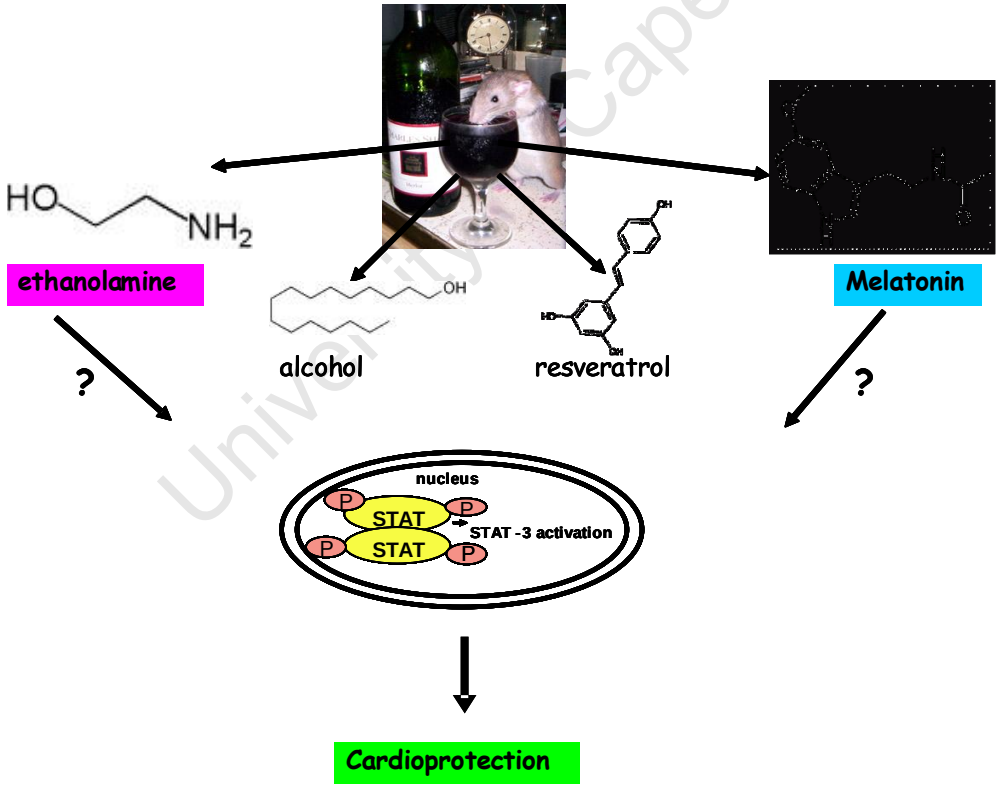


Figure 11: *Hypothetical protective pathway of red wine induced protection mediated by melatonin and ethanolamine via the activation of the JAK/STAT-3 pathway.*

To investigate this aim, Wistar rats were pre-treated for 10 days with a French Cabernet Sauvignon, resveratrol, alcohol, melatonin or ethanolamine (given at the concentration equivalent as the concentration found in 2 glasses of wine/day) and their hearts were subjected to an ischemia-reperfusion insult using the Langendorff perfusion system. The role of the JAK/STAT-3 pathway was studied using AG490, the JAK/STAT-3 pathway inhibitor and STAT-3 activation will be assessed by Western Blot technique.

CHAPTER 2: MATERIALS AND METHODS

2.1 Animals

All the experiments were conducted on male Wistar rats weighing 230-300g and were performed in accordance with the Guide for Care and Use of Laboratory animals published by the U.S. National Institutes of Health (NIH pulblication No. 85(23), revised 1996). All procedures were approved by the Animal Research Review Committee of the University of Cape Town (applications; 008/026 entitled “Delineation of mechanisms for wine-induced cardioprotection”, 007/012 “Delineation of resveratrol-induced cardioprotection” and 005/016 “The cardioprotective effect of alcohol-free wine”).

2.2 The Langendorff perfused isolated rat heart model

Rats were anaesthesized with 60 mg/kg intraperitoneal sodium pentobarbitone mixed with 200I.U. heparin. Hearts were excised rapidly, cannulated via the aorta, and perfused retrogradely using the Langendorff perfusion system at a constant pressure (100 cm H_20), at 37ºC, with Krebs-Henseleit buffer equilibrated with $0_2/CO_2$ 95:5%. The following composition of the Krebs- Henseleit perfusion buffer as follows in mM: (118.0 NaCl, 25.2 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$.7H2O, 1.2 KH_2PO_4, 1.2 CaCl.$2H_20$, and 11.0 Glucose, pH of 7.4).

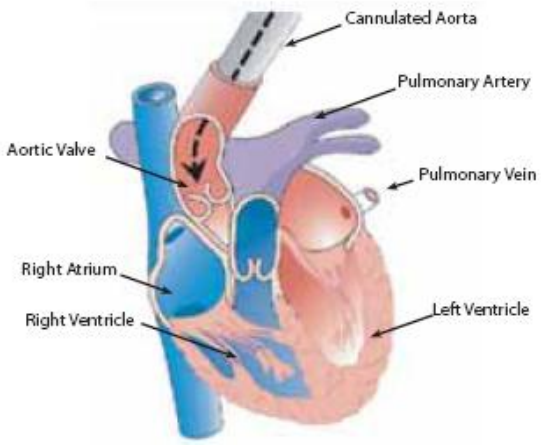


Figure 12: A graphic representation of retrograde perfusion in Langendorff mode (ADInstruments, London)

A balloon was inserted through the left atrium into the left ventricle and the left ventricular end diastolic pressure was adjusted between 4 and 12mmHg. Cardiac parameters were monitored throughout the experiments and included heart rate (HR), left ventricular developed pressure (LVDP: difference between left ventricular end systolic pressure (LVESP) and end diastolic pressure (LVEDP) and the coronary flow (CF). Rate pressure product was expressed as LVDP X HR.

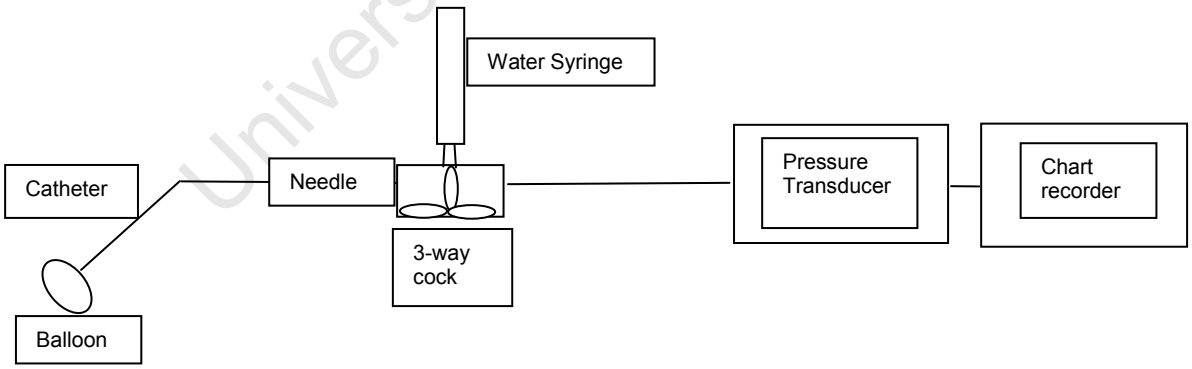


Figure 13: *Schematic representation of the balloon catheter (ADInstruments, London).*

Exclusion criteria

Rats that did not comply with the following criteria were removed from the study:

(1) Left ventricular pressure must be higher than 80mmHg.

(2) Coronary flow must be a minimum of 8mL/min and a maximum of 16mL/min.

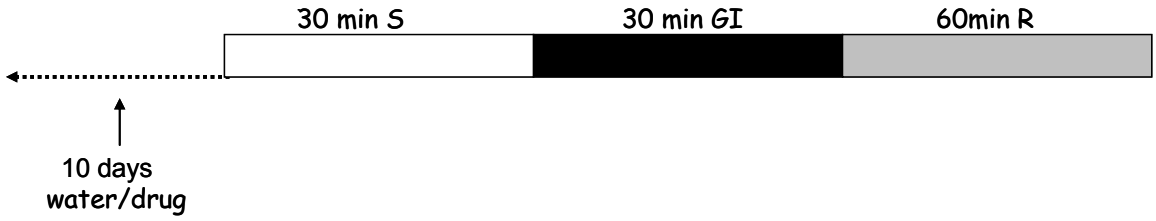
(3) Heart rate should be a minimum of 240beats/min and a maximum of 400 beats/min. A total of 10 out of 144 rats were excluded from the study.

2.3 Langendorff perfusion protocols

2.3.1. Chronic study

French Cabernet sauvignon was used in this study. The alcohol was removed by the lirisation® process and oenological expertise from approximately 12% alcohol by volume to a final concentration of 6% alcohol by volume. The lirisation® process presents the advantage of lowering the alcohol content of the original wine without any other alteration of the original composition of the wine. The drinking water used for controls was supplemented by wine (12%), wine (6%), alcohol (6%), resveratrol (check7mg/L), melatonin (0.75µg/L) or ethanolamine (21µg/L) before the dilution for 10 days. The different solutions were prepared by adding one part of red wine or drug solution to seven parts drinking water. Since the rats drank an average of 30mL/day and the final concentration received was the following: wine (1.50%), wine (0.75%), alcohol (0.75%), resveratrol (0.8mg/L), melatonin (0.94µg/L) or ethanolamine (2.60 µg/L). When the drugs were given, both body weight and the amount of drinking water consumed by each rat were 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 reality. Luzindole (5mg/kg/day), a melatonin receptor 1 and 2 antagonist, or AG490 (5mg/kg/day), a JAK-2 antagonist, was administered intraperitonelly once a day for 10 days. Thereafter, the rat hearts were removed and mounted onto the Langendorff perfusion system. All hearts were equilibrated for a period of 30 min and were consequently subjected to either 30 min global or regional ischemia followed by 30 min of reperfusion which was later increased to 60min reperfusion for improved visualisation of the infarct size. Global ischemia was used for all the chronic studies and was obtained by a cessation of perfusate together with additional KHB within the jacket to stabilise the temperature at 37ºC.

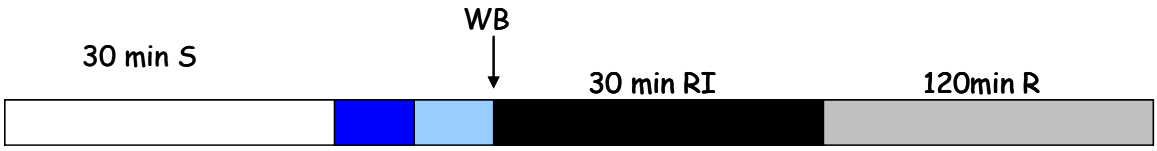
The functional parameters of the heart were monitored (LVEDP, LVESP, LVDP, heart rate and coronary flow). A preliminary study was performed to ensure that the amount of beverage drink/ day was unchanged with the addition of drug or wine.



S=stabilisation, GI=Global Ischemia, R= Reperfusion

2.3.2. Acute study

In a Langendorff perfusion system, all rat hearts were equilibrated for 30 min and consequently subjected to a standard 30 min of regional ischemia which was used for all the acute studies. In regional ischemia, a 3/0 silk suture was placed around the left coronary artery to form a snare. After the occlusion, the heart was reperfused for 120 min. Melatonin (0.75µg/L), ethanolamine (21µg/L) or resveratrol (2.30mg/L) at the concentration found in wine were perfused for 15 min followed by 10 min wash out period before regional ischemia. Additional groups were perfused with AG490 (100 nM), a STAT-3 inhibitor. AG490 was perfused for 3 min on its own followed by a 15 min co-administration with melatonin, ethanolamine or resveratrol followed by a further 5 min of AG490 alone and 10 min wash-out prior to regional ischemia (RI).

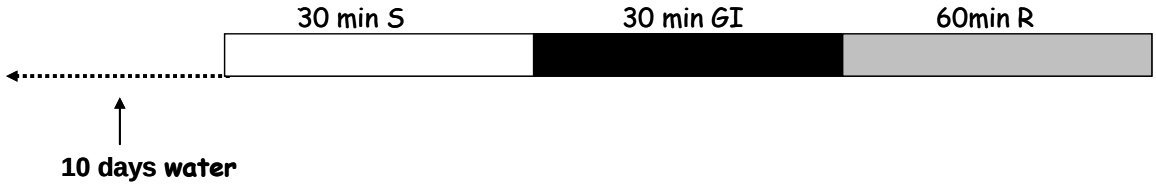


S=Stabilisation, WB= Western Blots, RI= Regional Ischemia, R= reperfusion

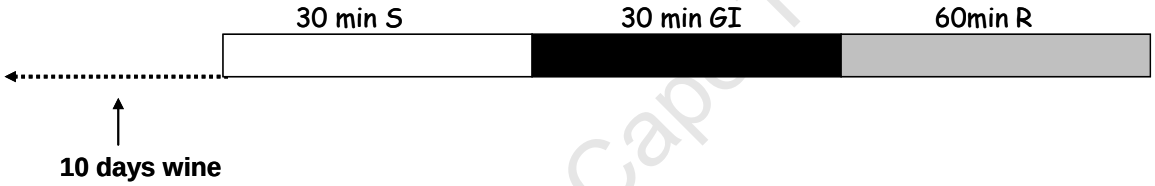
2.4 Different perfusion protocols

Group 1: Role of chronic moderate consumption of red wine for (10 days) protects against ischemia reperfusion injury (I/R) (Endpoint = Functional recovery and infarct size).

