# The Significance of Mitophagy in Myocardial Ischaemia/ Reperfusion: the effect of melatonin

by
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# ABSTRACT

Introduction:

Myocardial ischaemia and concomitant cell damage are caused by a reduction in the blood supply to the heart. To date, the most effective strategy to salvage the myocardium is timely reperfusion which is unfortunately associated with further tissue damage. This phenomenon, termed ischaemia reperfusion injury, is associated with mitochondrial structural damage which could lead to death of cells previously damaged by ischaemia. Damaged and dysfunctional mitochondria play a key role in mediating tissue damage in this setting, thus the swift yet selective removal of these damaged organelles by mitochondrial autophagy – mitophagy could be of importance in cell survival and therefore is a potential therapeutic target.

Studies have shown that upregulation of autophagy during ischaemia/reperfusion is cardioprotective, however, very little is known about the role of mitophagy in this setting. Subsequently, the aims of this study were to (i) characterise the effect of ischaemia/reperfusion on functional recovery during reperfusion and to correlate this with mitochondrial oxidative phosphorylation capacity, infarct size and mitophagy in the working heart model using male Wistar rats; (ii) evaluate the effect of mitophagy manipulation on cardioprotection using the parameters listed above. To achieve this, used was made of melatonin, the pineal hormone, which is well-known for its cardioprotective effects.

Methods:

Male Wistar rat hearts were perfused ex vivo in the working mode using Krebs-Henseleit buffer and glucose (10mM) as substrate. After a stabilization period of 30 min, hearts were subjected to 20min global ischaemia followed by 30min reperfusion during which time functional recovery was monitored. Mitochondria were isolated from hearts at different times during the perfusion protocol: after stabilization for 30min, after 20min global ischaemia and after 30min of reperfusion. The mitochondrial pellets were used for measurement of mitochondrial oxidative phosphorylation using an Oxygraph as well as for western blotting to evaluate a number of indicators of mitophagy. In addition, hearts were subjected to the perfusion protocol as described above and freeze-clamped at the same time intervals for subsequent Western blotting

for mitophagy markers in the cytosolic fraction. In a separate series melatonin (0.3, 50μM) was added to the perfusate for 10min before and 10 min after ischaemia and the same parameters evaluated as above. For evaluation of infarct size by the tetrazolium method, hearts were stabilized for 30min, followed by 35min of regional ischaemia and 60min reperfusion.

Results:

Exposure of hearts to either 35min regional ischaemia/ 60min reperfusion or 20min global ischaemia/ 30min reperfusion was associated with impaired recovery of myocardial function during reperfusion, characterized by significant reduction in several haemodynamic endpoints including coronary flow, aortic output and total work performed. Exposure to 20min global ischaemia *per se* had no effect on mitochondrial oxidative phosphorylation function, but a significant reduction in QO2 States 3 and 4 was observed after *reperfusion*, with glutamate/malate as substrates. Contrary to expectations, ischaemia/reperfusion did not upregulate mitophagy, as indicated by the reduced expression of PINK1, Parkin and TOM70 as well as markers of the alternative pathway (ULK1, DRP-1 and Rab9).

Melatonin at both concentrations studied, significantly reduced the myocardial infarct size ($p<0.0001$), but did not improve mechanical recovery during reperfusion. In the global ischaemia model, melatonin increased mitochondrial oxphos during reperfusion only. While not having marked effects on the conventional PINK1/Parkin pathway, melatonin caused significant increases in the expression and phosphorylation of ULK1 and DRP-1, suggesting upregulation of the alternative pathway of mitophagy.

Conclusion:

In this experimental model ischaemia/ reperfusion reduced (i) contractile function and (ii) oxidative phosphorylation during reperfusion. It also subdued both the (iii) conventional and alternative mitophagy pathways suggesting that mitochondrial fission, which is a prerequisite for mitophagy, may be impaired under these conditions. These changes may contribute to the impaired functional recovery during reperfusion. Melatonin's cardioprotective effects were associated with upregulation of a novel mitophagy signalling pathway, the significance of which in its cardioprotective actions needs to be further elucidated.

# ABSTRAK

Miokardiale iskemie en selbeskadiging word deur ‘n vermindering in die bloedvoorsiening aan die hart teweeggebring. Tot op hede, is herperfusie van die iskemiese hart die mees effektiewe strategie om selbeskadiging te voorkom. Ongelukkig gaan herperfusie ook met verdere selbeskadiging gepaard, die sogenaamde verskynsel van herperfusie beskadiging. Herperfusie gaan gepaard met verdere beskadiging van selle wat reeds deur die voorafgaande periode van iskemie affekteer is. Beskadigde en disfunksionele mitochondria speel ‘n belangrike rol in hierdie scenario, gevolglik is die spoedige en selektiewe verwydering van hierdie organelle deur die proses van mitochondriale outofagie/mitofagie van kardinale belang vir oorlewing. Mitofagie mag dus ‘n potensiële terapeutiese teiken wees vir beskerming van die iskemies/herperfuseerde hart.

Dit is voorheen getoon dat opregulering van outofagie tydens iskemie/herperfusie die hart teen beskadiging beskerm, maar min of geen inligting is beskikbaar aangaande die rol van mitofagie in hierdie verband nie. Die doelwitte van hierdie studie was dus (i) karakterisering van die effek van iskemie/herperfusie op funksionele herstel tydens herperfusie en die verband met mitochondriale oksidatiewe fosforilasie, infarkt grootte en mitofagie in ‘n model van die geïsoleerde, geperfuseerde rothart; (ii) evaluasie van mitofagie manipulasie op beskerming van die hart deur gebruik te maak van melatonien, ‘n hormoon afgeskei deur die pineaalklier.

Metodes

Harte van manlike Wistar rotte is *ex vivo* geperfuseer met Krebs-Henseleit buffer en glukose (10mM) as substraat, volgens die werkhart tegniek. Na ‘n stabilisasie periode van 30min, is harte onderwerp aan 20min globale iskemie gevolg deur 30min herperfusie waartydens funksionele herstel gemonitor is. Mitochondria is op verskillende tye tydens die perfusie protokol geïsoleer naamlik na stabilisasie, na 20min globale iskemie en na 30min herperfusie. Hierdie pellets is gebruik vir die meting van die oksidatiewe fosforilasie proses met behulp van ‘n Oksigraaf sowel as vir western blots vir evaluering van merkers van mitofagie. ‘n Addisionele series van harte is ook geperfuseer volgens bogenoemde protokol en die harte gevriesklamp vir

bereiding van sitosoliese fraksies vir daaropvolgende Western blots. Melatonien (0.3, 50mM) is by die perfusaat gevoeg 10min voor en 10min na globale iskemie en dieselfde parameters evalueer soos bo gelys. Vir bepaling van infarkt grootte met die tetrazolium metode, is harte gestabilseer vir 30min, gevolg deur 35min streeks iskemie en 60 min herperfusie.

Resultate

Blootstelling van die geperfuseerde hart aan of 35min streeks iskemie/60min herperfusie of 20min globale iskemie/30 min herperfusie het funksionele herstel tydens herperfusie beduidend onderdruk, soos aangedui deur die verlaging in koronêre vloei, aorta en kardiale omset. Blootstelling van die hart aan 20min globale iskemie *per se* het geen effek op mitochondriale oksidatiewe funksie gehad nie, maar ‘n beduidende onderdrukking in QO2 staat 3 en staat 4 is na herperfusie waargeneem, met glutamaat/malaat as substrate. In teenstelling met verwagtinge, het iskemie/herperfusie nie mitofagie opgereguleer nie, maar onderdruk, soos getoon deur die verminderde uitdrukking van mitochondriale PINK1, Parkin en TOM70 sowel as merkers van die alternatiewe pad van mitofagie (ULK1, DRP-1 en Rab9).

Beide hoë en lae melatonien konsentrasies het die infarkt grootte beduidend verlaag, maar nie funksionele herstel tydens herperfusie verbeter nie. Melatonien het mitochondriale oksidatiewe fosforilasie slegs tydens herperfusie na 20min globale iskemie verbeter. Melatonien het geen effek op die konvensionele pad van mitofagie gehad nie, maar het die uitdrukking en fosforilering van ULK1 en DRP-1 beduidend verhoog, wat dui op opregulering van die alternatiewe pad van mitofagie.

Gevolgtrekking

In die eksperimentele model wat gebruik is, het miokardiale iskemie/herperfusie die kontraktiele herstel en oksidatiewe fosforilasie proses onderdruk tydens herperfusie. Dit het ook beide die konvensionele en alternatiewe paaie van mitofagie onderdruk, wat moontlik dui op inhibisie van die mitochondriale fissie proses. Beide hierdie prosesse mag tot die onderdrukking in funksie bydra. Daarenteen was die beskerming deur melatonien verleen, geassosieer met opregulering van die alternatiewe mitofagie proses en dus moontlik mitochondriale fissie. Die belang van hierdie proses in die

beskerming teen iskemiese beskadiging deur melatonien moet egter nog verder ondersoek word.

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# ABBREVIATIONS

| | |
|---|---|
| L | Langendorff |
| WH | Working Heart |
| °C | Degrees Celsius |
| µg | Microgram |
| µL | Microliter |
| µM | Micro molar |
| 3-OHM | Cyclic-hydroxymelatonin |
| 5-MCA-NAT | 5-methoxy-carbonyl-N-acetyl-tryptamine |
| AAALAC | Association for Assessment and Accreditation of Laboratory Animal Care |
| ADP | Adenosine Diphosphate |
| AFMK | N1-acetyl-N2-formyl-5-methoxykynuramine |
| AIF | Apoptosis-inducing factor |
| AMK | N-acetyl-5-methoxykynuramine |
| AMPK | Adenosine monophosphate- activated protein kinase |
| ANOVA | Analysis of Variance |
| ANT | Adenine Nucleotide Translocator |
| APAF1 | Apoptotic protease activating factor 1 |
| AR | Area at Risk |
| Atg | Autophagy Related |

| | |
|---|---|
| ATP | Adenosine Triphosphate |
| bpm | Beats per minute |
| BSA | Bovine Serum Albumin |
| $Ca^{2+}$ | Calcium Ion |
| $CaCl_2$. $H_2O$ | Calcium Chloride Dihydate |
| CMA | Chaperone-Mediated Autophagy |
| CO | Cardiac Output |
| $CO_2$ | Carbon Dioxide |
| Cu | Copper |
| $CuSO_4.5H_2O$ | Copper (II) Sulphate Pentahydrate |
| CVD | Cardiovascular Diseases |
| CypD | Cyclophilin D |
| DIABLO | Direct Inhibitor of Apoptosis Binding Protein with Low Pi |
| DNA | Deoxyribonucleic Acid |
| DRP-1 | Dynamin-1-like Protein |
| ECL | Enhanced Chemiluminescence |
| EDTA | Ethylenediamine Tetraacetic Acid |
| EDTA | Ethylenediaminotetraacetic acid |
| ER | Endoplasmic Reticulum |
| ETC | Electron Transport Chain |
| $F_1F_0$ ATPase | F-type ATPase |

| | |
|---|---|
| $FADH_2$ | Flavin Adenine Dinucleotide |
| Foxo | Forkheadbox |
| g | grams |
| GABARAP | LC3/$\gamma$-aminobutyric acid receptor-associated protein |
| GPx | Glutathione Peroxidase |
| GSH | Glutathione |
| GSSG | Gluthathione Disulfide |
| GTP | Guanosine Triphosphate |
| $H^+$ | Hydrogen Ion |
| $H_2O$ | Dihydrogen monoxide |
| $H_2O_2$ | Hydrogen Peroxide |
| HDACs | Histone Deacetylases |
| HIF-1 | Hypoxia-inducible factor-1 |
| HIOMT | Hydroxyindole-O-methyltransferase |
| HPR | Horseradish peroxidase |
| HR | Heart Rate |
| HtrA2 | Omi/ High temperature requirement protein A2 |
| I/R | Ischaemia Reperfusion |
| *Ibid* | ibidem |
| IFS | Infarct Size |
| IHD | Ischaemic Heart Disease |

| | |
|---|---|
| IMM | Inner Mitochondrial Membrane |
| IRI | Ischaemia Reperfusion Injury |
| Isch | Ischaemia |
| $Ka^{+}$ | Potassium Ion |
| KCl | Potassium Chloride |
| KCL | Potassium Chloride |
| kDa | Kilo Dalton |
| $KH_2PO_4$ | Potassium Dihydrogen Phosphate |
| LAD | Left Anterior Descending |
| lb | Libra |
| LC3 | Microtubule- Associated Protein Light Chain 3 |
| LDL | Low Density Lipoprotein |
| LIR | LC3-interacting region |
| LV | Left Ventricular |
| M | Molar |
| mA | Milliamps |
| MFN2 | Mitofusin 2 |
| mg | milligram |
| mg/prot/min | Milligram Per Protein Per Minute |
| $MgSO_4.7H_2O$ | Magnesium Sulfate Heptahydrate |
| MI | Myocardial Infarct |

| | |
|---|---|
| min | Minute/s |
| mL | Millilitres |
| MLT | Melatonin |
| mmHg | Millimetres Mercury |
| MMP | Matrix Metalloproteinase |
| Mn | Magnesium |
| MOA | Monoamine Oxidase |
| MPTP | Mitochondrial Permeability Transition Pore |
| mtDNA | Mitochondrial Deoxyribonucleic Acid |
| mTOR | Mammalian Target Of Rapamycin |
| mW | milliWatts |
| n | Sample Size |
| $Na^+$ | Sodium Ion |
| $Na_2CO_3$ | Sodium Carbonate |
| $Na_2HPO_4.\ 2H_2O$ | Sodium Phosphate Dibasic Dihydrate |
| $Na_2S_2O_4$ | Sodium Dithionite |
| $Na_2SO_4$ | Sodium Sulfate |
| $Na_3VO_4$ | Sodium Orthovandate |
| NaCl | Sodium Chloride |
| NAD | Nicotinamide Adenine Dinucleotide |
| NADPH | Nicotinamide Adenine Dinucleotide Phosphate |

| | |
|---|---|
| $NaH_2PO_4$ | Monosodium Phosphate |
| $NaHCO_3$ | Sodium Biocarbonate |
| Na-K-Tartrate | Potassium Sodium Tartrate |
| NaOH | Sodium Hydroxide |
| NAT | N-acetyltransferase |
| nAtoms | NanoAtoms |
| ng | Nanogram |
| nm | Nanometre |
| nmoles | Nanomoles |
| NO | Nitric Oxide |
| NOS | Nitric Oxide Synthase |
| Nox2 | Nicotinamide Adenine Dinucleotide Phosphate Oxidase 2 |
| NRF-2 | Nuclear Respiratory Factor 2 |
| $O_2$ | Oxygen |
| $O_2^-$ | Superoxide |
| OD | Optical Density |
| $OH^-$ | Hydroxyl Radical |
| OMM | Outer Mitochondrial Membrane |
| *op.cit.* | *opera citato* |
| Oxphos | Oxidative Phosphorylation Rate/Capacity |
| PCI | Percutaneous Coronary Intervention |

| | |
|---|---|
| PE | phosphatidylethanolamine |
| PGC-1 alpha | Peroxisome Proliferator-activated receptor gamma coactivator 1-Alpha |
| PiC | Phosphate Carrier |
| PINK1 | Phosphate and Tensin Homologue-Induced Putative Kinase 1 |
| PMSF | Phenylmethylsulphonyl fluoride |
| POA | Aortic Pressure |
| PSP | Peak Systolic Pressure |
| PVDF | Polyvinylidene fluoride membranes |
| Px | Pinealectomized |
| Qa | Aortic Flow |
| Qe | Coronary Flow |
| RCI | Respiratory Control Index |
| Rep | Reperfusion |
| RIP1 | Receptor interacting protein 1 |
| RIP3 | Receptor interacting protein 3 |
| RIRR | ROS-induced ROS release |
| RISK | Reperfusion Injury Salvage Kinase |
| RNA | Ribonucleic Acid |
| RNOS | Reactive Nitrogen Oxide Species |
| RNS | Reactive Nitrogen Species |

| | |
|---|---|
| ROS | Reactive Oxygen Species |
| rpm | Revolutions Per Minute |
| S3 | State 3 |
| S4 | State 4 |
| SAFE | Survivor Activating Factor Enhancement |
| SDS | Sodium Dodecyl Sulfate |
| SDS-PAGE | Sodium Docecyl Sulfate Polyacrylamide Gel Electrophoresis |
| Sirt | Sirtuins |
| SMAC | Second Mitochondria-Derived Activator of Caspase |
| SOD | Superoxide Dismutase |
| SQSTM1 | p62/ Sequestosome-1 |
| SSA | Sub Saharan Africa |
| Stb | Stabilisation |
| TCA | Trichloroacetetic acid |
| TD | Terminally Differentiated |
| TFP | Triphenylformazan |
| TOM | Translocase of the Outer Membrane |
| Tris-HCL EGTA | Tris Hydrogen Chloride Ethylene glycol tetraacetic acid |
| Tris-HCl | Tris Hydrogen Chloride |
| TTC | Triphenyltetrazolium chloride |
| TW | Total Work |

| | |
|---|---|
| ULK | Unc-51-Like Kinase |
| UPS | Ubiquitin- Proteasome System |
| V | Volts |
| VDAC | Voltage-Dependent Anion Channel |
| VT-VF | Ventricular Tachycardia And / Or Fibrillation |
| Zn | Zinc |

# CHAPTER 1: BACKGROUND

## 1.1. Introduction

During embryogenic organogenesis, the heart, which is of mesodermal origin, is one of the earliest differentiating and functioning organs. Together with its conduits (arteries, veins and capillaries) the heart forms the cardiovascular system which has the core function of circulating blood which contains oxygen, nutrients, hormones and waste to and from cells in the body (Sandler, 2012). Like any other system in the body, the structure and function of the cardiovascular system can be altered and impaired by disease. Cardiovascular diseases (CVDs) are any disorders of the heart and blood vessels. There are several major CVDs and they include: ischaemic heart disease (IHD), cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease and deep vein thrombosis and pulmonary embolism (Steyn, 2007; WHO, 2017; Heart foundation). These major CVDs differ with regards to their underlying pathology, interaction with other organ systems and the population they occur in and are often the result of increased exposure to cardiovascular risk factors (Moran et al, 2013).

Cardiovascular risk factors, controllable and uncontrollable (Table 1), not only increase the likelihood of CVD development through past and current exposure but can also contribute to CVD progression. These known risk factors, which were introduced by the Framingham Heart Study are the same worldwide and explain the majority of CVD burden within the population (Mensah, 2013). In Sub-Saharan Africa (SSA), however, cardiovascular risk factors in conjunction with rapid urbanisation and epidemiological transition (i.e. changes in patterns of population age distributions, mortality, fertility, life expectancy and causes of death) are the key drivers of the upsurge in CVD development, progression and ultimately mortality (Morgan et al. 2013; McKeown, 2009).

Table 1: Controllable and Uncontrollable Cardiovascular Risk Factors (Adapted from Mensah, 2013; Kalogeries et al. 2012; The Heart Federation, 2017)

| Controllable | Uncontrollable |
|---|---|
| **High Blood Pressure** | Age |
| **High Total Blood Cholesterol** | Sex |
| **High Fasting Plasma Glucose and Diabetes** | Genetic |
| **Harmful Alcohol use** | Family History |
| **High Body mass index** | Poverty |
| **Nutrition: low dietary intake of fruits and vegetables, unhealthy diet** | Fetal programming |
| **Sedentary lifestyle** | |
| **Tobacco use/ smoking and exposure to second hand smoke** | |
| **Stress** | |
| **Pollution (household and ambient air)** | |
| **High Dietary Sodium intake** | |

CVDs have become a primary area of concern over the past few years since their swift incline to pandemic status. According to the World Health Organisation (WHO, 2017), CVDs account for 17.7 million deaths annually, which represents about 31% of all global deaths. Of these deaths 7.4 million were the result of IHD. In Africa communicable diseases, such as HIV/ AIDS and malaria, together with maternal, as well as neonatal risk factors are currently the leading cause of death. However, there is mounting evidence (see below) that the upsurge of non-communicable diseases such as CVDs will soon result in them being the leading cause of death and mortality in the region (Kenge et al. 2013; Mensah, 2013).

According to Keats and colleagues (2017), 38% of the deaths that occurred in Africa were related to CVD, reflecting the growing threat expressed earlier. A study conducted by Mensah and colleagues (2015) showed that SSA contributed to 5.5% of

the global deaths that were attributed to CVD. In addition, the SSA CVD contributed to 11.3% of all deaths. Three years later CVDs have been deemed one of the top three causes of death in the SSA (Mensah et al. 2013, Zühlke, 2016). Considering the above, this comes as no surprise and it is projected that by 2030 the age standard mortality rates for ischaemic heart disease will rise by an estimated 70 and 74% for men and women respectively in SSA (Onen, 2013). To further aggravate the matter, research shows that the average age at which CVD death occurs in the region is the youngest in the world (Moran et al. 2013, Kenge et al. 2013). In South Africa, this trend is very similar: data shows that CVD accounts for 18% of deaths and this comes as no surprise since about 225 people die from heart disease daily (WHO 2014; Heart Federation, 2017). Furthermore, Steyn (2007) states that more than half the deaths caused by chronic disease including heart disease occur before the age of 65 years. These premature deaths have dire implications on the country's workforce population and the economy at large.

Considering the alarming CVD morbidity and mortality rates above there is an urgent need not only for control and prevention but also for the development of new therapies or drugs that will protect the heart from damage. Over the past few years' research into interventions that can protect the heart against damage have been focused on the role of mitochondria in heart disease.

### 1.2. Research Focus

Myocardial ischaemia is characterised by reduced blood flow to the heart due to partial or complete occlusion of the coronary arteries due to atherosclerosis and is associated with necrosis and apoptosis (Steyn, 2007; Andalib, 2017). In an effort to decrease myocardial ischaemic injury, reduce the size of the infarct and improve clinical outcomes, the preferred treatment is timely myocardial reperfusion (restoration of blood flow to an ischaemic area) through primary percutaneous coronary intervention (PCI) or the use of thrombolytic therapy (Yellon and Hausenloy 2007). Although being essential for recovery, ironically, the process of reperfusion has been clearly associated with further tissue damage. This phenomenon, first described by Jennings and colleagues (1977), is known as ischaemia reperfusion injury (IRI).

In IRI, the impaired electron transport chains (ETC/s) of dysfunctional mitochondria (Zhou et al., 2015) cause free radical generation in the ischaemic/reperfused myocardium. Consequently, reperfusion is associated with huge bursts of free radicals resulting in oxidative stress, which is one of the critical role players in activating the opening of the mitochondrial permeability transition pore (MPTP), release of cytochrome c leading to apoptosis and aggravated IRI (Hausenloy and Yellon, 2003). This suggests that the mitochondrial redox state is an important modulator of cell survival (McFalls et al., 2003). For this reason it is important to explore how intrinsic processes such as mitophagy can be strengthened and manipulated to salvage cardiomyocytes in the setting of IRI.

Mitophagy is the selective removal of damaged and dysfunctional mitochondria. It is an imperative process in cells which have a high density of mitochondria such as cardiomyocytes as it enriches the pool of healthy organelles (Suen et al., 2010; Jimenez et al. 2014). Although relatively much information is available regarding the mitophagic process, little is known about its role in myocardial ischaemia/reperfusion (I/R) and cell survival, creating a need for the present study. Consequently, manipulation of the mitophagic process with melatonin, a potent antioxidant and known cardioprotectant, may provide more insight into the therapeutic potential of mitophagy in heart disease.

### 1.3. Research questions

- How does ischaemia with and without reperfusion affect mitochondrial oxidative phosphorylation function and the mitophagic process?
- Will manipulation of mitophagy affect functional recovery during reperfusion, infarct size (IFS) and mitochondrial oxidative phosphorylation capacity of isolated perfused rat hearts subjected to I/R?

### 1.4. Research Aims

Aim 1: To characterise the effect of I/R *per se* on the relationship between (i) functional recovery during reperfusion, (ii) mitochondrial oxidative phosphorylation capacity, (iii) IFS and (iv) mitophagy in the working heart model using male Wistar rats.

Aim 2: To evaluate the effect of manipulation of mitophagy by the pineal hormone, melatonin, on cardioprotection, using the parameters listed in aim 1.

## 1.5. Research Objectives

Aim 1: Objectives

i. Determination of the effect of global I/R on the mechanical function of isolated perfused working rat hearts.
ii. Isolation of mitochondria from hearts in objective (i) for evaluation of oxidative phosphorylation capacity and mitophagy.
iii. Evaluation of the functional recovery during reperfusion and IFS of isolated perfused hearts subjected to regional ischaemia.

Aim 2: Objectives

i. Examination of the effect of high (50µM) or low (0.3µM) melatonin concentrations on the functionality and recovery of isolated rat hearts subjected to global I/R.
ii. Isolation of mitochondria from hearts in objective (i) for evaluation of oxidative phosphorylation capacity and mitophagy.
iii. Evaluation of the effects of high (50µM) or low (0.3µM) melatonin concentrations on the functional recovery and IFS of isolated hearts subjected to regional ischaemia.

# CHAPTER 2: LITERATURE REVIEW

## 2.1. Myocardial Ischaemia/Reperfusion and Injury

Myocardial I/R embodies the transition of cardiomyocyte well-being from health to disease, adaptation and recovery. Theoretically speaking one would assume that treatment would restore cardiomyocyte well-being back to good health immediately. However, as this review reveals the intricacies of I/R, one will soon realise that the journey back to good health is a fight for survival.

This review focuses on cardiac metabolic as well as mitochondrial processes that are central to the proposed study. For a more detailed description of the pathophysiology of myocardial IRI, the following outstanding reviews (Murphy and Steenbergen 2008; Kalogeris et al. 2012; Mzezewa 2017) are recommended.

## 2.2. Pathophysiology of Myocardial Ischaemia/Reperfusion injury

The actin and myosin filaments in cardiomyocytes are crucial components of cross-bridge formation and ultimately contraction. Adenosine triphosphate (ATP), which is generated mainly through mitochondrial oxidative phosphorylation, an oxygen dependent process, and the metabolism of substrates such as glycogen and free fatty acids, are not only responsible for the various intracellular processes, but are also essential for the activation of myocardial contraction.

Under normal oxygen conditions, the heart maintains high oxygen extraction levels of about 70-80% to generate 95% of the ATP levels which are derived from mitochondrial oxidative phosphorylation (also known as aerobic respiration). The remaining 5% is derived primarily from glycolysis and the citric acid cycle (Dunker and Bache 2008; Doenst et al. 2013). Approximately 60-70% of the generated energy is used primarily for cardiac work while the rest is used for the functioning of several ion pumps, particularly the $Ca^{2+}$- ATPase located in the sarcoplasmic reticulum (Doenst et al. 2013). Considering that the heart is a continuously contracting organ and which is devoid of hefty energy reserves, a constant, stable supply of oxygen and nutrients is vital for ATP generation and subsequent cardiac function. In light of the above, it is evident that an episode of myocardial ischaemia will have serious implications not only for cardiomyocytes but the heart at large.

### 2.2.1. Effects of Ischaemia

Myocardial ischaemia, which is characterised by a reduction in blood supply, can be either global or regional. Global ischaemia is commonly associated with hospital procedures such as seen during a cardiac operation while regional ischaemia is characterised by complete or partial occlusion of coronary arteries as seen in ischaemic heart disease (Adapted from Boyle et al. 1996). An ischaemic myocardium is associated with a variety of metabolic and biochemical changes. At a molecular level, the lack of oxygen induces the reduction or cessation of oxidative phosphorylation, ATP depletion, depolarisation of the mitochondrial membrane and subsequently, depression of contractile function. In an effort to re-establish homeostasis, cardiomyocytes will invest in the following compensatory mechanisms described below (**Fig 1**).

Initially, cardiomyocytes will switch to and increase anaerobic glycolysis for ATP generation, which in turn, results in the production and accumulation of lactate and hydrogen ions and consequently acidification of the intracellular milieu which prevents opening MPTP and cardiomyocyte hypercontracture (Kalogeris et al. 2012). The accumulation of hydrogen ions triggers the activation of the $Na^+$-$H^+$ ion exchanger which will remove $H^+$ in exchange for $Na^+$ entry. In an attempt to maintain mitochondrial membrane potential, the F-type ATPase ($F_1$ $F_0$ ATPase) pump functions in reverse, resulting in the breakdown of the available ATP as well as the accumulation of mitochondrial inorganic phosphate leaving the cardiomyocyte with no ATP supply. This ATP deficiency interrupts the functioning of the $3Na^+$-$2K^+$ ATPase pump thus aggravating the intracellular $Na^+$ overload. Subsequently, the reverse action of the $2Na^+$-$Ca^{2+}$ ion exchanger is activated and $Na^+$ is extruded from the cell with concomitant $Ca^{2+}$influx, which results in intracellular $Ca^{2+}$ overloading. Research shows that, intracellular $Ca^{2+}$ overload induces a whole cascade of events that activate a wide range of systems that contribute to ischaemic cell death.

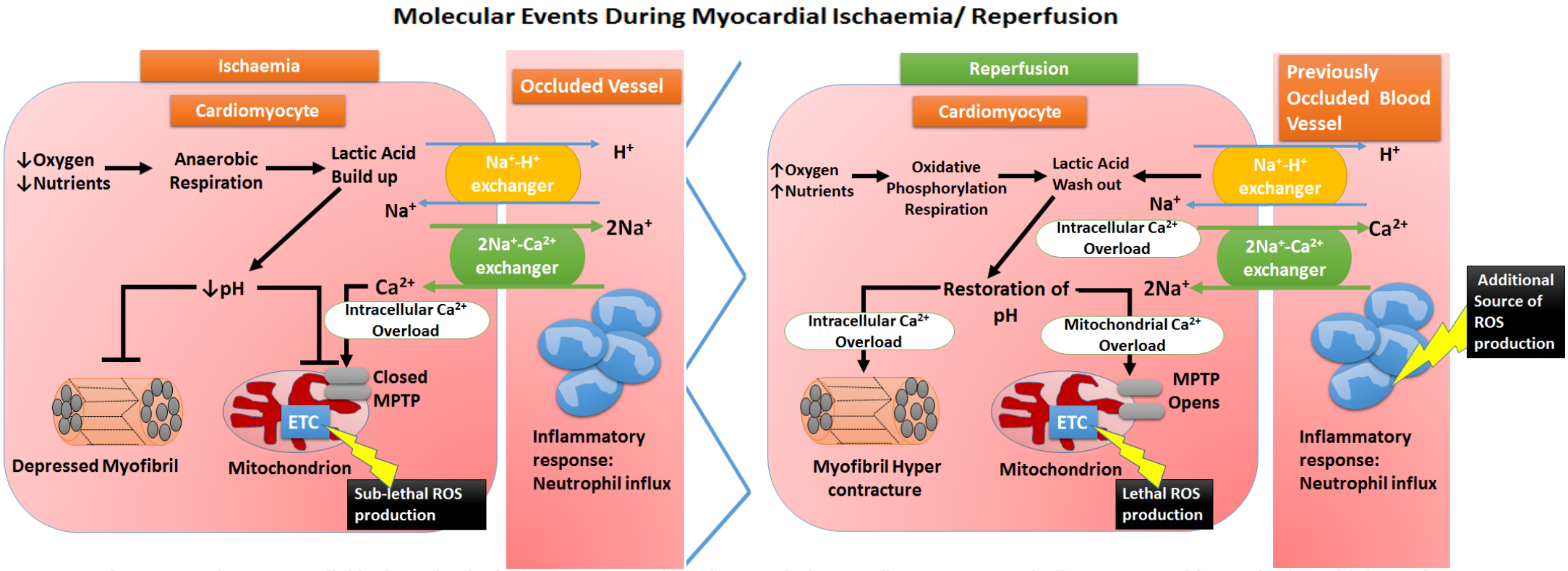



***Figure 1: During myocardial ischaemia, the lack of oxygen and nutrients switches cardiomyocyte metabolism to anaerobic respiration which results in the build-up of lactic acid and an acidic intracellular pH. This triggers activation of the Na+-H+ exchanger to extrude H+ and intrude Na+, which results in Na+ overload. Na+ overload activates the 2Na+-$Ca^{2+}$ exchanger to function in reverse by removing 2Na+ in exchange for $Ca^{2+}$, leading to intracellular calcium overload. The acidic milieu not only prevents myofibril contraction but it keeps the MPTP closed. During reperfusion, oxygen and nutrient levels are restored and this switches cell metabolism back to oxidative phosphorylation and leads to the wash out of lactic acid and the production of lethal ROS levels. Lactic acid wash out in turn restores the pH to physiological levels. Intracellular calcium overload triggers myofibril hypercontracture as well as mitochondrial $Ca^{2+}$ uptake leading to mitochondrial $Ca^{2+}$ overload. Lethal ROS production together with mitochondrial $Ca^{2+}$ overload trigger MPTP opening. IRI also triggers the activation of the inflammatory response (Adapted from Hausenloy and Yellon, 2013). Abbreviations: ROS- Reactive Oxygen Species; ETC- Electron Transport Chain; MPTP- Membrane Permeability Transition Pore; $Ca^{2+}$- Calcium Ion; Na+- Sodium Ion; H+ - Hydrogen Ion.***

In an attempt to deal with the lethal intracellular concentration of $Ca^{2+}$, cardiomyocytes trigger the uptake of $Ca^{2+}$ into the mitochondria via the mitochondrial $Ca^{2+}$ uniporter (Contreras et al. 2010; Szydlowska and Tymianski 2010). $Ca^{2+}$ overload in the mitochondria can in turn result in the formation and opening of the MPTP which ultimately leads to cell death (**Fig 1**). In addition, intracellular $Ca^{2+}$ overload can induce the activation of degrading enzymes such as nucleases, phospholipases and proteases.

When the ischaemic period is long enough, these changes can compromise the membrane integrity and ultimately lead to cell death (Halestrap and Pasdois 2009). Another deadly target of intracellular $Ca^{2+}$ overload is a family of cysteine proteases known as calpains. Their activation culminates in the degradation of mitochondrial and intracellular proteins. Calpain activity also modifies the contractile machinery by decreasing their $Ca^{2+}$ sensitivity and maintaining impaired contractility despite the upsurge in intracellular $Ca^{2+}$ (Buja et al. 1988; Buja 1991; Thandroyen et al. 1992; Avkiran and Marber 2002; Croall and Erfeld 2007). Furthermore, the calcium hypothesis suggests that $Ca^{2+}$ overload results in the damage of the mitochondrial ETC which in turn leads to the production of free radicals (Goldhaber and Weiss 1992; Maxwell and Lip 1997).

Changes induced by ischaemia are not only evident at a molecular level but are also imprinted in the cardiomyocyte ultrastructure. Ultrastructural changes can be induced by periods of ischaemia as short as 10min and deteriorate with increased ischaemic time (Edoute et al. 1983).

Ischaemic damage is characterized by cell swelling, swollen or disrupted mitochondria, sarcoplasmic reticulum as well as changes in nuclear chromatin. Prolonged ischaemia can also result in the wave front phenomenon which is the death of cardiomyocytes from the sub endocardium towards the epicardium over time (Reimer et al. 1977). Thus while the changes in ischaemia start at a molecular level, it is important to bear in mind that their effects extend to the tissue and organ levels.

It is evident that myocardial ischaemia results in an imbalance between the oxygen demand and supply and culminates in either the dysfunction or damage of the cardiac tissue or death with adequate ischaemic periods (Grover et al. 2004; Murphy and

Steenbergen 2008; Frank et al. 2012). Considering that ischaemia-induced changes are initially reversible, becoming irreversible after prolonged periods of ischaemia, it becomes important to act within the window of opportunity i.e. prior to the manifestation of irreversible changes. Thus currently, the most effective treatment strategy against myocardial ischaemia is timely myocardial reperfusion.

### 2.2.2. Effects of Reperfusion

Myocardial reperfusion is the revascularisation and subsequent restoration of blood flow to the ischaemic myocardium (Kalogeris et al. 2012). It is associated with reoxygenation and substrate delivery for oxidative phosphorylation and normalisation of the previously acidic intracellular milieu of cardiomyocytes. Paradoxically, while myocardial reperfusion is initiated with the intention to salvage the viable myocardium, limit the size of the myocardial infarct (MI), preserve left ventricular (LV) systolic function and to avert the start of heart failure, it also induces tissue damage (Yellon and Hausenloy, 2007). This phenomenon known either as IRI, reperfusion injury or lethal reperfusion injury, is one of the four types of cardiac tissue dysfunctions that can occur due to myocardial reperfusion. The other dysfunctions include myocardial stunning, reperfusion-induced arrhythmias and microvascular obstruction. These dysfunctions will be briefly explained below.

Myocardial stunning refers to the contractile dysfunction that continues during myocardial reperfusion regardless of the absence of irreversible damage and the restoration of normal or close to normal coronary flow (Braunwald and Kloner 1982; Duncker et al., 1998). This phenomenon has various triggers and modulators (Duncker et al., 1998) and the myocardium usually recovers from this damage after several days or weeks (Yellon and Hausenloy, 2007). The sudden reperfusion of the myocardium in patients that undergo PCI may experience ventricular arrhythmias, reperfusion-induced arrhythmias, which can self-terminate or be treated (Hearse et al., 1977; Hausenloy and Yellon, 2013). Microvascular obstruction, which was defined by Krug et al. in 1966, is the failure to reperfuse a previously ischaemic region. Some contributing factors are capillary damage accompanied by impaired vasodilation and neutrophil plugging (for reviews see Duncker et al. 1998; Moens et al. 2005).

There is a great deal of uncertainty about the contribution of ischaemic injury and reperfusion injury to cardiomyocyte death, which is partly due to the inability to accurately monitor the progress of necrosis during the change from myocardial ischaemia to reperfusion. This has sparked debates between researchers about whether IRI is an independent mediator of cardiomyocyte death separate from ischaemic injury or whether reperfusion only aggravates the cellular injury that was incurred during the ischaemic period (Yellon and Hausenloy, 2007; Kalogeris et al. 2012).A hypothetical scheme (**Fig 2**) by Yellon and Hausenloy (*ibid*) shows that while reperfusion has great therapeutic potential, this potential is masked by the effects of the sustained injury due to IRI. In addition, this scheme also argues in favour of the theory that reperfusion only aggravates the cellular injury that was incurred during the ischaemic considering that without reperfusion the ischaemic damage incurred is extensive and the introduction of reperfusion does decrease the damage. Furthermore, reperfusion with a potent cardioprotective intervention further reduces IRI and results in a significant reduction in IFS.

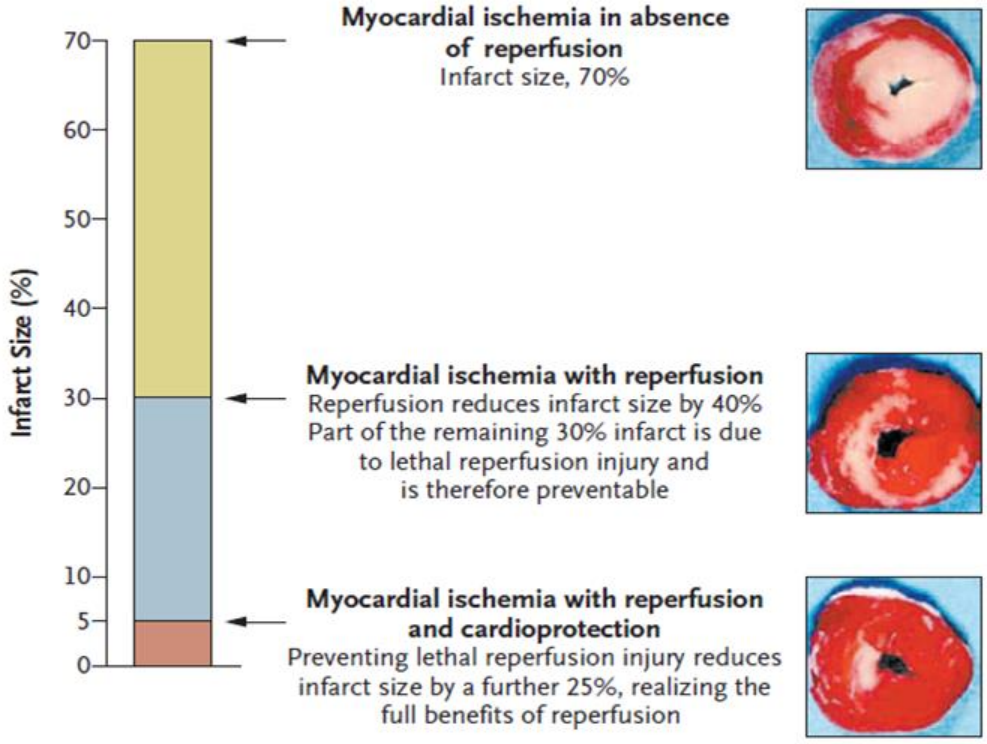



***Figure 2: Hypothetical illustration of the contribution of myocardial ischaemia, myocardial ischaemia with reperfusion and myocardial I/R plus a potent cardioprotectant to IFS due to IRI (Yellon and Hausenloy, 2007). Abbreviations: IFS- Infarct Size***

Despite this controversy, it is well established that during myocardial reperfusion IRI is the principal mediator of cardiomyocyte death. IRI has various mediators for example: intracellular $Ca^{2+}$ overload, opening of the MPTP, inflammation, pH and oxidative stress in the form of reactive oxygen species (ROS). All these mediators have variable contributions to IRI and while their mechanisms of action attempt to restore homeostasis, their combined effect often overwhelms the cell and culminates in IRI. While all these mediators result in deleterious effects, ROS remains the front-runner in mediating cell death (Anaya-Pardo et al. 2002). In light of the above, it is evident that there is a need for therapeutic interventions and strategies that will efficiently abrogate all the mediators of IRI or the chief mediator of IRI i.e. oxidative stress (as depicted in **Fig 3**).

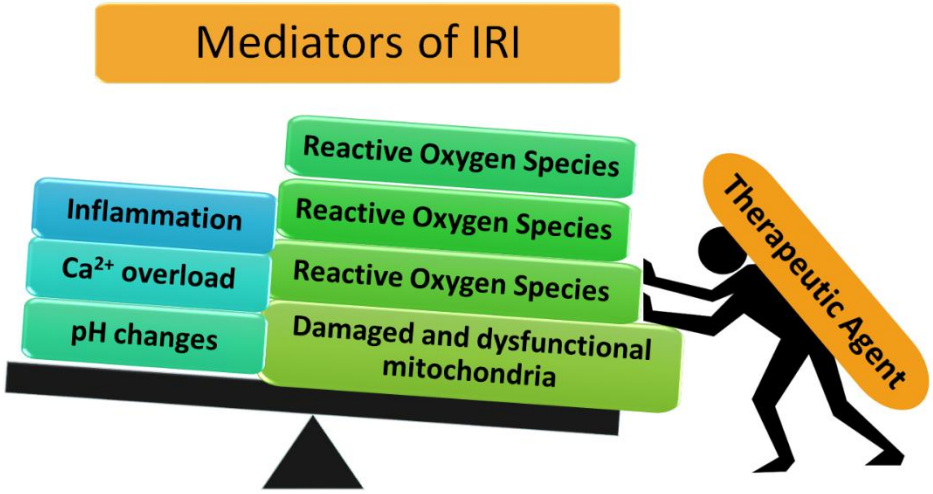



***Figure 3: Schematic representation of the various mediators of IRI. Much is known about the mediators of IRI such as inflammation, $Ca^{2+}$ overload and pH changes. However, it is evident that ROS production from damaged and dysfunctional mitochondria is at the forefront of mediating damage. Consequently, in the setting of IRI an efficient therapeutic agent is needed to restore the balance. Abbreviations: IRI- Ischaemia Reperfusion Injury; ROS- Reactive Oxygen Species; $Ca^{2+}$- Calcium Ion.***

### 2.2.3. Cardioprotection

A major breakthrough of a non-pharmacological intervention or mechanical intervention was in the form of ischaemic conditioning. The discovery of ischaemic preconditioning and later remote conditioning as well as postconditiong by Murray et al. (1986), Przyklenk et al. (1993) and Zhao et al. (2003) respectively, revealed that brief ischaemia/ reperfusion cycles prior to an ischaemic insult, as in preconditioning,

or to a remote organ, as in remote conditioning or at the onset of reperfusion, as with postconditioning can trigger adaptive responses that protect the heart against IRI. The discovery of preconditioning had far-fetched implications: not only did it prove to be the most effective intervention as yet to *reduce IFS*, but subsequent studies shed light on the *signalling mechanisms* involved in cardioprotection. This in turn, led to a shift towards pharmacological interventions such as adenosine, opioids and bradykinin receptor agonists, to name but a few. Experiments and clinical trials have focused on interventions ranging from increasing the bioavailability of nitric oxide (NO), scavenging of free radicals, inhibition of platelet aggregation to attenuating the accumulation of hydrogen peroxide in post ischaemic tissue, targeting the MPTP, metabolic modulation, temperature modulation, $Na^+/H^+$- exchange inhibitors, anti-apoptotic and anti-inflammatory drugs and more recently to anaesthetics (Grisham et al. 1998; Anaya-Pardo et al. 2002; Moens et al. 2004; Yellon and Hausenloy 2007; Yellon and Hausenloy 2013; Ibáñez et al. 2015; Xia et al. 2016). Advances in gene therapy and stem cell biology also brought new and exciting approaches that still need clinical evaluation (Isner, 2002; Buja and Vela, 2008; Spath et al., 2016). In addition to the above, new animal models have also been introduced to identify novel therapies (Asnani and Peterson, 2014).

