Investigating the Possible Cytoprotective Effects of A Melatonin Isomer Against Simulated Ischemic Injury

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ABBREVIATIONS

2-DG 2-Deoxy-d-Glucose

A2b Adenosine receptor

ACE Angiotensin converting enzyme

ADP Adenosine diphosphate

AIDS Acquired immune deficiency syndrome

Akt Protein kinase B

ANOVA Analysis of variance

AntA Antimycin-A

ANT Adenine nucleotide-translocase

Asc+TMPD Ascorbate and N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride

ATP Adenosine triphosphate

BP Blood pressure

BSA Bovine serum albumin

Ca^{2+} Calcium

$CaCl_2.2H_2O$ Calcium chloride dihydrate

CCCP Carbonyl cyanide p-(triflouromethoxy) phenylhydrazone

cGMP Cyclic guanosine monophosphate

CVD Cardiovascular disease

CytC Cytochrome c

D Digitonin

DASH Dietary approaches to stop hypertension

DMEM Dulbecco's Modified Eagle Medium

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

ECM Extracellular matrix

EDTA Ethylenediaminetetraacetic acid

EGTA Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

eNOS Nitric oxide synthase

Erk1/2 Extracellular regulated kinase 1 and 2

ETC Electron transport chain

ETS Electron transfer system

ETS_{CI+II} Electron system transport capacity through complex I and II

$FADH_2$ Flavin adenine dinucleotide

FCS Fetal Calf Serum

GC Guanylyl cyclase

GSK Glycogen synthase kinase

H_2O Water

H_2O_2 Hydrogen peroxide

HDL High density lipoprotein

HEPES 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfanic acid

HIOMT Hydroxyindole-O-methyltransferase

HIV Human immunodeficiency virus

HPLC-MS/MS High Performance Liquid Chromatography Tandem Mass Spectrometry

IHD Ischemic heart disease

JAK Janus Kinase

kATP ATP-dependent potassium channel

KCl Potassium chloride

KH_2PO_4 Potassium dihydrogen phosphate

Luz Luzindole

MARIA Melatonin Adjunct in the acute myocaRdial Infarction treated with Angioplasty

Mel Melatonin

MEK Mitogen activated protein kinase

$MgCl_2$ Magnesium chloride

$MgCl_2 \cdot 6H_2O$ Magnesium chloride hexahydrate

MI Melatonin isomer

MiR05 Mitochondrial respiration medium

MPTP Mitochondrial permeability transition pore

MT1 Melatonin receptor type 1

MT2 Melatonin receptor type 2

MT3 Melatonin 'receptor' 3

mtDNA Mitochondrial deoxyribonucleic acid

N_2 Nitrogen

Na^+ Sodium

NaCl Sodium chloride

NADH Nicotinamide adenine dinucleotide

NAT *N*-acetyl-transferase

NCD Non-communicable disease

NO Nitric oxide

O_2 Oxygen

O_2^- Superoxide

$^{\cdot}OH$ Hydroxyl radical

OXPHOS Oxidative phosphorylation

$OXPHOS_{CI+II}$ Complex I and II dependent OXPHOS capacity

PBS Phosphate buffered saline

PI3K Phosphatidylinositol 3-kinase

PKC Protein kinase C

PKG Protein kinase G

PM Pyruvate and malate

PREDIMED Prevencion con Dieta Mediterranea

PVC Premature ventricular contractions

RISK Reperfusion Injury Salvage Kinase

RNA Ribonucleic acid

ROS Reactive oxygen species

ROX Residual oxygen consumption

SAFE Survivor Activating Factor Enhancement

SCN Suprachiasmatic nucleus

SEM Standard error of the mean

SI Simulated ischemia

STAT-3 Transcription factor signal transducer and activator of transcription-3

Succ Succinate

TCA Tricarboxylic acid

TLR4 Toll-like receptor 4

TMPD N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride

TNFα Tumor necrosis factor alpha

TNFR2 Tumor necrosis factor receptor 2

VDAC Voltage-dependent anion channel

VF Ventricular fibrillations

WHO World Health Organisation

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ABSTRACT

Introduction: The presence of melatonin in wine contributes to the cardioprotective effect of regular and moderate consumption of wine against lethal ischemia/reperfusion injury. Recently, the presence of melatonin isomers has been identified in red wine, but whether or not these isomers confer any physiological properties is unknown.

Aim: The aim of our study was to establish a cell culture model of simulated ischemia to study and compare the possible cytoprotective effects of dietary melatonin and a melatonin isomer against an ischemic insult and to explore the possible role of melatonin receptors in this effect.

Methods: H9C2 cardiac fibroblast cells were subjected to simulated ischemia by exposure to 1mM H_2O_2 following a 30min pre-treatment with 75ng/L (dietary concentration), 1µM (pharmacological concentration, 0.232mg/L) melatonin or/and 75mg/L (dietary concentration) melatonin isomer. To determine the role of melatonin receptors, cells were pre-treated with the melatonin receptor inhibitor, luzindole (10 µM) for 1h prior to H_2O_2 treatment. At the end of the simulated ischemic insult, cell viability was assessed using trypan blue staining. Mitochondrial respiration in permeabilized H9C2 cells was measured using the Oroboros Instrument, at two different time points: at the end of a 30min pre-treatment with either 75ng/L melatonin or 75mg/L melatonin isomer, or the afore mentioned pre-treatments prior to a 15min treatment of 1mM H_2O_2.

Results: A simulated ischemic insult with 1mM H_2O_2 reduced cell viability from 92.9±1.5% to 28.4±1.4% ($p<0.001$ vs control). Pre-treatment with the dietary concentrations of melatonin or the melatonin isomer improved the cell viability to a similar extent as a pre-treatment with the pharmacological concentration of melatonin (74.4±3.1%, 73.9±2.7% and 69.0±1.2%, $p<0.001$ vs H_2O_2 and $p<0.01$ vs H_2O_2 respectively). A combined pre-treatment of melatonin and the melatonin isomer did not add further cytoprotective benefit. Addition of luzindole fully abolished the cytoprotective effect of dietary melatonin (29.7±2.4%, $p<0.001$ vs H_2O_2 + Mel), but only partially abolished the cytoprotective effect of the melatonin isomer (41.4±3.6%). Both dietary concentrations of melatonin and the melatonin isomer did not affect mitochondrial respiration in permeabilized H9C2 cells.

Conclusion: Our findings suggest that both dietary melatonin and the melatonin isomer confer cytoprotection against a simulated ischemic insult, an effect which is mediated, at least in part, via the activation of melatonin receptors. Both melatonin and melatonin isomers present the advantage to be potentially safe and inexpensive therapies against ischemic heart disease.

A) INTRODUCTION

1. Cardiovascular Disease

Cardiovascular disease (CVD) is a non-communicable disease (NCD) and is defined as all diseases of the heart and its circulatory system (World Heart Federation 2017). It is a huge burden worldwide with 80% of CVD deaths occurring in middle to low income countries (World Health Organisation 2011). In 2012, the leading cause of NCD deaths was CVD, responsible for 17.5 million deaths and accounted for 46.2% of the NCD deaths worldwide (WHO 2014a). The World Health Organisation (WHO) has estimated that by 2030, there will be more than 23 million deaths worldwide from CVDs annually and that it will remain the leading cause of death.

In Africa, there is evidence of a changing disease profile with the co-existence of communicable diseases, nutritional deficiencies and non-communicable diseases, whereas previously, it was predominantly infectious diseases and nutritional deficiencies only. This epidemiological transition results, at least in part, from societies undergoing industrialisation, economic development and globalisation (Mensah 2008). In South Africa, CVD accounts for the largest percentage of deaths amongst the NCD's (Figure 1) and there have been observed increases in the proportion of deaths, in both males and females, due to ischemic heart diseases between 2013 and 2015 (WHO 2014b)(Lehohla 2017).

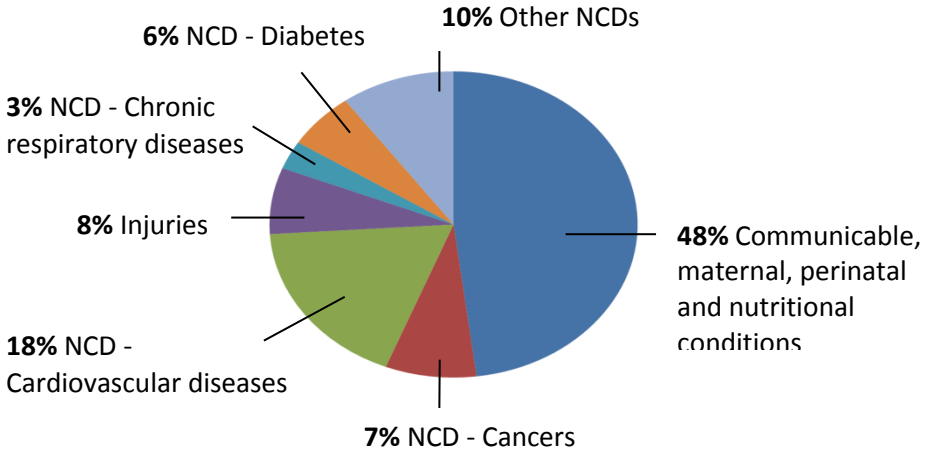


Figure 1: Proportional mortality in non-communicable and communicable diseases. Adapted from (WHO 2014b).

Following a similar trend, CVD in the Western Cape region accounts for 25% of deaths, making it the leading cause of death for both men and women in this region (Chopra et al. 2007). It is predicted that within the next 30 years, there will be an almost doubling of CVD deaths in South Africans between the ages of 35-64 (Chopra et al. 2007).

2. Ischemic heart disease

Ischemic heart disease (IHD), or myocardial infarction, is a CVD which most commonly results from the build-up of fatty, atherosclerotic plaque deposits on the inner wall of blood vessels supplying the muscle of the heart with blood. This results in the narrowing of the arteries, thus reducing blood flow to the heart muscle and the supply of oxygen and nutrients from reaching the heart (Fuster et al. 1992)(Pepine & Nichols 2007). In the event of a myocardial infarction, the tissue subjected to reduced flow is referred to as 'ischemic' tissue. There are several physiological changes associated with myocardial ischemia such as; the generation of reactive oxygen species (ROS), intracellular sodium (Na^+) accumulation and acidification amongst others (Kumar et al. 1990) (Regan et al. 1980)(Tani 1990) (Effros et al. 1975). When the ischemic insult is of sufficient duration, it will lead to irreversible injury of the cardiomyocytes as well as cell death (Bell & Yellon 2011). This necrotic area of myocardial tissue is known as the infarcted area.

IHD is the single largest cause of death worldwide and will be the leading cause of death in Africa by 2030 if no preventative action is taken (Figure 2) (Mensah 2008). In South Africa, IHD has remained the highest cause of death amongst the non-communicable diseases, and second overall only to human immunodeficiency virus and acquired immune deficiency syndrome (HIV/AIDS), from 2005 to 2015 (Figure 3) (IHME 2017).

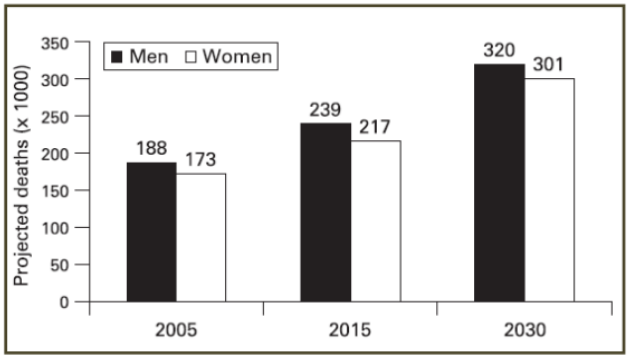


Figure 2: Projection of deaths, in Africa, from ischemic heart disease in men and women from 2005 to 2030 (Mensah 2008).

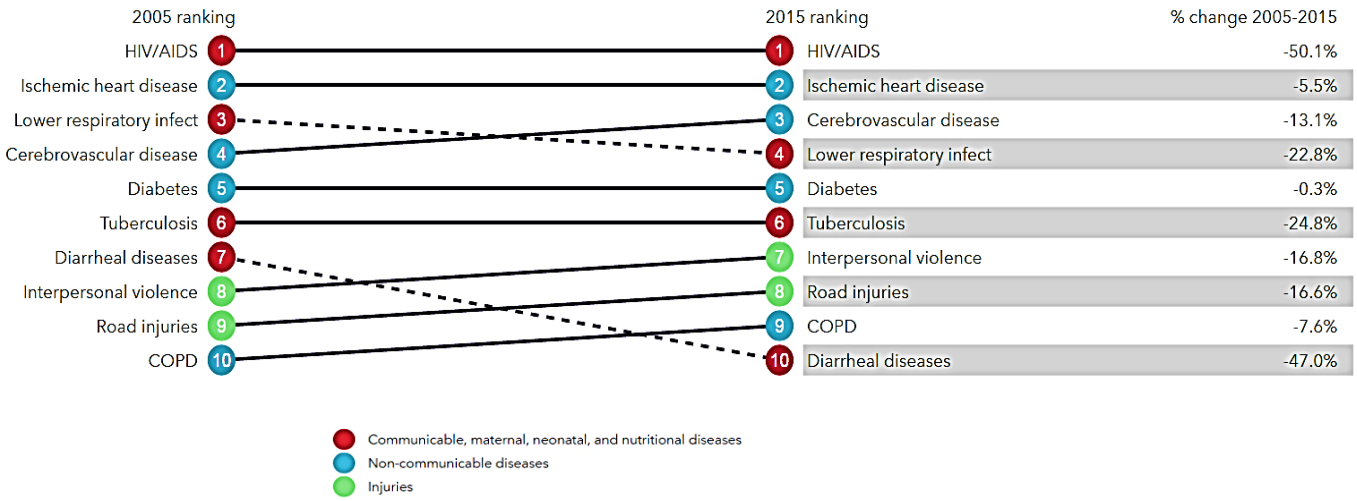


Figure 3: Top ten causes of premature death in 2005 and 2015 and the percentage change from 2005-2015 in South Africa (IHME 2017). COPD, Chronic obstructive pulmonary disease.

3. Therapies for IHD

3.1 Pharmacological therapies

As a result of the myocardial oxygen demand exceeding the oxygen supply, there is the occurrence of what is known as angina pectoris or simply angina, which is a condition marked by severe chest pain. Many IHD patients experience angina as a clinical manifestation of the CVD (Kannam et al. 2016). At present, there are four classes of anti-ischemic drugs which are used in the management of angina. The four classes are:

1. Calcium channel blockers (cause coronary and peripheral vasodilation and reduce contractility)
2. Beta blockers (reduce heart rate and contractility and prevent re-infarction)
3. Nitrates (used with calcium channel and beta blockers to produce greater anti-anginal and anti-ischemic effects)
4. Anti-platelet/anti-coagulants (to reduce blood clot formation) (Kannam et al. 2016).

Exacerbating factors such as hypertension, tachyarrhythmia's etc. need to be considered and treated, as these could aggravate myocardial ischemia (Kannam et al. 2016). Preventative therapies are also very important in the long-term care of patients with IHD.

These can include risk factor reductions and angiotensin converting enzyme (ACE) inhibitors, which have been shown to have particular benefit for patients with hypertension, diabetes mellitus or chronic kidney disease (Kannam et al. 2016).

When IHD occurs, the best way to salvage the heart is via reperfusion, which is the restoration of blood flow to the ischemic area (Barron et al. 1998). Function to severity and time of intervention after the first sign of myocardial infarction, one can perform reperfusion via: thrombolysis, balloon angioplasty and stenting (Steg et al. 2012)(Reiter & Tan 2003).

However, even after reperfusion, a large number of cells will still undergo apoptosis/necrosis and additional therapies are needed to improve cell survival and coronary flow in patients following IHD.

3.2 Targeting lifestyle factors

Three major studies organised by Harvard Medical School found that there are five key factors which, when combined in practice, reduce the risk of a heart attack or stroke by almost 80% (Chiuve et al. 2006)(Chiuve et al. 2008)(van Dam et al. 2008). These healthy lifestyle factors include; non-smoking, ideal body weight, exercise, diet and moderate alcohol consumption (Table 1)(Opie 2011).

Table 1. The lifestyle 'big five' that can reduce the risk of a heart attack or stroke. Adapted from (Opie 2011).

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

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

3.2.1 The Mediterranean diet and the heart

An ideal diet is one which best reduces weight as well as one which gives overall optimal health and longevity. The Western diet of today is one which includes a high intake of fat, sugars and calories and leads to arterial endothelial damage and the promotion of obesity, diabetes, heart attacks and strokes (Opie 2011). However, there are four validated diets that can counter the effects of the Western diet:

1. Prudent diet (emphasises a high intake of fruit, vegetables, poultry, fish, whole grains and legumes) (Zulyniak et al. 2016)
2. DASH BP-reducing diet (similar to the prudent diet, but adds a salt restriction – this is important for people with hypertension) (Saneei et al. 2014)

3. Healthy Eating diet (similar to above diets, but incorporates a numerical index for the seven components which are assigned scores from 0-10 where 10 stands for 'optimal dietary behaviour') (Schwingshackl & Hoffmann 2015)
4. Mediterranean diet (similar to above diets, but includes olive oil and moderate alcohol)

A number of observational studies have documented a lower risk of CVD/IHD in populations following Mediterranean dietary patterns (Keys 1980)(Tong et al. 2016)(Casas et al. 2016)(Estruch et al. 2013)(Mena et al. 2009)(Casas et al. 2014). The Prevencion con Dieta Mediterranea (PREDIMED) study showed that participants had lower cellular and plasma concentrations of inflammatory markers related to atherosclerosis, as well as a reduction in blood pressure, fasting glucose and insulin, and total cholesterol: high density lipoprotein (HDL)-cholesterol ratio (Casas et al. 2016)(Hernáez et al. 2017).

The Mediterranean diet is therefore suggested to have a dual effect against CVD which includes; the improvement of classic CVD risk factors, and a long-term maintenance of its anti-inflammatory effect (Casas et al. 2016).

3.3 Targeting cardioprotective signalling pathways

The discovery of pre- and post-conditioning, which is the brief exposure of tissue to cycles of ischemia/reperfusion, prior to or post the ischemic insult, has led to the discovery of two independent, intrinsic, pro-survival signalling cascades known as; the Reperfusion Injury Salvage Kinase (RISK) pathway and Survivor Activating Factor Enhancement (SAFE) pathway (Figure 4) (Ferdinandy et al. 2007)(Lacerda et al. 2009). These pathways can be additionally activated using pharmacological agents in a process known as pharmacological conditioning (Lecour et al. 2005)(Lamont et al. 2015)(Hausenloy et al. 2014).

The RISK pathway involves the activation of the pro-survival kinases, protein kinase B (Akt) and extracellular regulated kinase 1 and 2 (Erk1/2), at the onset of reperfusion (Tsang et al. 2004) (Hausenloy et al. 2005).