1. Ischemic control (n=6)

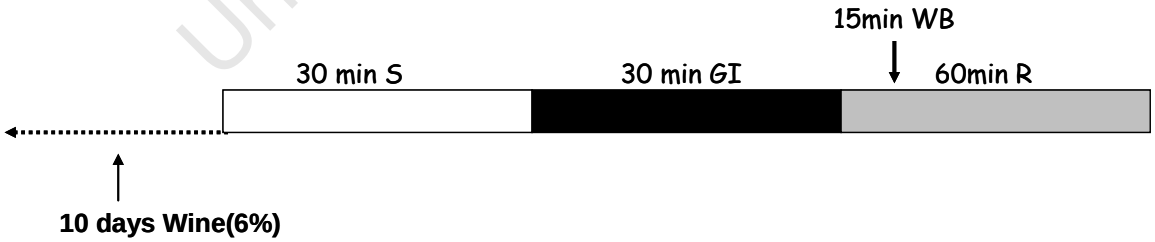


2. Red wine (12%) n=6

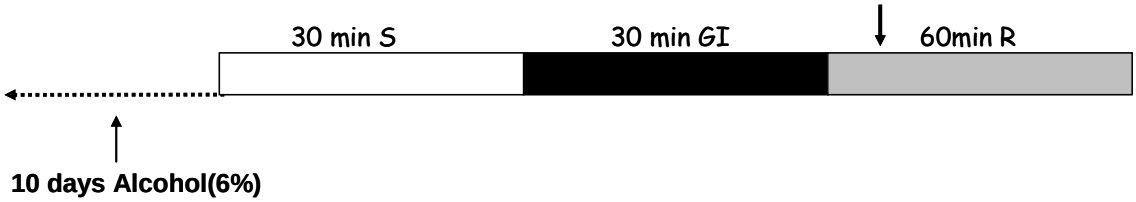


Group 2: Delineation of the active components in red wine (Endpoint = Functional recovery.

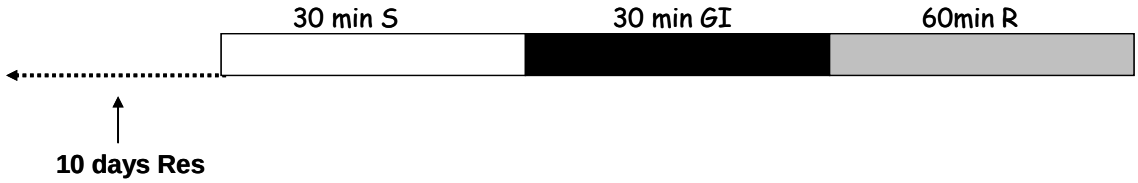
1. Wine (6%) n=6



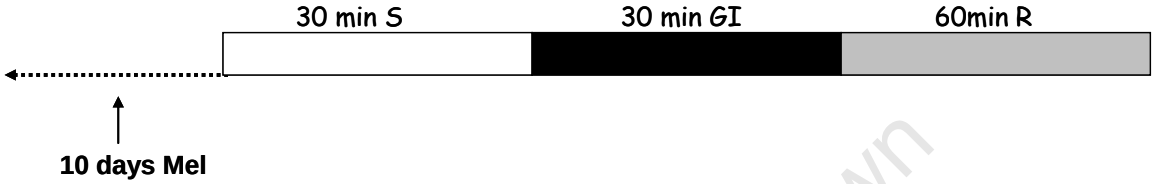
2. Alcohol(6%) n=6



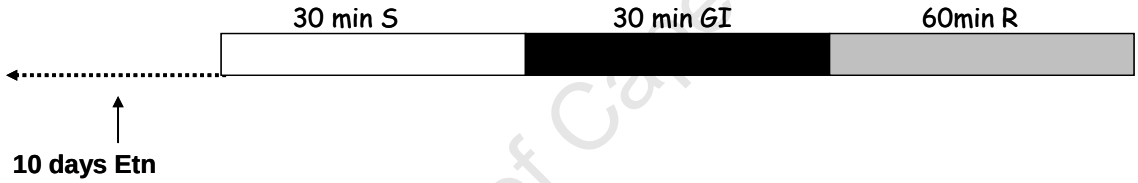
3. Resveratrol (Res 7mg/L) n=6



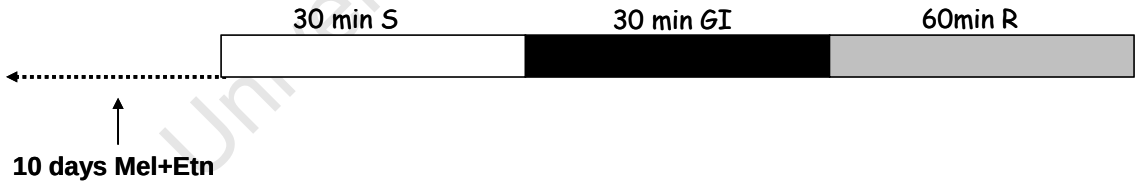
4. Melatonin (Mel 0.75µg/L) n=6



5. Ethanolamine (Etn 21µg/L) n=6

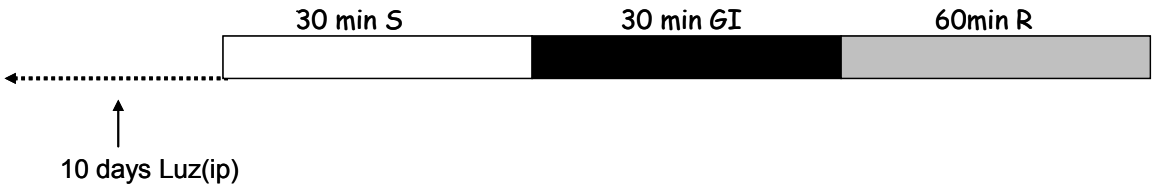


6. Pre-treatment with melatonin and ethanolamine (Mel+Etn) n=6

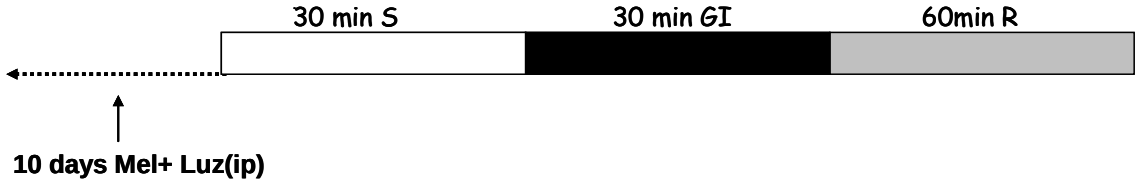


Group 3: Role of melatonin in red wine-induced cardioprotection (Endpoint = functional recovery and infarct size)

1. Luzindole (Luz 5mg/kg/day) n=6

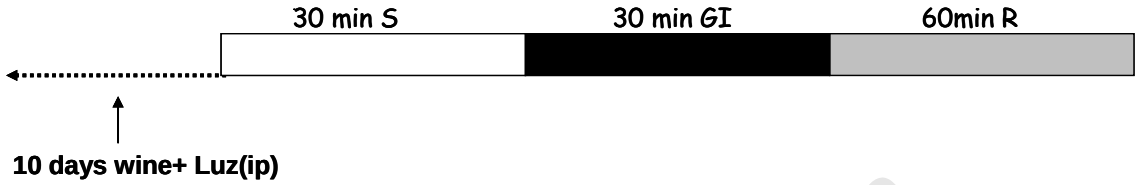


2. Co-administration of luzindole and melatonin (Mel+ Luz) (n=6)



10 days Mel+ Luz(ip)

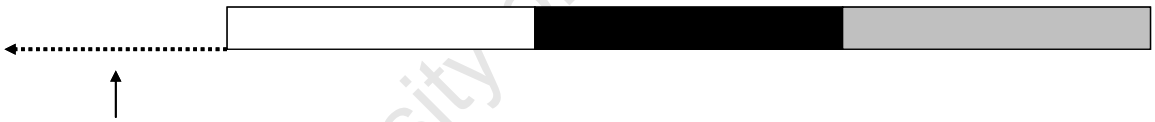
3. Co-administration of luzindole (Luz) and wine(n=6)



10 days wine+ Luz(ip)

Group 4: The role of STAT-3 in red wine-induced cardioprotection (Endpoint = Functional recovery and infarct size).

1. AG490 (5mg/kg/day) n=6



10 days AG490(ip)

2. Co-administration of red wine and AG490 n=6

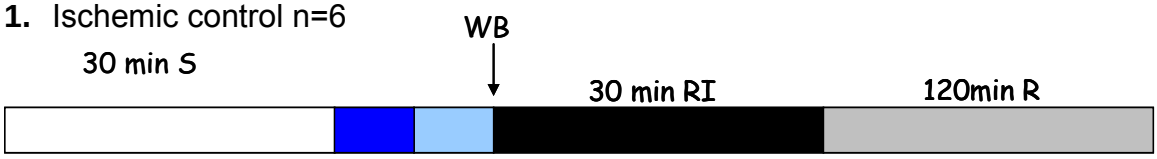
10 days wine+ AG490(ip)

Group 5: Melatonin, ethanolamine and resveratrol protect against I/R via the activation of STAT-3.

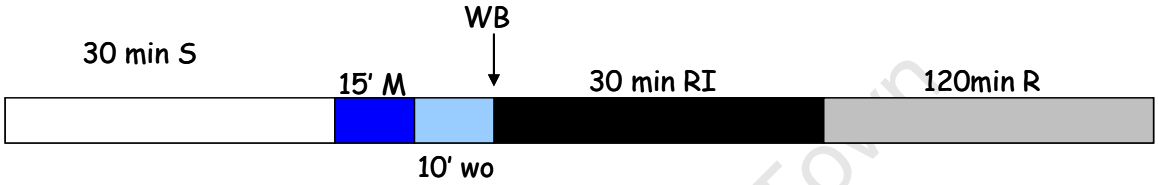
To explore whether ethanolamine, melatonin and resveratrol components may protect against I/R via the activation of STAT-3, we subjected rats to ischemia (RI)-reperfusion insult following an acute treatment of ethanolamine,

melatonin or resveratrol and the STAT-3 inhibitor, AG490. The endpoint for all the groups below was infarct size.

1. Ischemic control n=6

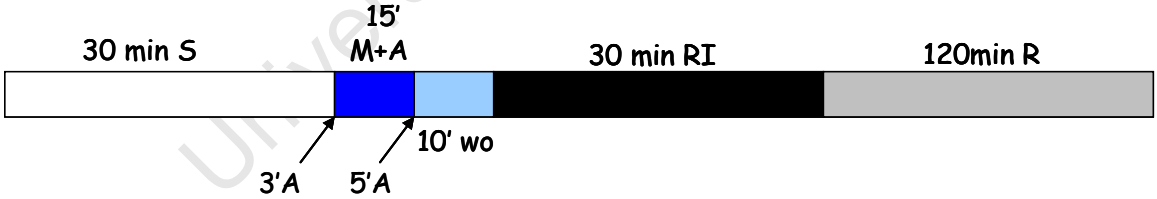


2. Acute administration of melatonin (M 0.75µg/L) n=6



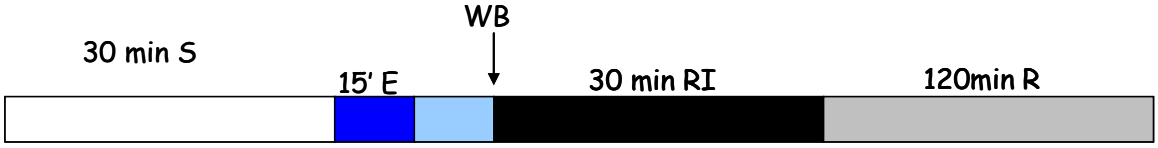
3. AG490 (A 100nM) n=6

4. Co-administration of melatonin and AG490 (M+A) n=6

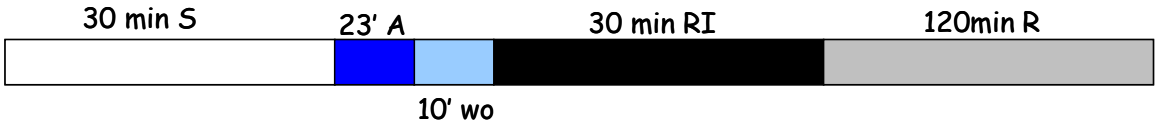


Group 6: The mechanism for ethanolamine-induced protection

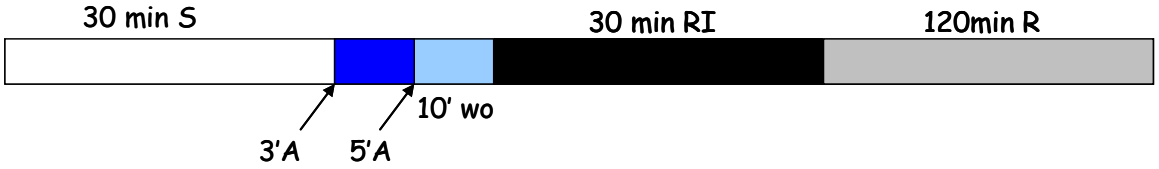
1. Acute administration of ethanolamine (E 21µg/L) n=6



2. Administration AG490 (A 100nM) n=6



3. Co-administration of ethanolamine + AG490 (E+A) n=6



The rat hearts were isolated and pre-treated either chronically or acutely with red wine, alcohol, resveratrol, melatonin or ethanolamine to examine the cardioprotective effect.