Thus the discovery of IRI paved the way for the investigation of various therapeutic strategies, some of which not only made their way to the clinical trial platform, but they also managed to unravel certain intricacies about IRI that were as yet unknown. Unfortunately a number of strategies that have been very successful in animal models failed to produce similar beneficial results in human patients.

Thus, since the translation of these treatments from the bench to the bedside yielded very little or no fruits, the quest for new therapeutic strategies continues. More recently, there has been great interest in the role of the mitochondrion as a regulator of energetics and cell viability (Murphy and Steenbergen, 2008). According to Buja and Weeransinge (2010) the most deleterious event in IRI is “the loss of mitochondrial integrity subsequent to opening of the membrane PTP”, which again reiterates the point that the mitochondrial redox state is an important modulator of cell survival in IRI (McFalls et al. 2003). Thus recent studies have become directed at regulating

mitochondrial function (Spath et al. 2016). The section below will attempt to unravel the role of mitochondria in the setting of IRI and their potential as a therapeutic target.

## 2.3. Mitochondria, ROS and Cell Damage

### 2.3.1. Mitochondria

Mitochondria are simplistic organelles that play a vital role in the cell. They consist of an outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM). The OMM is responsible for separating the entire organelle from the cytoplasm and also consists of channels known as porins for the transport of certain molecules (**Fig 4**). The IMM divides the internal components into various compartments namely the matrix core and the intermembranous space which is compartmentalised into plenty of cristae (Hwang and Kim 2013). Amongst other things, the selectively permeable IMM is responsible for maintaining the transmembrane potential (Turer and Hill 2010). In the myocardium mitochondria are packed into dense complex structures that account for about 35% of the myocardium cell volume (Hwang and Kim, 2013). They have a wide range of functions and play a role in NADPH, ATP and GTP production in the citric acid cycle, biosynthesis of amino acids, calcium signalling, stress response and cell death (Kühlbrant 2015). Despite their multiple functions mitochondria are well-known for their central role in ATP production and their infamous role in the production of ROS and cell death in certain pathological conditions.

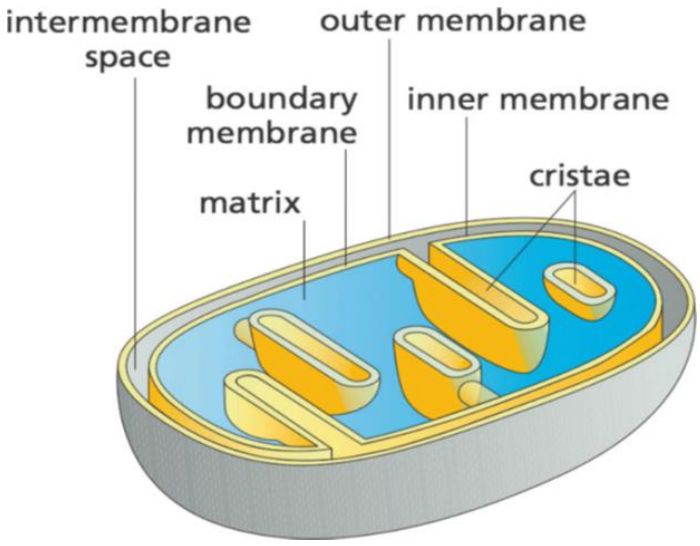



***Figure 4: Schematic of the mitochondrial structure and various compartments (Kühlbrant 2015).***

### 2.3.1.1. Oxidative phosphorylation

As previously mentioned, the bulk of ATP production is produced through mitochondrial oxidative phosphorylation. This process is localised in the IMM where the ETC and associated enzymes interact to meet the energy demands of the cardiomyocytes. Briefly, under normoxic conditions, the generation of ATP commences when the soluble carrier molecule NADPH and $FADH_2$ feed electrons from various metabolic processes into Complex I and Complex II of the ETC respectively. The electrons are transferred to a quinol in the membrane and subsequently to Complex III. From Complex III electrons are transferred to Complex IV and ultimately to oxygen to form water (**Fig 5**).

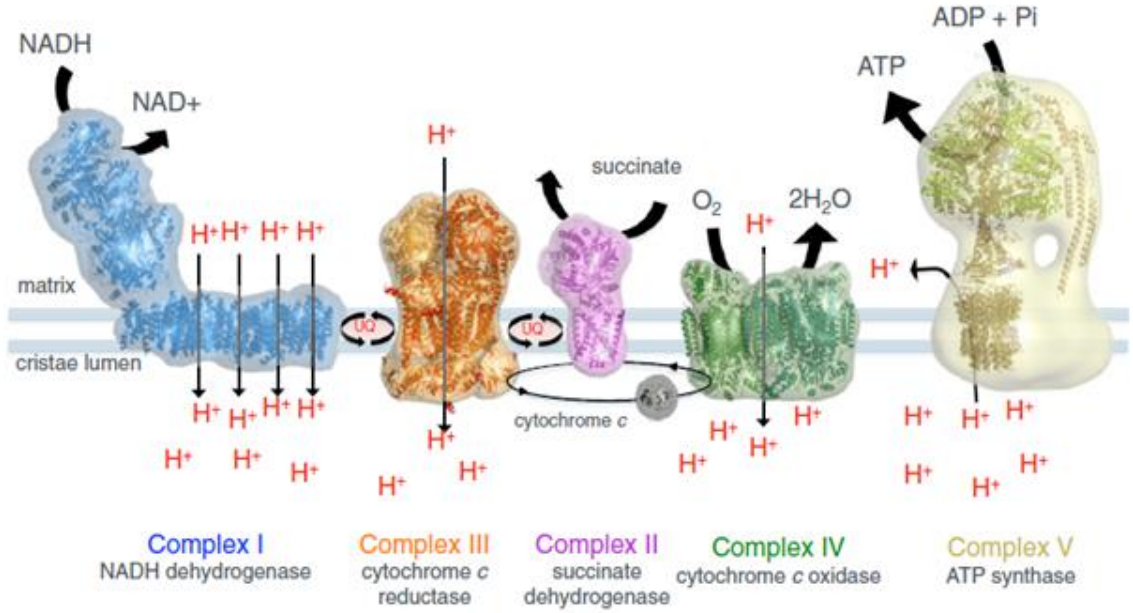



***Figure 5: Schematic representation of the ETC and the various enzymes (Kühlbrant, 2015).***

It is well established that while more than 90% of the oxygen that enters the cell will be used for the production of water about 1-2% of this oxygen will contribute to the formation of superoxide, a form of ROS which will be discussed in the next section (Chen Q et al. 2003; Murphy and Steenbergen, 2008; Kalogeris et al. 2012). Superoxide production is the result of the reaction between “leaking electrons” from complex I and III and the unreduced oxygen. Throughout the electron transfer process,

protons are being pumped from the matrix into the crista lumen creating the proton gradient that will be ultimately used for the phosphorylation of ADP to form ATP (Kühlbrant, 2015). According to Hwang and Kim (2013) the mitochondrial oxidative phosphorylation regulatory pathway remains to be fully understood, however studies do suggest that cytosolic calcium ($Ca^{2+}$) and ADP/ATP/inorganic phosphorous play a vital role in the regulation of energy generation.

For the purpose of this study the role of mitochondria in ROS production and cell death will be discussed briefly. (For more comprehensive and in-depth reviews on the structure and function as well as the role of calcium in the mitochondria see the following references: Hwang and Kim, 2013; Kühlbrant, 2015; Andalib et al. 2017; Mzezewa 2017).

#### 2.3.1.2. Mitochondria and ROS

While the ETC and concomitant production of ATP are essential for contractile activity, they are also associated with the generation of ROS. Reactive oxygen species are often referred to as free radicals, which are atoms or molecules that have unpaired electrons and exist independently (Raedshelders et al. 2012). This definition however does not encompass molecules such as hydrogen peroxide, which form part of ROS but are in fact non- free radicals. Thus, ROS should be defined as free radicals and non-free radicals which are produced from oxygen (Poljsak et al. 2013). ROS can be in the form of superoxide ($O_2^-$), which the most abundant form of ROS, hydrogen peroxide ($H_2O_2$) and the very toxic hydroxyl radical ($OH^-$).

Mitochondria are responsible for ~90% of cellular ROS production, with complexes I and III being the chief contributors to superoxide production (Balaban et al. 2005; Herrero and Barja 1997). The beneficial effects of ROS are evident at basal levels, where they are released in low or adequate amounts by the ETC and other cellular processes. This allows them to function as mediators for numerous cellular signalling cascades such as stress adaptation and cell growth (Zhou et al. 2015). The beneficial effects of ROS are also seen in ischaemic preconditioning and postconditioning, where ROS has been suggested to function as a secondary messenger in activation of conditioning mediators as well as the Reperfusion Injury Salvage Kinase (RISK) pathway, both of which confer cardioprotection in I/R (Hausenloy et al. 2005). Under

these conditions, excess ROS can also be efficiently removed by endogenous scavenger mechanisms located within cells; these include systems such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione (GSH), catalase, uricic acid, lipoic acid and coenzyme Q. In the event that the endogenous systems do not function efficiently, which is highly unlikely during homeostatic conditions, excess ROS can also be removed via exogenous antioxidants indigested through dietary or supplementary means. These include lipid soluble antioxidants such as vitamin E and C, carotenoids and polyphenols such as flavonoids (Poljsak et al. 2013).

**Table 2** below provides a summary of the major antioxidant systems as well their location and action in cardiomyocytes. For an in depth description on the antioxidant status of cardiomycytes please refer to the excellent review by Dhalla and colleagues (2000) as well as Luschchak (2014). This topic is not within the scope of the project and consequently is briefly highlighted.

***Table 2: Summary of some of the major antioxidants in cardiomyocytes together with their location and mechanism of action (adapted from Dhalla et al. 2000).***

| Name | Location | Mechanism of action |
|---|---|---|
| **Cu, Zn SOD** | Cytoplasm, cell surfaces and mitochondria | Catalyses $O_2^{\cdot -}$ dismutation to $H_2O_2$ <br> $2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$ |
| **Mn SOD** | Mitochondria | Catalyses $O_2^{\cdot -}$ dismutation to $H_2O_2$ <br> $2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$ |
| **Catalase** | Peroxisomes and mitochondrial membrane | $H_2O_2 \rightarrow 2H_2O + O_2$ |
| **Glutathione peroxidase** | Cytoplasm | $H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$ |
| **Glutathione** | Intracellular | Cellular reductant |
| **CoenzymeQ10 (unbiquinone)** | Cell membrane | Redox active electron carrier |
| **Vitamin E (α-tocopherol)** | Cytoplasm and plasma | Break lipid peroxidation chain and LDL reaction |
| **Pro-vitamin A (ß-Carotene )** | Plasma | Inhibits the oxidation of LDL |
| **Vitamin C (ascorbic acid)** | Cytoplasm and Plasma | Directly as an antioxidant or as a cofactor for vitamin E |

**Abbreviations:** LDL- Low density lipoprotein

The noxious effects of ROS are exhibited during I/R, when the myocardium is exposed to high concentrations of ROS over an extended period of time. Generally it is well accepted that reperfusion is associated with huge bursts of ROS upon reperfusion. However, Raedschelders and colleagues (2012) oppose this school of thought and consider it somewhat misleading since a large body of evidence demonstrated that ROS production also occurs at sub-lethal levels in the myocardium during ischaemia (Zweier et al. 1987; Vanden Hoek et al. 1997; Becker et al. 1999 ). This misleading statement stems from the perception that oxygen levels in the myocardium instantly fall to zero however, this could have not been further from the truth. According to Murphy and Steenbergen (*op.cit.*) "…even in a global ischaemia model of ischaemia

oxygen does not immediately fall to zero, so there is initially some oxygen to generate ROS".

This statement is also true in *vivo:* according to Merx et al. (2001), myoglobin serves as an initial oxygen reservoir during ischaemia however, this reservoir becomes swiftly depleted. Considering the above, it is important to bear in mind that ROS production *does* occur during ischaemia and the sub-lethal levels are escalated to lethal levels upon reperfusion. During ischaemia the impaired ETCs of damaged and dysfunctional mitochondria result in the incomplete reduction of the residual oxygen resulting in the formation of ROS (Turner and Hill, 2010). Damaged mitochondria produce about 10-fold the amount of ROS than healthy mitochondria (Jimenez et al. 2014).

***Other ROS Producing Systems***

In addition to the ROS produced by the ETC, there has been emerging evidence of mitochondrial ROS production through two other major systems namely $p66^{Shc}$ and monoamine oxidase (MOA) (Di Lisa et al. 2009). $p66^{Shc}$ belongs to the She proteins which play a role in Ras signalling. A spliced variant of the proteins are localised in the mitochondria; unlike its counterparts $p66^{Shc}$ is not associated with Ras signalling but with superoxide production. A recent study by Arany and colleagues (2010) shows that oxidative stress phosphorylates $p66^{Shc}$ at the serine residue resulting in its translocation to the OMM where it binds and oxidises cytochrome c. This process results in the production of ROS. MOAs are located in the OMM where they play a role in the removal of the amino group (deamination) from monoamine neurotransmitters and dietary tyramines resulting in the production of hydrogen peroxide and aldehydes. They are implicated in contributing to IRI with increased levels in the circulation as well as through the production of hydrogen peroxide (Di Lisa et al. 2009; Kaludercic et al. 2014).

In addition to mitochondrial ROS, other cellular processes or systems which contribute to ROS production during I/R include xanthine oxidase, cytochrome p450, NAD(P)H oxidase and the immune system. The xanthine oxidase enzyme is implicated in the production of ROS in the reperfused heart. Under normoxic conditions xanthine oxidase is in the form of xanthine dehydrogenase which is responsible for the reduction of NAD to NADH through xanthine. (Maxwell and Lip, 1997). During

ischaemia however, the substrates of xanthine oxidase, namely xanthine and ultimately hypoxanthine (which is the target substrate), accumulate and upon reperfusion they become oxidised to form superoxide (**Fig 6**) (Dewall et al. 1971; Xia and Zweier 1995).

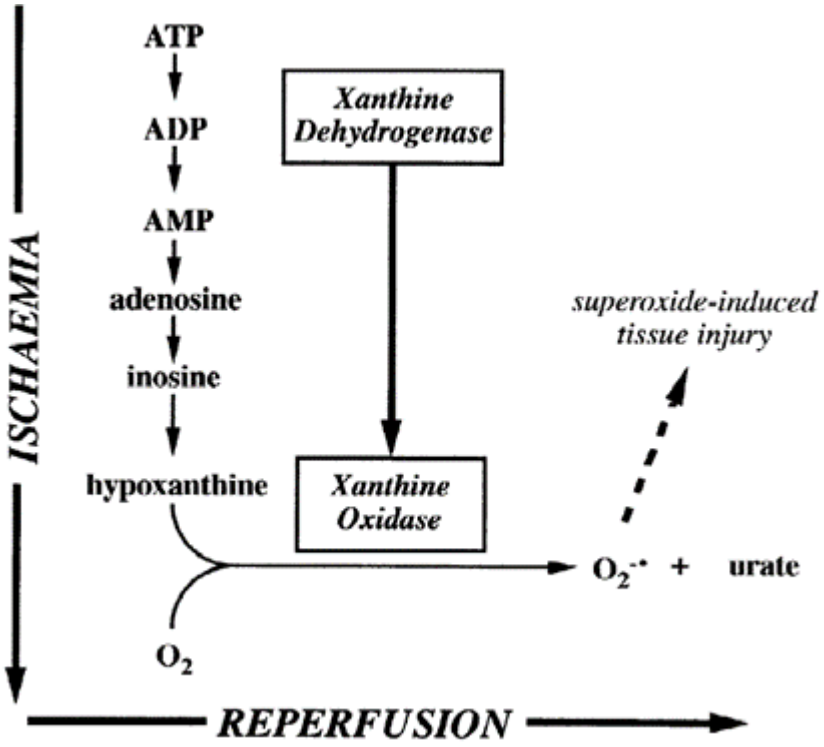



***Figure 6: Schematic illustration of the simultaneous conversion of xanthine dehydrogenase to xanthine oxidase, the breakdown of purine to nucleotides and ultimately the target substrate, hypoxanthine, during ischaemia. Its accumulation creates the perfect opportunity for superoxide production during ischaemia (Maxwell and Lip, 1997).***

Injury during ischaemia also leads to the activation of the endothelium, which becomes permeable, and the recruitment of inflammatory cells (such as neutrophils) which is facilitated by adhesion molecules and ultimately leads to myocardium toxicity. Protease secretion and ROS generation, through the NADPH oxidase system (Nox 2 in cardiomyocytes), are some of the factors that contribute to their toxicity (Kvietys and Granger 2012; Raedschelders et al., 2012). Furthermore the generation of ROS contributes to a positive feedback loop resulting in the accumulation of inflammatory cells which also increased ROS production (Maxwell and Lip 1997; Turner and Hill 2010; Ibáñez et al. 2015). Other potential sources of ROS during I/R include free metal

ions which produce hydroxyl radical from the Fenton and Haber-Weiss reactions (Maxwell and Lip 1997).

In conjunction with the above, two important concepts to also take into consideration when discussing ROS in I/R or IRI are the effects of NO and the concept of ROS-induced ROS release (RIRR). NO plays an important protective as well as regulatory role in the vasculature. According to Grisham and colleagues (1999) it has direct and indirect effects: the former is often beneficial and mostly protective for example prevention of the formation of radicals by hydrogen peroxide while the latter is detrimental and results in the interaction of ROS forming potent reactive nitrogen oxide species (RNOS) such as dinitrogen trioxide or peroxynitrate. The production of RNOS in I/R is the result of NO production, which is associated with mitochondrial cytochrome c oxidase during ischaemia, and ETC superoxide production (Golwala et al. 2009). The detrimental effects of RNOS production, particularly peroxynitrate, in I/R are associated with damage to proteins, lipids and DNA (Ferdinandy and Schulz 2003). Excess ROS leads to the induction of RIRR, which is a self-propagating phenomenon in which MPTP opening and ROS release from one mitochondrion could trigger ROS release as well depolarization of adjacent mitochondria (Zorov et al. 2000). Both these phenomena contribute to the escalating levels of reactive species which lead to the induction of cell damage and death which will be discussed below.

As the major contributor to ROS production it is inevitable that mitochondria will also become a target of ROS. Mitochondria are an ideal target of ROS for two reasons, firstly ROS are highly reactive and short lived molecules thus their effects will be substantial in their immediate area surrounding their site of production. Secondly, mitochondrial components such as the ETC, mitochondrial membrane and phospholipid constituents could be a major target due to biological composition; this concept will be further explored below with the mechanisms of ROS attack. There are various mechanisms of ROS attack and they include lipid peroxidation, DNA damage, protein oxidation and nitration, matrix metalloproteinases, and mitochondrial permeability transition. These mechanism will be discussed in detail below.

#### 2.3.1.3. Lipid peroxidation

Lipid peroxidation has been suggested to be the major mechanism of a ROS attack (Petrosillo et al. 2005), the mechanism which is associated with biological membranes which consist of phospholipid bilayers, responsible for encapsulating cells and organelles. To successfully fulfil their function the phospholipid bilayers need to maintain a balance between membrane integrity and fluid. This balance is dependent on the composition of unsaturated and saturated fatty acids. In lipid peroxidation, free radicals such as superoxide disrupt the double bonds of polyunsaturated acids resulting in the formation of conjugated dienes (double bonds separated by a single bond) (Adapted from Raedschelders et al. 2012). Consequently, the level of conjugated diene formation may be attributed to the level of oxygen radical production (Pertosillo et al., 2005). These fatty acid modifications not only affect the functional integrity of the membrane but can also yield carbon centred and peroxyl radicals which can further aggravate damage (Radi et al. 1991 a-b; Renner et al. 2005).

The mitochondrial phospholipid cardiolipin is a good example of lipid peroxidation damage: it is most abundant in the mitochondria and exclusively located in the IMM. It has a high unsaturated fatty acid content and is primarily associated with stabilising the ETC complex and mitochondrial bioenergetics (Chicco and Sparanga, 2006). Cardiolipin could possibly be amongst the earliest targets of ROS and ultimately lipid peroxidation either due to their location or abundant unsaturated fatty acid content (Petrosillo et al. 2003).

#### 2.3.1.4. Protein nitration and oxidation

According to Stadtman and Levine (2003), amino acid residues in proteins as well as free amino acids are highly susceptible to oxidation by several types of ROS that are generated through pollution, irradiation and normal metabolic processes. Oxidation, which is a direct form of ROS attack, denatures proteins by cross-linking functional groups, thereby altering the hydrophobicity of amino acids on protein surfaces and cleavage of peptide bonds. Previous studies unravelled the mechanisms of ROS-mediated oxidation and implicated abstraction (hydrogen removal by the hydroxyl radical) as the major form of attack. The initial sites of attacks include, but are not limited to, the α-carbons of amino acids and protein poly-peptide backbone (Stadtman

and Levine, 2003). In IRI cardiomyocytes can undergo cell death either through apoptosis or necrosis even when the subcellular and cellular membranes are functionally intact. In this instance, cell death is triggered by the non-enzymatic modifications of cellular proteins by ROS. In addition to the activation of necrosis and pro-apoptotic proteins, the contractile machinery also sustains damage contributing to the myocardial stunning. (Van Eyk et al. 1998; Kloner and Jennings 2001; Krijnen et al. 2002).

Proteins can also be subjected to indirect forms of ROS attack. In this setting ROS generation leads to the formation of reactive molecules such as reactive aldehydes, which are cleaved products of ROS-mediated lipid peroxidation. These reactive aldehydes become the mediators of indirect ROS attack (Raedschelders et al. 2012). Irrespective of the mechanism, it is evident that this type of damage will culminate in the death of cardiomyocytes.

#### 2.3.1.5. DNA Damage

In view of its role in the cell, DNA damage will have far-reaching consequences. DNA damage by ROS is similar to the manner by which it affects proteins. DNA can be damaged through hydrogen abstraction by the hydroxyl radical followed by modification and/or fragmentation of purines or pyrimidines such as guanine or cytosine respectively (Adapted from Kang and Kim 1997). This damage can activate apoptotic pathways and alter myocardial protein expression. Induction of these processes amongst other things, can deplete myocardial energy and disrupt the restoration of energy by inhibiting glycolysis as well as mitochondrial function (Szabó and Dawson, 1998). Raedschelders and colleagues (2012) do however bring to our attention that while DNA damage can trigger apoptotic cascades, they are actually more likely to occur from damage to proteins and cellular membranes. In addition, while DNA damage is often synonymous with mutations, ROS-mediated genetic mutations are generally not a characteristic of myocardial IRI (Adapted from Raedschelders et al. 2012).

Apart from DNA damage, other genetic changes that can cause I/R damage are epigenetic changes. These changes have been extensively reviewed by Kalogeris and colleagues (2012) and will not be discussed here as they do not contribute to any of the aspects covered in the scope of this study.

#### 2.3.1.6. Matrix metalloproteinases

As previously mentioned, the myocardium can undergo structural rearrangements. These rearrangements occur when the myocardium is subjected to prolonged sub-lethal ischemia and result in reduced contractility or myocardial stunning which was briefly discussed (**Section 2.2.2**). These changes can proceed either due to calpains or to extracellular matrix metalloproteinase (MMP) activity. Calpains and MMP activity target contractile proteins such as the myosin light chain, α-actin and cardiac troponin (Cohen et al. 2006; Sung et al. 2007; Zhang et al. 2011). Both of these intracellular proteolytic enzymes are activated through various mechanisms, for example calpains activity is triggered by calcium overload and MMPs are activated transcriptionally in the setting of chronic remodelling. In addition, they can also be induced by peroxynitrite and hydrogen peroxide ($H_2O_2$) mediated proenzyme cleavage (Viappiani et al. 2009). In essence, MMP activity upregulation is the result of ROS and reactive nitrogen species (RNS) generation during I/R.

#### 2.3.1.7. Mitochondrial Permeability Transition and MPTP opening

The upsurge of ROS production during myocardial I/R results in a cellular process known as mitochondrial permeability transition (Di Lisa et al. 2001; Halestrap et al. 2004; Kim et al. 2003). During ischaemia this process and consequently MPTP opening is strongly inhibited by the acidic intracellular milieu (Kim et al. 2003; Kalogeris et al. 2012). Induction of permeability transition occurs during the first few minutes of reperfusion when the intracellular pH begins to shift towards homeostatic levels. This, together with matrix calcium and oxidative stress, amongst other things, culminates in the formation and opening of the MPTP (Halestrap 2009; Ong and Gustafsson 2012).

The MPTP is considered to be an evolutionary conserved, non-selective pore located within the IMM. MPTP formation and opening results in the permeability for molecules which are smaller than 1.5 kDa in size. The molecular identity of the pore remains a mystery. According to Karch and Molkentin (2014) "… the molecular identity of the

MPTP has been the subject of a protracted scientific debate for nearly three decades". Despite all the debates, research from the past few years has proposed two MPTP models. The first model suggests that the MPTP is a single functional unit which spans the intermembrane space and is regulated by cyclophilin D (CypD). This model suggest that the MPTP consists of the voltage-dependent anion channel (VDAC) as an OMM component and adenine nucleotide translocator (ANT) as an IMM component (Halestrap 2009; Bernardi 2013; Karch and Molkentin 2014). In addition, the mitochondrial phosphate carrier (PiC) was hypothesised to also be an inner membrane component since it can bind to CypD.

Recently, this long standing model has been refuted by new findings which sparked renewed interested into the molecular identity of the MPTP (Baines et al. 2009; Kwong et al. 2014; Gutièrrez-Agullar et al. 2014) The current model of the MPTP suggests that it consists of inner and outer membrane components which function separately in membrane opening. Proapototic proteins Bax or Bak have been suggested to form the outer membrane component while the c-subunit of $F_1F_0$ ATP synthase forms the inner membrane pore-forming component (Karch et al. 2013; Alavian et al. 2014). This model also suggests that the oligomycin sensitivity- conferring protein facilitates pore opening while PiC and ANT function as c-subunit ATP synthase regulators (Karch and Molkentin *op.cit*.).

Despite the uncertainty surrounding MPTP identity, researchers consent that pore formation allows for the passage of $H^+$ ions back into matrix, which dissipates the mitochondrial membrane potential, disrupts ATP production by uncoupling oxidative phosphorylation and ultimately results in the depletion of the limited ATP stores. In addition, mitochondrial swelling and even rupture occur due to the colloid osmotic pressure exerted by large matrix proteins as well as water passing through due to its osmotic gradient. In conjunction with the above, cardiolipin peroxidation results in the release of cytochrome c which will initiate pro-apoptotic signalling cascades (Adapted from Kagan et al. 2005; Gustafsson and Gottlieb, 2008).

A cell can recover and survive mitochondrial permeability transition if the OMM remains intact. In the event of outer membrane rupture, a cell will undergo apoptosis if it has sufficient energy or in the absence of sufficient energy it will become necrotic

(Halestrap et al. 2004; Kim and Lemasters 2003; Tatsumi et al. 2003). While it is evident that ROS generation plays a role in MPTP pore opening, there is a blurred line. According to Petrosillo and colleagues (2005), it is unclear whether ROS generation is a consequence of pore opening or whether it plays a vital role in the signalling cascade that causes pore opening.

In conclusion, it is evident that the production of ROS is unavoidable and essential since they are by-products of metabolism and are required for key processes such as signalling (Adapted from Di Meo et al. 2016). Secondly, their cellular concentrations are highly dependent on the balance between the rate of their production and clearance by the various intrinsic antioxidant compounds and enzymes. In IRI, we see that these various systems are overwhelmed or in some cases such as with NO they become suppressed. This results in elevated ROS levels which can facilitate cardiomyocyte damage or death through mechanisms such as lipid peroxidation or necrosis respectively. Lastly, it seems that in I/R, ROS do not act alone, but it also contribute to the other IRI mediators (see **Table 3** below).

***Table 3: Summary of the various changes that cardiomyocytes are subjected to during the transition from normoxia to ischaemia and reperfusion (adapted from Raedschelders 2012; Lushchak, 2014).***

| Condition | Sources of ROS | Intensity of ROS Production | Molecular Outcome | Physiological Outcome |
|---|---|---|---|---|
| **Normoxic** | Mitochondrial ETC | Low | Antioxidant systems | Signalling, adaptive response |
| **Ischaemia** | Mitochondrial ETC, Cellular Xanthine | Intermediate/ Sub-lethal | Antioxidant Systems,<br><br>Inflammation Proteins,<br><br>Heat Shock Proteins | Adaptive response vs. damage to cellular components |
| **Reperfusion** | Mitochondrial ETC, Cellular Xanthine Oxidase, Immune System, NOS uncoupling | High/ Lethal | Overwhelmed/ suppressed antioxidant systems, lipid peroxidation, protein nitration and oxidation , DNA damage, MMP damage, mitochondrial permeability transition | Autophagy, apoptosis,<br><br>necrosis, |

Considering that in the setting of IRI, the upsurge of ROS production is primarily due to damaged and dysfunctional mitochondria it becomes important to enhance intrinsic systems such as mitophagy which selectively removes damaged and dysfunctional mitochondria. Autophagic processes, such as mitophagy, and the various cell death pathways, such as apoptosis, play an important role in cell health and survival. However, they could also be detrimental. This dual role will be elucidated in the next section.

### 2.3.2. Autophagy and Cell death- the fight for survival

#### 2.3.2.1. Autophagy

Currently there are two schools of the thought about the proliferative ability of mature cardiomyocytes. The first one, which has been widely accepted, states that cardiomyocytes are long-lived, terminally differentiated (TD) cells. This means that the heart consists of a fixed number of cardiomyocytes which will be maintained until the death and this notion is attributed to the stance that once cardiomyocytes became specialized they lose their proliferative ability (adapted from Sacco et al. 2013). The second one not only challenges the simple definition of TD cells as well as their proliferative ability but it argues that cardiomyocytes can proliferate to a certain extent and even goes as far as suggesting five potential sources of cardiomyocytes in the adult heart (Leri et al. 2016). Regardless of which premises one chooses to support, it is generally accepted that autophagy is essential for housekeeping.

Autophagy is one of the two protein systems found in cells. The second system, which will not be discussed in detail as it does not form part of our study, is the Ubiquitin-Proteasome System (UPS) which is responsible for degradation of short-lived proteins. Briefly, the lysine residues of the short-lived proteins that are targeted for degradation are tagged with ubiquitin molecules and are ultimately delivered to the proteasome for degradation (Hochstrasser 1996). Autophagy on the other hand, is the intracellular process that is responsible for the degradation of long-lived proteins and organelles that are ageing, damaged or in excess by delivery to the lysosomes (adapted from Mei et al. 2015).

There are three types of autophagy namely: microautophagy, chaperone-mediated autophagy (CMA) and macroautophagy. They differ in terms of how they deliver their cargo to the lysosome for degradation (Sridhar et al. 2012). The degraded products of all the forms of autophagy, which include but are not limited to amino acids and fatty acids, can be used for anabolic processes such as protein synthesis and energy production (adapted from Rabinowitz and White, 2010; Mei et al.2015). Consequently, autophagy not only serves as a system for the elimination of cellular cargo and misfolded proteins but it also serves as a recycling system which provides energy and building materials (Mei et al. 2015).

Microautophagy involves the direct uptake of substrates by the lysosomes through membrane invagination, while in CMA the substrates that are targeted for degradation contain a KFERQ-like motif i.e. a pentapeptide sequence. In this form of autophagy a chaperone heat shock cognate 71-kDa protein will bind to the KFERQ-like motif on the substrate and will then translocate to the lysosome where it will interact with the lysosomal membrane receptor lysosomal – associated membrane protein 2A ultimately resulting in degradation ( Adapted from Orenstein and Cuervo 2010) . The rest of this review will focus on macroautophagy which forms the core of this study and is the most predominant and best studied type of autophagy.

Macroautophagy (referred to as autophagy hereafter) is characterised by the *de novo* synthesis of a cup-shaped double membrane vesicle (autophagosome) which will engulf the target substrates or organelles and deliver them to the lysosome for degradation. Genetic screening has identified approximately 30 autophagy-related (Atg) genes, which encode the Atg proteins that orchestrate autophagy (Sciarretta et al. 2011; Subramani and Malhotra, 2013). To date, two types of autophagy have been identified namely selective and non-selective autophagy. Non- selective autophagy, which is inducted during starvation, occurs to supply cells with vital components for energy and anabolic processes until nutrients are available again. Selective autophagy, such as mitophagy, occurs during nutrient rich conditions as well as stressful conditions such as I/R. It involves the removal of damaged and dysfunctional organelles as well as protein aggregates that could be toxic to the cell (Adapted from Youle and Narendra, 2011).

According to Maejima and colleagues (2015) autophagy can be separated into several steps and they include induction, recognition and selection of the cytoplasmic substrate, formation of the autophagosomes around the substrate, autophagosome-lysosome fusion, degradation of the autolysosomal contents and release of the degraded substrates into the cytoplasm. A detailed description of the molecular mechanisms as well as signalling cascades that are involved in some of these steps can be found in the excellent reviews by He and Klionsky (2009) Hamacher-Brady (2012) and Mei et al. (2015). This review will highlight the key aspects.

Under basal conditions autophagy occurs at low levels and serves to maintain a healthy content of long lived proteins and cellular organelles. When the cell is devoid of the appropriate signals for autophagy induction, the mammalian target of rapamycin (mTOR) maintains ULK1 in the inactivated phosphorylated form. In the presence of the appropriate cellular stimuli mTOR is inactivated and ULK1 is stimulated allowing it to activate the Beclin-1 complex. The Beclin-1 complex together with other Atg proteins will facilitate the formation of the double membrane autophagosome (Hamacher-Brady, 2012; Linton et al. 2015; Mei et al. 2015). This process is characterised by the formation of a small isolation membrane known as a phagophore. Researchers acknowledge that the origin of the phagophore is unknown however, it is speculated that it could originate from the endoplasmic reticulum (ER) which is the site of autophagosome assembly or it could be derived from the Golgi complex, mitochondrial or plasma membrane, alternatively it could be synthesised *de novo* (Reggiori et al. 2005; Juhasz and Neufeld 2006; Linton et al. 2015).

Elongation of the phagophore requires the coordinated assembly of two complexes. The assembly of the first complex is mediated by Atg 7 and Atg 10 to form a covalent linkage between Atg 12 and Atg 5. The Atg 12-Atg 5 complex will then associate non-covalently to Atg16L1 and this complex is responsible for membrane elongation as well as the recruitment of the second complex (Sciarretta et al. 2011; Harmacher-Brady; 2012). The second complex is responsible for integrating LC3-II into the autophagosome. To begin with Atg 4 will cleave terminal amino acid(s) from pro-LC3, an immature precursor, to form LC3-I. Next, Atg 7 will mediate the activation of the exposed LC3-I C- terminal glycine residue and transfer the product to Atg 3 which will facilitate its conjugation to the lipid phosphatidylethanolamine (PE) to form LC3-II. LC3-II integration into the elongating autophagosome is directed by the Atg 12- Atg5-Atg 16L1 complex. LC3-II is integrated into both the inner and outer membrane of the autophagosome. Once the autophagosome elongation has been completed the Atg 12- Atg5- Atg 16L1 complex disbands and the LC3-II which is located on outer membrane is converted back to LC3-I by Atg 4. LC3-II which is incorporated into the inner surface of the membrane is present right thought autophagosome maturation and will be degraded together with the cargo in the lysosome (Gustafsson and Gottlieb 2008; Gottlieb et al, 2009; Jimenez et al. 2014; Linton et al. 2015). Degradation in the

lysosome occurs through lipases, proteases, nucleases and glycosidases to yield the products (e.g. lipids and nucleosides) which will be recycled back into the cell for anabolic processes (Mei et al. 2015). In addition to the proteins mentioned above, the Rabs, which are small GTPases, play a role in autophagy. They are associated with the regulation of the steps in vesicle transport e.g. budding of the autophagosome and targeting it for fusion to the lysosome. Of importance to this study is Rab9 which is involved in autophagosome maturation and is associated with the so-called non-canonical autophagy pathway. This pathway is unique in that it can occur independent of some of the Atg proteins involved in the classical autophagy pathway described above (Adapted from Amaya et al. 2015).

A large body of evidence has shown that autophagy is induced by the deprivation of energy, nutrients and hormones ( such as growth factor) and stress signals such as hypoxia, ER stress, DNA damage, damage or pathogen molecular associated patterns, oxidised lipids, ROS and mitochondrial damage (Gurusamy et al. 2009; Kroemer et al. 2010; Tang et al. 2012). Of particular interest to this study is the induction of autophagy by ROS and mitochondrial damage in I/R.

#### 2.3.2.2. Autophagy and I/R

It is well established that autophagy is upregulated during I/R. However, there is much controversy about the role of autophagy in this setting. We know that ischaemia is mainly characterised by hypoxia and a deficiency of ATP and in this setting a wide number of studies implicate and emphasise the roles of adenosine monophosphate-activated protein kinase (AMPK) and Hypoxia-inducible factor-1 (HIF-1) in authophagic cardioprotection. AMPK is responsible for sensing cellular energy and during ischaemia it is activated by the low levels of ATP and the increased AMP/ ATP ratio. AMPK can promote cardioprotection through various cellular pathways and processes including glycolysis and fatty acid oxidation (Russell et al. 2004). In addition, AMPK can mediate cardioprotection through autophagy by indirect or direct modifications to ULK1 (Takagi et al. 2007; Matsui et al. 2009).

HIF-1 is responsible for monitoring the oxygen levels in cells and mediating the relevant adaptive responses during hypoxia or increased oxidative stress through gene expression (Adapted from Semenza 2015; Ma et al. 2015). HIF-1 consists of α

and ß subunits of which the protein stability of the former is dependent on cellular oxygen levels while the latter is independent (Bellanti, 2017). During normoxic conditions HIF-1 α levels are kept low through degradation, consequently inhibiting its adaptive effects (adapted from Bellanti, 2017). HIF-1 mediates oxygen delivery through regulation of angiogenesis, glucose metabolism and redox homeostasis amongst other things (Semenza, 2014). The effects of HIF-1 and its adaptive effects during hypoxic stress have been well studied in cancer. Currently various researchers such as Zhang's group (2008) and Bellot's group (2009) have provided evidence for HIF-1 mediated induction of autophagy/mitophagy in various cell lines such as fibroblasts and human cancer cell lines. Consequently, researchers speculate that these findings would also be true for myocardial I/R. Existing evidence from Lee et al. (2000), Ockaili et al. (2005) and Cai et al. (2013) supports the expression of HIF-1 in I/R as an adaptive response and links its protective effects to mechanisms such as inflammation and not autophagy. He and Klionsky (2010) acknowledge that "the area of study is at the beginning stage" consequently more research needs to be done to elucidate the link between HIF-1, hypoxia and autophagy in myocardial I/R.

To conclude, one needs to bear two things in mind when looking at autophagy during ischaemia. Firstly, HIF-1 mediates hypoxia-induced autophagy (i.e. this would be applicable in a regional ischaemia disease model), however, anoxia-induced autophagy (i.e. such as in a global ischaemia disease model) would have to occur independently of HIF-1 (Adapted from Majmundar et al. 2010). Secondly, some processes in autophagy such as autophagosome formation are dependent on ATP, consequently in a global ischaemia model autophagy could be inhibited once oxygen levels ultimately reach zero (Adapted from Sciarretta et al. 2011). This could partly explain why during prolonged ischaemia cells are subjected to cell death since the absence of protective mechanisms such as autophagy probably inhibits a large number of cellular processes, if not all, due to the complete lack of ATP amongst other things. This would then force cardiomyocytes to opt for necrotic cell death, which does not require energy (the mechanisms of cell death such as necrosis will be discussed later in the review). During reperfusion, hypoxia and energy deficiency are no longer a problem for the cell as oxygen return to normoxic levels and oxidative

phosphorylation is induced once again, consequently AMPK and HIF-1 can no longer mediate autophagy in this setting.

During reperfusion it is evident that ROS and mitochondrial damage or dysfunction predominantly induces authophagic cell death or apoptosis through various signalling cascades. Thus the swift and yet selective removal of mitochondria (mitophagy) seems to be pertinent in this setting. Mitophagy would not only increase the pool of healthy mitochondria but it would also indirectly contribute to cardiomyocyte survival.

#### 2.3.2.3. Mitophagy

The half- life of mitochondria, which is tissue dependent, has been reported to range from days to weeks (Menzies and Gold 1971; Kim et al. 2012). The accumulation of damaged and dysfunctional mitochondria has been associated with the pathophysiology of cardiovascular, neurodegenerative and pulmonary diseases, amongst others. This once again emphasises the important role that autophagy, particularly mitophagy, plays in organ homeostasis. Considering that cardiomyocytes are long lived, terminally differentiated cells with a high density of mitochondria that have a half-life of approximately 17.5 days, the role of mitochondrial dynamics and mitophagy in cardiac homeostasis is of the utmost importance.

The term mitochondrial dynamics refers to two processes, namely fusion and fission which are adaptive responses that mitochondria utilise to maintain their function in the fluctuating cellular environment (Adapted from Ikeda et al. 2015). Although mitochondrial dynamics have been implicated as regulators of mitophagy, Ikeda and colleagues (2015) disclose the following:

“It should be noted that the continuous occurrence of mitochondrial fusion and fission has not been tracked in normal adult ventricular cardiomyocytes and, thus, their roles have been inferred based on pharmacological or genetic manipulation. Although we discuss molecular mechanisms controlling fission and fusion of mitochondria…, almost all works have been conducted using non-cardiac cell types. Thus, caution should be exercised regarding whether the findings from other cell types can be applicable to adult ventricular cardiomyocytes.”

Considering the above, this review will briefly highlight the single aspects of mitochondrial dynamics that is likely to contribute to the induction of mitophagy. For a more extensive review on this topic refer to Dorn II (2013).

Mitochondrial fusion, is considered to protect mitochondria against mitophagy since it involves the union and mixture of recoverable depolarized mitochondria with intact and highly functional ones in an effort to maintain membrane potential amongst other things. Fission on the other hand, is considered to be the central mediator of mitophagy (in mitochondrial dynamics) since it involves the exclusion of unrecoverable and damaged mitochondria, which are characterised by low mitochondrial potential and ATP production as well as high levels of ROS (Adapted from Twig et al. 2008; Ikeda et al. 2015).

DRP-1 (Dynamin-1-like Protein) is the primary regulator of fission. Under basal conditions most of DRP-1 is found in the cytosol while the remaining portion is associated with the OMM (Smirnova et al. 2001). When DRP-1 is activated (under basal and stressful conditions) it translocates to the OMM (Varadi et al. 2004). Inhibition of fission interferes with mitophagy and culminates in mitochondrial dysfunction (Twig et al. 2008). It is evident that mitochondrial fusion and fission play an important role in maintaining a healthy pool of mitochondria by sorting the “good from the bad and ugly”. The induction of mitophagy, through fission, further enhances this role by ensuring that the “bad and ugly” not only get excluded but are also degraded.

Mitophagy, as previously mentioned is the selective autophagy of damaged and dysfunctional mitochondria. There are several mitophagy pathways and they can be classified as traditional or alternative. The traditional pathway, is the phosphate and tensin homologue-induced putative kinase 1 (PINK1)/ Parkin pathway which is the best characterised. The alternative pathways involve BNIP3/ NIX, FUNDC1 and more recently Rab 5. The mechanisms of the traditional pathway will be discussed first followed by the alternative pathways.