It was demonstrated that ischemic pre-conditioning acts as a primer for kinase phosphorylation and thus protects the heart by increasing Akt and Erk1/2 phosphorylation at reperfusion, but it was also suggested that directly targeting the reperfusion phase would have the same Akt and Erk1/2 induced protective effect (Hausenloy et al. 2005). This indicates that the RISK pathway can be activated via both pre- and post-conditioning. This pathway then ultimately leads to the inhibition of the opening of the mitochondrial permeability transition pore (MPTP), which is a vital determinant of cell death in ischemia/reperfusion injury, and prevents the release of cytochrome C from the mitochondria, ultimately preventing apoptosis (Hausenloy et al. 2004).

The SAFE pathway alternatively activates tumor necrosis factor alpha (TNFα) and the transcription factor signal transducer and activator of transcription-3 (STAT-3) (Lecour et al. 2005)(Lecour 2009). Myocardial TNFα, a pro-inflammatory cytokine, is expressed in direct response to several different forms of cardiac injury, such as myocardial infarction and heart failure. Paradoxically, TNFα can be either detrimental or protective to the heart, depending on whether the cardiotoxic TNFα receptor 1 or the cardioprotective TNFα receptor 2 is activated, as well as being dependent on the concentration of TNFα activated (Lecour 2009). Studies have shown protective effects at lower concentrations, with higher concentrations having detrimental effects (Lecour 2009).

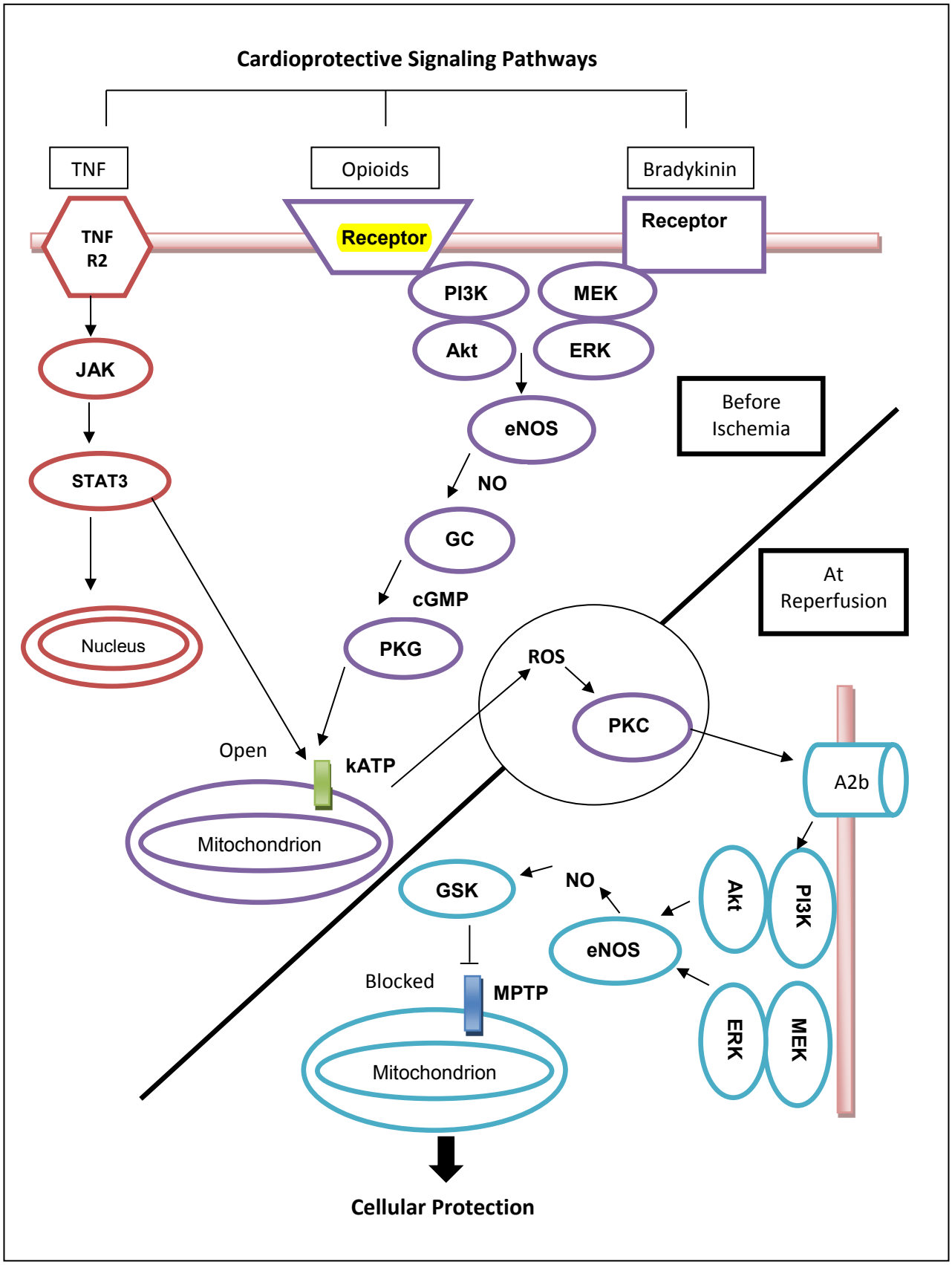


Figure 4: Proposed signalling scheme of the SAFE and RISK pathways. Tumor necrosis factor (TNF), Tumor necrosis factor receptor 2 (TNFR2), Janus Kinase (JAK), Signal transducer and activator of transcription-3 (STAT3), Phosphatidylinositol 3-kinase (PI3K), Protein kinase B (Akt), Mitogen activated protein kinase (MEK), Extracellular signal-regulated kinase (ERK), Nitric oxide synthase (eNOS), Nitric oxide (NO), Guanylyl cyclase (GC), cyclic guanosine monophosphate (cGMP), Protein kinase G (PKG), ATP-dependent potassium channel (kATP), Reactive oxygen species (ROS), Protein kinase C (PKC), Adenosine receptor (A2b), Glycogen synthase kinase (GSK), Mitochondrial permeability transition pore (MPTP). Modified from (Cohen & Downey 2011).

A recent focus of preventative strategies for IHD is the use of pharmacological agents to induce protection via the activation of the SAFE and RISK pathways. One of the actively investigated pharmacological agents, for protection against ischemia/reperfusion injury, is melatonin.

4. Melatonin as a therapy against IHD

4.1 Melatonin structure and biosynthesis

Melatonin (N-acetyl-5-methoxytryptamine) is a neurohormone produced mainly by the pineal gland. Its structure (Figure 5) includes an indole ring with two distinguishable side chains; the N-acetylaminoethyl group at position 3 (known as Chain A) and the methoxy group at position 5 (known as Chain M)(Tan et al. 2012).

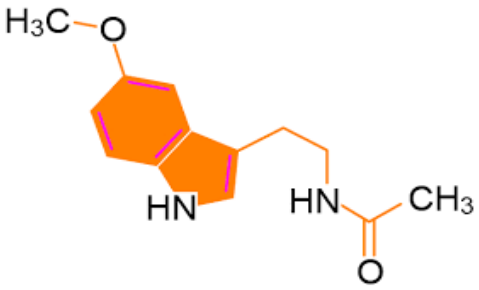


Figure 5: Melatonin structure

Although melatonin is referred to as a neurohormone, it exhibits features which distinguish it from a classic hormone including; its production from non-endocrine organs, gastrointestinal tract, retina, its non-receptor mediated actions, as well as it being present in foodstuffs which are ingested, thus affording it the added title of a vitamin (Slominski et al. 2008) (Messner et al. 2001)(Konturek et al. 2007) (Tosini & Menaker 1998)(Tan et al. 2003).

The biosynthesis of melatonin involves the uptake of tryptophan from the blood, by pinealocytes, and its conversion to serotonin through hydroxylation and decarboxylation. Serotonin is converted into *N*-acetyl-serotonin by the enzyme *N*-acetyl-transferase (NAT).

The enzyme hydroxyindole-O-methyltransferase (HIOMT) methylates the N-acetyl-serotonin to produce melatonin (Figure 6) (Fernández-Mar et al. 2012)(Reiter 1991). Melatonin has a bi-exponential half-life where the first distribution half-life is 2 minutes and the second metabolic half-life is 20 minutes (Pandi-Perumal et al. 2006)(Claustrat et al. 2005).

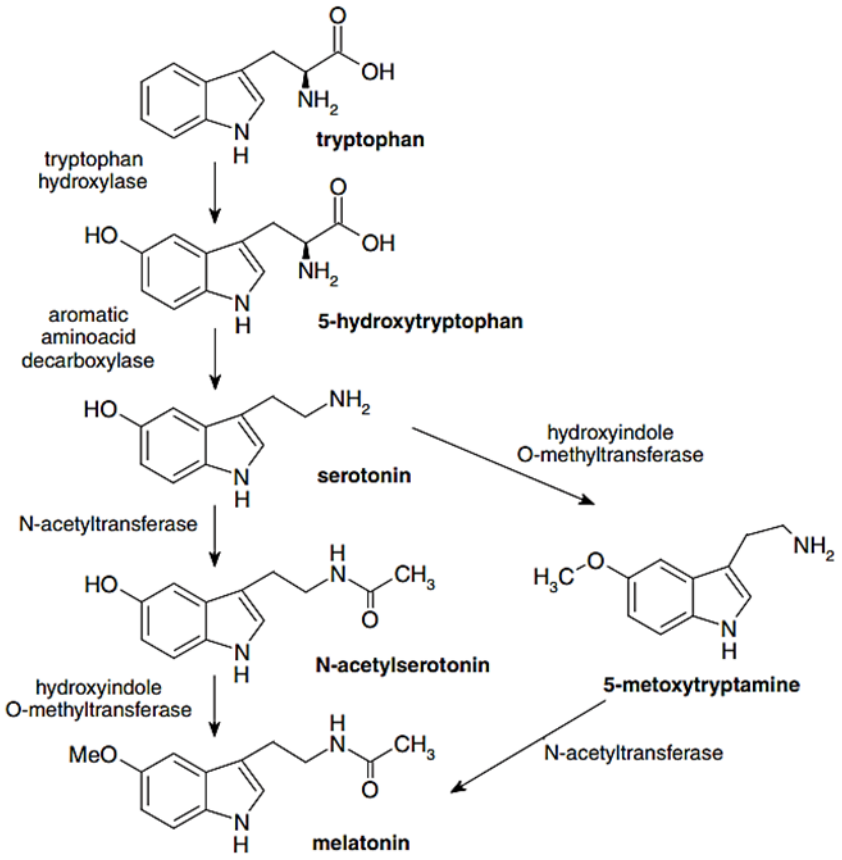


Figure 6:Biosynthetic pathway of melatonin (Fernández-Mar et al. 2012).

The retinohypothalamic tract, which projects from the retina to the suprachiasmatic nucleus (SCN), mediates the regulation of pineal melatonin biosynthesis (Pandi-Perumal et al. 2006). The SCN is a major circadian oscillator and therefore, during the light phase of the daily photoperiod, the output from the retinohypothalamic tract results in the inhibition of melatonin production (Moore 1997). However, during the dark phase of the daily photoperiod, there is the stimulation of the synthesis of melatonin by the pineal gland (Claustrat et al. 2005). Once melatonin has been synthesized, it is released into the blood stream with concentrations varying between ±10-110pg/ml depending on the

time of day (Figure 7) and it will eventually be metabolized primarily in the liver and secondarily in the kidney (Iguchi et al. 1982)(Videnovic et al. 2014). The resulting products, 6-hydroxymelatonin and *N*-acetyl-5-hydroxytryptamine, are excreted in urine as their conjugates; sulphate and glucuronide respectively (Young et al. 1984).

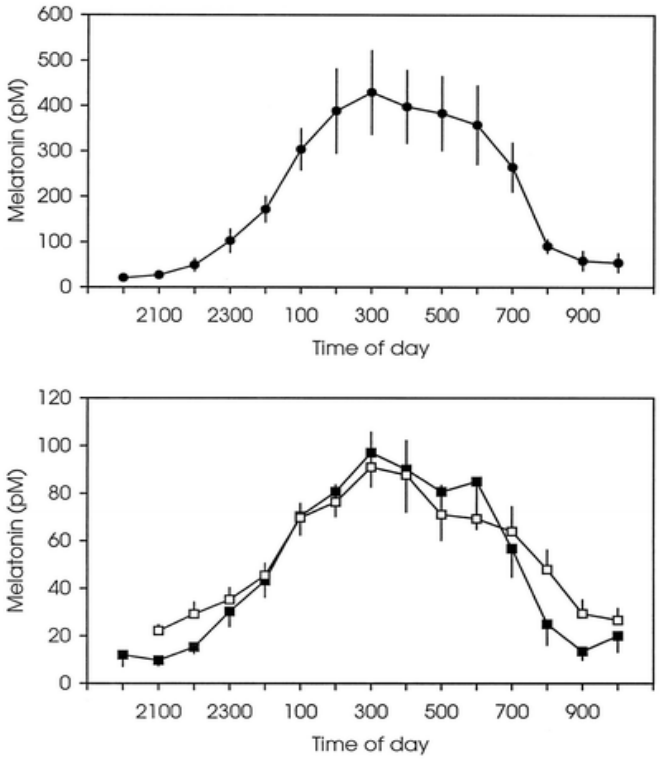


Figure 7: The corresponding plasma and saliva melatonin levels of 10 healthy individuals, between the hours of 21h00 and 09h00 (Kennaway & Voultsios 1998).

4.2 Melatonin receptors

Melatonin modulates its effects either via G-protein coupled receptors; melatonin receptor type 1 (MT1) and melatonin receptor type 2 (MT2), or independently of the receptors by binding intracellular proteins (Dominguez-Rodriguez et al. 2012). They are unique receptors in that they have distinct molecular structures, chromosomal localisation, and pharmcological characeristics (Reppert et al. 1996)(Reppert et al. 1995)(Slaugenhaupt 1995) (Dubocovich et al. 1997).

Fully characterized and functional MT1 and/or MT2 receptors have been identified in the following organs/tissues: brain and retina, cardiovascular system, kidney and liver, intestine, adipocytes, immune cells, prostate, testes, avaries, breast epithelial cells and skin to name a few (Ekmekcioglu 2006)(Dubocovich & Markowska 2005).

MT1 and MT2 are 350 and 362 amino acids long, respectively and can be present as monomeric receptors or as homo- and heterodimers, which are functionally distinct (Liu et al. 2016). This was demonstrated in mouse rod photoreceptors, where the MT1/MT2 heterodimers modulated light sensitivity via a pathway not normally triggered by activation of the monomeric receptors (Baba et al. 2013).

A third melatonin 'receptor', MT3, was discovered and characterised as a protein that is part of the quinone reductase family (Nosjean et al. 2000)(Mailliet et al. 2005). It was identified that MT3 and quinone reductase II both bind to melatonin with the same pharmacological profile and that MT3 is actually quinone reductase II which is present as a cystolic enzyme (Nosjean et al. 2001)(Nosjean et al. 2000)(Dubocovich & Markowska 2005). This enzyme is said to protect against oxidative stress by preventing electron transfer reactions of quinones (Pandi-Perumal et al. 2008). It was also shown to possibly be involved in the regulation of inflammatory responses in the microvasculature (Lotufo et al. 2001). MT3 is expressed in the kidney and liver, brain, heart, lung, intestine, muscle and brown adipose tissue (Nosjean et al. 2001)(Pandi-Perumal et al. 2008).

The ability of melatonin to bind, acivate and modulate its receptors, is dependent on the interaction of melatonin with the specific amino acids and/or domains of the G-protein coupled receptors (Witt-Enderby et al. 2003). These interactions can include amino acids/domains that are unique to each receptor, or they can be similar between the receptors. It is simply dependent on the types of responses that are being elicited (Witt-Enderby et al. 2003).

4.3 Main physiological functions of melatonin

Melatonin is amphipathic in nature which allows it to diffuse to cells and cellular compartments, therefore, making it effective as a powerful anti-oxidant and free radical scavenger (Dominguez-Rodriguez et al. 2012). Melatonin presents a wide spectrum of other physiological functions such as a role in circadian rhythm, immune defence, anti-hypertension, anti-inflammation and cardioprotection (Dominguez-Rodriguez et al. 2012).

4.3.1 Melatonin and the Circadian rhythm

Melatonin is well known for its role in sleep and the circadian rhythm, which is defined as physical, mental and behavioral changes which follow a cycle that is roughly 24 hours and which responds primarily to light and darkness (Cajochen et al. 2003)(National Institute of General Medical Sciences (NIH) 2012). Lighting conditions control the synthesis of melatonin via the transmission of photosensory information to the pineal gland by way of the retinohypothalamic tract (Moore 2007). The circadian rhythm of endogenous melatonin is closely associated with the circadian component of the sleep propensity rhythm and therefore, the idea is that melatonin facilitates sleep in humans (Cajochen et al. 2003)(Fisher et al. 2013). Exogenous melatonin is said to entrain as well as phase shift the circadian rhythm and as such, has been termed a regulator of circadian rhythms (Lockley et al. 2000)(Sack et al. 2000)(Lewy et al. 1992)(Elbaz et al. 2013). For example, this has important implications for shift workers who's sleep cycles are disrupted, but show improvements upon consistent melatonin therapy (Sadeghnilat-Haghighi et al. 2016).

4.3.2 Melatonin and hypertension

Hypertension is a major risk factor of CVD and more specifically, of hypertensive heart disease which is characterised by left ventricular hypertrophy and fibrosis (Tengattini et al. 2008)(Masuyama et al. 2006). Hypertensive heart disease patients are shown to present with disturbed day-night rhythms in vasodilation and suppressed levels of night-time melatonin (Scheer et al. 2004)(Shaw et al. 2001)(Brugger et al. 1995).

A number of animal models have shown the benefits of melatonin treatment in hypertension. Results from pinealectomized rat studies showed that melatonin prevented a rise in blood pressure and a similar trend was seen in spontaneously hypertensive rats, where melatonin treatment resulted in the decrease of blood pressure (Holmes & Sugden 1976)(Nava et al. 2002). A study performed by Scheer and colleagues on hypertensive men, showed that repeated bedtime intake of melatonin, for a duration of three weeks, significantly reduced blood pressure during sleep (Scheer et al. 2004). Melatonin has also been shown confer cardioprotection against pulmonary hypertension, which is characterised by elevated pulmonary arterial pressure leading to right ventricular hypertrophy and failure (Maarman et al. 2015). In their study, Maarman et al showed that chronic melatonin treatment reduced right ventricular hypertrophy, improved right ventricular function and reduced plasma oxidative stress in a rat model of pulmonary hypertension (Maarman et al. 2015).