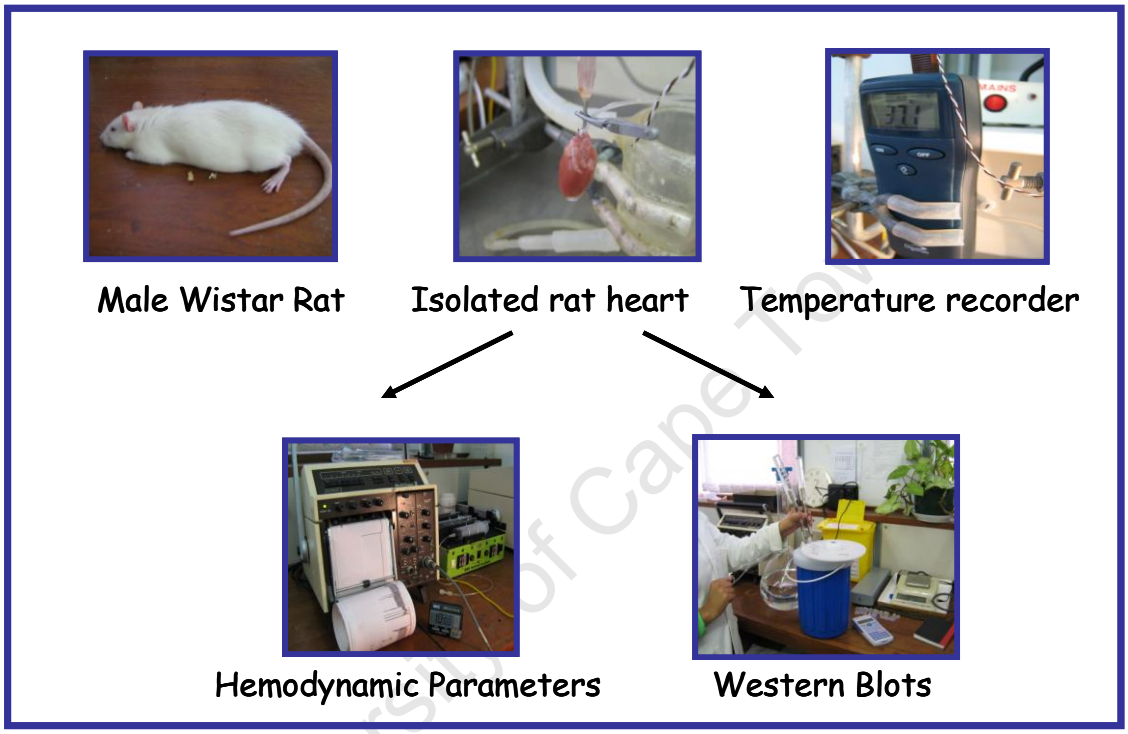


Figure 13: *Pictures taken during the perfusion protocol.*

2.5 Infarct size

At the end of the reperfusion period, the coronary artery was reoccluded and 0.5 mL of 2% Evans blue was perfused slowly through the heart to delineate the area at risk. Hearts were frozen for 24 hours at -20° C and cut into 2 mm thick slices. The slices were stained and incubated in sodium phosphate buffer containing 1% w/v of triphenytetrazolium chloride pH7.4 at 37º C for 15 min. Slices were fixed in 10% v/v formaldehyde solution for 24 hours for visualisation. Infarct and the area at risk were determined with planimetry (Summa Sketch II; Summa Graphics) and infarct size was expressed as a percentage of the area at risk.

2.6 Western Blots

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

2.6.1 Protein extraction

Frozen tissue was pulverized with a hammer. The powdered tissue (200mg) were homogenized with 900µl lysis buffer (20mM HEPES, 2.5mM MgCL2, 100µM EDTA, 20mM β- glycerophosphate, 0.05% Triton x-100 (cytosolic extract), 1% Triton x-100(nuclear extract), 500µM dithiothereitol (DTT),1mM phenylmethylsulfonyl fluoride (PMSF) and 75mM NaCl and centrifuged at 10000g for 5min. The supernatant (crude cytosolic extract) was removed and corresponded to the crude cytosolic extract. Thereafter, 500µl lysis buffer was added to the homogenized and centrifuged at 15000g for 30min. The supernatant was removed and corresponded to the crude nuclear extract.

2.6.2 Protein quantification

The Lowry Assay was used to quantify the concentration of proteins (Lowry *et al*., 1951). Bovine Serum Albumin (BSA) standard curve ranged from a concentration of 5-200mg/L and the absorbance was measured at 250 nm.

2.6.3 SDS PAGE of extracted proteins

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

2.6.4 Immuno-blotting and detection

The membranes were stained with a Ponceau Red stain (Ponceaus solution sigma, USA P7170) to check for equal loading. Membranes were blocked with 5% milk in tri-buffered saline (TBS-Tween) (0.1% Tween) for 3 hours. The membranes were probed with primary antibodies phospho-STAT and total-STAT or β-actin overnight at 4°C. The primary antibody (total STAT (H-190) sc-7179 Santa Cruz Biotechnology rabbit polyclonal, phospho-STAT-3 (Tyr 705) sc-7993 Santa Cruz Biotechnology goat polyclonal, phospho-STAT(serine 727) # 91365 Cell signaling technology, USA Mouse mAb) was washed off with TBS -T (0.1% Tween),3 times for 5 min, and the membranes were probed with the secondary antibody (Biotechnology, Donkey anti-rabbit IgG-HRP sc-2313 Santa Cruz Biotechnology, USA) for 1 hour. Detection was accomplished with enhanced chemiluminescents (ECL). The emission of light was based on the interaction between the luminol and the horse radish peroxides (HRP) conjugated to the secondary antibody for qualitative or semi-quantitative analysis. Relative densitometry was determined with use of a computerized software package, UVIBAND.

2.7 Statistical analysis

All values are expressed as the mean ± SEM. Multiple comparisons were made with one-way analysis of variance (ANOVA) followed by the post-hoc Tukey-Kramer. The statistical analyses were implemented in INSTAT programme. Statistical significance was set at *p< 0.05.

2.8 Pharmacologic Agents

Luzindole, a melatonin inhibitor was obtained from Tocris bioscience (London, England) and if not mentioned in the text all drugs were obtained from Sigma Chemicals Company (St Louis, MO, USA).

CHAPTER 3: RESULTS

3.1 Chronic moderate consumption of red wine confers protection against ischemia reperfusion

3.1.1 Effect of red wine on hemodynamic parameters

To explore the cardioprotective effect of chronic moderate consumption of red wine, the isolated hearts from rats pretreated with wine for 10 days were exposed to 30 min global ischemia followed by 30 min of reperfusion. Pre-treatment with red wine had no significant effect on the pre-ischemic values of LVDP, heart rate and coronary flow compared to the control group. After 30 min of reperfusion, the control hearts had a LVDP of 15.3±2.4mmHg. Pre-treatment with red wine improved LVDP to 38.0±3.0mmHg ($p<0.001$ vs. control) without any significant changes in the heart rate (HR for red wine: 253.0±29.0 beats/min ns vs. control) nor the coronary flow (8.8±0.3mL/min vs. 10.0±1.6mL/min ns vs. control)

Table 3: *The effect of chronic red wine consumption in the isolated rat hearts subjected to ischemia- reperfusion*

	Hemodynamic parameters		
	Pre-ischemic	***Reperfusion 5 min***	***Reperfusion 30 min***
LVDP (mmHg)			
Control	89.3 ± 3.0	7.33 ± 2.4	15.3 ± 2.4
Wine	94.5 ± 2.4	14.5 ± 2.6	38.0 ± 3.0***
Heart Rate (beats/min)			
Control	265.3 ± 52.5	200.0 ± 9.5	227.0 ± 20.0
Wine	292.5 ± 8.7	180.1 ± 26.1	253.0 ± 29.0
Coronary flow (mL/min)			
Control	11.60 ± 1.12	10.0 ±1.63	10.0 ±1.63
Wine	10.0 ± 0.27	6.6 ± 0.90	8.8 ± 0.26

*Parameters measured prior to ischemia (pre-ischemic), parameters measured after ischemia at 5 or 30 min of reperfusion (post-ischemic). LVDP=left ventricular developed pressure, ***p<0.001 vs. the control group at 30 min of reperfusion*.

3.1.2 Effect of chronic moderate consumption of red wine on functional recovery

After 30 min of reperfusion the control hearts had a rate pressure product (RPP) of 15.3±2.4% (which is expressed as a percentage of a baseline value). This is in accordance with findings previously described in the literature (Lecour *et al.,* 2002). Pre-treatment with red wine improved the functional recovery of the heart to 36.9±5.0% (**p<0.01 vs. control).

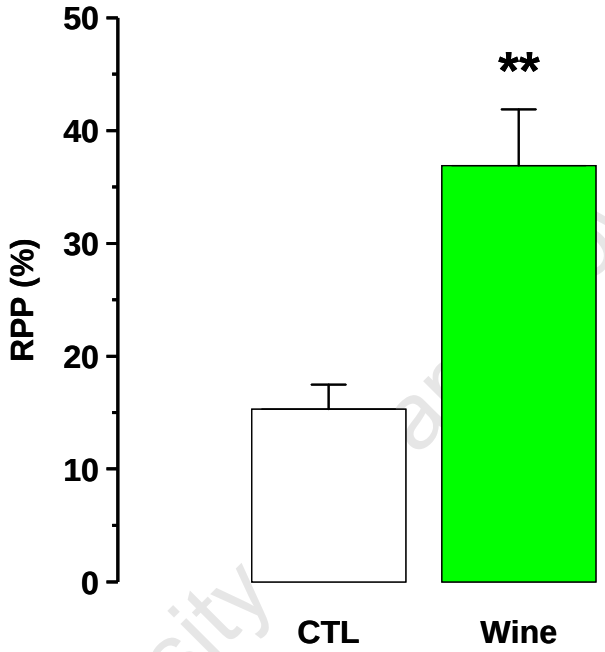


***Figure 14**: The effect of chronic to moderate consumption of red wine on rate pressure product (RPP), CTL: control **p<0.01.n=6 per group.*

3.1.3 Effect of chronic moderate consumption of red wine on infarct size

The control hearts subjected to 30 min global ischemia followed by 60 min of reperfusion had an infarct size of 60.8±5.9%. Pre-treatment with red wine improved the infarct size to 21.5±7.8% (**p<0.01 vs. control group).

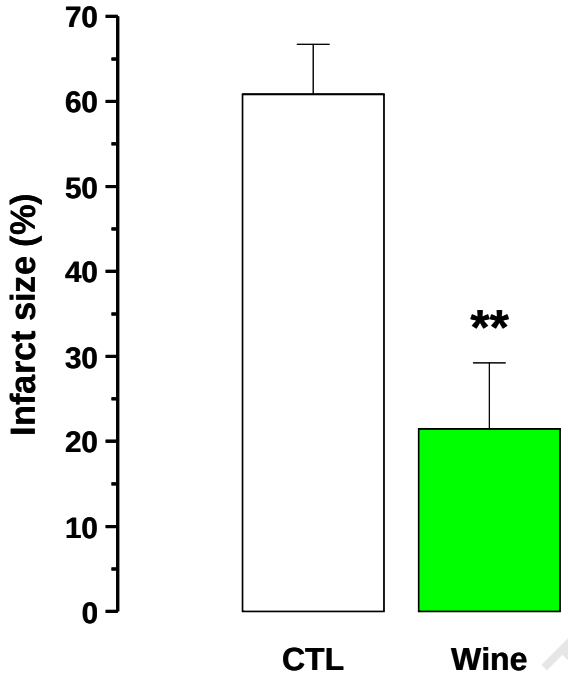


Figure 15: *The effect of chronic to moderate consumption of red wine on infarct size. CTL: control, **p<0.01 n=6 per group.*

3.2 Delineation of the active components found in red wine

To explore the role of alcohol in the cardioprotective effect of red wine, rats were pre-treated with wine 12%, wine 6% or alcohol 6% for 10 days and subjected to 30 min of ischemia followed by 30 min of reperfusion.

3.2.1 Role of alcohol in the cardioprotective effect of red wine

Of note, the alcohol 6% used in this study was in fact the alcohol extracted from the wine. Pre-treatment with wine (12%), wine (6%) or alcohol (6%) had no significant effect on the pre-ischemic values for LVDP, heart rate and coronary flow compared to the control group. After 30 min of reperfusion, the LVDP of wine (6%) improved to 36.6±3.0mmHg (***p<0.001 vs. control) and protected to a similar extent than the wine (12%). The pre-treatment with wine (6%) did not change heart rate (270.0±18.0 beats/min ns vs. control) or the coronary flow (6.9±0.3mL/min vs. 5.5±0.3mL/min).

Alcohol 6% on its own, did not significantly improve LVDP at 5 min of reperfusion. However, after 30 min of reperfusion the LVDP of alcohol

improved to 26.4±2.2mmHg (** p<0.01) and there was no difference in heart rate or coronary flow (ns vs. control).

Table 4: *The effect of wine (12%), wine (6%) or alcohol (6%) on the isolated rat heart subjected to ischemia-reperfusion*

Hemodynamic parameters			
	Pre-ischemic	***Reperfusion 5min***	***Reperfusion 30min***
LVDP (mmHg)			
Control	89.3 ± 3.0	7.33 ± 2.4	15.3 ± 2.4
Wine	94.5 ± 2.4	14.5 ± 2.6	38 ± 3.0 ***
Wine (6%)	87.3 ± 2.0	27.0 ± 5.0	36.6 ± 3.0***
Alcohol	101.2 ± 5.6	33.4 ± 21.9	26.4 ± 2.2**
Heart Rate (beats/min)			
Control	265.3 ± 52.5	200.0 ± 9.5	227.0 ± 20.0
Wine	292.5 ± 8.7	180.1 ± 26.1	253.0 ± 29.0
Wine (6%)	307.0 ± 13.3	260.3± 34.0	270.0 ±18.0
Alcohol	304.6 ± 9.7	228.0 ± 26.5	280.0 ± 0.0
Coronary Flow (mL/min)			
Control	11.60 ± 1.12	10.0 ±1.63	5.5 ± 0.3
Wine	10.0 ± 0.27	6.6 ± 0.90	5.6 ± 0.2
Wine (6%)	13.0 ± 1.1	7.3 ± 0.4	6.9 ± 0.3
Alcohol	11.6 ± 0.2	9.8 ± 0.6	6.8± 0.48

*Parameters measured prior to ischemia (pre-ischemic), parameters measured after ischemia at 5 or 30 min of reperfusion (post-ischemic). LVDP=left ventricular developed pressure, HR=heart rate, CF= coronary flow **p<0.01 vs. the control group, ***p<0.001 vs. the control*.

3.2.1.1 Effect of wine (12%), wine (6%) and alcohol on functional recovery

The treatment with wine (6%) enhanced the functional recovery compared with control groups (**p<0.01) and protected to a similar extent to wine 12% (RPP: 36.8±5.0% vs. 36.9±5.0%). Alcohol (6%) given on its own did not significantly improve the functional recovery after 30 min of reperfusion compared with control 24.3±2.3% (ns vs. control).