#### 2.3.2.4. PINK1/ Parkin Pathway

PINK1/ Parkin mediated mitophagy predominantly depends on low mitochondrial membrane potential. Under basal conditions Parkin, an E3 ubiquitin ligase, is located in the cytosol. While PINK1, a serine/threonine kinase, is imported into highly functional healthy mitochondria by the translocase of the outer membrane (TOM) complex where it will be degraded by presenilin-associated rhomboid-like (PARL) protease. During stressful conditions, such as in I/R, where mitochondrial dysfunction and loss of mitochondrial membrane potential have occurred, PINK1 anchors and accumulates on the OMM (Narendra et al. 2010). PINK1 accumulation results in the recruitment of Parkin from the cytosol to the mitochondria (**Fig 7)**.

Initially, it was thought that PINK1 is essential for the recruitment of Parkin and induction of mitophagy. However, evidence from Kubli's group (2015) show that in the absence of PINK1, Parkin recruitment still correlated with increased mitochondrial protein ubiquitination and a functional mitophagy pathway. There are various proposed pathways for the progression of mitophagy after Parkin recruitment. The discrepancies seem to be dependent on the role of Parkin and to a certain degree PINK1 not being fully elucidated.

Once recruited it seems as if though Parkin is still to be activated. PINK1 facilitates the activation of Parkin through several steps that involve the phosphorylation of mitofusin 2 (MFN2) and ubiquitin. Upon phosphorylation MFN2 functions as a receptor for Parkin while ubiquitin phosphorylation activates the E3 ubiqitin ligase activity of Parkin (Chen and Dorn, 2013; Moyzis et al. 2015; Maejima et al. 2015). Subsequently, Parkin will ubiquinate various proteins on the OMM such as the voltage-dependent anion channel (VDAC) 1, TOM, MIRO etc. (Geisler et al. 2010). Ubiquitination of these proteins signals for p62/ Sequestosome-1 (SQSTM1) binding to these ubiquitinated mitochondrial proteins and LC3 on the autophagosome for ultimate degradation (Pankiv et al. 2007; Narendra et al. 2008; Geisler et al. 2010; Jimenez et al. 2014).

Increasing lines of evidence suggest that PINK1-Parkin mediated autophagy participates in mitochondria quality control and maintenance of heart function at baseline. Parkin-KO mice exhibit normal cardiac function at baseline, but their cardiomyocytes exhibit morphologically disorganized mitochondria with or without

dysfunction (Kulbi et al. 2013; Piquereau et al. 2013). It was also suggested that lack of Parkin-mediated mitophagy in heart specific MFN2 mice is compensated for by activation of non-selective autophagy in cardiomyocytes (Song et al. 2014). This suggested that both mitophagy and general autophagy cooperatively participate in removal of mitochondria in cardiomyocytes. It would be interesting to clarify whether these two forms of autophagy degrade the same targets or qualitatively or quantitatively different populations of mitochondria

Whether or not endogenous PINK1 plays an essential role in mediating mitophagy in the heart remains to be elucidated. Genetic deletion of PINK1 induces more severe cardiac effects than that of Parkin (Billa et al. 2011), despite the fact that PINK1 and Parkin are thought to work together to mediate mitophagy. The role of MFN2 in the heart is also not clear: it has been shown that mice hearts lacking MFN2 develop cardiac dysfunction with age (Chen and Dorn II 2013), while another study showed that downregulation of MFN2 protects the heart against I/R damage (Billa et al 2011). It is also possible that PINK1 has additional functions besides Parkin-mediated autophagy.

#### 2.3.2.5. Mitochondrial receptor-mediated mitophagy

**BNIP and NIX**

Another pathway involved in mitophagy occurs through the BCL-2-related proteins BNIP3 and BNIP3L/NIX (**Fig 7**). These atypical BH3-only proteins are well-known activators of cell death, for example BNIP3 activates BAX/BAK in the OMM and causes opening of the MPTP (Regula et al. 2002; Kulbi et al. 2007) and NIX activates cell death via the mitochondrial apoptotic pathway (Yussman et al. 2002). Both these proteins are located on the OMM where they act as receptors for targeting autophagosomes to mitochondria (Novak et al. 2010; Hanna et al. 2012). Using their LC3-interacting region (LIR) motifs they directly bind to the LC3/$\gamma$-aminobutyric acid receptor-associated protein (GABARAP) which is located on the autophagosome. BNIP3 has been identified as a vital redox sensor of mitochondrial oxidative stress and it has been suggested that it mediates cell death in response to increased oxidative stress (Kulbi et al. 2008). Hamancher-Brady and colleagues (2006) implicated BNIP3 overexpression in myocardial IRI. In their model of I/R BNIP3

overexpression resulted in fragmented mitochondria i.e. mitochondrial dysfunction. In addition to mitochondrial dysfunction, BNIP3 overexpression is associated with mitochondrial permeability transition, MPTP opening and subsequent release of cytochrome c which in turn leads to cell death.

Since both BNIP3/NIX and PINK1/Parkin play a role in mitophagy, the question arises of whether they participate in the same pathway to clear mitochondria. However, the signals that activate these pathways are different. While Parkin-mediated mitophagy requires loss of mitochondrial membrane potential, BNIP3 promotes mitophagy even when mitochondria retain their potential (Rikka et al. 2011). The respective roles of these two pathways and potential cross-talk in the regulation of mitophagy needs to be further investigated.

**FUNDC1**

FUNDC1 is an integral outer membrane mitochondrial protein which is implicated in mediating mitophagy during hypoxic conditions (**Fig 7**). The study which was conducted by Liu's group (2012) showed that FUNDC1 functions as a receptor and mediates mitophagy by interacting with LC3 through its LIR motif. This will allow it to couple to core authophagic machinery such as Atg 5. More studies are being conducted to elucidate the mechanisms of FUNDC1.

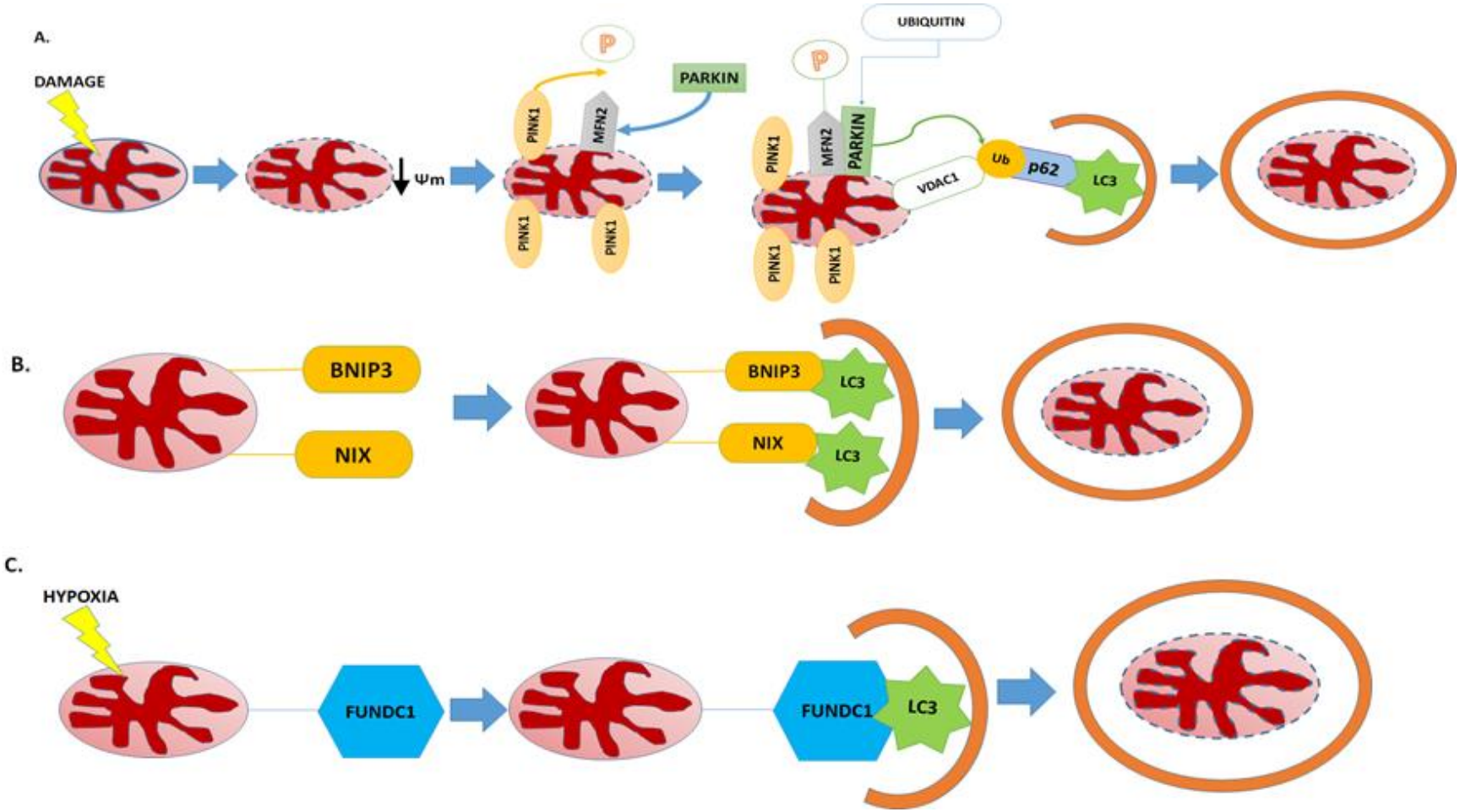



***Figure 7: Schematic Illustration of the PINK1/PARKIN Pathway and receptor mediated pathways. In (A) Damage to the mitochondria that results in decreased membrane potential activated the PINK1/PARKIN mitophagy pathway. PINK1 accumulates on the outer membrane where it recruits PARKIN and activates MFN2. MFN2 acts as a receptor for PARKIN. Ubiquitin activates PARKIN, PARKIN will ubiquitinate a wide range of proteins on the mitochondria such as VDAC1. This results in p62 and LC3 interaction and autophagosome formation. The autophagosome will ultimately be targeted for degradation. (B) Shows the BNIP3 and NIX pathway which is activated even when the mitochondria retain their membrane potential. BNIP3 and NIX interact with LC3 through their LIR motif. (C) Illustrates the FUNDC1 which is inducted by hypoxia. FUNDC1 interacts with LC3 through its LIR motif. LIR interaction with LC3 in both (B) and (C) leads to autophagosome and ultimately degradation (Adapted from Moyzis et al. 2015).***

#### 2.3.2.6. Alternative pathway

Another alternative pathway for mitophagy has recently attracted much attention. It is well-established that the lowering of cellular ATP, leads to activation of AMPK and initiation of autophagy. AMPK phosphorylates and activates the Unc-51-Like Kinases (ULK), which, in turn, activates the BECLIN1/VPS34/VPS15 complex to initiate autophagy (Kim et al. 2011). ULK then translocates to the mitochondria where it phosphorylates the mitophagy receptor FUNDC1 (Wu et al. 2014). It has also been recently reported that ULK phosphorylates cytosolic Rab9 which, in turn leads to the phosphorylation of DRP-1 and subsequent mitophagy (Sadoshima 2017, personal communication).

#### 2.3.2.7. Mitophagy and cardioprotection

As discussed above, mitochondrial degradation is critical for cardiac homeostasis and any interference in this process could lead to mitochondrial as well as cardiac dysfunction. Studies thus far showed that autophagy is upregulated during IRI and is initially a protective response activated by the cell (Hamacher-Brady et al. 2006; Matsui et al. 2007; Tannous et al. 2008), which eventually could lead to cell death, while downregulation of the process protects against cell injury (Hamacher-Brady et al. 2006; Eckele et al. 2012; Eltzschig et al. 2013). To date, very few studies focused on the specific role of mitophagy during I/R in the heart, but initial reports support a protective effect for mitophagy in response to stress. Increased mitophagy was initially described in myocytes overexpressing BNIP3 and in hearts subjected to ex vivo I/R (Hamacher-Brady et al. 2007).

Studies using knockout mice confirmed the importance of mitophagy in cardioprotection. For example, PINK1 deficiency increased the susceptibility of the heart to ex vivo IRI (Lee et al. 2011), while Parkin-deficient mice accumulate dysfunctional mitochondria (Kulbi et al. 2013), which leads to increased mortality. Interestingly, Parkin plays a role in ischaemic preconditioning, which may indicate that removal of unstable mitochondria during the preconditioning process may play a role in cardioprotection (Haung et al. 2011)

In contrast, augmented autophagy can also promote cardiomyocyte loss, particularly during reperfusion (Matsui et al. 2007). Studies assessing the effects of chronic and excessive mitophagy in the heart are lacking, but it stands to reason that excessive loss of mitochondria will be harmful to the myocardium. The threshold for mitophagy in cardiomyocytes are still unknown and it is unclear how many mitochondria can be removed from the cell before it becomes energy-deficient and undergo necrosis.

It is evident from the above that while cardiomyocytes are subjected to damage there are systems in place such as autophagy / mitophagy that will attempt to salvage and promote cell survival. However, cardiomyocyte cell death do occur, which is clinically manifested as an infarct. At molecular level cell death exhibits in the form of authophagic cell death (discussed previously), apoptosis, necrosis and necroptosis.

### 2.4. Cell death- an unhappy ending

It is evident that in a healthy heart, mitochondria are the core energy producers for contraction and ROS generators for signalling. During I/R however, we see that mitochondria are at the heart of injury. This section will unravel the intricacies of the unhappy ending i.e. cell death.

According to literature there are three forms of cell death namely: **autophagy**, **apoptosis** and **necrosis**. In the latter classification cell death and necrosis are considered to be two different things (**Fig 8**). Cell death is considered to be invisible to the naked eye i.e. occur at a molecular level and can be reversible. Necrosis on the other hand is defined as the process of degradation, which follows irreversible injury via oncosis or apoptosis, which is visible to the naked eye (Adapted from Manjo and Joris 1972; Buja and Velera, 2008). For example in IRI cardiomyocytes can die due to excessive autophagy i.e. autophagic cell death or through apoptosis both of which occur at molecular level. Both these forms of cell death are reversible if an intervention is administered on time. Considering that some authors use the term oncosis and necrosis interchangeably, while others clearly distinguish the two terms, we will be discussing both these concepts and highlight key aspects of both.

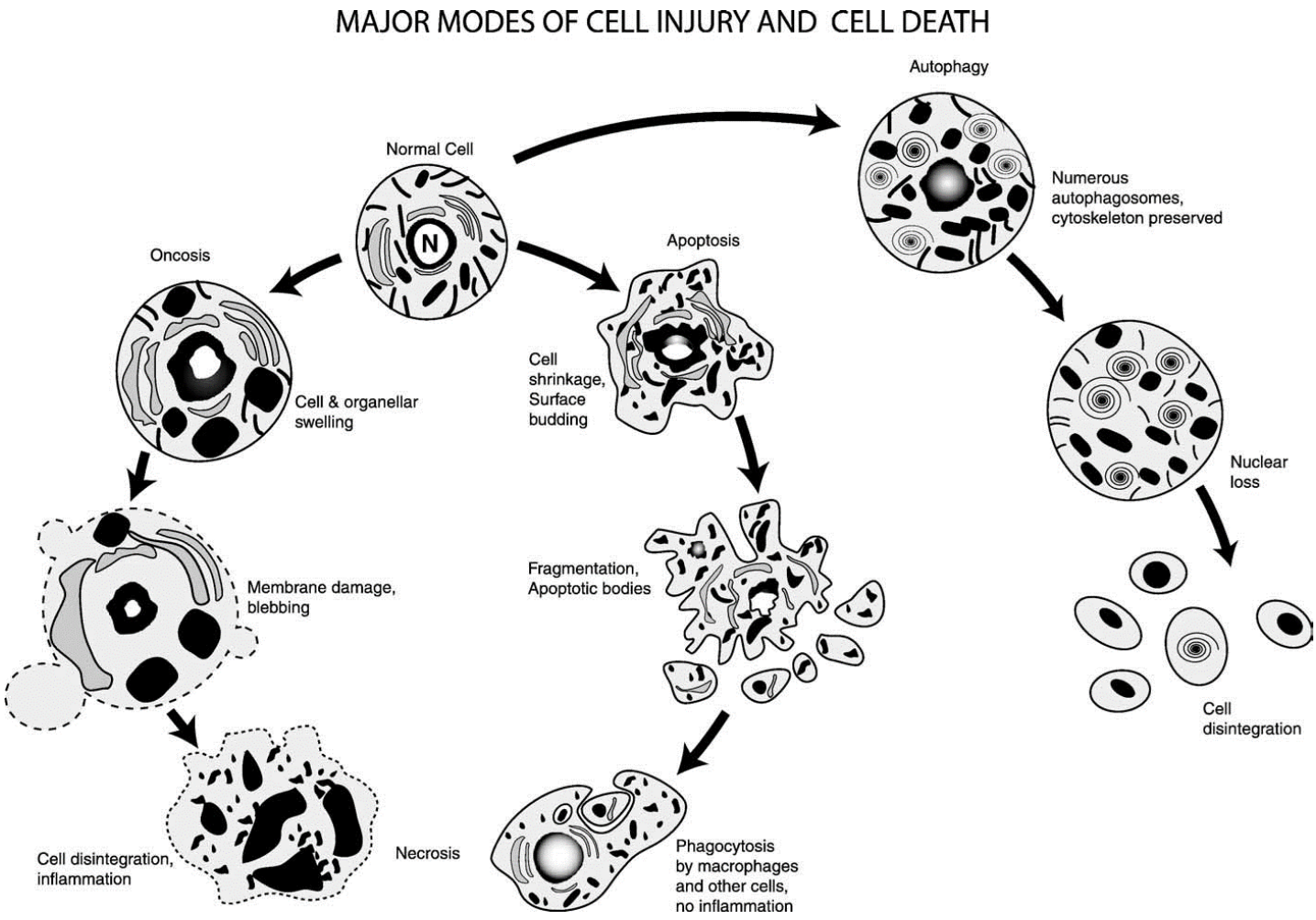



***Figure 8: Illustration of the various cell death pathways and necrosis. (Buja and Valera, 2008).***

### 2.4.1.1. Apoptosis

Apoptosis is considered to be a tightly regulated, evolutionary conserved, programmed and energy dependent process which consists of an **extrinsic** and **intrinsic** pathway, both of which culminate in cell death (Adapted from Kalogeris et al. 2012; Orogo and Gustafsson, 2014). The **extrinsic pathway** is mediated by death receptors while the intrinsic pathway is predominantly associated with the mitochondria.

Apoptosis is characterised by gene activation, activation of the cytosolic aspartate-specific cysteine proteases (caspases), mitochondrial alterations such as loss of membrane potential which culminates in membrane permeability transition and cytochrome c release. This is followed by endonuclease activation, which results in the fragmentation of DNA, membrane and morphological alterations. Buja, 2005).

The morphological changes observed during apoptosis can be distinguished into two stages. The **first stage** is characterised by nuclear condensation, DNA and nuclear fragmentation, plasma blebbing and cell shrinkage. While the **second stage** is characterised by the formation of apoptotic bodies that are engulfed by surrounding cells or macrophages to prevent an inflammatory response (Kerr et al. 1972).

**The Extrinsic Apoptosis Pathway**

The extrinsic apoptotic pathway triggers cell death through the activation of death domain receptors - localised on the plasma membrane. Ligands such as fatty acid synthetase ligand (FasL) and tumour necrosis factor alpha (TNF-α) will bind to their death receptors such as Fas receptor and TNF receptor, respectively. This leads to the recruitment of a number of death domain containing proteins such as the Fas-associated death domain (FADD) or TNF-receptor associated death domain (TRADD), to form the death-inducing signalling complex (DISC) (Kalogeries et al. 2012; Orogo and Gustafsson 2013). The DISC complex will activate caspase 8, which then initiates apoptosis according to one of two pathways which are dependent on the cell. In a **type I cell**, caspase will initiate apoptosis directly by cleaving executioner caspases 3 and 7. In a **type II cell,** caspase will have to initiate the intrinsic apoptotic pathway to induce cell death (Samraj et al. 2006). Type I and II cells are distinguished by the intracellular composition of the inhibitor of apoptosis proteins (IAPs), which deactivate the executioner caspase function (Jost et al. 2010).

**The Intrinsic Apoptosis Pathway**

The intrinsic apoptotic pathway is also known as the mitochondrial pathway because it relies on factors released from the mitochondria. This pathway is activated by **extracellular** and / or **intracellular** stress stimuli such as hypoxia, DNA damage and oxidative stress which induce the activation and translocation of the pro-apoptotic Bcl2 proteins such as BNIP3, NIX, and Bad from the cytosol to the mitochondria where they abolish the effects of the anti-apoptotic proteins or they directly activate Bax and Bak. Bax and Bak amalgamate to form pores on the OMM that will allow the translocation of proapoptotic proteins namely: cytochrome c, second mitochondria-derived activator of caspase (SMAC), direct inhibitor of apoptosis binding protein with low pI (DIABLO), Omi/ High temperature requirement protein A2 (HtrA2), apoptosis-inducing factor

(AIF) and endonuclease G from the intermembrane space into the cytosol. Cytochrome c binds to the apoptotic protease activating factor 1 (APAF1) which leads to the formation of the apoptosome which will recruit caspase 9 which is an activator caspase. Activated caspase 9 cleaves the executor caspases 3 and 7 (Orogo and Gustafsson, *op.cit*.). SMAC, DIABLO and Omi/HtrA2 activate the caspases by ingesting or engulfing caspase inhibitors while endonuclease G mediates DNA fragmentation (Kalogeries et al. 2012).

#### 2.4.1.2. Oncosis

Oncosis is the term used to define cell injury accompanied by swelling (Buja 2005). This form of cell death is induced by various insults and occurs in response to direct cell membrane damage or dysfunction and / or loss of energy which culminates in swelling and cell death (Buja and Entman 1998; Buja, 2005). Oncosis can be induced by various stimuli such as toxic chemicals, ischaemia, hypoxia, drugs and inflammation. To date, the disruption of cellular homeostasis in oncosis can be attributed to membrane damage which could occur in at least four ways: **(i)** direct damage to the membrane which can occur as a result of toxic elements such as chemicals or osmotic fluctuations as in calcium overloading, **(ii)** impairment of the respiratory chain which is accompanied by acidosis and decreased ATP production, **(iii)** the breakdown of membrane associated cytoskeletal proteins and **(iv)** the unregulated degradation and lipid peroxidation of membrane phospholipids due to ROS. Membrane damage due to injury progresses from reversible to irreversible based on these three stages: small alterations to the ionic transport system, increases in the permeability of the phospholipid bilayer, and lastly physical disruption of the membrane (Buja 2005; Buja and Vela 2008). The morphological changes that can occur due to these forms of injury include membrane blebbing, swelling and cell rupture. Oncosis is considered to evolve into necrosis within 24 hours (Manjo and Joris, 1995). The leakage of intracellular components of oncotic cells into the extracellular environment often activates an inflammatory response.

#### 2.4.1.3. Necrosis and Necroptosis

Prior to the discovery of apoptosis in mammalian cardiac cells it was thought that ischaemia predominantly resulted in necrosis (Suleiman et al. 2001). Necrosis is

considered to be an accidental, passive form of uncontrolled cell death which is independent of energy and can be toxic to the cell (Adapted from Orgo and Gustafsson, 2013). The role of mitochondria in mediating cell death through necrosis is associated with prolonged/ irreversible MPTP opening and ATP depletion. However, apoptosis is associated with moderate insults that lead to reversible pore opening and maintained ATP production. To date, there is evidence suggesting that necrosis could also be a tightly regulated form of cell death which has been called necroptosis (Golstein and Kroemer, 2007). The effector receptors that are responsible for the initiation of necroptosis are the receptor interacting proteins (RIPs). RIP1 and RIP3 have been implicated as the mediators of necroptosis (Cho et al. 2009). The evidence from Cho's group speculates that activation of RIP 3 and 1 culminates in phosphorylation of MPTP components, increased ROS production and ultimately ends in cell death. Evidence also shows that caspase 8 seems to have a dual role. Apart from induction of the extrinsic and intrinsic apoptotic pathways, it has also been implicated in the regulation of necrosis as a suppressor (McIlwain et al. 2013). Although there is still much to be uncovered about the necroptosis pathway, Kalogeries and colleagues (2012) suggest that it is gaining acceptance in the field.

Considering the above, it is evident that much is known about the various cell death/ injury pathways. However, there are a number of aspects which need to be clarified. For example, it remains unclear which mechanism of cell injury/ death is triggered and how much each form of cell injury contributes to the myocardial infarct. Both these questions have therapeutic implications and answering them would enable us to target the most predominant form of cell death when triggered. What we do know at this point is that mitochondria are the central energy generators but they are also the central mediators of injury and cell death. Consequently, they are an important therapeutic target for I/R. Manipulating and enhancing intrinsic mechanisms such as **mitophagy** through pharmacological interventions could potentially salvage the myocardium and promote cardiomyocyte survival. For our study we employed melatonin and in the next section we will review various properties of melatonin that may substantiate its usefulness in the context of mitophagy.

## 2.5. Melatonin

Melatonin is an indole amine which has been detected in bacteria, plants and humans (Hardeland, 2016). Melatonin, also known as N-acetyl-5-methoxytryptamine, was identified and structurally characterised by Lerner and colleagues in 1958 (Lener et al. 1960) as a product of the pineal gland. Since its discovery melatonin was thought to be exclusively synthesised in the pineal gland however, it is now known to be produced in extra- pineal tissues including the testis, ovary, bone marrow and lymphocytes (Tijmes et al. 1996; Itoh et al. 1997; Tan et al. 1999; Carrillo-Vico et al. 2004). In the pineal gland, melatonin synthesis occurs through several well characterised enzymatic steps in pinealocytes from the precursor tryptophan. Synthesis commences with the uptake of tryptophan, from circulation, and its conversion to 5-hydroxytryptophan and ultimately serotonin. Serotonin is converted to N-acetylserotonin which is converted to melatonin through the action of N-acetyltransferase (NAT) and hydroxyindole-O-methyltransferase (HIOMT), respectively (Axelrod 1974).

Primarily known for its role in the regulation of circadian rhythm and seasonal reproduction, melatonin is now known for a wider range of physiological functions including its role in obesity, immunology and asthma amongst other things. These functions have been extensively reviewed by Pandi-Perumal et al. (2006), Calvo et al. (2013); Cipolla-Neto et al. (2014) and Marsegila et al. (2014). Of particular interest to our study is melatonin's protective mechanisms offered by its antioxidant, free radical scavenging and signalling pathways.

#### 2.5.1.1. Melatonin as Free Radical Scavenger and Antioxidant

ROS production in cells is not only inevitable but it is essential for various processes including signalling and induction of autophagy. Under basal conditions, if the equilibrium between ROS production and antioxidant defences is tilted in favour of oxidants it can easily be rectified by endogenous cellular and mitochondrial antioxidant systems. These enzymatic and non-enzymatic systems (previously highlighted) are considered the first line of defence and are able to catabolise toxic oxidants into neutral products. Under these very same conditions moderate increases in ROS result in the upregulation of antioxidant expression as well as activity (Gechev et al. 2002; Rodriguez et al. 2004). This adaptive mechanism not only offers protection against

ROS damage but it keeps ROS levels at homeostatic levels allowing these molecules to still fulfil their regulatory role.

Under stressful conditions such as in I/R where ROS levels are elevated and antioxidant systems are overwhelmed, the adaptive mechanism which is activated under basal conditions may fail. This failure, which is also evident in ageing, can be attributed to damage and instability of the molecular machinery such as mitochondrial DNA (mtDNA) which is responsible for increased antioxidant expression and activity (Wei and Lee, 2002).

Melatonin's indirect beneficial effects on antioxidant regulation are well exhibited in such conditions. There are several characteristics that make melatonin such an effective antioxidant. It is amphiphilic and it can easily cross various bio-barriers (Coto-Montes et al. 2012). These characteristics not only make melatonin highly accessible to all tissue types and cells but it also ensures that it can be distributed to subcellular compartments such as the cell membrane, nucleus and mitochondria. In addition, melatonin is highly concentrated in the mitochondria and recent evidence shows that melatonin is also produced in the mitochondria (Tan et al. 2016). This unlimited accessibility means that melatonin can localise to sites where ROS production is augmented.

Evidence of melatonin's role in antioxidant activity dates as far back as the 90's. It has been implicated in stimulating the upregulation of SOD (Okanti et al. 2000; Liu and TB, 2002), GPx (Barlow-Walden et al. 1995; Okatani et al. 2000) and to a lesser extent glutathione reductase and catalase (Tomas-Zapico et al. 2002; Liu and Ng, 2002). These effects have been predominantly studied in neuronal tissue. In addition to enhancing antioxidant systems, melatonin also decreases prooxidant enzymes and several lipoxygenases (Pozo et al. 1994; Hardeland and Pandi-Perumal, 2005). The exact mechanism through which melatonin upregulates antioxidants and downregulates prooxidants remains to be elucidated. However, evidence point to gene expression either through melatonin receptor function or other interactions (Antolin et al. 1996; Mayo et al. 2002).

Apart from mediating the efficient removal of ROS indirectly by influencing antioxidant systems, melatonin and its metabolites can directly scavenge several ROS and RNS.

Melatonin's scavenging abilities are attributed primarily to its core structure and its side chains (Tan et al. 2002). Structural changes to either the indole moiety to yield structurally similar analogues such as benzofurane resulted in decreased antioxidant capacity in comparison to melatonin (Gozzo et al. 1999). The same was also true when the side chains of melatonin were analysed (Poeggeler et al. 2002). The chemical reactions that have been associated with melatonin's actions include electron donation, hydrogen donation, addition and substitution amongst others (Tan et al. 2002). Melatonin's structural properties allow it to neutralize various ROS and RNS directly or indirectly. The ROS and RNS targeted by melatonin include hydrogen peroxide, hydroxyl radical, singlet oxygen, peroxyl radical, NO radical, peroxynitrite and hyperchlorous acid (Zhang and Zhang, 2014).

Melatonin's ability to scavenge free radicals and improve antioxidant systems has important implications for the mitochondria especially in stressful settings such as in I/R. As previously mentioned complex I and III are the major contributors of superoxide production during mitochondrial oxidative phosphorylation. The production of NO could be due to the activity of nitric oxide synthase (NOS): under basal conditions its contribution to NO production is minimal however, during pathophysiological conditions production is upregulated and can lead to the production of peroxynitrate. The toxicity of peroxynitrite is considered to be equivalent to that of the hydroxyl radical (Pryor and Squadrito, 1995). Thus swift removal of both radicals could save the cell from extensive ROS and RNS damage.

Hydrogen peroxide neutralisation is also of great importance, since it is the principal precursor of hydroxyl radical formation. Peroxyl radical formation is a consequence of the peroxidation of polyunsaturated fatty acids. Induction of peroxyl formation often culminates in a positive feedback loop where the reaction is repeated due to the presence of certain products (Paradies et al 2015). An added dimension of complexity regarding most of these reactive species is that they are capable of not only damage but modifying neutral molecules into potential reactive species. This could induce a whole irreversible cascade of reactions with far reaching consequences. Thus it is evident that the swift and yet selective removal of excessive free radicals plays an important role in promoting cell survival.

Fortunately circulating melatonin or exogenous administration of the hormone at pharmacological concentrations could trigger the uptake of these species through melatonin's scavenging cascade reaction. In this reaction, a single melatonin molecule [together with its major metabolites, such as cyclic-hydroxymelatonin (3-OHM), N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N-acetyl-5-methoxykynuramine (AMK) ] can scavenge up to 10 ROS or RNS molecules (Adapted from Tan et al. 2002, 2007). For a detailed description of melatonin's chemical reaction with the various reactive species, see Allegra et al. (2003) for a detailed review. This reaction allows melatonin to swiftly restore ROS and RNS levels back to homeostatic levels. Protection offered by melatonin is mainly associated, but not limited to, the safeguarding of proteins, DNA and lipids from ROS and RNS damage (previously discussed) as well as preservation of mitochondrial function and quality control (to be discussed below).

#### 2.5.1.2. Melatonin and the Mitochondrial Function

Mitochondrial function is essential for the survival of cells. Highly functional mitochondria are characterised by efficient oxidative phosphorylation, low levels of ROS and oxidative modification of proteins ( Adapted from Tan et al. 2016). As previously mentioned, melatonin is highly lipophilic and consequently it can easily permeate cellular membranes such as the mitochondrial membrane where it can accumulate in high concentrations and influence mitochondrial homeostasis by actively contributing to the existence of highly functional mitochondria. Evidence has shown that melatonin contributes to mitochondrial homeostasis by stabilising the ETC and cardiolipin which in turn enhances oxidative phosphorylation capacity which is a key component of mitochondrial bioenergetics (Petrosillio et al. 2009).

It has been shown that melatonin increases the activity of complex I and IV in the ETC in a time-dependent manner in isolated brain and liver mitochondria (Martin et al. 2000). In the same study, evidence showed that melatonin counteracted the pro-oxidant effects of ruthenium red by restoring complex I and IV to control levels and by partially restoring the activity of GPx. The most likely scenario is that melatonin interacts with the ETC by accepting and donating electrons as well as hydrogen ions, which could in turn increase the electron and proton gradient (Adapted from Paradies et al. 2015).

López and co-workers (2009) also investigated the effects of melatonin on mitochondria. In their model they found that melatonin slightly uncoupled oxidative phosphorylation, which was associated with decreased oxygen consumption and consequently ROS formation and oxidative damage. This slight uncoupling was associated with preserved function of the ETC function and ATP production. This mechanism of uncoupling is seen as protective, since neuronal cells also employ it to prevent oxidative stress and death. In their study melatonin improved the activity of complexes I, III and IV (López et al. 2009). The effects seen above can be attributed to its direct interaction with the mitochondria.

Melatonin may also indirectly contribute to the mitochondrial function by stabilising cardiolipin. Cardiolipin is a polyunsaturated fatty acid which is exclusively and abundantly found in the IMM (Paradies et al. 2009). Apart from maintaining membrane integrity, cardiolipin is associated with the optimal activity of various electron transport complexes and enzymes. This comes as no surprise considering the close association with the IMM where the ETC components are situated.

Cardiolipin has also been implicated in the formation of “supercomplexes”. The term supercomplexes refers to the clustering of related components of the ETC to form units that decrease electron leakage by efficiently channelling the transfer of electrons ( Adapted from Houtkeeper and Vaz, 2008; Paradies et al. 2009, 2015). By preventing cardiolipin oxidation and subsequent loss, melatonin indirectly preserves mitochondrial function. Three studies from Petrosillo’s laboratory (2006, 2009a, 2009b) have shown the effect of melatonin on cardiolipin as well as mitochondrial homeostasis. The first study demonstrated that melatonin’s protective effects in I/R were associated with improved mitochondrial respiration, membrane integrity as well as function (Petrosillo’s et al. 2006). In addition, they observed that melatonin in this setting prevented the loss of complex I and III activity, increased production of hydrogen peroxide and cardiolipin loss through oxidation. The subsequent studies showed that by averting cardiolipin loss melatonin prevented mitochondrial permeability transition and ultimately cytochrome c release (Petrosillo et al. 2009a,) in I/R (Petrosillo et al. 2009b).

Despite the differences in dose, time and route of administration in the various studies melatonin consistently preserved and enhanced mitochondrial function even in the setting of I/R. Melatonin's ability to localise in subcellular compartments gives it an added advantage compared to other molecules with similar therapeutic potential.

#### 2.5.1.3. Melatonin, Cardioprotection and Signalling

Melatonin's cardioprotective properties have been an area of intense research for many years. Studies have used various models of cardiovascular disease, specifically I/R, in various animal and experimental models to elucidate the role of melatonin and test its robustness as a cardioprotectant. Ex-vivo and in situ experimental heart models have shown that melatonin's cardioprotective actions are predominantly associated with a reduction in IFS, ventricular fibrillation and arrhythmias, morphological damage and ROS production (Adapted from Reiter and Tan, 2003).

Tan and co-workers (1998) examined the effects of melatonin at 1, 10 or 50μM on I/R-induced arrhythmias in isolated hearts. The data showed that melatonin at all three concentrations was able to reduce the frequency as well as the length of premature ventricular contractions and ventricular fibrillation in comparison to the control hearts. In the same study Vitamin C, which is a classical antioxidant, was used as positive control and for comparison of efficacy. Vitamin C had no effects on the measured parameters and was less potent than melatonin.

Melatonin's effects in regional ischaemia/ reperfusion were also compared to a structurally similarly indole compound [5-methoxy-carbonyl-N-acetyl-tryptamine (5-MCA-NAT)]. In this study by Lagneux and colleagues (1999), melatonin and 5-MCA-NAT were administered through intraperitoneal injection. IFS as well as ventricular tachycardia and / or fibrillation (VT-VF) were measured together with other haemodynamic endpoints. Both compounds significantly reduced VT-VF and IFS. This not only demonstrated melatonin's cardioprotective characteristics but also showed that its structure played an irreplaceable role in its mechanism of action.

Sahna et al. (2002) conducted a study to evaluate melatonin's physiological role on I/R arrhythmias. In this study rats that were pinealectomized (Px) two months before experimentation. It was found that Px rats had a higher incidence irreversible

ventricular fibrillation which resulted in an increased mortality rate compared to controls. In a second set of experiments the group investigated the effect of 0.4mg/kg melatonin administration either prior to ischaemia or reperfusion in Px rats. In these experiments they found that melatonin administration decreased the occurrence of ventricular fibrillation.

All these studies, regardless of differences in I/R protocols, routes of melatonin administration etc., have shown melatonin's therapeutic potential. The cardioprotective actions of melatonin have been thoroughly reviewed (Reiter et al. 2003; Yang et al. 2014). Current research has shifted to focusing on the signalling mechanisms involved in cardioprotection. Although there are various signalling molecules and pathways that have been identified, for the purpose of this review the pathways/ molecules that will be discussed below are those pertinent to the present study.

**Melatonin and the Sirtuins (Silent information Regulators)**

Sirtuins (Sirt1-7) belong to the superfamily of histone deacetylases (HDACs). These molecules are responsible for regulating transcriptional modification by removal of the acetyl group (deacetylation). Generally, sirtuins have a wide range of functions and are associated with DNA repair, cell survival, cell cycle and apoptosis (Chalkiadaki and Leonard 2015). Sirtuins also play a role in metabolic regulation, because they consume one molecule of $NAD^+$ per reaction they are highly sensitised to cellular stress that decreases the nicotinamide levels (Adapted from Cattlen et al. 2015; Mayo et al. 2017). Sirt1 and Sirt3 are associated with the nucleus / cytosol and mitochondria, respectively and are well-known as metabolic regulators.

Sirt1 activation can result in metabolic changes (e.g. gluconeogenesis) and mitochondrial biogenesis by activating PGC-1 alpha. Although PGC-1 alpha overexpression is sufficient to stimulate mitochondrial biogenesis (Wende et al. 2007), it can be activated through several signalling pathways and various post translational modifications including deacetylation by Sirt1 (Soutland and Gottlieb, 2015). Once activated, together with nuclear respiratory factor 2 (NRF-2), PGC-1 alpha activates NRF-1. The NRFs are responsible for directing the transcription of mitochondrial nuclear proteins, import machinery and cofactors and regulatory factors required for

the assembly of the ETC complexes and mtDNA transcription and translation (Gottlieb and Gustafsson, 2010). In this study PGC-1 alpha activation through Sirt1 was assessed and it was found that Sirt1 can also deacetylate the Forkheadbox (Foxo) transcription factors to induce resistance to oxidative stress (Nogueiras et al. 2012).

Since melatonin's actions are related to mitochondrial function, Sirt3 would be one of melatonin's major mitochondrial targets. Sirt3, located in the mitochondrial matrix, regulates mostly the lysine modifications related to its function. In mitochondria, Sirt3 activation is associated with strengthened antioxidant defence via the upregulation of mitochondrial SOD, improved oxidative phosphorylation via several complexes and fatty acid and acetyl CoA metabolism (Ahn et al. 2008; Sack, 2012; Bause and Haigis et al. 2013; Mayo et al. 2017). The sirtuins are also associated to autophagy regulation of autophagy (Gottlieb and Gustafsson, 2011) and in I/R, the caridioprotective effects of melatonin have been linked to both Sirt1 and Sirt3.

A study conducted by Yu's laboratory (2014), showed that melatonin offers protection in myocardial I/R via its receptors. In this study, rats were exposed to melatonin treatment in the absence or presence of either luzindole (a melatonin receptor agonist) or EX527 (a Sirt1 inhibitor). Melatonin's protective functions were associated with decreased IFS and apoptotic index, improved cardiac function and protection from oxidative damage. These effects were attributed to the activation of Sirt1 in a receptor-dependent manner. The same group has also reported melatonin's protective effects to Sirt1 signalling in a type 2 diabetic model (Yu et al. 2015).

More recently, Yu and colleagues (2017) showed that in a type-1 diabetic model of I/R melatonin exhibited its classical protective functions, through signalling. The study showed that melatonin improved cardiac performance, mitigated mitochondrial oxidative damage and stress, improved ATP production and reduced apoptosis. All these actions were attributed to the induction of AMPK-PGC-1 alpha-Sirt3 signalling. In this same study the authors showed that blocking AMPK, using a specific AMPK signalling blocker, abolished these effects.

Melatonins has been well studied in various disease models and organs. Multiple pathways of melatonin protection are being/ have been discovered and elucidated some of them include the RISK, SAFE and Notch pathways. In addition, there are

emerging studies that are focusing on elucidating melatonin's unknown characteristics such as potential pro-oxidant effects.

From the above it is evident that melatonin is a promising therapeutic agent that has a wide range of functions. Its cardioprotective effects have been thoroughly studied (See **Fig 9**) however, so much more still remains to be uncovered. In the context of this study, manipulating mitophagy with melatonin will not only unravel the role of mitophagy in I/R but it will allow us to observe the effects of melatonin in this setting.

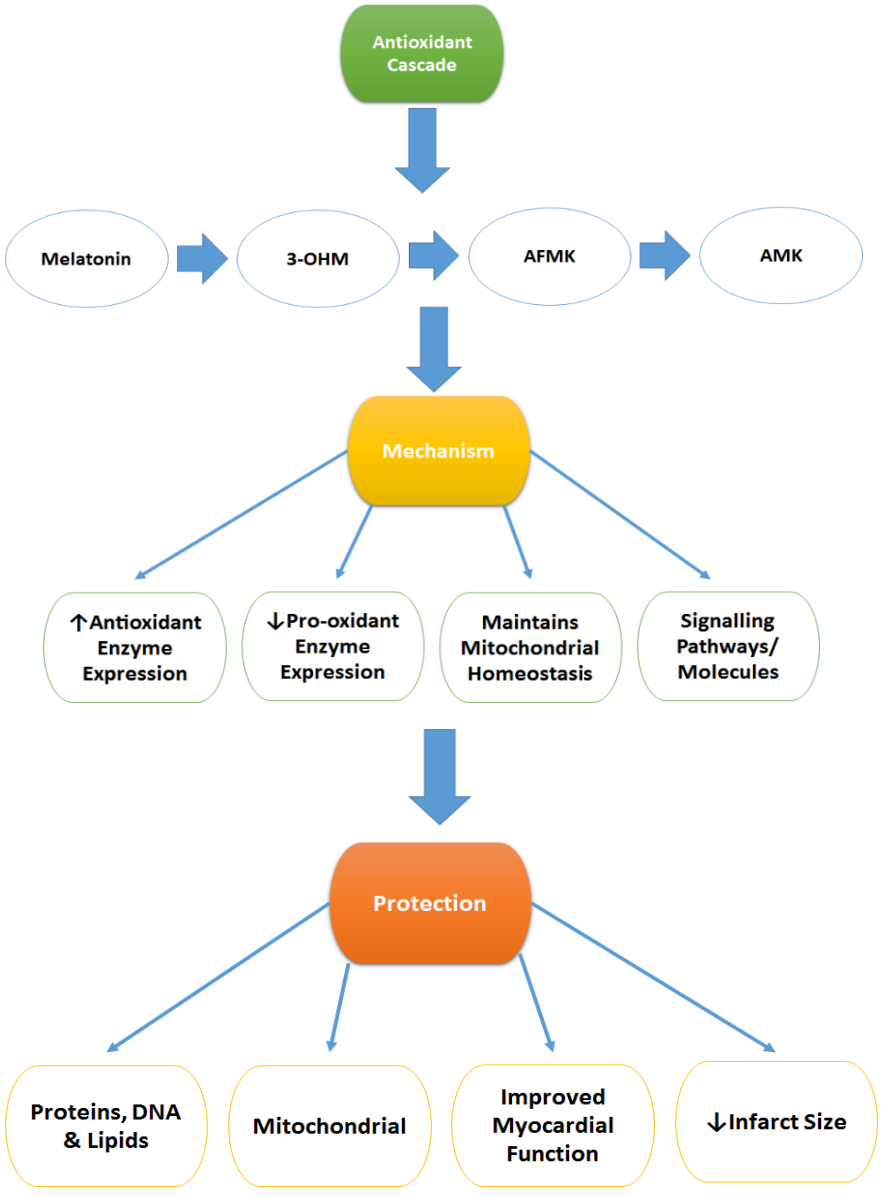



***Figure 9: Schematic illustrating melatonin's actions from the cascade reaction to conferring protection. (Modified from Zhang & Zhang, 2014).***

## 2.6. Concluding remarks

I/R is the transient disruption of blood supply which is associated with oxygen and nutrient deprivation. Subsequent reperfusion is associated with mainly four types of injury (stunning, microvascular obstruction i.e. no-flow phenomenon, reperfusion arrhythmias and IRI). IRI is the most complex, since it involves various structural and molecular changes that ultimately contribute to the other types of injury.