4.4 Melatonin and cardioprotection

Experimental data suggest that melatonin is implicated in cardioprotection with special reference to ischemia/reperfusion injury as studied in isolated rodent hearts (Reiter & Tan 2003)(Tan et al. 1998). This study performed by Tan and colleagues involved the induction of ischemia via the ligation of the descending coronary artery followed by reperfusion, during which, most of the hearts displayed premature ventricular contractions (PVC) and ventricular fibrillations (VF). However, the infusion of melatonin during the occlusion and after the reopening of the coronary artery, significantly reduced PVC and VF (Tan et al. 1998). Another study used melatonin as a pre-treatment, followed by either regional ischemia or global ischemia and reperfusion. Their results showed that melatonin was protective against both ischemia/reperfusion models (Lagneux et al. 1999). These results, obtained from *ex vivo* models, were confirmed by Lee and colleagues who were the first group to perform an *in vivo* study exploring the effects of melatonin against ischemia/reperfusion injury in rats (Lee et al. 2002). These promising melatonin studies led to a prospective ongoing trial; Melatonin Adjunct in the acute myocaRdial Infarction treated with Angioplasty (MARIA), which is aimed at testing the hypothesis that melatonin administration confers cardioprotection against ischemia/reperfusion injury (Dominguez-Rodriguez et al. 2007).

The results of the trial, however, showed that intravenous and intracoronary administration of melatonin was not associated with a reduced infarct size (Dominguez-rodriguez et al. 2017). Although the trial was well conducted, the extraphysiological amount of melatonin given to the patients may be questioned.

4.4.1 Melatonin found in wine confers cardioprotection

In France, wine consumption is an important part of the heritage where it is habitually drunk, in preference to other alcohols, during meals (Heath 1995). Epidemiological studies showed that in France there is an inverse association between moderate red wine consumption and CVD when compared to other European countries, Australasia and North America, even though the French consume foods high in saturated fat (St Leger et al. 1979). A possible reason for this observed phenomenon is the regular consumption of red wine with meals which was consequently termed as the French Paradox (Renaud & de Lorgeril 1992).

Sato and colleagues then suggested that red wine has a cardioprotective effect against ischemia/reperfusion injury and performed experiments where rats were acutely treated with red wine extract followed by an ischemic insult and reperfusion. They observed a significant reduction in infarct size compared to the rats which had only received water (Sato et al. 2000). Subsequent experiments were performed where non-alcoholic red wine extract was tested for protective effects and the results were positive, indicating that red wine potentially contains non-alcoholic components that are cardioprotective (Fantinelli & Mosca 2007). Among numerous other components, melatonin's presence was detected in wine (Kocadağli et al. 2014)(Vitalini et al. 2013). The cardioprotective effects of melatonin against ischemia/reperfusion injury have been reported in *ex vivo* and *in vivo* experiments as mentioned above. However, the concentration of melatonin used ranged from 1µM to 50µM, which is up to 1000 times higher than the physiological concentration found in human blood levels and the concentration found in wine (Lagneux et al. 1999)(Tan et al. 1998)(Kennaway & Voultsios 1998)(Vitalini et al. 2013).

Novel *ex vivo* experiments on rats and mice, performed by Lamont et al, showed that acute perfusion of melatonin, at a dietary concentration (as found in wine), is sufficient to confer cardioprotection against a model of myocardial infarction (Lamont et al. 2011). Lamont et al also demonstrated that the presence of melatonin contributes to the cardioprotective effect of red wine, consumed regularly and moderately, against lethal ischemia/reperfusion injury and that this protection is most likely mediated via MT3 and the activation of the SAFE pathway (Lamont et al. 2015).

4.4.2 Mechanisms involved in melatonin induced cardioprotection

The cardioprotective effect of melatonin can be mediated via receptor independent actions by efficiently interacting with reactive oxygen (hydroxyl radical ($^{\cdot}OH$) and superoxide (O_2^{-})) and nitrogen (Nitric oxide (NO)) species, or via receptor dependent actions by upregulating anti-oxidant enzymes and downregulating pro-oxidant enzymes (Kaneko et al. 2000)(Tengattini et al. 2008). Reactive oxygen species have a significant role in the pathogenesis of ischemia/reperfusion injury and one of the main sources of ROS in cardiomyocytes is a faltering electron transport chain in mitochondria (Kloner et al. 1989)(Petrosillo et al. 2006b). The Hatter Institute for Cardiovascular Research in Africa conducted research in animal models which has led to the delineation of mechanisms involved in the cardioprotective effect of melatonin.

They suggest that the reduction in infarct size, with dietary melatonin, is mediated via the activation of the SAFE pathway (Lamont et al. 2011).

The receptor-dependent cardioprotective effect has been demonstrated by Lochner and colleagues with the administration of luzindole, a high-affinity melatonin receptor antagonist. Luzindole reversed the protective effects afforded by a high concentration of melatonin by causing an increase in infarct size (Lochner et al. 2006). Recently, melatonin was shown to activate the SAFE pathway via the toll-like receptor 4 (TLR4), which in turn activates TNFα and STAT3 (Figure 8)(Nduhirabandi et al. 2016). The SAFE pathway ultimately converges on the mitochondria of the cell, preventing the opening of the mitochondrial permeability transition pore (MPTP), which promotes cell survival (Petrosillo et al. 2009a).

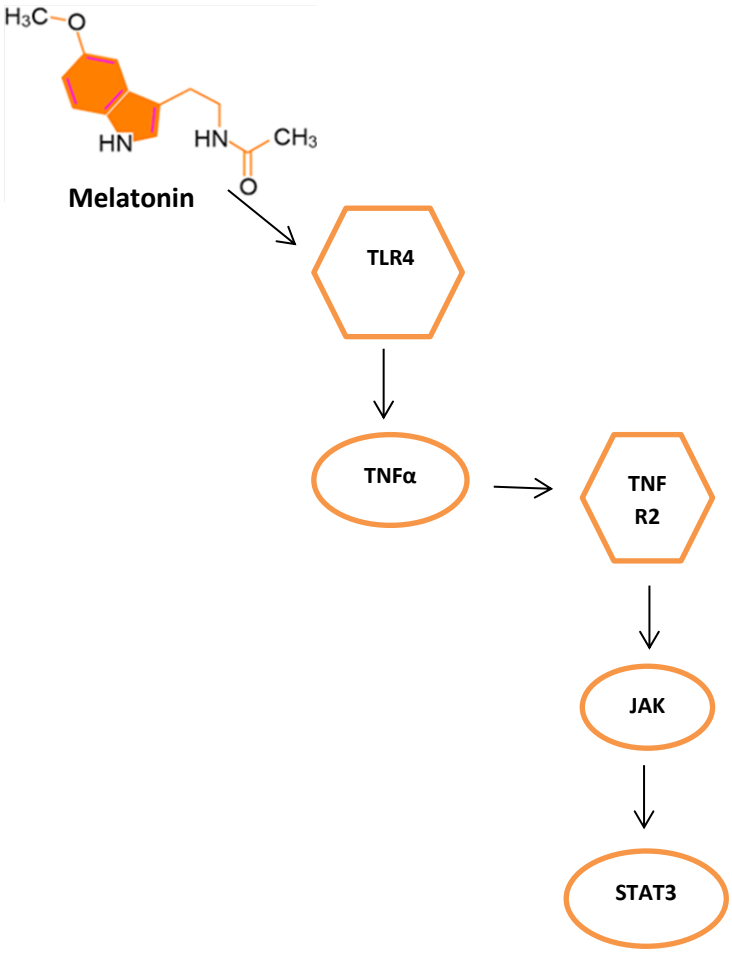


Figure 8: Melatonin activation of the SAFE pathway (Nduhirabandi et al. 2016).

5. Melatonin and mitochondria

5.1 Normal mitochondrial physiology

Under physiological conditions, mitochondria are essential for cell survival as they are central to energy production and regulate programmed cell death (Vakifahmetoglu-Norberg et al. 2017). There are two functionally distinct populations of cardiac mitochondria; the subsarcolemmal mitochondria which are located directly under the plasma membrane and the interfibrillar mitochondria which are situated among the myofibrils (Palmer et al. 1985)(Lesnefsky et al. 2001).

The mitochondrion has an outer membrane containing the voltage-dependent anion channel (VDAC) which allows metabolic substrates (pyruvate and malate) and nucleotides (adenosine diphosphate (ADP) and adenosine triphosphate (ATP)) to move into the intermembrane space (Lesnefsky et al. 2017). From here, the substrates are moved across the selectively impermeable inner mitochondrial membrane and are metabolised by enzymes in the tricarboxylic acid (TCA) cycle and fatty acid oxidation, amongst others. The products of the above processes; nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide ($FADH_2$), then undergo oxidation by the electron transport chain (ETC) (Lesnefsky et al. 2017). The ETC, found on the cristae of the inner mitochondrial membrane, is made up of four multi-protein complexes and also contains mobile electron carries; co-enzyme Q and cytochrome C. Firstly, NADH is oxidised by complex I, leading to the reduction of co-enzyme Q, which is oxidised by complex III and results in the reduction of cytochrome c. Cytochrome c is oxidised by complex IV which transfers the electrons to O_2 to form H_2O. Succinate, produced in the TCA cycle, is oxidised by complex II and co-enzyme Q is reduced and enters the ETC at complex III. The cristae also house the phosphorylation apparatus, which consists of complex V, a phosphate transporter, and adenine nucleotide-translocase (ANT) (Lesnefsky et al. 2001). Complex V uses the proton gradient to phosphorylate ADP to ATP. These processes couple respiration to phosphorylation – known as oxidative phosphorylation (OXPHOS) – and the myocyte energy demand determines the rate of OXPHOS (Lesnefsky et al. 2017).

5.2 Mitochondria during cardiac ischemia

However, mitochondrial dysfunction is a key component in a variety of pathophysiological conditions including cardiovascular and neurodegenerative diseases, diabetes and aging (Beal 1998)(Wallace 1999)(Schon et al. 2010). Mitochondria are suggested to be the main source of intracellular ROS, which are generated at low levels during physiological mitochondrial respiration, but increase under a number of pathological conditions (Vanden Hoek et al. 1997)(Becker et al. 1999). Mitochondrial ROS, produced by the ETC, attach to a number of mitochondrial constituents including; proteins, lipids and mitochondrial deoxyribonucleic acid (mtDNA) (Paradies et al. 2015).

Thus, the aetiology of a variety of pathological conditions, including myocardial ischemia/reperfusion, may be explained by ROS-induced alterations to the mitochondrial membrane constituents which could lead to a decline of the mitochondrial bioenergetics function (Chen & Zweier 2014). One of the major components involved in mitochondrial injury is the MPTP. During ischemia, ROS oxidises cardiolipin, which is found on the inner mitochondrial membrane and provides a binding site for cytochrome c (Ott et al. 2002). This causes the mobilisation and loss of cytochrome c to the intermembrane space, while the oxidised cardiolipin is translocated to the outer mitochondrial membrane. Here, it participates in the opening of the MPTP, along with elevated matrix Ca^{2+} levels which induce the collapse of the transmembrane ion gradients, resulting in membrane depolarisation and uncoupling of OXPHOS (Petrosillo et al. 2006a)(Petrosillo et al. 2009b)(Paradies et al. 2015). The opening of the MPTP is detrimental to the cells survival as it allows cytochrome c, along with other pro-apoptotic factors, to be released from the mitochondria into the cytosol of the cell where apoptosis is triggered (Paradies et al. 2015).

5.3 Melatonin can modulate mitochondrial function

The duration and severity of ischemic injury results in a varying mixture of apoptotic, necrotic and normal tissue and this injury can be abrogated by identifying potentially protective targets. As mitochondria are involved in cell death following an ischemic event, they become targets for protective intervention. Studies show that melatonin accumulates in mitochondria and can influence their homeostasis by stabilizing their inner membrane and therefore, improve ETC activity and mitochondrial function (Paradies et al. 2015)(López et al. 2009)(Martín et al. 2002). Melatonin has multiple mechanisms that provide protection to mitochondria. These include; a reduction of mitochondrial oxidative stress, the preservation of the mitochondrial membrane potential, the upregulation of antiapoptotic and downregulation of pro-apoptotic mitochondrial proteins, an increased efficiency of ATP production and a reduction in the release of cytochrome c into the cytosol (Figure 9)(Ganie et al. 2016)(Waseem et al. 2016)(Liu et al. 2015)(Yang et al. 2015)(Carretero et al. 2009)(Jumnongprakhon et al. 2014)(Tan et al. 2016).

As mentioned above, one of the main targets of ROS is mtDNA, which encodes polypeptides essential for ETC and ATP generation by OXPHOS and therefore, oxidative damage of mtDNA can lead to cell death through the disruption of electron transport, mitochondrial membrane potential and ATP generation (Paradies et al. 2015). Mitochondrial membrane lipids are also highly susceptible to oxidative damage which alters the structural and functional organization of the lipid bilayer. This causes a change in membrane fluidity and permeability, thus affecting respiration and OXPHOS processes, mitochondrial membrane potential and mitochondrial Ca^{2+} buffer capacity (Pamplona 2008). Under these circumstances, melatonin has an important role as an anti-oxidant, where it reduces free radical generation and thus reduces the damage they inflict on the mitochondria. Following cardiac ischemia/reperfusion injury in isolated rat hearts, mitochondria exhibit a decreased rate of oxygen consumption and reduced activity of complex I and III, but treatment with melatonin had a protective effect against the bioenergetic alterations (Petrosillo et al. 2003)(Petrosillo et al. 2006b). This effect is possibly attributed to melatonin's direct improvement of the activity of the respiratory chain complexes (Paradies et al. 2015). Melatonin also has a vital role in the modulation of MPTP opening. A study has shown that melatonin can directly inhibit the opening of the MPTP via a low-affinity binding site, which may contribute to the anti-apoptotic nature of melatonin (Andrabi et al. 2004). Melatonin can also act indirectly to inhibit the opening of the MPTP, by preventing the oxidation of cardiolipin and desensitising the MPTP to Ca^{2+} (Petrosillo et al. 2006a). These numerous beneficial effects of melatonin on mitochondria highlight its therapeutic potential against mitochondrial-mediated apoptosis in ischemic heart disease.

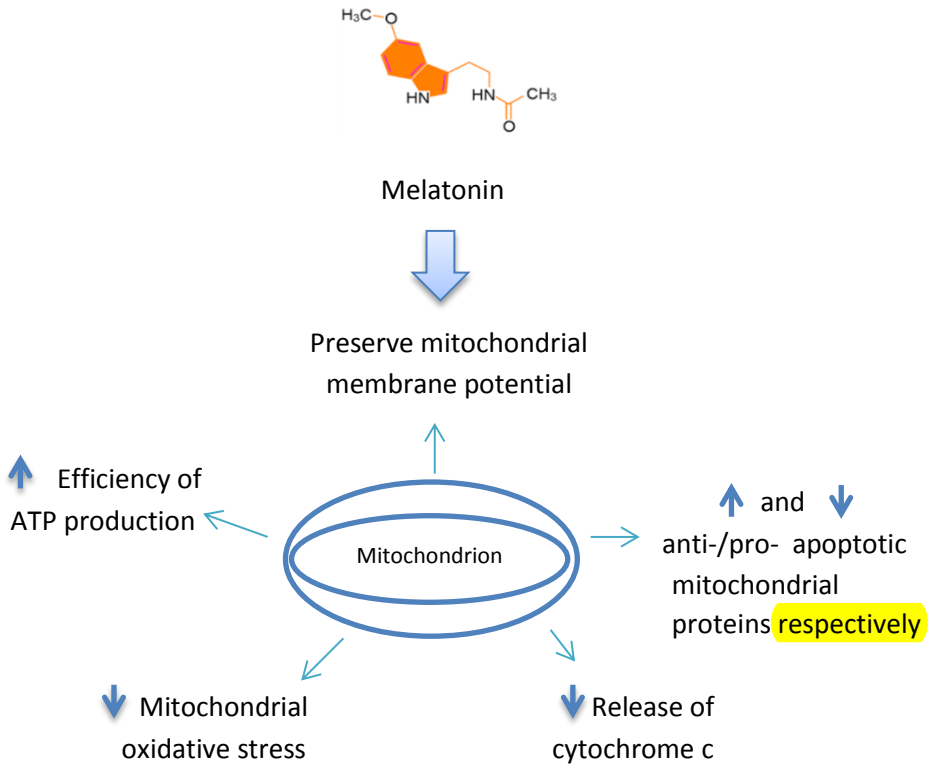


Figure 9: Melatonin's protective mechanisms on mitochondria.

6. Melatonin and melatonin isomers

In 2011, Rodriguez-Naranjo and colleagues found, in red wine, a supposed melatonin fragmentation that did not have the characteristic minor fragments that coincide with those of the melatonin standard. Upon detailed analysis, they identified that these molecules were melatonin isomers (Rodriguez-Naranjo et al. 2011). Since then, naturally occurring melatonin isomers were discovered in plant/plant products as well as in fermented products such as wine and bread (Kocadağli et al. 2014). Their delayed identification was due to insufficient detection capacity by conventional methods. The more advanced, High Performance Liquid Chromatography Tandem Mass Spectrometry (HPLC-MS/MS) method was used for accurate characterisation (Kocadağli et al. 2014). Interestingly, the concentration of the melatonin isomers in fermented products such as wine, are much higher than those of melatonin.

This is demonstrated in figure 10 where there is an exponential increase in the concentration of the isomers as the fermentation time increase, whereas melatonin concentrations remain relatively stable (Tan et al. 2012).

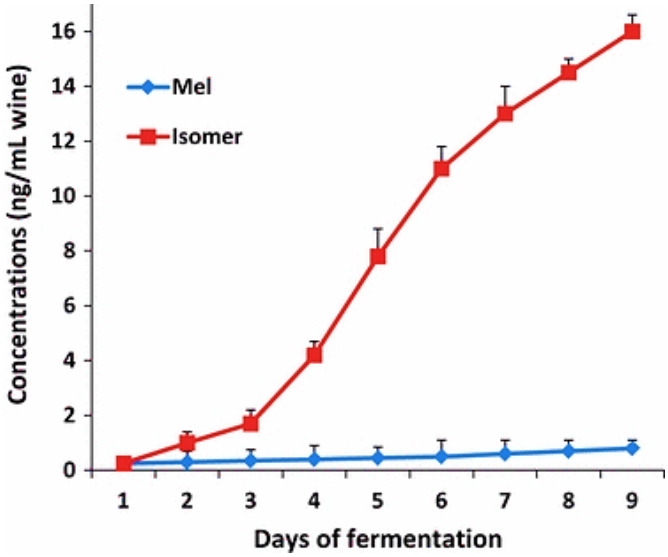


Figure 10: Concentrations of melatonin and melatonin Isomers over a period of fermentation of wine (Tan et al. 2012).