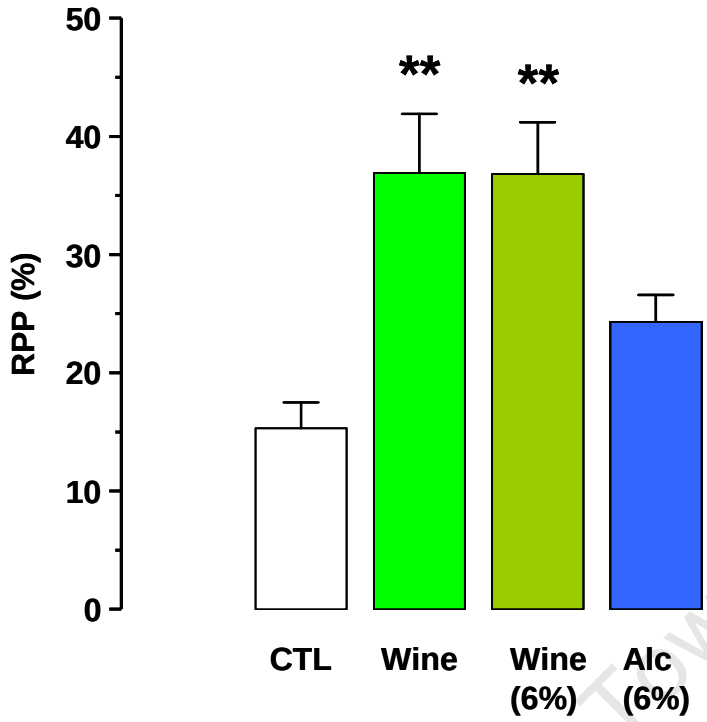


Figure 16: *The role of alcohol in wine induced cardioprotection. CTL: control, wine (6%): wine with reduced alcohol, alc (6%): alcohol on its own from the red wine. **p<0.01 n=6 per group.*

3.2.2 Role of resveratrol in cardioprotective effect of red wine

3.2.2.1 Effect of resveratrol on hemodynamic parameters

To explore the possible contribution of resveratrol in red wine-induced cardioprotection, we pretreated rats with resveratrol (7mg/L) for 10 days, a concentration equivalent to the concentration found in red wine.

Chronic pre-treatment with resveratrol had no significant effect on the pre-ischemic values of LVDP, heart rate and coronary flow compared to the control groups. After 5 min of reperfusion the LVDP improved to 18.8±2.0mmHg (**p<0.01 compared to control). However, pre-treatment with resveratrol after 30 min of reperfusion did not improve the LVDP compared to control (16.7±2.4mmHg vs.15.3±2.4mmHg; ns vs. control). The pre-treatment with resveratrol did not change the heart rate during the reperfusion period (242±29.7beats/min ns vs. control) nor the coronary flow (10±1.63mL/min vs.8.2±0.86mL/min; ns vs. control).

Table 5: *The effect of chronic pre-treatment with resveratrol on the isolated rat hearts against ischemia-reperfusion*

Hemodynamic parameters			
	Pre-ischemic	***Reperfusion 5min***	*Reperfusion 30min*
LVDP(mmHg)			
Control	89.3 ± 3.0	7.33 ± 2.4	15.3 ± 2.4
Res	94.8 ± 3.3	18.8 ± 2.0 **	16.7 ± 2.4
Heart Rate(beats/min)			
Control	340.3 ± 17.0	200.0 ± 9.5	260.0 ± 8.7
Res	304.5 ± 7.4	242.1 ± 29.7	276.0 ± 13.2
Coronary Flow(mL/min)			
Control	11.60 ± 1.12	10.0 ± 1.63	10.0 ± 1.63
Res	12.8 ± 0.80	10.4 ± 1.60	8.2 ± 0.86

*Parameters measured prior to ischemia (pre-ischemic), parameters measured after ischemia at 5min or 30 min of reperfusion (post-ischemic). Res=resveratrol LVDP=left ventricular developed pressure, **p<0.01 vs. the control.n=6 per group.*

3.2.2.2 Effect of resveratrol on functional recovery

At the end of reperfusion period, control hearts presented a functional recovery of 16.4±6.4%. The pre-treatment with resveratrol did not improve the functional recovery compared with the controls (16.7±2.4%; ns vs. control).

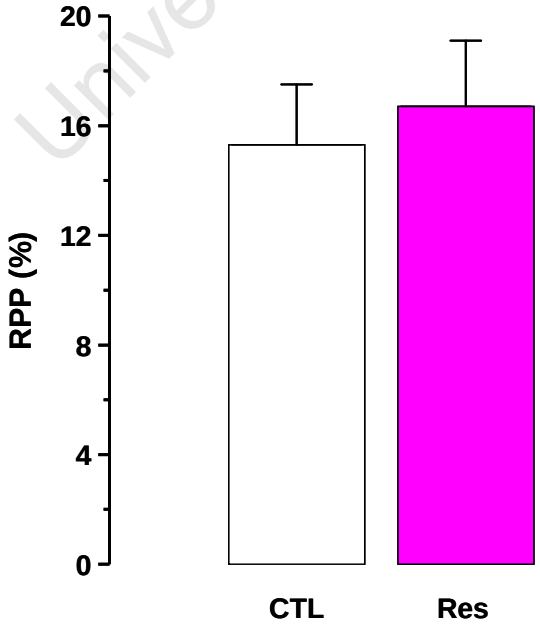


Figure 17*: The effect of chronic treatment with resveratrol, with an equivalent concentration to that found in red wine. CTL: control, Res: Resveratrol. n=6 per group.*

3.2.3 Role of melatonin in red wine-induced cardioprotection

3.2.3.1 Effect of melatonin on hemodynamic parameters

To explore the possible contribution of melatonin in red wine induced cardioprotection, we pretreated rats with melatonin (0.75µg/L), for 10 days, a concentration was equivalent to the concentration found in red wine, and we subjected the hearts to 30 min of global ischemia followed by 60 min of reperfusion. The pre-treatment with melatonin had a significant effect after 5min of reperfusion when compared to the control (26.0±3.0; **p<0.01). The pre-ischemic values for heart rate and coronary flow compared to the control group.

After 30 min of reperfusion the LVDP improved to 27.44mmHg (**p<0.01 vs. control). This protection persisted at 60 min of reperfusion (34.9±5.1mmHg vs. 16.5±3.2mmHg; **p<0.01). The pre-treatment with melatonin had no effect on heart rate (HR for melatonin: 286.6±16.0 beats/min ns vs. control) nor coronary flow before or after ischemia-reperfusion.

***Table 6**: The effect of chronic melatonin pre-treatment in the isolated rat hearts subjected to ischemia- reperfusion*

Hemodynamic parameters				
	Pre-ischemic	***Reperfusion 5min***	***Reperfusion 30min***	***Reperfusion 60min***
LVDP (mmHg)				
Control	89.3 ± 3.0	7.33 ± 2.4	15.3 ± 2.4	18.0 ± 3.2
Mel	95.9 ± 4.4	26.0 ± 3.0**	27.4 ± 5.1**	34.9 ± 5.1**
Heart rate (beats/ min)				
Control	265.3 ± 52.5	200.0 ± 9.5	260.0 ± 8.7	268.6 ± 34.6
Mel	268.6 ± 11.4	217.1 ±11.9	262.8 ± 34.7	286.6 ±16.0
Coronary flow (mL/min)				
Control	11.6 ± 1.12	10.0 ± 1.63	10.0 ±1.63	10.6 ± 1.639
Mel	10.4 ± 0.4	9.4 ± 0.5	8.6 ± 0.3	8.6 ± 0.3

*Parameters measured prior to ischemia (pre-ischemic), parameters measured after ischemia at 5 or 30 min of reperfusion (post-ischemic). Mel=melatonin LVDP=left ventricular developed pressure, **p<0.01 vs. the control group at 30 min or 60 min of reperfusion.*

3.2.3.2 Effect of melatonin on functional recovery

At the end of the reperfusion period control hearts presented a functional recovery of 16.4±6.4%. The pre-treatment with melatonin enhanced the functional recovery to 30.6±2.3% (**p<0.01).

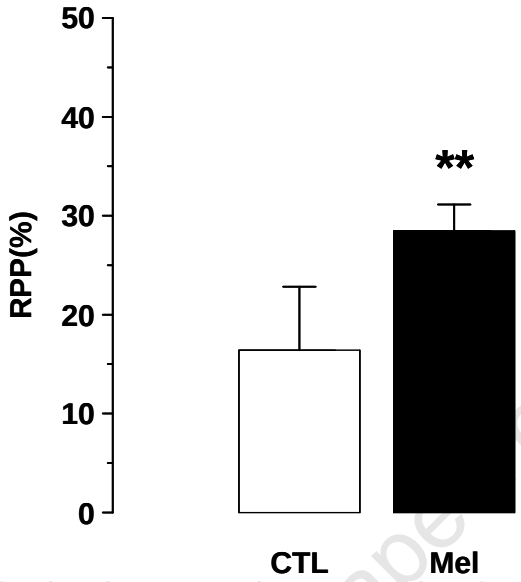


Figure 18*: The effect of melatonin on RPP. The concentration of melatonin was equivalent to the concentration found in red wine. RPP: rate pressure product, CTL: control and Mel: melatonin **p<0.01. n=6 per group*

3.2.3.3 Effect of melatonin on infarct Size

The control hearts presented an infarct size of 60.8±5.9%. The treatment with melatonin decreased infarct size to 27.5±7.8% (***p<0.001 vs. control).

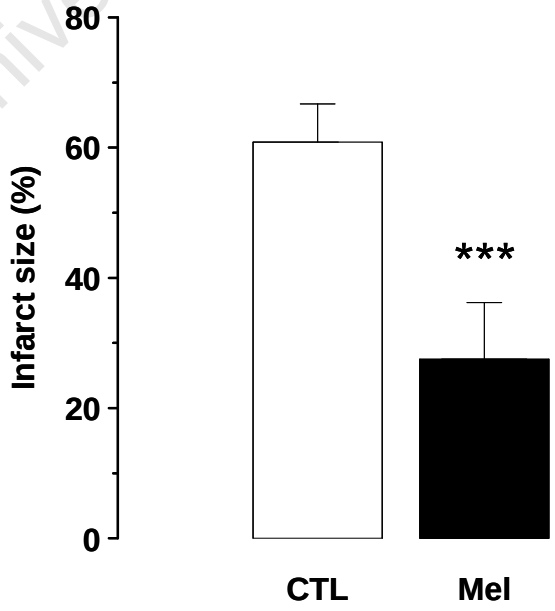


Figure 19*: The effect of chronic to moderate treatment with melatonin on infarct size. CTL: control and Mel: melatonin ***p<0.001. n=6 per group.*

3.2.3.4 Effect of luzindole combined with wine or melatonin on functional recovery

To explore the possible role of melatonin in red wine-induced cardioprotection, we pretreated rats with melatonin or wine in the presence of luzindole (5mg/kg, ip), a melatonin inhibitor, for 10 days prior to ischemia-reperfusion.

Hearts were pre-treated with luzindole alone presented a functional recovery of 16.0±1.0% at the end of the reperfusion period. The co–administration of luzindole with melatonin abolished the protective effect of melatonin 15±1.3% (ns vs.control). Surprisingly, the co-administration of wine and luzindole improved functional recovery to 40.0±1.5% (***p<0.001 vs. control).

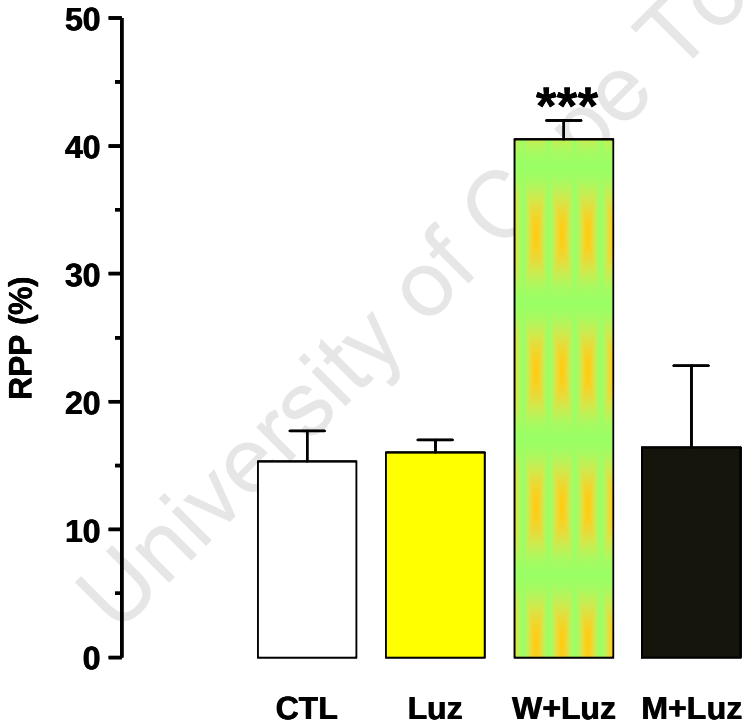


***Figure 20**: The role of melatonin in wine induced cardioprotection. RPP: rate pressure product, CTL: control, Luz: luzindole; melatonin inhibitor, W+L: wine + luzindole and M+Luz: melatonin + luzindole ***p<0.001. n=6 per group*

3.2.3.5 Effect of luzindole combined with wine or melatonin on infarct size

Hearts treated with chronic administration of luzindole had an infarct size of 60.7±1.2% (ns vs. control). The co–administration of melatonin with luzindole abolished the protective effect and presented an infarct of (55.9±1.3%; ns vs.

control). However, the co-administration of wine and luzindole decreased infarct size to 22.0±1.3% (***p<0.001 vs. control).