Mitochondrial protection have been identified as a therapeutic target and it has become imperative to find a therapeutic agent or drug that will prevent mitochondrial damage and offer cardioprotection upon reperfusion. In this study, melatonin has been identified as the cardioprotectant and it has been coupled to mitophagy (the intrinsic mechanism) which is related to the target of interest i.e. damaged and dysfunctional mitochondria. Manipulating mitophagy pharmacologically in the well-established working heart model of I/R may give us more insight on the role of mitophagy in I/R and the therapeutic potential of melatonin in this setting.

# CHAPTER 3: MATERIALS AND METHODS

## 3.1. Ethics Approval and Experimental Animals

Ethics approval was obtained from Research Ethics Committee: Animal Care and Use (REC: ACU) of Stellenbosch University (Faculty of Medicine and Health Science; Protocol #: SU-ACUM14-00039). Animals were handled and cared for according to the accepted standards and use of animals in research and teaching as reflected in the South African National Standards document (SANS 10386:2008; available at www.sun.ac.za/research ) of the South African Bureau of Standards. All research procedures and conduct was in line with the standard operating procedures and guidelines of the REC: ACU.

Male Wister rats (250-300g) were used for this study. All animals were housed in and obtained from Stellenbosch University's Central Animal Research Facility located in the Faculty of Medicine and Health Sciences, Tygerberg. This facility was previously accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The animals were fed a standard rat chow diet and received access to food and water *ad libitum.* They were exposed to 12-hour dark/light cycles (with light from 6am-6pm) at a constant temperature of 20-21°C.

## 3.2. Isolated Heart Perfusions

Isolated heart perfusion are one of the most popular experimental models in cardiovascular research. Pioneered by Oscar Langendorff in 1885, the technique was developed from the isolated frog heart perfusion model which was previously established by Elias Cyon (Bell et al. 2011). Since its establishment, the technique has been modified and refined over time. Despite this, the principle has remained the same. Perfusion of the isolated mammalian heart has unravelled the fundamental physiology of the heart. While the technique is old, it still continues to give valuable insight into cardiovascular physiology and pharmacology. This experimental model remains attractive to researchers for a number of reasons. Firstly, the isolated heart of small mammals e.g. rats is highly reproducible, secondly the preparation is quick and thus allows for large numbers to be studied at a relatively low cost. Lastly, the quality and quantity of the data that are produced or that can be measured are valuable

(Sutherland and Hearse, 2000). Although irreplaceable, this model has two limitations. Firstly, studying the heart ex vivo means that one cannot observe the effects of other factors such as the systemic circulation and neurohormonal factors. However, this limitation can be seen as an advantage since measurements can be made in the absence of the confounding effects of the above (Sutherland and Hearse, 2000; Skrzypiec-Spring et al., 2007). Lastly, the model is clinically less relevant (Hearse and Sutherland, 2000) and it can be challenging to translate findings to humans. Despite this, it is important to bear in mind that while there may be decreasing clinical relevance, animal models are the cornerstone for the identification and development of novel therapies. For this study, the isolated working rat heart perfusion technique, established by Neely and Morgan (1967) was used. This model, which is simply a conversion of the retrogradely perfused (Langendorff) rat heart into working mode, allows for the study of cardiac contractile function, pharmacology, vascular biology, morphology and vascular anatomy as well as biochemistry (Zimmer, 1998; Sutherland and Hearse 2000).

### 3.2.1. Reagents

Filtered modified Krebs-Henseleit buffer (KHB) was the perfusate of choice. The buffer contained: 119.00mM NaCl, 25.00mM $NaHCO_3$, 4.75mM KCl, 1.2mM $KH_2PO_4$, 0.6mM $MgSO_4.7H_2O$, 0.6mM $Na_2SO_4$, 1.25mM $CaCl_2$. $H_2O$ and 10mM glucose. The buffer has a pH of 7.4 at 37°C when gassed with a 95% $O_2$/5% $CO_2$ mixture and mimics the key ionic components of blood.

### 3.2.2. Procedure

Rats were anaesthetised by the intraperitoneal injection of sodium pentobarbitone (Bayer, Johannesburg, South Africa) (30mg/rat). Absence of the pedal pain withdrawal reflex (foot pinch) was indicative of deep anaesthesia.

Subsequently, the heart was cannulated via the aorta onto the aortic cannula. The aorta was secured with a crocodile clip and a suture was used to tie it down. The cannulation of the left atrium using one of the pulmonary orifices is the last and most critical step of the working heart perfusion.

Stabilization and wash out of the blood, attained by perfusing the hearts in retrograde mode, was done at a constant hydrostatic pressure of 100cm $H_2O$. Following stabilization, hearts were perfused in the working mode (also known as anterograde, which will be referred to as working mode from here onwards) at a preload and afterload of 15cm $H_2O$ and 100cm $H_2O$ respectively (Marais et al. 2005).

After stabilization, the hearts were subjected to either global or regional ischaemia. In the treatment groups, melatonin was administered prior to and post ischaemia i.e. at the end of the stabilisation and beginning of reperfusion periods (**Refer to section 3.2.6**).

To monitor myocardial temperature, a small incision made through the coronary sinus allows for the placement of a temperature probe. Myocardial temperature was kept constant at 34.5- 36°C, 36.6-36.8 °C and 36.5 °C during retrograde, working heart and ischaemic period respectively. In order to determine myocardial contractile function, for both the global and regional ischaemia protocols, haemodynamic measurements (discussed below) were recorded prior to the induction of ischaemia and post ischaemia, during reperfusion.

### 3.2.3. Haemodynamic Data for Isolated Heart Perfusions

Myocardial functional performance was measured during perfusion in the working mode at two time points namely prior to the induction of ischaemia as well as during reperfusion. The parameters were: coronary flow (Qe), aortic output (Qa), peak systolic pressure (PSP), heart rate (HR) and total work (TW).Qe and Qa, in mL/minute, were measured manually. Cardiac output (mL/min) was calculated as the sum of the coronary flow and aortic output. The aortic pressure (peak systolic pressure and diastolic pressure; mmHg) and HR (bpm) were measured using a Viggo- Spectramed pressure transducer which was inserted into the aortic perfusate line and coupled to a computer with specialized software (Lochner et al. 2006). Hearts that displayed impaired myocardial function and had an aortic output of <28mL/minute were excluded from the study.

The mean external power produced by the left ventricle (Total Work- TW) in mWatts (mW) was calculated according to the formula described by Kannengieser and

colleagues (1979) as follows: TW= 0.002222(PAO-11.25) (CO), where POA= aortic pressure and CO= cardiac output. The functional recovery of the hearts was determined by expressing the post-ischaemic TW as a percentage (%) of the pre-ischaemic TW.

### 3.2.4. Melatonin Administration

Melatonin (Sigma Aldrich, St Louis, Missouri, United States), was first dissolved in 250µL of absolute ethanol and diluted in modified KHB to a final concentration of 0.3µM or 50µM (see **Appendix A**). Based on previous studies in our laboratory the 50µM concentration of melatonin was selected. In view of the study done by Lamont and co-workers (2011) the 0.3µM concentration was selected. Melatonin was perfused into the heart for a duration of 10min.

### 3.2.5. Experimental Protocols, Groups, Sample sizes

For this study 155 male Wistar rats were used and all the experimental groups were randomized. All hearts were subjected to a stabilization period which consisted of 10 min retrograde perfusion; 10 min working heart perfusion; 10 min retrograde perfusion prior to either global ischaemia or regional ischaemia, followed by reperfusion. Hearts subjected to global ischaemia (n=123) were perfused according to either one of three perfusion protocols namely: stabilisation, ischaemia or reperfusion, which refers to the time periods in which melatonin was applied, i.e. melatonin applied at the end of stabilisation period (stabilisation group) or melatonin applied at the end of stabilisation period prior to global ischaemia (ischaemic group) or melatonin applied at the end of stabilisation prior to global ischaemia and at the onset of reperfusion (reperfusion group).

Hearts exposed to global ischaemic experimental protocols were used either for mitochondrial phosphorylation studies and Western blotting of mitochondrial markers (n=69) or freeze-clamped for the analysis of cytosolic markers using western blotting (n=54) (**Fig 10 and 11**).

Hearts subjected to regional ischaemia (n=32) were used for the analysis of IFS. In the treatment group, melatonin was applied at the end of the stabilisation period prior to regional ischaemia as well as at the start of reperfusion. IFS analysis followed at

the end of reperfusion (**Fig 10 and 11**). All protocols are discussed and depicted in detail in subsequent sections (see **section 3.2.6**).

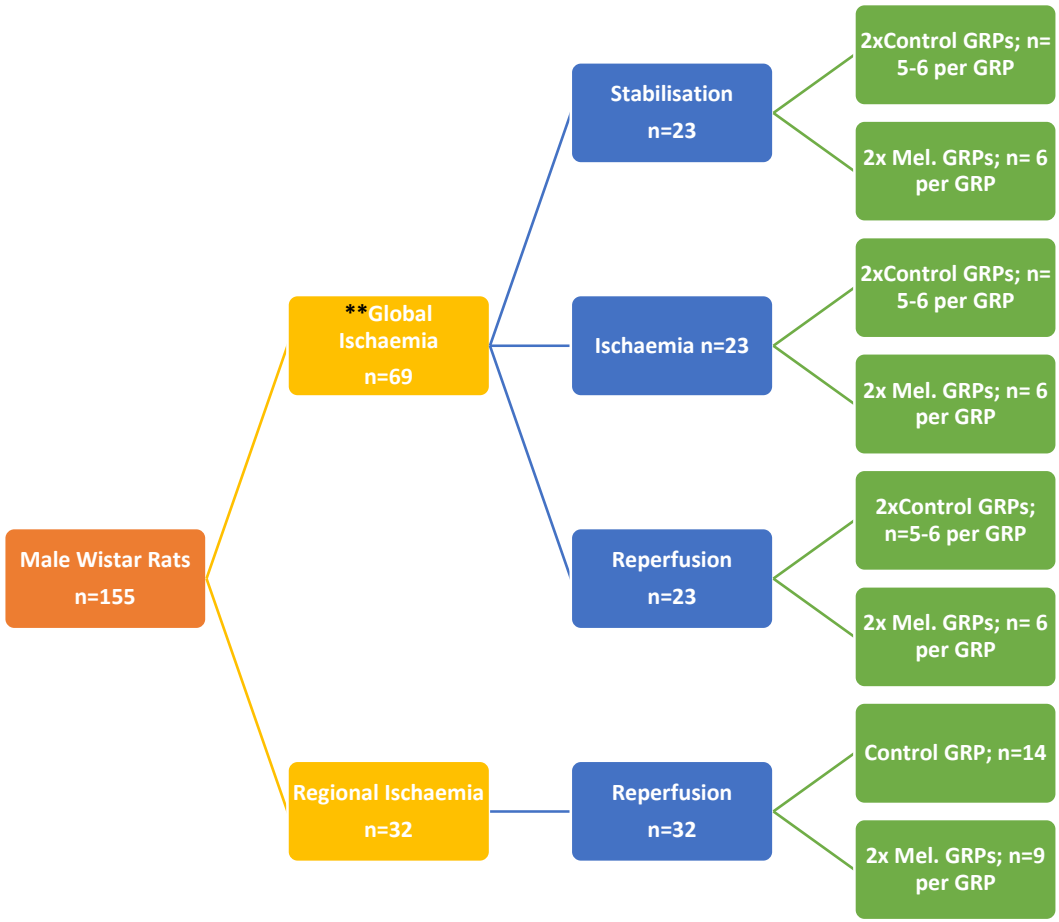



***Figure 10:*** ***Outline of experimental groups and protocol. ** Indicates the omission of the 54 hearts that were subjected to global ischaemia and freeze clamped; this omitted group consisted of a single control group (n=6) and 2 melatonin groups (n=6 per group) for the stabilisation, ischaemia and reperfusion protocols. Abbreviations: n- Sample Size; GRP/s- Group/s.***

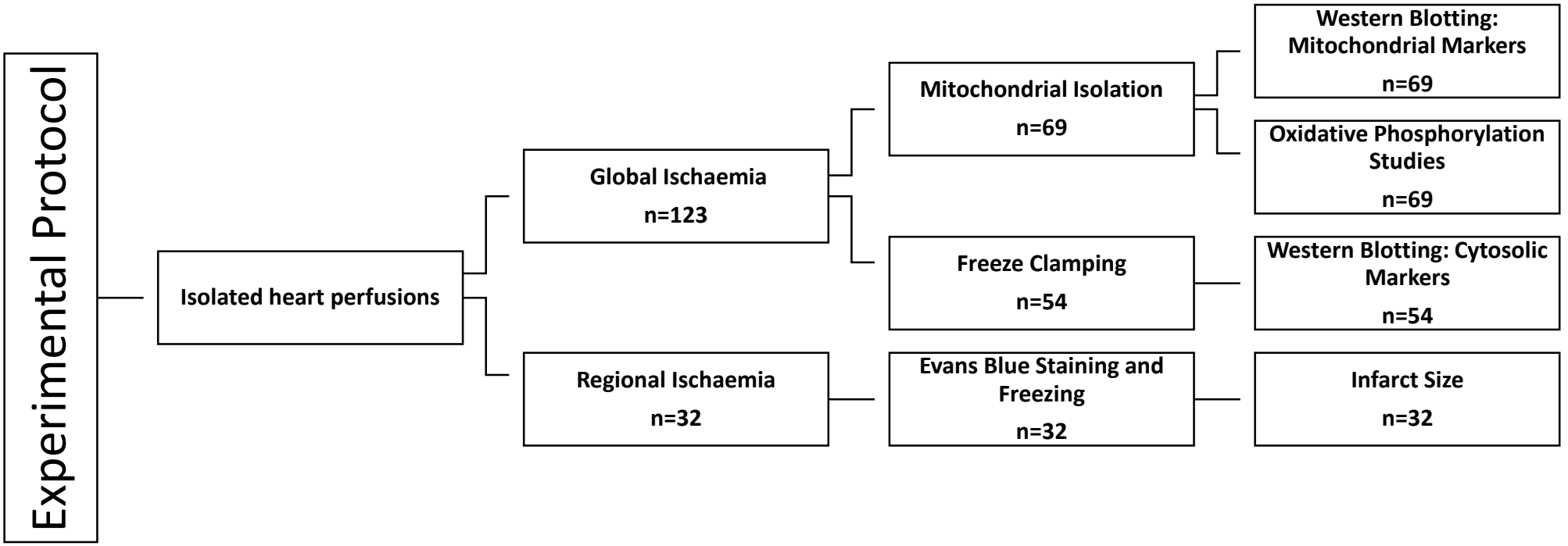



***Figure 11: Outline of the experimental study design,***

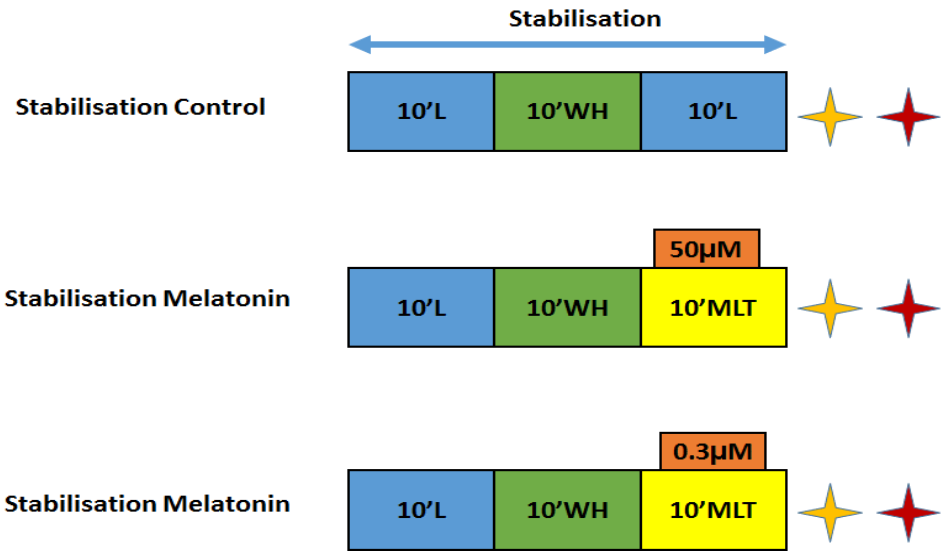
### 3.2.6. Experimental Protocols:

#### 3.2.6.1. Global Ischaemia

The induction of global ischaemia was achieved by closing off the aortic and the atrial cannulas (no flow ischaemia, Qe: 0mL/min). Melatonin was administered directly to the heart prior to or post ischaemia (as indicated in **Fig 12** below) using a separate drug line. For a detailed description of the melatonin calculations as well as preparation please refer to **Appendix A**. The myocardial temperature was monitored and kept at 36.5°C during the 20 minutes of ischaemia. Reperfusion was initiated by opening the aortic cannula once the 20 minute ischaemic period had elapsed. Hearts were either freeze clamped or used for mitochondrial isolation at the end of the protocol as depicted in figure 12 below. The global ischaemia protocols were as follows:

- **Stabilisation**: 10 minutes retrograde perfusion; 10 minutes working heart perfusion; 10 minutes retrograde perfusion. For the stabilisation melatonin groups, melatonin was administered at a concentration of 50µM or 0.3µM during the last 10 minutes of the retrograde perfusion.
- **Ischaemia**: 10 minutes retrograde perfusion; 10 minutes working heart perfusion; 10 minutes retrograde perfusion and 20 minutes global ischaemia. For the ischaemia melatonin groups, melatonin was administered at a concentration of 50µM or 0.3µM prior to ischaemia (during the last 10 minutes of retrograde perfusion).
- **Reperfusion**: 10 minutes retrograde perfusion; 10 minutes working heart perfusion; 10 minutes retrograde perfusion; 20 minutes global ischaemia; 30 minutes of reperfusion which consisted of 10 minutes of retrograde perfusion followed by 20 minutes of working heart perfusion. For the reperfusion melatonin groups, melatonin was administered at a concentration of 50µM or 0.3µM (10 minutes prior to and 10 minutes post global ischaemia).

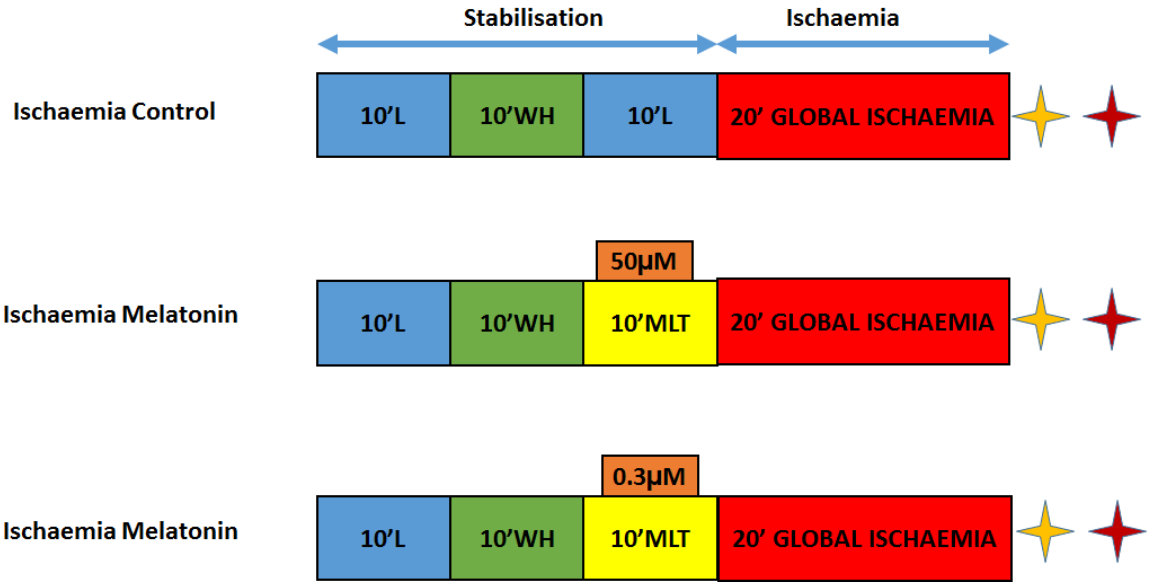
**Stabilisation (Stb)**




**Keys:**

| | |
|---|---|
| 'L | Minutes Retrograde |
| 'WH | Minutes Working Heart |
| MLT | Melatonin |
| | Freeze Clamping |
| | Mitochondrial Isolation |

**Ischaemia (Isch)**



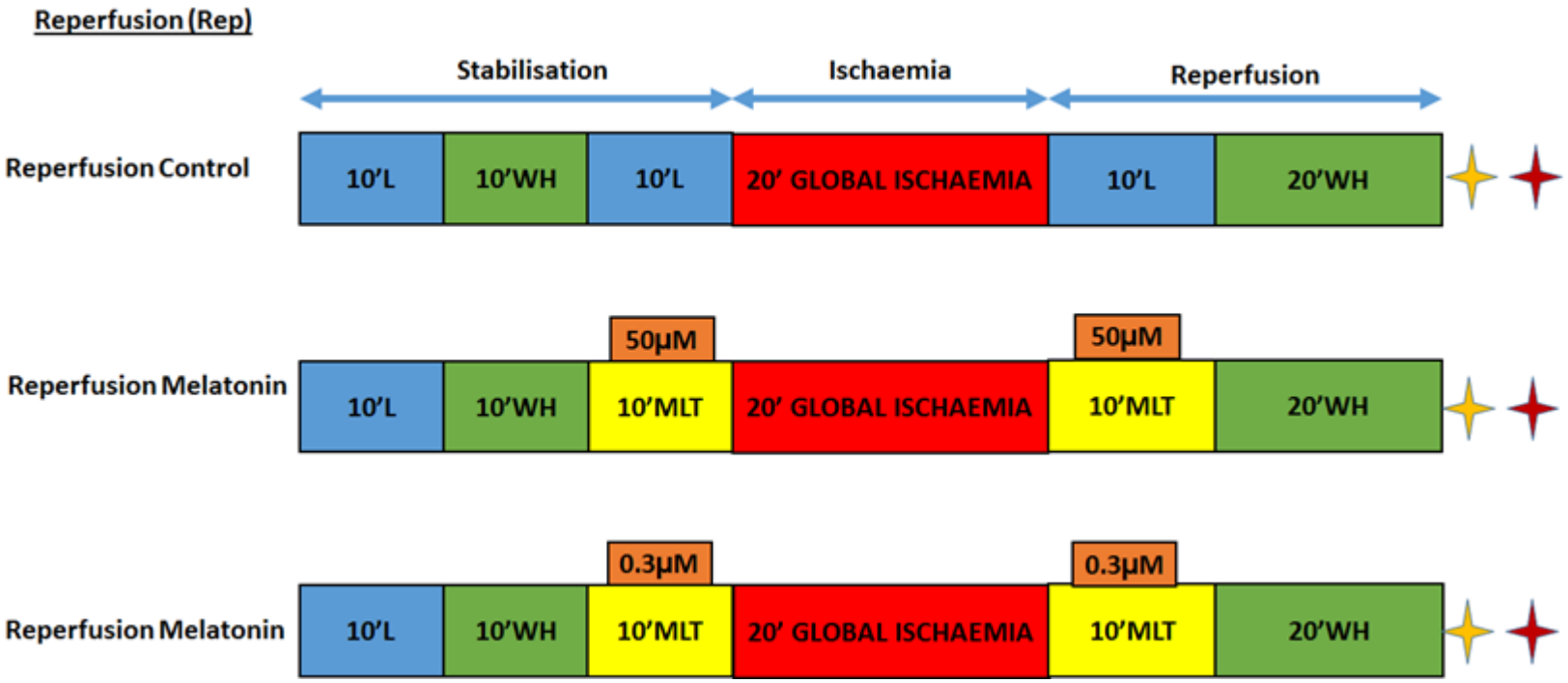



***Figure 12: Outline of stabilisation, ischaemia and reperfusion perfusion protocols. Each protocol consists of three groups: Control, with no melatonin treatment; Melatonin, with 50µM of melatonin and Melatonin, with 0.3µM of melatonin administered. For the Ischaemia protocol melatonin was administered prior to ischaemia for 10 minutes. For the reperfusion protocol melatonin was administered prior to ischaemia and at the onset of reperfusion for 10 minutes. Abbreviations: L-Langendorff; WH- Working Heart; MLT- Melatonin; µM-Micromolar.***

### 3.2.6.2. Regional Ischaemia

Regional ischaemia was induced by ligation of the left anterior descending (LAD) coronary artery using an Ethicon silk suture. Initiation of regional ischaemia commenced at the end of the stabilization period, for 35 minutes with the myocardial temperature maintained at 36.5ºC. Successful induction of regional ischaemia was indicated by a ~33-40% reduction of the coronary flow in comparison to the pre-ischaemic coronary flow as well as cyanosis on the surface of the myocardium. The 60 minute reperfusion period started at the end of ischaemia when the suture was released (**Fig 13**).

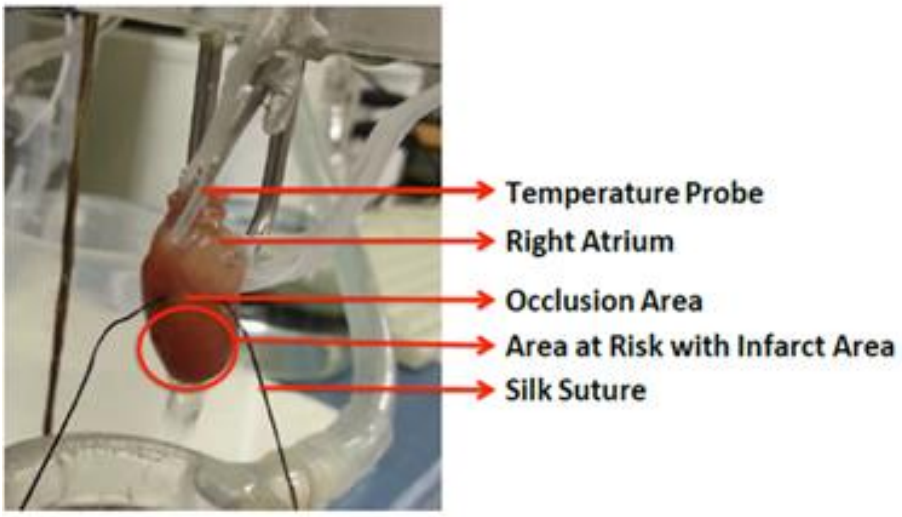



***Figure 13: Schematic representation of the heart post regional ischaemia (Everson, 2016),***

The regional ischaemia-reperfusion protocol was as follows:

Stabilisation consisted of 10 minutes retrograde perfusion; 10 minutes working heart perfusion; 10 minutes retrograde perfusion; followed by 35 minutes regional ischaemia; reperfusion consisted of 10 minutes retrograde perfusion followed by 20 minutes of working heart perfusion and 30 minutes of retrograde perfusion. For the melatonin treatment groups, melatonin was administered 10 minutes prior to and 10 minutes post regional ischaemia (**Fig 14**).

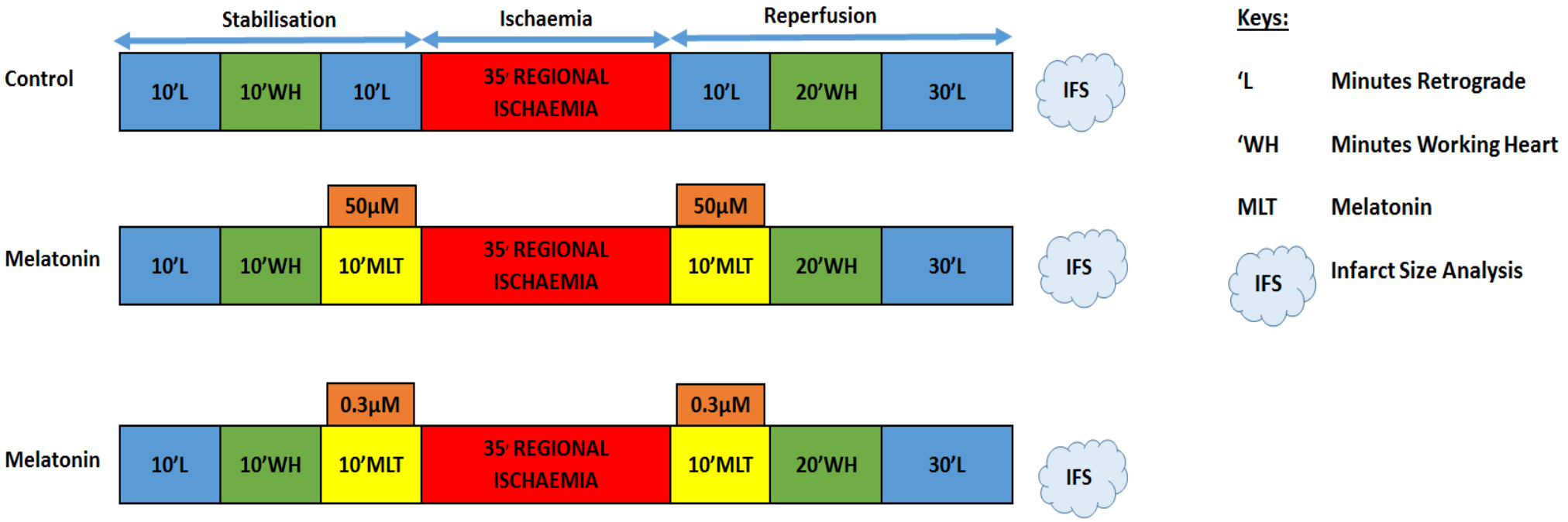



***Figure 14: Outline of regional ischaemia perfusion protocol. Control, with no melatonin treatment; Melatonin, with 50µM of melatonin administered prior to ischaemia and at the onset of reperfusion, Melatonin, with 0.3µM of melatonin administered prior to ischaemia and at the onset of reperfusion. Abbreviations: L- Langendorff; WH- Working Heart; MLT- Melatonin; IFS- Infarct Size; µM-Micromolar.***

## Infarct size determination

According to Holmbom et al. (1992) there are several methods that have been described for IFS determination however, two methods are used *viz.* histological evaluation to determine necrotic tissue and the macroscopic evaluation using tetrazolium dyes to stain viable tissue. Initially histological evaluation was considered to be the definite way to measure IFS however, this technique is costly and time consuming amongst other limitations (Holmbom et al. 1992). Over time both these techniques have been modified and new techniques have come into practice (Minicucci et al. 2007; Price et al. 2011). For this study myocardial IFS was determined using a tetrazolium dye. The triphenyltetrazolium chloride (TTC) staining method which is fast, inexpensive, popular and well established allows one to delineate between viable and nonviable tissue. This technique is based on the enzymatic reaction of mitochondrial dehydrogenases, in viable tissue, with TTC. Ultimately the reaction results in the reduction of TTC to triphenylformazan (TFP), with nicotinamide adenine dinucleotide (NADH) as an electron donor, giving viable tissue a distinct brick red pigment. The necrotic tissue remains unstained/ white or pale due to the absence of dehydrogenase activity and cofactors i.e. NADH (Redfors et al. 2012)

## Reagents

0.25% Evans blue (Sigma Aldrich, St Louis, Missouri, United States) in distilled water (0.025g/10mL), Ethion silk suture (25 type needle size, 3/0, 26mm ½ Taper, Johnson and Johnson Medical (PTY) LTD, South Africa).

$NaH_2PO_4$ (20mL of 100mM) and $Na_2HPO_4.\ 2H_2O$ (80mL of 100mM) were mixed to obtain a phosphate buffer, pH7.4; this phosphate buffer was mixed with 2, 3, 5 – TTC. Phosphate buffer (5mL) and TTC (0.05g) were used per heart; a 10% formaldehyde solution was used for fixing.

## Procedure

To delineate the area at risk at the end of the experimental protocol, the aortic cannula was used to gradually infuse 1mL of 0.25% Evans blue into the heart during retrograde perfusion. Evans blue is a water soluble azo dye which stains the perfused tissue dark

blue. The stained hearts were cut above the atrium and frozen at -20ºC overnight and would later be analysed for IFS as depicted in **Fig 15**.

The hearts were cut into 2mm slices from the apex to the base. They were stained with a 1% 2, 3, 5- TTC in phosphate buffer solution (pH 7.4) at room temperature for 15 minutes. Subsequently, heart slices were drained and fixed in a 10% v/v formaldehyde solution at room temperature for an hour. The formaldehyde is used to fix and accentuate the contrast between the necrotic and viable tissue. The heart slices are then neatly arranged between two Perspex glasses plates and scanned. The left ventricle area at risk (AR), the infarcted and viable areas were drawn using computerized planimetry (UTHCSA Image Tool programme, University of Texas Health Center at San Antonio, Tx, USA). The areas of each heart were used to calculate and express the IFS as a percentage of the area at risk (I/AR %).

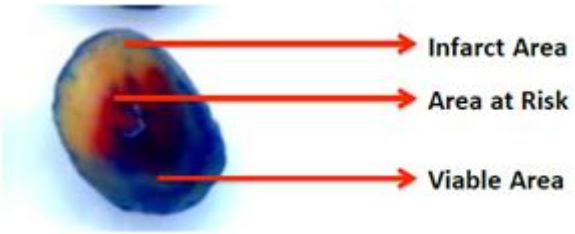



***Figure 15: Diagram depicting a stained section of the heart. The white tissue represents the infarct area, the red tissue represents the area at risk and the blue represents the viable tissue (Everson, 2016).***

### 3.3. Mitochondrial studies

The isolation of mitochondria using differential centrifugation was pioneered by individuals such as George Palade and co-workers as well as Chance and Williams. This technique lead to numerous discoveries such as defining the mitochondrial ultrastructure and the chemiosmotic theory of oxidative phosphorylation (Frezza et al. 2007; Picard et al. 2011). Mitochondrial isolation consists of three stages namely: mechanical disruption of the tissues or cells, the use of low speed centrifugation to separate the cellular debris and nuclei and the use of high speed centrifugation to extract the mitochondria. Initially two populations of mitochondria were isolated from rat cardiac muscle namely: subsarcolemmal mitochondria, which are located below the sarcolemma, and interfibrillar mitochondria which are located between the

myofibrils (Palmer et al. 1977). Recently Ong and colleagues (2013) described a third population of mitochondria located adjacent to the nucleus known as perinuclear mitochondria. **Figure 16** below shows all three mitochondrial populations. The protocol described below is for the isolation of subsarcolemmal mitochondria. The primary goal of mitochondrial isolation is to obtain organelles that are functional and as pure as possible i.e. with minimal contamination with other cellular components that can be used for the analysis of mitochondrial bioenergetics.

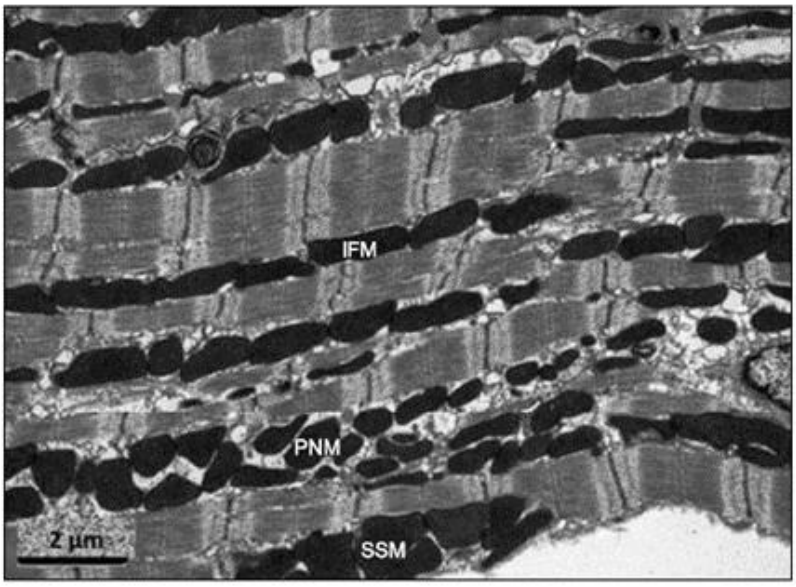



***Figure 16: Electron micrograph of a cardiomyocyte showing three populations of mitochondria. Abbreviations: IFM- interfibrillar; PNM-perinuclear; SSM- subsarcolemmal. (Ong et al. 2013).***

Oxidative phosphorylation, which is a key component of bioenergetics, is being studied extensively in order to elucidate mitochondrial mechanisms and systems that occur in health and disease (Gnaiger, 2014). There are a wide variety of methods using either fluorescence or a Clarke-type oxygen electrode (Zhang et al. 2012). Some well-known platforms available for oxidative phosphorylation analysis include the: Oroboros: 2k Oxygraph, Seahorse XF analyser and the Hansatech Oxygraph. Each platform has its respective advantages and disadvantages (Zhang et al. 2012). However, the platform that one selects is dependent on factors such as the endpoints of interest and the nature of the research question. For the present study the Hansatech Clarke type oxygen electrode (Oxygraph; Hansatech Instruments, Pentney, England), which

measures oxygen tension, was readily available in our facility and sufficient for the purpose of this study. It measures oxidative phosphorylation polarographically.

### 3.3.1. Reagents

The mitochondrial isolation medium (KE buffer) consisted of 0.18M KCL and 0.01M ethylenediaminotetraacetic acid (EDTA; Sigma Aldrich, St Louis, United States) (pH 7.4, using 2M Tris); 10% trichloroacetetic acid (TCA; BDH laboratory Supplies, Poole England); lysis buffer (See **section 3.4.1.1**).

### 3.3.2. Mitochondrial isolation procedure

Hearts allocated for mitochondrial isolation were cut above the atrium and placed into a Sorvall tube containing ice-cold KE buffer. Using scissors, the ventricles were cut up finely and then homogenised using a Polytron (PT-10 homogenizer, Kinematica, Fisher Scientific, Germany) (2x4 seconds, speed 4). The homogenate was equally distributed between two Sorvall tubes and tubes were topped up half way with KE buffer before being centrifuged (Sorvall SS34 rotor, Thermo Electron Cooperation, United States) for 10 minutes at 2500rpm (755g) at 4°C. At the end of the first spin, the supernatants were carefully decanted into two clean Sorvall tubes and centrifuged for 10 minutes at 4°C at 12 500 rpm (18 800g). At the end of the spin, the supernatant was discarded and the two mitochondrial pellets gently suspended in KE buffer using a Glass- Teflon homogenizer (Teflon® pestle PYREX® Potter-Elvehjem tissue grinders).

The first mitochondrial pellet was resuspended in 250µL of KE buffer. 50µL of this suspension was precipitated in 1mL of 10% TCA and refrigerated for subsequent protein determination using the Lowry assay (Lowry et al. 1951; **see Section 3.3.4**). The rest of the suspension was placed on ice to be used for analysis of the mitochondrial oxidative phosphorylation potential. The second pellet was suspended in 200µL of lysis buffer (**see Section 3.4.1.1**) and stored at -80°C for Western blotting (**see Section 3.4**).

### 3.3.3. Mitochondrial Oxidative Phosphorylation

#### 3.3.3.1. Reagents

250mM Sucrose , 10mM Tris-HCl, pH7.4, 8.5mM $KH_2PO_4$, 5mM glutamate*, 4.5mM palmitoyl-L-carnitine*, 2mM malate* (See **Table 4** below for medium composition), ±350nM of adenosine-5'-diphosphate monopotassium salt hydrate (ADP) (see **Appendix B** for procedure and formula), ±3500nM ADP (10x ADP; see **Appendix B** for procedure), 50% KCL and sodium dithionite ($Na_2S_2O_4$; BDH Laboratory Supplies, Poole, England) were used for the Oxygraph setup and calibration (procedure explained below). Reagents indicated with * were obtained from Sigma Aldrich, St Louis, United States.

***Table 4: Composition of the (A) glutamate /malate and (B) palmitoyl-L-carnitine/ malate medium.***

**A) Glutamate (Carbohydrate) and malate medium**

| **Reagent** | Stock Concentration (M) | Volume of Stock Used (mL) | Final Concentration in Incubation Medium (mM) | Rationale of medium composition |
|---|---|---|---|---|
| **Sucrose** | 1.25 | 2 | 250 | Supplies correct osmotic milieu to keep mitochondria intact |
| **Tris-HCL** | 0.1 | 1 | 10 | Tris: buffer of pH for the solution |
| **Potassium dihydrogen phosphate ($K_2HPO_4.H_2O$)** | 0.085 | 1 | 8.5 | $KH_2PO_4$: Supplies the inorganic phosphate ( Pi) for the phosphorylation of ADP to ATP |
| **Glutamate** | 0.05 | 1 | 5 | Electron donor in the ETC |
| **Malate** | 0.02 | 1 | 2 | Electron transporter |
| **Distilled water** | - | 4 | - | - |
| **Total** | - | 10 | pH adjusted to 7.4 with HCL before use | |

**B) Palmitoyl-L-carnitine (Fatty acid) and malate medium**

| **Reagent** | Stock Concentration (M) | Volume of Stock Used (mL) | Final Concentration in Incubation Medium (mM) | Rationale of medium composition |
|---|---|---|---|---|
| **Sucrose** | 1.25 | 2 | 250 | Supplies correct osmotic milieu to keep mitochondria intact |
| **Tris-HCL** | 0.1 | 1 | 10 | Tris: buffer of pH for the solution |
| **Potassium dihydrogen phosphate ($K_2HPO_4.H_2O$)** | 0.085 | 1 | 8.5 | $KH_2PO_4$: Supplies the inorganic phosphate ( Pi) for the phosphorylation of ADP to ATP |
| **Palmitoyl- L –Carnitine** | 0.045 | 1 | 4.5 | Electron donor in the ETC |
| **Malate** | 0.02 | 1 | 2 | Electron transporter |
| **Distilled water** | - | 4 | - | - |

**Abbreviations**: M- Molar; mL- Mililitres, mM-Milimolar; Tris-HCL- Tris Hydrochloride; ADP- Adenosine Diphosphate; ATP- Adenosine Triphosphate; ETC- Electron Transport Chain; HCL- Hydrogen Chloride.

Oxygraph setup, calibration and procedure for determination of mitochondrial oxidative phosphorylation rates:

Before use, the Oxygraph electrode (See **Fig 17** below) was cleaned with clay. Drops of 50% KCL were put around the base of the electrode dome (to the anode) and to the

cathode located on the top of the electrode. An oxygen permeable membrane was placed over the cathode and secured with an inner O-ring using an applicator shaft. An outer O-ring was used to seal the electrode disk into the oxygen electrode chamber. The Oxygraph system is coupled to a computer with specialised software and a water bath which maintains the temperature in the Oxygraph chamber.

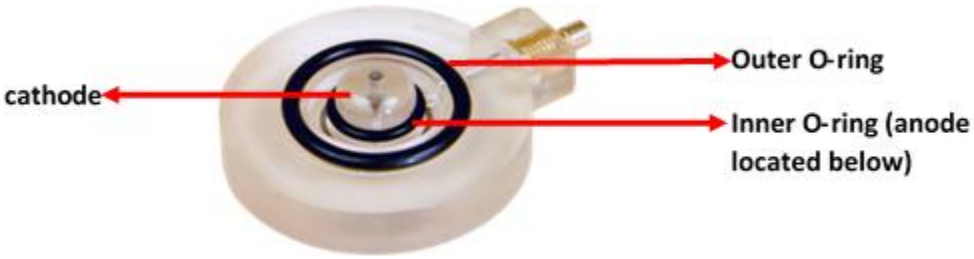



***Figure 17: Oxygraph electrode with accessory rings (Adapted from Hansatech Instruments, 2017)***

Separate Oxygraph chambers (See **Fig 18** below) were used for the two substrate mediums A [A: Glutamate (Carbohydrate)/malate medium] or B [B: Palmitoyl-L-carnitine (Fatty acid)/malate medium] to compare mitochondrial oxidative phosphorylation rates between carbohydrate and fatty acid substrates, respectively. The electrodes were calibrated before experimentation: 650µL of incubation medium [Glutamate/malate medium] and a magnetic stirrer were added to the Oxygraph chamber (temperature $25^0$ C, stirrer speed 100). Once the oxygen levels had reached ambient levels (100%), a small amount of $Na_2S_2O_4$ was added to remove all oxygen in the chamber and record zero oxygen levels. The calibration settings were saved and distilled water was used to thoroughly rinse the chamber before 650µL of substrate medium A [A: Glutamate/malate medium] was added. The same calibration procedures were followed with substrate medium B [B: Palmitoyl-L-carnitine/ malate medium] and allowed to stabilise to 100% ambient oxygen at 25°C.