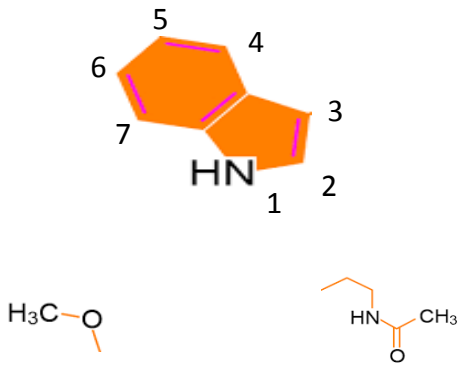
In collaboration with Dr Marcello Iriti (University of Milan, Italy), we have been able to measure the concentration of a melatonin isomer in different South African wines (Table 2). The mean concentration of this melatonin isomer (the exact formula of this isomer is kept undisclosed by the group in Italy) in South African wines was very similar to the concentrations found in other international wines (75mg/L) (Vitalini et al. 2013).

Table 2. Melatonin and melatonin isomer concentrations in South African wines (unpublished data from the Hatter Institute for Cardiovascular Research in Africa).

	Melatonin (ng/ml)	Melatonin isomer (ng/ml)
Idiom Nebbiolo (2010)	0.70 ± 0.03	34.25 ± 1.76
Pinot Noir – Springfield (2010)	1.19 ± 0.08	70.89 ± 1.96
Bon Courage Pinotage (2009)	2.19 ± 0.12	37.65 ± 0.74
Sauvignon Blanc – Springfield (2010)	0.62 ± 0.03	24.19 ± 0.75
Pinot Noir – Paul Cuvier (2009)	0.80 ± 0.06	45.83 ± 3.25

6.1 Melatonin isomer structure and biosynthesis

Isomers are molecules with the same chemical formula, but different arrangement of atoms in space. Therefore, melatonin isomers are formed when either one, or both, of the following side chains are moved to any of the seven positions on the indole ring; the methoxy group (Chain M), normally at position 5, and the N-acetylaminoethyl group (Chain A), normally at position 3 (Figure 11) (Tan et al. 2014).



Methoxy Group
Position 5
(Chain M)

N-acetylaminoethyl Group
Position 3
(Chain A)

Figure 11: Possible melatonin isomer structures.

Since melatonin isomer research is in its infancy, the synthetic/metabolic pathways of the isomers are currently unknown and there is no existing evidence showing that isomers are present in humans. Vigentini et al have, however, suggested that melatonin isomers are derived from yeasts and bacteria during fermentation (Vigentini et al. 2015).

6.2 Physiological functions of melatonin isomers

There is very limited information regarding the functions of melatonin isomers, but due to their presence in numerous nutritional products that are widely consumed by humans, the benefits of the isomers could be substantial. Iriti and Vigentini have recently shown that a particular melatonin isomer evokes a rapid and transient dose-dependent decrease in mean arterial pressure and heart rate, as well as causing significant vasodilation in small mesenteric arteries by blocking voltage-operated calcium channels on vascular smooth muscle cells (Iriti & Vigentini 2015).

These findings hint at the possibility that melatonin isomers may exhibit physiological functions similar to those of melatonin and therefore, may offer cardioprotective effects. However, there is no knowledge as to whether the melatonin isomer measured in our South African wines may confer any physiological properties.

7. Models used to study the cardioprotective effects of melatonin

7.1 *Ex vivo* (Langendorff model)

A number of different models have been used in order to study the protective effects of melatonin against ischemia/reperfusion injury. *Ex vivo* experiments, with isolated perfused hearts, are performed using the Langendorff model which involves of the anaesthetisation of rodents and the rapid excision of their hearts. The hearts are mounted on the Langendorff apparatus and perfused retrogradely at a constant flow or pressure (Lamont et al. 2011)(Kaneko et al. 2000). Ischemia is induced either regionally via coronary ligation or globally via partial or complete reduction of flow (Lecour & Hausenloy 2014). The Langendorff-perfused heart allows one to measure physiological, morphological, biochemical and pharmacological parameters in a single experiment, therefore, providing a vast amount of information in a time efficient manner (Skrzypiec-spring et al. 2007). However, as this is an isolated organ, it lacks the influence of other organs, the systemic circulation as well as signals from the nervous system which could result in the misrepresentation of whole-system outcomes (Skrzypiec-spring et al. 2007). In this model, dietary melatonin was shown to be protective when given prior to an ischemic insult (Nduhirabandi et al. 2016).

7.2 *In vivo*

To observe outcomes in a physiologically correct manner, researchers use *in vivo* models. Traditionally dogs were used, but due to high costs and social pressure, the pig became the more favourable model (Swindle et al. 1994). The animals are anaesthetized and ischemia is induced in one of the following ways: the open-chest procedure involving the occlusion of the coronary artery by inflating a balloon which is placed around the vessel, or the closed-chest procedure involving fluoroscopy and the inflation of a balloon at the tip of a catheter (Zaragoza et al. 2011). In this model, using rats or mice, pharmacological and dietary concentrations of melatonin were shown to be protective when given prior to an ischemic insult (Yu et al. 2015)(Lamont et al. 2015)(Lochner et al. 2006). *In vivo* models are important for translational studies, but due to strict animal ethics regulations, it is exceedingly challenging to obtain approval for such research and preliminary data using cell culture models may be required.

7.3 *In vitro* (cell culture)

Cell culture is defined as the removal of cells, from an organism, which are then subsequently grown in a favourable artificial environment (Life Technologies n.d.). This makes cell culture an excellent model system when studying the physiology and biochemistry of cells, as specific variables can be altered in a controlled manner during experiments in order to fully understand and explore biological mechanisms. There are two types of cell culture namely; Primary culture and Cell line culture. Primary culture contains cells that are morphologically similar to that of the parent tissue and can only be maintained for a limited amount of time *in vitro* (Lonza 2017). They are believed to retain many of the characteristics of the cells *in vivo*, therefor making them the more favourable cells for research. However, these cells require direct animal use by the researcher to be extracted and ethical considerations need to be taken into account.

This research project was performed using a finite cell line, otherwise known as secondary culture. These cells are formed from a primary cell line after the first sub-culturing and are only able to undergo a limited number of cell divisions (passages) before senescence.

This cell line is commercially available and it allows one to obtain a large population of similar cells, where most cellular characteristics are maintained, with which to perform experiments (Lonza 2017). The protective effect of dietary melatonin has not, to our knowledge, previously been tested using this model.

7.3.1 Advantages and setbacks in cell culture model

Besides the above-mentioned advantage of being able to vigorously explore and understand biological mechanisms using cell culture, it is also an ethically sound tool for experimental research. The age long debate over the decency and value of animal research, as well as the way in which experimental procedures are conducted, is still under heavy scrutiny and this makes approval for animal research a tough and tedious feat. Cell culture also has the advantage of being a more cost effective way of conducting research. This is an important factor to consider as funding can often be a challenge to obtain and this, in conjunction with the rising cost of animal care and maintenance, can be a major setback (Murphy 1991). The testing of drugs using cell culture, as performed in this project, has the distinct benefit of providing individualised organ, tissue or cellular responses without the interference of a number of other endogenous factors (Lecour et al. 2014). This could, however, act in a detrimental manner when information regarding interactions between whole systems is required (Lecour et al. 2014).

B) HYPOTHESIS, AIM and OBJECTIVES

CVD is a serious burden in both developed and developing countries, with IHD being the single largest cause of death worldwide. A continual increase in CVD and IHD is expected unless serious preventative action is taken and additional therapies are made available. The use of dietary melatonin, as found in regular and moderate consumption of red wine, has shown very promising results in animal models as a possible therapy against IHD, but the mechanisms of this protection are not yet fully understood. Recently, the presence of melatonin isomers has also been detected in food products, but whether these isomers confer any cardiovascular benefit is unknown. Both melatonin and melatonin isomers present the advantage to be potentially safe and inexpensive therapies against IHD and this would be of great benefit for patients in developing countries.

Hypothesis

Therefore, we hypothesize that melatonin isomers given at the concentration found in regular and moderate consumption of red wine, may exhibit similar functions to those of melatonin and thus, may offer cardioprotection against ischemic injury.

Aim

The aim of our project is to establish a cell culture model of simulated ischemia to study and compare the possible cytoprotective effects of dietary melatonin and the melatonin isomer and to explore possible mechanisms involved in this effect.

Important note: melatonin isomers are not commercially available, but we were fortunate enough to obtain one melatonin isomer from Dr Iriti (University of Milan) who has identified and isolated this isomer in red wine.

The objectives of this study were as follows:

1. To validate a suitable model of simulated ischemia using H9C2 cardiac fibroblast cells.
2. To compare the cytoprotective effect of both pharmacological and dietary concentrations of melatonin against simulated ischemia.
3. To determine whether the dietary concentration of melatonin isomer confers cytoprotection against simulated ischemia.
4. To explore if there is a possible additive cytoprotective effect against simulated ischemia when dietary melatonin and melatonin isomer are combined.
5. To determine whether dietary melatonin and melatonin isomer protect via the activation of melatonin receptors
6. To explore whether the cytoprotective effect of dietary melatonin and melatonin isomer protect against simulated ischemia by modulation of mitochondrial function.

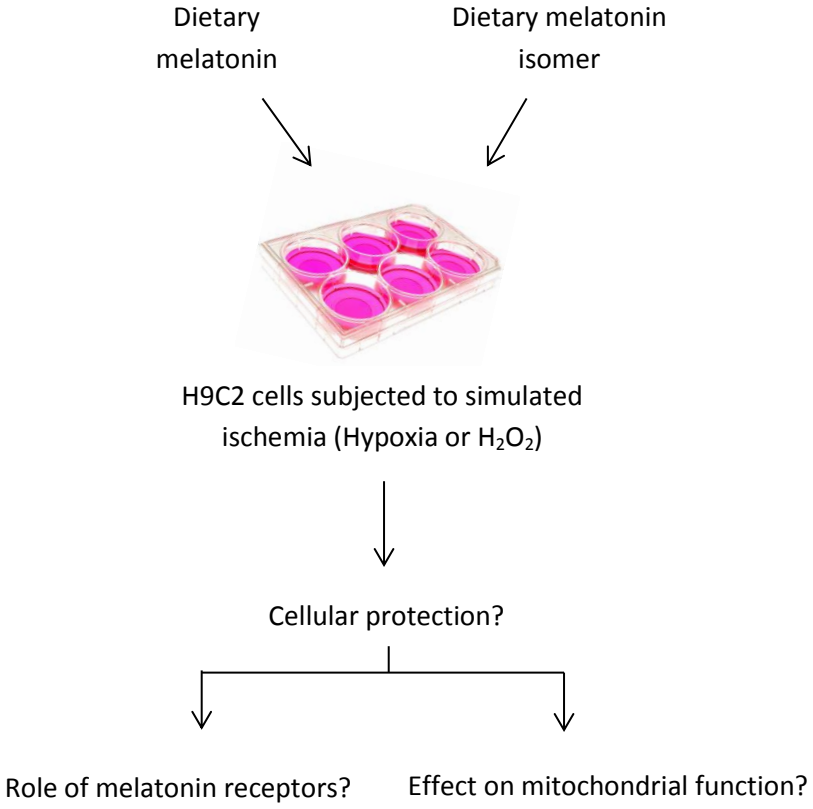


Figure 12. Schematic representation of the aim of the study.

C) METHODS

1. Cell Culture

For this study, H9C2 myoblasts (obtained from ATCC, USA) derived from embryonic rat hearts were used. The cells were stored in cryovials, containing $1x10^6$ cells/ml and 10% dimethyl sulfoxide (DMSO), in liquid nitrogen at -196°C. The cells were transferred to a $25cm^2$ tissue culture flask containing Dulbecco's Modified Eagle Medium (DMEM) with 4,5g/L glucose, 0,11g/L sodium pyruvate and L-glutamine supplemented with 10% Fetal Calf Serum (FCS). The H9C2 cells were incubated at 5% CO2, 37 °C and 95% humidity until they were 80% confluent. The cells were trypsinised with 0,25% trypsin supplemented with 0,2% ethylenediaminetetraacetic acid (EDTA) for 3min at 37 °C before it was neutralised with 10% FCS DMEM. The suspended cells were transferred into a 50ml polypropylene conical tube and centrifuged for 5min at 1000rpm at 4°C. The cells were counted, before centrifugation, in a Neubauer haemocytometer. The cell pellet was resuspended in a sufficient volume of 10% FCS DMEM to give $1x10^6$ cells/ml. $75cm^2$ tissue culture flasks or 6-well plates were seeded with a sufficient number of cells to achieve 80% confluency, for the experiments within 2 days' time, with fresh 10% FCS DMEM fed to the cells every second day. Passages 7-24 were used for experiments.

2. Mycoplasma test

Mycoplasmas are a group of the smallest, free-living organisms which lack a rigid cell wall and are therefore, resistant to most of the antibiotics that are commonly used in cell cultures (Drexler & Uphoff 2002). They are also extremely small in size (+/- 100nm) and can thus go undetected for extended periods of time (InvivoGen 2011). Mycoplasmas can attach themselves to eukaryotic cells and eventually fuse with the cell membrane, invading the host cell, where they will evade defences in order to survive. Once inside, mycoplasmas compete with the host for nutrients and biosynthetic precursors which can have the following consequences (InvivoGen 2011):

- Alter deoxyribonucleic acid (DNA) and ribonucleic acid (RNA)
- Alter protein synthesis
- Reduce amino acid levels
- Reduce ATP levels
- Introduce chromosomal changes
- Alter host-cell plasma membrane antigens

The mycoplasma test was performed using a modified version of the Hoechst stain which is a DNA staining method (Chen 1977). Cells were seeded in a 12-well plate with 1ml DMEM and incubated. After 24 hours the DMEM was removed and cells were washed with 1ml phosphate buffered saline (PBS) for 5min.

Cells were incubated in 1ml formalin for 30min, followed by two 5min washes with 1ml PBS. 1ml PBS with 0.5µl Hoechst nuclei stain was added for 5min, followed by three 5min washes with 1ml PBS. The cells were viewed on a fluorescent microscope where all cell nuclei fluoresce, with mycoplasma contaminated cells showing small, morphologically uniform fluorescent bodies in the cytoplasm and extracellular matrix (ECM). The mycoplasma tests performed throughout the experiments were all negative.

3. Experimental protocols

3.1 Simulated ischemia with the hypoxic chamber

Under physiological ischaemic conditions, the pH becomes mildly acidic, there is an increase in the potassium concentration and metabolic activity is disturbed. To simulate these ischemic conditions in the H9C2 model, an adapted protocol from Esumi et al was used (Esumi et al. 1991). The Esumi buffer contained 137mM NaCl, 12mM KCl, 0.5mM $MgCl_2$, 0.9mM $CaCl_2.2H_2O$, 4mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfanic acid (HEPES) and 20mM 2-Deoxy-d-Glucose (2-DG) and was adjusted to a pH of 6.4. The 2-DG serves to inhibit glycolysis which disrupts metabolic activity. To mimic the low O_2 concentration occurring during ischemia, a multi-gas incubator (Gentronics) supplying 1% O_2 and the balance liquid N_2, was used to house the 6-well plates.

The cells were maintained in the hypoxic environment for 8 hours, targeting ±50% cell viability measured using trypan blue and the Countess II FL system (Life Technologies).

Two experimental groups were tested; the normoxic control group and the hypoxic control group.

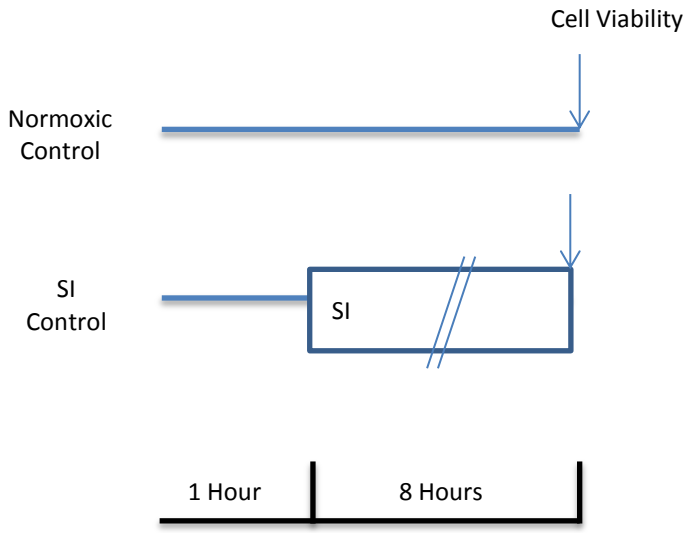


Figure 13: Schematic representation of the experimental protocol with simulated ischemia performed in the hypoxic chamber. (SI= simulated ischemia).

3.2 Simulated ischemia with hydrogen peroxide

Hydrogen peroxide (H_2O_2) is a reactive oxygen species and an abnormal accumulation causes oxidative stress and cellular damage (Lee et al. 2015). As ROS plays an important part in the pathogenesis of cardiac ischemic injury, exposure of the cells to H_2O_2 was used to simulate the ischemic event. The disadvantage of this model, when compared to the hypoxic chamber, is that it does not take into account the exact ischemic conditions that the Esumi buffer mimics and most importantly does not have a hypoxic environment.

H9C2 cells, at 80% confluency, were treated with three different concentrations of H_2O_2 for 2h to target a ±40% cell viability: 0.25mM, 0.5mM and 1mM prepared in DMEM (Lim et al. 2013). Cell viability was determined using trypan blue and the Countess system.

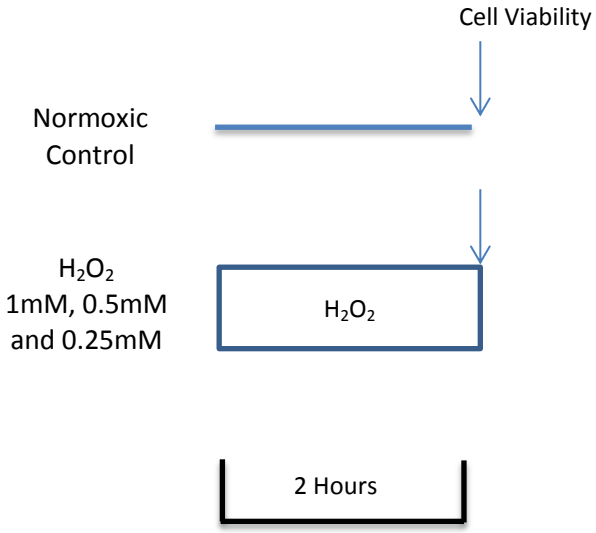


Figure 14. Schematic representation of the experimental protocol with simulated ischemia induced by H_2O_2 treatment.

3.3 Testing the protective effect of melatonin and the melatonin isomer against simulated ischemia

Initially, a pharmacological concentration of 1µM (0.232mg/L) melatonin was used to optimise the treatment period, as this concentration corresponds to the pharmacological range of melatonin (Lochner et al. 2006). Of note, we will continue to refer to this concentration as 1µM and not 0.232mg/L, as 1µM is how the pharmacological concentration of melatonin is usually referred to in the literature. 1µM melatonin was given as a pre-treatment for 30min, prior to the optimal 1mM H_2O_2 treatment chosen, and continued or not during the H_2O_2 treatment. Melatonin and the melatonin isomer, both at dietary concentrations of 75ng/L and 75mg/L respectively, were given as a pre-treatment for 30min prior to the simulated ischemic insult with 1mM H_2O_2. Cell viability was determined using trypan blue and the Countess system.