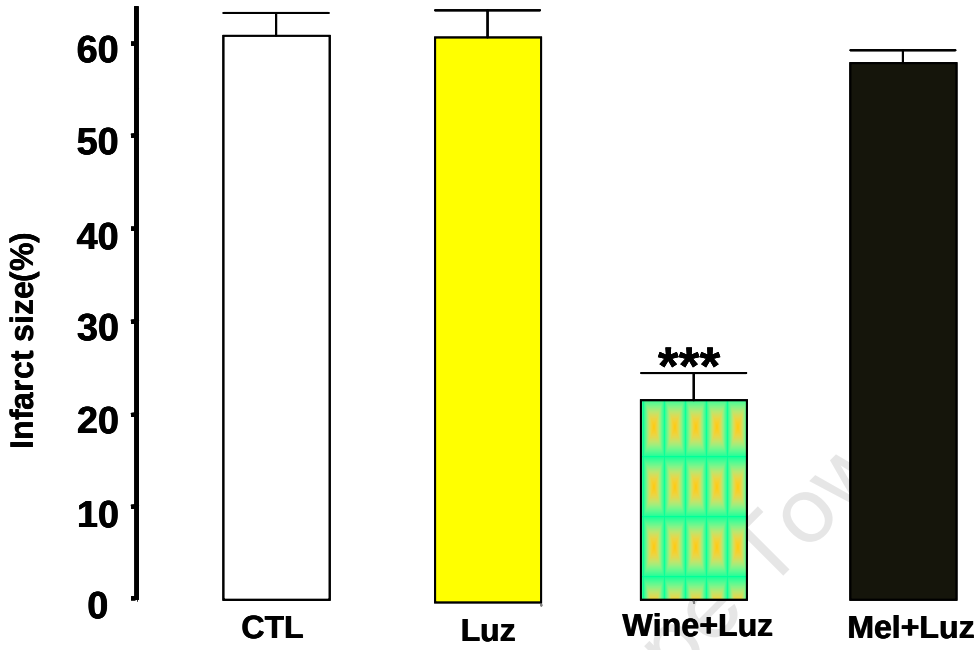


***Figure 21**: The effect of melatonin on red wine induced cardioprotection on infarct size. CTL: control, Wine + Luz: wine + luzindole, Luz: luzindole, Mel + Luz: melatonin + luzindole ***p<0.001. n=6 per group.*

3.2.4 Role of ethanolamine in the cardioprotective effect of red wine

3.2.4.1 Effect of ethanolamine on hemodynamic parameters

To explore the role of ethanolamine as a cardioprotective agent in red wine, rats were pre-treated for 10 days with ethanolamine (21µg/L), a concentration equivalent to the concentration found in French red wine. The control hearts presented an LVDP of 15.3±2.4mmHg after an I/R insult. Pre-treatment with ethanolamine did not change pre-ischemic values for LVDP, heart rate and coronary flow compared to control. The pre-treatment with ethanolamine improved LVDP at 60 min of reperfusion to 33.5±6.0mmHg (**p<0.01 vs. control). Interestingly, the combination of melatonin and ethanolamine increased LVDP at 60 min of reperfusion to 38.3±6.8mmHg (**p<0.01 vs. control). The pre-treatment with ethanolamine alone, or a combination with melatonin, had no effect on heart rate (ns vs. control) or coronary flow throughout the protocol (ns vs. control).

Table 7: *The effect of ethanolamine and a combination of ethanolamine and melatonin consumption in the isolated rat hearts subjected to ischemia reperfusion*

Hemodynamic parameters				
	Pre-ischemic	***Reperfusion 5min***	***Reperfusion 30min***	***Reperfusion 60min***
LVDP(mmHg)				
Control	89.3 ± 3.0	7.33 ± 2.4	15.3 ± 2.4	16.0 ± 3.2
Etn	98.6 ± 4.2	15.3 ± 5.3	24.6 ± 7.0	33.5 ± 6.0**
Mel+Etn	89.3 ± 4.2	20.6 ± 6.9	28.0 ± 6.3	38.3 ± 6.8***
Heart rate(beats/min)				
Control	265.3 ± 52.5	200.0 ± 9.5	260.0 ± 8.7	260.0 ± 35.4
Etn	286.6 ± 19.0	206.0 ± 41.7	280.0 ± 40.0	286.6 ±16.0
Mel+Etn	280.0 ± 25.3	253.3 ± 22.1	226.4 ± 8.4	300.0 ± 26.8
Coronary flow(mL/min)				
Control	11.60 ±1.12	10.0 ±1.63	10.0 ±1.63	10.6 ±1.639
Etn	10.3 ± 0.3	8.3 ± 0.3	8.0 ± 0.0	8.0 ± 0.0
Mel+Etn	10.6 ± 0.42	10.0 ± 0.0	8.3 ± 0.5	9.0 ±1.1

*Parameters measured prior to ischemia (pre-ischemic), parameters measured after ischemia at 5 min or 30/60 min of reperfusion (post-ischemic). Etn=ethanolamine Mel+Etn=melatonin and ethanolamine LVDP=left ventricular developed pressure, **p<0.01, ***p<0.01 vs. the control at 60 min of reperfusion n=6 per group.*

3.2.4.2 Effect of ethanolamine on functional recovery

At the end of the reperfusion control hearts presented a functional recovery of 16.4±6.4%. The pre-treatment with ethanolamine improved functional recovery to 28.5±2.7% (**p<0.01 vs. control). Interestingly, the combination of ethanolamine and melatonin further enhanced the functional recovery to 40.0±2.6% (***p<0.001).

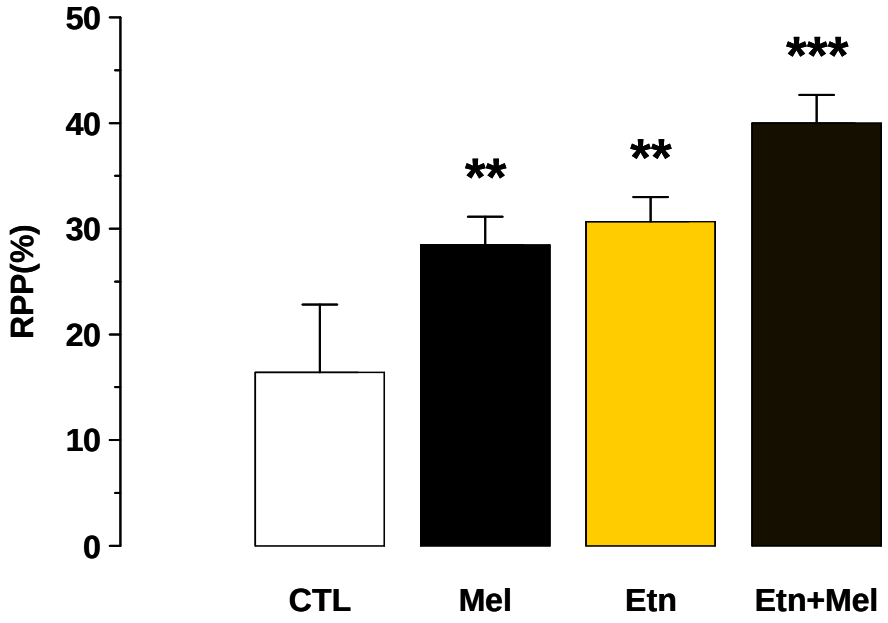


***Figure 22**: The effect of ethanolamine or its combination with melatonin on RPP. RPP: rate pressure product, CTL: control, Etn: ethanolamine, Etn+Mel; ethanol and melatonin. **p<0.01 and ***p<0.001 vs. CTL. n=6 per group.*

3.2.4.3 Effect of ethanolamine on infarct size

Control hearts presented an infarct size of 60.8±5.9%. The pre-treatment with ethanolamine for 10 days improved functional recovery to 22.6±5.7% (***p<0.001 vs. control). Interestingly, the combination of ethanolamine and melatonin further enhanced the protection and decreased the infarct size to 5.8±1.6% (***p<0.001 vs. control).

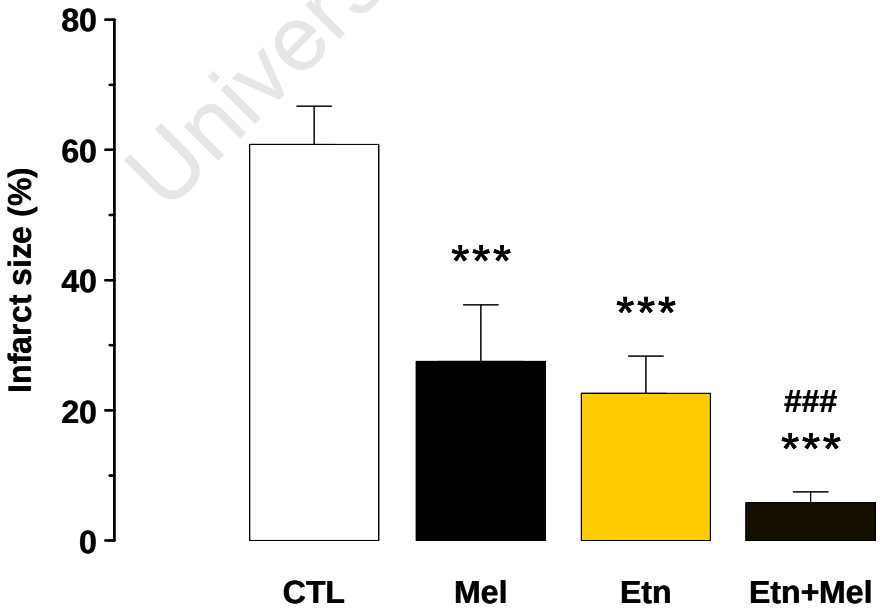


***Figure 23**: The effect of ethanolamine or its combination with melatonin on infarct size, CTL: control, Etn: ethanolamine, Etn+Mel; ethanolamine and melatonin. ###p<0.001vs.Etn and ***p<0.001 vs. CTL. n=6 per group.*

3.2.5 Role of STAT-3 in red wine-induced cardioprotection

To explore whether the cardioprotective signal transducer and activator of transcription (STAT-3) may be involved in red wine-induced cardioprotection, rats were treated with AG490 (5mg/kg/day; ip) a STAT-3 inhibitor for 10 days, alone or in combination with treatment of red wine.

3.2.5.1 Effect of the co-administration of red wine and AG490, a STAT-3 inhibitor, on hemodynamic parameters

The pre-treatment with AG490 or the combination of wine and AG490 did not alter the pre-ischemic values for LVDP, heart rate and coronary flow compared to controls. Hearts that were pre-treated with red wine and AG 490, the STAT-3 inhibitor had a LVDP of 14.7±1.7mmHg at 60 min of reperfusion (ns vs. control). The pre-treatment with AG490, or a combination of wine and AG490, had no effect on heart rate (ns vs. control) nor in coronary flow (ns vs. control).

Table 8: *Role for STAT-3 in wine-induced cardioprotection in the isolated rat heart subjected to ischemia-reperfusion*

Hemodynamic parameters				
	Pre-ischemic	***Reperfusion 5min***	***Reperfusion 30min***	***Reperfusion 60min***
LVDP(mmHg)				
Control	89.3 ± 3.0	7.3 ± 2.4	15.3 ± 2.4	16.5 ± 3.2
Wine	94.5 ± 2.4	14.5 ± 2.6**	38.0 ± 3.0	39.1 ± 3.3***
AG490	93.3 ± 3.8	7.3 ± 2.3	10.6 ± 1.7	10.6 ± 1.7
Wine+AG490	96.6 ± 1.3	4.0 ± 1.4	9.3 ± 0.8	14.7 ± 1.7
Heart Rate(beats/min)				
Control	265.3 ± 52.5	200.0 ± 9.5	260.0 ± 8.7	286.6 ± 16.0
Wine	292.5 ± 8.7	180.1 ± 26.1	290.0± 13.4	317.5 ± 9.3
AG490	320.0 ± 27.3	173.3± 77.4	273.3 ± 49.9	360.0 ± 27.3
Wine+AG490	296.6 ± 48.0	160.0 ± 52.6	226.0 ± 16.8	306.0 ± 16.8
Coronary Flow(mL/min)				
Control	11.6 ± 1.1	10.0 ± 1.6	10.0 ± 1.6	10.6 ± 1.6
Wine	10.0 ± 0.3	6.6 ± 0.9	8.8 ± 0.3	8.9 ± 0.3
AG490	10.3 ± 0.3	8.3 ± 0.3	8.0 ± 0.0	8.0 ± 0.0
Wine+AG490	10.0 ± 0.1	6.0 ± 1.9	8.6± 0.4	8.6 ± 0.4

*Parameters measured prior to ischemia (pre-ischemic), parameters measured after ischemia at 5min or 30/60 min of reperfusion (post-ischemic). Wine = Wine (2 glasses/day), AG490=a STAT-3 inhibitor, wine+AG= co-administration of wine and AG LVDP=left ventricular developed pressure **p<0.01 vs. the control group, ***p<0.01 vs. control. n=6 per group.*

3.2.5.2 Effect of the co-administration of red wine and AG490, a STAT-3 inhibitor, on hemodynamic parameters

Hearts pre-treated with AG490 presented a functional recovery of 11.4±3.1% at the end of reperfusion period (ns vs. control). Interestingly, the combination of wine and AG490 abolished the protective effect of red wine on functional recovery (14.4 ± 2.9; ns vs. control).