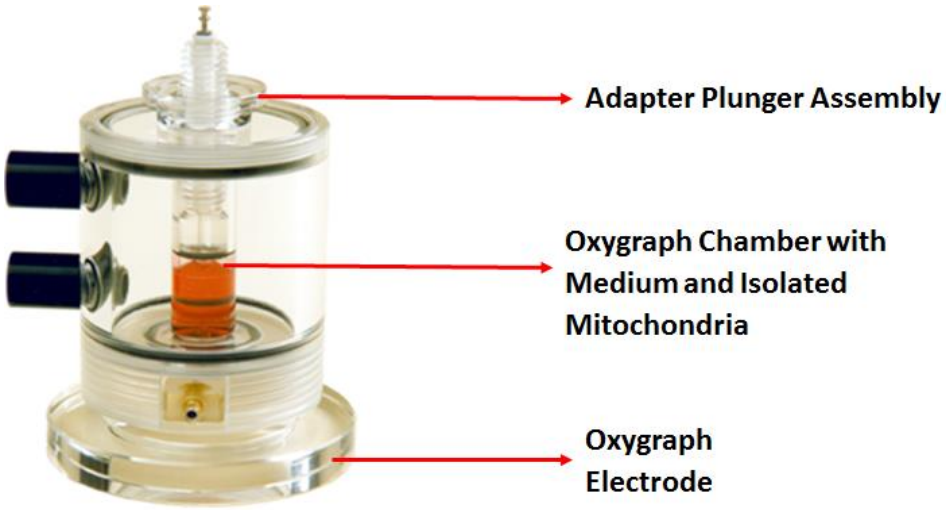



***Figure 18 Oxygraph chamber (Adapted from Hansatech Intruments, 2017)***

100µL of the isolated mitochondrial suspension in KE was added to each chamber, which was then sealed with the adapted plunger assembly and the recording was allowed to run for several minutes. This represented **state 2** (S2, See **Fig 19**), which is the respiration of the mitochondria in the presence of substrate but in the absence of ADP. A known concentration of 50µL ADP was then injected into the chamber using a Hamilton syringe to prevent the entry of any external oxygen into the system. This phase represented **state 3** (S3) respiration (also known as active respiration), which occurs in the presence of both substrate and ADP and allows for the conversion of ADP to ATP. After the added ADP was converted to ATP, the respiration rate slows down significantly (S4; respiration in the presence of substrate and ATP). **State 4** was allowed to run for approximately 1 minute. 50µL of 10x ADP was subsequently injected into the chamber using a Hamilton syringe to induce anoxia. The system was considered anoxic when all the oxygen in the system consumed (registering 0 on the Oxygraph). The mitochondria were exposed to 20 minutes of anoxia followed by re-oxygenation, after which state 3 respiration was determined.

From the graph generated by the Oxygraph the following parameters were calculated (for detailed calculations please refer to **Appendix C-E**) in conjunction with the mitochondrial protein concentrations obtained from the Lowry assay (**see Section 3.3.4**):

**$QO_2$ (S3)**: nAtoms oxygen taken up in the presence of ADP/mg mitochondrial protein/ min

**$QO_2$ (S4)**: nAtoms oxygen uptake in the absence of ADP/mg mitochondrial protein/min

**Respiratory control index (RCI)**: $QO_2$ (S3)/ $QO_2$ (S4) (an indicator of the tightness of coupling between respiration and phosphorylation)

**ADP/O**: ratio of ATP production to total oxygen uptake during state 3 (nmoles ATP/nAtom of oxygen consumed)

**Oxidative phosphorylation rate**: $QO_2$ (S3) X ADP/O (nmoles ATP produced/mg mitochondrial protein/min)

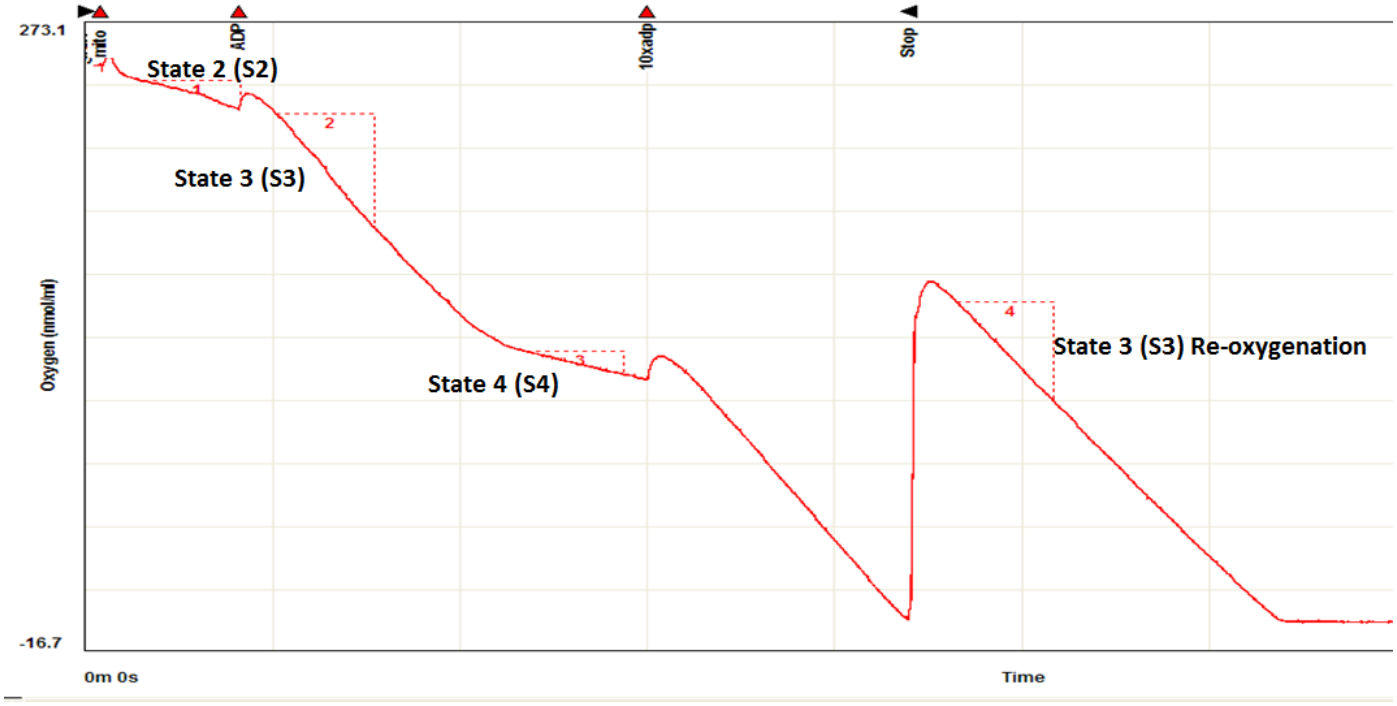



***Figure 19: Graph obtained from Oxygraph recording depicting the various stadia (indicated in red) of mitochondrial respiration. State 2 represents respiration in the presence of substrates only; State 3 represents respiration in the presence of substrate and ADP; State 4 represents respiration once all ADP has been converted to ATP. State 3 Re-oxygenation represents respiration during reoxygenation after the addition of 10x ADP and 20 minutes of anoxia.***

### 3.3.4. Lowry Assay

The Lowry assay, which is one of three copper-based colorimetric assays used to quantify total protein, was developed by Lowry and colleagues (1951) based on the technique described previously by Wu (Sapan et al. 1999; Krohn, 2011).

The method is based on the Biuret reaction and Folin-Ciocalteau reaction. In the Biuret reaction, the peptide bonds of proteins interact with copper under alkaline conditions to produce $Cu^{+}$ which then reacts with the Folin reagent to produce the latter reaction (Waterborg, 2002). This results in the reduction of phosphomolybdotungstate to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids. The reactions ultimately result in a vivid blue colour, which depends on the content of tryptophan and tyrosine (Sapan et al. 1999; Waterborg, 2002). The detection wave length for the assay is 750nm however, other investigators (in Sapan et al. 1999) have described the use of wavelengths at 600 and 650nm particularly in substances that may interfere with the Lowry reaction. Selected concentrations of Bovine Serum Albumin (BSA) were used to generate a standard curve for calculation of sample protein concentrations. BSA was selected because it is a well-known and popular choice for a protein standard which is relatively inexpensive and is widely available in high purity (Krohn, 2011).

#### 3.3.4.1. Reagents

2% $Na_2CO_3$, 2% Na-K-Tartrate, 1% $CuSO_4.5H_2O$, Folin Ciocalteus phenol reagent, 1N NaOH, 0.5N NaOH.

#### 3.3.4.2. Preparation of Standards

A stock solution of BSA (Roche Diagnostics, Indianapolis, United States) was made by dissolving 0.5g of BSA in 10 mL of distilled water. 50µL of the stock solution was diluted in 5mL of distilled water (1:101). The Heλios Ultra violet spectrophotometer (Unicam) was used to read the OD at 280nm using quarts cuvettes and distilled water as a blank. The BSA stock concentration was calculated (See **Appendix F** for formula). Standards were prepared in volumetric flasks according to **Table 5** below:

*Table 5: BSA standard dilutions and dilution ratios*

| Standard Number | Amount of BSA stock solution (0.5g in 10ml) used | Amount of 0.5 N NaOH used for standard dilution | Dilution Ratio |
|---|---|---|---|
| **1** | 1mL | 200mL | 1: 200 |
| **2** | 2mL | 200mL | 1:100 |
| **3** | 2mL | 100mL | 1:50 |

### 3.3.4.3. Procedure

Mitochondrial samples (100 µl), precipitated in 10% TCA, were centrifuged (Haraeus Megafuge 16R, Thermo Fisher Scienctific, Massachusetts , United States) at 3000 rpm for 10 minutes at 4°C, the supernatant discarded and the samples allowed to air dry. 500µL of 1 N NaOH was pipetted into each sample and placed in a 50°C water bath until the proteins were dissolved. Afterwards 500µL of distilled water was added to the samples (final sample volume 1mL; NaOH concentration 0.5N). Standards and samples were analysed in triplicate using a volume of 50µL. For the blank 50µL of 0.5 N NaOH was used. 1mL of Solution 1 (49mL 2% $Na_2CO_3$ and 0.5mL each of 2% Na-K-Tartrate and 1% $CuSO_4.5H_2O$), was added to each tube at 10 second intervals to the blank, standards and samples. After 10 minutes 100µL of Solution 2, (Folin Ciocalteus reagent diluted 1:2 in distilled water), was added to the samples as described above. The reaction was left to incubate for 30 minutes at room temperature. OD readings were measured using a spectrophotometer (Spectronic® 20 Genesys™, Spectronic Instruments, United States at 750nm). The protein concentrations of the mitochondrial samples were calculated from the generated standard curve (Absorbance vs. protein concentration).

## 3.4. Western Blotting technique

Western Blotting is a popular technique used to identify, quantify and determine the size of specific proteins from extracted cellular protein mixtures. This technique evolved from the Northern and Southern Blots which are used to detect RNA and DNA, respectively. The technique is based on the separation of native or denatured proteins followed by transfer to a membrane for detection using antibodies that are specific to the protein of interest (Jensen, 2012). **Fig 20** provides an outline of the Western Blotting process.

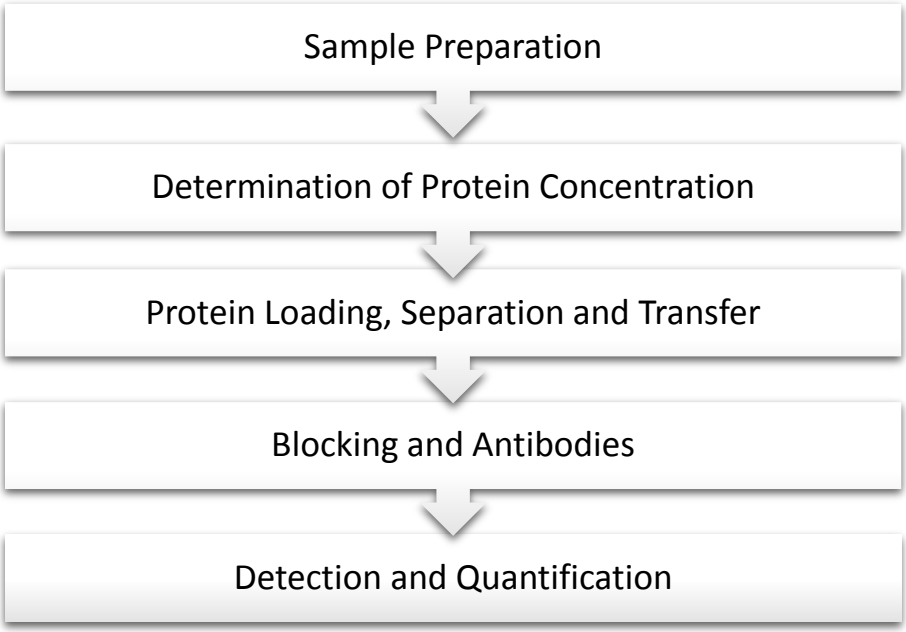



***Figure 20: Overview of Western Blotting process.***

### 3.4.1. Sample Preparation: Protein Extraction

#### 3.4.1.1. Reagents

**Table 6** below gives a summary of all the reagents used to make heart and mitochondria lysis buffer.

***Table 6: Lysis Buffer composition for heart and mitochondria***

| Reagent | Stock | Final Concentration/ Content | Amount in 30mL |
|---|---|---|---|
| **Tris-HCL EGTA (pH 7.5)** | 200mM | 20mM | 3mL |
| **EDTA** | 100mM | 1mM | 300µL |
| **Sodium Chloride (NaCl)** | 1M | 150mM | 4.5mL |
| **ß-glycerophosphate** | - | 1mM | 0.006g |
| **Tetra-Sodium Pyrophosphate** | - | 2.5mM | 0.03g |
| **Sodium Orthovandate ($Na_3VO_4$)** | 10mM | 1mM | 3mL |
| **Triton** | 10% | 1% | 3mL |
| **Leupeptin** | 10mg/mL | 10µg/mL | 30µL |
| **Aprotinin** | 10mg/mL | 10µg/mL | 30µL |
| **PMSF** | 100mM | 50µg/mL | 90µL |
| **Distilled water** | - | - | Used to fill up to 30mL |

**Abbreviations:** EGTA- Ethylene glycol tetraacetic acid, EDTA- Ethylenediamine tetraacetic acid, PMSF-Phenylmethylsulphonyl fluoride.

### 3.4.1.2. Protein Extractions from Heart Tissue

Freeze clamped hearts were pulverized and approximately 150mg of heart tissue was compressed into Eppendorf tubes containing 700µL of lysis buffer. One scoop of 1.6 mm, 1lb stainless steel zirconium oxide beads (Next Advance Inc., United States) and four 3.2mm, 1/8 inch stainless steel balls (Next Advance Inc., United States) were put

into each sample. Samples were then placed in the Bullet Blender (Next Advance Inc., United States) at setting 10 for two 2minute intervals with a 1minute resting time in between at 4°C. If samples were not completely blended, they were subjected to one more blending cycle. The samples were then allowed to rest for 15 minutes before they were centrifuged (Sigma-1-14k bench top refrigerated centrifuge, Thermo Scientific Fisher, Massachusetts, United States) for 20 minutes (15 000rpm, 4°C). Following centrifugation, the supernatant was transferred into clean Eppendorf tubes and kept on ice for protein concentration determination using the Bradford assay (see **Section 3.4.2**).

#### 3.4.1.3. Protein Extractions from Mitochondrial Samples

As previously mentioned the mitochondrial samples that were designated for blotting were stored at -80°C. In preparation for processing samples were thawed on ice. Next, a scoop of 0.15mm 1lb Zirconium oxide beads (Next Advance Inc., United States) were added to samples. The samples were placed into the Bullet Blender (Next Advance Inc., United States) (temperature $4^0$C, speed setting 5) for 3 minutes. The samples were allowed to stand for 15 minutes before they were centrifuged (Sigma-1-14k bench top refrigerated centrifuge, Thermo Scientific Fisher, Massachusetts, United States) for 20 minutes (15 000rpm, 4°C). The supernatant was aspirated into clean Eppendorf tubes in preparation for the Bradford assay described below.

### 3.4.2. Protein Determination: Bradford assay

#### 3.4.2.1. Reagents

500mg of Coomassie Brilliant Blue G-250 in 50mL 95% Ethanol, add 500mL Phosphoric Acid make up to 1L with distilled water (Bradford Reagent), Bovine Serum Albumin (BSA).

A 5x dilution (20mL Bradford reagent plus 80mL distilled water) of the Bradford reagent was made and filtered through a double layer of Whatman filter paper (125mm, Schleicher and Schuell Microsciences, Germany). For the standard curve a 5 times dilution (100µl of BSA stock in 400µL of distilled water) of BSA stock (5mg/mL in distilled water) was prepared. The BSA dilution was serially diluted (see **Table 7**) in duplicate test tubes resulting in concentrations ranging from ±1- 19µg/ 100µL.

***Table 7: BSA serial dilution for Bradford assay***

| Diluted BSA | Distilled water (µL) |
|---|---|
| **0 (Blank)** | 100 |
| **5** | 95 |
| **10** | 90 |
| **20** | 80 |
| **40** | 60 |
| **60** | 40 |
| **80** | 20 |

For the samples two dilutions were made in preparation for the Bradford assay. Frist, a 1:10 dilution of the centrifuged samples was made (10µL supernatant plus 90µL distilled water). For the second dilution a 1:20 dilution was made for the heart tissue samples (5µl of the first dilution plus 95µL distilled water. Conversely, for the mitochondrial samples the second dilution was a 1:10 dilution (10µL of the first dilution plus 90µL distilled water). For each sample, the second dilution was prepared in duplicate and used for the assay. All tested tubes were vortexed thoroughly.

After preparation of the standards and samples for the assay, 900µL of the diluted Bradford reagent was dispensed into each tube starting with the blank, followed by the standards and samples. The initial volume of the standards and samples was 100µL, together with the Bradford reagent the tubes will now have a total volume of 1000 µL. All the tubes were vortexed and left to incubate for 20 minutes at room temperature to allow colour development. At the end of the incubation period the absorbance was measured at 595nm using a spectrophotometer (Spectronic® 20 Genesys™, Spectronic Instruments, United States). A standard curve of absorbance vs. protein concentration, was generated from the readings and used to calculate the protein content of each sample and ultimately the lysates.

### 3.4.3. Lysate preparation

Lysates were prepared by adding protein sample (as per Bradford assay calculations) to Laemmli sample buffer (850µL Western sample buffer and 150µL mercaptoethanol) and lysis buffer according to respective amounts as calculated. Lysates were boiled for five minutes to denature the proteins and stored at -80°C.

### 3.4.4. Protein Loading, Separation and Transfer

#### 3.4.4.1. Reagents

Criterion™ TGX Stain-free™ Precast Gels (Bio-rad), running buffer stock (250mM Tris, 192mM Glycine, 1% SDS), transfer buffer (250mM Tris, 192mM Glycine, 20% (v/v) Methanol).

#### 3.4.4.2. Procedure

The lysates which were previously stored at -80°C were boiled again for five minutes and centrifuged (Minispin Microcentrifuge, Eppendorf, Sigma Aldrich, St Louis, United States) for 10 seconds using the short spin function. Next lysates were loaded into 26 well Criterion™ TGX Stain-free™ 4-20% gradient precast gels. The gels were then placed into the dual Biorad Criterion™ cell (Bio-rad Laboratories Inc., United States) and filled with running buffer (100mL running buffer stock in 900mL of distilled water) up to the demarcated lines on the tank. 7.5µL of the PagerRuler™ Prestained Protein Ladder (Thermo Scientific, Massachusetts, United States) was loaded into the first well followed by an unperfused or a baseline perfused heart and the samples. The unperfused/ baseline sample was used as an internal control for the normalisation of the blots. 15µL of each sample containing 30µg of protein for the heart samples (used for the analysis of cytosolic markers) and 12µL of each sample containing 25µg of protein for the mitochondrial samples (used for the analysis of mitochondrial markers) were loaded. Empty wells were loaded with western sample buffer to prevent the samples from running crooked. Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gels were run for 10 minutes at 100V and 200mA, followed by a 50 minute run at 200V and 200mA using the Biorad PowerPac Basic. Gels were activated using the ChemiDoc™ MP system (Bio-rad Laboratories Inc., United States). Gels were kept in running buffer before and after imaging to prevent drying out and breaking.

Once gels were activated, proteins were transferred to polyvinylidene fluoride membranes (PVDF, Immobilon®-P Transfer membranes, pore size 0.45µm, Merk Millipore, Mass, United States) using the Biorad Criterion™ Blotter. The sponges and filter papers used for the wet transfer were soaked in cold transfer buffer and refrigerated approximately an hour before transfer.

The transfer cassettes were packed from black to red i.e. positive to negative (**Fig 21**) as follows:

- 1 Sponge/ Fibre Foam Pad
- 2 Filter papers
- 1 gel activated with separated proteins
- 1 PVDF membrane
- 2 Filter papers
- 1 Sponge/ Fibre Foam Pad

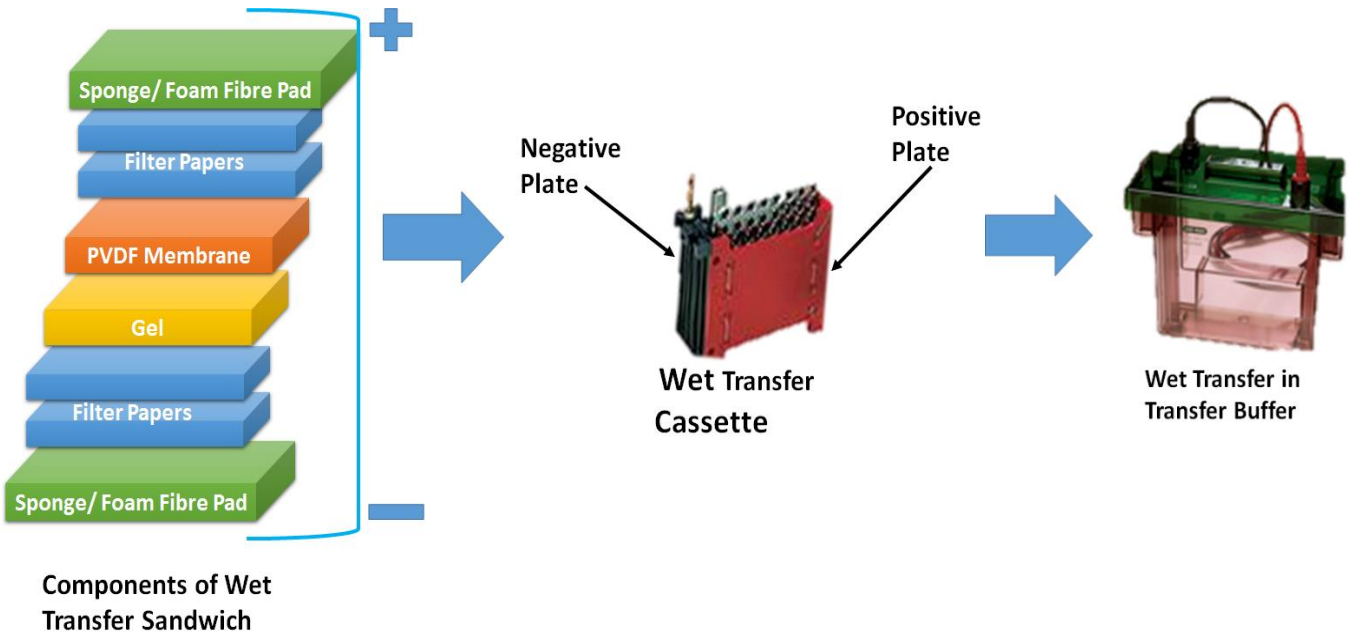



***Figure 21: Assembly of Western Blot Sandwich***

During the packing process a roller was used to remove air bubbles as each layer of the sandwich was introduced. The cassette was closed and placed inside the tank. A thermo-safe Polar Pack was placed in the ice holder compartment of the cell. Ice cold transfer buffer was used to fill the cell up to the demarcated markers. The transfer process took place at 200V, 200mA for 30-35 minutes. At the end of the transfer period the PVDF membrane which contained the separated proteins was visualized using the ChemiDoc™ MP system (Bio-rad Laboratories Inc., United States). Membranes were kept in transfer buffer before and after visualisation to prevent drying.

### 3.4.5. Blocking and Antibodies

#### 3.4.5.1. Reagents

10x TBS stock (200mM Tris, pH 7.6, 1.37 M NaCl), Tween-20, long life fat free milk, antibodies. All antibodies were obtained from Cell Signalling Technologies (Mass, United States) except for TOM70 which was obtained from Santa Cruz Biotechnologies (Texas, United States). All the antibodies were monoclonal except for SQSTM1/p62, PARKIN and phosphorylated DRP-1 which were polyclonal.

Membranes were rinsed once with 1x TBS Tween (100mL of 10x TBS buffer in 900mL distilled and 1 mL Tween-20) and blocked for two hours, shaking at room temperature in 50mL 5% milk (5ml long life fat free milk in 95mL 1x TBS Tween) per membrane. After blocking the non-specific binding sites with 5% milk, the membranes were thoroughly washed for 30 minutes (3x10 minute washes on the shaker, with fresh buffer for each wash). Next the membranes were incubated overnight at 4°C on the shaker with polyclonal primary antibody solution (1:1000 dilution of antibody in TBS-Tween). Please refer to **Table 8** below for a description of all the antibodies that were used for the study.

***Table 8: Summary of the mitophagy proteins analysed for** (A) **PINK1/ PARKIN Pathway** (B) **Alternative Pathway and** (C) **Cytosolic Proteins: molecular weight, percentage milk in primary antibody, ECL incubation period and Chemi Doc exposure period.***

| A. Mitophagy Proteins: PINK1/PARKIN Pathway | | | | |
|---|---|---|---|---|
| Proteins | Molecular Weight (kDa) | Milk in Primary antibody (%) | ECL incubation period prior to detection (min) | Exposure period in the Chemi Doc system (min) |
| PARKIN | 52 | 2.5 | 3-5 | <1 |
| PINK1 | 60, 50 | 0 | 3-5 | <1 |
| SQSTM1/p62 | 60 | 0 | 3-5 | <1 |
| TOM70 | 70 | 0 | 3-5 | 1-2 |
| Sirt3 | 28 | 0 | 3-5 | <1 |

| B. Mitophagy Proteins: Alternative Pathway | | | | |
|---|---|---|---|---|
| Proteins | Molecular Weight (kDa) | Milk in Primary antibody (%) | ECL incubation period prior to detection (min) | Exposure period in the Chemi Doc system (min) |
| Phosphorylated ULK** ( Ser 555) | 140,150 | 0 | 3 | 2 |
| Total ULK | 150 | 0 | 3 | <1 |
| Rab9 | 23 | 0 | 3 | <1 |
| Phosphorylated DRP-1 (Ser 637) | 78-82 | 0 | 3 | 2 |
| Total DRP-1 | 78-82 | 0 | 3 | <1 |

****** Indicates the addition of signal boost to the primary and secondary antibody

| C. Cytosolic Proteins | | | | |
|---|---|---|---|---|
| Proteins | Molecular weight (kDa) | Milk in Primary antibody (%) | ECL incubation period prior to detection (min) | Exposure period in the Chemi Doc system (min) |
| PGC-1 alpha | 130 | 2.5 | 3-5 | 10-15 |
| Sirt1 | 120 | 0 | 3-5 | 3 |

### 3.4.6. Detection and Quantification

Subsequent to overnight incubation, membranes were washed thoroughly for 30 minutes as described above. The membranes were then incubated for an hour, shaking at room temperature, in anti-rabbit, immunoglobulin G, conjugated Horseradish peroxidase (HPR) secondary antibody (dilution in TBS-Tween 1:4000), followed by another 30 minute wash with TBS-Tween.

The proteins were visualised by covering the membrane with Clarity ™ Western ECL Substrates (1.5mL of the Luminol/Enhancer solution and 1.5mL of the Peroxidase solution) and incubated and exposed as indicated in **Table 8**. Membranes with ECL were placed in the Bio Rad ChemiDoc system and allowed to develop. Pictures were taken and saved for the quantification of the blots.

The finest images from the exposed membranes were normalised to the corresponding PVDF membranes images obtained after the transfer step. The normalisation process compares the signal intensity of the protein of interest, which is unknown, to an internal loading control, which is known. In this study baseline hearts that were unperfused or perfused for 5 minutes were used as internal loading controls. This process corrects for sample-to-sample and lane-to-lane variation which is unavoidable during blotting. The Bio Rad ChemiDoc Image Lab 5.0 series software was used to analyse all the blots.

### 3.4.7. Stripping of membranes

In the event that a membrane was not probed correctly it was stripped. The membrane was incubated twice in distilled water for 5 minutes. This was followed by a single incubation step in 0,2M NaOH for 7 minutes and two five minute incubations in distilled water once again. All the incubation steps took place on the rotor shaker V1.00. Subsequent to stripping, the membrane was blocked, probed with antibody and lastly visualised and quantified as described above.

## 3.5. Statistical Analysis

All data points were expressed as mean ± standard error of mean (SEM) .GraphPad Prism® 6 was used for statistical analysis. In some instances Student's t-test was used for the comparison of two groups in the same perfusion condition/protocol. A one-way analysis of variance (ANOVA) followed by a Bonferroni post hoc correction test was used for the comparison of all the groups within the same perfusion condition/protocol. A p-value of $<0.05$ was considered to be statistically significant.

# CHAPTER 4: RESULTS

## 4.1. Introduction

The aims of this study were to characterise (i) the effect of I/R *per se* on the relationship between functional recovery during reperfusion, mitochondrial oxidative phosphorylation capacity, IFS and mitophagy in the working heart model using male Wistar rats and (ii) the effect of manipulation of mitophagy by the pineal hormone, melatonin, on cardioprotection, using the parameters listed in aim 1. The following chapter provides a detailed description of the data obtained from the isolated heart perfusions, mitochondrial oxidative phosphorylation capacity and Western Blotting for important mitochondrial and cytosolic markers involved in various processes (**Fig. 22** below). For each of the above the effect of high (50µM) or low (0.3µM) melatonin concentrations were examined.

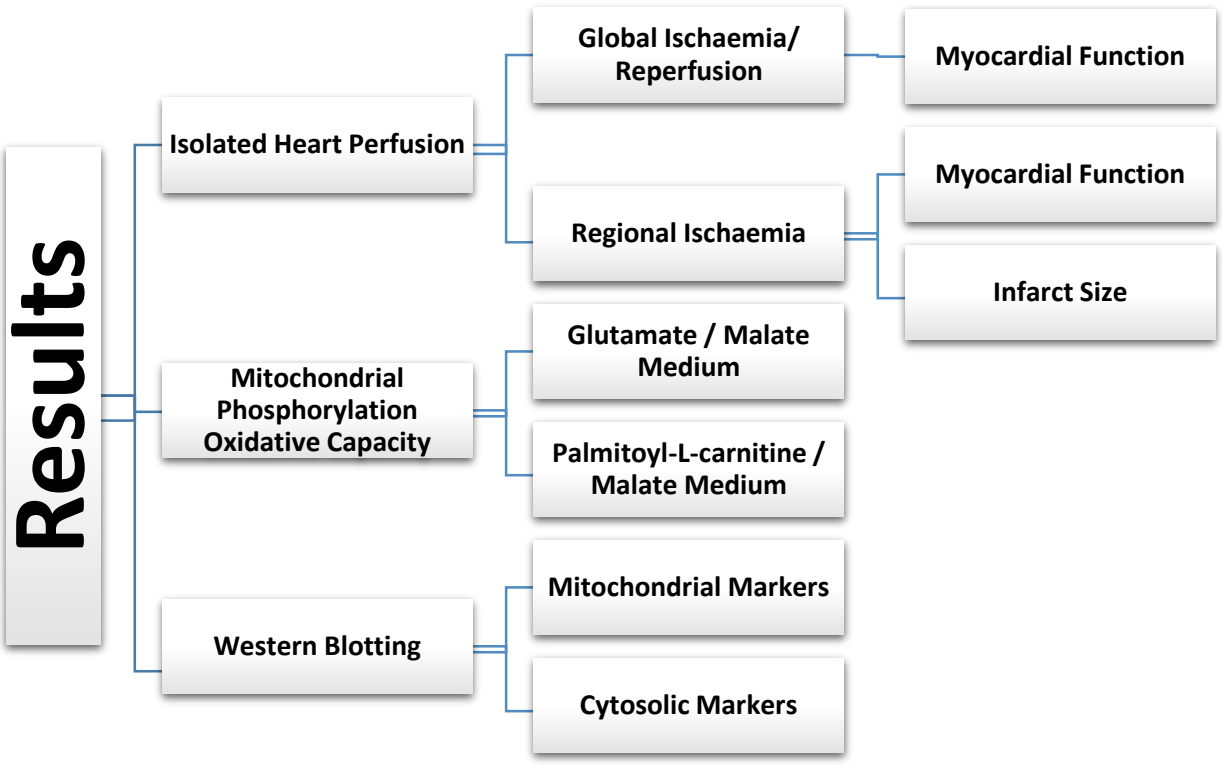



***Figure 22: Outline of results format***

## 4.2. Isolated heart perfusions

### 4.2.1. Global Ischaemia

Effect of melatonin on myocardial functional recovery after exposure to global ischaemia

The isolated perfused rat heart was used for all experimentation. Hearts subjected to global ischaemia were subsequently used for immediate preparation of mitochondria or were freeze-clamped for Western blotting at a later stage. Since these two series of experiments were performed independently, the mechanical data obtained will be presented separately (**Tables 9** and **10**). Hearts subjected to regional ischaemia, were used for determination of IFS.

For evaluation of functional performance, six haemodynamic endpoints (refer to **Section 3.2.3**) namely coronary flow (Qe; mL/min), aortic output (Qa; mL/min), cardiac output (CO; mL/min), peak systolic pressure (PSP; mmHg), heart rate (HR; beats/min) and total work (TW; mW) were measured during stabilisation prior to global ischaemia and during reperfusion post global ischaemia as indicated on **Fig 23** below. At least two measurements were made at each of the time points indicated. Melatonin was administered 10min before induction of global ischaemia and during the first 10min of reperfusion.

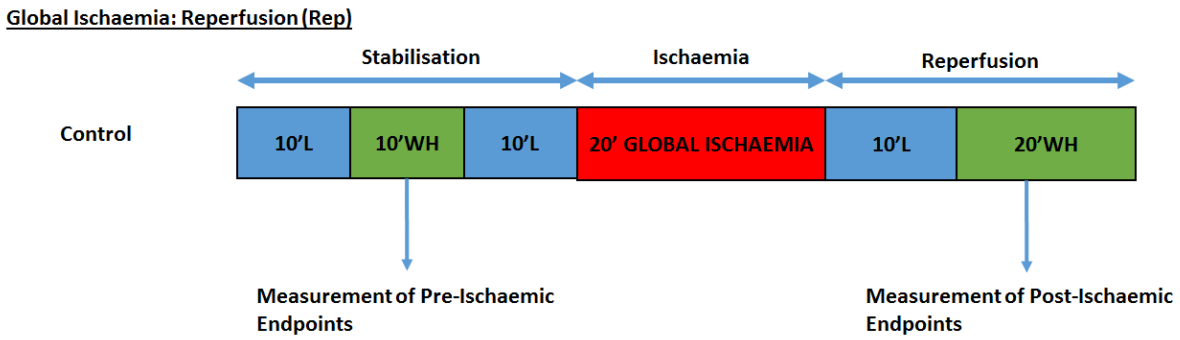



***Figure 23: Overview of the global I/R protocol which indicates the time points where the haemodynamic endpoints used to determine mechanical function were measured. Abbreviations: L- Langendorff; WH- Working heart.***

***Table 9**: Myocardial performance of hearts subjected to 20min global ischaemia and 30min reperfusion: effect of melatonin (50µM). (Hearts subsequently used for mitochondrial isolation).*

| Endpoints | | Control<br><br>Pre: n= 6 ; Post n=6 | Melatonin 50µM<br><br>Pre: n=6 ; Post n= 6 |
|---|---|---|---|
| Coronary Flow (mL/min) | Pre | 10.00 ± 0.90 | 17.00 ± 4.17 |
| | Post | 7.25 ± 1.12 * p=0.0035 | 17.00 ± 3.81 # p=0.0339 |
| Aortic Flow (mL/min) | Pre | 28.33± 1.89 | 32.33 ± 1.41 |
| | Post | 7.00 ± 2.30 * p<0.0001 | 13.67 ± 4.05 *p=0.0014 |
| Cardiac Output (mL/min) | Pre | 37.40 ± 2.69 | 49.33 ± 5.01 |
| | Post | 14.25± 3.17 * p=0.002 | 30.67± 5.42<br><br>*p=0.0063; #p=0.0015 |
| Heart Rate (beats/min) | Pre | 286.50±22.78 | 250.80± 23.12 |
| | Post | 161.20± 52.59 | 189.30± 43.60 |
| Peak Systolic Pressure<br><br>(mm Hg) | Pre | 83.83± 1.45 | 86.17± 0.83 |
| | Post | 53.17± 16.88 | 69.00± 13.94 |
| Total work (mW) | Pre | 7. 39± 0.51 | 9.54±0.88 |
| | Post | 2.26±0.89 * p= 0.002 | 4.58±1.00 *p= 0.041 |
| Total Work Recovery % | | 34±11 | 53±12 |

Results are expressed as means±SEM. * pre vs post; # control vs melatonin; only values with significance are shaded.

#### 4.2.1.1. Mitochondrial hearts

**Effects of ischaemia/reperfusion**

In this series of experiments all perfused hearts were subsequently used for **mitochondrial isolation**. All parameters of mechanical performance before exposure to ischaemia were similar in the two groups. I/R caused a significant reduction in aortic flow ($p<0.0001$; $p=0.0014$), cardiac output ($p=0.002$; $p=0.0063$) and total work ($p=0.002$; $p=0.041$) in both control and melatonin-treated groups, while heart rate and peak systolic pressure remained unchanged (**Table 9**).

**Effects of melatonin**

The only significant difference between the groups is the observation that coronary flow during reperfusion was significantly reduced in the control hearts, but remained unchanged in the melatonin-treated hearts (see **Table 10**). Furthermore, post-ischaemic cardiac output was significantly higher in the melatonin-treated group ($p=0.0015$).

***Table 10**: Myocardial performance of hearts subjected to 20min global ischaemia/30min reperfusion: effect of melatonin (0.3µM, 50µM). (Hearts subsequently used for Western blotting).*

| Endpoints | | Reperfusion Control Pre: n= 6; Post: n=6 | Reperfusion Melatonin 50µM Pre: n=6; Post: n= 6 | Reperfusion Melatonin 0.3µM Pre: n=6; Post: n=6 |
|---|---|---|---|---|
| Coronary Flow (mL/min) | Pre | 15.75 ± 1.89 | 11.75 ± 1.52 | 14.67 ± 0.67 |
| | Post | 10.00 ± 2.84 | 5.250 ± 1.27 *p=0.0083 | 13.50 ± 0.50 |
| Aortic Flow (mL/min) | Pre | 42.00 ± 4.26 | 39.33 ± 6.71 | 44.83 ± 2.71 |
| | Post | 7.00 ± 3.64 * p<0.0001 | 8.33 ± 3.40 * p=0.0021 | 22.00 ± 6.00 *p=0.0010 |
| Cardiac Output (mL/min) | Pre | 57.75 ± 5.22 | 51.08 ± 7.24 | 59.50 ± 3.03 |
| | Post | 17.00 ± 5.17 *p=0.002 | 13.58 ± 4.53 * p=0.0014 | 35.50 ± 6.29 *p=0.0026 |
| Heart Rate (beats/min) | Pre | 288.20 ± 7.94 | 240.20 ± 7.47 #p= 0.0013 | 294.30 ± 7.19 |
| | Post | 125.2 ± 56.75 *p=0.0174 | 141.20 ± 44.93 | 266.5 ± 13.67 |
| Peak Systolic Pressure (mm Hg) | Pre | 93.50 ± 1.46 | 91.83 ± 1.85 | 83.17 ± 1.66 #p=0.0009 |
| | Post | 42.00 ± 18.89 *p=0.0216 | 57.67 ± 18.25 | 77.75 ± 1.03 |
| Total work (mW) | Pre | 10.81 ± 1.12 | 9.43 ± 1.50 | 11.07 ± 0.77 |
| | Post | 2.36 ± 1.07 *p=0.0003 | 2.20 ± 0.85 * p= 0.0018 | 6.17 ± 1.16 *p=0.0020 |
| Total Work % Recovery | | 26±13 | 24±11 | 38±12 |

Results are expressed as means±SEM. * pre vs post; # control vs melatonin; only values with significance are shaded.

#### 4.2.1.2. Freeze-clamped hearts

**Effects of ischaemia/reperfusion**

In the second series of experiments hearts were perfused as described above, but these hearts were freeze-clamped at the end of reperfusion for **subsequent Western blotting**. The parameters of mechanical performance of these hearts were summarized in **Table 10**.

As was observed in **Table 9**, the mechanical performance of the hearts before induction of global ischaemia was similar in the three groups studied, except for a lower heart rate in the 50µM melatonin group (see below). Similarly, exposure to I/R caused a significant reduction in aortic flow, cardiac output and total work during reperfusion in all three groups. However, control untreated hearts had a significant reduction in post-ischaemic heart rate and peak systolic pressure. A reduction in coronary flow during reperfusion was observed in the 50µM melatonin treated hearts.

**Effects of melatonin**

Comparing the pre and post-ischaemic values of the control and melatonin (50µM and 0.3µM) groups showed no significant differences in all the endpoints except the pre-ischaemic heart rate ($p=0.0013$) and pre-ischaemic peak systolic pressure ($p=0.0009$) were lower in hearts treated with 50µM melatonin and 0.3µM, respectively in comparison to the controls. This however, may be due to differences between the hearts when being perfused, since these measurements were made before addition of melatonin. No other differences were observed except a reduction in coronary flow during reperfusion in the 50µM melatonin treated hearts.

### 4.2.2. Regional Ischaemia

Effect of melatonin on myocardial function and IFS during reperfusion after 35min regional ischaemia

Similar pre- and post- ischaemic haemodynamic endpoints as obtained for the hearts subjected to global ischaemia, were collected for all hearts subjected to the regional ischaemia-reperfusion protocol. Endpoints were measured at the indicated time points in **Fig 24** below. Melatonin (0.3 or 50 µM) was administered 10 min before induction of regional ischaemia and for 10 min at the onset of reperfusion.