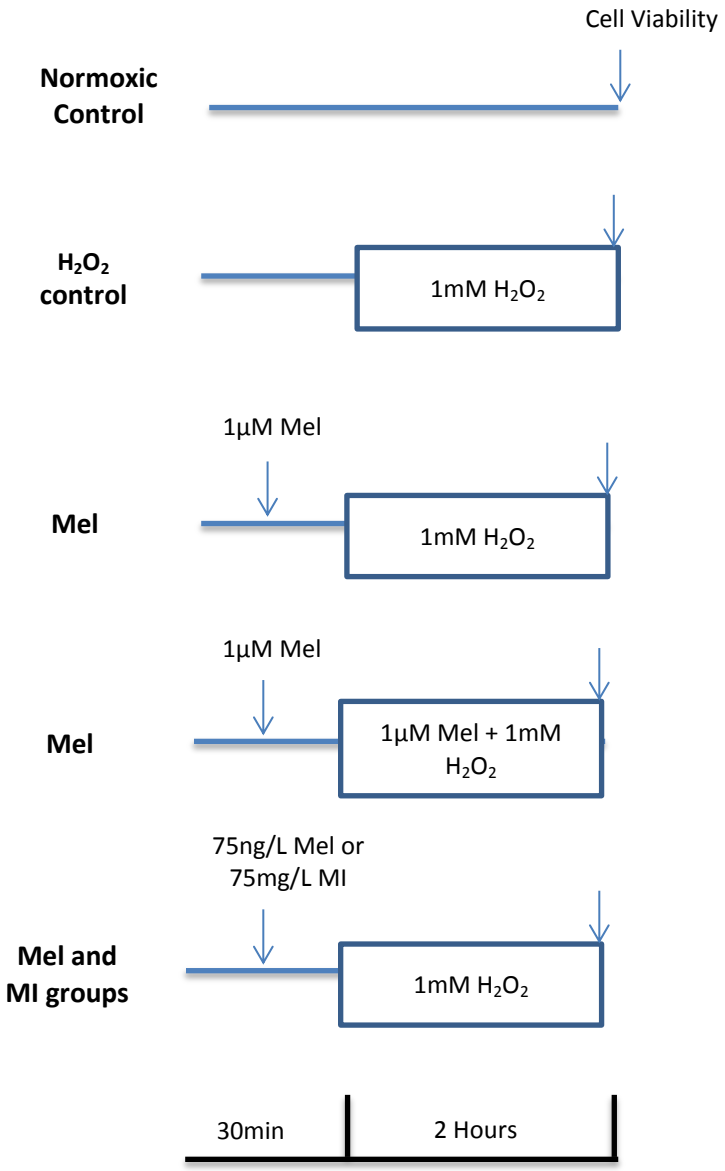


Figure 15. Schematic representation of the experimental protocol testing the protective effect of melatonin (1µM and 75ng/L) and the melatonin isomer (75mg/L) against simulated ischemia. (Mel= melatonin, MI= melatonin isomer).

3.4 Testing the role of the melatonin receptor in melatonin and melatonin isomer induced cytoprotection

To determine whether protection was mediated via the melatonin receptors (MT1 and MT2), luzindole which is an inhibitor of both receptors, was used. 5mg luzindole was dissolved in 1ml DMSO and added to DMEM to achieve a final concentration of 10µM (Quan et al. 2015). The cells were pre-treated with 10µM luzindole for 30min followed by a combined pre-treatment of 30min with 75ng/L melatonin and 75mg/L melatonin isomer, prior to the 2h simulated ischemia with 1mM H_2O_2. Cell viability was determined using trypan blue and the Countess system.

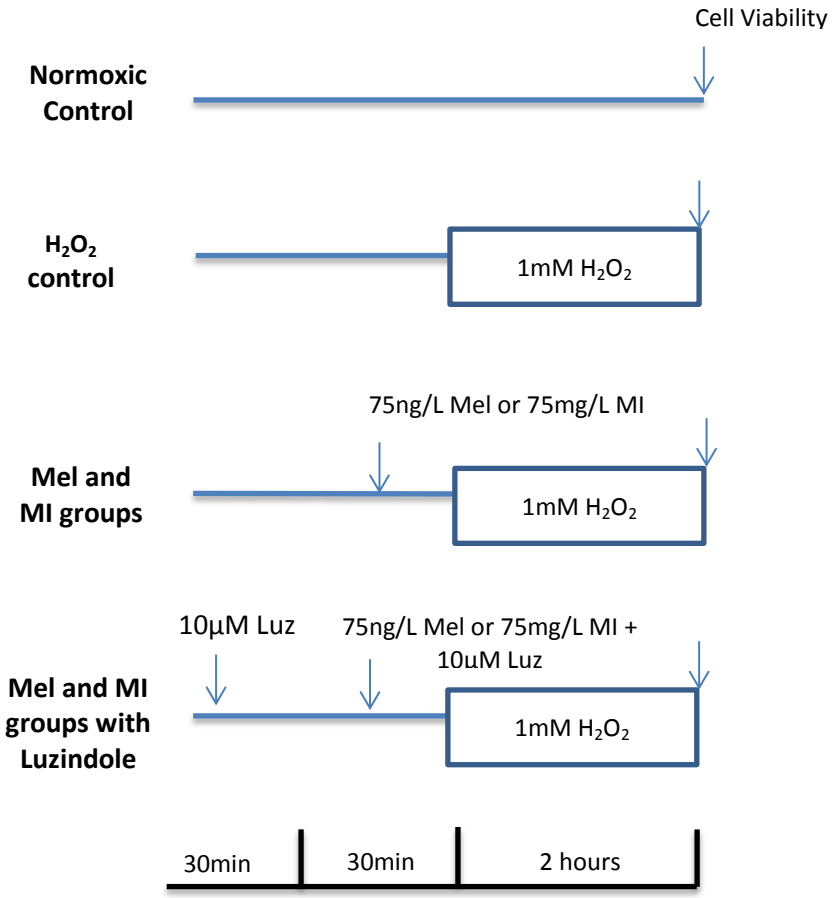


Figure 16: Schematic representation of the experimental protocol testing the role of melatonin receptors in melatonin and melatonin isomer induced protection against simulated ischemia with 1mM H_2O_2. (Mel= melatonin, MI= melatonin isomer, Luz = luzindole).

4. Cell Viability Analysis

4.1 Trypan blue

Cell viability was evaluated using the trypan blue exclusion method (Kitakaze et al. 1997). This method works on the principle whereby the dye enters through disrupted cell membranes of dead or dying cells and stains them blue. Cells were trypsinized, post 8 hours hypoxia, centrifuged and resuspended in PBS. The cell suspension and trypan blue are mixed in a 1:1 ratio (10µl each) and the cell viability was measured using the Countess system.

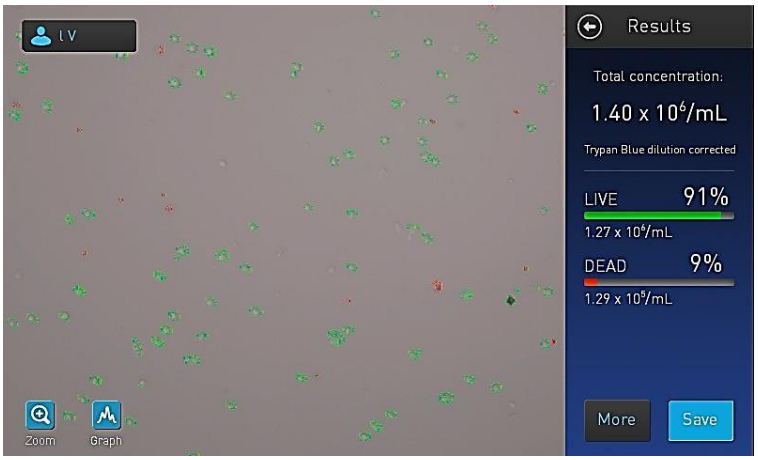


Figure 17: Actual picture of H9C2 cells, control group, with 91% cell viability as calculated by the Countess system.

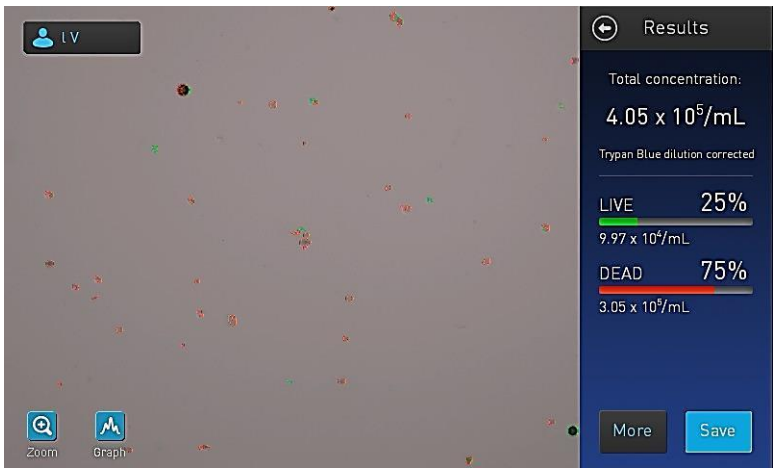


Figure 18: Actual picture of H9C2 cells, H_2O_2 group, with 25% cell viability as calculated by the Countess system.

5. High resolution respirometry

Real time respirometric measurements were performed with a polarographic oxygen sensor in 2ml glass chambers of an Oxygraph 2K (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria). For data recording, DatLab software version 6 was used with sampling intervals of 2 seconds. Settings, daily calibrations and instrumental background corrections were conducted according to the manufacturer's instructions. Respiratory measurements were performed after cells were suspended in a mitochondrial respiration medium (MiR05, 0.5mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 3mM $MgCl_2 \cdot 6H_2O$, 60mM potassium lactobionate, 20mM taurine, 10mM KH_2PO_4, 20mM HEPES, 110mM sucrose and 1g/L bovine serum albumin (BSA), pH 7.1). Before all experiments, calibration of the respirometer was performed at air saturation, 37°C, using MiR05. Following permeabilization of the H9C2 cells with digitonin (1.5mM), pyruvate (5mM) and malate (2mM) were added to determine the LEAK state. Complex I linked OXPHOS was measured by addition of ADP (2.5mM) and combined complex I and II linked OXPHOS was determined by titration of succinate (10mM) in the presence of ADP. Electron transfer system (ETS) capacity was determined through subsequent titrations of carbonyl cyanide p-(triflouromethoxy) phenylhydrazone (CCCP) (0.5µM), until a maximal oxygen flux was reached, which was taken as the maximal ETS capacity. Correcting for residual oxygen consumption (ROX), involved the addition of complex III inhibitor antimycin-A and the resulting flux was subtracted from all final values. Complex IV activity was used as a surrogate marker for mitochondrial number and therefore, subtracted from all the final ETS and OXPHOS capacities (Larsen *et al*., 2012). To achieve this, titration of N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD, 0.5mM) and ascorbate (2mM) to assess complex-IV-linked respiration was performed as a proxy for mitochondrial content/number. Oxygen flux at all respiratory states was normalized to the complex IV flux to correct for variations in cell mitochondrial content in the oxygraph chambers.

5.1 Experimental protocol for mitochondrial studies

5.1.1 Testing the effect of melatonin (75ng/L) and the melatonin isomer (75mg/L) on mitochondrial respiration in permeabilized H9C2 cells

At confluence, cells were treated with melatonin (75ng/L) or the melatonin isomer (75mg/L) for 30min. Cells were then trypsinised, centrifuged (1000rpm for 5min at 4°C), resuspended in MiR05 medium (2ml for ±700 000 cells) and transferred into the oxygraph chambers. A protocol was applied using digitonin permeabilization of H9C2 cells and sequential addition of respiratory complex-specific substrates and inhibitors in MiR05 medium, adapted from (Piel et al. 2015).

After stabilization of the oxygen flux signal, the endogenous ROUTINE respiration of the cells was evaluated. This was achieved by the titration of NADH-linked substrates, pyruvate and malate (5mM). This was followed by digitonin (1µL 10^{-6} cells) in order to induce maximal cell membrane permeabilization. The signal of ROUTINE respiration was allowed to reach a stable point and a subsequent titration of ADP (2.5mM) was made. This allowed us to measure complex I linked OXPHOS. Hereafter, the integrity of the outer mitochondrial membrane was examined by adding cytochrome c (10µM).

Subsequently, to determine convergent complex I and II dependent OXPHOS capacity ($OXPHOS_{CI+II}$), the $FADH_2$-linked substrate succinate (10mM) was added. Maximal uncoupled respiratory electron transport system capacity supported by convergent input through complex I and II (ETS_{CI+II}) was evaluated by subsequent titration of CCCP (0.05µM steps).

To correct for ROX, the complex III inhibitor, antimycin-A (2.5µM), was titrated and the resulting flux was subtracted from all final values. This allowed us to ensure that the oxygen consumption measured from our cells was due to actual mitochondrial respiration and not side chain reactions. Lastly, the artificial complex IV substrate TMPD (0.5mM) was titrated along with ascorbate (2mM) to measure complex IV linked respiration.

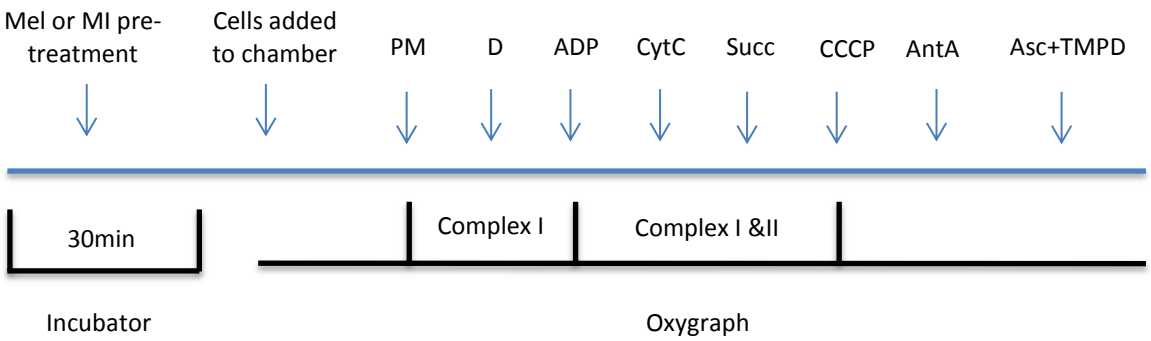


Figure 19: Schematic representation of the experimental protocol exploring the effect of melatonin (75ng/L) and the melatonin isomer (75mg/L) on mitochondrial respiration. (PM= pyruvate and malate, D= digitonin, ADP= adenosine diphosphate, CytC= cytochrome C, Succ= succinate, CCCP= carbonyl cyanide p-(triflouromethoxy) phenylhydrazone, AntA= antimycin-A, Asc+TMPD= ascorbate and N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride).

5.2 Testing the effect of melatonin (75ng/L) and the melatonin isomer (75mg/L) on mitochondrial respiration in permeabilized H9C2 cells subjected to H_2O_2 treatment

At confluence, cells were treated with melatonin (75ng/L) and the melatonin isomer (75mg/L) for 30min, followed by a 15min treatment with H_2O_2 (1mM). 15min exposure was chosen for this set of experiments as the 2h period damaged the cells to the point where they were no longer respiring efficiently and could not be used for the OXYGRAPH experiments. The cells were then trypsinised, centrifuged (1000rpm for 5min at 4°C), resuspended in MiR05 medium (2ml for ±700 000 cells) and transferred into the oxygraph chambers. The same protocol, as previously described, was followed.

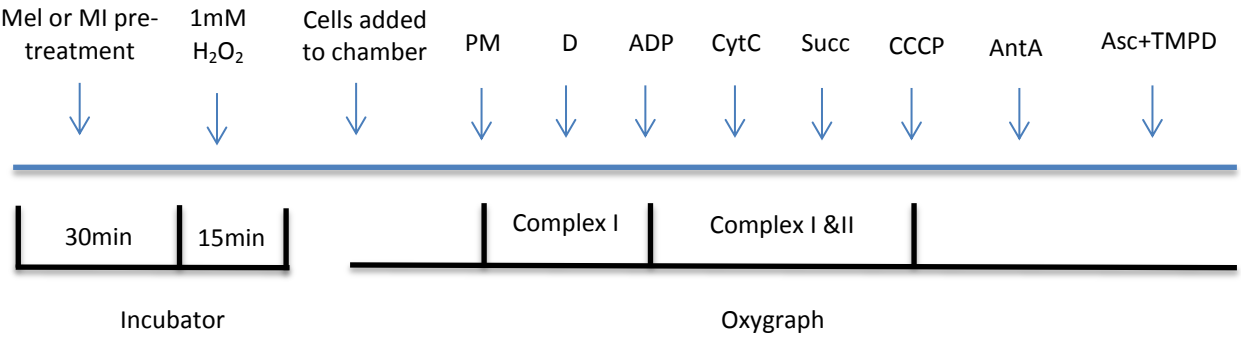


Figure 20: Schematic representation of the experimental protocol exploring the effect of melatonin (75ng/L) and the melatonin isomer (75mg/L) on mitochondrial respiration in permeabilized H9C2 cells subjected to H_2O_2 treatment. (PM= pyruvate and malate, D= digitonin, ADP= adenosine diphosphate, CytC= cytochrome C, Succ= succinate, CCCP= carbonyl cyanide p-(triflouromethoxy) phenylhydrazone, AntA= antimycin-A, Asc+TMPD= ascorbate and N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride).

5.3 Drugs

Unless specified, all drugs were obtained from Sigma-Aldrich, South Africa. The melatonin isomer was obtained from Dr Marcello Iriti (University of Milan, Italy) and the exact formula of the isomer was undisclosed.

6. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical significance between multiple groups was determined by one-way analysis of variance (ANOVA) followed by the Tukey posthoc test (GraphPad InStat). A value of $P<0.05$ was considered significant.

D) RESULTS

1. Validation of the experimental model

1.1 H9C2 cells subjected to simulated ischemia in the hypoxic chamber

H9C2 cells cultured in normoxic conditions, presented a cell viability of 86.8±1.3%. In cells subjected to hypoxic conditions for 8h, the cell viability was reduced to 48.0±15.0%. The non-reproducibility of the hypoxic chamber could be explained by the unreliability of the chamber to control the feeding of the gases. Due to this, another simulated ischemic model (oxidative stress with H_2O_2) had to be considered.