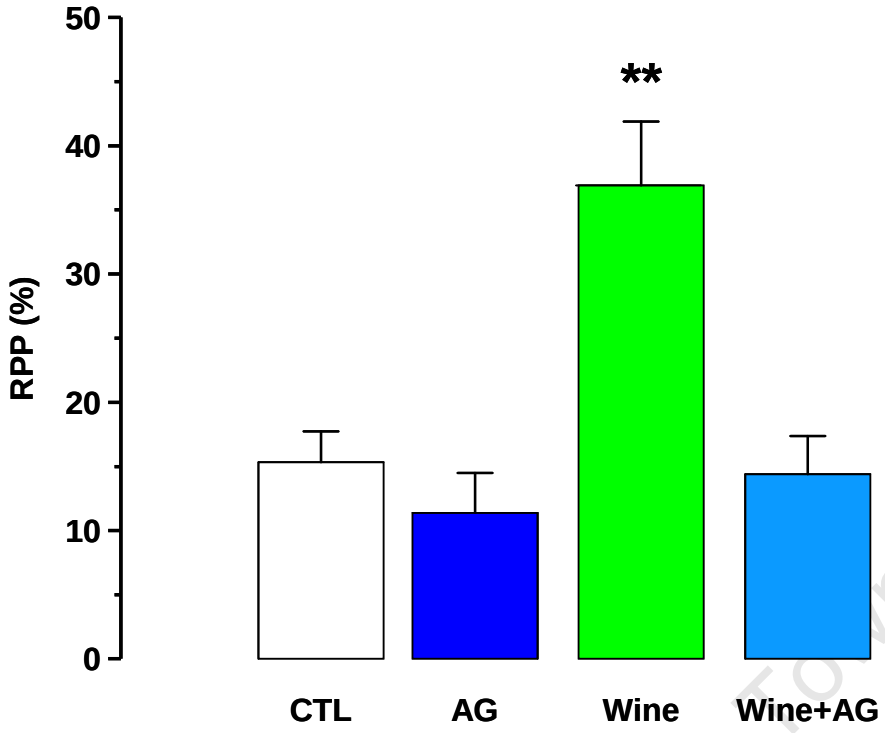


***Figure 24**: The effect of red wine in the presence of AG490; a STAT-3 inhibitor on RPP. RPP; rate pressure product, CTL: control, AG: AG490, Wine+AG: wine + AG490 ***p<0.001 vs. control. n=6 for per groups.*

3.2.5.3 Effect of the co-administration of red wine and AG490, a STAT-3 inhibitor, on infarct size

Hearts pre-treated with AG490 for 10 days presented an infarct size of 60.9±7.3% (ns vs. control). However, the co-treatment of red wine with AG490 abolished the infarct-sparing effect of red wine to (57.9±3.4%; ns vs. control).

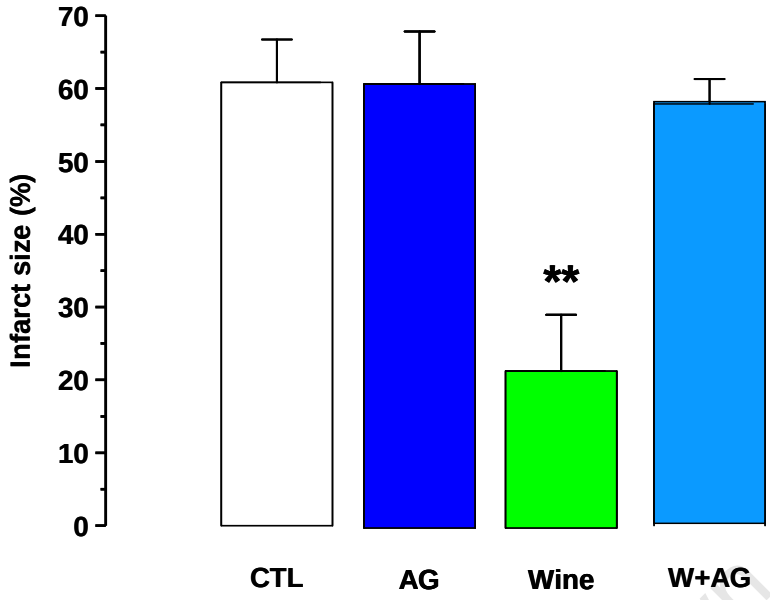


Figure 25: *The effect of red wine in the presence or the absence of AG490; a STAT-3 inhibitor on infarct size. CTL: control, AG: AG490, W+AG: wine + AG490 **p<0.01 vs. control. n=6 per group*

3.2.5.4 Levels of pSTAT-3 after chronic and moderate consumption of red wine

To investigate the role of STAT-3 in red wine-induced cardioprotection, we measured the levels of phosphorylated STAT-3 in rat hearts pre-treated with red wine and subjected to 30 min global ischemia and 15 min of reperfusion. The graph below shows that the pre-treatment with red wine increased STAT-3 phosphorylation in both the cytosol (0.5±0.2 A.U; *p<0.05 vs. control) and the nucleus (**p<0.01 vs. control).

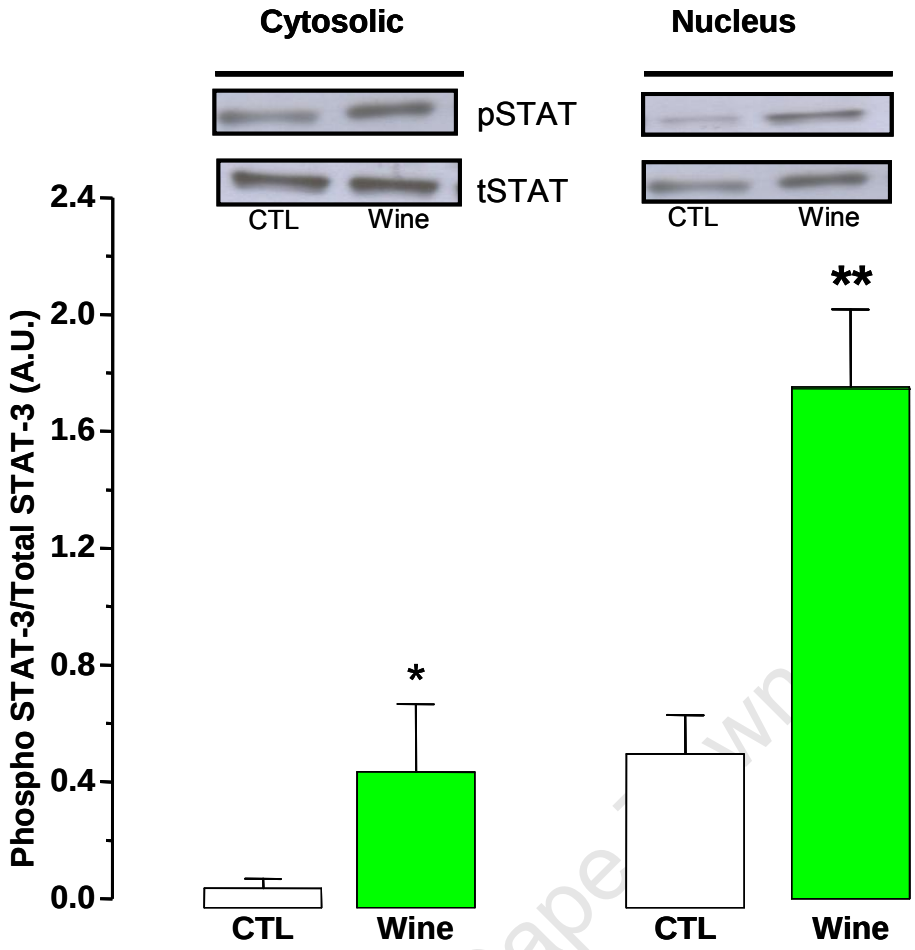


Figure 26*: The effect of red wine on levels of pSTAT-3/Total STAT-3 in the cytosol and in the nucleus pSTAT:phosphorylated STAT, tSTAT: total STAT. *p<0.05 **p<0.01 vs. control. n=4 per groups.*

3.2.6 Role of ethanolamine in wine induced cardioprotection

To explore whether ethanolamine, melatonin and resveratrol may protect against I/R via the activation of STAT-3, we subjected rats to an acute treatment of ethanolamine, melatonin or resveratrol with/without AG490, a STAT-3 inhibitor before being subjected to 30 min of regional ischemia and 120 min of reperfusion (all drugs were given directly to the perfused heart).

3.2.6.1 Effect of the co-administration of ethanolamine and AG490, a STAT-3 inhibitor, on hemodynamic parameters

The pre-treatment with ethanolamine, AG490 or the combination of ethanolamine and AG490 prior to the ischemia reperfusion insult had no significant effect on both the pre-ischemic and post ischemic values.

Table 9: *Role for STAT-3 in ethanolamine induced cardioprotection in the isolated rat heart subjected to ischemia-reperfusion.*

Hemodynamic parameters						
	Pre-ischemic	***Ische mic***	***Reperfusion 5min***	***Reperfusion 30min***	***Reperfusion 60min***	***Reperfusion 120min***
LVDP (mmHg)						
Control	95.0 ± 4.4	52.4 ± 5.5	65.2 ± 6.8	62.2 ± 6.0	54.4 ± 4.4	44.8 ± 5.7
Etn	90.9 ± 3.0	41.7 ± 6.3	56.0 ± 6.4	45.7 ± 3.5	38.6 ± 4.3	35.1 ± 3.4
Etn+AG490	89.0 ± 3.2	43.0 ± 4.6	67.3 ± 4.1	56.6 ± 6.6	53.3 ± 4.0	53.0 ± 8.0
AG490	92.6 ± 4.0	38.6 ± 2.6	53.1 ± 6.3	53.1 ± 6.3	46.3 ± 5.4	40.0 ± 3.8
Heart Rate (beats/min)						
Control	348 ± 6.1	308 ± 12.0	312 ± 10.0	308 ± 10.4	292 ± 14.7	292 ± 14.7
Etn	314.0 ± 10.4	268.6 ± 11.4	285.7 ± 18.4	297.1 ± 4.8	297.1 ±14.8	308.6 ± 14.2
Etn+AG490	326.6 ± 16.0	333.3 ± 8.4	320.0 ± 20.7	313.0 ±19.0	306.7 ± 22.3	367 ± 24.6
AG490	320.0 ± 21.4	302.9 ± 24.5	274.3 ± 28.1	291.4 ± 24.2	302.9 ± 22.9	302.9 ± 19.2
Coronary Flow (mL/min)						
Control	11.6 ± 0.6	7.0 ± 0.7	11.2 ± 0.7	12.4 ± 0.6	10.6 ± 0.9	10.0 ± 0.9
Etn	11.4 ± 0.4	7.4 ± 0.8	11.4 ± 0.4	10.6 ± 0.7	10.6 ± 0.7	8.9 ± 0.9
Etn+AG490	12.7 ± 1.2	8.3 ± 1.7	10.8 ± 1.0	11.0 ± 1.5	11.7 ±1.2	8.0 ± 1.7
AG490	11.4 ± 0.57	8.0 ± 0.7	11.5 ± 0.4	11.1 ± 0.6	11.1 ± 0.6	9.4 ± 0.7

Parameters measured prior to ischemia (pre-ischemic), parameters measured after ischemia at 5 or 30-120 min of reperfusion (post-ischemic). Etn= Ethanolamine, AG490=a STAT-3 inhibitor, Etn+AG= co-administration of ethanolamine and AG490 LVDP=left ventricular developed pressure, HR=heart rate, CF= coronary flow ns vs. the control group at 120 min of reperfusion.n=6 per group.

3.2.6.2 Effect of the co-administration of ethanolamine and AG490 on infarct size

The control hearts presented an infarct size of 39.9±10.7%. The acute administration of ethanolamine before ischemia improved infarct size compared to the control (***p<0.001 vs. control). AG490 on its own did not change infarct size compared with the control group (33.9 ± 8.7%; ns vs. control). However, the co-administration of ethanolamine with AG490 abolished the infarct sparing effect of ethanolamine (36.8 ±8.7%; ns vs. control).

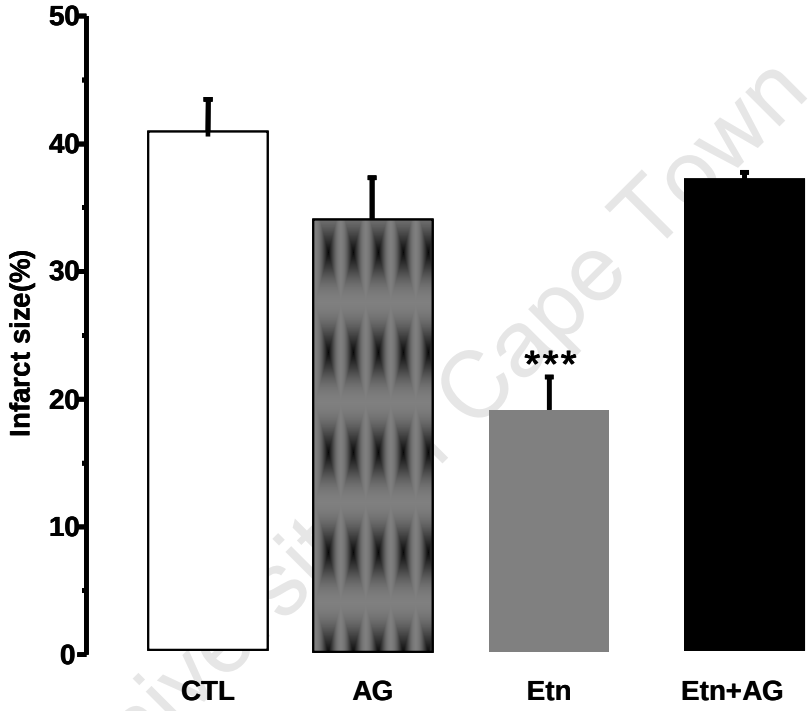


Figure 27: *The effect of ethanolamine with or without the STAT-3 inhibitor AG490 on infarct size. CTL= Control, Etn= Ethanolamine, Etn+AG=Ethanolamine+STAT-3 inhibitor, AG=AG490 ***p<0.001. n=6 per group.*

3.2.6.3 Levels of pSTAT-3 after a pre-treatment of ethanolamine

The hearts were pre-treated with ethanolamine for 15 min followed by a 10 min wash out and were collected before index ischemia to investigate STAT-3 phosphorylation. The graph below shows that the pre-treatment with ethanolamine decreased STAT-3 phosphorylation in the cytosol (0.5±0.2 A.U; **p<0.01 vs. control). However, there was a 57% increase of pSTAT in the nucleus (***p<0.01 vs. control).