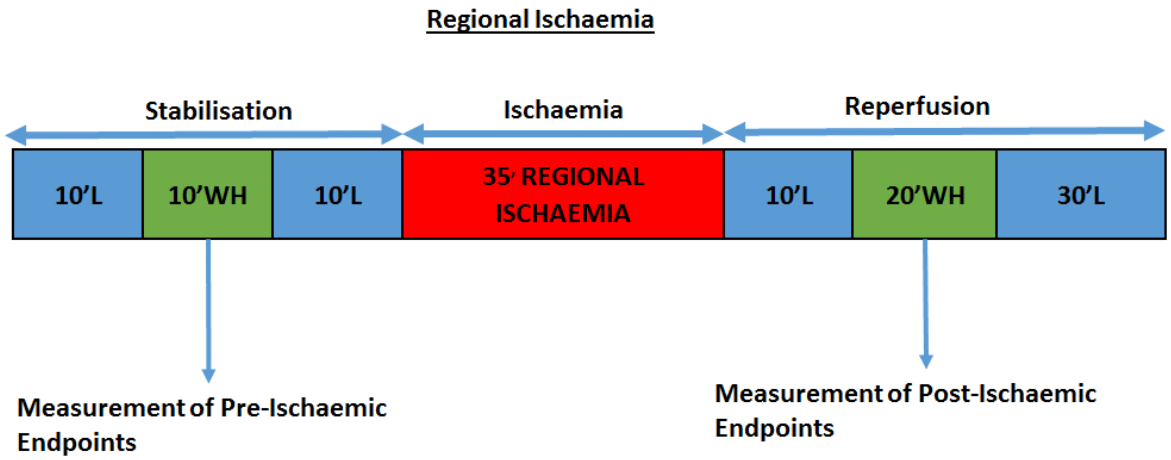



***Figure 24: Outline of regional ischaemia- reperfusion protocol indicating the time points for measurement of haemodynamic endpoints. Abbreviations: L- Langendorff; WH- Working heart***

***Table 11: Myocardial performance of hearts subjected to 35 min regional ischaemia/60min reperfusion: effect of melatonin (0.3µM, 50µM). (Hearts were used for IFS measurements).***

| Endpoints | | Reperfusion Control Pre: n= 14; Post: n= 10 | Reperfusion Melatonin 50µM Pre: n=9; Post: n= 9 | Reperfusion Melatonin 0.3µM Pre: n=9; Post: n=9 |
|---|---|---|---|---|
| Coronary Flow (mL/min) | Pre | 12.32 ± 1.22 | 17.67 ± 2.79 | 13.17 ± 1.14 |
| | Post | 13.82 ± 1.57 | 7.42 ± 1.97 *p 0.0085; #p= 0.0190 | 5.50 ± 1.25 *p= 0.003 ; #p= 0.0011 |
| Aortic Flow (mL/min) | Pre | 41.14 ± 3.17 | 46.00 ± 1.94 | 41.33 ± 4.10 |
| | Post | 15.14 ± 3.61 *p<0.0001 | 8.94 ± 4.49 *p<0.0001 | 3.56 ± 3.56 *p<0.0001 ; #p=0.0415 |
| Cardiac Output (mL/min) | Pre | 54.30 ± 3.93 | 63.89 ± 3.70 | 54.47 ± 4.62 |
| | Post | 28.96 ± 4.61 *p=0.0003 | 15.70 ± 6.13 *p<0.0001 | 9.05 ± 4.47 *p<0.0001 ; #p= 0.0080 |
| Heart Rate (beats/min) | Pre | 276.00 ± 6.93 | 281.20 ± 7.69 | 260.10 ± 10.99 |
| | Post | 242.40 ± 29.23 | 128.70 ± 51.21 *p=0.00095 | 100.60 ± 39.82 *p= 0.0014 ; #p= 0.0080 |
| Peak Systolic Pressure (mm Hg) | Pre | 80.29 ± 1.26 | 81.56 ± 0.96 | 86.11 ± 1.94 |
| | Post | 62.29 ± 7.48 *p=0.0253 | 33.67 ± 13.32 *p=0.0025 | 27.67 ± 12.14 *p= 0.0002 ; #p= 0.00176 |
| Total work (mW) | Pre | 9.77 ± 0.77 | 11.55 ± 0.7718 | 9.76 ± 0.92 |
| | Post | 4.74 ± 0.91 *p=0.0003 | 2.16 ± 1.12 *p< 0.0001 | 1.53±0.85 *p<0.0001 ;#p=0.0117 |
| Total Work % Recovery | | 49.00±9.00 | 16.00±7.80 #p= 0.0452 | 11±8.90 #p=0.0152 |

Results expressed as mean±SEM. *pre vs post; # control vs melatonin; only values with significance are shaded.

#### 4.2.2.1. Effects of ischaemia/reperfusion

Exposure to 35 min regional ischaemia caused a significant reduction in aortic flow, cardiac output, peak systolic pressure and total work during reperfusion in all three groups (see **Table 11**).

#### 4.2.2.2. Effects of melatonin

The melatonin group (50µM) showed that I/R resulted in decreases in the coronary flow (p=0.0085), heart rate (p= 0.0095), peak systolic pressure (p=0.0025), aortic output (p<0.0001), cardiac output (p<0.0001) and consequently total work (p<0.0001) during reperfusion. Melatonin (50µM) treatment resulted in a significant reduction in coronary flow during reperfusion (p=0.0190) compared to the control group, while all other parameters measured were similar to those of the untreated control hearts. Interestingly, the lower concentration of melatonin (0.3µM) resulted in a significantly lower coronary flow (p=0.0011), aortic output (p=0.0415), cardiac output (p=0.0080), heart rate (p=0.0080), peak systolic pressure (p=0.0176) and total work (p=0.0117) during reperfusion when compared to the control group.

#### 4.2.2.3. Infarct size determination

At the end of the regional ischaemia-reperfusion protocol, hearts were stained and used for IFS as previously described in detail in chapter 3 (**Section 3.2.6.2**). The AR and IFS were determined using computerised planimetry. AR was expressed as the % of the total area while IFS was expressed as a % of the area at risk.

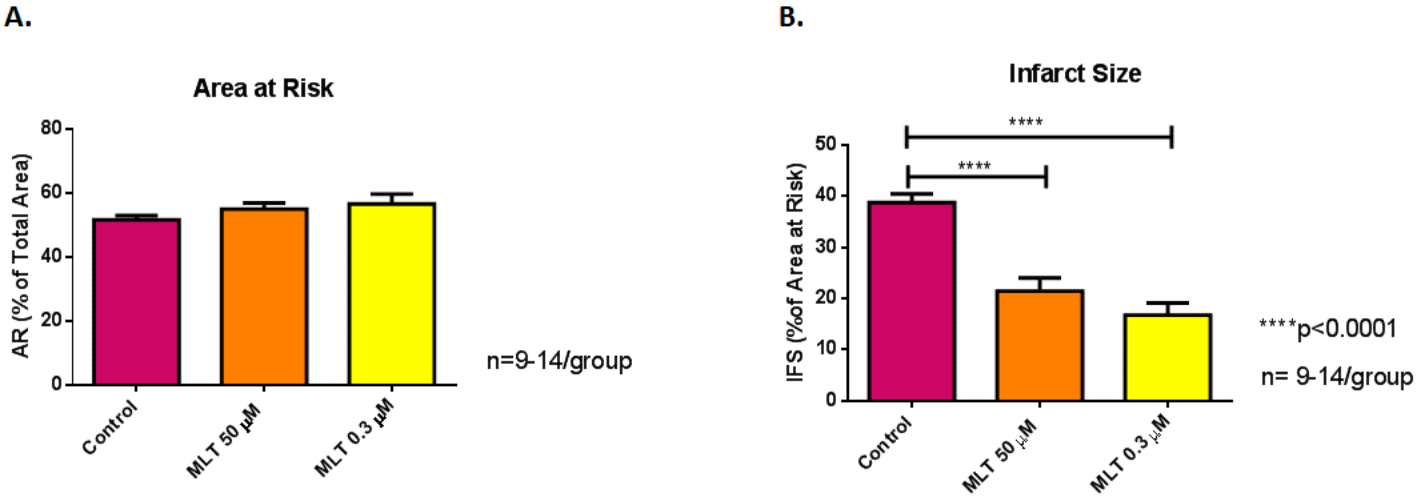



***Figure 25: (A) Area at risk (expressed as % of the total area): effects of melatonin and (B) IFS (expressed as a % of the area at risk): effects of melatonin. (n= 9-14/group). Abbreviations: AR- Area at Risk, IFS- Infarct Size, MLT- Melatonin.***

There were no significant differences between the AR of the various groups (**Fig 25** -A). (Control: 51.69±1.30%, MLT 50µM: 55.01±1.97%, MLT 0.3µM: 56-65±3.03%; n= 9-14/group). Melatonin at both 50µM and 0.3µM ($p<0.0001$) significantly decreased the infarct size by 45% (21.44±2.59) and 64% (13.78±2.35), respectively compared to the control group (38.73±1.73) (**Fig 25** -B).

### 4.2.3. Summary: Global Ischaemia, Regional Ischaemia and Infarct Size

In summary, exposure of hearts to either 20 minutes of global ischaemia or 35 minutes of regional ischaemia resulted in decreases in several haemodynamic endpoints during reperfusion. In the global ischaemia experimental model, melatonin (50µM) maintained the coronary flow and increased the cardiac output. In the regional ischaemia experimental model, melatonin (at both concentration) significantly reduced the IFS. However, this was associated with a reduction, rather than improvement in mechanical performance during reperfusion.

## 4.3. Oxidative phosphorylation capacity: effects of ischaemia/reperfusion and melatonin

As stated in the aims and objectives, hearts were subjected to a global I/R protocol to allow correlation of the mitochondrial oxidative phosphorylation process with mitophagy and the effects of manipulation with melatonin on these parameters. To achieve these aims, in the first set of experiments mitochondria were isolated from hearts at different stages of a global I/R protocol to allow evaluation of the effects of ischaemia *per se* and also that of reperfusion in the absence or presence of melatonin (0.3 or 50 µM), on mitochondrial oxidative phosphorylation and function.

Mitochondrial phosphorylation oxidative capacity was assessed using two substrates [A: Glutamate (Carbohydrate)/malate medium] or B [B: Palmitoyl-L-carnitine (Fatty acid)/malate medium]. The subsequent parameters examined, were the $QO_2$ State 3 (S3; nAtoms oxygen/mg prot/min), QO2 State 4 (S4; nAtoms oxygen/mg prot/min), respiratory control index (RCI), ADP/O and the oxidative phosphorylation rate (Oxphos, nmoles ATP/mg prot/min). For control purposes, mitochondria isolated from a 5min retrograde perfused heart was included each day to serve as baseline.

### 4.3.1. QO2 State 3 (S3)

Exposure of the heart to 20min global ischaemia had no effect on QO2 (S3), but a significant reduction was observed in the mitochondria isolated from untreated reperfused hearts: I/R decreased the QO2 (S3) in the glutamate/malate medium, (**Fig 26** -A) in the reperfusion control group compared to the ischaemic control group (p=0.0467; Isch Control: 209.30±30.57 vs. Rep Control: 133.40±13.56). However, the palmitoyl-L-carnitine/malate substrate (**Fig 26-** B, D), had no significant effects on any of the groups. Melatonin at 50µM and 0.3µM was without effect in all groups.

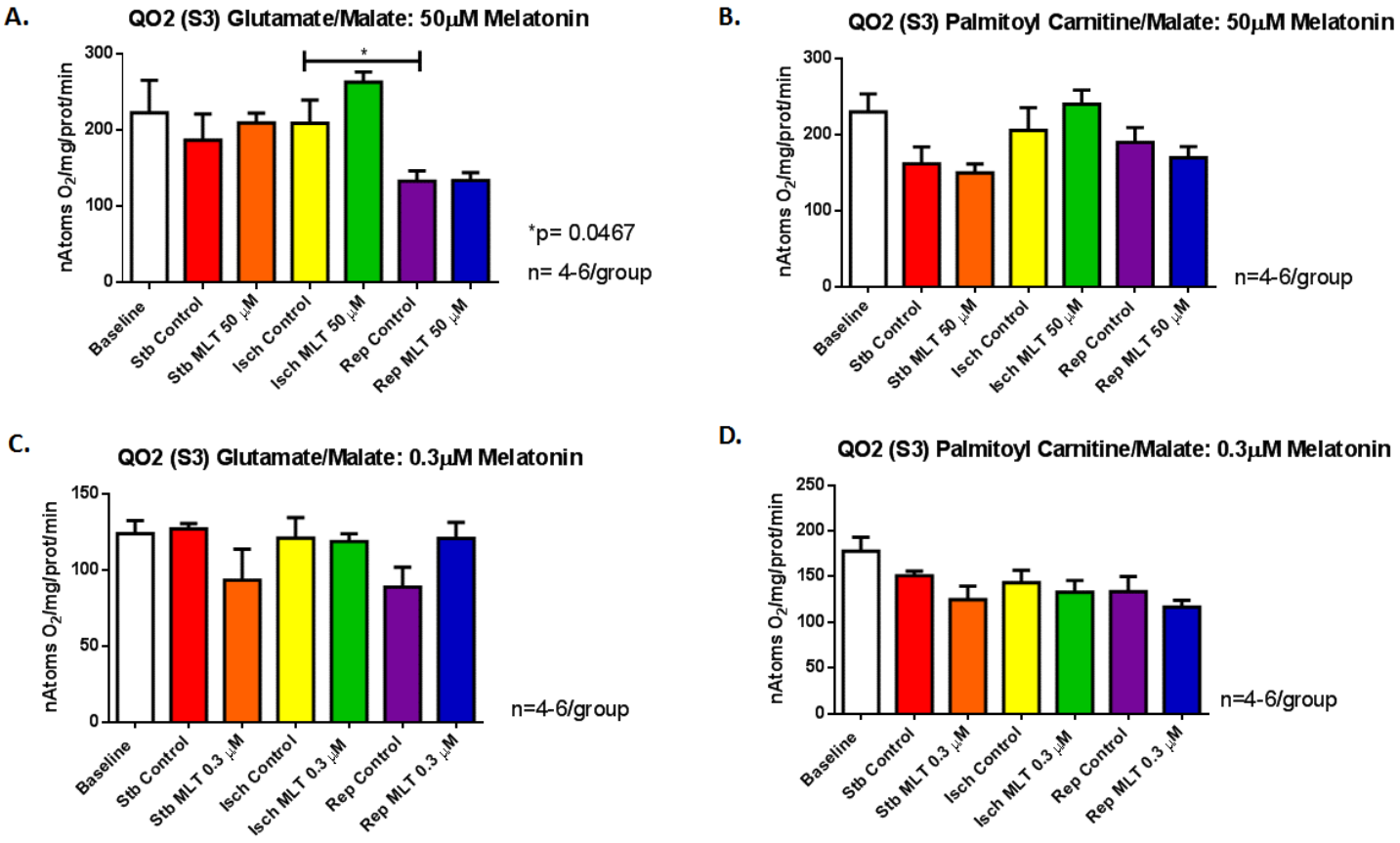



***Figure 26: The effect of I/R and melatonin on (A) QO2 (S3) Glutamate/malate: melatonin 50µM; (B) QO2 (S3) Palmitoyl-L-carnitine/malate: 50µM melatonin; (C) QO2 (S3) Glutamate/malate: 0.3µM melatonin; (D) QO2 ( S3) Palmitoyl-L- carnitine/malate: 0.3µM melatonin. Abbreviations: Stb-Stabilisation, Isch – Ischaemia, Rep- Reperfusion, MLT- Melatonin.***

### 4.3.2. QO2 State 4 (S4)

Ischaemia per se had no effect on the QO2 (S4), with both substrates. However, as with QO2 (S3), the QO2 (S4) of mitochondria isolated after reperfusion, was significantly lower with glutamate/malate (p=0.0300; Isch Control- 44.69±2.108 vs.

Rep Control- 32.82±4.20) as well as palmitoyl-L- carnitine/malate (p=0.0066; Isch Control-50.15± vs. Rep Control-35.69±2.961) as substrates (**Fig 27** A and B respectively). Melatonin (50µM) had a lowering effect on QO2 (S4) during reperfusion in the glutamate/malate medium (**Fig 27**- A), when compared to the values obtained during stabilisation (p=0.0020; Stb MLT-46.00±3.32 vs. Rep MLT-26.20±2.16) and after subjecting the hearts to ischaemia (p=0.0001; Isch MLT-50.99±3.91 vs. Rep MLT-26.20±2.16). This was not observed with the lower concentration.

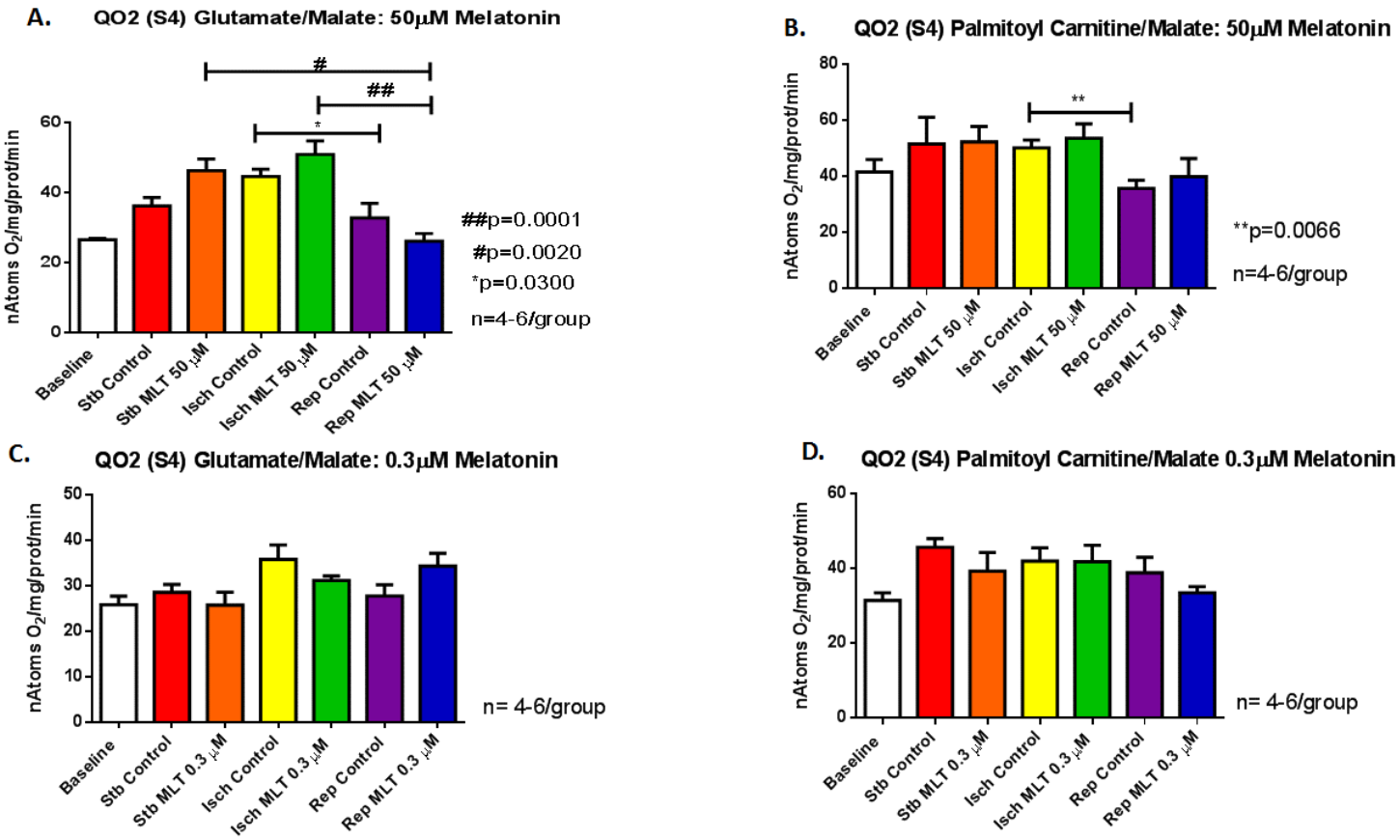



***Figure 27: The effect of I/R and melatonin on (A) QO2 State 4 (S4) Glutamate/malate: melatonin 50µM; (B) QO2 State 4 (S4) Palmitoyl-L- carnitine/malate: 50µM melatonin; (C) QO2 State 4 (S4) Glutamate/malate: 0.3µM melatonin; (D) QO2State 4 (S4) Palmitoyl-L- carnitine/malate: 0.3µM melatonin. Abbreviations: Stb-Stabilisation, Isch – Ischaemia, Rep- Reperfusion, MLT-Melatonin.***

### 4.3.3. Respiratory Control Index (RCI)

Exposure of the heart to ischaemia had no effect on the mitochondrial RCI, regardless of the substrate used. Reperfusion however, significantly decreased the RCI in the glutamate/malate medium (**Fig 28** -C; p= 0.0123; Stb Control-4.48±0.19 vs. Rep

Control- 3.144±0.27). Melatonin, at a concentration of 0.3µM in the glutamate/malate medium decreased the RCI during stabilisation (**Fig 28** -C; p=0.0326; Stb Control- 4.48±0.19 vs. Stb MLT- 3.46±0.34).

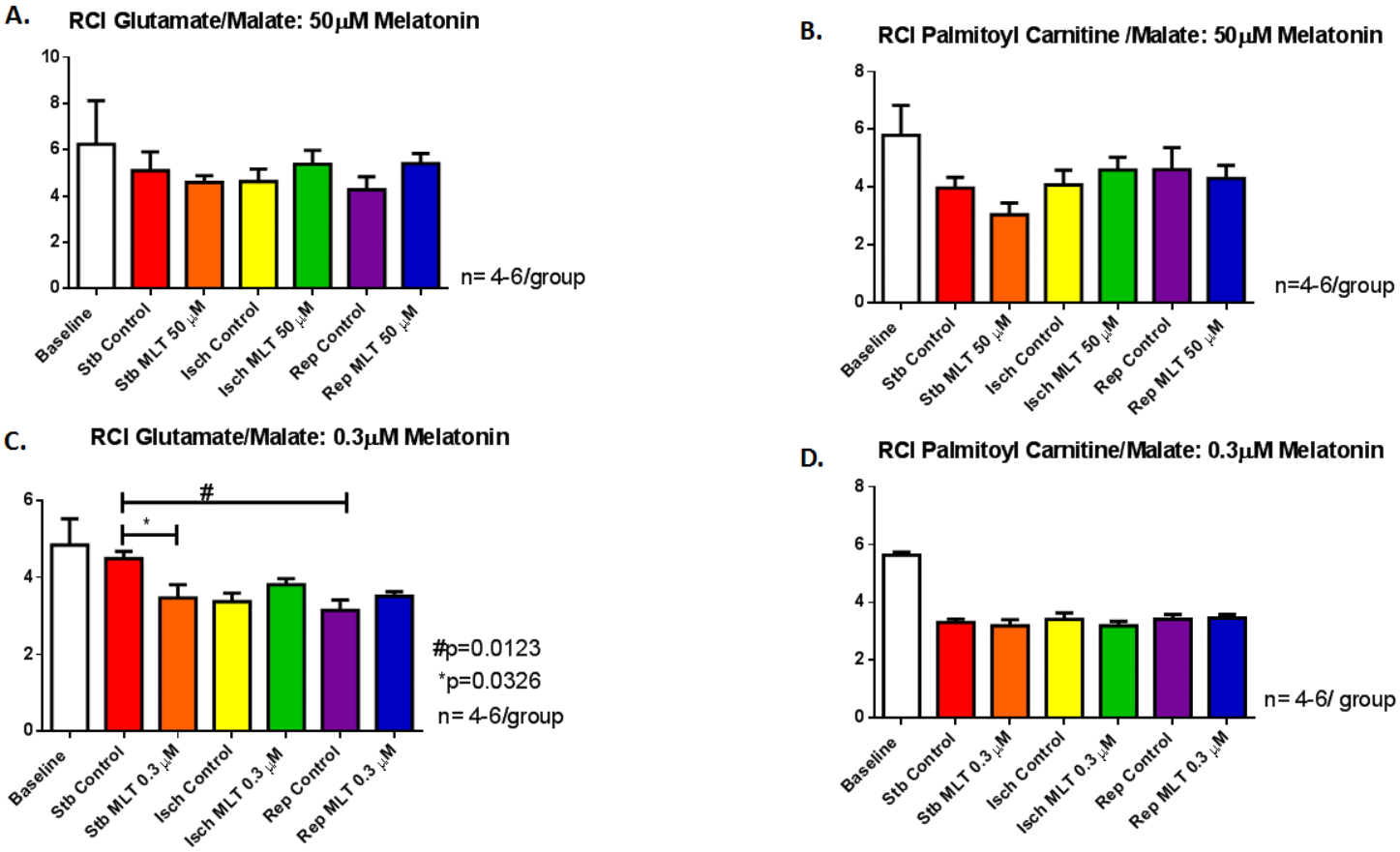



***Figure 28: The effect of I/R and melatonin on (A) RCI Glutamate/malate: melatonin 50µM; (B) RCI Palmitoyl-L- carnitine/malate: 50µM melatonin; (C) RCI Glutamate/malate: 0.3µM melatonin; (D) RCI Palmitoyl-L- carnitine/malate: 0.3µM melatonin. Abbreviations: Stb-Stabilisation, Isch – Ischaemia, Rep- Reperfusion, MLT- Melatonin.***

### 4.3.4. ADP/O ratio

Only in one set of experiments, a reduction on ADP/O ratio was observed in hearts subjected to I/R in comparison to ischaemia alone (p=0.0294; Isch Control-2.58±0.13 vs. Rep Control-1.99±0.18), with glutamate as substrate. (**Fig 29** -C). However no differences in this parameter were observed in any of the other groups, with both substrates.

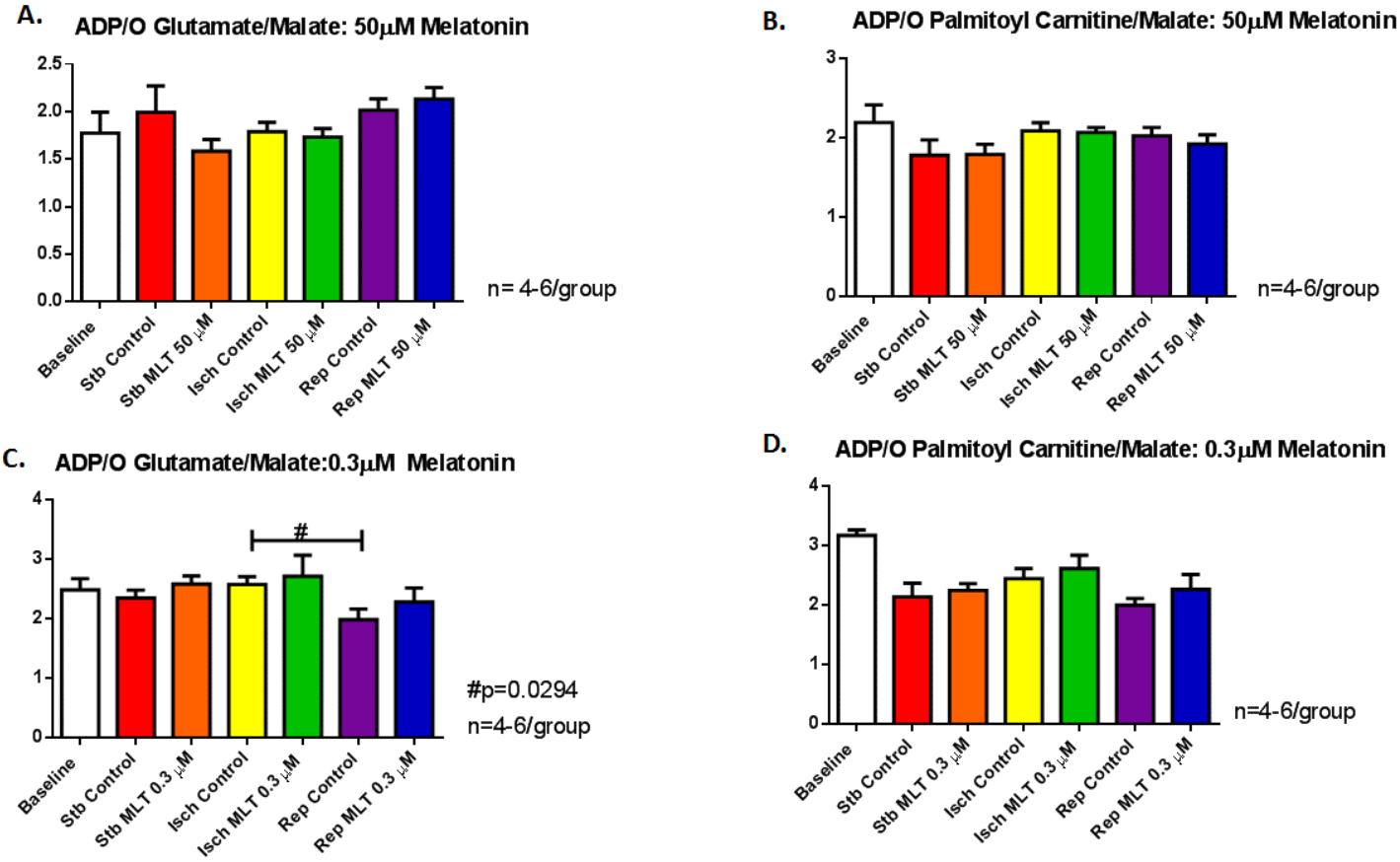



***Figure 29: The effect of I/R and melatonin on (A) ADP/O Glutamate/malate: melatonin 50µM; (B) APO/O Palmitoyl-L- carnitine/malate: 50µM melatonin; (C) ADP/O Glutamate/malate: 0.3µM melatonin; (D) ADP/O Palmitoyl-L-carnitine/malate: 0.3µM melatonin. Abbreviations: Stb- Stabilisation, Isch – Ischaemia, Rep- Reperfusion, MLT- Melatonin.***

### 4.3.5. Oxidative Phosphorylation Rate (Oxphos)

Analysis of the Oxphos with both the glutamate/malate and palmitoyl-L-carnitine/malate substrates (**Fig 30** A-B), showed considerable variations in the data obtained in the different groups. However with untreated hearts and glutamate as substrate, ischaemia was without effect while reperfusion reduced the oxphos rate when compared with ischaemia alone. Melatonin at 50µM decreased this parameter

during reperfusion with glutamate/malate (**Fig 30-** A; p=0.0122; Isch MLT- 460±45.72 vs. Rep MLT- 284.60±27.65) and significantly increased it during ischaemia with palmitoyl-L -carnitine/malate (**Fig 30**-A; p=0.0225; Stb MLT-273.60±36.89 vs. Isch MLT-495.60±39.67) as substrates. Melatonin at 0.3µM also significantly increased the oxphos rate observed after reperfusion in comparison with the untreated control group (p=0.0043; Rep Control- 171.00±20.89 vs. Rep MLT- 267.80±12.99), using glutamate/malate as substrate (**Fig 30**- C). No significant difference was observed in the palmitoyl-L- carnitine/malate medium (**Fig 30** D).

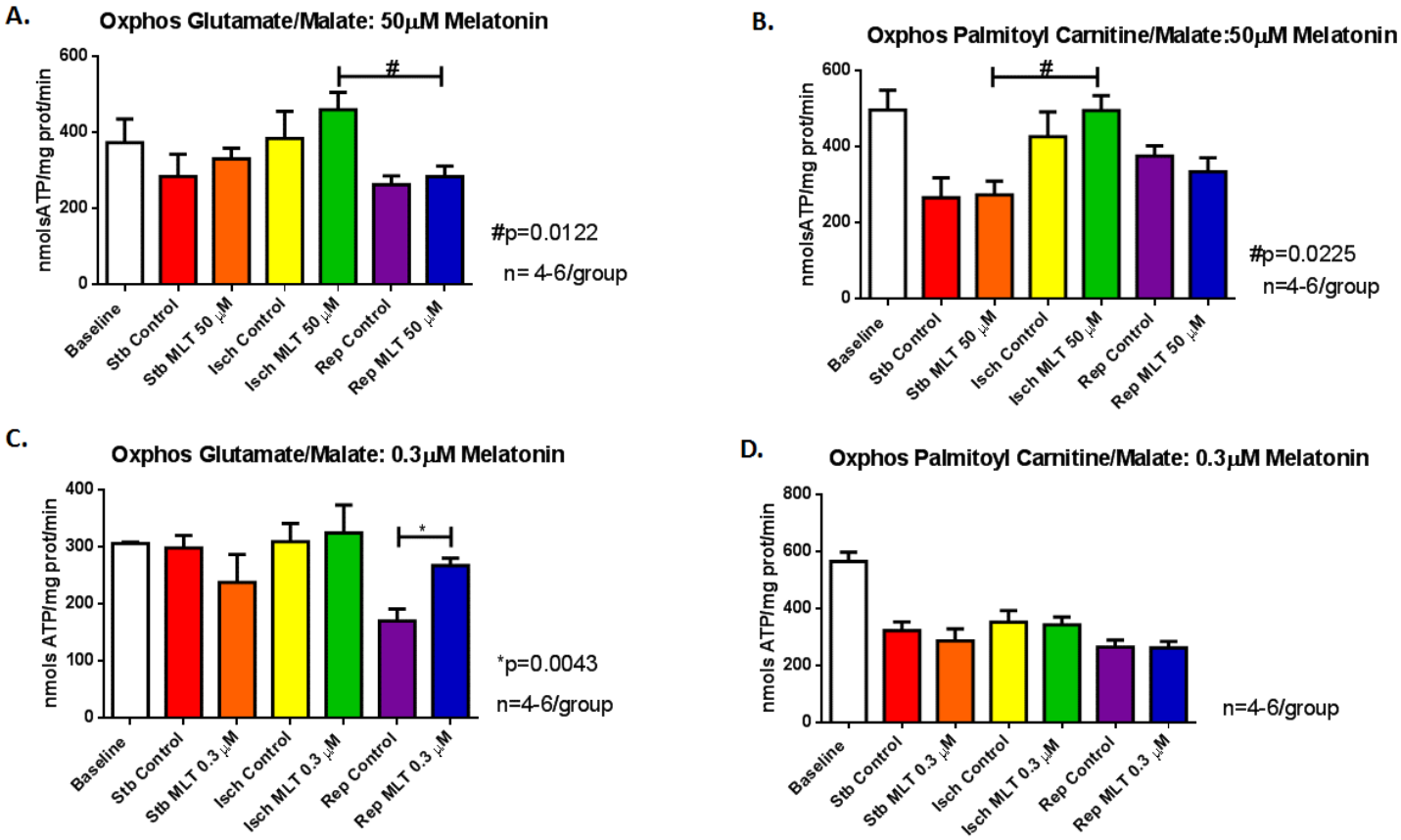



***Figure 30: The effect of I/R and melatonin on (A) Oxphos Glutamate/malate: melatonin 50µM; (B) Oxphos Palmitoyl-L-carnitine/malate: 50µM melatonin; (C) Oxphos Glutamate/malate: 0.3µM melatonin; (D) Oxphos Palmitoyl-L- carnitine/malate: 0.3µM melatonin. Abbreviations: Stb- Stabilisation, Isch – Ischaemia, Rep- Reperfusion , MLT- Melatonin.***

### 4.3.6. Summary: Oxidative Phosphorylation Capacity

In summary, exposure of the heart to 20 min global ischaemia followed by reperfusion, caused significant changes in mitochondrial QO2 state 4, RCI, ADP/O and Oxphos

rate with glutamate/malate as substrates. Palmitoyl/ malate on the other hand had small or non –existent effects on these parameters.

Melatonin, at both concentrations, had few significant effects, regardless of the substrate used. Melatonin did however, decrease State 4 and the oxphos rate during reperfusion with glutamate/malate as substrate.

### 4.4. Evaluation of Mitophagy by Western Blot Analysis

In order to allow a meaningful correlation between mitochondrial oxidative phosphorylation function and mitophagy after exposure of the hearts to ischaemia and reperfusion as well as the effect of manipulation thereof by melatonin, mitochondrial pellets obtained after experimentation were divided in two: one half was used for evaluation of mitochondrial oxidative phosphorylation function (results shown above) and the other half was used for preparation of lysates for Western blotting. To allow evaluation of markers of mitophagy in the cytosol, an additional series of experiments were performed following the same protocols as depicted in **Section 3.2.6.1**- **Fig 12**, with the hearts being freeze-clamped at the designated intervals for subsequent preparation of cytosolic fractions. Two additional proteins, known to be associated with the effects of melatonin, were also included namely PGC-1 alpha and Sirt1. While in most of the studies the effects of two melatonin concentrations were evaluated namely 50µM and 0.3µM, the effects of the low concentration of melatonin only were evaluated in a number of the studies on cytosolic proteins.

The proteins that were analysed included:

- Mitophagy proteins
  - PARKIN, PINK1, TOM70, p62/ SQSTM1, Sirt3 ,ULK (phosphorylated and total), Rab9, DRP-1 (phosphorylated and total)
- Cytosolic proteins
  - PGC-1 alpha, Sirt1

In view of the limitation on the number of samples (24) which could be compared on one blot, we ran two series of perfusions and blots, using 0.3µM and 50µM melatonin respectively (See **Appendix G and H** for templates). In this section the markers from the PINK1/PARKIN pathway will be discussed first. Subsequently, the mitophagy

markers from the alterative pathway will be presented followed by the cytosolic markers (See **Fig 31** below).

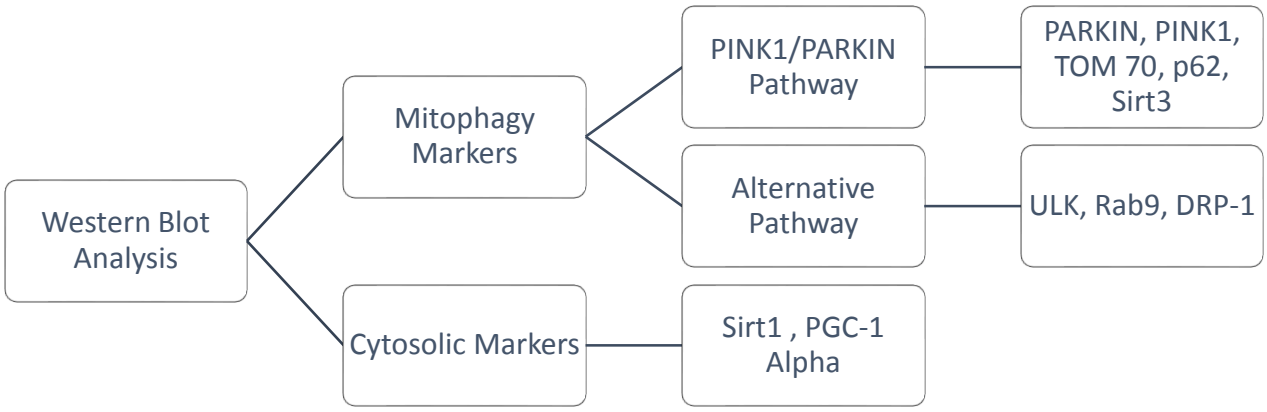



***Figure 31: Schematic Illustration of Western Analysis Presentation.***

### 4.4.1. Mitophagy Markers

#### 4.4.1.1. PINK1/PARKIN Pathway

**PARKIN**

Neither, ischaemia, reperfusion nor melatonin at 50µM had a significant effect on the expression of PARKIN in the different groups (**Fig 32-** A). Analysis of Parkin expression in the series treated with melatonin at 0.3µM (**Fig 32** -B), showed that in this series the expression of PARKIN in the untreated stabilisation group differed significantly from control hearts when subjected to ischaemia (p=0.0090; Stb Control-0.77±0.09 vs. Isch Control 0.50±0.02) or reperfusion (p<0.0001; Stb control-0.77±0.09 vs. Rep Control-0.81±0.02). Melatonin (0.3µM) decreased Parkin expression during stabilisation (p=0.0262; Stb Control- 0.77±0.09 vs. Stb MLT-0.53±0.014) compared to its control counterparts, but it had no effects during I/R.

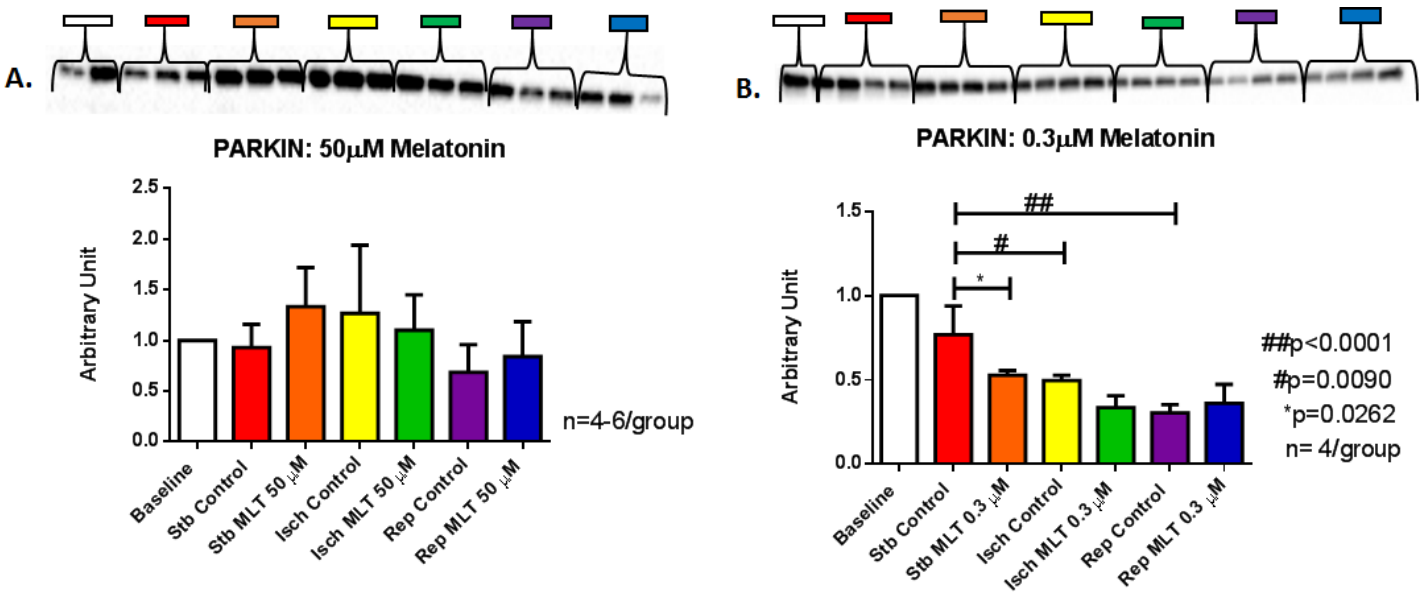



***Figure 32: Parkin levels in the mitochondria of hearts perfused according to the various global ischaemia/ reperfusion protocols. (A) effects of 50µM melatonin (B) effects of 0.3µM melatonin. Abbreviations: Stb- Stabilisation, Isch- Ischaemia, Rep- Reperfusion, MLT- Melatonin***

**PINK1**

As was seen with PARKIN, PINK1 expression remained unchanged in the mitochondria of hearts subjected to ischaemia alone, but reperfusion decreased mitochondrial PINK1 significantly when compared with the stabilization controls (p= 0.0023; Stb Control- 0.94±0.23 vs. 0.27±0.03). Melatonin, at both concentrations studied, was without effect on PINK1 levels (**Fig 33** -A and B).

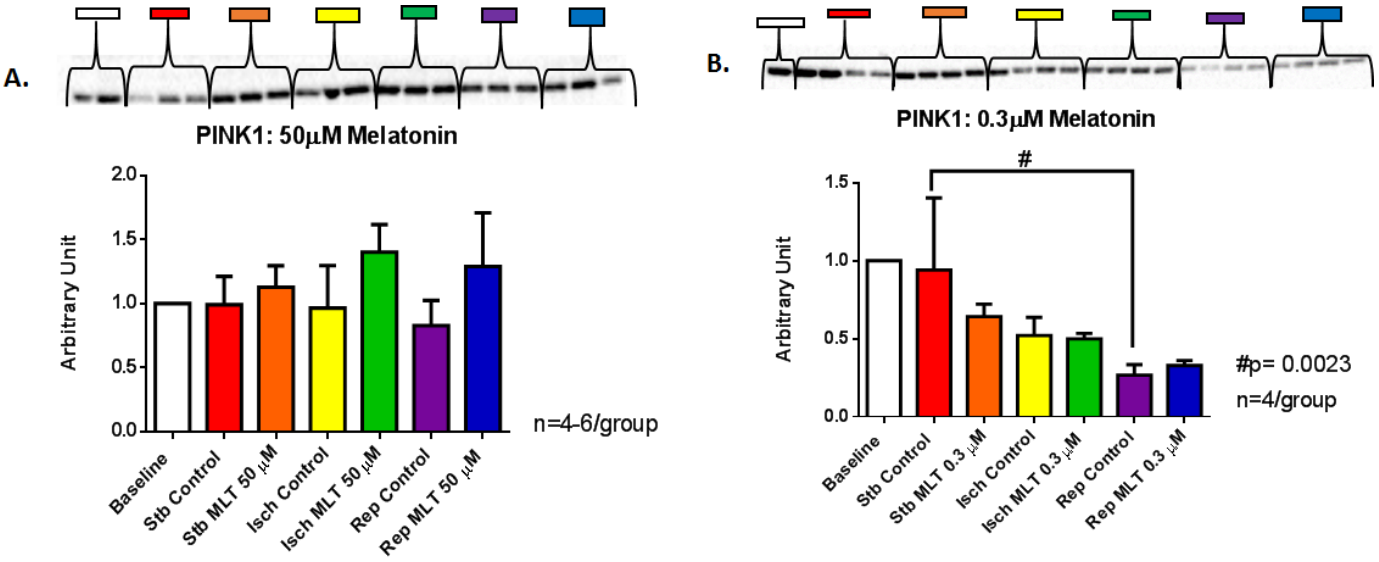



***Figure 33: PINK1 levels in the mitochondria of hearts perfused according to the various global ischaemia protocols. (A) effects of 50µM melatonin (B) effects of 0.3µM melatonin. Abbreviations: Stb- Stabilisation, Isch- Ischaemia, Rep- Reperfusion, MLT- Melatonin.***

**TOM70**

Compared with the untreated stabilization control, mitochondrial TOM70 expression was significantly decreased by ischaemia alone (p=0.0343; Stb Control- 1.60±0.19 vs. Isch Control- 0.95±0.075) and by reperfusion (p=0.0462; Stb Control- 1.6±0.19 vs. 0.97±0.12), (**Fig 34**- A). Melatonin at 50µM decreased the expression of TOM during stabilisation (p=0.0235; Stb Control- 1.6±0.19 vs. Stb MLT- 0.92±0.12), whereas melatonin at both concentrations had no effect on the expression of TOM70 in either, ischaemia or I/R when compared with their respective untreated controls.