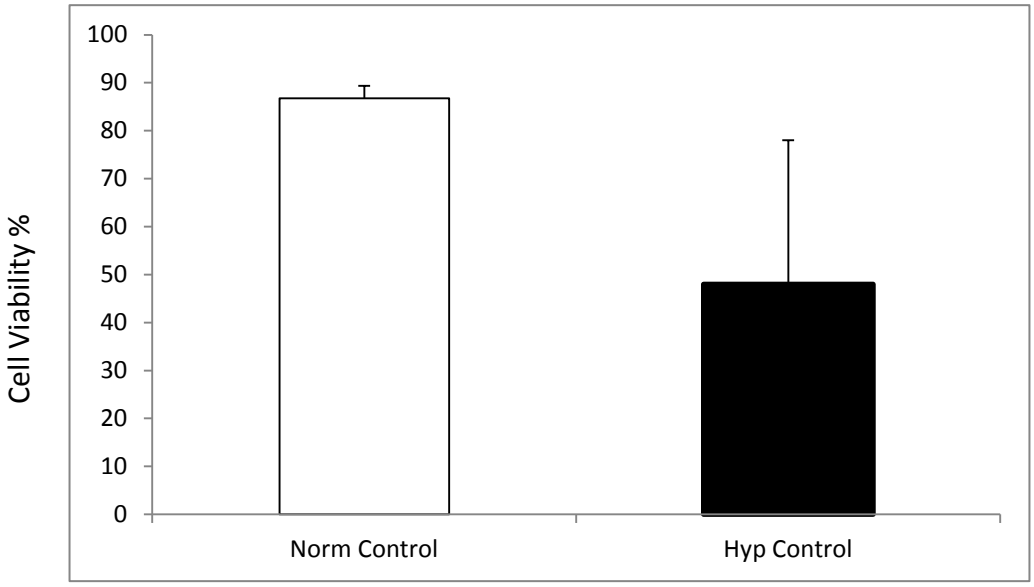


Figure 21: Cell viability expressed as percentage in H9C2 cells subjected to 8h simulated ischemia in the hypoxic chamber (n=3, values as mean ±SEM)

1.2 H9C2 cells subjected to simulated ischemia by exposure to H_2O_2

H9C2 cells cultured in normoxic conditions, presented a cell viability of 92.9±1.5%. The addition of 1mM H_2O_2 for 2h, to mimic an ischemic insult, significantly reduced cell viability to 28.4±1.4% ($p<0.001$ vs control), while 0.5mM and 0.25mM H_2O_2 reduced cell viability to 49.5±11.0% and 67.6±9.5% ($p<0.01$ vs control) respectively. In conclusion, as 1mM H_2O_2 presented the best reproducibility in the results and a cell viability close to target, the experiments to follow will be conducted with H_2O_2 at the concentration of 1mM for 2h.

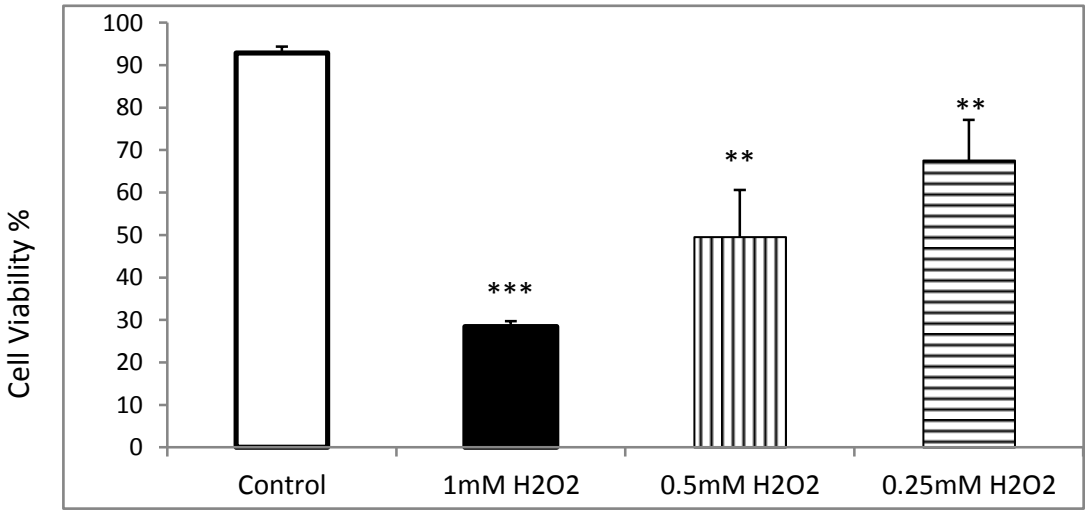


Figure 22: Cell viability expressed as percentage in H9C2 cells subjected to simulated ischemia by exposure to H_2O_2 at different concentrations for 2h (n=6, values as mean ±SEM). ***p<0.001 vs control, **p<0.01 vs control.

1.3 Delineation of the time of exposure for melatonin to protect H9C2 cells against a simulated ischemic insult

To explore the maximal cytoprotective effect of melatonin against a simulated ischemic insult, 1µM melatonin (which falls within the protective pharmacological concentration range given in the literature (Lochner et al. 2006)) was given for 30min prior to the ischemic insult and continued or not during the simulated ischemic insult.

H9C2 cells cultured in normoxic conditions, presented a cell viability of 97.0±0.6%. The addition of 1mM H_2O_2 for 2h significantly reduced cell viability to 36.0±4.7% ($p<0.001$ vs control). The pre-treatment of the cells with 1µM melatonin for a period of either 30min or 30min with continued use during the 2h H_2O_2 treatment, showed a similar trend in protection with a cell viability of 69.0±1.2% and 65.0±9.6% ($p<0.01$ vs H_2O_2) respectively. The 30min pre-treatment period presented better reproducibility and was chosen for use throughout the experiments to follow.

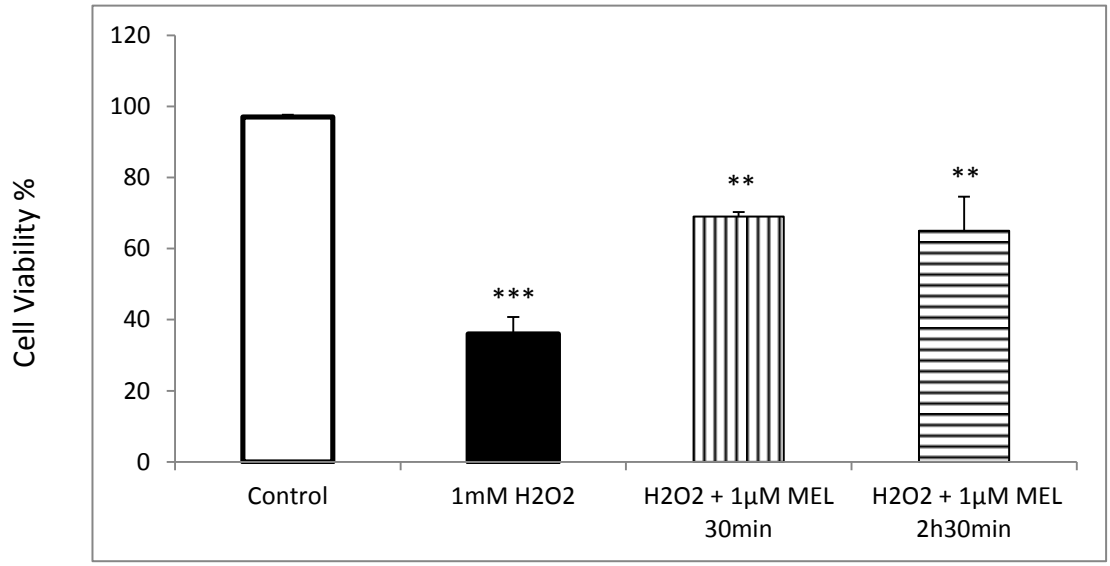


Figure 23: Cell viability expressed as percentage in H9C2 cells subjected to simulated ischemia by exposure to 1mM H_2O_2 for 2h and 1µM Mel for different exposure times (n=3, values are mean ±SEM). ***$p<0.001$ vs control, **$p<0.01$ vs H_2O_2.

In conclusion, the model of simulated ischemia to be considered to study the possible cytoprotective effect of the melatonin isomer in H9C2 cells will be as follows:

- Exposure of the cells to 1mM H_2O_2 for 2 hours
- Pre-treatment of melatonin/melatonin isomer for 30min prior to the simulated ischemic insult.

2. Cytoprotective effect of melatonin and melatonin isomer in H9C2 cells subjected to a simulated ischemic insult

In the following experiments, we compared the cytoprotective effect of 1µM melatonin in H9C2 cells subjected to a simulated ischemic insult to the cytoprotective effect of 75ng/L melatonin and 75mg/L melatonin isomer, which both correspond to a concentration found in the diet (wine).

H9C2 cells cultured in normoxic conditions, presented a cell viability of 96.3±0.4%. The addition of 1mM H_2O_2 for 2h significantly reduced cell viability to 49.7±4.4% ($p<0.001$ vs control). Pre-treatment of the cells with 75ng/L melatonin or 75mg/L melatonin isomer, dietary concentrations, significantly improved cell viability to 74.4±3.1% and 73.9±2.7% respectively ($p<0.001$ vs H_2O_2), which is on par with the protection provided by pharmacological concentration of melatonin at 69.0±1.2% ($p<0.01$ vs H_2O_2).

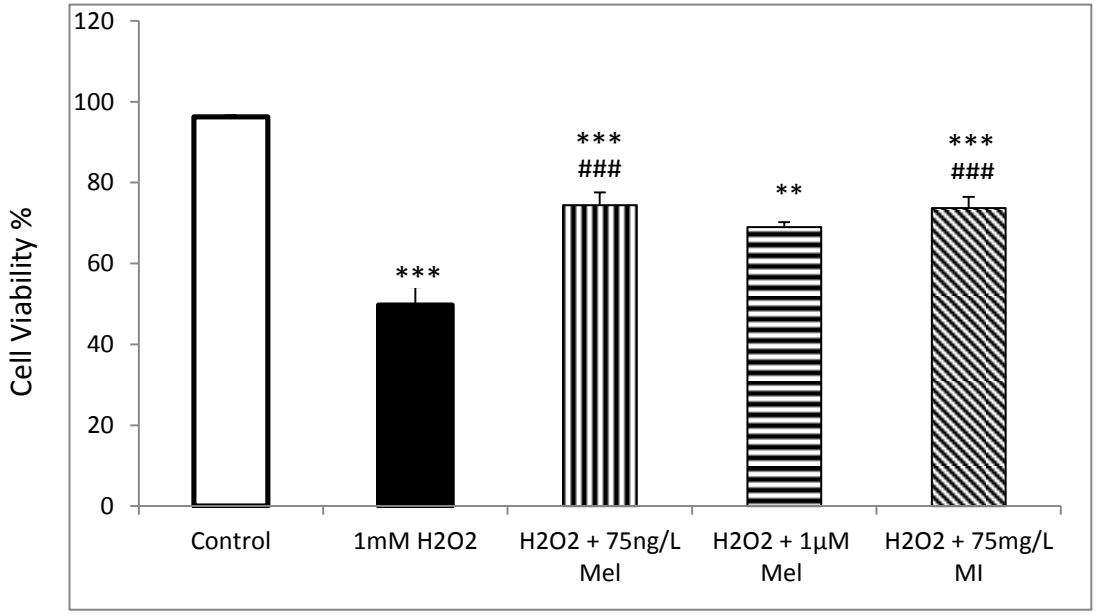


Figure 24: Cell viability expressed as percentage in H9C2 cells subjected to simulated ischemia by exposure to 1mM H_2O_2 for 2h following a 30min pre-treatment with dietary concentrations of Mel (75ng/L) and MI (75mg/L) compared with pharmacological concentration of Mel (1µM). (n=6 except for H_2O_2 + 1µM Mel where n=3, values as mean ±SEM). ***$p<0.001$ vs control, **$p<0.01$ vs H_2O_2, $^{###}p<0.001$ vs H_2O_2.

3. Combined treatment of melatonin and the melatonin isomer did not have an additive protective effect

H9C2 cells cultured in normoxic conditions, presented a cell viability of 96.3±0.7%. The addition of 1mM H_2O_2 for 2h significantly reduced cell viability to 42.5±4.5% ($p<0.001$ vs control). A combined treatment of 75ng/L melatonin and 75mg/L melatonin isomer, significantly improved cell viability to 65.0±6.2% ($p<0.01$ vs H_2O_2), but did not have an additive protective effect compared to melatonin or the melatonin isomer given alone (59.7±6.9% and 60.3±8.4% respectively).

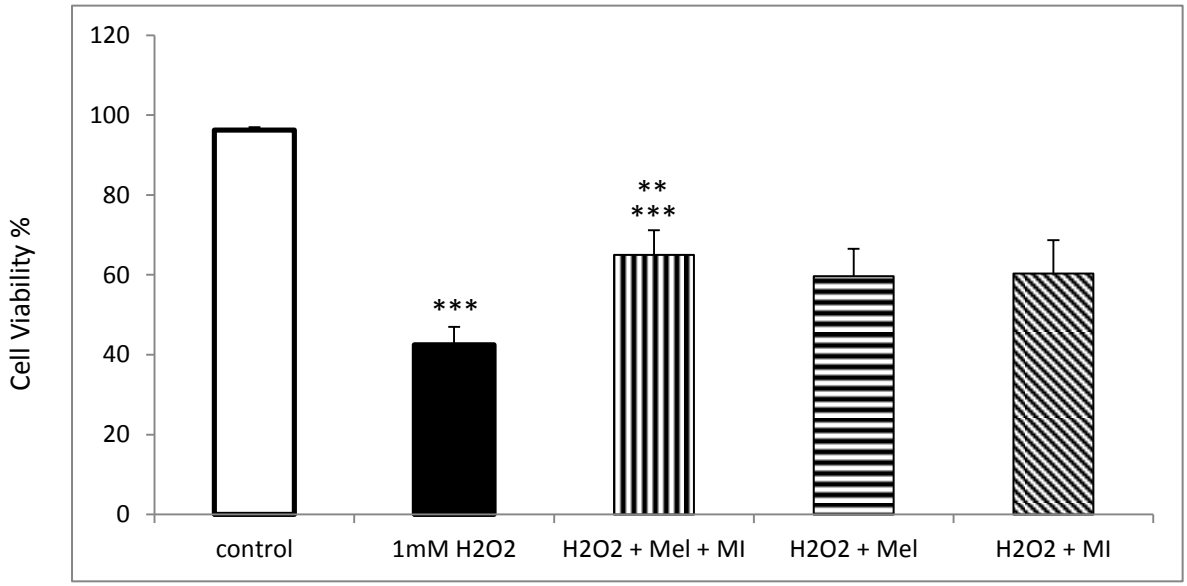


Figure 25: Cell viability expressed as percentage in H9C2 cells subjected to simulated ischemia by exposure to 1mM H_2O_2 for 2h following a combined pre-treatment of Mel (75ng/L) and MI (75mg/L), compared to individual pre-treatments (n=6, except for H_2O_2+Mel and H_2O_2+MI where n=3, values as mean ±SEM). ***$p<0.001$ vs control, **$p<0.01$ vs H_2O_2.

4. Luzindole only partially reduces the protection provided by the melatonin isomer

To test whether the cytoprotective effect of 75ng/L melatonin and 75 mg/L melatonin isomers, in H9C2 cells subjected to a simulated ischemic insult is mediated via the melatonin receptors (MT1 and MT2), the experiments were repeated in the presence of the melatonin receptor inhibitor luzindole (10µM).

H9C2 cells cultured in normoxic conditions, presented a cell viability of 95.2±2%. The addition of 1mM H_2O_2 for 2h significantly reduced cell viability to 26.7±2.2% ($p<0.001$ vs control). Pre-treatment with 75ng/L melatonin and 75mg/L melatonin isomer significantly improved cell viability to 51.9±3% and 53.3±4.3% respectively ($p<0.001$ vs H_2O_2). Luzindole significantly reduced cell viability to 29.7±2.4% when used in combination with 75ng/L melatonin ($p<0.001$ vs H_2O_2 + melatonin). There was, however, no significant reduction in cell viability when luzindole was used in combination with 75mg/L melatonin isomer, only a trend towards a decrease in protection with the cell viability at 41.4±3.6%.

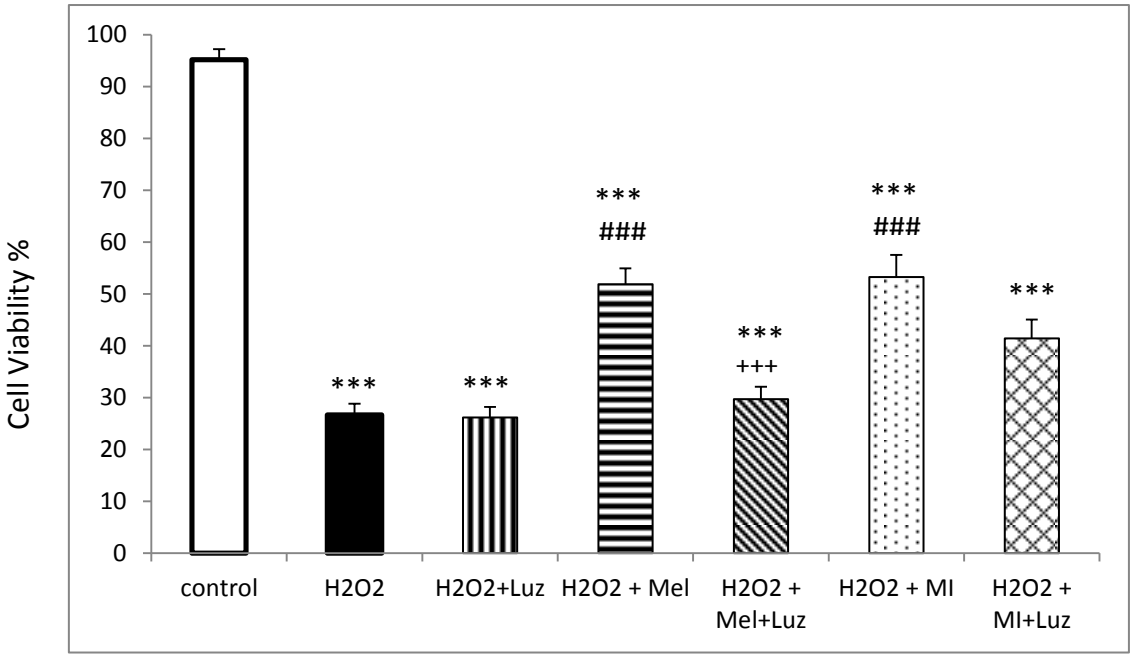


Figure 26: Cell viability expressed as percentage in H9C2 cells subjected to simulated ischemia by exposure to 1mM H_2O_2 for 2h following a combined pre-treatment of 10µM luz with 75ng/L Mel or 75mg/L MI. (n=6, values as mean ±SEM). ***$p<0.001$ vs control. $^{###}p<0.001$ vs H_2O_2. $^{+++}p<0.001<$ vs Mel.