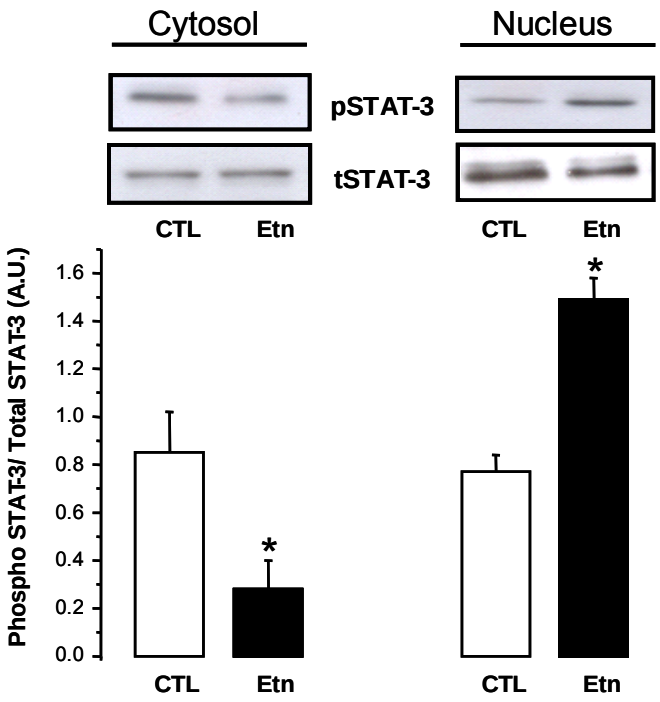


Figure 28: *The effect of Etn on Phospho STAT-3/Total STAT-3 in the cytosol or the nucleus with Etn. CTL: control, Etn: ethanolamine, pSTAT: phosphorylated STAT, tSTAT: total STAT **p <0.01, ***p<0.001. n=4 for all groups.*

3.2.7 Role of STAT-3 in melatonin induced cardioprotection

3.2.7.1 Effect of the co-administration of melatonin and AG490, a STAT-3 inhibitor on hemodynamic parameters

To explore whether melatonin protects against I/R via the activation of STAT-3, we subjected the hearts to an acute treatment of melatonin with/without AG490, a STAT-3 inhibitor before 30 min of regional ischemia and 120 min of reperfusion.

Table 10: *Role for STAT-3 in melatonin induced cardioprotection in the isolated rat heart subjected to ischemia-reperfusion.*

Hemodynamic parameters						
	Pre-ischemic	***Ischemic***	***Reperfusion 5min***	***Reperfusion 30min***	***Reperfusion 60min***	***Reperfusion 120min***
LVDP(mmHg)						
Control	97.0 ± 6.9	49.3 ± 7.5	58.2 ± 7.8	56.3 ± 5.0	52.6± 4.9	44.6 ± 6.6
Mel	101.3 ± 4.3	71.3 ± 17.3	101.3± 36.4	86.0 ± 15.6	75.3 ± 18.3	71.3 ± 19.0
Mel + AG490	94.3 ± 5.2	65.7 ± 24.4	50.3 ± 12.5	55.1 ± 14.6	40.0 ±11.6	34.9 ± 10.5
AG490	99.3 ± 3.9	52.3 ± 6.2	63.1 ± 5.3	65.5 ± 5.1	52.0 ±5.6	50.0 ± 5.9
Heart rate (beats/min)						
Control	340 ± 13.6	320 ± 17.9	313 ± 19.1	320± 10.3	306.6±16.9	300± 22.5
Mel	300.0 ± 13.3	240.0 ±17.9	213.3 ±19.7	213.1 ±19.7	240.1 ±34.3	276.6 ±30.3
Mel+AG 490	268.6 ± 13.6	240.3 ± 17.8	222.9 ±19.7	234.0 ±34.3	211.4 ±43.4	200±41.8
AG490	305.0 ± 21.3	275.0 ± 27.7	260.0 ±23.9	280.4 ±26.2	275.0 ±26.6	275.0 ±26.6
Coronary flow (mL/min)						
Control	11.0 ±0.7	8.3.0 ±0.7	8.3 ±2.1	9.6 ±1.3	9.3 ±0.9	11.0 ±0.9
Mel	11.0 ±0.4	9.0 ±0.7	9.6 ±0.6	10.3 ±0.6	10.3 ±0.6	10.3 ±0.6
Mel+AG 490	10.9 ±1.4	9.5 ±0.4	8.9±0.4	9.4 ±0.4	8.9 ±0.6	8.9 ±0.6
AG490	11.8 ± 0.3	9.0 ± 0.5	11.0 ±0.5	11.1 ±0.5	11.1 ±0.6	10.0 ±0.7

Parameters measured prior to ischemia (pre-ischemic), parameters measured after ischemia at 30 min of reperfusion (post-ischemic). Mel= melatonin, AG490=a STAT-3 inhibitor, Mel+AG= co-administration of melatonin and AG490. LVDP=left ventricular developed pressure, HR=heart rate, CF= coronary flow. All groups ns vs. the control group at 120 min of reperfusion

3.2.7.2 Effect of the co-administration of melatonin and AG490, a STAT-3 inhibitor on infarct size

The control hearts presented an infarct size of 44.4±2.8%. Administration of melatonin reduced infarct size to 25.0±2.9% (**p<0.01 vs. control). AG490 on its own did not change infarct size 33.9±8.7% compared to the control group

(ns vs. control). However, the co-administration of melatonin with AG490 abolished the infarct sparing effect of melatonin (36.83 ±8.7; ns vs. control).

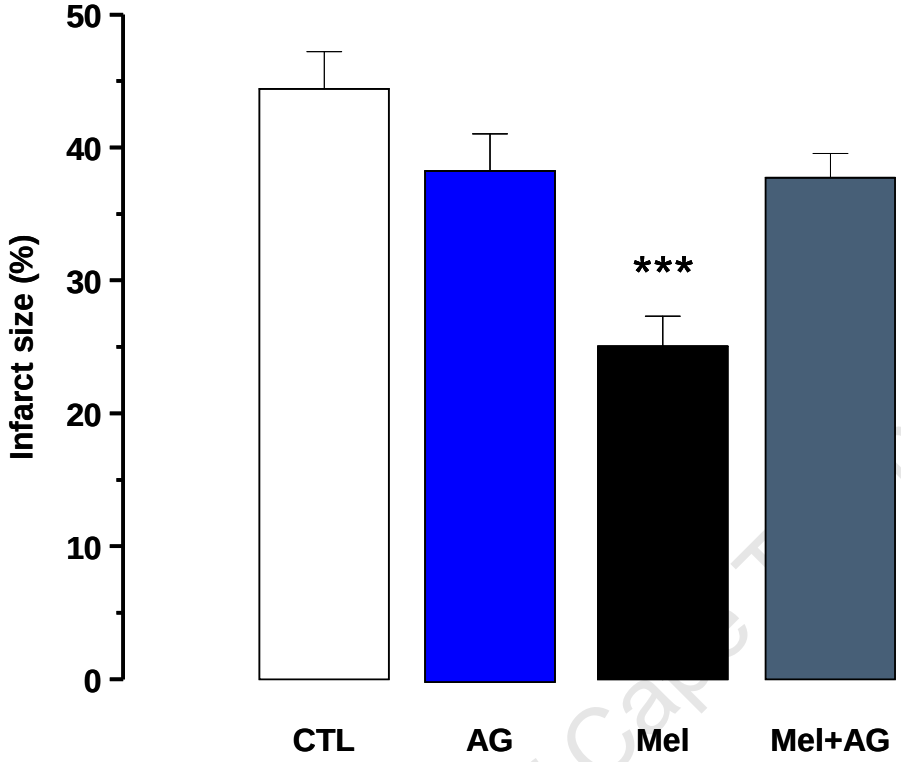


Figure 29: *The effect of melatonin with or without AG490 on infarct size. AG: AG490 and Mel + AG: melatonin + AG490 ***p<0.001. n=6 per group.*

3.2.7.3 Levels of pSTAT-3 after a pre-treatment with melatonin

The hearts were pre-treated with melatonin for 15 min followed by a 10 min wash-out and were collected before index ischemia to investigate STAT-3 phosphorylation. The graph below shows that the pre-treatment with melatonin had no effect on STAT-3 phosphorylation in the cytosol (ns vs. control). In the nucleus there was a 79% increase of pSTAT (***p<0.01 vs. control).

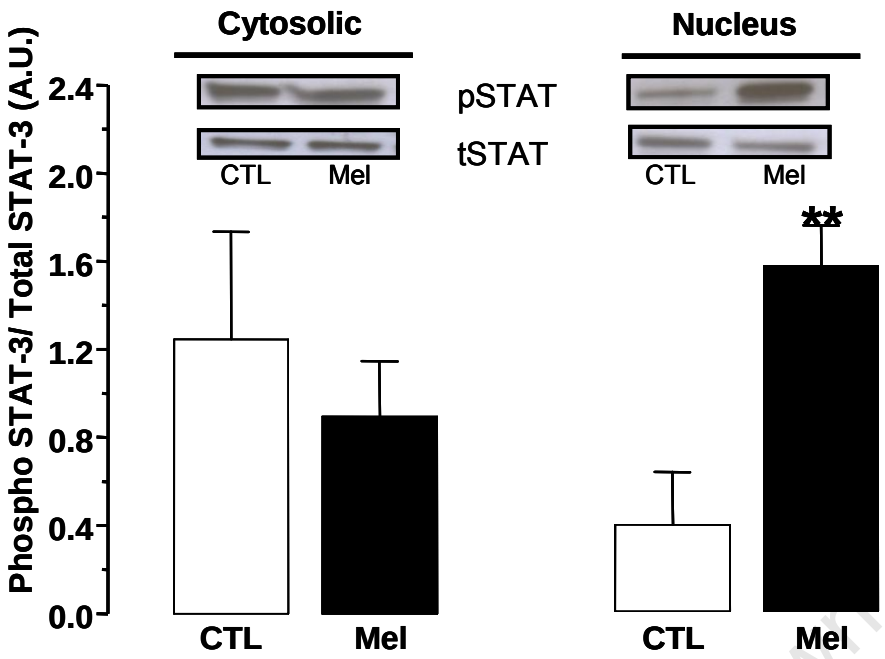


Figure 30: *The effect of melatonin on Phospho STAT-3/Total STAT-3 in cytosol or nucleus. CTL: control, Mel: melatonin, pSTAT: phosphorylated STAT, tSTAT: total STAT **p<0.01. n=4 for all groups.*

CHAPTER 4: DISCUSSION AND CONCLUSIONS

4.1. Summary of results

The aim of our study was to examine whether chronic moderate consumption of red wine equivalent to 2-3 glasses per day could protect isolated rat hearts from ischemia-reperfusion injury and to identify the cardioprotective components which are found in red wine and which are responsible for its cardioprotective effect.

In this study, we have shown that the chronic and moderate consumption of red wine for 10 days has the ability to protect the heart against ischemia-reperfusion. Our study did not support the statement that alcohol and resveratrol contribute to the cardioprotective effect of red wine. Hence, a chronic treatment with either alcohol or resveratrol, given at the concentration equivalent to the concentration found in red wine did not protect the heart against ischemia-reperfusion.

However, our results show for the very first time to our knowledge, the presence of two key biogenic amines in red wine that may contribute to the cardioprotective effect of wine. A chronic moderate treatment with either melatonin or ethanolamine for 10 days, given at the concentration found in red wine, protected the heart against ischemia-reperfusion to a similar extent to red wine itself.

Furthermore, our data suggest that red wine and its cardioprotective components (melatonin and ethanolamine) protect the heart via the activation of the JAK/STAT-3 pathway.

4.2. Red wine can protect isolated rat heart against ischemia-reperfusion.

Chronic moderate consumption of red wine is thought to contribute to cardioprotection and many experimental studies using the isolated rat heart models have explored the role of red wine against ischemia-reperfusion. However, all these studies were performed by giving acutely either red wine

polyphenols extracts (Das et al. 1999, Fuhrman et al. 2001) or some components of the wine such as resveratrol or ethanol (Ray et al. 1999a). At the time we started this study, no previous study had given an oral administration of red wine (12% or 6%) to study its cardioprotective effect. The other components besides the alcohol content of the wine 6% were not altered. In our experiments, rats were pre-treated for 10 days with a French Carbernet Sauvigonon. The red wine was diluted into the drinking water to an equivalent of 2 glasses of red wine per day. This protocol originally used by Wollny and Collaborators successfully prevented thrombosis in rats (Wollny et al. 1999). Our data show that the red wine treatment improved the functional recovery and decreased the infarct size in hearts subjected to an ischemia-reperfusion insult.

Recently, a study performed by Das's group demonstrated that rats pre-treated for 14 days with red wine (Reunite Lambrusco) by gavage (6.5mg/kg), were protected against an ischemia-reperfusion (Dudley et al. 2008, Mukherjee et al. 2009). It would be of interest to compare whether the same wine, given chronically either by gavage or just diluted in the drinking water could confer a similar protection.

4.3. Role of alcohol in red wine-induced cardioprotection

Alcohol is thought to contribute to the cardioprotective effect of red wine against ischemia-reperfusion injury but no study has been able to compare the wine and its alcohol content together. Here, we were able to compare the wine 12% with the same wine after the removal of 6% alcohol (with no alteration of other components in the wine). Alcohol 6% was also administered on its own, and this alcohol corresponded to the alcohol removed from the wine directly (the alcohol in wine contains ethanol and other types of alcohol). Interestingly, the wine 12% and 6% protected to a similar extent while alcohol 6% did not protect, therefore suggesting alcohol does not play a role in the cardioprotective effect of red wine.

Recent studies have shown that mice treated with 10% alcohol for 12 weeks protected the hearts against ischemia reperfusion injury (Zhou, Karliner & Gray 2002a, Collins et al. 2009). Similarly, rats that were treated with 18% alcohol for 8 weeks in drinking water improved post-ischemic systolic and diastolic pressure and it also reduced cardiovascular resistance via the activation of PKCε, see review (Collins et al. 2009). However, in our study the chronic moderate consumption of alcohol (6%) for 10 days at the concentration found in wine did not improve the functional recovery or the infarct size. Perhaps if the pre-treatment of alcohol was extended over a longer period of time or if the concentration of alcohol was increased to 12%, cardioprotection may have occurred. However, data strongly suggest a cardioprotective effect of red wine beyond alcohol.