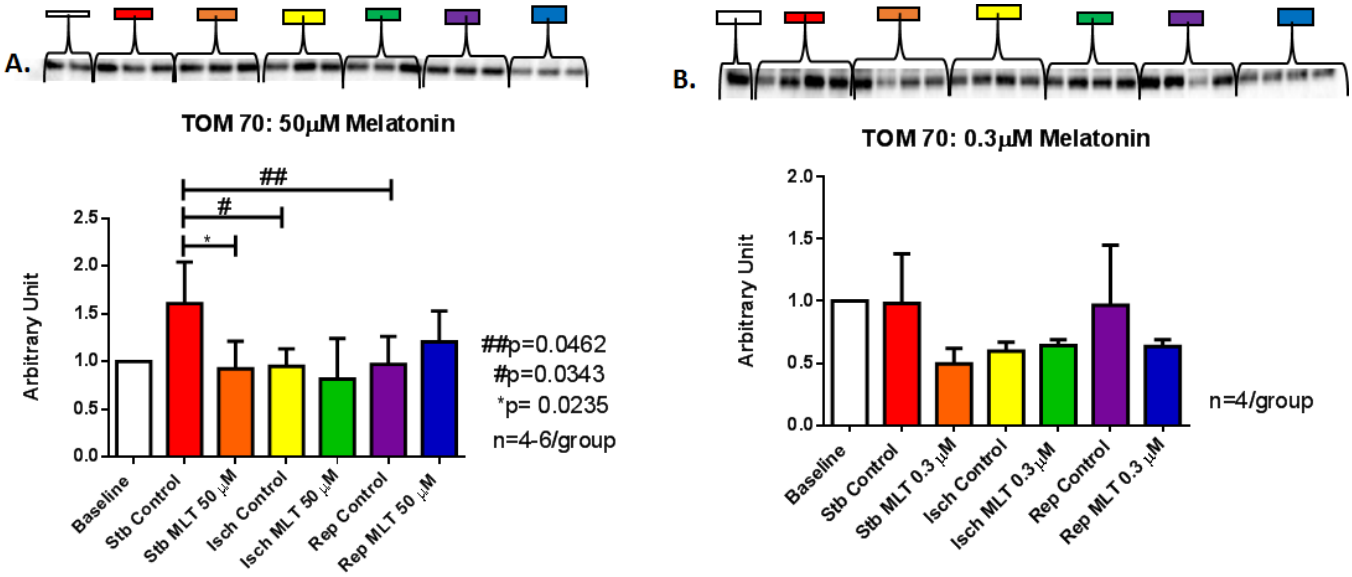



***Figure 34: TOM70 levels in the mitochondria of hearts perfused according to the various global ischaemia protocols. (A) effects of 50µM melatonin (B) effects of 0.3µM melatonin. Abbreviations: Stb- Stabilisation, Isch- Ischaemia, Rep- Reperfusion, MLT- Melatonin.***

## SQSTM1/p62

Exposure to ischaemia alone as well as reperfusion had no effects on the expression of mitochondrial p62 from untreated hearts, when compared with the stabilization group (**Fig 35** A and B). Interestingly, in the melatonin treated groups ischaemia and reperfusion decreased p62 expression in comparison to stabilisation (p= 0.0103; Stb MLT- 2.4±0.20 vs. Rep MLT- 0.76±0.19). While melatonin at 50µM was without effect when compared with their corresponding control untreated groups (**Fig 35**– A), 0.3µM of melatonin (**Fig 35**- B) did result in a significant decrease in the expression of p62 in comparison to the untreated reperfused control group (p=0.0030; Rep Control- 2.6±0.16 vs. 0.76±0.19).

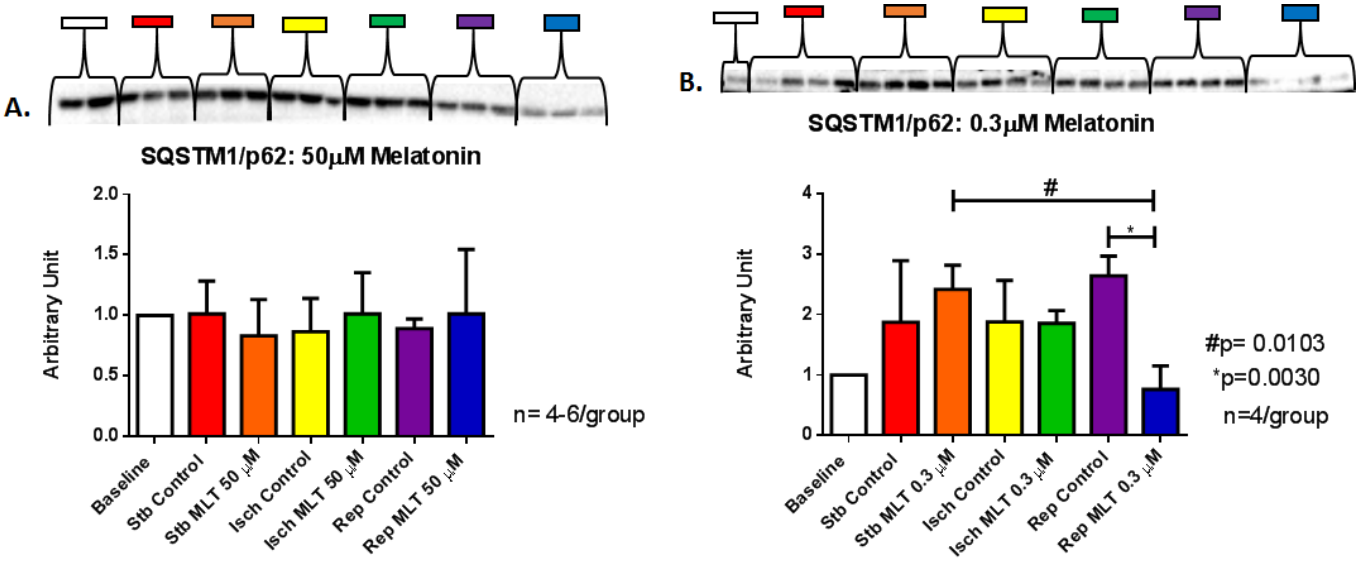



***Figure 35: SQSTM1/p62 levels in the mitochondria of hearts perfused according to the various global ischaemia protocols. (A) effects of 50µM of melatonin (B) effects of 0.3µM melatonin. Abbreviations: Stb- Stabilisation, Isch- Ischaemia, Rep- Reperfusion, MLT- Melatonin***

### Sirt3

Exposure of untreated hearts to ischaemia and reperfusion did not have a significant on Sirt3 expression, when compared with stabilization. Treated melatonin (50µM; **Fig 36**-A) groups showed increased Sirt3 expression when exposed to reperfusion, compared to the stabilisation (p=0.0003; Stb MLT- 1.10±0.13 vs. 2.1±0.20) and ischaemia (p<0.0001; Isch MLT- 0.84±0.066 vs. Rep MLT- 2.1±0.20). Melatonin at a concentration of 50µM caused a reduction in the expression of Sirt3 (p=0.0113; Stb control-1.9±0.16 vs. Stb MLT- 1.1±0.13) in the stabilisation group. Sirt3 expression was significantly upregulated in the melatonin reperfusion group in comparison to the control reperfusion group (p=0.0025; Rep Control-1.3±0.10 vs. Rep MLT- 2.1±0.20). Melatonin at concentration of 0.3µM also increased expression of Sirt3 in the reperfusion group, but the change was not significant, due to a rather large standard error.

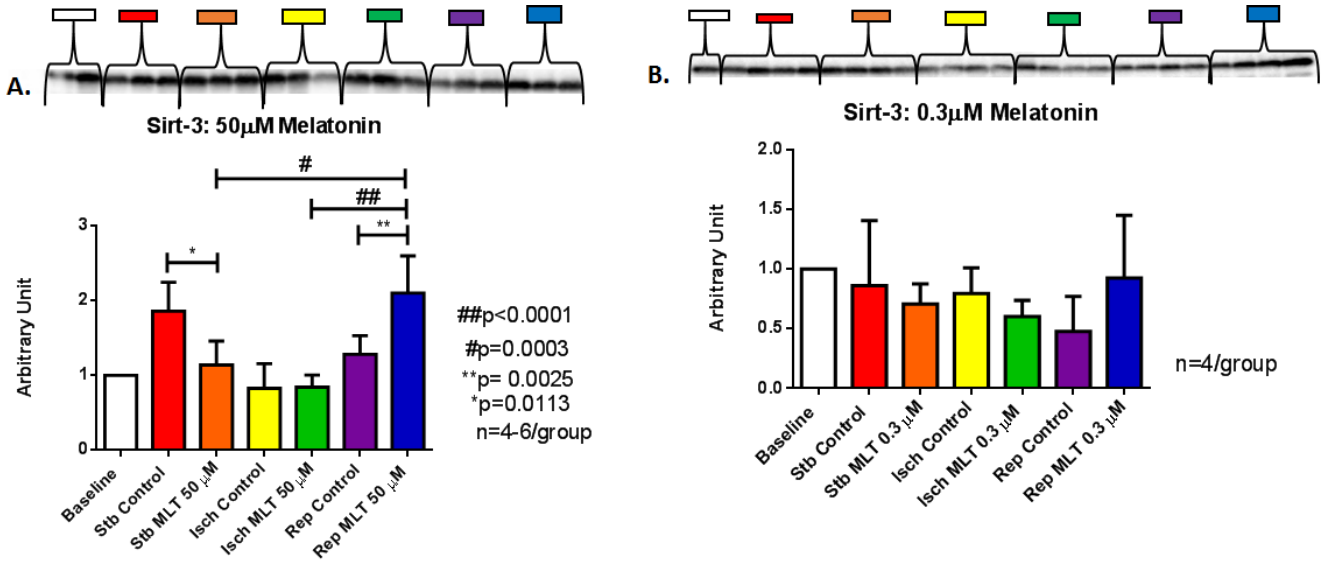



***Figure 36: Sirt3 levels in the mitochondria of hearts perfused according to the various global ischaemia protocols. (A) effects of 50µM of melatonin (B) effects of the 0.3µM.*** *Abbreviations:* ***Stb- Stabilisation, Isch- Ischaemia, Rep- Reperfusion, MLT- Melatonin.***

### Summary: PINK1/PARKIN Pathway

In summary, ischaemia alone or ischaemia plus reperfusion decreased the mitophagy markers of the PINK1/PARKIN pathway (PARKIN, PINK1, TOM70 and p62). Melatonin either at 50 or 0.3µM decreased the expression of the mitophagy markers during stabilisation (PARKIN, TOM70 and Sirt3). Melatonin, 0.3 µM and 50µM

respectively, decreased p62 expression and increased Sirt3 expression during reperfusion.

#### 4.4.1.2. Alternative Pathway Markers

In the analysis of the alternative pathway only the effect of the 0.3µM melatonin was evaluated. In this section the mitochondrial and cytosolic expression of Rab9 and DRP-1 are presented together.

**Cytosolic Phosphorylated and Total ULK**

The expression of total ULK (**Fig 37-** A) was significantly decreased by exposure to both ischaemia (p=0.0015; Stb Control- 1.5±0.11 vs. Isch Control- 0.17±0.05) and reperfusion (p=0.0314; Stb Control- 1.5±0.11 vs. Rep Control- 0.53±0.076) in untreated control groups in comparison to their stabilisation counterparts. In untreated controls, the expression of phosphorylated ULK was significantly decreased by ischaemia (p=0.00193; Stb Control- 0.21±0.014 vs. Isch Control- 0.45±0.073) when compared to stabilisation (**Fig 37**-B).

Melatonin administration during stabilisation (p=0.0358; Stb Control- 1.5±0.11 vs. Stb MLT- 2.4±0.38), ischaemia (p=0.0449; Isch Control-0.17±0.05 vs. Isch MLT-0.51±0.13) as well as during reperfusion (p=0.002; Rep Control- 0.53±0.076 vs. Rep MLT-2.1±0.16) significantly increased total ULK expression (**Fig 37-**B). Phosphorylated ULK levels (**Fig 37** -B) during stabilisation (p=0.0003; Stb Control-0.21±0.014 vs. Stb MLT- 1.1±0.16) as well as during reperfusion (p=0.0098; Rep Control- 0.24±0.025 vs. Rep MLT- 0.89±0.14) were significantly increased by melatonin, compared to their untreated counterparts (**Fig 37-**B).

The phosphorylated/total ULK ratio (**Fig 37-** C) was increased in the ischaemia control group (p=0.0024; Stb Control- 0.15±0.017 vs. Isch Control-3.5±1.20) in comparison to the stabilisation control group. Reperfusion in turn caused a significant reduction in the phosphorylated /total ULK ratio (p=0.0064; Isch Control- 3.5±1.2 vs. Rep Control-0.46±0.05), in comparison with the ischaemic control group. Melatonin decreased the phosphorylated/ total ULK ratio (p=0.0196; Stb Control-0.15± 0.017 vs. Stb MLT-0.52±0.12). No further differences were observed.

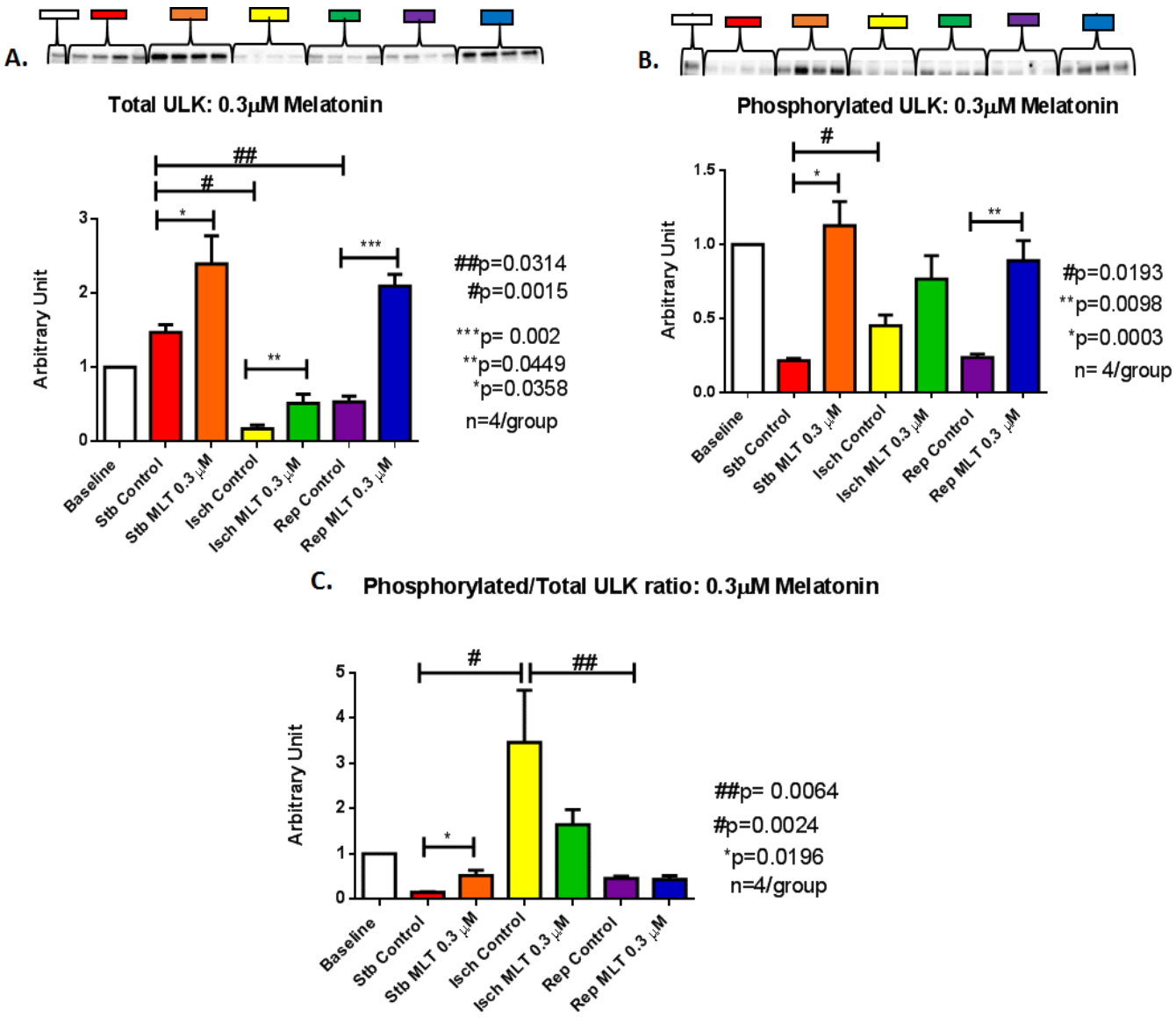



***Figure 37: Expression of (A) Total ULK, (B) Phosphorylated ULK and (C) Phosphorylated and Total ULK ratio in the freeze clamped hearts perfused according to the various global ischaemia protocols. Treated groups were perfused with 0.3µM melatonin. Abbreviations: Stb- Stabilisation, Isch- Ischaemia, Rep- Reperfusion, MLT- Melatonin***

## Mitochondrial and cytosolic Rab9

In the mitochondria (**Fig 38-** A) exposure to ischaemia caused a significant reduction in Rab expression (p=0.0026; Stb Control- 1.5±0.23 vs. Isch Control- 0.71±0.067), when compared with its corresponding stabilisation control. Compared to the untreated stabilisation group, Rab9 expression was significantly decreased by 0.3µM

melatonin (p=0.0023; Stb Control- 1.5±0.23 vs. Stb MLT- 0.69±0.040). No further differences were observed between the groups.

In the cytosol (**Fig 38-** B) Rab9 expression was increased in the control ischaemic group in comparison to the stabilisation control group (p=0.0062; Stb Control- 0.75±0.03 vs. Isch Control-1.1±0.01). On the other hand, the control reperfusion group had significantly lower Rab9 levels in comparison to the ischaemic control (p=0.0002; Isch Control- 1.1±0.01 vs. Rep Control-0.62±0.03). In the melatonin treated groups, ischaemia and reperfusion decreased Rab9 expression in comparison to stabilisation (p=0.0267; Stb MLT- 1.1±0.04 vs. Rep MLT- 0.82±0.06). The stabilisation (p=0.0052; Stb Control-0.75±0.03 vs. Stb MLT- 1.1±0.04) and reperfusion melatonin groups (p=0.0251; Rep Control- 0.62±0.02 vs. Rep MLT-0.82±0.06) had increased Rab9 levels in comparison to their untreated counterparts.

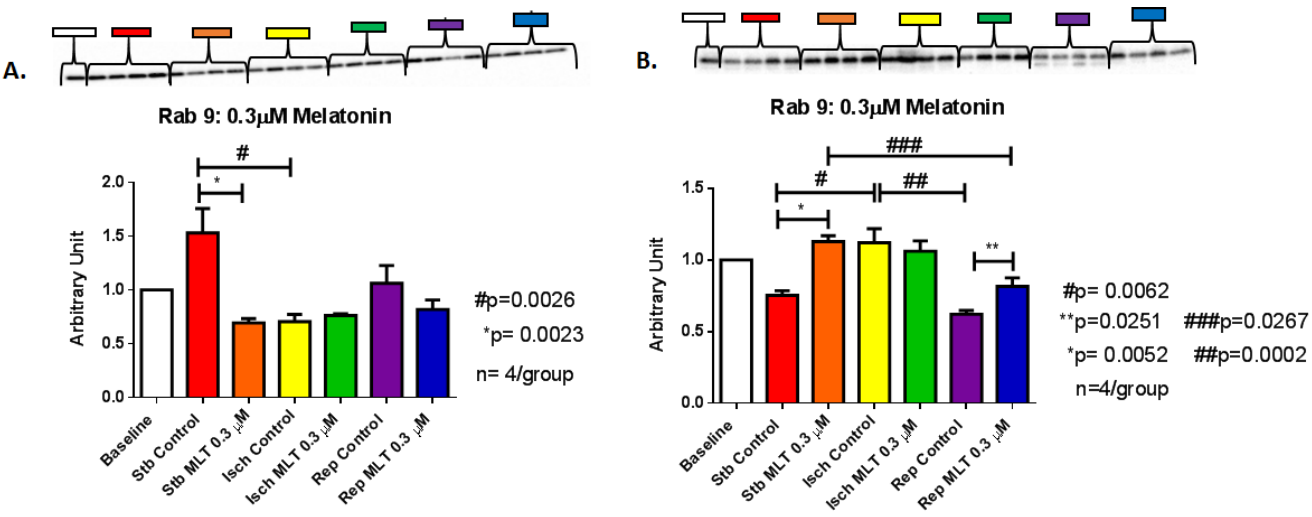



***Figure 38: Expression of (A) Rab9 in the mitochondria and (B) Rab9 in the cytosolic fraction of freeze clamped hearts perfused according to the various global ischaemia protocols. Treated groups were perfused with 0.3µM melatonin. Abbreviations: Stb- Stabilisation, Isch- Ischaemia, Rep- Reperfusion, MLT- Melatonin.***

**Mitochondrial Phosphorylated and Total DRP-1**

Total DRP-1 levels (**Fig 39-** A) were decreased in the stabilisation melatonin group compared to its untreated control (p=0.0137; Stb Control- 2.5±0.42 vs. Stb Mel-0.93±0.16). Melatonin significantly decreased phosphorylated DRP-1 (**Fig 39-** B) in the stabilisation (p=0.0249; Stb Control- 8.0±2.1 vs. Stb Mel-1.5±0.44) and ischaemic groups (p=0.0458; Isch Control-6.5±1.10 vs. Isch MLT- 2.5±1.1) group compared to their respective control counterparts.

Interestingly, melatonin decreased the phosphorylated/total DRP-1 ratio (**Fig 39-** C) in the stabilisation (p=0.0211; Stb Control-3.00±0.41 vs. Stb MLT- 1.6±0.20), ischaemic (p=0.0320; Isch Control- 2.8±0.14 vs. Isch MLT- 1.8±0.34) and reperfusion (p=0.0065; Rep Control-2.3±0.29 vs. 0.44±0.35) groups compared to their control counterparts. Comparison of the melatonin treated groups showed phosphorylated/total DRP-1 ratio (**Fig 39-** C) was significantly decreased during reperfusion in comparison to stabilisation (p= 0.0331; Stb MLT- 1.6±0.20 vs. Rep MLT-0.44±0.35) and ischaemia (p=0.0333; Isch MLT-1.80±0.34 vs. Rep MLT- 0.44±0.35). No further differences were observed.

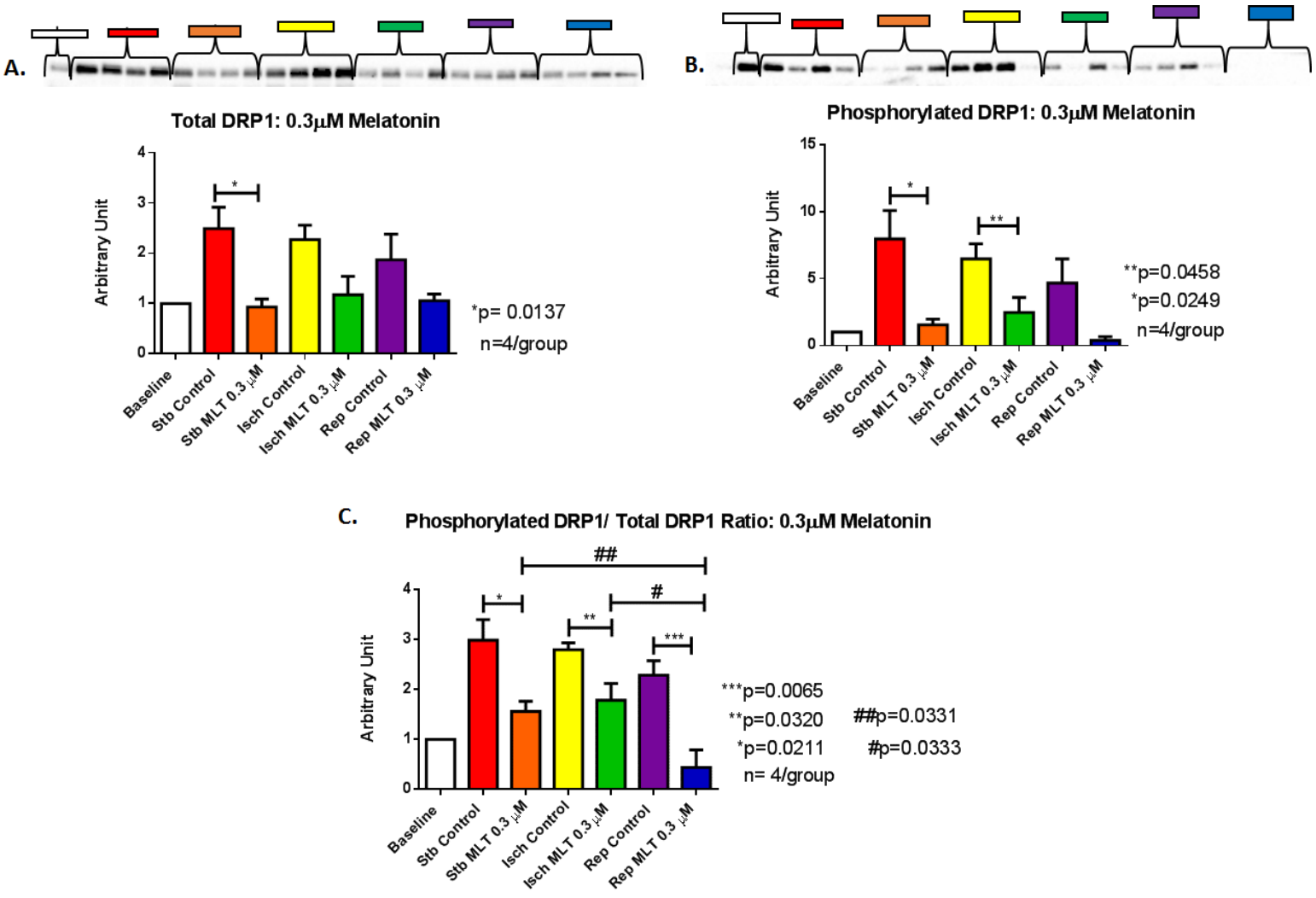



***Figure 39: (A) Total and (B) Phosphorylated DRP-1 expression, together with (C) the phosphorylated/total DRP-1 ratio in the mitochondria of hearts perfused according to the various global ischaemia protocols. Melatonin groups were treated with 0.3µM of melatonin. Abbreviations: Stb- Stabilisation, Isch- Ischaemia, Rep- Reperfusion, MLT- Melatonin.***

## Cytosolic Phosphorylated and Total DRP-1

In the melatonin treated groups, ischaemia increased the total DRP-1 expression (p=0.0001; Stb MLT-0.77±0.05 vs. Isch Mel- 1.3±0.02) while reperfusion had no effect (**Fig 39**-A). During ischaemia, melatonin increased the total DRP-1 expression (p=0.0021; Isch Control- 0.91±0.064 vs. Isch MLT- 1.30±0.02) in comparison to its control counterpart. No further differences were observed.

While phosphorylated DRP-1 expression was not affected by exposure to ischaemia or reperfusion in the untreated control groups, melatonin significantly increased this parameter in the stabilisation (p=0.0069; Stb Control-1.3±0.26 vs. Stb MLT- 4.2±0.69), ischaemic (p=0.0373; Isch Control- 0.90±0.26 vs. Isch MLT- 4.3±1.20) and reperfusion (p=0.0003; Rep Control- 0.32±0.06 vs. Rep MLT- 4.3±0.53) melatonin groups in comparison to their respective control groups (**Fig 40**-B). Similarly, the phosphorylated/total DRP-1 ratio was significantly increased in the stabilisation (p=0.0069; Stb Control- 1.6±0.39 vs. Stb MLT- 5.5±0.89) and ischaemic (p=0.0492; Isch Control- 0.96±0.23 vs. Isch MLT-3.4±0.96) melatonin group compared to the control group (**Fig 40-**C).

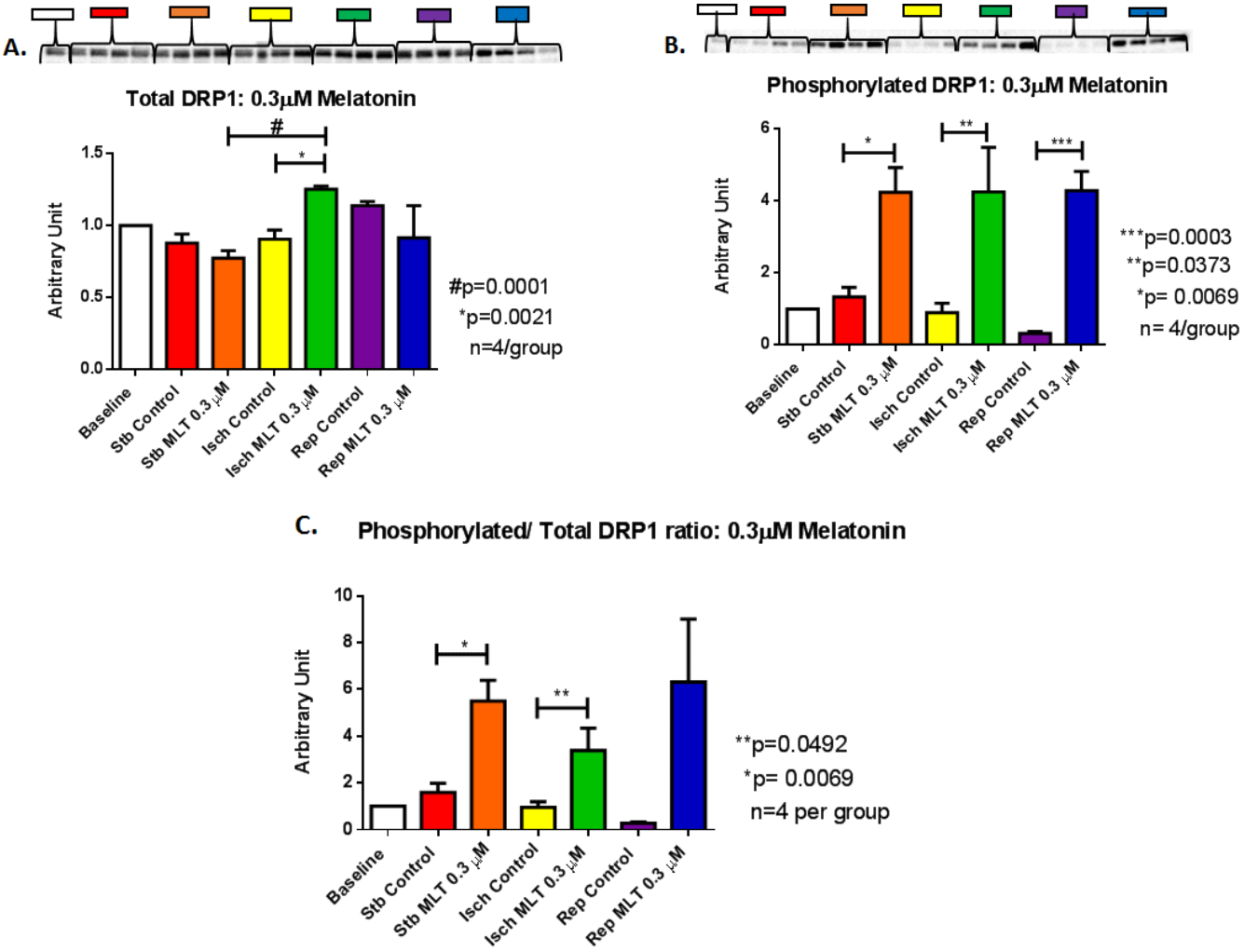



***Figure 40: Expression of (A) Total DRP-1, (B) Phosphorylated DRP-1 and (C) Phosphorylated/Total DRP-1 ratio in the freeze clamped hearts perfused according to the various global ischaemia protocols. Treated groups were perfused with 0.3µM melatonin. Abbreviations: Stb- Stabilisation, Isch- Ischaemia, Rep- Reperfusion, MLT- Melatonin.***

**Summary: Alternative Pathway**

In summary, either ischaemia alone or I/R had an effect on the markers of the alternative pathway. Changes in the mitochondria were often associated with corresponding changes in the cytosol. Melatonin had effects often in all three conditions (stabilisation, ischaemia and reperfusion).

#### 4.4.1.3. Cytosolic Markers: Mitochondrial Biogenesis

**Sirt1**

In both series of experiments exposure of untreated control hearts to ischaemia (p=0.001; Stb Control – 0.86± 0.08 vs. Isch Control- 0.36±0.03) (p= 0.0088; Stb Control- 0.75±0.05 vs. Isch Control- 0.021±0.04) as well as reperfusion (p=0.0001; Stb Control- 0.86±0.08 vs. Rep Control- 0.32±0.04) (p=0.0005; Stb Control-0.86±0.08 vs. Rep Control- 0.039±0.02) significantly decreased cytosolic Sirt1 expression, compared to the corresponding stabilisation control groups (**Fig 41** A-B).

Sirt1 was significantly decreased in the 50µM melatonin stabilisation group (p=0.0178; Stb Control- 0.86±0.08 vs. Stb MLT- 0.53±0.005) and increased in the reperfusion group (p= 0.0009; Rep Control- 0.32±0.04 vs. Rep MLT-0.74±0.09) in comparison to their corresponding control groups (**Fig 41**-A)

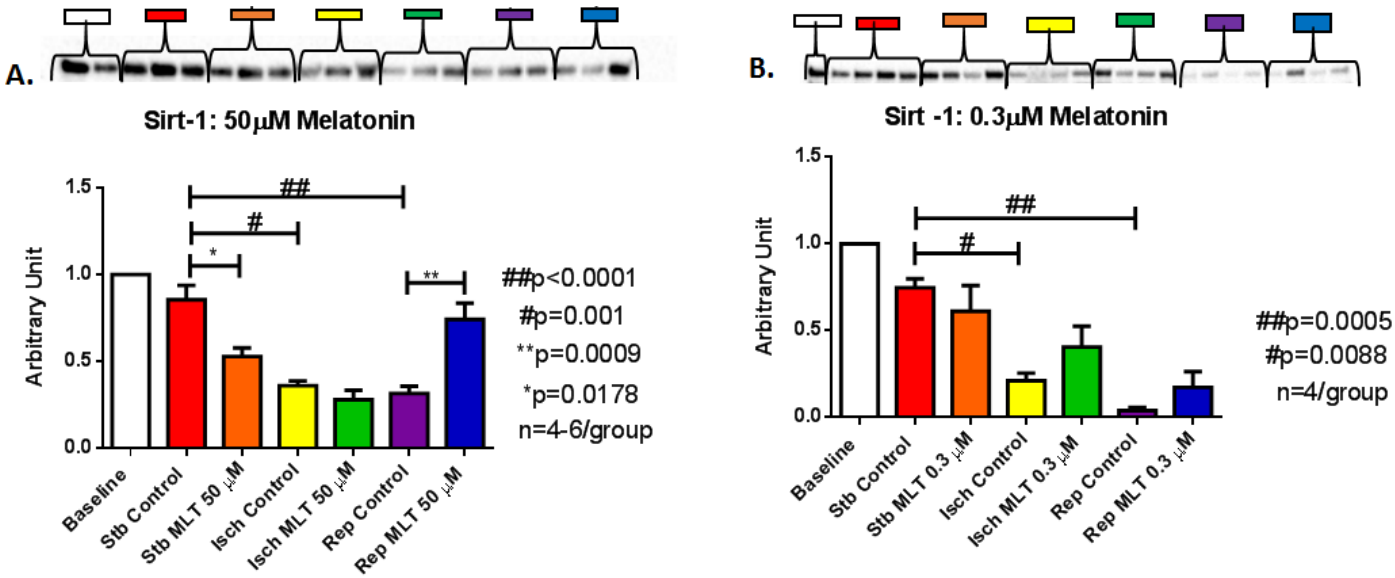



***Figure 41: Expression of Sirt1 in the freeze clamped hearts perfused according to the various global ischaemia protocols. Melatonin groups were treated with either (A) 50µM or (B) 0.3µM of melatonin. Abbreviations: Stb- Stabilisation, Isch- Ischaemia, Rep- Reperfusion, MLT- Melatonin***

## PGC-1 alpha

Exposure to ischaemia alone or reperfusion was without effect on the expression of cytosolic PGC-1 alpha untreated control groups (**Fig 42**- A-B). Similarly melatonin at a concentration of 50µM melatonin resulted in no significant differences in the expression of PGC-1 alpha between groups. However, PGC-1 alpha expression in melatonin (0.3µM; **Fig 42**- A) treated groups was increased during reperfusion (p=0.0041; Isch MLT-0.58±0.05 vs. Rep MLT- 0.88±0.08). Similarly, PGC-1 alpha expression was increased in the reperfusion melatonin group (0.3µM) in comparison to the reperfusion control group (p=0.0010; Rep Control-0.054±0.03 vs. Rep MLT-0 .88±0.08).

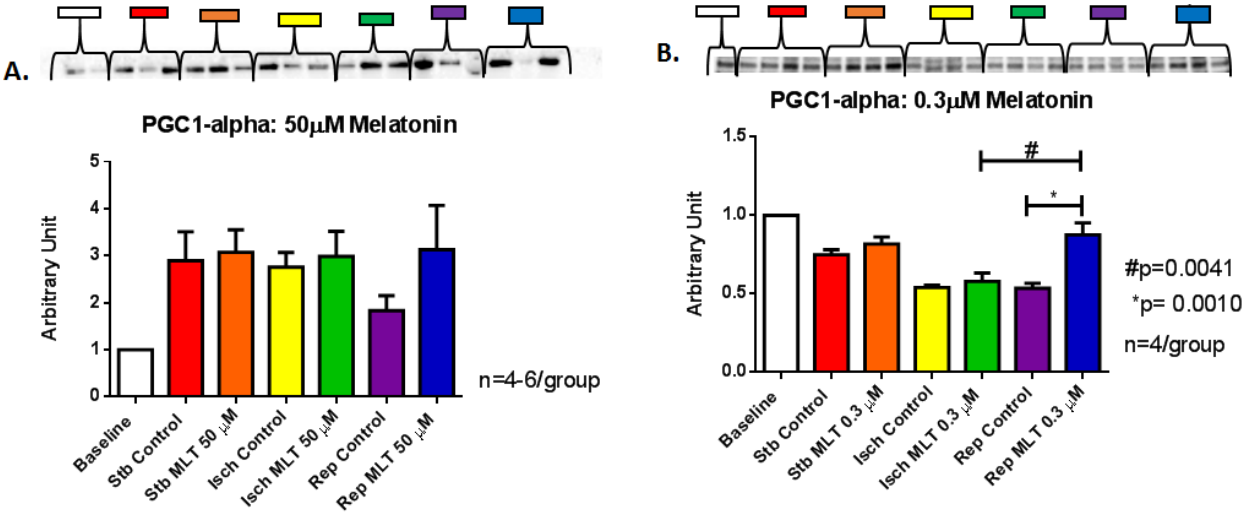



***Figure 42: Expression of PGC-1 alpha in the cytosol of freeze-clamped hearts perfused according to the various global ischaemia protocols. Melatonin groups were treated with either (A) 50µM or (B) 0.3µM of melatonin. Abbreviations: Stb- Stabilisation, Isch- Ischemia, Rep- Reperfusion, MLT- Melatonin***

## Summary: Cytosolic Markers of Mitochondrial Biogenesis

In summary, ischaemia alone and I/R decreased Sirt1 expression. Melatonin decreased Sirt1 expression during stabilization and increased it significantly during reperfusion. Melatonin increased PGC-1 alpha expression significantly during reperfusion only.

# CHAPTER 5: DISCUSSION

The aims of this study were to characterise the effect of I/R *per se* on functional recovery, mitochondrial oxidative phosphorylation and IFS using the working rat heart as experimental model and to correlate these parameters with the mitochondrial mitophagy process. In addition, to further evaluate the contribution of mitophagy to cardiomyocyte survival, we made use of the pineal hormone, melatonin (0.3μM and 50μM), well-known for its cardioprotective properties, to investigate its effects on the mitophagic process in the context of IRI.

The results obtained demonstrated the well-established reduction in mechanical recovery during reperfusion which was associated with a reduction in mitochondrial oxidative phosphorylation particularly during reperfusion. Interestingly, the conventional PINK1/Parkin mitophagy pathway was not upregulated, while an alternative cytosolic pathway which involves ULK, Rab9 and DRP-1 appeared to be affected to a larger degree. Melatonin, which caused a major reduction in IFS, while having no effect on the conventional mitophagy pathway, induced major changes in the alternative pathway for mitophagy. The alternative pathway for mitophagy involves the ULK phosphorylation of Rab9 which leads to DRP-1 phosphorylation and subsequently mitophagy, as recently proposed by Sadoshima and coworkers (Sadoshima 2017, Personal Communication). Since Nishida et al. (2009) also reported an alternative pathway which involves ULK, Beclin 1 and Rab-9 dependent autophagosome formation, it was decided to evaluate Rab9 as a marker of autophagosome formation in the alternative pathway.

## 5.1. Experimental model

The well-characterized working rat heart was used as experimental model throughout the study since it allows correlation of haemodynamic endpoints (which form an important basis for the evaluation of myocardial contractile function) with intracellular processes such as mitochondrial function, signalling and also mitophagy. The effects of different periods of ischaemia and reperfusion on mechanical recovery during reperfusion using this experimental model, mitochondrial function, tissue high energy phosphates and ultrastructure have previously been studied in our laboratory as described in detail by Edoute and coworkers (1983).

Subsequently, for the present study it was decided to use a period of ischaemia which caused contractile dysfunction, but not complete failure during reperfusion and based on our previous experimentation, 20 min global and 35 min of regional ischaemia respectively, fit these criteria.

## 5.2. Myocardial Function: Effect of Global Ischaemia/ Reperfusion and Melatonin

In our model of I/R, exposure of the heart to 20 minutes of normothermic global ischaemia followed by 30 minutes of reperfusion, significantly decreased coronary flow, aortic flow, cardiac output and total work in all groups (Results, **Table 9 and 10**). Furthermore, the control group experienced a reduction in heart rate and peak systolic pressure. These findings are consistent with the knowledge that I/R impairs myocardial contractile function (Buja, 2005). Impairment of function during reperfusion can be attributed to the no flow phenomenon (Kloner et al. 1974), myocardial stunning (Braunwald and Kloner, 1982; Bolli, 1990 ), reperfusion arrhythmias (Scherlag et al. 1986) and reperfusion injury (Murry et al. 1986) caused by intracellular $Ca^{2+}$ overload. Intracellular $Ca^{2+}$ overload can induce the activation of nucleases, phospholipases as well as proteases such as calpains which not only degraded cellular and mitochondrial proteins but impair contractile machinery (Buja et al. 1988; Croall and Erfeld 2007). In our model the reduction in coronary flow during reperfusion and concomitant stunning appears to be largely responsible for the very significant reduction in cardiac output and work performance. The contribution of stunning in this model, was the subject of investigation previously. The study showed that treatment with isoproterenol during reperfusion restored heart function, which shows that the contractile machinery is intact (Lochner et al 1999) but impaired upon reperfusion by concomitant stunning.