5. Mitochondrial respirometry measurements

High resolution respirometry using an Oroboros 2K oxygraph was used to measure ROUTINE respiration in intact H9C2 cells and capacities of ETS, OXPHOS and LEAK, in digitonin permeabilized cells supplied with substrates for complex I and complex I + II. Representative oxygraph traces are shown in figure 27.

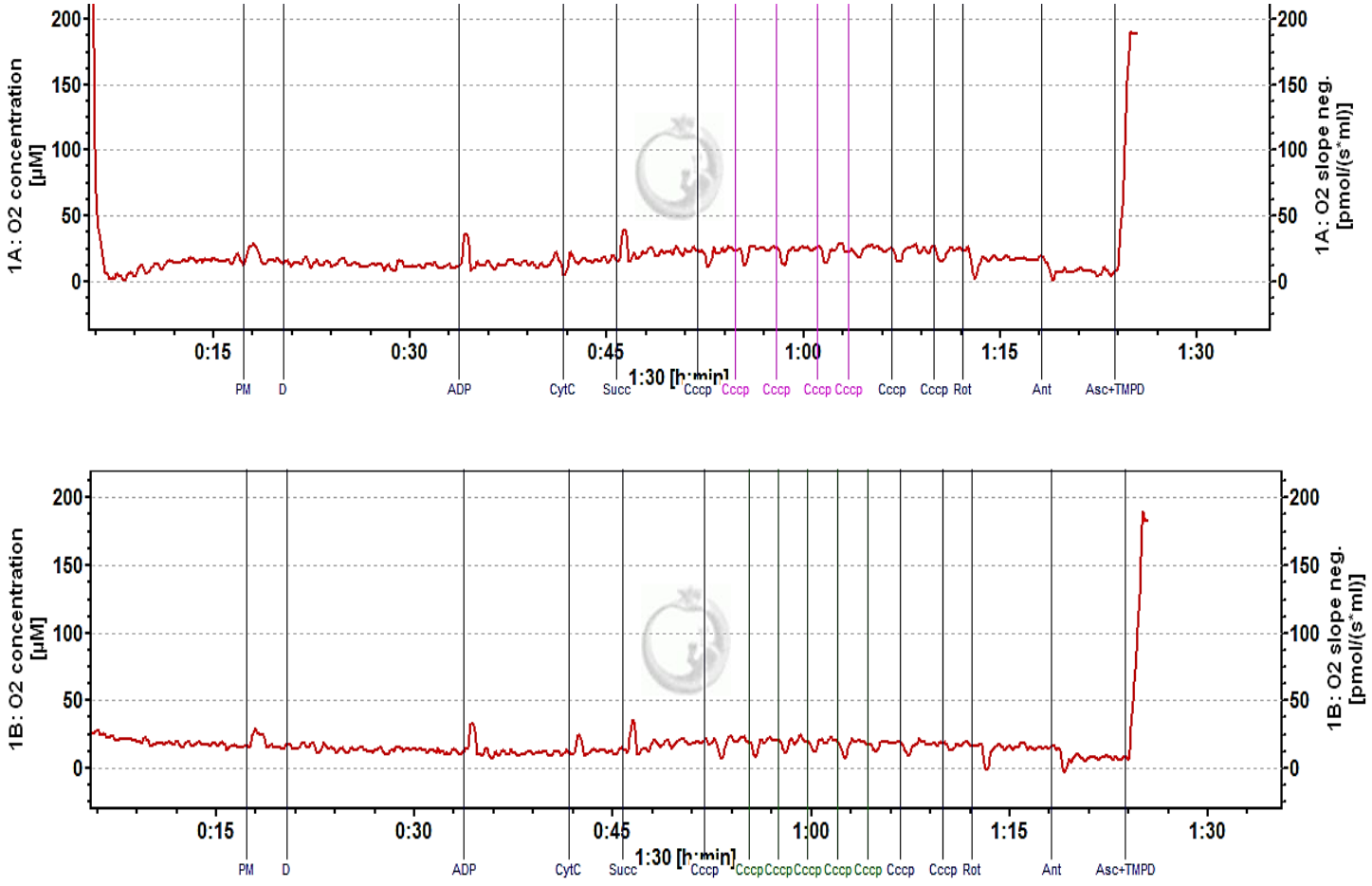


Figure 27: Representative oxygraph traces for control (1A) and H_2O_2 (1B). (PM= pyruvate and malate, D= digitonin, ADP= adenosine diphosphate, CytC= cytochrome C, Succ= succinate, CCCP= carbonyl cyanide p-(triflouromethoxy) phenylhydrazone, AntA= antimycin-A, Asc+TMPD= ascorbate and N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride).

5.1 Endogenous ROUTINE respiration

To determine the endogenous substrate and ADP availability within the H9C2 cells at the start of the experiments, ROUTINE respiration was measured.

H9C2 cells pre-treated for 30min with either 75ng/L melatonin or 75mg/L melatonin isomer, presented a ROUTINE respiration of 4.4±1.2 pmolO_2/S*ml/CIV and 6.0±0.7 pmolO_2/S*ml/CIV respectively, which did not significantly differ from the untreated control of 5.8±0.6 pmolO_2/S*ml/CIV (Figure 28A). H9C2 cells pre-treated for 30min with 75ng/L melatonin or 75mg/L melatonin isomer prior to a 15min treatment of 1mM H_2O_2, presented with a ROUTINE respiration of 5.7±1.0 pmolO_2/S*ml/CIV and 4.9±0.6 pmolO_2/S*ml/CIV respectively, with no significant difference when compared to the untreated control of 6.1±0.6 pmolO_2/S*ml/CIV and the 1mM H_2O_2 treated control of 5.9±0.5 pmolO_2/S*ml/CIV (Figure 28B).

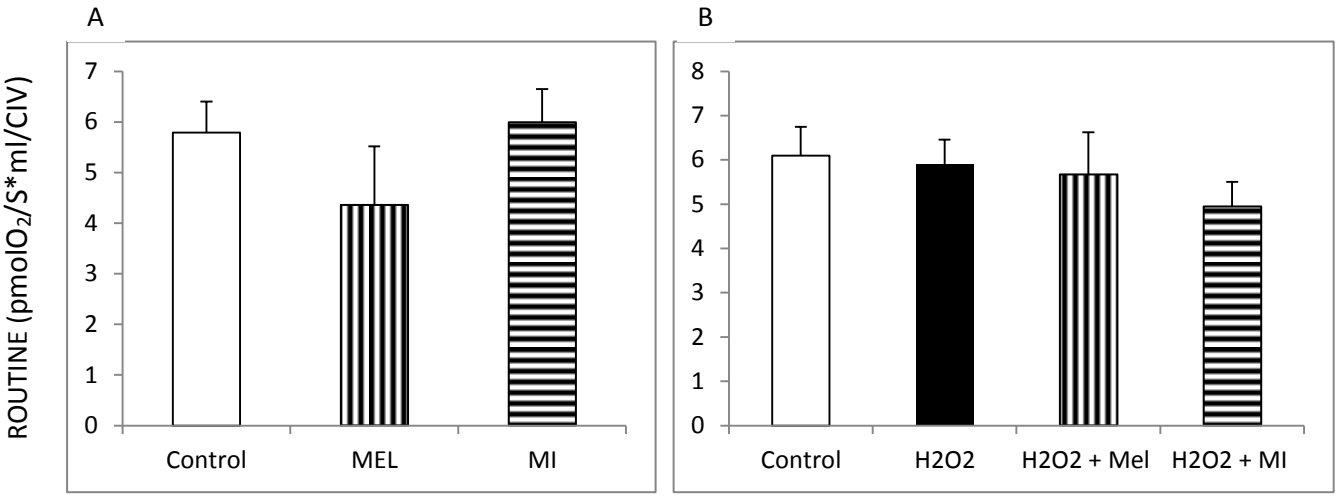


Figure 28: (A) ROUTINE respiration expressed as pmolO_2/S*ml/CIV in H9C2 cells subjected to a 30min pre-treatment with either 75ng/L Mel or 75mg/L MI (n=6, values as mean ±SEM). **(B)** ROUTINE respiration expressed as pmolO_2/S*ml/CIV in H9C2 cells subjected to simulated ischemia by exposure to 1mM H_2O_2 for 15min following a 30min pre-treatment with either 75ng/L Mel or 75mg/L MI (n=6, values as mean ±SEM).

5.2 ETS capacities

To observe changes in maximal oxygen consumption capacity, we measured the ETS capacity. This was achieved by uncoupling the respiration with CCCP in permeabilized cells in the presence of pyruvate, malate and succinate.

H9C2 cells pre-treated for 30min with either 75ng/L melatonin or 75mg/L melatonin isomer, presented an ETS of 12.2±1.4 pmolO_2/S*ml/CIV and 12.4±2.0 pmolO_2/S*ml/CIV respectively, which did not significantly differ from the untreated control of 14.0±1.8 pmolO_2/S*ml/CIV (Figure 29A). H9C2 cells pre-treated for 30min with 75ng/L melatonin or 75mg/L melatonin isomer prior to a 15min treatment of 1mM H_2O_2, presented with an ETS of 10.5±1.5 pmolO_2/S*ml/CIV and 9.2±0.8 pmolO_2/S*ml/CIV respectively, with the H_2O_2 + MI group showing a significant difference when compared to the untreated control of 15.5±2.1 pmolO_2/S*ml/CIV ($p<0.05$ vs control), but with no significant difference when comparing both the H_2O_2 + Mel and H_2O_2 + MI groups to the 1mM H_2O_2 treated control of 12.7±1.2 pmolO_2/S*ml/CIV (Figure 29B).

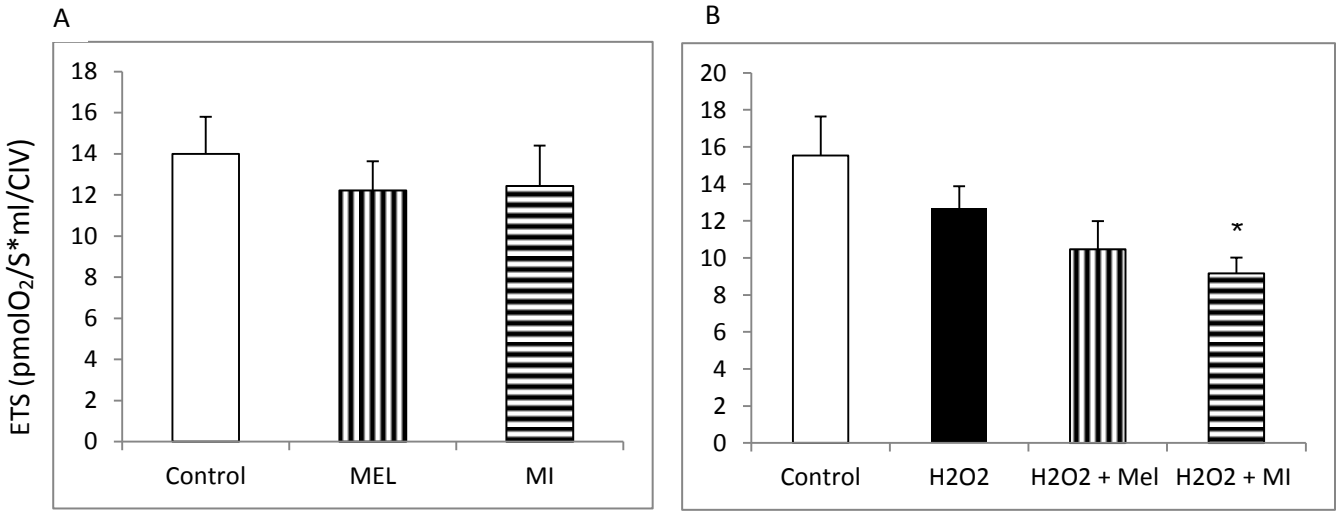


Figure 29: (A) ETS expressed as pmolO_2/S*ml/CIV in H9C2 cells subjected to a 30min pre-treatment with either 75ng/L Mel or 75mg/L MI (n=6, values as mean ±SEM). **(B)** ETS expressed as pmolO_2/S*ml/CIV in H9C2 cells subjected to simulated ischemia by exposure to 1mM H_2O_2 for 15min following a 30min pre-treatment with either 75ng/L Mel or 75mg/L MI (n=6, values as mean ±SEM).

*$p<0.05$ vs control

5.3 LEAK respiration

To determine LEAK respiration via complex I, H9C2 cells were permeabilized with digitonin and supplied with pyruvate and malate.

H9C2 cells pre-treated for 30min with either 75ng/L melatonin or 75mg/L melatonin isomer, presented a LEAK respiration of 1.7±0.4 pmolO_2/S*ml/CIV and 2.0±0.3 pmolO_2/S*ml/CIV respectively, which did not significantly differ from the untreated control of 2.0±0.3 pmolO_2/S*ml/CIV (Figure 30A). H9C2 cells pre-treated for 30min with 75ng/L melatonin or 75mg/L melatonin isomer prior to a 15min treatment of 1mM H_2O_2, presented with a LEAK respiration of 2.2±0.5 pmolO_2/S*ml/CIV and 2.1±0.3 pmolO_2/S*ml/CIV respectively, with no significant difference when compared to the untreated control of 2.1±0.4 pmolO_2/S*ml/CIV and the 1mM H_2O_2 treated control of 2.6±0.4 pmolO_2/S*ml/CIV (Figure 30B).

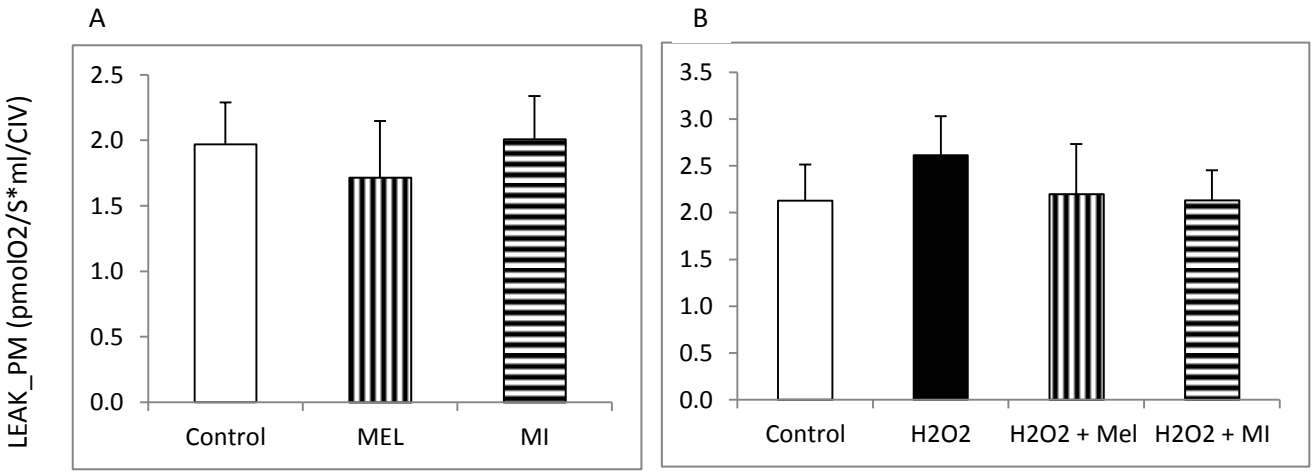


Figure 30: (A) LEAK respiration expressed as pmolO_2/S*ml/CIV in H9C2 cells subjected to a 30min pre-treatment with either 75ng/L Mel or 75mg/L MI (n=6, values as mean ±SEM). **(B)** LEAK respiration expressed as pmolO_2/S*ml/CIV in H9C2 cells subjected to simulated ischemia by exposure to 1mM H_2O_2 for 15min following a 30min pre-treatment with either 75ng/L Mel or 75mg/L MI (n=6, values as mean ±SEM).

5.4 Complex I linked OXPHOS capacity

To determine the complex I linked OXPHOS capacity of permeabilized H9C2 cells, they were supplied with pyruvate and malate in the presence of ADP. These substrates produce NADH when oxidised and therefore supply electrons to complex I only.

H9C2 cells pre-treated for 30min with either 75ng/L melatonin or 75mg/L melatonin isomer, presented an oxygen flux of 3.5±0.7 pmolO_2/S*ml/CIV and 4.1±0.4 pmolO_2/S*ml/CIV respectively, which did not significantly differ from the untreated control of 4.5±0.8 pmolO_2/S*ml/CIV (Figure 31A). H9C2 cells pre-treated for 30min with 75ng/L melatonin or 75mg/L melatonin isomer prior to a 15min treatment of 1mM H_2O_2, presented with an oxygen flux of 3.1±1.0 pmolO_2/S*ml/CIV and 2.8±0.6 pmolO_2/S*ml/CIV respectively, with no significant difference when compared to the untreated control of 5.4±1.1 pmolO_2/S*ml/CIV and the 1mM H_2O_2 treated control of 3.6±0.6 pmolO_2/S*ml/CIV (Figure 31B).

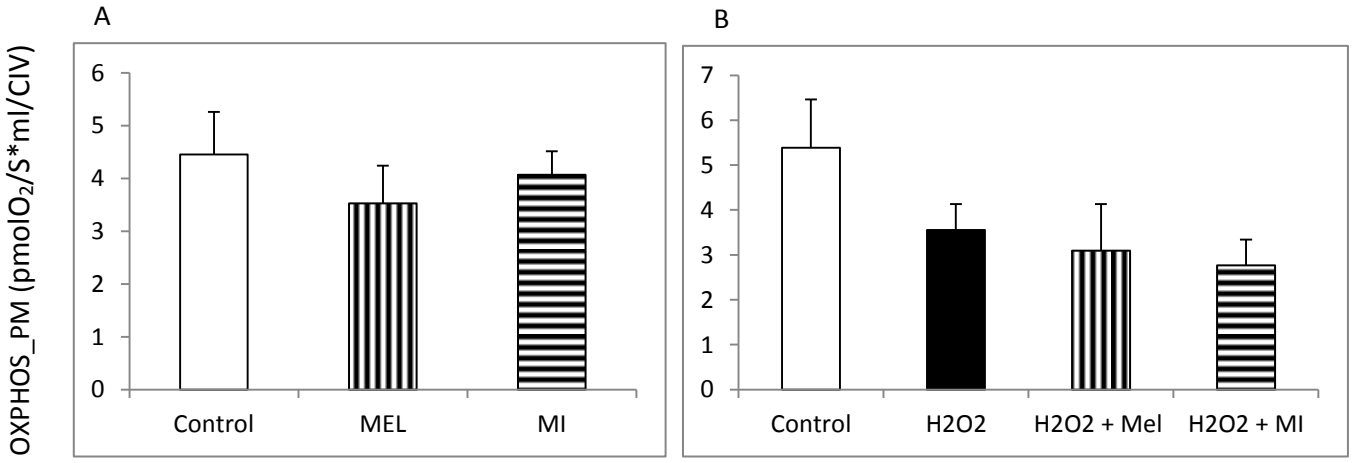


Figure 31: (A) Oxygen flux expressed as pmolO_2/S*ml/CIV in H9C2 cells subjected to a 30min pre-treatment with either 75ng/L Mel or 75mg/L MI (n=6, values as mean ±SEM). **(B)** Oxygen flux expressed as pmolO_2/S*ml/CIV in H9C2 cells subjected to simulated ischemia by exposure to 1mM H_2O_2 for 15min following a 30min pre-treatment with either 75ng/L Mel or 75mg/L MI (n=6, values as mean ±SEM).