4.4. Role of resveratrol in red wine-induced cardioprotection

In the literature, numerous studies have demonstrated that an acute administration of resveratrol at 10μM provided cardioprotection against an ischemia-reperfusion insult, as evidenced by improved postischemic ventricular recovery, reduced infarct size and decreased cardiomyocytes apoptosis (Hattori et al. 2002).

Although we have been able to reproduce this cardioprotective effect in our laboratory when resveratrol was given at this concentration and acutely, we have not been able to protect the heart when resveratrol was given chronically, at a concentration similar to the concentration found in 2 glasses of wine per day. No previous studies have explored the cardioprotective effect of a chronic administration of resveratrol against ischemia-reperfusion. The concentration given chronically was 3.8μM in the drinking water. Although the bioavailability of resveratrol is unknown, it is reasonable to suggest that the concentration of resveratrol after 2 glasses of wine is much lower than 2.3mg/L. The difference in concentrations between the acute and the chronic studies is therefore likely to explain why resveratrol can protect acutely but not chronically.

As a consequence, our data do not support the fact that resveratrol contributes to the cardioprotective effect of red wine.

4.5. Role of melatonin in red wine-induced cardioprotection

Interestingly, Guerrero et al. demonstrated that serum melatonin was significantly increased in humans, one hour after an intake of 100 mL of red wine and that the mean quantity of melatonin in red wine was 0.75µg/L (Guerrero *et al.,* 2008).

In previous studies, melatonin was administered acutely in an isolated rat heart preparation. The concentration of melatonin ranged from 1 to 50µM, which corresponded too much higher concentrations than the concentration of melatonin found in chronic moderate consumption of red wine (Tan et al. 1998). In our experiments, an acute administration of melatonin, given at concentration of 0.75µM was enough to improve the functional recovery and reduce the infarct size in isolated hearts subjected to an ischemia-reperfusion insult.

The long-term effects of melatonin were evaluated 24 hours after melatonin administration (2.5 or 5.0 mg/kg, ip) or after the oral supplementation of melatonin in the drinking water (20 or 40µg/mL) (Lochner et al. 2006). In our study, a chronic pre-treatment of melatonin protected the heart against ischemia-reperfusion. The final concentration of melatonin in the drinking water was equivalent to 0.94µg/L, which is far lower than the 40 µg/mL administered in previous studies.

Luzindole, a non-selective competitive melatonin receptor antagonist, did not protect the heart when given alone. Co-administration of luzindole and melatonin abolished the protective effect of melatonin against ischemia-reperfusion, therefore suggesting that the protective effect of melatonin is dependent on the activation of its receptors.

However, we were surprised to observe that wine-induced cardioprotection was not abolished in the presence of luzindole. Instead, the protective effect

of wine was further enhanced. Luzindole is a non selective inhibitor which binds to melatonin receptor 1 and 2 but not receptor 3. Inhibiting the binding of melatonin to its receptor may lead to the accumulation of endogenous melatonin in the cells. Despite this excess, some of the melatonin may compete to bind to receptor 3, thereby conferring protection. We speculate that the remaining melatonin in the cell may interact with the red wine administered; therefore, increasing the antioxidant activity which may result in enhanced cardioprotection.

Another explanation for the accrued protective effect of red wine with luzindole may be the fact that luzindole itself possesses antioxidant properties (Mathes, Wolf & Rensing 2008).

4.6. Role of ethanolamine in red wine-induced cardioprotection

The novel compound ethanolamine has never been studied in the setting of ischemia-reperfusion in the heart. Using the isolated rat heart model, our novel data demonstrate for the first time that the acute administration of ethanolamine, a component found in wine and food products can reduce infarct size and mediate cardioprotection against ischemia-reperfusion insult. The chronic pre-treatments of ethanolamine, given at a concentration equivalent to 2 glasses of red wine per day, protected the isolated heart against ischemia-reperfusion. The final concentration of ethanolamine in the drinking water was 2.6µg/mL.

Interestingly, the co-administration of melatonin and ethanolamine, given at concentration similar to the concentrations found in red wine, had an additive effect and protected the heart against ischemia reperfusion injury by improving functional recovery and decreasing infarct size to a better extent than wine on its own. These data led us to suggest the presence of other protective as well as harmful components present in red wine which intuitively counter regulates each other.

4.7. Red wine-induced cardioprotection via the JAK/ STAT-3 pathway

Red wine and it major components alcohol, resveratrol, melatonin and ethanolamine are generally thought to protect against ischemia-reperfusion via their powerful antioxidant properties. Here, we have delineated a novel prosurvival pathway that can be activated within the heart by chronic moderate consumption of red wine. Our data suggest that this pathway is activated in the red wine by melatonin and ethanolamine as the two components were able to activate STAT-3 and their cardioprotective effect was lost in the presence of the STAT-3 inhibitor, AG490. The melatonin receptor and the JAK/STAT pathway may be stimulated by TNF (Xuan et al. 2001, Lecour et al. 2005a) and possible downstream targets may lead to the inactivation of pro-apoptotic factors such as Bax and Bad and enhances the activation of Bcl-2 see review (Lecour 2009a, Lecour 2009b). However, the role of STAT-3 in melatonin- and ethanolamine-induced cardioprotection has been studied after an acute treatment of these biogenic amines in the isolated heart. Further experiments will be required to confirm that a chronic administration of ethanolamine and/or melatonin protects via the activation of STAT-3.

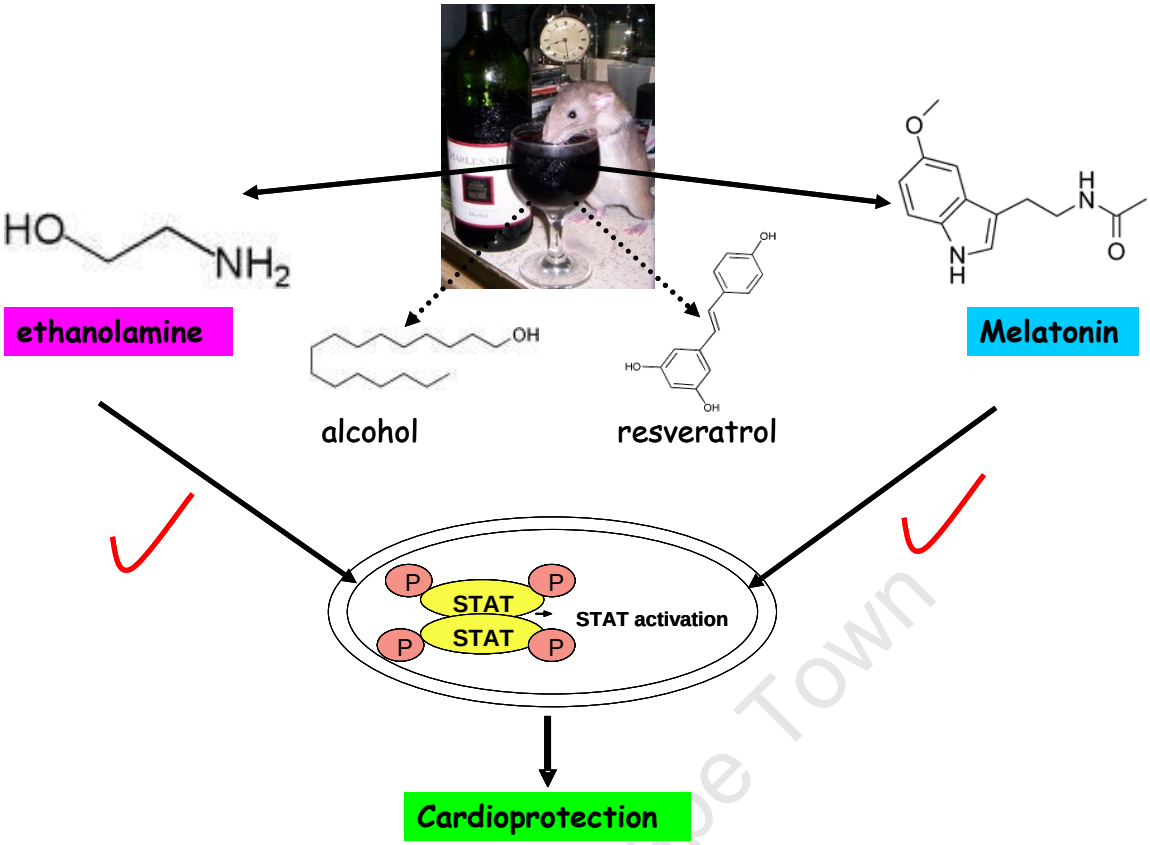


Figure 31: *The key components of red wine that may be individually or cordially implicated in red wine induced cardioprotection.*

4.8. Future implications and limitations of the study

Our study was performed on a French red Cabernet Sauvignon. It would be of interest to test whether wines from another grape variety or another country (such as South Africa) would confer a similar protective effect.

Also, an important limitation of our study is the fact that we were unable to quantify the exact concentrations of melatonin, resveratrol and ethanolamine in the wine that we have used. The concentrations that we had chosen corresponded to the mean concentrations in red wine reported in the literature. We are hoping to obtain the exact concentrations of melatonin and ethanolamine in a near future.

In addition, it would be of interest to repeat our experiments using an in vivo model of ischemia-reperfusion as the isolated heart system does not take into account the role of the blood and other organs.

Similarly, repeating our experiments in cardiomyocyte specific STAT-3 deficient mice would be useful to confirm the role of STAT-3 in red wine-induced cardioprotection. Preliminary experiments that we have performed in the laboratory have shown that STAT-3 knockout mice failed to be protected with resveratrol or ethanolamine.

Finally, measuring STAT-3 activation in humans following a chronic moderate consumption of red wine would be of interest to confirm our statements.

4.9. Conclusion

In conclusion, our data suggest for the first time that melatonin and ethanolamine account for the cardioprotective effect of chronic moderate consumption of red wine against an ischemia-reperfusion insult and this effect is mediated via the activation of STAT-3. Our data provide a novel opportunity for the development of new therapeutic drugs against ischemic heart disease. In addition, the use of red wine or melatonin as a therapeutic approach against heart disease is facilitated by the fact that they are natural compounds freely available in most countries.

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PUBLICATIONS AND ABSTRACTS

Kelly R. F., Lamont K.T., King J. C., Somers S., Hacking H., Opie L. H., Lecour S. Ethanolamine is a downstream product of sphingosine-1-phosphate that confers cardioprotection via activation of STAT-3. Currently in revision in Cardiovascular Research, 2009.

Lamont K.T., Opie L. H., Lecour S. Melatonin found in red wine: just a sleep away from cardioprotection. SA Heart, Sun City (oral presentation), 2009.

Lamont K.T., Opie L. H., Lecour S. Melatonin activates the transcription factor STAT-3: its role in red wine-induced cardioprotection. PSSA, Stellenbosch (oral presentation) 2009.

Lamont K.T., Kelly R. F., Opie L. H., Lecour S. Rooting out the active cardioprotective components in red wine. SA Heart, Wild Coast (oral presentation) 2008.

Lamont K.T., Kelly R. F., Opie L. H., Lecour S. Revisiting the cardioprotective components in red wine. MRC Research day, (oral presentation) 2008.

APPENDICES: SOLUTIONS AND BUFFERS

1. HEMODYNAMIC PARAMETERS OF ISOLATED PERFUSED RAT HEARTS

1.1. KREBS HENSELEIT BUFFER FOR LANGENDORFF PERFUSION (5L)

NaCl	**34.63g**
$NaHCO_3$	**10.5g**
Glucose	**10.99g**
KCl	**1.77g**
$MgSO_4.7H_2O$	**1.47g**
$KH_2\ PO_4$	**0.8g**
$CaCl_2.2H_2O$	**1g**

2. SOLUTIONS AND BUFFERS FOR PROTEIN EXTRACTION AND QUANTIFICATION

2.1. PROTEIN EXTRACTION

2.1.1. LYSIS BUFFER FOR PKB/IRSI (30ml)

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

2.2. PROTEIN QUANTIFICATION

2.2.1. CTC REAGENT FOR PROTEIN ASSAY

(a) Na_2CO_3	**20g**
(b) $CuSO_4.5H_2O$	**0.2g**
K_2 Tatrat	

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

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

2.2.2. 10% SDS

SDS **20g**

Add dH_2O up to 200ml

2.2.3. 0.2M NaOH

NaOH **0.4g**

Add dH_2O up to 200ml

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

3.1. 10% SDS

SDS **20g**

Add dH_2O up to 200ml

3.2. 10% AMMONIUM PERSULPHATE (APS)

APS **20g**

Add dH_2O up to 200ml

3.3. 3X Laemeli (loading buffer)

Tris **3.03g**

SDS **8.8g**

Glycerol **20g**

Bromophenol Blue **0.025g**

Na in ddH_2O **75ml**

Adjust pH to 6.6 with HCl

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

3.4. 10X Running (Tank) buffer

Tris **60.6g**

Glycine **288g**

SDS **20g**

Add dH_2O up to 2L

3.5. 10% Resolving gel

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

3.6. 10% Stacking gel

dH_2O	**7.5ml**
0.5M Tris-HCl, pH 8.8	**3ml**
20% SDS	**60µl**
Acrylamide	**1.5ml**
10% APS	**30µl**
TEMED	**40µl**

4. SOLUTIONS AND BUFFERS FOR WESTERN BLOTTING AND IMMUNODETECTION

4.1. Transfer buffer

Tris	**6.06g**
Glycine	**28.8g**
20% methanol	**400ml**
Add dH_2O up to 1L	

4.2. 10X Tris-buffered saline (TBS)

Tris	**48.4g**
Dissolve in 500ml dH_2O and adjust pH to 7.6 by adding HCl	
NaCl	**160g**
Add dH_2O up to 2L	

4.3. TBS-Tween 20 (0.01%)

Tween 20	**1ml**
TBS	**1000ml**

4.4. Blocking solution (5% milk powder)

Powdered milk	**5g**
TBS solution	**100ml**