In the present study the effects of melatonin on functional recovery varied from time to time: in the first series of experiments (Results, **Table 9**), melatonin at 50 µM improved functional recovery after 20 min global ischaemia. However this was not seen in subsequent experiments where both concentrations of melatonin did not significantly improve the parameters of function evaluated. In fact melatonin at 0.3µM caused a reduction in function after regional ischaemia, when compared to untreated controls. A reason for this discrepancy is not readily available, except sample/

population variation (Results, **Table 10**). Despite the fact that all the rats are from the same species, weight and sex, being subjected to the same experimental conditions, they do not always exhibit the same response even under stringently controlled perfusion conditions. In our previous experiments melatonin treatment also had no significant effects on functional recovery, except in hearts from animals which were treated with melatonin for 18 weeks prior to experimentation (Nduhirabandi et al. 2011). The slight effects of melatonin on myocardial contractile function as well as recovery in this model of ischaemia/ reperfusion could possibly be attributed to the duration of melatonin administration. A study by Dobsak and colleagues (2003) showed that melatonin administration throughout the entire 45 minutes of reperfusion, after 30 minutes of normothermic global ischaemia, resulted in 93% functional recovery. Thus further studies are required to clarify whether there is a direct relationship between the duration of melatonin administration and myocardial performance and recovery.

### 5.3. Myocardial Function and Infarct Size: Effect of Regional Ischaemia/Reperfusion and Melatonin

For further evaluation of the effects of melatonin on the ischaemic/reperfused heart, IFS was determined after exposure of the hearts to 35 minutes of normothermic regional ischaemia and 60 minutes of reperfusion. Regional ischaemia, similar to global ischaemia, was associated with impaired myocardial function during reperfusion which was characterized by a significant reduction in coronary flow, aortic flow, cardiac output, peak systolic pressure and total work in both untreated and melatonin treated groups ( Results, **Table 10**). This model once again echoes the detrimental effects of ischaemia/ reperfusion on myocardial contractile function. As was also observed after global ischaemia, melatonin at both concentrations did not improve mechanical recovery during reperfusion (Results; **Table 10**)

Interestingly, in this model of I/R melatonin at 50μM and 0.3μM resulted in a significant reduction of IFS, which is consistent with existing evidence (Results, **Fig 25 B**; Lagneux et al. 1999; Shana et al. 2005). The dosage administered did not affect the outcomes, with 0.3μM being as effective as 50μM melatonin in reducing IFS (Results; **Fig 25** B).The decrease in IFS supports the evidence that myocardial

ischaemia/reperfusion with a cardioprotectant can decrease lethal reperfusion injury (Yellon and Hausenloy, 2007). Interestingly, this reduction in IFS was not correlated with enhanced myocardial function or recovery (Results; **Table 10**). Evidence from several studies (Cohen et al. 1999; Lochner et al. 2003) have shown that IFS reduction is not necessarily directly related to improved myocardial function. Ibáñez and colleagues (2015) also agree with the above, stating that haemodynamic endpoints influence IFS only to a limited extent.

The data obtained in this study again demonstrate the potent cardioprotective properties of melatonin against IRI. What makes this more remarkable is the fact that this was obtained with high pharmacological doses as well as very low dosages (50 and 0.3µM, Results; **Fig 25** A-B respectively). These results confirm the data reported by Lamont et al. (2011) using even lower concentrations of melatonin (75ng/ml). These beneficial actions of melatonin are known to be receptor dependent with its anti-oxidant actions probably playing an important role. However recent evidence showed that a multitude of signalling pathways are activated by melatonin, most of which are involved in cardioprotection (Lochner et al.; submitted for publication). Subsequently, as pointed out in the motivation for the present study, it deems necessary to explore the effects of melatonin on the mitophagic process in the context of IRI.

## 5.4. Oxidative phosphorylation capacity: Effect of Ischaemia/ Reperfusion and melatonin

It is well-established that the mitochondrial oxidative phosphorylation process plays an important role in the supply of energy for the continuously contracting myocardium and that any interruption in the supply of oxygen to the cardiomyocyte has severe consequences for cell survival. It is also well-known that the mitochondria are implicated in the pathophysiology of various diseases including myocardial IRI.

Analysis of Oxphos in the setting of ischaemia and reperfusion will allow us to effectively assess the mitochondrial oxidative phosphorylation potential and evaluate its contribution to the pathophysiology of global IRI in the working heart model. Five endpoints [QO2 (S3), QO2 (S4), RCI, ADP/O and Oxphos] were measured in a carbohydrate (glutamate /_malate) and fatty acid (palmitoyl-L-carnitine / malate) medium (See **Section 3.3.3**). For the present study use was made of the

subsarcolemmal mitochondrial fraction, rather than the intermyofibrillar fraction, since ischaemic damage was shown to occur more rapidly in the former (Lopaschuk et al. 2007). It is also known that complexes I, III and IV are more vulnerable to ischaemic damage, with complex II being more resistant (Lesnefsky 2016).

Ischaemia is associated with nutrient and oxygen deprivation which halts oxidative phosphorylation (Hausenloy and Yellon, 2013) and it was expected to have profound effects on mitochondrial function. Interestingly, in the present study exposure of the heart to 20min global ischaemia alone (without reperfusion) had no effect on any of the parameters of mitochondrial function studied, regardless of the substrate present in the incubation medium( Results; **Figs 26-30** ). In fact, compared to mitochondria isolated directly after the stabilization phase, the data obtained after 20min global ischaemia, shows preserved mitochondrial function. Evidence from various studies has shown that in global ischaemia oxygen levels do not immediately fall to zero (Murphy and Steenbergen, 2008) and this could also be true for nutrient levels. Consequently, oxidative phosphorylation can proceed although it may be for a limited period of time. Another factor to consider is the fact that once mitochondria are isolated, they are incubated under ideal conditions, with sufficient oxygen and substrates available and will only display dysfunction if the damage persist even under ideal isolation and incubation procedures. The danger exist therefore that mitochondrial function may be overestimated under these experimental conditions.

However the finding that 20min global ischaemia had no effect on mitochondrial function is in contrast to other studies in this regard, including our own (Edoute et al; 1983). It is known that mitochondrial damage occurs mainly in the ischaemic period (Papa et al. 1997; Koonen et al. 2007; McLeod et al. 2005 Lopaschuk et al. 2007; Lopaschuk et al. 2010; Fillmore et al. 2014), induced by generation of ROS by the ETC. It is possible that the period of ischaemia was too short to induce sufficient damage since it has been reported that in both animal and human models irreversible cardiomyocyte damage only occurs after 20 minutes of ischaemia (Kalogeries et al. 2012).

Significant changes in mitochondrial function were observed during *reperfusion* namely a significant reduction in QO2 (S3) and (S4), RCI, ADP/O and Oxphos in one

or both of the substrates (Results; **Figs 26** A; **27** A-B; **28** C; **29** C; **30** A-B). It is well established that the detrimental changes that occur during ischaemia are escalated upon reperfusion due to the generation of ROS during early reperfusion (Lopaschuk et al. 2010; Fosslien 2001). This is consistent with the evidence that I/R leads to the reduction of mitochondrial bioenergetics parameters ( Petrosillo et al. 2003; Paradies et al. 2004; Lesnefsky et al. 2004; Petrosillo et al. 2006) which was partially attributed to a loss of mitochondrial cardiolipin and increased peroxidation of this phospholipid (Petrosillo et al. 2006, 2009). The effect of I/R on mitochondrial function, is however, also controversial, since Petrosillo and coworkers (*op.cit.*) reported an increase in mitochondrial QO2 (S3) and (S4) after I/R. These discrepancies could be due to the perfusion models used (retrograde vs working heart), periods of ischaemia and reperfusion, mitochondrial isolation and incubation media. The finding that tissue ATP content is not increased by reperfusion after ischaemia (Edoute et al. 1983; Moolman, 1994), also indicate impaired mitochondrial oxphos upon reperfusion. As previously mentioned, oxygen levels in a global ischaemia model do not immediately fall to zero and while this may preserve mitochondrial function during ischaemia, it also creates the ideal environment for the sub-lethal production of ROS which becomes lethal upon reperfusion (Zweier et al. 1987; Van den Hoek et al. 1997; Becker et al. 1999). These lethal levels of ROS result in damaged and dysfunctional mitochondria that produce 10-fold the amount of ROS and have reduced mitochondrial oxidative phosphorylation capacity (Twig et al. 2008; Jimenez et al. 2014; Ikeda et al. 2015). Interestingly in this study, the decrease in QO2 (S3) and (S4) in the glutamate/malate medium (Results, **Fig 26** A**; 27** A) was not associated with a decreased RCI (Results; **Fig 28** A), suggesting that the mitochondria are still coupled.

Effects of melatonin: Melatonin's effects on mitochondrial function appears to be minimal. Melatonin had no significant effect on QO2 (S3), (S4) and the ADP/O. Mel at 0.3μM did however exert effects during stabilisation and reperfusion, where it decreased the RCI during stabilisation and increased the Oxphos during reperfusion in comparison to its corresponding control counterparts (Results, **Fig 30**).

These results are surprising: in view of melatonin's free radical scavenging and anti-oxidant potential, it was expected to improve mitochondrial function, particular during reperfusion when the main generation of ROS occurs. As far as we are aware, this is

the first demonstration of melatonin being able to reduce mitochondrial oxygen uptake during reperfusion. Petrosillo and coworkers (2006) reported that melatonin induced a marked increase in mitochondrial respiration after exposure of hearts to I/R which was attributed to preservation of the integrity of mitochondrial cardiolipin (Petrosillo 2006, 2009). Petrosillo and coworkers (2009) suggested that these changes in cardiolipin may inhibit opening of the MPTP and loss of cytochrome C. It is clear that the results obtained in the present study are in direct contrast to those obtained by Petrosillo and his team, using a retrogradely perfused heart model and different periods of I/R. Recently, there has been evidence of melatonin exhibiting pro-oxidants in various cell types at various concentrations in certain conditions, although this has not been studied in I/R or in cardiomyocytes. This possibility cannot be completely excluded. It is clear that these discrepancies need to be further investigated.

In summary, in the present study the poor mechanical recovery of the hearts during reperfusion after exposure to I/R was associated with decreased oxidative phosphorylation capacity. In contrast to its marked cardioprotective effects, as evidenced by the significant reduction in IFS, melatonin either preserved or resulted in marginal improvements in myocardial function and recovery, while having no beneficial effect on mitochondrial oxidative phosphorylation after exposure to ischaemia /reperfusion. It can be concluded that the cardioprotective effects of melatonin clearly was not associated with improvement in mitochondrial oxphos capacity.

### 5.5. Mitophagy: effects of ischaemia/reperfusion and melatonin

It is well-established that the mitochondria are key regulators of cell fate, controlling survival (via ATP production which fuels cellular processes) and conversely, cell death. It is thus essential to have stringent control mechanisms regulating the quality of mitochondria to avoid the pathological effects of dysfunctional mitochondria. The mitochondrial quality control cycle involves a dynamic cycle of fission, fusion, mitophagy and biogenesis (for a detailed review see Gottlieb and Gustaffson, 2011; Anzell et al. 2017). It is therefore expected that stress conditions such as acute I/R with its known effects on mitochondrial ultrastructure (for example swelling, disruption of cristae) should affect the removal of dysfunctional mitochondria by mitophagy. However, to date the role of mitophagy in the setting of I/R is poorly understood.

Furthermore, as far as we are aware, no information is available regarding the effects of melatonin on the mitophagy process.

Thus far, three mitophagic pathways (the so-called conventional pathways) have been described namely PINK/Parkin, BNIP3/NIX, FUNDC1 (see Chapter 2), while cardiolipin may also be involved (Anzell et al. 2017). Recently another pathway (the so-called unconventional or alternative pathway) involving AMPK, ULK, Rab9 and DRP-1 received much attention. For the purpose of the present study, it was decided to first evaluate the conventional pathway using the expression of mitochondrial markers such as PINK1, Parkin, TOM70, p63/SQSTM1and Sirt3 as markers in hearts subjected to ischaemia and reperfusion. For evaluation of the unconventional pathway, ULK1, Rab9, and DRP-1 expression were evaluated. In addition, we also evaluated mitochondrial biogenesis, using PGC-1 alpha and Sirt1 as markers.

### 5.6.1. Mitochondrial Markers

#### 5.6.1.1. PINK1/PARKIN Pathway: Effect of global ischaemia and reperfusion

In contrast to expectations of upregulation of mitophagy (due to the reduction in mitochondrial QO2 (3) and (S4) observed in reperfused hearts), ischaemia *per se* as well as reperfusion were found to be associated with decreased PARKIN and TOM70 expression levels, while PINK1 was reduced after I/R only (**Fig 32** B**; 34** B**, 33** A). SQSTM1/p62 levels were changed by I/R (Results, **Fig 35** A -B).

It is well-established that PARKIN and PINK1 play an important role in regulating mitochondrial function and mitophagy and loss of either protein can result in detrimental changes in mitochondrial structure and function (Narenda et al 2008; Gottlieb and Gustafsson, 2010). These changes include suboptimal mitochondrial respiration as well as oxidative damage (Gautier et al. 2008; Kubli et al. 2013). It has also been shown that during cellular stress (such as in ischaemia) PINK1 accumulates on the depolarized mitochondrial outer membrane and results in the increased expression as well as recruitment of PARKIN from the cytosol. PARKIN recruitment in turn leads to the ubiquitination of various proteins and recruitment as well as assembly of the autophagosome by SQSTM1/p62 (see Chapter 2; Narendra et al. 2010; Calo et al. 2013). In view of these events, we expected mitophagy to be upregulated after I/R.

The decreases in PINK1, Parkin and TOM70 expression suggest that instead of mitophagy being activated by the stressful stimuli in this setting, it was impaired by both ischaemia and particularly during ischaemia/ reperfusion. This could be attributed to the fact that certain processes in autophagy such as autophagosome formation require ATP production (Sciarretta et al. 2011), which is severely depressed during global ischaemia.

Although PINK1 is highly expressed in the heart (Unoki and Nakamura, 2001), the role of PINK1 in this organ is not yet clear (Siddall et al. 2008a; Siddall et al. 2008b ). Using mice overexpressing PINK1 as well as knockouts, Siddall and coworkers (2013) showed that loss of this protein increases the vulnerability of the heart to IRI, probably due to mitochondrial dysfunction. However, the importance of PINK1 in mitophagy has been questioned since Parkin was shown to be able to function without PINK1 (Kulbi et al. 2015).

The TOM complexes have been suggested to be involved in mitochondrial biogenesis and bioenergetics to meet metabolic demands under stress. TOM70 has been shown to be essential for importing PTEN –induced kinase 1 (PINK1) into mitochondria, reducing myocardial vulnerability to ischaemic injury (Kato et al. 2013). TOM70 was also reported to suppress oxidative stress in myocardial hypertrophy (Li et al. 2014). However, in the model of the reperfused working rat heart, TOM70 expression was reduced lending support to the notion that myocardial I/R suppresses mitophagy.

As far as we know, this is the first demonstration of a reduction in mitophagy (as indicated by the mitochondrial PINK1, Parkin, TOM70) occurring simultaneously with a decrease in mitochondrial oxygen uptake during reperfusion. Since PINK1/PARKIN mediated mitophagy is mainly associated with depolarized mitochondria, it is possible that the 20 minute ischaemic period was too short to elicit a more significant upregulation of mitophagy.

Whether failure to upregulate mitophagy contribute to the loss of contractile function during reperfusion, remains to be established. Unpublished data by Gottlieb reported that overnight fasting in mice decreased mitochondrial content by 50%, suggesting that mitophagy is a very rapid process (Gottlieb and Gustafsson, 2010). Clearly more work is required to elucidate the contribution of the mitophagy process, as indicated

by the PINK1/Parkin pathway, to events known to occur in the ischaemic/reperfused myocardium. Coupling Western Blotting and the analysis of mitophagic flux through techniques such as mRNA and fluorescence markers (Gottlieb et al. 2015) could better elucidate our findings since Western Blotting provides only snap shots of very dynamic cellular processes.

#### 5.6.1.2. Alternative pathway: Effect of ischaemia and reperfusion

Indications are that the non-conventional or alternative pathway of mitophagy is also affected by exposure of the heart to I/R: the cytosolic pDRP-1 as well as pDRP-1/totalDRP-1 ratio were lowered by both ischaemia and I/R, suggesting a reduction in mitophagy (Results; **Figs 40** A and C). Interestingly, a very similar pattern was seen in the mitochondrial fractions, namely a lowering in phospho, total and pDRP-1/total DRP-1 ratio (albeit not significantly) by ischaemia and reperfusion. The significant loss of mitochondrial Rab9, as well as total ULK1 (phosphorylation unchanged) proteins occurring in hearts exposed to I/R, also suggest downregulation of mitophagy and strengthens the premise that autophagy/mitophagy is depressed during I/R (Results; **Fig 37-38**). Thus the data suggest that mitophagy during ischaemia as well as during reperfusion is reduced, albeit to a limited extent.

ULK protects cells from starvation and promotes survival by autophagy induction (Russell et al. 2013). Our data showed that total ULK was decreased by ischaemia and I/R. The changes in total ULK were reflected by an increased phosphorylated/total ULK ratio during ischaemia and a decreased ratio during reperfusion. This suggests that ischaemia was associated with increased ULK activation while conversely, reperfusion was associated with decreased activation (Results; **Fig 38** A and C). This is consistent with the knowledge that ULK is phosphorylated by AMPK during nutrient deprived conditions (such as in ischaemia) and inactivated by mTOR during nutrient rich conditions (Kim et al. 2011). Inactivation of pULK during reperfusion may be attributed to the restoration of nutrients as well energy. However, AMPK has been shown to remain activated during reperfusion (Hermann et al. 2017).

Cytosolic Rab9 was increased by ischaemia and decreased by reperfusion (**Fig 38 B**). It is expected that phosphorylated Rab will follow a similar pattern, but the appropriate phosphorylated Rab antibodies were not available commercially. The increased in

total Rab could suggest that ischaemia was associated with the accumulation of matured autophagosomes while reperfusion would be associated with clearance. This would be consistent with evidence from Hirota and colleagues (2015) which showed that knockdown of Rab9 suppressed mitophagy but not macroautophagy. In addition, Rab9 is required for autophagosome maturation (Amaya et al. 2015) thus increased expression could be indicative of accumulating maturing autophagosomes in this setting.

Furthermore, the significant reduction in the expression of mitochondrial Sirt3 is also an indicator of disrupted autophagy/mitophagy regulation (Results, **Fig 36**). Evidence suggests that Sirt3 is associated with metabolism, protection from apoptosis, improved ETC function, enhanced endogenous antioxidant defences and autophagy regulation (Tao et al. 2010; Rardin et al. 2013). SIRT3 also protects against oxidative stress related diseases, including myocardial IRI (Porter et al. 2014; Botchaton et al. 2015; Klishadi et al. 2015; Zhai et al. 2017). This sirtuin acts mainly through deacetylation of mitochondrial proteins, including cyclophilin D (CypD), Ku70, and complex I, independently of transcription. Sirt3 moreover deacetylates FOXO3, leading to transcriptional upregulation of MnSOD and catalase and inhibition of cardiac hypertrophy (Daitoku et al. 2004; Zhai et al. 2017). A reduction in its expression in mitochondria could therefore only have harmful effects on the outcome of myocardial I/R.

Recent observations by Sadoshima and his group suggested that the unconventional pathway, initiated by ischaemia-induced activation of AMPK and ULK1, is a major form of mitophagy and more important than the PINK1/Parkin pathway and that particularly during stress this alternative pathway becomes more important (Sadoshima et al 2017, personal communication). Rab9, which has a wide range of functions (Lombardi et al. 1993), has also been suggested to be associated with non-canonical autophagy (Codogno et al. 2012) as well as mitophagy (Amaya et al. 2015). Our results also confirm a role for the alternative non-conventional pathway in I/R. Whether the changes observed have an effect on mitochondrial fission remains to be further evaluated. In addition, the significance of these changes and whether they contribute indirectly to the impaired mitochondrial function and loss of contractile function during reperfusion, remain to be established.

### 5.6.2. Effect of Melatonin on mitophagy during global Ischaemia/ Reperfusion

The results thus far suggested that I/R reduced both mitochondrial oxidative function and the removal of damaged mitochondria by mitophagy. In view of the marked cardioprotective effects of melatonin and the fact that it is a scavenger of ROS which, in turn, is required to initiate mitophagy (Scherz-Shouval and Elzar, 2007), it was decided to evaluate the effects of melatonin treatment on the mitophagic process. Interestingly, melatonin decreased PARKIN, TOM70, Sirt3 and Rab9 expression during the stabilization period (Results**; Figs 32** B**, 34** A**, 36,** A**, 38** A-B), the mechanism of which is still unknown. It could be due to the free radical scavenging effects of the hormone. During I/R it decreased SQSTM1/p62 levels and had no effects on PINK1 expression. Considering the functions of these proteins as described above, these findings suggest that melatonin had very little effect on the PINK1/Parkin pathway during I/R. While ROS is damaging, it is required in small amounts at basal levels (such as during stabilisation) and in stressful conditions (such as in I/R) for the induction of autophagy/mitophagy (Scherz-Schouval and Elazar, 2007). Thus melatonin's free radical scavenging properties could have removed most if not all the cellular and mitochondrial ROS that was generated and subsequently, having a little effect on mitophagy.

Although our results did not show an effect of melatonin on mitochondrial TOM70 expression after exposure of the heart to I/R (Results; **Figs 35** A**)**, melatonin *pretreatment* of the mice (10mg/kg/day) was able to increase expression of PGC-1 alpha as well as TOM70 and attenuated myocardial injury in control, but not in TOM70 deficient mice. These actions could be abolished by luzindole, indicating melatonin receptor dependence of TOM70 effects (Pei et al. 2017). The links between the melatonin receptor, PGC-1 alpha and TOM70 need to be further investigated. However these observations suggest that TOM70 and its ability to regulate oxidative stress, were also dependent on the experimental conditions and need to be further examined. However the results obtained by Pei et al. (*ibid*) provided evidence that TOM70 and its ability to regulate oxidative stress, were critical to the observed beneficial effects of melatonin.

Despite the ROS scavenging actions of melatonin and the negative effects observed with the PINK1/Parkin pathway, our results suggested that melatonin does affect

mitophagy and that it exerts its most marked effects on the *alternative mitophagic* pathway: melatonin treatment caused a significant upregulation of total ULK throughout the perfusion protocol at all-time intervals studied while phospho-ULK was significantly elevated throughout the stabilisation and reperfusion intervals studied (Results, **Fig 37-B**).

In the present study similar striking changes were observed with DRP-1 (see Results **Fig 39 and 40**). It is known that under basal conditions most of DRP-1 is found in the cytosol while the remaining portion is associated with the OMM (Smirnova et al. 2001). When DRP-1 is activated (under basal and stressful conditions), it translocates to the OMM (Varadi et al. 2004). DRP-1 is known to be involved in mitochondrial fission which is an essential stimulus for mitophagy (Smirnova et al. 2001). The data obtained in the present study showed that melatonin significantly affects not only the expression of, but also the distribution of DRP-1 during I/R. It significantly increased phosphorylation (and thus activation) of cytosolic DRP-1 at the end of stabilisation, ischaemia and after reperfusion; in contrast melatonin treatment caused a significant reduction in mitochondrial pDRP-1 as well as in the pDRP-1/total DRP-1 ratio (See Results**, Figs 39** B-C). These results are difficult to explain: while the significant increase in pULK suggest upregulation of the alternative pathway, this was associated with a significant increase in cytosolic, but not mitochondrial pDRP-1, which is an indicator of mitochondrial fission. It is possible that phosphorylation of cytosolic DRP-1 precedes its translocation to the mitochondria, but this remains to be demonstrated. As far as we know, this is the first demonstration of stimulation by melatonin of the alternative pathway and may indicate a novel mechanism for melatonin-induced cardioprotection.

Melatonin's beneficial effects were also seen during I/R where it upregulated Sirt3 expression. Sirt3 is an essential mitochondrial deacetylase which regulates mitochondrial function and biogenesis via the modulation of various acetyl-lysine containing proteins. The effect of the hormone on Sirt3 was previously investigated by Zhai and coworkers (2017) who used a model of *in vivo* coronary artery ligation as well as H9c2 cells, with 3-TYP as selective Sirt3 inhibitor. Melatonin pretreatment improved postischaemic function, reduced IFS and ameliorated oxidative damage and reversed the IRI induced reduction in Sirt3 expression and activity. These changes

were associated with a reduction in the acetylation of SOD2 which was largely abolished by the Sirt3 inhibitor 3-TYP. In addition, Sirt3-targeted siRNA abolished the beneficial effects of melatonin in H9c2 cells subjected to simulated I/R, lending support to their suggestion that melatonin ameliorates I/R induced oxidative stress by activating the Sirt3 pathway. It was also found that melatonin reduces the translocation of Bax to the mitochondria via activation of Sirt1, indicating that the protective effect of the hormone against I/R induced apoptosis is mediated, at least in part, by Sirt3 signalling. Increases in Sirt3 are also associated with increased transcription of SOD and catalase (Tao et al. 2010; Rangarajan et al. 2015) as well as increased oxidative phosphorylation (Cimen et al. 2010).

### 5.6.3. Effect of Melatonin on mitochondrial biogenesis

Melatonin resulted in decreased Sirt1 expression during stabilisation and ischaemia which was increased significantly during I/R (Results, **Fig 41 A**). Involvement of Sirt1 in IRI has previously been shown by the observation that Sirt1 deficient mice exhibit increased injury in response to I/R while cardiac damage was reduced in Sirt1 transgenic mice (Hsu et al. 2010). In addition, upregulation of Sirt1 (Hsu et al. 2010; Tong et al. 2013) signalling has been shown to protect against IRI. The role of Sirt1 in melatonin-induced cardioprotection was further investigated by Yu and coworkers (Yu et al. 2014), using a model of *in vivo* coronary artery ligation after seven days of melatonin administration (10mg/kg/day) to rats, with or without co-administration of luzindole and EX527, a Sirt1 inhibitor. The results showed the expected melatonin-induced reduction in I/R damage, associated with increased Sirt1 and BcL2 expression and a reduction in BAX and caspase 3 cleavage by melatonin. The results furthermore suggested that melatonin-induced cardioprotection involved activation of Sirt1 which may, via decreased acetylation of FOXO1, promote anti-apoptotic signalling in the heart.

In the present study increased Sirt1 expression was associated with elevated PGC-1 alpha levels during reperfusion (Results, **Fig 42** B). In this setting Sirt1 upregulated the deacetylation of PGC-1 alpha consequently stimulating mitochondrial biogenesis (Houtkooper et al. 2010). In addition, Sirt1 upregulation is associated with protection against apoptosis and regulation of autophagy (Sengupta et al. 2009; Cattelan et al. 2015)

Mitochondrial biogenesis and mitophagy are intimately linked (Stotland and Gottliebb, 2015). The data obtained in the present study confirm that pretreatment with melatonin upregulates mitochondrial biogenesis and that this occurred concomitantly with upregulation of the alternative pathway of mitophagy.

In summary, the above results propose a novel mechanism for melatonin-induced cardioprotection. Further proof for this proposal however, should be obtained using appropriate experimental approaches such as knockout models.

# CHAPTER 6: CONCLUSION

The incidence of deaths due to CVD and more specifically ischaemic heart disease has increased substantially over the years. Evidence shows that 80% of CVD deaths occur in low /middle- income countries (LMIC e.g. SA) and they also occur at a younger age in comparison to higher income countries (e.g. United States of America) (WHO, 2011). In addition, economists predict that not investing in CVD prevention and treatment over the next few years will result in economic losses estimated at $47 trillion globally and $3.64 trillion at the LIMC level (Laslett et al. 2012). This loss can be prevented and the burden of CVD reduced with the implementation of feasible and cost effective cardioprotective interventions (WHO, 2011). In the present study we investigated the feasibility of using melatonin, an inexpensive and in many countries an over-the-counter product, for the management of I/R damage by targeting mitophagy in the hearts of male Wistar rats.

Subsequently, the overall aims of this study were to characterise the effect of ischaemia as well as reperfusion on mitochondrial oxidative phosphorylation and mitophagy and to evaluate their significance in myocardial IRI. Using the isolated working rat heart as model, the results demonstrated that (i) exposure of the isolated rat heart to I/R caused a significant reduction in contractile function during reperfusion (Results, **Tables 9-11**) (ii) the most significant change in mitochondrial oxidative phosphorylation was a reduction in oxygen uptake (States 3 and 4) which occurred during *reperfusion* (Results, **Figs 26** A, **27** A-B,) (iii) contrary to expectations, both the conventional ( Results, **Figs 32-36**)as well as alternative ( Results, **Figs 37-40** ) pathways of mitochondrial mitophagy were inhibited partially, suggesting impaired fission during I/R (iv) the changes in mitophagy occurred simultaneously with the reduction in mitochondrial States 3 and 4 respiration.

The role of ROS in I/R damage was evaluated using the well-established ROS scavenger and anti-oxidant, melatonin. As has been shown previously in our own as well as in other laboratories, melatonin's potent cardioprotective actions were confirmed by the significant reduction in IFS, measured after exposure of the heart to 35 min regional ischaemia/60min reperfusion. Although melatonin had no major effects on mitochondrial oxidative phosphorylation function, the results showed

marked effects on the alternative pathway of mitophagy ( Results, **Figs 26-30**), as well as mitochondrial biogenesis ( Results, **Figs 41-42**). As far as we know, this is the first demonstration of this particular effect of melatonin.

Finally, the role of mitophagy in the setting of I/R is still poorly understood and it is not known whether manipulation of this process could play a role in cardioprotection. The major changes induced by melatonin are associated with a reduction in IFS, but whether they play a causal role, remains to be established.

**Strengths, Limitations and Future recommendations**

Although much information is available regarding the process of mitophagy and the different pathways involved (for recent reviews see Saito and Sadoshima, 2015; Gottlieb and Thomas, 2017), not much is known about this process in I/R.

It is recognized that the present study has a number of limitations. Firstly, although the aims were to evaluate the effects of ischaemia and reperfusion on the mitochondrial mitophagy process and to correlate this with mitochondrial function, this approach proved to be more difficult than realized initially. Since the most significant changes in mitochondrial oxidative phosphorylation and mitophagy occurred during reperfusion, co-incidence does not necessarily indicate causality.

Despite these shortcomings, the data obtained gave a good indication of the effects of ischaemia *per se* on mitochondrial mitophagy and the effects of subsequent reperfusion on this process. Most articles in the literature focussed on the combined effects of I/R on this process. Our approach certainly has added to current knowledge regarding the effect of ischaemia on mitophagy and its association with mitochondrial oxidative function. However, it is realized that the signalling pathways associated with mitophagy are complicated with the many participants in the process. The newly proposed non-conventional or alternative pathway further complicated the experimental approach. Clearly by looking at more intermediates in both pathways would certainly have given more insight into the exact sequence of events. Another complicating factor in a study of this kind is the fact that by freeze-clamping the heart at fixed time intervals only gives a snapshot of events at that particular time, but does not give an indication of flux. Gottlieb and co-workers (2016) have suggested that

pretreatment of hearts with chloroquine before exposure to I/R would give a better indication of flux. Such studies are in progress.

Another unexpected complicating factor was that exposure of the heart to 20 min global ischaemia caused less damage to mitochondrial function than described before (see for example Edoute et al. 1986). It is clear that to fully appreciate the effects of I/R on mitochondrial function and mitophagy, more and longer periods of ischaemia have to be studied, in combination with varied periods of reperfusion, both in the absence and presence of chloroquine pretreatment. In addition, it would be helpful to also evaluate autophagy as well under these conditions. It will also be interesting to evaluate the role of the mitochondrial phospholipid cardiolipin in the mitophagy process. Previous work by Petrosillo and coworkers (*op. cit.*) showed not only involvement of this phospholipid in mitochondrial damage, but also the beneficial effects of melatonin treatment. Clearly a topic for further study.

The finding of the effects of melatonin on the alternative pathway of mitophagy was one of the highlights of the study and will certainly form the basis of further studies in this regard. Whether these events have any effects on the myocardial response to I/R and whether they can be manipulated, may lead to discovery to new cardioprotective strategies.

# APPENDIX A

**Preparation of 50µM of Melatonin**

To prepare 500mL melatonin solution with a concentration of 50µM the formula together with the information below was used to obtain the mass of melatonin to be weighed off (m):

Molecular weight (Mw) of melatonin: 232.28

Volume to be prepared (v): 0.5L

Concentration to be prepared: 50µM which is $50x10^{-6}M$

$m = cxvxMr$

$m = 50x10^{-6}\ x\ 0.5\ x\ 232{,}28$

$m = 0.005807g$

$m = 0{,}006g\ melatonin.$

6mg of melatonin was weighed and dissolved in 250µL of absolute ethanol. The solution was made up to 500mL with KHB.

For preparation of 0.3µM melatonin 0.075g of melatonin was weighed off and dissolved in 1mL of ethanol. This solution was poured into a 1000mL volumetric flask and diluted into 999mL of distilled water. 10µL from the stock was then diluted in 9.99mL of distilled water (dilution 1). 500µL of the first dilution (dilution 1) was diluted in 499.5mL of Krebs buffer and use for perfusions.

Since melatonin is light sensitive, it was weighed in a dark room and all containers used to prepare and administer the melatonin were covered in foil. Melatonin was prepared fresh daily.

# APPENDIX B

## Making up ADP

0.02g of ADP was first dissolved in 5mL distilled water. Next, 0.1mL of the ADP solution was diluted in 25mL of distilled water using a volumetric flask (dilution 1:250 dilution) to be used for the spectrophotometric determination of the concentration.

The Helios Ultra violet spectrophotometer (Unicam) was used to determine the OD of the diluted ADP solution in a quartz cuvette at 259nM. Distilled water was used as a blank. The OD was recorded and the ADP concentration determined using the molar extinction coefficient of ADP at 259nM(15.4)

**nmoles of ADP/0.1mL** = $\frac{Recorded\ OD}{15.4} \times \frac{250\ (ADP\ dilution\ factor)}{1}$

## Making up 10x ADP

To make 10XADP ten times the amount of ADP was used i.e. 0.200g of ADP was dissolved in 5mL distilled water. The exact concentration of the 10xADP solution was not calculated as it was not used to calculate any of parameters used for mitochondrial function.

# APPENDIX C

## Mitochondrial Calculations

As previously mentioned the image below is a typical Oxygraph reading at the end of the experiment.

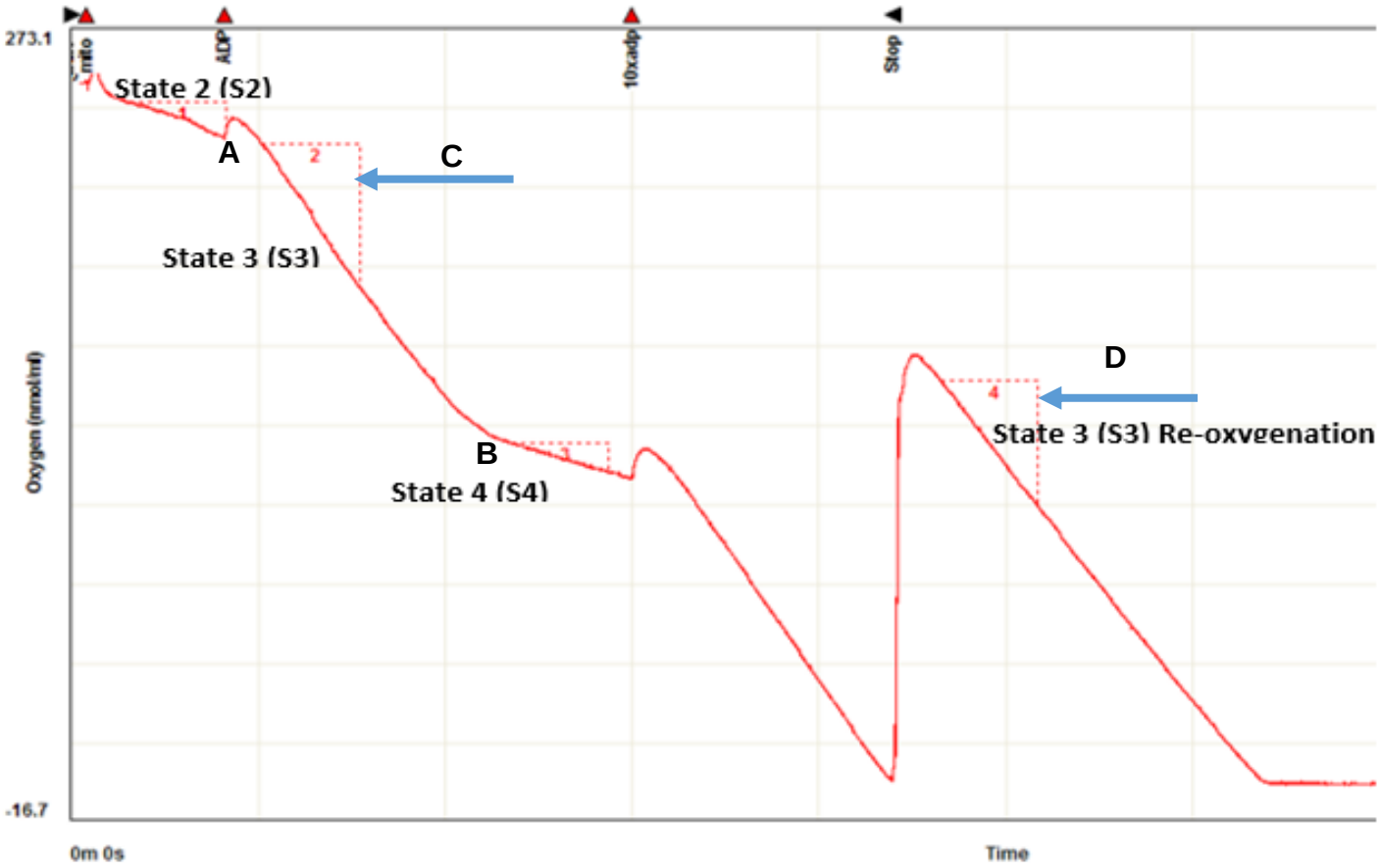



*Figure 43: Oxygraph Recording*

## ADP/O ratio

To calculate the ADP/O ratio the numbers from point A and B on the curves would be used. These numbers are displayed as one hovers over the points with the arrow. Point A and B represent the oxygen levels at the start and end of S3 respectively. To give an example of how the values would be calculated let us assume that point A= 233.5nmol/ml/min and point B= 83.7nmol/ml/min and that the ADP concentration which was calculated using the formula form **Appendix B** was determined to be 379.06 nmoles of ADP/50µL. The ADP/O ratio would be calculated as follows:

# APPENDIX D

## Mitochondrial Calculations (continued)

$Oxygen\ uptake\ during\ State\ 3: 233.5 - 83.7 = 149.9\ nmoles/mL$

$149.8x\ 0.8 = 119.84\ nmoles/800\mu L$ (This step converts the answer to nmoles/800µL; the Oxygraph chamber during S3 contains a volume of 800µL which consists of 650µL incubation medium, 100µL of the isolated mitochondria and 50µL of ADP)

$119.84x2 = 239.68\ nAtoms\ of\ oxygen$

$ADP/O\ ratio = 379.06 \div 239.68$

$ADP/O\ ratio = 1.58\ nmoles\ ATP/nAtom\ of\ oxygen\ consumed$

## $QO_2$ (State 3)

To calculate the QO2 (State 3) one would use point C which is the rate of oxygen consumption measure during state 3 respiration (**Fig** 44). Using the mouse and the rate option on the Oxygraph you draw in the red slope. The software will then determine the rate of C. For this example let us assume that C= 22.33 nmol/ml/min ( **Fig 45**; highlighted in orange).

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 1 | | | | QO2 state 3: oxygen taken up as natoms oxygen/mg prot/min | | | | | |
| 2 | | | | | | | | | |
| 3 | | | | | | | Stage 3 $O_2$ | | |
| 4 | Sample | Perfusior | Medium | mg Protein (800µL) | µg Protein (800µL) | µg Ptotein (mL) | O2 nmol/ml/min | O2 nmol/mg/min | nAtoms O2/mg/min |
| 5 | B5 | REP- 80 | GLU+MA | 0.194 | 194.48 | 243.10 | 22.33 | 91.86 | 183.71 |

***Figure 44:Example of QO2 (S3) calculation on an Excel Spreadsheet.***

# APPENDIX E

**Mitochondrial Calculations (continued)**

Mitochondrial oxygen uptake is expressed as nAtoms oxygen uptake/mg protein in 100µl mitochondrial sample/ min..

**$QO_2$ (State 4)**

To calculate the $QO_2$ (State 4) the same procedure and calculation applies as for the $QO_2$ (State 3). The primary difference is that point D from the Oxygraph recording of the mitochondrial sample will be used and the final answer will be expressed as the nAtoms oxygen uptake in the absence of ADP/mg mitochondrial protein/min.

**Respiratory control index (RCI):**

Once the $QO_2$ (S3) and $QO_2$ (S4) have been calculated the two values can be used to calculate the RCI. The RCI is calculated according to the formula below which was shown previously:

$$RCI: QO_2\,(S3) \div QO_2\,(S4)$$

**Oxidative phosphorylation rate**

To calculate the oxidative phosphorylation rate the ADP/O ratio as well as the $QO_2$ (State 3) have to be calculated. The oxidative rate is as previously mentioned is calculated as follows:

$(\,QO_2\,(S3)\;X\;ADP/O)$ nmoles ATP produced /mg mitochondrial protein/min

# APPENDIX F

## BSA Stock Concentration Formula

As previously mentioned the BSA stock was prepared by dissolving 0.5g of BSA in 10 mL of distilled water. 50µL of the stock solution was diluted in 5mL of distilled water (1:101). The Heλios Ultra violet spectrophotometer (Unicam) was used to read the OD at 280nm using quarts cuvettes and distilled water as a blank. The BSA stock concentration (to be referred to as $p$ was calculated as follows:

$$[BSA] = OD\ x\ dilution\ 1.51$$

$$= ODx\ 101x\ 1.51$$

$$= p \text{ mg protein/mL (stock concentration)}$$

# APPENDIX G

Western Blotting Template (n=4-6)

| Lanes | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Blot 1 | U2 | U4 | B1 | B2 | B4 | B3 | B5 | B10 | C1 | C5 | C6 | C12 | C13 | 16 | A4 | A8 | A10 | A2 | A3 | A5 |
| Blot 2 | U5 | U7 | B7 | B9 | B17 | B11 | B13 | B15 | C11 | C14 | C17 | C19 | C20 | C21 | A13 | A17 | A18 | A6 | A14 | A16 |
| Group | Baseline | | Stabilisation Control | | | Stabilisation Melatonin 50µM | | | Ischaemia Control | | | Ischaemia Melatonin 50µM | | | Reperfusion Control | | | Reperfusion Melatonin 50µM | | |

***Figure 45: Western Blotting Template showing Blot 1 and Blot 2 with control and 50µM melatonin groups n=4-6.***

# APPENDIX H

Western Blotting Template (n=4)

| Lanes | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Blot 1 | J1 | G7 | G1 | G3 | G5 | G2 | G4 | G6 | H8 | H3 | H5 | H2 | H7 | H4 | H6 | I1 | I3 | I5 | I3 | I1 | I6 | I8 | I10 | I12 | I7 |
| Group | Baseline | Stabilisation Control | | | | Stabilisation Melatonin 0.3µM | | | | Ischaemia Control | | | | Ischaemia Melatonin 0.3µM | | | | Reperfusion Control | | | | Reperfusion Melatonin 0.3µM | | | |

***Figure 46: Western Blotting Template for controls groups and 0.3µM Melatonin groups.***

# APPENDIX I

**Research Outputs Associated With This Study**

Conference Contributions:

Year: 2017

Authors: K, Dube, R Salie, A Lochner.

Reference: The Significance of Mitopphagy in Myocardial Ischaemia/ Reperfusion: the effect of melatonin (Oral Presentation)

Physiological Society of Southern Africa Annual Congress, Pretoria, 2017

Other Outputs:

Year: 2017

Authors: K, Dube, R Salie, A Lochner.

Reference: The Significance of Mitopphagy in Myocardial Ischaemia/ Reperfusion: the effect of melatonin (Oral Presentation)

South African Medical Research Council Biomedical Research and Innovation Platfom: Research Symposium, 2017

Year: 2017

Authors: K, Dube, R Salie, A Lochner.

Reference: The Significance of Mitopphagy in Myocardial Ischaemia/ Reperfusion: the effect of melatonin (Oral Presentation)

Annual Research Day, Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Stellenbosch University.

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