5.5 Complex II linked OXPHOS linked capacity

To determine the complex II linked OXPHOS capacity of permeabilized H9C2 cells, they were supplied with pyruvate and malate in the presence of ADP and succinate.

H9C2 cells pre-treated for 30min with either 75ng/L melatonin or 75mg/L melatonin isomer, presented an oxygen flux of 9.6±1.2 pmolO_2/S*ml/CIV and 9.6±1.3 pmolO_2/S*ml/CIV respectively, which did not significantly differ from the untreated control of 10.2±1.5 pmolO_2/S*ml/CIV (Figure 32A). H9C2 cells pre-treated for 30min with 75ng/L melatonin or 75mg/L melatonin isomer prior to a 15min treatment of 1mM H_2O_2, presented with an oxygen flux of 8.7±1.3 pmolO_2/S*ml/CIV and 8.0±1.0 pmolO_2/S*ml/CIV respectively, with no significant difference when compared to the untreated control of 12.3±1.9 pmolO_2/S*ml/CIV and the 1mM H_2O_2 treated control of 10.6±1.2 pmolO_2/S*ml/CIV (Figure 32B).

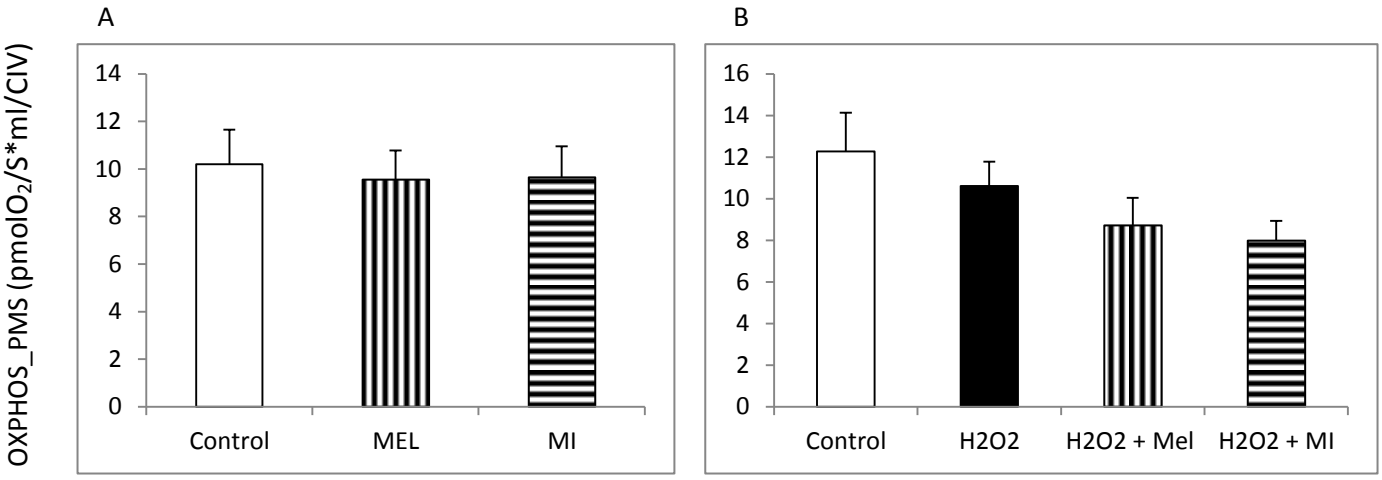


Figure 32: (A) Oxygen flux expressed as pmolO_2/S*ml/CIV in H9C2 cells subjected to a 30min pre-treatment with either 75ng/L Mel or 75mg/L MI (n=6, values as mean ±SEM). **(B)** Oxygen flux expressed as pmolO_2/S*ml/CIV in H9C2 cells subjected to simulated ischemia by exposure to 1mM H_2O_2 for 15min following a 30min pre-treatment with either 75ng/L Mel or 75mg/L MI (n=6, values as mean ±SEM).

5.6 Mitochondrial respiration control ratios and coupling efficiency

We further investigated the impact of the various treatment regimens on mitochondrial contributions to electron transport and OXPHOS capacity control ratios and coupling efficiency. To achieve this, we expressed the various oxygen fluxes as fractions of OXPHOS or ETS: LEAK control ratio (LEAK/complex I linked OXPHOS), OXPHOS control ratio (OXPHOS/ETS) and OXPHOS coupling efficiency (1-LEAK/OXPHOS)(Table 3).

Table 3. Basal mitochondrial respiratory parameters for H9C2 cells with pyruvate and malate as substrates.

	Control	Mel	MI	Control	H_2O_2	H_2O_2 + Mel	H_2O_2 + MI
LEAK control ratio (CI)	0.50±0.08	0.53±0.14	0.53±0.11	0.42±0.08	0.74±0.07	0.94±0.26	0.80±0.06
OXPHOS control ratio (CI)	0.33±0.05	0.29±0.06	0.36±0.06	0.34±0.04	0.27±0.03	0.31±0.08	0.29±0.04
OXPHOS control ratio (CII)	0.41±0.04	0.49±0.05	0.43±0.05	0.45±0.02	0 .55±0.04	0.52±0.08	0.57±0.03
OXPHOS coupling Efficiency	0.79±0.03	0.83±0.03	0.79±0.03	0.82±0.03	0.74±0.04	0.72±0.06	0.73±0.02

Values are mean ± SEM, n=6.

H9C2 cells pre-treated for 30min with either 75ng/L melatonin or 75mg/L melatonin isomer, presented with the above control ratios and coupling efficiency, which did not significantly differ from the untreated control. H9C2 cells pre-treated for 30min with 75ng/L melatonin or 75mg/L melatonin isomer prior to a 15min treatment of 1mM H_2O_2, presented with the above control ratios and coupling efficiency, with no significant difference when compared to the untreated control the 1mM H_2O_2 treated control.

E) DISCUSSION

1.1 Summary of results

The aim of this study was to establish a cell culture model of simulated ischemia to study and compare the possible cytoprotective effects of dietary melatonin and the melatonin isomer and to explore possible mechanisms involved in this effect. Our data reveal, for the very first time to our knowledge, that both dietary concentrations of melatonin and the melatonin isomer are cytoprotective in an H9C2 cardiac fibroblast cell culture model of simulated ischemia. Combining the melatonin and melatonin isomer together did not add further benefit. Furthermore, our data suggest that the cytoprotective effect of the melatonin isomer may be only partially via the activation of the melatonin receptors, whereas the cytoprotective effect of melatonin is fully dependent on the activation of the melatonin receptors. Our data measuring mitochondrial respiration did not provide evidence that melatonin and the melatonin isomer affect mitochondrial function.

1.2 Validation of the model

In order to establish a model upon which the efficacy of melatonin and the melatonin isomer could be tested against a simulated ischemic insult, we needed to validate the 8 hour duration of the hypoxic insult which would result in ±40% cell viability, as previously described in the laboratory (Lecour et al. 2006). Indeed, this percentage of viability allows for the detection of a possible protective or toxic effect of the drug which is tested. Unfortunately, the non-reproducibility of the data, as a result of a faulty hypoxic chamber, meant that another model of simulated ischemia had to be considered.

Due to H_2O_2 being widely described in the literature as a model of oxidative stress, it was chosen for our model of simulated ischemia (Lecour et al. 2014)(Lim et al. 2013)(Wang et al. 2014). This is because oxidative stress is largely caused by ROS which plays an important part in the pathogenesis of cardiac ischemic injury (Paradies et al. 2015). To validate the concentration of H_2O_2 for the experiments, H9C2 cells were exposed to three different concentrations of H_2O_2 for 2h (0.25mM, 0.5mM and 1mM).

1mM H_2O_2 was chosen to simulate ischemia as it presented with better reproducibility compared to 0.5mM H_2O_2 and it allowed us to still be able to detect a possible toxicity of the drugs tested in our model.

The protective effects of melatonin, using *ex vivo* and *in vivo* models, against myocardial ischemia/reperfusion injury is well established in the literature (Lochner et al. 2006)(Lamont et al. 2015)(Yu et al. 2017). In our setting, we tested for the first time to our knowledge, the possible cytoprotective effect of dietary melatonin on a cell culture model. To do this, the protective effect of a pharmacological concentration of melatonin on H9C2 cells had to be validated. A concentration of 1µM (0.232mg/L) was chosen, as it falls within the range of pharmacological concentrations (1µM – 50µM) used for melatonin research in the literature (Tan et al. 1998)(Lochner et al. 2006). A 30min pre-treatment with 1µM melatonin presented with a protective effect on cell viability in cells subjected to a simulated ischemic insult and thus going forward, our model to test the protective effect of dietary melatonin and the melatonin isomer on H9C2 cells included a simulated ischemia model induced by 1mM H_2O_2 for 2h and a 30min melatonin and melatonin isomer pre-treatment prior to the insult.

1.3 Dietary concentrations of melatonin and the melatonin isomer are protective against simulated ischemia

The regular and moderate consumption of red wine, containing physiological concentrations of melatonin, has shown to be cardioprotective against ischemia/reperfusion injury in *ex vivo* and *in vivo* models (Nduhirabandi et al. 2016)(Yu et al. 2015). Using our cell culture model of simulated ischemia, we compared the cytoprotective effects of the pharmacological and dietary concentrations of melatonin. Both concentrations of melatonin confer a similar protective effect against the simulated ischemic insult. Our data therefore demonstrate that an acute treatment of dietary melatonin is enough to confer full benefits, although the concentration is much lower than the pharmacological concentration of melatonin. Pharmacological melatonin is known to present very little side effects, but anti-oxidants, including melatonin, may turn into pro-oxidants in a concentration dependent manner (Cristofanon et al. 2009). In contrast, dietary melatonin raises levels of blood melatonin at a

physiological concentration and is therefore unlikely to provide any unexpected side effects (Maldonado et al. 2009).

Having confirmed that dietary melatonin has the same protective effect as a pharmacological concentration of melatonin against a simulated ischemic insult in a cell culture model, we tested whether a dietary concentration of the melatonin isomer would also confer protection. The melatonin isomer tested in our study was extracted from the wine by our colleague, Marcello Iriti. Unfortunately, the exact formula of the isomer was kept undisclosed. Our data have shown for the first time that a melatonin isomer, given at the dietary concentration as found in red wine, confers cytoprotection against a simulated ischemic insult in the cell culture model and that it protects to the same extent as a pharmacological or a dietary concentration of melatonin. As the dietary concentration of the melatonin isomer in red wine is the only concentration we tested, it is unknown whether the protection obtained is the maximum protection afforded by the isomer.

As both compounds are found in wine, melatonin and the melatonin isomer were combined during the pre-treatment to see whether they would confer an additive protective effect. Unfortunately, no additional protection was observed, therefore suggesting that each drug already offers maximal protection.

1.4 Possible mechanisms of protection

The cardioprotective effect of pharmacological concentrations of melatonin in animals has mainly been explained by its binding to the melatonin receptors (MT1 and MT2) (Genade et al. 2008). Therefore, to explore whether dietary concentrations of melatonin and the melatonin isomer were conferring protection via the melatonin receptors in our cell culture model of simulated ischemia, the cells were treated with luzindole (an MT1 and MT2 inhibitor). The combined treatment of melatonin and luzindole inhibited the protective effect of melatonin. This suggests that melatonin's actions are being mediated via the MT1 and MT2 receptors. However, the combined treatment of the melatonin isomer and luzindole only partially inhibited the protection conferred by the isomer against the simulated ischemic insult. This partial inhibition could be explained by a number of reasons: 1) Firstly, the concentration of the melatonin isomer used, as found in red wine, is 1000 times higher than that

of dietary melatonin. Therefore, the concentration of luzindole used could be insufficient to cause complete inhibition (Kocadağli et al. 2014)(Tan et al. 2014).

This explanation however, is not the most likely reason as luzindole competitively binds MT1 and MT2, meaning that, if the concentration used is sufficiently saturating the melatonin receptors in the melatonin treated group, the same should apply for the melatonin isomer group (Dubocovich & Markowska 2005). 2) Another possible reason is that the melatonin isomer's protective effects are being initiated partially via MT3 in the cell culture model. MT3 is an enzyme, quinone reductase II, which plays an important role in balancing the generation of free radicals in organisms (Tan et al. 2007). Therefore, the possible binding of the melatonin isomer to MT3 may allow it to function as a co-factor of the enzyme and to confer protection via this alternative pathway (Tan et al. 2007). Previous experiments conducted in the laboratory have already suggested the role of MT3 in the protective effect of dietary melatonin (Lamont et al. 2015). 3) The melatonin isomer could also potentially have strong anti-oxidant and cytoprotective effects which are involved in its mediation of protection. It was reported that the melatonin isomers anti-oxidant and cytoprotective properties can be altered depending on the position of either side chain A or M in the indole ring, where chain M in position 4 gives the isomer the strongest anti-oxidant capacity and chain A in position 3 makes the isomer the most effective as a cytoprotective agent (Spadoni et al. 2006)(Vitalini et al. 2013). Unfortunately, the exact formula of the isomer that we have tested is unknown to us and we are therefore unable to conclude on that point.

1.5 Role of melatonin and the melatonin isomer in mitochondrial respiration

Mitochondria are intimately involved in cell death during ischemia/reperfusion injury, which makes them vital targets in the attempt to improve cell survival and overall functional recovery of the heart. Previous work suggested that pharmacological melatonin protects against simulated ischemia by targeting the modulation of the mitochondrial permeability transition pore (Liu et al. 2015)(Andrabi et al. 2004). In our study, dietary melatonin and dietary melatonin isomer did not appear to have any significant physiological effect on mitochondrial respiration. There was also no significant protection afforded to mitochondrial respiration when melatonin and the melatonin isomer were given to cells

exposed to pathological conditions. Although the mitochondria have been described as a key element in the cardioprotective signalling paths against ischemia, our data here are inconclusive.

1.6 Limitations of the study and the way forward

Our mitochondrial respiration experiments had a relatively short pre-treatment period with melatonin and the melatonin isomer compared to recently available literature, which could have prevented the drugs from reaching their full protective capacity (Maarman et al. 2016). Further mitochondrial experiments, with different treatment periods, will need to be tested to identify the optimum time of treatment. The time of exposure (15min) to the simulated ischemic insult (H_2O_2) may have been too short to observe the adverse effect of H_2O_2 on mitochondrial function, thus preventing any protective effects of melatonin and the melatonin isomer from being seen.

In addition to our study, it would be of interest to test the melatonin isomer using an *ex vivo* or *in vivo* model of simulated ischemia as our model does not take into account the isolated heart or the role of whole systems.

Our model described an acute treatment of dietary melatonin isomer and it would be of interest to study whether this protective effect is also observed with a chronic treatment of the melatonin isomer. Indeed, this would allow us to better understand whether the melatonin isomer effectively contributes to the cardioprotective effect afforded with chronic and moderate consumption of red wine.

Finally, further experiments are needed to delineate the protective mechanisms of the melatonin isomer as well as to investigate other available melatonin isomers to determine whether their protective properties differ.

F) CONCLUSION

In conclusion, our data suggest for the first time to our knowledge that a dietary concentration of a melatonin isomer, as found in wine, is cytoprotective against a simulated ischemic insult. Moreover, we have shown that this protection is mediated, at least in part, via the activation of the melatonin receptors. Our data may lead to the development of a safe, efficient and inexpensive therapy against ischemic heart disease.

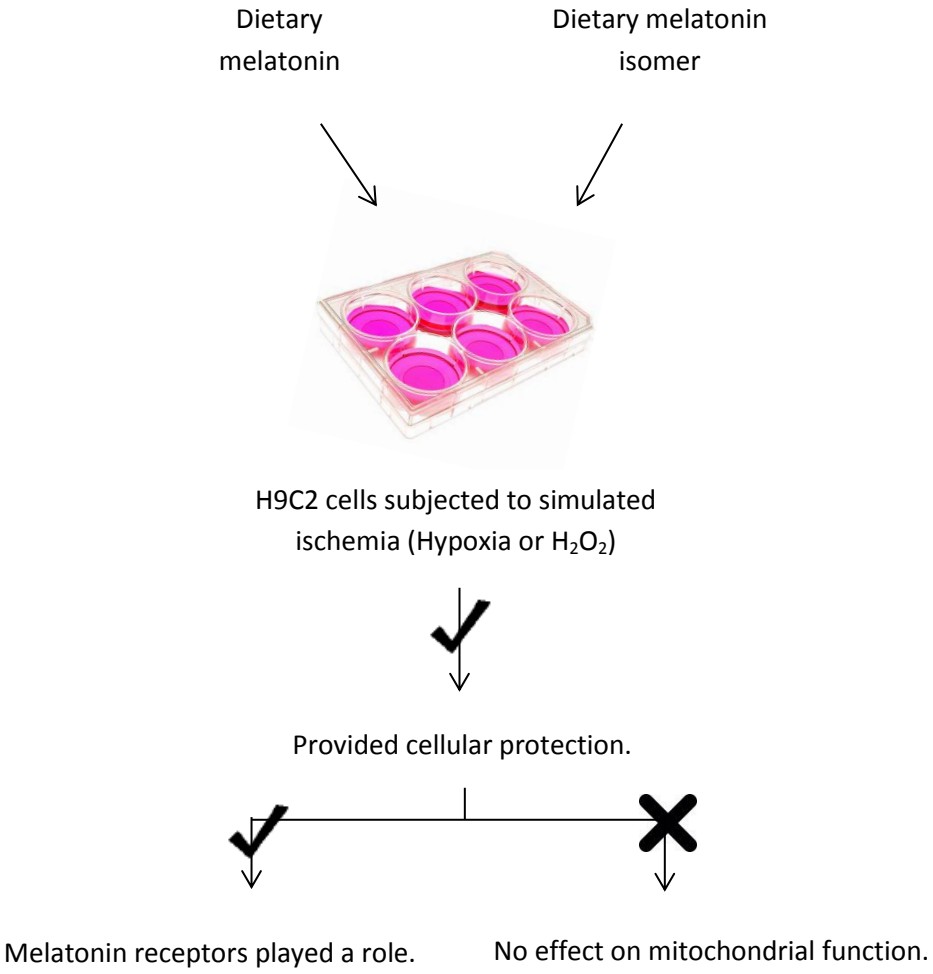


Figure 33: Schematic representation of the outcomes of the study.

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